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Original Article

Comparison of 2,3,5,4′-tetrahydroxystilbene-2-O-b-D-glucoside-induced proliferation and differentiation of dental pulp stem cells in 2D and 3D culture systems—gene analysis

Yen Wu [a,b,c†], Yao-Yu Chung [a,c†], Yu-Tang Chin [a,c], Chi-Yu Lin [a,c], Po-Jan Kuo [d], Ting-Yi Chen [a,b,c], Tzu-Yu Lin [a,b,c], Hsien-Chung Chiu [d], Haw-Ming Huang [a], Jiiang-Huei Jeng [e,f], Sheng-Yang Lee [a,b,c*]

[a] School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan
[b] Department of Dentistry, Wan-Fang Medical Center, Taipei Medical University, Taipei, Taiwan
[c] Center for Tooth Bank and Dental Stem Cell Technology, Taipei Medical University, Taipei, Taiwan
[d] Department of Periodontology, School of Dentistry, National Defense Medical Center and Tri-Service General Hospital, Taipei, Taiwan
[e] School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[f] Department of Dentistry, Kaohsiung Medical, University Hospital, Kaohsiung, Taiwan

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Dental pulp stem cells;
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Expansion

Abstract *Background/purpose:* Culture environments play a critical role in stem cell expansion. This study aimed to evaluate the effects of 2,3,5,4′-tetrahydroxystilbene-2-O-b-D-glucoside (THSG) on the proliferation and differentiation of human dental pulp stem cells (DPSCs) in 2-dimensional (2D) and 3-dimensional (3D) culture systems.
Materials and methods: Human DPSCs were seeded in T25 flasks for 2D cultivation. For the 3D culture system, DPSCs were mixed with microcarriers and cultured in spinner flasks. Cells in both culture systems were treated with THSG, and cell proliferation was determined using a cell counter and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. In THSG-treated DPSCs, the genes associated with proliferation, adipogenesis, neurogenesis, osteogenesis, pluripotency, oncogenesis, and apoptosis were analyzed using real-time polymerase chain reactions.
Results: The spinner flask time-dependently improved cell numbers, cell viability, and expansion rates in THSG-treated DPSCs. In both the T25 and spinner flasks, the messenger RNA (mRNA) levels

* Corresponding author. School of Dentistry, College of Oral Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei, 11031, Taiwan.
E-mail address: seanlee@tmu.edu.tw (S.-Y. Lee).
† Both authors contributed equally to this study and share first authorship.

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of proliferation, osteogenesis, and pluripotent-related genes had a significant maximum expression with 10 μM THSG treatment. However, 0.1 μM of THSG may be the most suitable condition for triggering neurogenesis and adipogenesis gene expression when DPSCs were cultured in spinner flasks. Furthermore, the number of oncogenes and apoptotic genes decreased considerably in the presence of THSG in both the T25 and spinner flasks.
Conclusion: The spinner flask bioreactor combined with THSG may upregulate proliferation and lineage-specific differentiation in DPSCs. Thus, the combination can be used to mass-produce and cultivate human DPSCs for regenerative dentistry.

Introduction

The prospective fields of stem cell–based therapies and research have garnered increasing attention.[1] Based on their self-renewal and specific-lineage differentiation, embryonic stem cells, induced pluripotent stem cells (iPSCs), and adult stem cells have been used to study numerous severe diseases, such as heart disease.[2] However, producing large quantities of stem cells remains an immense challenge for clinical applications.

Human dental pulp stem cells (DPSCs), the first isolated mesenchymal stem cells (MSCs) from extracted human teeth, are usually regarded as medical waste after orthodontic treatment.[3] Because of their immune modulating and multidifferentiation capabilities,[4] DPSCs are considered possible candidates for regenerative medicine. Two clinical studies on pulp regeneration by DPSC transplantation have been reported,[5,6] and the results have revealed that DPSCs may serve as promising biomaterial for dental regenerative practices. However, obtaining equivalent cell numbers is the critical concern for systemic regenerative applications. A previous study reported that approximately $1–2 \times 10^5$ DPSCs were harvested from a single adult third molar.[7] Nevertheless, the clinical applications of human MSCs (hMSCs) require approximately $1–10 \times 10^9$ functional cells per patient to replace disease-induced cell loss.[8] Thus, DPSC production for clinical use must be intensified.

Three-dimensional (3D) cultures were regarded as a potent approach to expand large-scale stem cell production. Presently, spinner flask bioreactors are widely used to increase cell numbers.[9,10] These stirred bioreactors are typically equipped with a glass vessel and internal impeller that efficiently mixes cells and microcarriers.[11–13] These spinner flask bioreactors have facilitated the proliferation of stem cells and triggered their specific-lineage differentiation.[14–16] For example, Wang and colleagues observed that compared with 2-dimensional (2D) culture systems, 3D bioreactors enhanced the osteogenic and chondrogenic differentiation of MSCs.[17] Hence, these rotary bioreactors have a pivotal role in switching stem cells. After long-term culturing in vitro, bone marrow MSCs were observed to undergo several critical transformations, such as rapid aging, phenotype changes, and stemness loss.[18] Therefore, increasing the number of cell pools and maintaining a high quality of stem cells that meet therapeutic requirements are crucial.

2,3,5,4′-Tetrahydroxystilbene-2-O-b-D-glucoside (THSG), the major component of the herbal medicine *Polygonum multiflorum* Thunb., is water soluble and biologically active.[19] Because of its strong antioxidant capacity and free radical scavenging activity,[19] THSG has protective potential to treat human diseases and disorders, including aging[20] and cardiovascular diseases.[21] In our prior studies, THSG has improved various capabilities of DPSCs, including proliferation,[22] osteogenic

Differentiation,[23] osteogenesis,[24] and reprogramming potential.[25] The aforementioned evidence, has demonstrated that THSG is a reliable and powerful modulator for DPSCs. Therefore, its effects on cell growth and the differentiation of DPSCs cultured in a 3D environment should be investigated.

The aim of this study was to examine the effects of THSG on the proliferation- and multilineage differentiation–related genes of DPSCs in 2D and 3D culture systems.

Materials and methods

Reagents

THSG was kindly provided by Dr Ching-Chiung Wang (Graduate Institute of Pharmacognosy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan) and was extracted and dissolved in dimethyl sulfoxide (Invitrogen, Grand Island, NY, USA) for cell treatment, as previously described.[22,23]

Cultivation of DPSCs

Human DPSCs were purchased from Lonza Bioscience (P1, PT-5025, Basel, Switzerland). According to the supplier's instructions, DPSCs were guaranteed to express CD105, CD166, CD29, CD90, and CD73 and to not express CD34, CD45, or CD133. All cells tested negative for mycoplasma, bacteria, yeast, and fungi. The cells were maintained in low-glucose Dulbecco"s modified Eagle medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The culture medium was changed every 2 to 3 days. The cells at 70%–80% confluence in the T75 flask were detached and subcultured. Prior to the experiments, the cells were stored overnight in the medium without FBS.[3,22,23,26]

Cultivation and treatment of human DPSCs in T25 flask

Human DPSCs were cultured in low-dose DMEM to reach 80% confluence in T75 flasks. The cells were washed once with phosphate-buffered saline (PBS) and were then trypsinized, centrifuged, and suspended with low-glucose DMEM. After 10^5 cells with a cell density of 4000 cells/cm^2 were seeded into T25 flasks, the medium was replaced with DMEM without FBS overnight for starvation. Subsequently, cells were treated with 0.1 and 10 μM THSG in low-glucose DMEM with 2.5% FBS, and the medium for treatment was refreshed daily. Then, cells were collected for quantitative real-time polymerase chain reaction (qPCR), cell counting, and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTS) assay.

