## Title

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# Biological Function and Potential Applications of Garcinol in Human Dental Pulp Stem Cells

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## SIGNIFICANCE

Our research discovered that garcinol enhances odontogenic differentiation while inhibiting osteogenic differentiation in hDPSCs, supported by both *in vitro* and *in vivo* experiments. Furthermore, garcinol treatment promotes endothelial differentiation and neurogenesis in hDPSCs, consistent with prior studies. Therefore, this study is a crucial step toward distinguishing between odontogenic and osteogenic differentiation and analyzing the influence of garcinol on related genes, cell signals, and factors, considering their interactions, and the results bring garcinol closer to clinical applicability in the dental field, especially for regenerative endodontic treatment.

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## ABSTRACT

**Introduction:** The regeneration of pulp tissue is crucial for true regenerative endodontic treatment, which requires a reduction in osteogenic differentiation. Garcinol, a histone acetyltransferase inhibitor, is a natural regulator that is known to suppress the osteogenic differentiation of dental pulp stem cells. In this study, the inhibitory effect of garcinol on the osteogenic differentiation of human dental pulp stem cells (hDPSCs) was evaluated using three-dimensional culture under *in vitro* and *in vivo* conditions. **Methods:** hDPSCs were obtained from caries-free third molars and cultured with 10 μM garcinol for 7 days in an ultra-low attachment plate. The cell stemness and expression of osteogenic differentiation-related genes were analyzed using reverse transcription-polymerase chain reaction and single-cell analysis. A transplantation experiment was performed in mice to investigate whether garcinol-treated hDPSCs showed restrained osteogenic differentiation. **Results:** hDPSCs cultured in the U-shaped ultra-low attachment plate showed the highest expression of stemness-related genes. Garcinol-treated hDPSCs demonstrated downregulation of osteogenic differentiation, with lower expression of bone sialoprotein, which is related to bone formation, and higher expression of dentin sialophosphoprotein, which is related to dentin formation. However, the garcinol-treated hDPSCs did not show any alterations in their stemness. Consistent results were observed in the transplantation experiment in mice. **Conclusions:** Garcinol reduced the osteogenic differentiation of hDPSCs, which can contribute to true regenerative endodontic treatment. *(J Endod 2023;49:1652–1659.)*

## KEY WORDS

Gene expression; odontogenesis; osteogenesis; pulp biology; single cell sequencing; stem cell

The goal of regenerative endodontic treatment (RET) is for the regrown dentin pulp complex to maintain blood flow, sensory nerve fiber innervation, and defensive ability, which enables recognition and protection from external attacks, such as bacteria, via the immune system[1]. Prolongation of the lifespan of natural teeth is also an advantage, and endodontic treatment can be extended as long as necessary. However, fibrous connective tissue, sporadic bone tissue, and cementum have been observed histologically in teeth that have undergone RET, unlike normal pulp tissue[2]. Furthermore, regenerated hard tissue has been found to predominate over soft tissue, indicating calcification under clinical conditions[3].

Most studies examining the differentiation of dental pulp stem cells (DPSCs) in RET have targeted histone-deacetylase inhibitors[4]. More recently, researchers have explored modulating histone acetylation by targeting the histone acetyltransferase (HAT) enzyme. However, studies have not been conducted actively because HAT modulators have a large molecular size with a bisubstrate structure[5]. Garcinol is a natural regulator obtained from the fruit of *Garcinia indica* that has been examined as a stimulator of self-renewal in stem cells[6] and an anticancer drug[7,8] because of its relatively small molecular size, which renders it a more suitable candidate for drug development. Garcinol is also known to reduce the osteogenic differentiation of DPSCs[9], making it potentially suitable for RET. Despite these properties,

designing the study using HAT inhibitors such as garcinol was considered difficult because of possible inconsistencies between *in vitro* and *in vivo* studies, which are attributed to the oxidation process of the phenol group and would require the verification of cytotoxic properties[10].

