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Original Article

2,3,5,4′-tetrahydroxystilbene-2-O-b-D-glucoside triggers the pluripotent-like possibility of dental pulp stem cells by activating the JAK2/STAT3 axis: Preliminary observations

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KEYWORDS
Dental pulp stem cells;
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Abstract
Background/Purpose: Although 2,3,5,4′-Tetrahydroxystilbene-2-O-beta-glucoside (THSG) reportedly has anti-inflammatory properties, its role in inducing the dedifferentiation of human dental pulp stem cells (DPSC) into pluripotent-like stem cells remains to be determined. The purpose of this study is to evaluate the effects of THSG on the pluripotent-like possibility and mechanism of DPSC.
Materials and methods: DPSCs were treated with THSG, and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTS) assay. Real-time polymerase chain reaction was used to analyze the mRNA expression levels of pluripotency-associated genes and oncogenes and to detect telomerase activity in the cells. Embryoid body formation assay was conducted, and pluripotency-related proteins were identified using

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Western blotting. Data were analyzed using one-way analysis of variance.
Results: Cell viability, telomerase activity, and embryoid body formation were enhanced in THSG-treated DPSCs. The mRNA expression levels of pluripotent-like genes (including Nanog homeobox [*NANOG*], SRY-box 2 [*SOX2*], and POU class 5 homeobox 1 [*POU5F1/OCT4*]) significantly increased after THSG treatment. The expression levels of pluripotency-related genes (Janus kinase-signal transducer 2 [*JAK2*] and signal transducer and activator of transcription 3 [*STAT3*]) increased, whereas those of oncogenes (*Ras*, *SRC*, *HER2*, and *C-sis*) decreased. Furthermore, the expression levels of the phosphorylated JAK2 and STAT3 proteins significantly increased after THSG treatment.
Conclusion: THSG treatment may enhance the pluripotent-like possibility of DPSC through the JAK2/STAT3 axis. Hence, it may be used as an alternative cell-based therapeutic strategy in regenerative dentistry.
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Introduction

Stem cell therapies and studies are expected to provide substantial benefits for a wide range of diseases and severe conditions. For instance, embryonic stem cells (ESCs), a type of pluripotent stem cell derived from the inner cell masses of mammalian blastocysts, are being used in clinical trials owing to their self-renewal and differentiation abilities.[1] However, immune rejection and ethical concerns are the two major challenges with regard to the clinical use of ESCs.

Based on previous studies, Takahashi and Yamanaka successfully established mouse-induced pluripotent stem cells (iPSCs) by introducing four critical genes (*OCT4*, *SOX2*, *c-Myc*, and *Klf4*) into mouse embryonic or adult fibroblasts.[2,3] iPSCs are similar to ESCs in various aspects, including proliferation, morphology, gene expression, surface antigens, epigenetic status of pluripotent cell-specific genes, and telomerase activity.[4] Therefore, scientists are considerably optimistic about the therapeutic potential of both ESCs and iPSCs in tissue repair and replacement due to their pluripotency.[4] As determined in previous studies, the critical transcription factors, including *OCT4*, *SOX2*, and *NANOG*, governs the pluripotency of both mouse and human ESCs.[5] In addition, these key transcription factors closely interact with and auto-regulate each other. Thus, it is essential to investigate the signaling transduction pathways of these three factors. According to previous studies, the activation of Janus kinase-signal transducer 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) may play a key role in maintaining the pluripotency of ES and iPSCs.[6] The STAT3-JAK2 pathway may be involved in the induction of *OCT4*, *SOX2*, and *NANOG* expression; however, additional studies are required to confirm this finding.

Dental pulp stem cells (DPSCs) are the first type of mesenchymal stem cell to isolated from human extracted teeth which are usually regarded as medical waste after orthodontic treatment.[7] These cells feature both immunomodulatory characteristics and multi-differentiation capability.[8] Therefore, these cells have been considered a potential candidate for the restoration of lost periodontal tissues.[9] Nonetheless, although the therapeutic capacity of DPSCs has been investigated, their therapeutic efficiency and potential for use in the dental setting need to be explored.