Preparation of spinner flasks

To prevent the adherence of the attachment-dependent cells and proteinaceous materials to glassware, siliconizing spinner flasks are necessary, and a siliconizing reagent (Sigmacote, Sigma, city, USA) was prepared for this study. First, the siliconizing reagent was added and swirled carefully to coat the surface of all spinner flasks. Then, any excess silicon-based product was poured from the

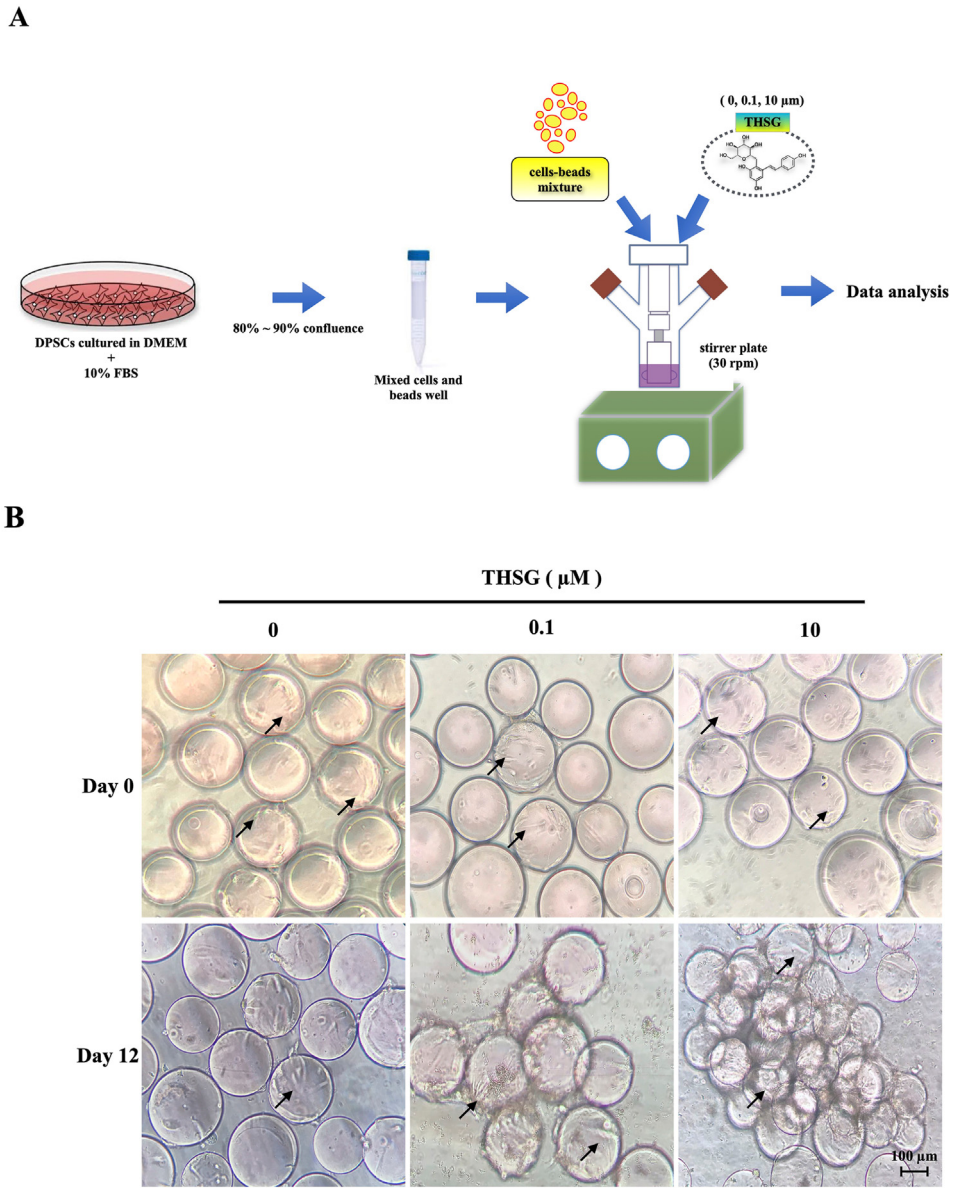

Figure 1 Experimental protocol for the culture system of a spinner flask with microcarriers For the 3D culture, human DPSCs were first cultured in a 10 cm dish until they reached 80%—90% confluence. **(A)** The cells were detached and well mixed with microcarrier beads in a tube. Then, the cell—bead mixture was placed into a spinner flask in DMEM containing 2.5% FBS. After 24 h' starvation, cells were treated with 0.1 and 10 μM THSG in low-glucose DMEM with 2.5% FBS at 30-rpm agitation. The medium was refreshed every day, and cells were collected on days 0, 3, 6, 9, and 12 for further analysis. **(B)** Optical microscope image of DPSCs attached to cytodex1 microcarriers. Cells were observed on days 0 and 12 under a light microscope (× 100 magnification).

Table 1 Primers used for qPCR. To examine the proliferation and multi-differentiation effects of 2D and 3D systems on THSG-treated DPSC, the mRNA associated with proliferation (*PCNA*, *Cyclin D1*, *RRM2*, and *SIRT1*), adipogenesis (*LPL* and *PPAR*), neurogenesis (*PAX6*, *DCX*, and *SOX1*), osteogenesis (*ALP*, *RUNX2*, and *BGLAP*), pluripotency (*NANOG*, *POU5F1*, *SOX2*, *LIN28*, *JAK2 and STAT3*), oncogenesis (*Ras*, *HER2*, *SRC*, and *C-Sis*), and apoptosis (*p53*, *BAD*, and *p21*) were measured. All genes expression levels are normalized to 18S reference genes.

Primer	Forward sequence	Reverse sequence	Accession No.
PCNA	TCTGAGGGCTTCGACACCTA	TCATTGCCGGCGCATTT TAG	NM_002592.2
CCND1	CAAGGCCTGAACCTGAGGAG	GATGACTCTGGAGAGGAAGCG	NC_000011.10
RRM2	GGAATCCCTGAAACCCGAGG	TGGCTAAA TCGCTCCACCAAG	NM_001165931.1
SIRT1	TTGGGTACCGAGATAACCTTCT	TTGCATGTGAGGCTCTATCC	NM_012238.4
ALP	CCGCGCCCGCTATCCT	GGTCAGATGTTAACTGATGTTCCAA	NM_000478.5
RUNX2	CCGGAATGCCTCTGCTGTTA	AACTCTTGCCTCGTCCACTC	XM_011514961.2
BGLAP	GGATGACCCCCAAATAGCCC	TTATACCCTCTGGGCTGTGC	NM_199173.5
LPL	AGTAGCAGAGTCCGTGGCTA	ATTCCTGTTACCGTCCAGCC	NM_000237.3
PPAR	ACGAGAGTCAGCCTTTAACGAA	CCACGGAGCTGATCCCAAAG	NM_138711.3
PAX6	CGTGGGAGAAGTTGGAATCTGAG	GAGAGTTTTCTCCACGGATGTTG	NM_001310158.1
DCX	TGACTCAGCAAACGGAACCT	GCGTAGAGATGGGAGACTGC	NM_178153.3
SOX1	AATACTGGAGACGAACGCCG	AACCCAAGTCTGGTGTCAGC	NM_005986.3
NANOG	ACCACGTGTTCTGGTTTCCAT	AAC CTCGCTGATTAGGCTCC	XM_011520852.1
POU5F1	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
p53	AAGTCTAGAGCCACCGTCCA	CAGTCTGGCTGCCAATCCA	NM_000546.5
BAD	CTTTAAGAAGGGACTTCCTCGCC	AAGTTCCGATCCCACCAGGA	NM_004322
p21	CTGGGGATGTCCGTCAGAAC	CATTAGCGCATCACAGTCGC	BT006719.1
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	NR_003286

PCNA: H. sapiens proliferating cell nuclear antigen, CCND1: H. sapiens cyclin D1, RRM2: H. sapiens ribonucleotide reductase M2, SIRT1: H. sapiens sirtuin 1, ALP: H. sapiens alkaline phosphatase, RUNX2: H. sapiens runt-related transcription factor 2, BGLAP: H. sapiens bone gamma-carboxyglutamate protein, LPL: H. sapiens lipoprotein lipase, PPAR: H. sapiens peroxisome proliferator activated receptor, PAX6: H. sapiens paired box 6, DCX: H. sapiens doublecortin, SOX1: H. sapiens SRY-box transcription factor 1, NANOG: H. sapiens Nanog homeobox; POU5F1/OCT4 : H. sapiens octamer-binding transcription factor 4; SOX2: H. sapiens SRY-box 2; JAK2:H. sapiens Janus kinase 2; STAT3:H. sapiens signal transducer and activator of transcription 3; Ras: H. sapiens HRas proto-oncogene, GTPase ; HER2: H. sapiens erb-b2 receptor tyrosine kinase 2; SRC: H. sapiens proto-oncogene tyrosine-protein kinase Src; C- Sis: H. sapiens c-sis protooncogene; p53: H. sapiens tumor protein; BAD: H. sapiens Bcl-2-associated death promoter; p21:H. sapiens cyclin-dependent kinase inhibitor 1A; 18S: H. sapiens ribosomal RNA.

glassware, which air dried for 24 h. The siliconized spinner flasks were completely cleaned with distilled water and autoclaved at 121 °C for 20 min.