The maintenance of stem cells' intrinsic properties and functions is affected by culture methods and the environment[11]. In two-dimensional (2D) culture methods, DPSCs attach to the plate bottom and proliferate in a stellate shape, leading to morphological changes and, consequently, limited functionality[12]. To solve these problems, three-dimensional (3D) culture methods that allow stem cells to float and form a spherical cluster via self-renewal have been introduced. As these methods more closely reflect the *in vivo* environment, higher viability and proliferative capacity have been observed in human DPSCs (hDPSCs) cultured via 3D culture methods than those obtained using conventional culture methods[13]. When Odot et al evaluated the potential of curcumin, a HAT inhibitor, as a drug for melanoma, the concentration corresponding to the $IC_{50}$ varied depending on the culture method, showing that the culture method is an important factor in the study design[14]. Amaral et al compared the effects of various drugs on tumor cells and confirmed that the *in vitro* results obtained using 3D culture methods mimicked the *in vivo* results[15]. These findings demonstrate that an appropriate environment is important for maintaining the properties of stem cells[16], and the 3D culture method is the simplest and most effective way to reduce the gap between experiments and make predictions. Therefore, the purpose of this study was to evaluate the inhibitory effects of garcinol on the osteogenic differentiation of hDPSCs using 3D culture under *in vitro* conditions and transplantation under *in vivo* conditions.

## MATERIALS AND METHODS

A detailed description of the materials and methods can be found in the appendix.

### Isolation and Primary Culture of hDPSCs

Primary DPSCs were isolated from human caries-free third molars extracted at Yonsei University Dental Hospital. Informed consent was obtained from each patient before extraction, and the study was approved by the Institutional Review Board of the Yonsei University Dental Hospital (Institutional Review Board number: 2-2020-0018). Freshly extracted teeth were rinsed with Dulbecco's modified Eagle's medium (Gibco, Waltham, MA) supplemented with 3% penicillin-streptomycin (Gibco). Minced tissues were seeded with a complete medium (Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum [Gibco]) in a humidified atmosphere containing 5% $CO_2$ at 37°C. hDPSCs were subcultured, and cells collected at passages 3 and 4 were used in this study.

### Treatment with Various Concentrations of Garcinol

The viability of hDPSCs cultured with 5, 7.5, 10, 15, 20 μM garcinol (Cayman, MI) was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a spectrophotometer (VersaMaxMultiplate Reader, Thermo Fisher Scientific, Waltham, MA).

### Characteristics of hDPSCs

Cells were seeded in a 6-well adherent monolayer plate (Corning, Kennebunk, ME) for 2D culture and in a 24-well plate with a flat bottom and a 96-well plate with a U-shaped bottom (Corning) for 3D culture. Garcinol was added on media after 1 day and they were changed every 3 days. Cultured cell morphology was examined under a microscope.

To analyze the expression of cell surface markers of hDPSCs, flow cytometry (FACS Verse; BD Biosciences, Franklin Lakes, NJ) was performed using the BD Stem Flow Human MSC Analysis Kit (BD Biosciences). The expression of mesenchymal stem cell surface markers (CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR) was analyzed using the v.10.0 FlowJo software (FlowJo, BD Biosciences).

hDPSCs were incubated in osteogenic, chondrogenic, and adipogenic induction medium for 21 days, 28 days, and 6 weeks, respectively. To compare differentiation rates in all media, Alizarin Red S, Alcian Blue, and Oil Red O staining were used, and photographs were taken using a light microscope (Olympus BX43, Tokyo, Japan).

### Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using the Trizol Reagent (Life Technologies Corporation, Carlsbad, CA) on day 7 of induction. Complementary DNA (cDNA) was generated from 1,000 ng of extracted RNA using SuperScript™ IV first-strand complementary DNA synthesis reaction (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction was performed using the Quantstudio 3 System (Applied Biosystems, Waltham, MA) and TaqMan gene expression assays (Applied Biosystems). *GAPDH* was used as a housekeeping gene. SRY-Box transcription factor 2, nanog homeobox, octamer-binding transcription factor 4, and estrogen related receptor beta were used as indicators of cell stemness. Bone sialoprotein (*BSP*), osteocalcin (*OCN*), collagen type I alpha 1 (*COL1A1*), dentin sialophosphoprotein (*DSPP*), runt-related transcription factor 2 (*RUNX2*), and alkaline phosphatase (ALPL) were used as indicators of osteogenesis. Reactions were performed in triplicate, and changes relative to the expression of the *GAPDH* gene were calculated using the $2^{-\Delta\Delta Ct}$ method.