2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (THSG), the main component of the Chinese herb Polygonum multiform, is water-soluble and biologically active.[10] Due to its strong antioxidant capacity and free radical scavenging activity,[10] THSG may play a vital role in the treatment of various human health issues and diseases, such as aging[11] and cardiovascular diseases.[12] In our previous studies,[9,13] we explored that THSG enhanced the proliferation and self-renewal of DPSCs, the promotion of osteoblast differentiation and alveolar bone regeneration. In addition, THSG increased the expression levels of the pluripotent stem cell-associated genes in DPSCs, indicating the possibility of the reprogramming of these cells.[13] Although these previous studies have demonstrated that THSG enhanced cell proliferation and differentiation, its role in inducing the dedifferentiation of DPSCs into pluripotent-like stem cells remains to be determined.

The aim of this study was to determine whether THSG induces pluripotent-like behavior in DPSCs.

Materials and methods

Reagents

THSG (purity: 95%) was kindly provided by Dr. Ching-Chiung Wang (Graduate Institute of Pharmacognosy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan). It was extracted[10,14] and dissolved in dimethyl sulfoxide (DMSO; Invitrogen, Grand Island, NY, USA) for cell treatment, as described previously.[9,13]

Isolation and culture of DPSC

DPSCs were obtained from four adult patients between 2017 and 2019 (one man and two women; age, 20—30 years; mean age, 24 years) and were cultured separately. All experiments were performed as described previously.[9,13] Briefly, normal human premolars were extracted from the

adults at the Department of Orthodontics, Wan-Fang Medical Center, Taipei Medical University, Taipei, Taiwan. Approval for all procedures was obtained from the Taipei Medical University Joint Institutional Review Board (TMU-JIRB, N201812044). All participants included in the study provided written consent before the procedures. The isolation and culture methods of DPSCs were described previously.[7,9,13,15]

Cell viability test

The viability of human DPSC cells was tested after treatment with THSG (0, 1, 10, and 100 μM) in 1% FBS-containing PluriSTEM™ Human ES/iPS cell medium (Merk, Darmstadt, Germany) for 48 or 96 h. The medium with different THSG concentrations was replaced daily. Cell viability was determined according to the instructions in the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay[(a)] kit (Promega, Madison, WI, USA) for 4 h at 37 °C. Quintuplicate wells were assayed for each experiment, and three independent experiments were performed.

Telomerase activity assay

The telomerase activity of human DPSCs was tested after treatment with THSG (0, 1, 10, and 100 μM) in 1% FBS-containing PluriSTEM™ Human ES/iPS cell medium (Merk, Darmstadt, Germany) for 24 h. Telomerase activity was measured according to the instructions in the TRAPEZE RT Telomerase Detection Kit (S7710, Merck Millipore, MA, USA). Preheated samples were used as negative controls, and PC was used as the positive control.

Embryoid body formation assay

To determine the effects of THSG on embryos body forming in DPSCs, The embryoid body formation assay (STEMCELL Technologies, Inc., Vancouver, Canada) was used. In brief, 9×10^5 cells were loaded into each well of an AggreWell™ plate and then treated with an anti-adherence solution. The cells were cultured in AggreWell™ EB Formation Medium containing THSG (0, 1, 10, and 100 μm) and a ROCK inhibitor (Y-27632) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 2 days. The medium was changed daily. Images of the cells were obtained using a microscope (Leica, Microsystems, Wetzlar, Germany).

Quantitative real-time polymerase chain reaction (qPCR)

To examine the effects of THSG on the mRNA expression levels of pluripotent SC-like genes, human DPSCs were treated with the 0, 1, 10, and 100 μM THSG for 6 h mRNA extraction and qPCR were performed as described previously.[13] The sequences of the primers used in this study were shown in Table 1. Calculations of the relative gene expression levels (normalized to the 18 S reference gene) were performed according to the Delta-Delta-Ct method, and PCR fidelity was determined using melting temperature analysis.

AG490 treatment

AG490, purchased from Sigma—Aldrich (St Louis, MO, USA), was dissolved in DMSO to obtain a concentration of 5 mM and was stored at −20 °C until further use. 5 μM of AG490 was used to inhibit the JAK2 in this study.

