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Original Article

The regulation of Oct4 in human gingival fibroblasts stimulated by cyclosporine A: Preliminary observations

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KEYWORDS
Cyclosporine A;
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Oct4;
Nanog

Abstract *Background/purpose:* Oct4, a key transcription factor, could reprogram human somatic fibroblasts into embryonic stem cell-like pluripotent cells. The exact mechanism of cyclosporine A (CsA)-induced gingival overgrowth is still unclear. The aim of this study was to investigate the effects of CsA on the expression of Oct4 in cultured human gingival fibroblasts (HGFs) *in vitro*.
Materials and methods: The effects of CsA on HGFs were used to elucidate whether Oct4 expression could be induced by CsA by using quantitative real-time reverse transcription-polymerase chain reaction and western blot. Cell growth in CsA-treated HGFs with Oct4 lentiviral-mediated shRNAi knockdown was evaluated by tetrazolium bromide reduction assay.
Results: CsA was found to upregulate Oct4 transcript in a dose-dependent manner ($p < 0.05$). CsA also dose-dependently increased Oct4 protein expression ($p < 0.05$). The lentivirus expressing sh-Oct4 successfully prevented the CsA-induced Oct4 mRNA and protein in HGFs ($p < 0.05$). However, knockdown of Oct4 was insufficient to inhibit CsA-stimulated cell growth in HGFs. Furthermore, double knockdown with pluripotency-associated transcription factor Nanog showed that the down-regulation of Oct4/Nanog by lentiviral infection significantly inhibited CsA-stimulated cell growth ($p < 0.05$).
Conclusion: Taken together, CsA was first found to upregulate Oct4 mRNA and protein expression in HGFs. The silencing Oct4 could not suppress cell growth unless Nanog was repressed simultaneously.

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Introduction

Cyclosporine A (CsA), a hydrophobic fungal metabolite form *Tolypocladium inflatum*, is an immune-suppressant widely used to prevent transplant rejection and manage several autoimmune conditions.[1] Like the calcium channel blockers, CsA is associated with gingival overgrowth with the wide range of prevalence rate estimated between 8% and 70%.[2] Despite the intensive studies investigating the cellular and molecular basis of the development of CsA-induced gingival overgrowth, the exact mechanism underlying this condition is still unclear. The imbalance of extracellular matrix molecules accumulation,[3–9] upregulation of epithelial–mesenchymal transition (EMT) makers,[10–12] and even the overexpression of embryonic stem cell (ESC) marker Nanog[13] are believed to attribute the pathogenesis of CsA-induced gingival overgrowth.

Oct4, a key transcription factor, is involved in the maintenance of pluripotency and self-renewal in undifferentiated ESCs.[14,15] Recently, Oct4 was found in fibroblasts derived from the cesarean scar tissue[16] and keloid.[17] In the present study, the effects of CsA on normal human gingival fibroblasts (HGFs) were determined through quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blot to determine the expression of Oct4. In addition, cell growth in CsA-treated HGFs with lentiviral-mediated shRNAi knockdown was evaluated by tetrazolium bromide reduction assay.

Materials and methods

Cell culture

Normal HGFs derived from three healthy male individuals undergoing surgical crown lengthening were cultured according to previous criteria and an explant technique as described previously.[4,13] This study was approval by the Institutional Review Board permission at the Chung Shan Medical University Hospital.

Oct4 expression analyses

HGFs were arrested in G_0 by serum deprivation according to our previous experiments.[3,5] Nearly confluent monolayers of HGFs were washed with serum-free medium and immediately exposed to 0, 100, 500, and 1000 ng/ml CsA (Sigma–Aldrich, St. Louis, MO, USA). Cell lysates were collected at 24 h for qRT-PCR and western blot assays. Cultures without fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD, USA) were used as negative control.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA of cells was purified using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to our previous publication.[10,11] Oct4 primers were designed: (forward) GTGGAGAGCAACTCCGATG and (reverse) TGCTC CAGCTTCTCCTTCTC. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) housekeeping gene was amplified as a reference standard. *GAPDH* primers were designed: (forward) CATCATCCCTGCCTCTACTG and (reverse) GCCTGC TTCACCACCTTC.

Western blot

The extraction of proteins from cells and immunoblotting procedure were performed as described previously.[8,9] The primary antibodies were anti-Oct3/4 (SC-9081, Santa Cruz, CA, USA) and anti-GAPDH (#MA1-16757, ThermoFisher Scientific, Waltham, MA, USA) antibodies.

