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ORIGINAL ARTICLE

Upregulation of embryonic stem cell marker Nanog in human gingival fibroblasts stimulated with cyclosporine A: An *in vitro* study

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KEYWORDS
cyclosporine A;
embryonic stem cell marker;
gingival overgrowth;
Nanog

Abstract *Background/purpose:* Gingival overgrowth is a common side effect of medication with the immunosuppressant cyclosporine A (CsA). This study proposed to verify whether Nanog, an embryonic stem cell marker, contributes to gingival overgrowth stimulated with CsA in human gingival fibroblasts (HGFs).
Materials and methods: The effect of CsA on HGFs was used to elucidate whether Nanog expression could be induced by CsA using quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. Cell growth in CsA-treated HGFs with Nanog lentivirus-mediated short hairpin RNA interference knockdown was evaluated by tetrazolium bromide reduction assay.
Results: CsA upregulated Nanog transcript in HGFs in a dose-dependent manner ($P < 0.05$). CsA was also shown to increase Nanog protein expression in HGFs in a dose-dependent manner ($P < 0.05$). In addition, downregulation of Nanog by lentiviral infection significantly inhibited CsA-stimulated cell growth in HGFs ($P < 0.05$).
Conclusion: CsA upregulated Nanog expression and cell growth in HGFs, while silencing Nanog effectively reversed these phenomena. Nanog may act as a major switch in the pathogenesis of CsA-induced gingival overgrowth.
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Introduction

Cyclosporine A (CsA) is an immunosuppressant widely used to avoid allograft rejection and treat several immune-related conditions. Unfortunately, gingival overgrowth is one of the significant adverse effects of medication with CsA,[1] with a quoted prevalence of between 8% and 100% in transplant patients.[2] The pathogenesis of CsA-induced gingival overgrowth is still not entirely understood. It is believed that the imbalance of extracellular matrix (ECM) molecules accumulation may contribute to CsA-induced gingival overgrowth.[3–9] Recently, the upregulation of epithelial–mesenchymal transition (EMT) makers Snail[10] and Slug[11] were also found to play an important role in the pathogenesis of CsA-induced gingival overgrowth.

The pluripotency-associated transcription factor Nanog was identified based on its ability to support embryonic stem cell (ESC) self-renewal,[12] and is involved in the maintenance of the undifferentiated state of pluripotent stem cells.[13] Recently, ESC marker Nanog was found in keloid scar[14] and liver fibrosis derived from alcohol-related hepatocellular carcinoma.[15] However, it is unclear whether Nanog is involved in the pathogenesis of CsA-induced gingival overgrowth.

In this study, the effect of CsA on normal human gingival fibroblasts (HGFs) was used to elucidate the possible role of Nanog in the pathogenesis of CsA-induced gingival overgrowth. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting were used to determine the effects of CsA on the expression of Nanog in HGFs. In addition, cell growth in CsA-treated HGFs with Nanog lentivirus-mediated short hairpin RNA interference (shRNAi) knockdown was evaluated by tetrazolium bromide reduction assay.

Materials and methods

Cell culture

After approval by the Institutional Review Board at Chung Shan Medical University Hospital, Taichung, Taiwan, normal gingival tissue samples were obtained from three healthy male individuals (mean age, 26.7 years; range, 20–32 years) undergoing routine surgical crown lengthening, with little if any evidence of inflammation and no systemic medication. HGFs were cultured by using an explant technique as described previously.[3,9]

Nanog expression analysis

HGFs were arrested in G_0 by serum deprivation according to our previous experiments.[4,7] Nearly confluent monolayers of HGFs were washed with serum-free medium and immediately thereafter exposed at the indicated incubation times to 0 ng/mL, 100 ng/mL, 500 ng/mL, and 1000 ng/mL CsA (Sigma–Aldrich, St. Louis, MO, USA). Cell lysates were collected at 24 hours for qRT-PCR and western blot assays. Cultures without fetal calf serum (Gibco BRL, Gaithersburg, MD, USA) were used as negative controls.

qRT-PCR

Total RNA of cells was purified using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously.[10,11] Nanog primers were designed: (forward) ATTCAGGACAGCCCTGATTCTTC and (reverse) TTTTTGCGACACTCTTCTCTGC. The *GAPDH* housekeeping gene was amplified as a reference standard. *GAPDH* primers were designed: (forward) CATCATCCCTGCCTCTACTG and (reverse) GCCTGCTTCACCACCTTC.