Preparation of microcarriers

Cytodex 1 microcarriers purchased from GE Healthcare (Uppsala, Sweden) were prepared and sterilized according to the manufacturer's instructions. Microcarriers were washed with PBS twice and autoclaved at 121 °C for 20 min. Prior to use, the microcarriers were rinsed in DMEM.

Spinner flask microcarrier culture and treatment

To create the 3D cell cultures, 25-mL spinner flasks (LEFOSCIENCE, Taipei, Taiwan) and microcarrier beads were used. In the spinner flasks, 2×10^6 cells were well mixed with 0.125 g of microcarrier beads with a surface area of 550 cm^2 in low-glucose DMEM containing 10% FBS. This method was conducted using a microcarrier surface area of 22 cm^2/mL and an inoculation density of 3000—4000 cells/cm^2.[27] After seeding, the cells were incubated at 37°C in 5% CO_2 for 24 h for attachment. The cells were then replaced with the medium without FBS and incubated at

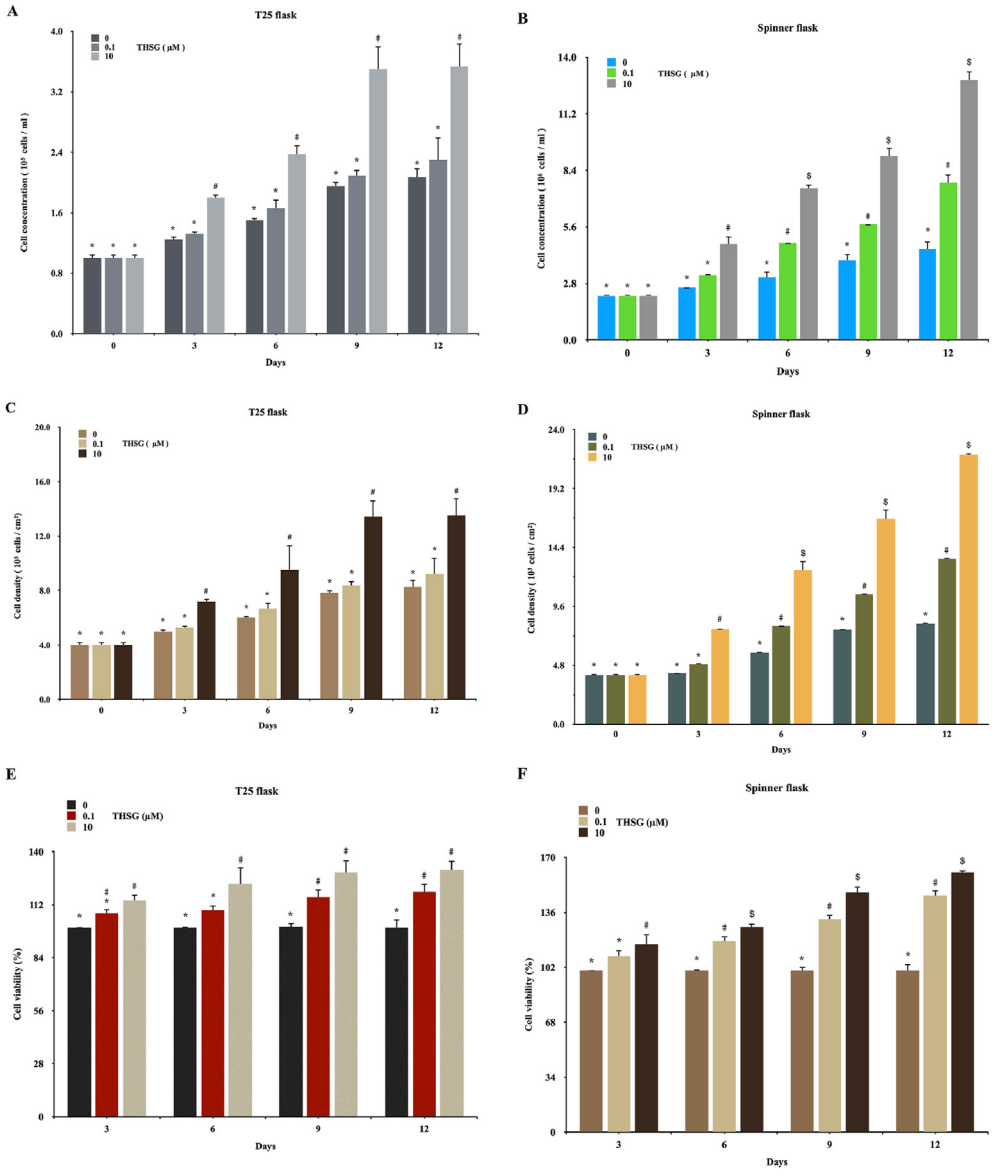

Figure 2 Comparisons of THSG-triggered proliferation in 2D and 3D culture systems. (A, B) Concentrations of THSG-treated human DPSCs in T25 flasks and spinner flasks. **(C, D)** Density of THSG-treated human DPSCs in T25 flasks and spinner flasks. **(E, F)** MTS assay. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

Table 2 The hDPSCs' expansion rate in different culture system.

Days	THSG (μM)					
	0		0.1		10	
	T25	Spinner	T25	Spinner	T25	Spinner
0	1 ± 0.01	1 ± 0.05	1 ± 0.01	1 ± 0.05	1 ± 0.01	1 ± 0.05
3	1.24 ± 0.04	1.18 ± 0.01	1.32 ± 0.04	1.45 ± 0.03	1.8 ± 0.04	2.13 ± 0.3
6	1.5 ± 0.03	1.4 ± 0.03	1.66 ± 0.11	2.00 ± 0.02*	2.37 ± 0.12	3.41 ± 0.04*
9	1.95 ± 0.05	1.85 ± 0.3	2.08 ± 0.08	3.05 ± 0.07*	3.5 ± 0.3	4.14 ± 0.1*
12	2.07 ± 0.12	2.05 ± 0.06	2.3 ± 0.3	4.00 ± 0.06**	3.53 ± 0.31	5.85 ± 0.02**

*Significant differences from T25, $p < 0.05$; **Significant differences from T25, $p < 0.01$.