Western blot analysis

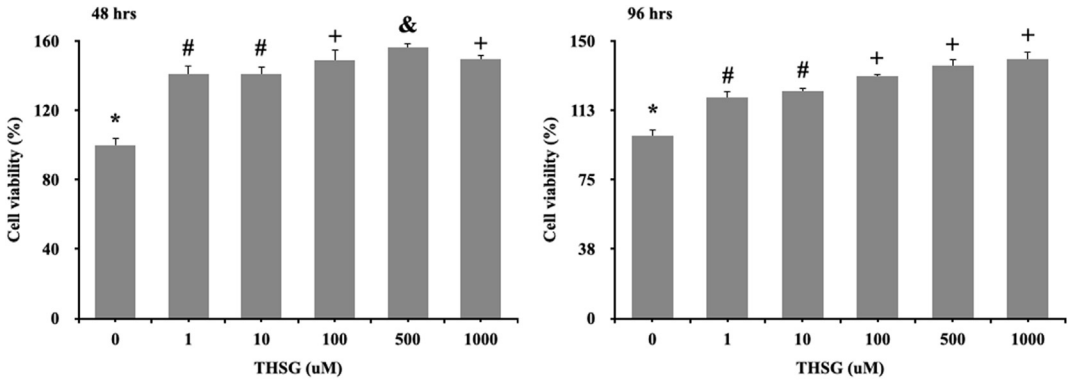
After the cells were treated with 0, 1, 10, and 100 μM THSG in PluriSTEMTM ES/iPS Medium (Merk) for 3 h, protein extraction and Western blotting were performed as described previously.[13] The resolved proteins were then incubated with rabbit anti-JAK2, rabbit anti-phospho-JAK2, rabbit anti-STAT3, rabbit anti-phospho-STAT3 (Cell Signaling Technology, Beverly, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Merk, Darmstadt, Germany) at 4 °C overnight. Signals were detected using the LumiFlas™ Femto Chemiluminescent Substrate, HRP kit (ENERGENESIS BIOMEDICAL, Taipei, Taiwan). Images of the Western blots were visualized and analyzed using the Chemi-Doc™ XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

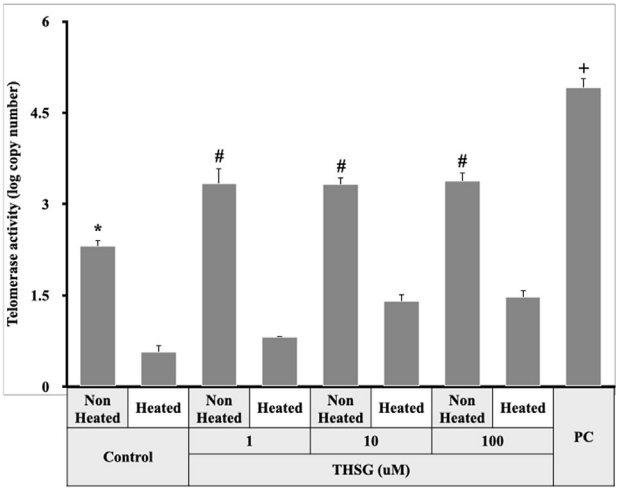
All data were analyzed using IBM SPSS Statistics Software 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of

Table 1 Primers used for qPCR.

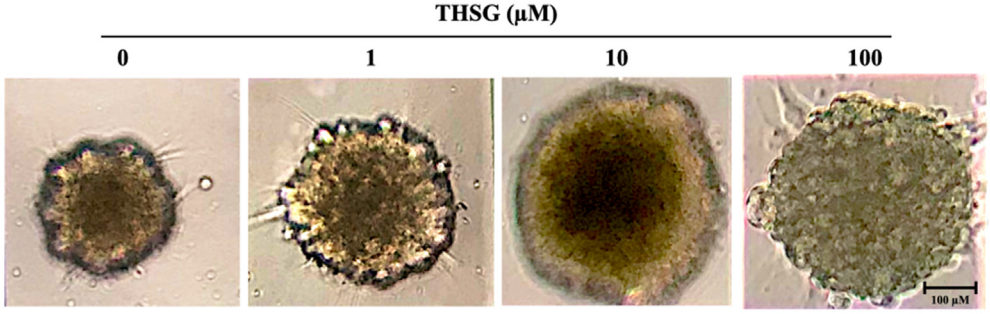
Primer	Forward sequence	Reverse sequence	Accession number
NANOG	ACCACGTGTTCTGGTTTCCAT	AAC CTCGCTGATTAGGCTCC	XM_011520852.1
OCT4	CCTTCGCAAGCCCTCATTTC	AAATCCGAAGCCAGGTGTCC	NM_002701.5
SOX2	ACAGCATGTCCTACTCGCAG	GAC TTGACCACCGAACCCAT	NM_ 003106.3
JAK2	TCTGGTGCCTTTGAAGACCG	TACCCTTGCCAAGTTGCTGT	NM_001322195.1
STAT3	GAAACAGTTGGGACCCCTGA	TACCGTGTGTCAAGCTGCTG	NM_139276.2
Ras	TGGTGGGGAACAAGTGTGAC	CGAGGTCTCGATGTAGGGGA	NM_005343.4
HER2	GCTCCTCCTCGCCCTCTT	CGCAGCTTCATGTCTGTGC	XM_024450641.1
SRC	TGGTGGGGAACAAGTGTGAC	CGAGGTCTCGATGTAGGGGA	BC_0011566.1
C-Sis	CAGCGGCCTCCGGTCTT	CCCAAGTTCTTGGAGTTAAGGGA	M12783.1
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	NR_003286