Western blotting

The extraction of proteins from cells and immunoblotting procedure were performed as described previously.[8,9]

Nanog knockdown in CsA-treated HGFs by lentivirus-mediated short hairpin RNA interference

The pLV-RNAi vector was purchased from Biosettia (San Diego, CA, USA). The method of cloning the double-stranded shRNA has been described previously.[10,11] Lentiviral vectors expressing shRNA that targets human Nanog (sh-Nanog-1: 5'-AAAAGCATCCGACTGTAAAGAATTTGGATCCAAATTCTTTACAGTCGGATGC-3'; sh-Nanog-2: 5'-AAAAGCTGTGTGTACTCAATGATTTGGATCCAAATCATTGAGTACACACAGC-3') were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. shRNA that targets luciferase (sh-Luc: 5'-CCGGACTTACGCTGAGTACTTCGAACTCGAGTTCGAAGTACTCAGCGTAAGTTTTTTG-3') was utilized for an experimental control.

Cell growth

HGFs were placed in 96-well plates, washed with phosphate-buffered saline, and cultured without fetal calf serum for starvation overnight. After treatment with 500 ng/mL CsA for 24 hours, cell growth was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Sigma–Aldrich) as described previously.[10,11]

Statistical analysis

Three replicates of each experiment were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out by one-way analysis of variance. Tests of differences of the treatments were analyzed by Duncan's test and a value of $P < 0.05$ was considered statistically significant.

Results

To examine the effect of CsA on Nanog expression, HGFs were treated with CsA and the levels of transcript and protein were measured by qRT-PCR and Western blotting, respectively. CsA increased Nanog transcription in HGFs in a dose-dependent manner ($P < 0.05$; Figure 1). CsA also upregulated Nanog protein expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA), the amount of

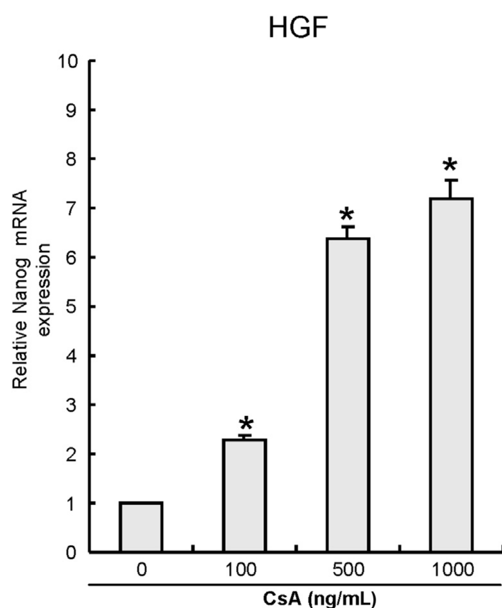

Figure 1 Nanog mRNA expression was examined by quantitative real-time reverse transcription-polymerase chain reaction. Human gingival fibroblasts were exposed for 24 hours in medium containing various concentrations of cyclosporine A as indicated. The relative Nanog mRNA expression represents the mean $\pm$ standard deviation. * Represents significant difference from control values with $P < 0.05$.

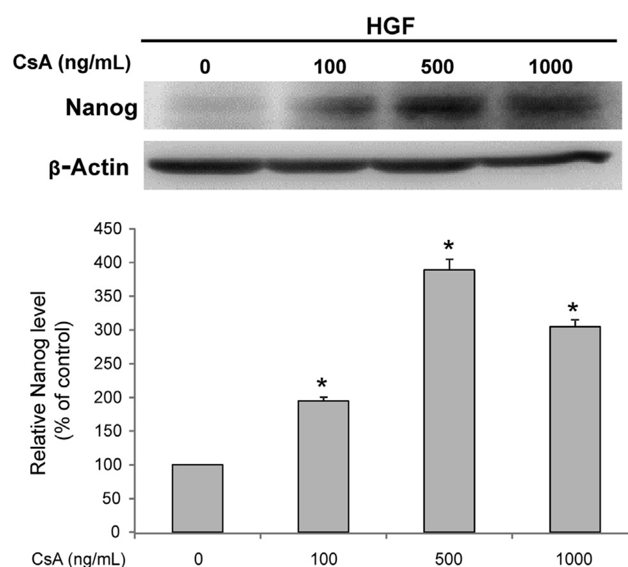

Figure 2 Nanog protein expression was examined by western blotting. Human gingival fibroblasts were treated with indicated concentration of CsA for 24 hours. β-Actin was used as protein loading control (Upper panel). Levels of Nanog protein treated with CsA were measured by AlphaImager 2000. The relative level of Nanog protein expression for each sample was normalized against β-actin signal, and the control was set as 1.0. Triplicate experiments were performed. * Represents significant difference from control values with $P < 0.05$ (Lower panel). CsA = cyclosporine A.