Oct4/Nanog knockdown in CsA-treated HGFs by lentiviral-mediated shRNAi

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described according to our recent publications.[11,13] Lentiviral vectors expressing short hairpin RNA (shRNA) that targets human Oct4 (sh-Oct4-1: 5′-AAAAGCTGGGGAGAGTATATATTTTGGATCCAAAATATATACTCT CCCCAGC-3′; sh-Oct4-2: 5′- AAAAGCTCTCCCATGCATTCAAA TTGGATCCAATTTGAATGCATGGGAGAGC -3′); Nanog (sh-Nanog: 5′-AAAAGCATCCGACTGTAAAGAATTTGGATCCAAATT CTTTACAGTCGGATGC-3′) were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. shRNA that targets luciferase (sh-Luc: 5′-CCGGACTTACGCTGAGTACTTC-GAACTCGAGTTCGAAGTACTCAGCGTAAGTTTTTTG-3′) was utilized for an experimental control.

Cell growth

HGFs placed in 96-well plates washed with phosphate-buffered saline and then cultured without FCS for starvation overnight. After treatment with 500 ng/ml CsA for 24 h, cell growth was tested using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit (Sigma–Aldrich, St. Louis, MO, USA) as described previously.[13]

Statistical analysis

All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test. $P < 0.05$ was considered statistically significant.

Results

As shown in Fig. 1, CsA was found to increase Oct4 transcript in HGFs in a dose-dependent fashion ($p < 0.05$). CsA also upregulated the protein expression of Oct4 in a dose-dependent manner ($p < 0.05$) (Fig. 2). From the AlphaImager 2000, the amount of Oct4 was elevated about 1.2, 4.3, and 4.8 fold at concentrations of 100, 500, and 1000 ng/ml CsA, respectively, as compared with control.

Down-regulation of Oct4 in CsA-treated HGFs was achieved by viral transduction with lentiviral vector expressing shRNA targeting Oct4. In addition, lentiviral vector expressing sh-Luc was used as control. qRT-PCR confirmed

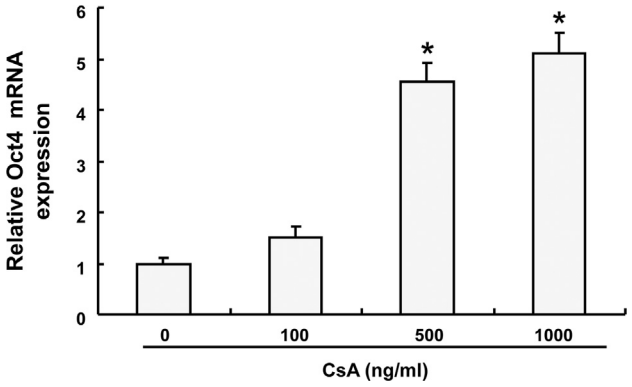

Figure 1 HGFs were treated with indicated concentration of CsA for 24 h. The Oct4 mRNA expression was examined by qRT-PCR. The relative Oct4 mRNA expression represent the mean ± SD. * represents significant difference from control values with $p < 0.05$.

that lentivirus expressing both sh-Oct4 markedly reduced the expression level of CsA-induced Oct4 expression in HGFs ($p < 0.05$) (Fig. 3). As shown in Fig. 4, western blot confirmed that the knockdown of Oct4 could reduce Oct4 expression in CsA-stimulated HGFs. The quantitative measurement of Nanog protein expression by the AlphaImager 2000 is shown in the lower panel of Fig. 4.

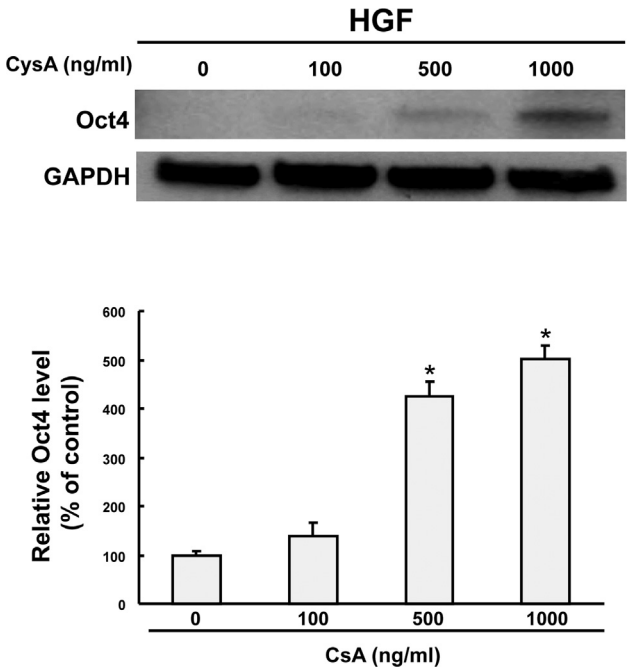

Figure 2 The Oct4 protein expression was examined by western blot. HGFs were treated with indicated concentration of CsA for 24 h. GAPDH was used as protein loading control (Upper panel). Levels of Oct4 protein treated with CsA were measured by AlphaImager 2000. The relative level of Oct4 protein expression for each sample was normalized against GAPDH 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).