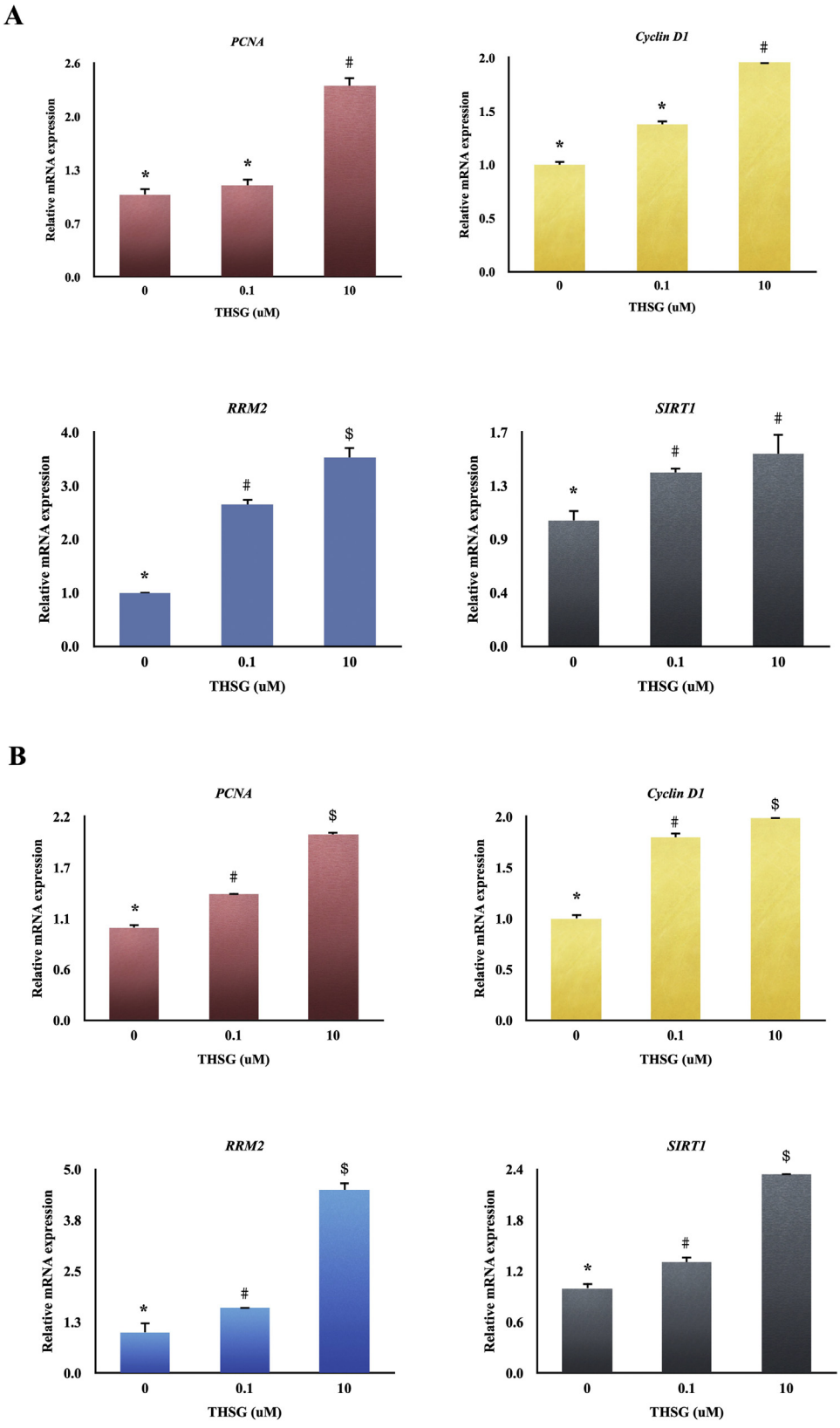

Figure 3 Effects of THSG on proliferation-related genes of DPSC in 2D and 3D culture systems. The expressions of *PCNA*, *Cyclin D1*, *RRM2*, and *SIRT1* were measured through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

Table 3 The hDPSCs' expansion rate in different culture system.

Days	THSG (μM)					
	0		0.1		10	
	T25	Spinner	T25	Spinner	T25	Spinner
0	1 ± 0.2	1 ± 0.09	1 ± 0.2	1 ± 0.09	1 ± 0.2	1 ± 0.09
3	1.24 ± 0.16	1.19 ± 0.02	1.31 ± 0.15	1.24 ± 0.09	1.8 ± 0.15	2.01 ± 0.06
6	1.5 ± 0.12	1.46 ± 0.05	1.66 ± 0.05	2.10 ± 0.04*	2.37 ± 0.02	3.13 ± 0.07*
9	1.95 ± 0.06	1.92 ± 0.05	2.08 ± 0.03	3.4 ± 0.04*	3.25 ± 0.02	4.18 ± 0.03*
12	2.07 ± 0.09	2.05 ± 0.07	2.3 ± 0.12	4.11 ± 0.07**	3.38 ± 0.03	5.49 ± 0.1**

*Significant differences from T25, $p < 0.05$; **Significant differences from T25, $p < 0.01$.

30 rpm overnight for starvation.[28] Subsequently, cells were treated with 0.1 and 10 μM THSG in low-glucose DMEM with 2.5% FBS (Fig. 1). Half of the medium was refreshed every day, and the agitation rate remained at 30 rpm. Then, the cells were harvested for subsequent experiments.

Cell counting

To determine the effects of THSG treatment on cell concentrations in T25 and spinner flasks, trypan blue dye exclusion assays were conducted. The cell numbers were measured at each time point. Cells were detached from the T25 flasks and microcarriers by using 0.5% trypsin/EDTA and resuspended with fresh medium. For viable cell calculation, the cells were stained with trypan blue and counted using a Countess 3 Automated Cell Counter (ThermoFisher, Washington, USA).

Cell viability test

To determine cell viability, human DPSCs were treated with 0.1 and 10 μM THSG in 2D and 3D culture systems for 3, 6, 9, and 12 days. Half of the medium with different THSG concentrations was refreshed daily. After treatment, cells were harvested from the T25 and spinner flasks at each time point. Then, cell viability was determined by incubating the cells in 100 mL of DMEM containing 1 mg/mL MTS solution (CellTiter 96″ AQueous One Solution Cell Proliferation Assay Kit; Promega, Madison, WI, USA) for 2 h at 37 °C. The results of the MTS assay were determined using an enzyme-linked immunosorbent assay reader (Thermo Labsystems, Waltham, MA, USA) at a wavelength of 490 nm.

Real-time qPCR

To examine the effects of THSG on the mRNA expression levels of proliferation, differentiation, oncogenesis, and apoptosis genes, human DPSCs were treated with 0.1 and 10 μM THSG for 24 h mRNA extraction, complementary DNA synthesis, and qPCR were performed as described previously.[22] In this study, the genes associated with proliferation (*PCNA, Cyclin D1, RRM2,* and *SIRT1*), adipogenesis (*LPL* and *PPAR*), neurogenesis (*PAX6, DCX,* and *SOX1*), osteogenesis (*ALP, RUNX2*, and *BGLAP*), pluripotency (*NANOG, POU5F1, SOX2, LIN28, STAT3, and JAK2*), oncogenesis (*Ras, HER2, SRC,* and *C-Sis*), and apoptosis (*p53, BAD,* and *p21*) were measured. Table 1 shows the sequences of the primers used in this study. Calculations of the relative gene expression levels, normalized to the 18S reference gene, were performed in accordance with the delta—delta Ct method, and polymerase chain reaction fidelity was determined using a melting temperature analysis.

Statistical analyses

All data were analyzed using IBM SPSS 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Bonferroni's test for post hoc analysis were used to evaluate the data obtained from the cell numbers, MTS assay, and qPCR. A *P* value of <0.05 was considered statistically significant. A two-tailed student's t-test was conducted to evaluate expansion rate (*P-value < 0.05, ** P- value < 0.01)

Results

Synergy of 3D cultivation and THSG enhanced cell proliferation in both T25 and spinner flasks

In the T25 flask (Fig. 2A), a substantially higher cell concentration, measured in cells/mL, was obtained at in the 10 μM THSG group at every time point ($P < 0.05$), when compared with that of cells in 0 and 0.1 μM THSG groups. However, no significant difference occurred in the cell numbers of the 0 and 0.1 μM THSG groups. In the spinner flask culture systems (Fig. 2B), the 10 μM THSG group had more cells than the 0 and 0.1 μM THSG groups did during 12 days of cultivation ($P < 0.05$) relative to the number of cells per milliliter at 0 days, the spinner flask culture had a faster expansion rate ($P < 0.05$, Table 2) than that of the T25 flask culture.

Similar findings were found regarding cell density, measured in cells/cm.[2] The highest cell density was found in the 10 μM THSG group at every time point ($P < 0.05$) when compared with the cell densities of the other groups during 12 days of cultivation in T25 flasks. However, cells stopped growing after 9 days of cultivation in T25 flasks (Fig. 2C). In spinner flasks, the highest cell density was observed in the 10 μM THSG group at every time point. Cell growth in all groups occurred in a time-dependent manner (Fig. 2D). Relative to the 10^3 cells/cm^2 at 0 days, the spinner flask culture had a significantly higher expansion rate than that of the T25 flask ($P < 0.05$, Table 3).