Nanog was elevated about 1.9-, 3.8-, and 3.1-fold at concentrations of 100 ng/mL, 500 ng/mL, and 1000 ng/mL CsA, respectively, as compared with the controls (Figure 2).

Downregulation of Nanog in CsA-treated HGFs was achieved by viral transduction with lentiviral vector expressing shRNA targeting Nanog. In addition, lentiviral vector expressing sh-Luc was used as a control. qRT-PCR confirmed that lentivirus expressing both sh-Nanog markedly reduced the expression level of CsA-induced Nanog expression in HGFs (Figure 3). Western blot confirmed that knockdown of Nanog reduced Nanog expression in CsA-stimulated HGFs (Figure 4). The quantitative measurement of Nanog protein expression by the AlphaImager 2000 is shown in the lower panel of Figure 4.

In addition, knockdown of Nanog inhibited CsA-stimulated cell growth in HGFs (Figure 5). These results suggested that Nanog expression acts as a major switch in the pathogenesis of CsA-induced gingival overgrowth.

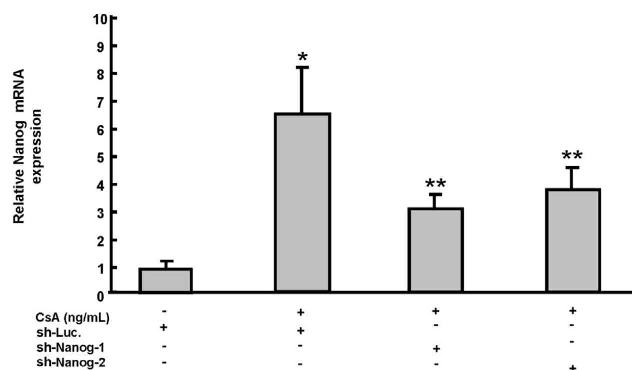

Figure 3 The silencing effect of Nanog short hairpin RNA in CsA-treated HGFs was validated transcriptionally by quantitative real-time reverse transcription-polymerase chain reaction. Single cell suspension of HGFs was transduced with sh-Luc or sh-Nanog lentivirus, individually or concurrently, and treated with or without CsA (500 ng/mL) as indicated. The relative Nanog mRNA expression represents the mean $\pm$ standard deviation. * $P < 0.05$, Sh-Luc + CsA group versus control group. ** $P < 0.05$, Sh-Nanog-1 + CsA or Sh-Nanog-2 + CsA versus Sh-Luc + CsA group. CsA = cyclosporine A; HGF = human gingival fibroblast.

Discussion

The pathogenesis of CsA-induced gingival overgrowth is not entirely understood. Nanog constitutes the core transcriptional network of pluripotency and has shown to reprogram human somatic fibroblasts into ESC-like pluripotent cells, termed inducible pluripotent stem cells.[16] Recently, Nanog was found to induce hyperplasia without initiating tumors in tet-Nanog mice.[17] In this study, to the best of our knowledge, CsA was first found to upregulate Nanog mRNA and protein expression in HGFs. In addition, qRT-PCR and Western blotting confirmed that lentivirus expressing both sh-Nanog-1 and sh-Nanog-2 significantly inhibited the levels of CsA-induced Nanog transcription and protein expression in HGFs. Taken together, these results suggest that CsA contributes to the pathogenesis of gingival overgrowth via the augmentation of Nanog expression in HGFs. In addition,