A



B



C

Figure 1 Proliferation, self-renewal, and embryoid body formation in DPSCs treated with THSG. (A) MTS assay in cells treated with various concentrations of THSG. THSG-enhanced cell proliferation at concentrations of >1 μM*, #, &, and + indicate significantly different subsets obtained by the post hoc Duncan test if a significance of $p < 0.05$ was reached by one-way ANOVA. **(B)** Telomerase activity assay was performed to detect the THSG-induced self-renewal of DPSCs (Samples were preheated as negative control; PC, positive control; *, #, and + indicate significantly different subsets obtained by the post hoc Duncan test if a significance of $p < 0.05$ was reached by one-way ANOVA.) **(C)** Optical image of THSG-triggered embryoid body formation on day 2. Scale bar: 100 μm.

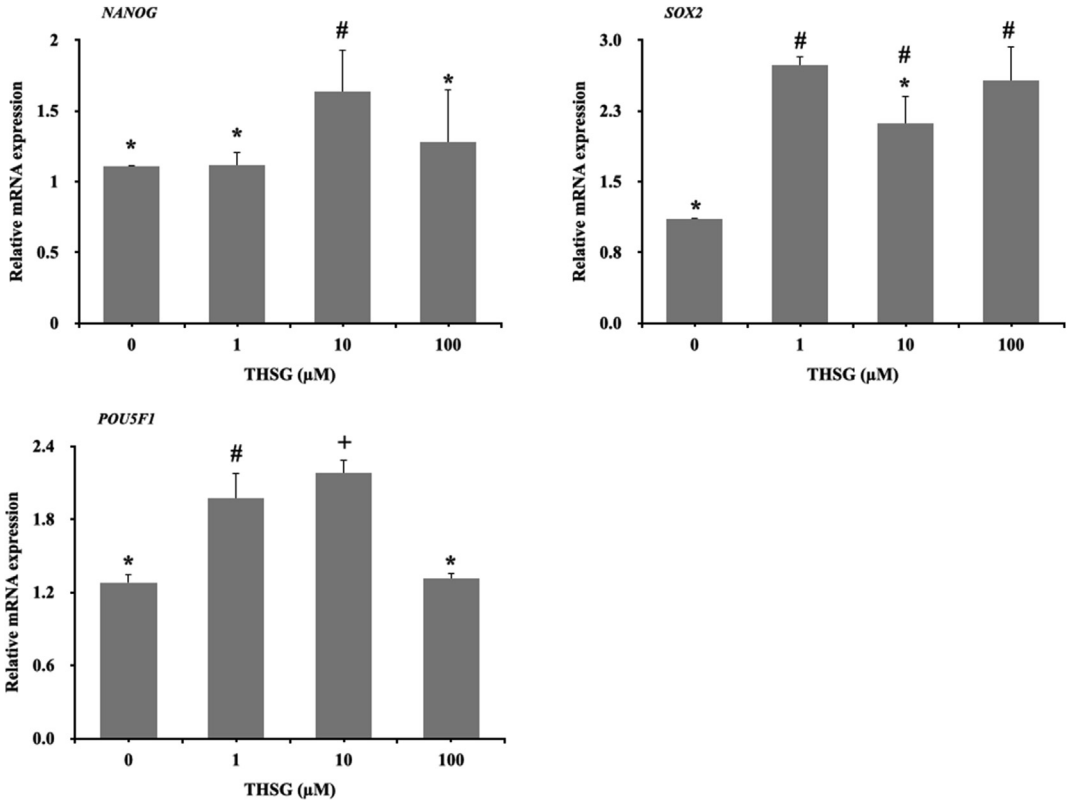


Figure 2 THSG treatment led to the up-regulation of pluripotency-associated genes. The expression levels of *NANOG*, *POU5F1*, and *Sox2* in DPSCs treated with various concentrations of THSG. Data are presented as means and standard deviations. The experiment was repeated three times. *, #, and + indicate significantly different subsets obtained by the post hoc Bonferroni test if a significance of $p < 0.05$ was reached by one-way ANOVA.