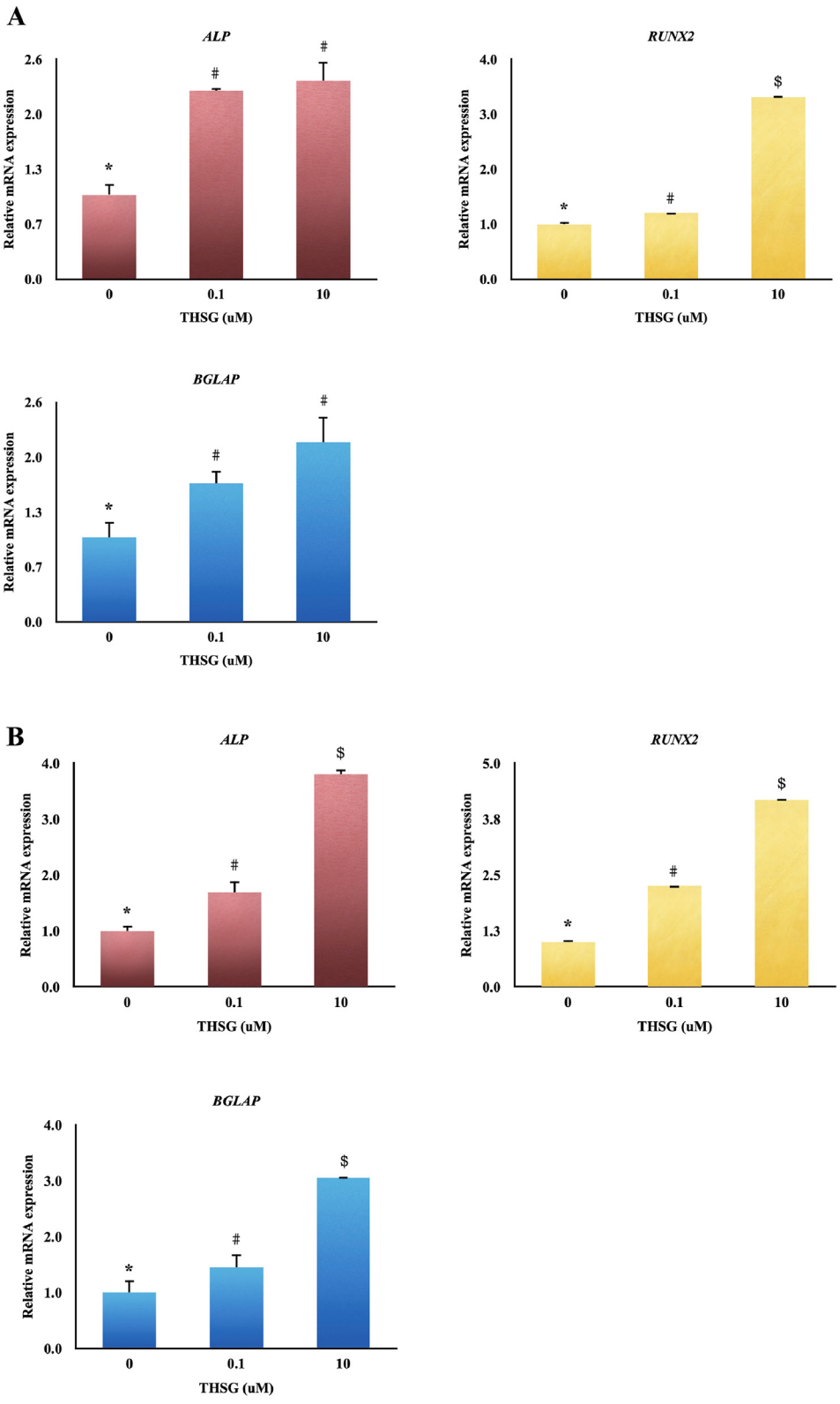

Figure 4 Osteogenic effects of THSG on DPSCs in 2D and 3D cultures. The expressions of genes associated with osteogenesis (*ALP*, RUNX2, and *BGLAP)* were measured through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

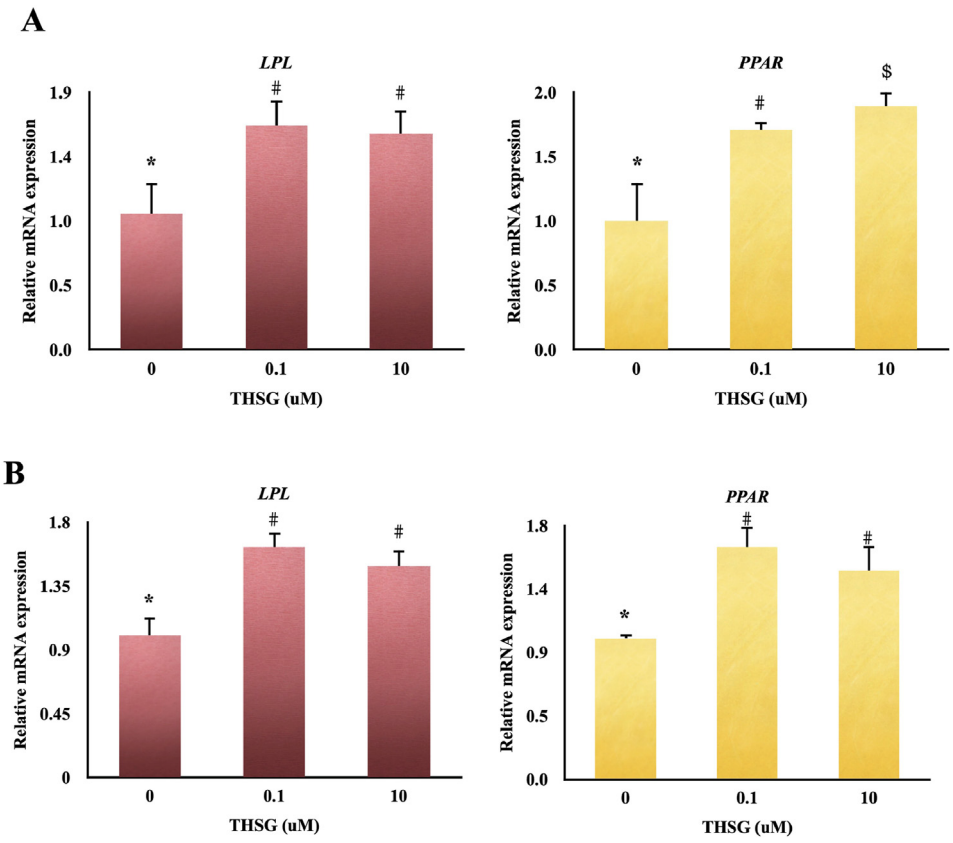

Figure 5 Adipogenic effects of THSG on DPSCs in 2D and 3D cultures. The mRNA expressions of *LPL* and *PPAR* were examined through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

Both the 0.1 and 10 μM groups had significantly higher mitochondrial activity ($P < 0.05$) at every time point compared with that of the 0 μM group. However, the mitochondrial activity of each group in T25 flasks stopped increasing after 9 days of cultivation (Fig. 2E), and the mitochondrial activity of each group in the spinner flasks increased in a time-dependent manner and peaked on the 12th day of cultivation. Additionally, mitochondrial activity in the 0.1 and 10 μM groups was significantly higher than that of the 0 μM group ($P < 0.05$, Fig. 2F).

In the T25 flask cultivation, THSG significantly upregulated the expressions of the 4 proliferative genes when the concentration was greater than 0.1 μM. Furthermore, the increase pattern of all genes was dose dependent ($P < 0.05$; Fig. 3A), and similar mRNA expression patterns appeared in the spinner flask cultivations ($P < 0.05$; Fig. 3B).

THSG triggered differentiation genes in DPSCs in both 2D and 3D culture systems

As Fig. 4A demonstrates, 10 μM THSG significantly induced peak expressions of osteogenic genes (*ALP*, *RUNX2*, and *BGLAP*) when cells were incubated in T25 flasks ($P < 0.05$). Similarly, all osteogenic genes were enhanced slightly with 0.1 μM THSG, but they dramatically increased when treated with 10 μM THSG in spinner flasks ($P < 0.05$, Fig. 4B).