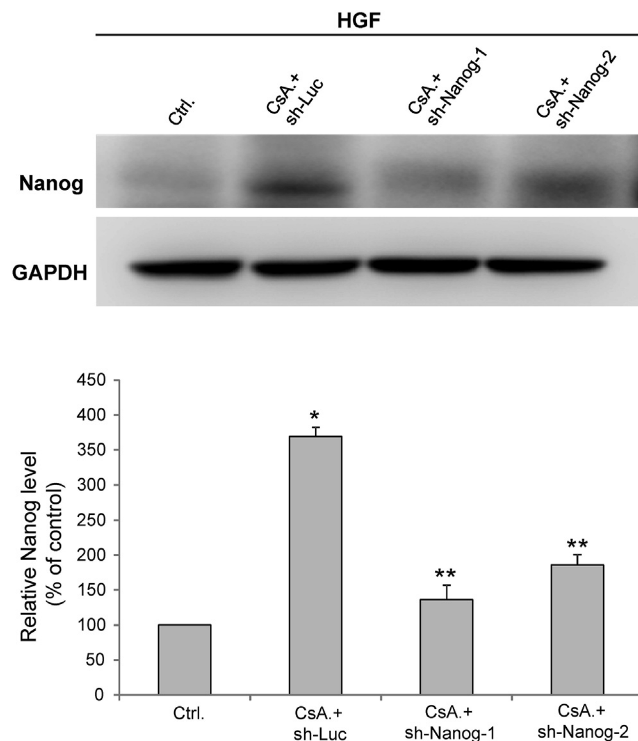

Figure 4 Total proteins prepared from single cell suspension of human gingival fibroblasts transduced with sh-Luc or sh-Nanog lentivirus, individually or concurrently, and treated with or without CsA were analyzed for CsA expression by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase was used as protein loading control. Levels of Nanog protein from HGFs transduced with sh-Luc or sh-Nanog lentivirus were measured by AlphaImager 2000. The relative level of Nanog protein expression for each sample was normalized against β-actin signal, and the control was set as 1.0. Triplicate experiments were performed. * Represents significant difference from control values with $P < 0.05$. CsA = cyclosporine A. ** $P < 0.05$, Sh-Naong-1 + CsA or Sh- Naong-2 + CsA vs. Sh-Luc + CsA group.

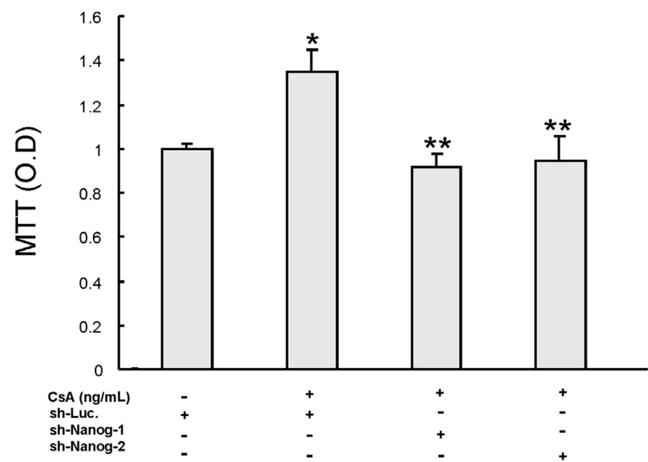

Figure 5 Silencing of Nanog expression suppresses CsA-stimulated growth of HGFs. Viability of the control or Nanog-knockdown CsA-treated HGFs was evaluated by MTT assay. Cells were exposed for 24 hours in medium containing various concentrations of CsA as indicated. * $P < 0.05$, Sh-Luc + CsA group versus control group. ** $P < 0.05$, Sh-Nanog-1 + CsA or Sh- Nanog-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.

the time-dependent effect may be also an important issue for CsA-induced gingival overgrowth, and it merits further study.

EMT, a transdifferentiation program that converts epithelial state into mesenchymal state, is critical for development and disease including drug-induced gingival overgrowth.[18] Researchers have shown that EMT can promote and maintain stemness properties of stem cells.[19] Recently, we reported that the upregulation of Snail[10] and Slug[11] may play an important role in the pathogenesis of CsA-induced gingival overgrowth. Thus, the detailed molecular mechanisms involved in the regulatory links between Nanog and EMT properties are worthy of further investigation.

CsA-induced gingival overgrowth consists of several cell types, with fibroblasts being one of the most predominant cells. The aberrant expression of Nanog has been linked to increased cell growth and proliferation in pancreatic cancer cells[20] and human dental pulp cells.[21] In this study, knockdown of Nanog suppressed CsA-stimulated cell growth in HGFs. Our results provide new avenues for the design of novel antifibrotic therapies for CsA-induced gingival overgrowth through targeting Nanog.

As far as we know, this is the first attempt to evaluate the role of Nanog expression in HGFs stimulated with CsA. CsA upregulated Nanog expression and cell growth in HGFs, while silencing Nanog reversed these phenomena. Nanog may play an important role in the pathogenesis of CsA-induced gingival overgrowth. Therefore, the design of novel antifibrotic therapies for CsA-induced gingival overgrowth through targeting Nanog may be considered. We aim to verify these current *in vitro* data in a future *in vivo* study on clinical samples of gingival overgrowth from patients receiving CsA. In addition to Nanog, other ESC markers such as Oct4, SSEA-4, and Sox2 will be investigated in the future to verify the possible roles of ESCs in CsA-induced gingival overgrowth.

Conflict of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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