variance (ANOVA) and Bonferroni's test for post hoc analysis were used to evaluate the data obtained from the MTS assay, telomerase activity assay, qPCR, and Western blot. A p value less than 0.05 was considered statistically significant.

Results

The proliferation, self-renewal and embryoid body formation of DPSCs were improved by THSG

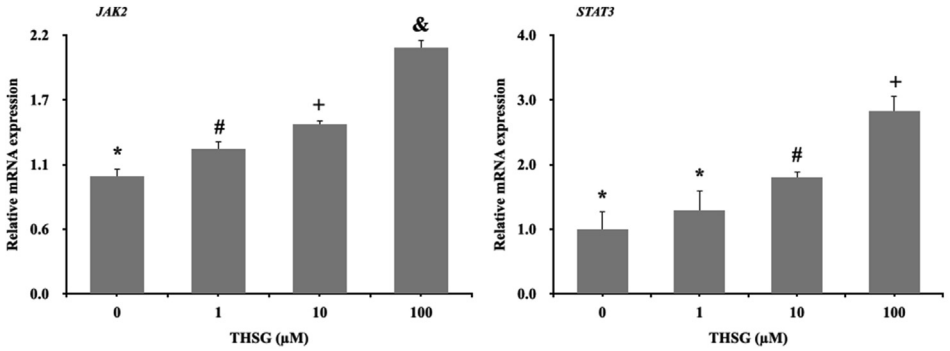
THSG significantly enhanced the proliferation of DPSCs when the concentration was higher than 1 μM, with no cytotoxic effects at 48 and 96 h ($p < 0.05$, Fig. 1A). In addition, THSG did not significantly affect the proliferation of the cells when the concentration was over 100 μM. Therefore, we used 1, 10, and 100 μM THSG for subsequent experiments. Significantly higher telomerase activity, indicating higher levels of cell renewal, was observed in DPSCs treated with 1, 10, and 100 μM THSG than in the controls (0 μM THSG; Fig. 1B). Larger and condensed embryoid bodies were observed in the THSG group (1, 10, and 100 μM) compared with the control group. The biggest embryoid body was observed after treatment with 10 μM THSG, and the most condensed one was observed after treatment with 100 μM THSG (Fig. 1C).

THSG led to the up-regulation of pluripotency-associated genes

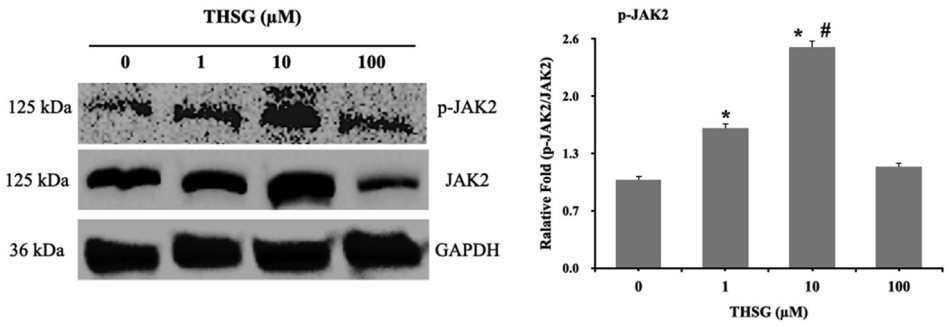
THSG significantly up-regulated the expressions of all the genes (Fig. 2). The expressions of *NANOG* and *POU5F1* were particularly high in the 10 μM treatment group ($p < 0.05$). Furthermore, the expression of *SOX2* was higher in all the THSG treatment group than in the solvent control group, although a slight decrease in expression was noted in the 10 μM treatment group.