In the T25 flasks, the expression of the adipogenic gene *LPL* peaked with 0.1 μM THSG and slightly decreased with 10 μM THSG. However, a significant increase in *PPAR* expression was observed in a dose-dependent manner ($P < 0.05$; Fig. 5A). In Fig. 5B, *LPL* and *PPAR* reached peak expressions in the 0.1 μM THSG group ($P < 0.05$).

Figure 6A indicates the mRNA expression of neurogenic genes *PAX6* and *DCX* significantly peaked with 0.1 μM THSG then slightly decreased at a THSG concentration of 10 μM. By contrast, the expression of *SOX1* increased in a dose-dependent manner. In the spinner flasks, however, 0.1 μM THSG may be the optimal concentration for inducing neurogenic genes of DPSCs (Fig. 6B).

Figure 7A demonstrates that 4 pluripotent-associated genes were significantly upregulated and peaked at 10 μM of THSG treatment in T25 flasks ($P < 0.05$). In the spinner flasks, however, *NANOG* expression was particularly high in the 0.1 μM THSG group, although a slight decrease in expression was noted in the 10 μM group ($P < 0.05$). Conversely, the expressions of *POU5F1*, *SOX2*, and *LIN28* increased dose dependently ($P < 0.05$; Fig 7B). Moreover, the pluripotent regulators *JAK2* and *STAT3* were significantly induced in both the T25 and spinner flasks ($P < 0.05$; Fig. 7C).

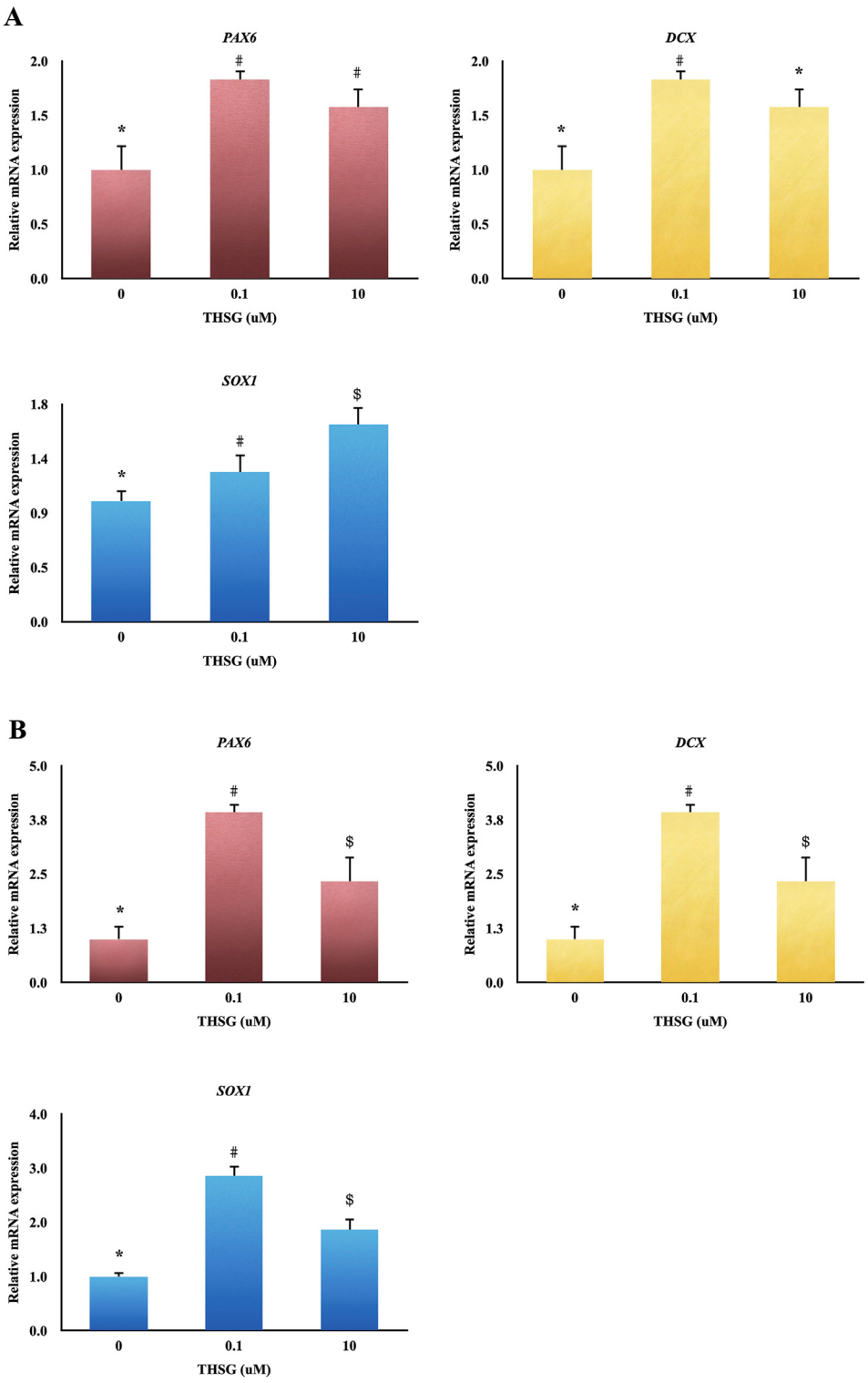

Figure 6 Neuronal differentiation effects of THSG on DPSCs in 2D and 3D cultures. The. expression levels of *PAX6*, *DCX*, and *SOX1* were examined through qPCR. (A) Cells were cultured. In T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