### Western Blot Analysis

Whole-cell extracts were isolated using RIPA buffer (BRI-9001, Tech & Innovation, Gangwon, Korea). Samples containing protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% bovine serum albumin at room temperature for 1 hour and incubated with the following primary antibodies: anti-DSPP, ALPL, RUNX2, COL1A1, and GAPDH at 4°C overnight. They were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 hours. Immunoreactive bands were

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Garcinol-treated hDPSCs demonstrated downregulation of osteogenic differentiation and higher expression of dentin sialophosphoprotein, which is related to dentin formation. Garcinol-treated hDPSCs did not show any alterations in their stemness. Garcinol can contribute to true regenerative endodontic treatment.

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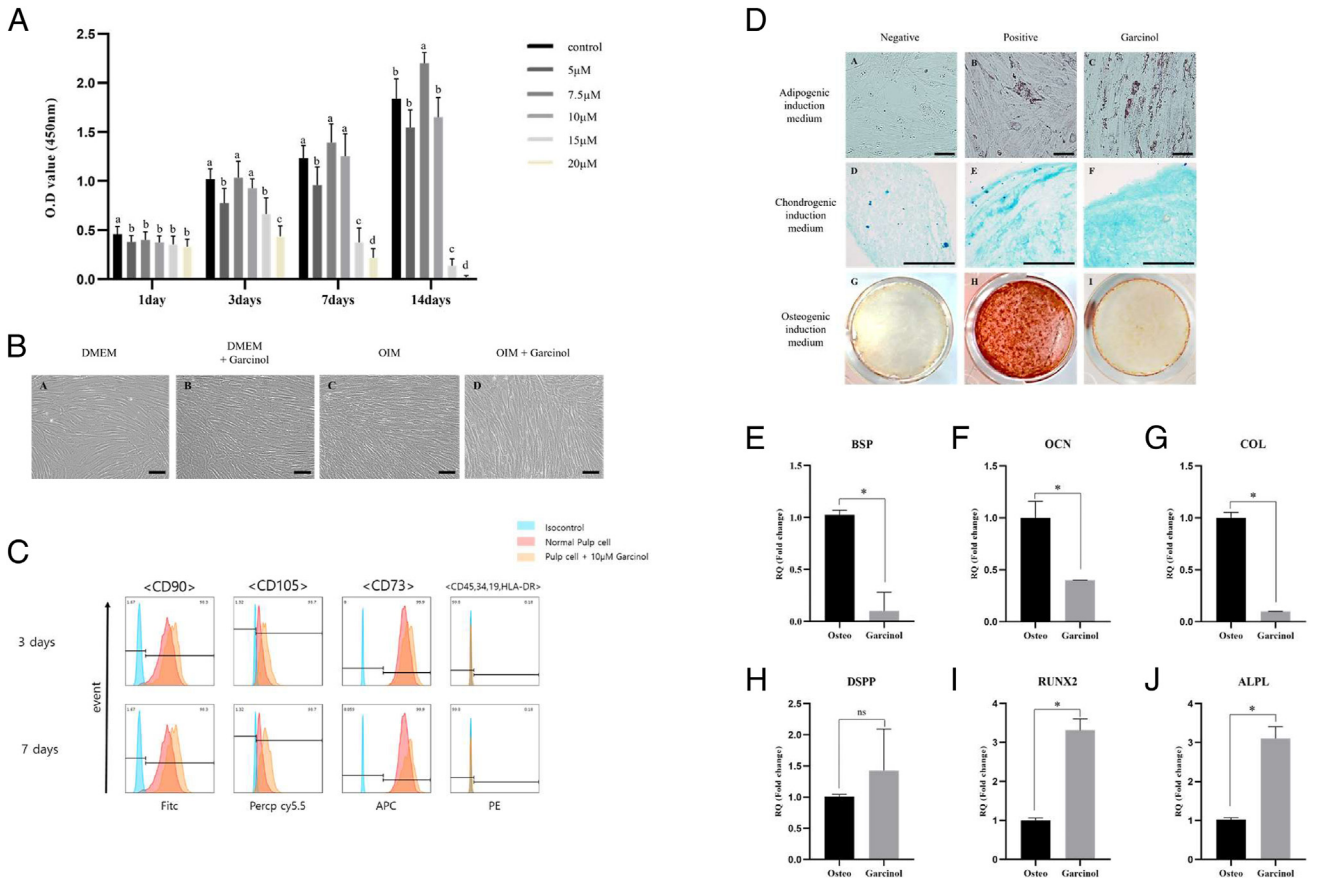