THSG up-regulated the genes and phosphorylated proteins levels of JAK2 and STAT3 in DPSCs

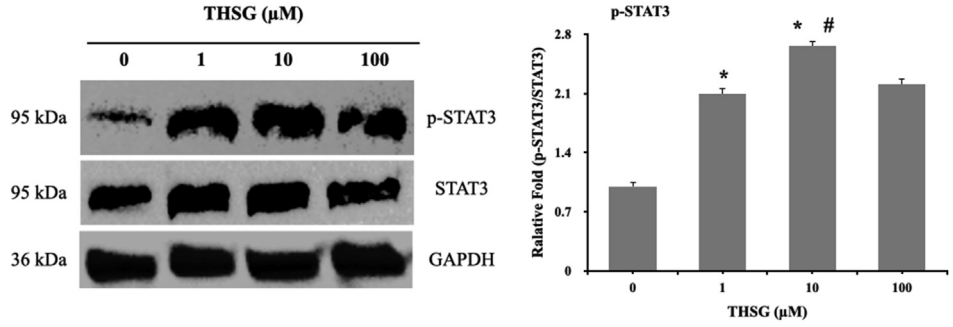
Both *JAK2* and *STAT3* mRNA levels were significantly increased after treatment with THSG in a dose-dependent manner. Compared with controls, JAK2 and STAT3 protein levels were escalated in DPSCs treated with 1 and 10 μM THSG (Fig. 3A). In addition, the phosphorylated forms (active form) of JAK2 and STAT3 were significantly enhanced compared with the total forms of the two proteins (Fig. 3B and C). However, the JAK2 inhibitor AG490 significantly suppressed the phosphorylation of JAK2 and STAT3 in THSG-treated DPSCs (Fig. 3D). The aforementioned results indicated that THSG improved the possible process of reprogramming through the JAK2-STAT3 axis in DPSCs.


JAK2
Relative mRNA expression
2.2
1.7
1.1
0.6
0.0
*
#
+
&
0
1
10
100
THSG (μM)
STAT3
Relative mRNA expression
4.0
3.0
2.0
1.0
0.0
*
*
#
+
0
1
10
100
THSG (μM)

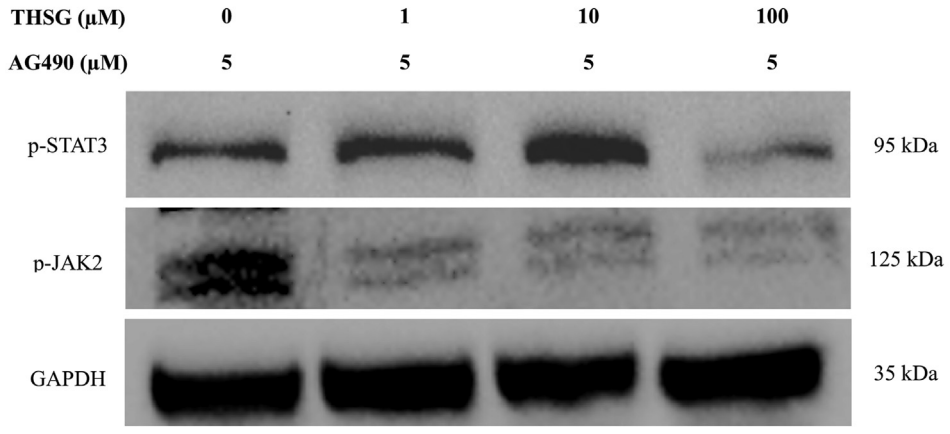
A


THSG (μM)
0
1
10
100
125 kDa
p-JAK2
125 kDa
JAK2
36 kDa
GAPDH
p-JAK2
Ralative Fold (p-JAK2/JAK2)
2.6
2.0
1.3
0.7
0.0
*
*
#
0
1
10
100
THSG (μM)

B


THSG (μM)
0
1
10
100
95 kDa
p-STAT3
95 kDa
STAT3
36 kDa
GAPDH
p-STAT3
Ralative Fold (p-STAT3/STAT3)
2.8
2.1
1.4
0.7
0
*
*
#
0
1
10
100
THSG (μM)

C


THSG (μM)
0
1
10
100
AG490 (μM)
5
5
5
5
p-STAT3
95 kDa
p-JAK2
125 kDa
GAPDH
35 kDa