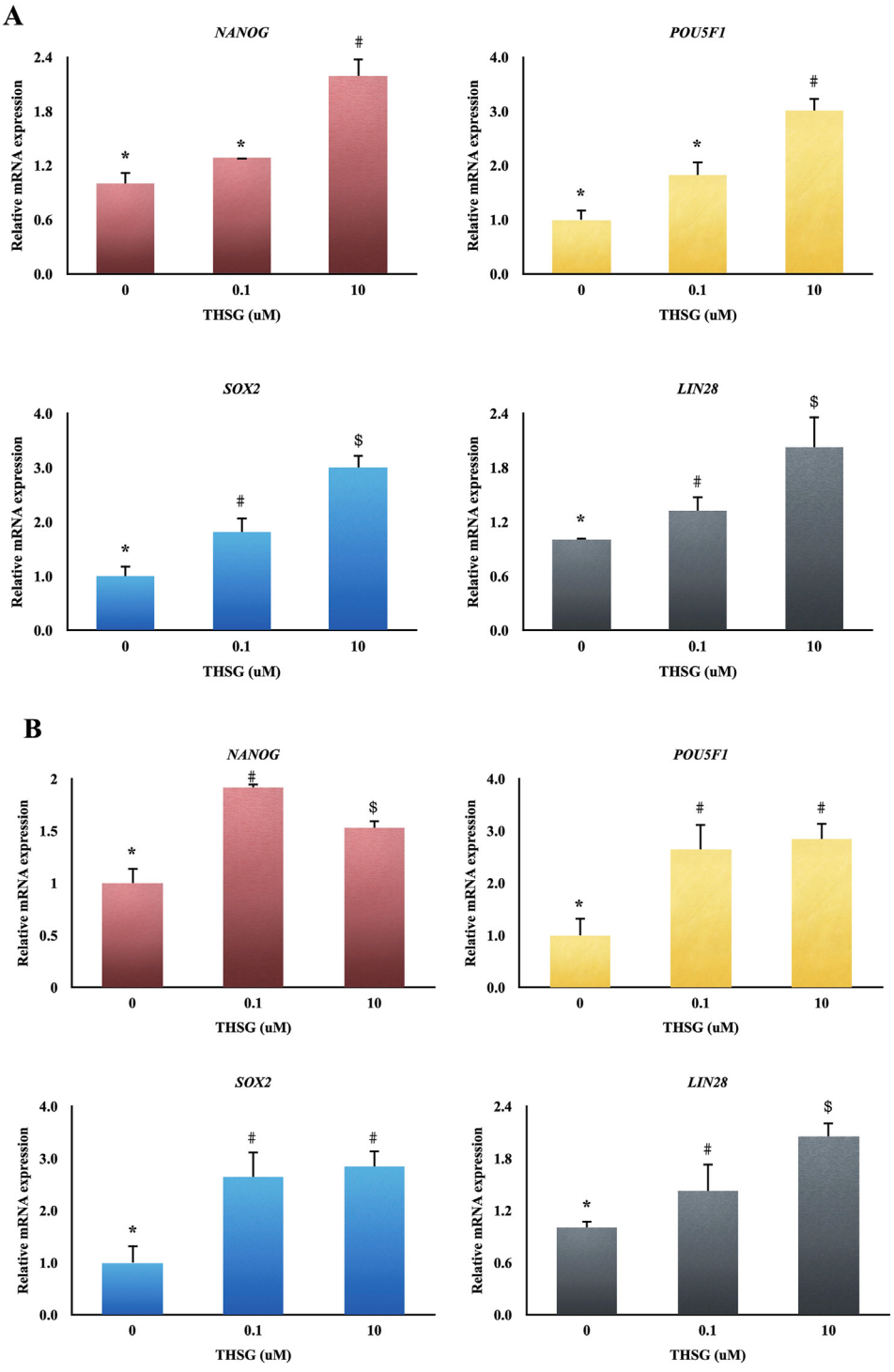

Figure 7 Pluripotent effects of THSG on DPSCs in 2D and 3D cultures. The mRNA expressions of *NANOG*, *POU5F1(OCT4)*, *SOX2*, and *LIN28* were examined through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. **(C)** The expression level of *JAK2* and *STAT3* of DPSCs treated with THSG in T25 and spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

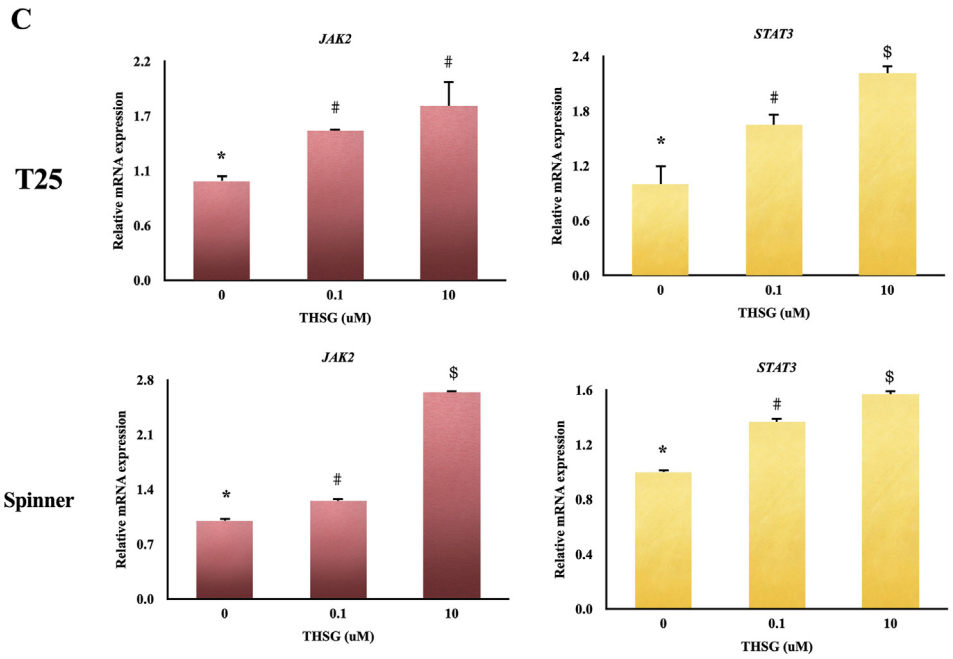

Figure 7 (*continued*).

THSG failed to enhance the oncogenes and apoptotic genes in DPSCs.

In the T25 flask cultivations, THSG significantly reduced the expressions of 4 oncogenes (Fig. 8A; $P < 0.05$) Likewise, lower expressions of the 4 oncogenes in the spinner flasks were observed in the THSG treatment group compared with the solvent control group (Fig. 8B; $P < 0.05$). As Fig. 9A shows, THSG significantly reduced all apoptotic genes in DPSCs in T25 flask cultivations. In the spinner flasks, however, *p53* and *p21* expression decreased significantly in a dose-dependent manner. Nevertheless, no significance was found among any of the groups for the *BAD* genes (Fig. 9B).

Discussion

Expansion of human DPSCs for regenerative medicine is attracting much attention. In this study, the synergy of THSG and the spinner flask bioreactors revolutionized the proliferation and multidifferentiation capabilities of DPSCs.

For industrial and clinical applications, the expansion of human MSCs can be performed through 2D and 3D cultivations.[29] The cell population resulting from 2D cultivation relies on adding containers to increase the culture surface. However, this increase in time, labor, and space intensive in a laboratory. Furthermore, 3D cultivation allows cell yields to reach therapeutic grades by using microcarrier–cell aggregates.[29] Among all 3D bioreactors, spinner flask systems (stirred tank systems) have been extensively applied in the biopharmaceutical industry for the large-scale production of recombinant proteins.[28,30] Furthermore, microcarriers and cell-assembling aggregates have been used to construct suitable environments for 2D-dependent stem cells.[31] In this system, the stirred rate was the key factor for cell yield because human MSCs seeded on microcarriers are sensitive to agitation speed.[28] For example, an agitation speed of less than 20 rpm in one study may have induced cell attachment to the microcarriers; therefore, the agitation speed was increased to 30 rpm for the subsequent culture.[28] In the present study, an agitation speed of 30 rpm was used for DPSC cultivation.

Subsequently, THSG-induced DPSCs in spinner flasks were observed to have a faster expansion rate than that of cells in T25 flasks (Tables 2 and 3). Both 2D and 3D culture systems can improve cell growth (Fig. 1A, B, C, D), mitochondrial activity (Fig. 1E and F), and proliferative gene expression (Fig. 2A and B) in THSG-treated DPSCs. Cells stopped growing after 9 days of cultivation in T25 flasks because of the small surface area. Therefore, spinner flask bioreactors overcome the limitation of surface area to provide more space for cell yield.

Additionally, several studies have reported the osteogenic, adipogenic, and pluripotent differentiation of MSCs in 3D bioreactors.[32] Although 3D culture improved the differentiation and dedifferentiation in MSCs, prolonged in vitro expansion causes various types of damage, such as rapid aging and stemless loss.[28] Our previous studies have demonstrated that THSG improves self-renewal and osteogenic differentiation in DPSCs.[22–24] In the present study, 10 μM THSG maximized the expression of osteogenesis (Fig. 4) and pluripotency (Fig. 7) genes in DPSCs under both T25 and spinner flask cultivation, demonstrating that both 2D and 3D culture systems may differentiate THSG-treated DPSCs into osseous and pluripotent lineages.