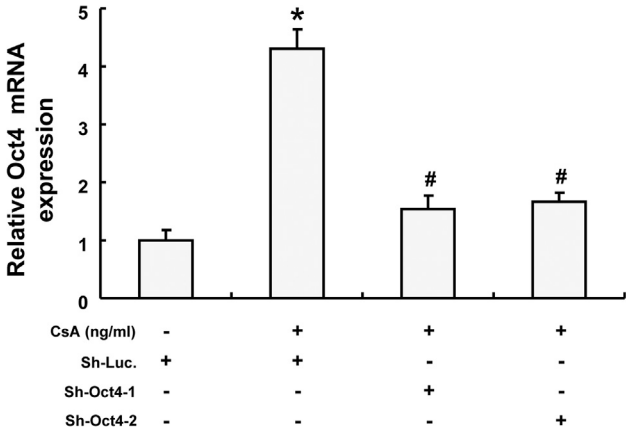

Figure 3 The silencing effect of Oct4 shRNA in CsA-treated HGFs was validated transcriptionally by qRT-PCR. Single cell suspension of HGFs was transduced with sh-Luc or sh-Oct4 lentivirus, individually or concurrently, and treated with or without CsA (500 ng/ml) as indicated. The relative Oct4 mRNA expression represent the mean ± SD. *$p < 0.05$, Sh-Luc + CsA group vs. control group; #$p < 0.05$, Sh-Oct4-1 + CsA or Sh-Oct4-2 + CsA vs. Sh-Luc + CsA group.

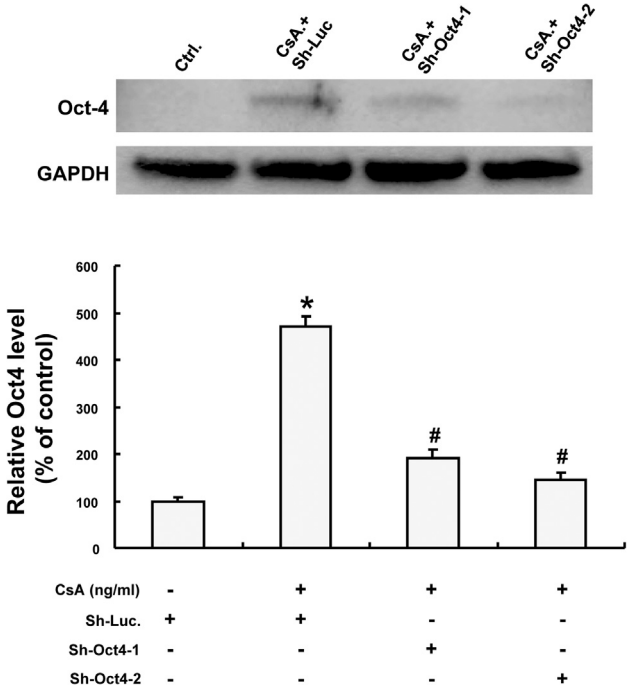

Figure 4 Total proteins prepared from single cell suspension of HGF transduced with sh-Luc or sh-Oct4 lentivirus, individually or concurrently, and treated with or without CsA were analyzed for CsA expression by western blot. GAPDH was used as protein loading control. Levels of Oct4 protein from HGFs transduced with sh-Luc or sh-Oct4 lentivirus were measured by AlphaImager 2000. Triplicate experiments were performed. * represents significant difference from control values with $p < 0.05$. # $p < 0.05$, Sh-ct4-1 + CsA or Sh-Oct4-2 + CsA vs. Sh-Luc + CsA group.

As shown in Fig. 5, the suppression of Oct4 was insufficient to prevent the CsA-induced cell growth. Our previous study[13] has found that Nanog was associated with CsA-induced gingival overgrowth. Both Oct4 and Nanog are crucial pluripotency-associated transcription factors, we therefore utilized higher efficient sh-Oct4-2/sh-Nanog-1[13] lentivirus and conducted the double knockdown experiments. The results demonstrated that the down-regulation of Oct4/Nanog significantly inhibited CsA-stimulated cell growth ($p < 0.05$).