D

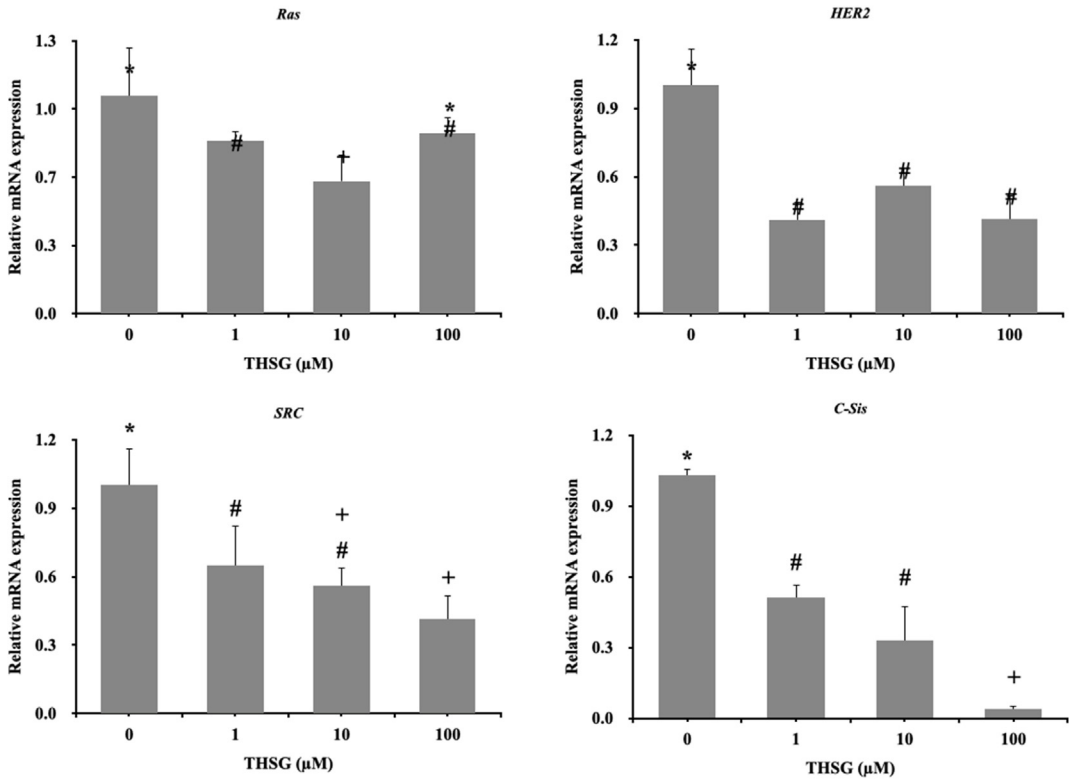


Figure 4 THSG treatment decreased the expression levels of oncogenes. The expression levels of *Ras*, HER2, *SRC*, and *C-Sis* in DPSCs treated with various concentrations of THSG. Data are presented as means and standard deviations. The experiment was repeated three times. *, #, and + indicate significantly different subsets obtained by the post hoc Duncan test if a significance of $p < 0.05$ was reached by one-way ANOVA.

THSG failed to induce the expression of oncogenes in DPSCs

THSG significantly decreased the expression levels of all oncogenes in DPSCs (Fig. 4; $p < 0.05$). The expression levels of *SRC* and *C-sis* in THSG-treated cells dramatically decreased in a dose-dependent manner. Lower *Ras* and *HER2* expression levels were also observed in the THSG treatment group than in the solvent control group.

Discussion

iPSCs represent a uniquely valuable resource for basic research and clinical applications.[22] However, the efficiency of these cells in reprogramming and signaling transduction remains unclear. The aim of this study was to determine whether THSG induces pluripotency in DPSC. These results demonstrated its potential to induce the proliferation and self-renewal of DPSCs and the formation of embryoid bodies in the cells. The genes expressions of *Oct4*, *Sox2*, and *NANOG* were significantly increased in THSG-treated DPSCs. THSG up-regulated the genes and phosphorylated proteins of JAK2 and STAT3 in DPSCs. In addition, we did not observe any increase in the expressions of the oncogenes in THSG-treated DPSCs.

It has been reported that THSG treatment significantly impedes cancer growth and invasion *in vitro* and *in vivo*.[16] However, in the present study, the MTS assay results indicated that THSG significantly enhanced the proliferation of DPSCs when the concentration was higher than 1 μM, with no cytotoxic effects at 48 and 96 h (Fig. 1A). No difference was observed in the effect of THSG at concentrations below 100 μM on the proliferation of DPSCs; hence, concentrations less than 100 μM were used in the subsequent experiments.