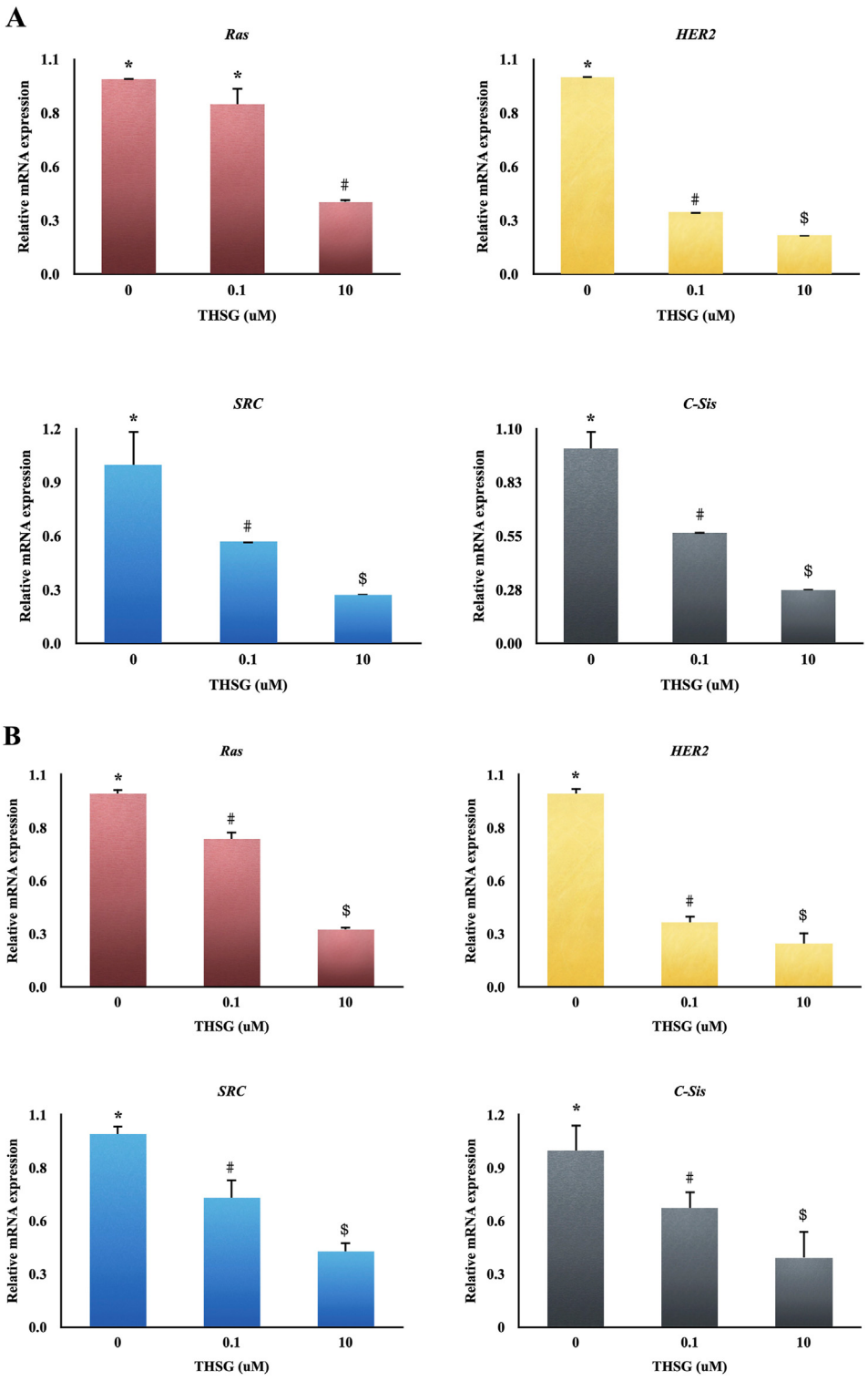

Figure.8 Expression levels of oncogenes on THSG-treated DPSCs in 2D and 3D cultures. The expression levels of *Ras*, *HER2*, *SRC*, and *C-Sis* were examined through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached in one-way ANOVA.

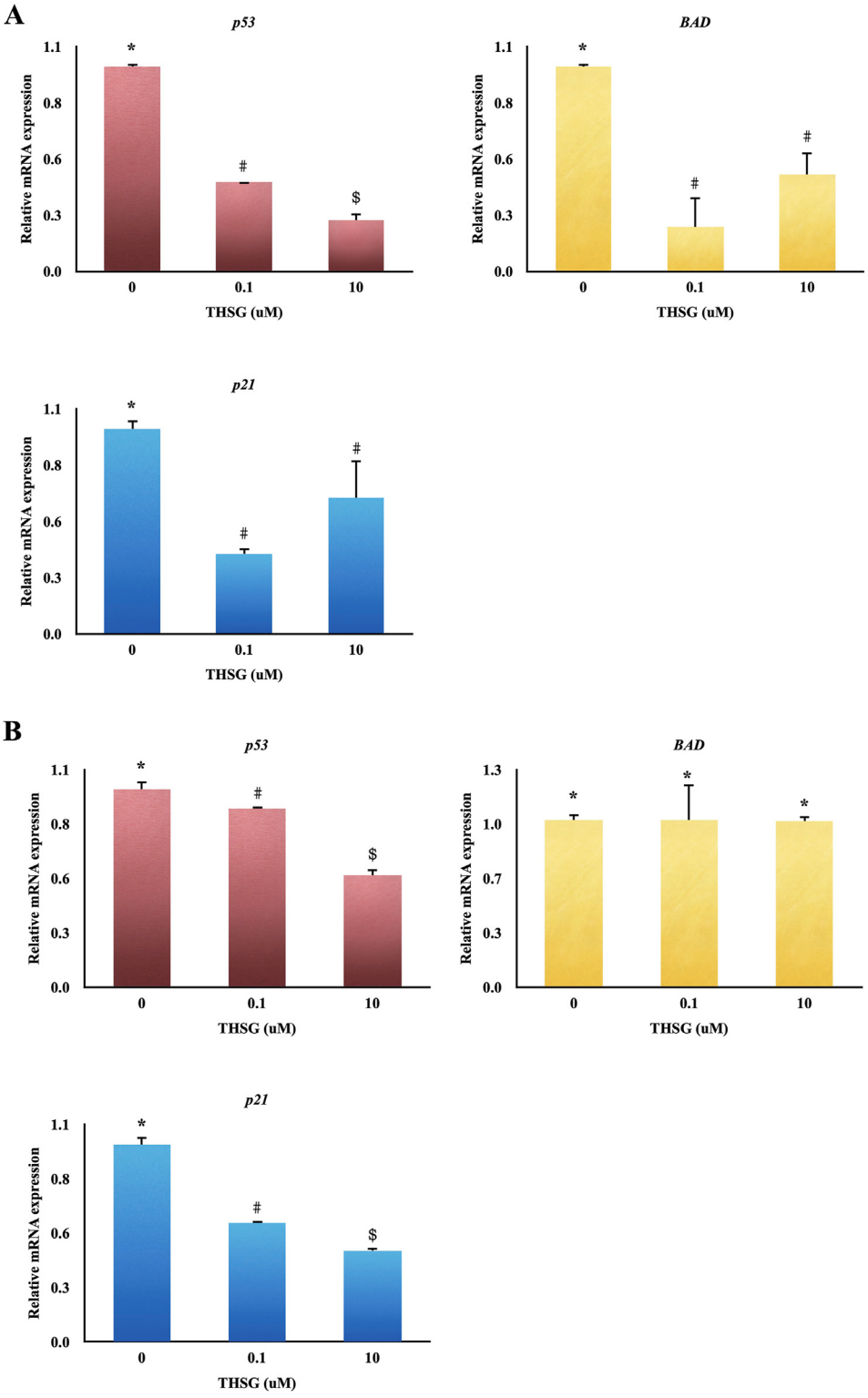

Figure 9 Expression levels of apoptotic genes on THSG-treated DPSCs in 2D and 3D cultures. The expression levels of *p53*, *BAD*, and *p21* in DPSCs were examined through qPCR. **(A)** Cells were cultured in T25 flasks. **(B)** Cells were cultured in spinner flasks. Data are presented as means and standard deviations. *, #, and $ indicate significantly different subsets obtained using the post hoc Bonferroni test if a significance of $P < 0.05$ was reached by one-way ANOVA.

Researchers have reported that 3D bioreactors differentiate DPSCs into different cell types, including neural progenitor cells and adipose-like structures.[32–34] In our results, 2D and 3D cultures treated with THSG induced neurogenic (Fig. 6) and adipogenic (Fig. 5) mRNA expression in DPSCs. Uniquely, the neurogenic and adipogenic effects of low-dose THSG were observed in the

Spinner flask bioreactors, possibly due to the different culture environments' influence on these 2 lineages.

Several risk factors, including tumorigenesis and apoptosis, influence cell expansion and differentiation in subsequent cell cultures.[35,36] In the current study, spinner flask cultivation significantly suppressed the expression of oncogenes (Fig. 8) and apoptosis genes (Fig. 9) in THSG-treated DPSCs, indicating that 3D culture systems did not increase the risk of tumorigenesis or apoptosis. These results reveal that spinner flask systems can serve as valuable and reliable devices for regenerative applications of THSG-treated DPSCs.

Although more evidence, such as protein analysis, for 3D-cultured DPSCs is required, this study nonetheless illustrated that the combination of a 3D bioreactor and THSG cultivation is a reliable method that promotes dental regenerative medicine.

Declaration of competing interest

The authors report no conflicts of interest related to this study.

Acknowledgments

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