Discussion

Gingival overgrowth is a widely reported side-effect of CsA therapy. Until now, the pathogenesis of CsA-induced gingival overgrowth still remains to be elucidated. Recently, *in situ* expression of OCT4, SOX2, KLF4, and C-MYC (OSKM) transcription factors during wound healing could diminish fibrotic activity and lead to reduce scar tissue formation in a mouse model.[18] To the best of our knowledge, this is the first report showed that Oct4 mRNA and protein expression was increased after CsA treatment in HGFs. These findings may raise a question of whether Oct4 is involved in the enhanced cell proliferation following CsA administration.

Oct4 was reported to regulate tumor initiating property and EMT traits.[19] EMT is critical for the development and the diseases including drug-induced gingival overgrowth.[20] Recently, the upregulation of Snail[10] and Slug[11,12] were found to play an important role in the pathogenesis of CsA-induced gingival overgrowth. Thus, the detailed molecular mechanisms involved in the regulatory links between Oct4 and EMT properties are worthy of further investigation.

Moreover, overexpression of Oct4 was found to enhance cell proliferative activity, invasiveness and colony formation in oral squamous cell carcinoma cell lines *in vitro*.[19] In addition, Oct4 knockdown treatment could significantly slow down the tumor growth mediated in subcutaneous xenografts nude mice model.[19] These studies indicated that Oct4 may participate in the regulation of oral cell growth and the inhibition of Oct4 could attenuate their excessive growth. Hence, we utilized the lentivirus expressing sh-Oct4 to inhibit the levels of CsA-induced Oct4 transcript and protein expression in HGFs after CsA treatment and examine cell proliferation to assess the effect of Oct4 on CsA-induced gingival overgrowth. Surprisingly, we found that knockdown of Oct4 alone could not suppress CsA-stimulated HGFs growth. Our previous study has revealed that Nanog was increased in CsA-treated HGFs.[13] Nanog and Oct4 have been reported to work in concert to support stem cell potency and self-renewal.[15] Therefore, we conducted the double knockdown experiments with Oct4 and Nanog. The data revealed that co-knockdown with Oct4 and Nanog was able to attenuate the CsA-stimulated HGFs growth. Although the detailed mechanism regarding the interaction between these two factors requires further experiments to unveil, our results suggested that Oct4 may be just one of the downstream factors affected by CsA treatment during the progressive change of gingival overgrowth instead of the crucial initiator to control cell proliferation. However, further exploration of the mechanisms through which Oct4/Nanog regulates cell proliferation is still necessary to shed light on the role of Oct4/Nanog in the pathogenies of CsA-induced gingival overgrowth.

In summary, this study demonstrated that the pluripotency-associated transcription factor Oct4 was elevated after CsA treatment, but this upregulation was not directly associated with excessive cell proliferation. It is reasonable to assume that the increase in Oct4 with CsA treatment may lead to the enhanced stemness in HGFs. Further studies are required to confirm this hypothesis. The interplay between Nanog and Oct4 in CsA-treated HGFs is worthy of further investigation.

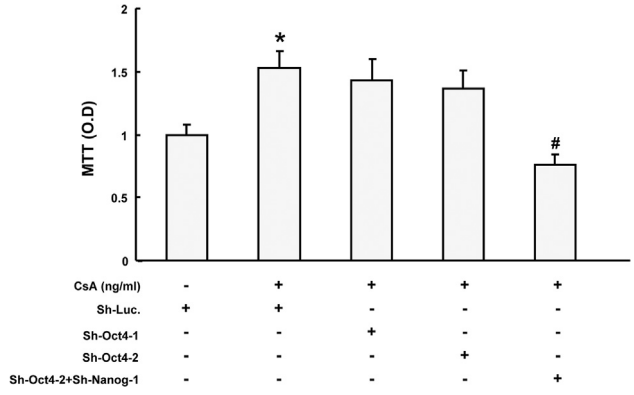

Figure 5 The cell viability of the control or Oct4/Nanog-knockdown CsA-treated HGFs was evaluated by an MTT assay. Cells were exposed for 24 h in medium containing 500 ng/ml CsA. * $p < 0.05$, Sh-Luc + CsA group versus control group. # $p < 0.05$, Sh-Oct-4-2 + Sh- Naong-1 + CsA vs. Sh-Luc + CsA group.

Declaration of Competing Interest

None declared.

Acknowledgment

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