Since Takahashi and Yamanaka successfully developed iPSCs by introducing four genes (*OCT4*, *SOX2*, *c-Myc*, and *Klf4*),[2,3] this has attracted growing attention in maintaining

Figure 3 THSG increased the expression levels of the genes and phosphorylated proteins of JAK2 and STAT3 in DPSCs. (A) The expression levels of *JAK2* and *STAT3* in DPSCs treated with various concentrations of THSG. Data are presented as means and standard deviations. The experiment was repeated three times.*, #, and + indicate significantly different subsets obtained by the post hoc Bonferroni test if a significance of $p < 0.05$ was reached by one-way ANOVA. **(B)** JAK2 and phosphorylated-JAK2 protein levels in DPSC treated with THSG. **(C)** STAT3 and phosphorylated-STAT3 protein status in DPSC treated with THSG. **(D)** The phosphorylated proteins of JAK2 and STAT3 levels in DPSC treated with THSG and JAK2 inhibitor, AG490. Data are presented as relative fold changes. The experiment was repeated three times. *, #, and + indicate significantly different subsets obtained by the post hoc Bonferroni test if a significance of $p < 0.01$ was reached by one-way ANOVA.

the pluripotency of stem cells and iPSCs. The self-renewal ability of human ES and iPSCs could be induced by different factors, such as hypoxia[17] and small molecules compounds.[18] However, few or none of these studies have considered the potential of herb medicine in maintaining stem cell self-renewal. In our study, THSG, an extract of Polygonum multiform, triggered an increase in telomerase activity, which promoted the self-renewal of DPSCs (Fig. 1B). In addition, the possibility for pluripotency was observed through the embryoid body formation assay (Fig. 1C). These results indicated that THSG might play a key role in maintaining the self-renewal and possible pluripotency of DPSCs.

NANOG, *POU5F1 (OCT4)*, and *SOX2* are well-known not only involved in pluripotent stem cell formation in mammalian embryos, but also to mediate germline development.[19–23] Thus, they may play crucial roles in maintaining stem cell pluripotency. In the current study, the expressions of *NANOG* and *POU5F1 (OCT4)* were up-regulated in DPSCs following THSG treatment at concentrations as low as 10 μM; surprisingly, a decrease in this effect was noted at a concentration of 100 μM. Although *SOX2* expression was slightly down-regulated, the results demonstrated that low concentrations of THSG treatment would be the optimal condition for the induction of the three key genes.

The JAK2-STAT3 signaling axis may act as a major pathway for maintaining the pluripotency of mouse iPSCs.[5,24,25] Leukemia inhibitory factor (LIF), which is involved in activating both JAK2 and STAT3, is also required to maintain the pluripotency of the cells.[26] Specifically, LIF-JAK2-STAT3 signaling is essential for the demethylation and deacetylation of pluripotency-associated gene promoters.,[5,24,25] resulting in the reprogramming of somatic stem cells and the self-renewal of ESCs.[6,26–28] In the present study, THSG enhanced *JAK2* and *STAT3* expression in DPSCs (Fig. 3A). In addition, the phosphorylated-JAK2 and -STAT3 protein levels peaked when the concentration of THSG was 10 μM (Fig. 3B and C); however, JAK2 inhibitor, AG490 dramatically contained the phosphorylation of the two proteins (Fig. 3D). Thus, THSG not only strengthened the possible process of reprogramming through the JAK2-STAT3 axis, but also possessed the potential to serve as a substitute for LIF.

Several risk factors, such as tumorigenesis, could occur during reprogramming in subsequent cell cultures.[29,30] In the current study, THSG treatment significantly suppressed the expressions of the oncogenes (*Ras*, *HER2*, *SRC*, and *C-Sis*) in DPSCs, indicating that THSG treatment did not carry the risk of tumorigenesis. These results indicated that THSG-triggered DPSCs may serve as an important and reliable resource for regenerative medicine.

This study provided the preliminary results that THSG might induce pluripotent-like possibility through the STAT3-JAK2 axis in DPSCs, which may be an alternative cell-based therapeutic tactic in regenerative dentistry.

Declaration of Competing Interest

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

Acknowledgments

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