**FIGURE 1 –** Characteristics of garcinol-treated 2D hDPSCs. (*A*) Determination of garcinol concentration on hDPSCs viability. Absorbance values of 2D hDPSCs were measured at 450 nm using the Cell Counting Kit-8 assay. a > b, c, d; b > c, d; c > d. Different alphabet letters represent statistically significant differences between garcinol concentrations within the same period group. The control group of hDPSCs was not treated with garcinol. ($n = 9$). (*B*) Morphology of 2D-cultured hDPSCs under a microscope. DMEM, Dulbecco's Modified Eagle's Medium; OIM, osteogenic induction medium. The scale bar is 50 μm. (*C*) The expression of stem cell markers CD90, CD105, CD73, CD45, CD34, CD19, and HLA-DR of 2D hDPSCs after garcinol application on day 3 and 7. (*D*) (a-c) The adipogenic, (d-f) chondrogenic, and (g-i) osteogenic differentiation potential of hDPSCs after garcinol application. The scale bar is 50 μm. (E-J) The expression of lineage-specific genes and transcription factors in 2D-cultured hDPSCs on day 7. The *P* values of all comparisons indicate significant differences between groups ($n \geq 9$). $^*P < .05$, ns; not significant. 2D, two-dimensional; hDPSC, human dental pulp stem cells.

visualized and analyzed using an electrochemiluminescence detection system (K-12049-D50, Advansta, San Jose, CA).

## RNA Sequencing Process

According to the protocol reported in a previous study[17], hDPSCs, beads, and droplet generation oil were injected into the Dolomite microfluidic system (Dolomite Microfluidics, Royston, UK). The droplets were collected, and sequencing alignment was read using cell barcodes and a unique molecular identifier. Clustering algorithms and visualization of hDPSCs were performed using the Seurat package in R.

## Transplantation of Differentiated hDPSCs

The pellet combination for transplantation was prepared with fibroblasts, endothelial cells, and neuronal cells according to the calculated ratio by single-cell RNA-sequencing analysis. All animal experiments were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Yonsei University, and were approved by the committee (protocol number 2020-0173). Mice were anesthetized with 3% isoflurane (Hana Pharm, Seoul, Korea) provided with 100% oxygen, and further anesthetized with Rompun (Bayer Korea, Seoul, Korea). Then, the pellet combination was transplanted into the kidney capsule. The mice were sacrificed 4 weeks following surgery, and the volume of hard tissue was quantified using a high-resolution micro-CT scanner (SkyScan 1173, Bruker, MA). For histological processing, slides were stained with hematoxylin and eosin (Cancer Diagnostics, Durham, NC) and images were obtained using a light microscope (BX43, Olympus, Tokyo, Japan). For immunofluorescence staining, slides were incubated with antibodies against HLA, DSPP, BSP, dentin matrix protein, platelet and endothelial cell adhesion molecule, vascular endothelial growth factor 2, neural cell adhesion molecule, and S100 calcium binding protein B at 4°C overnight. The specimens were incubated with Alexa Fluor secondary antibodies for 1 hour at room temperature and counterstained with DAPI to visualize the nuclei. The sections were examined using a confocal laser microscope (LSM 700, Carl Zeiss Microscopy, Oberkochen, Germany).

## Statistical Analysis

One-way analysis of variance was performed using SPSS version 26.0 (IBM Corp., Armonk, NY). The significance of differences among groups was analyzed using the Kruskal-Wallis test. The significance was set at $P < .05$.

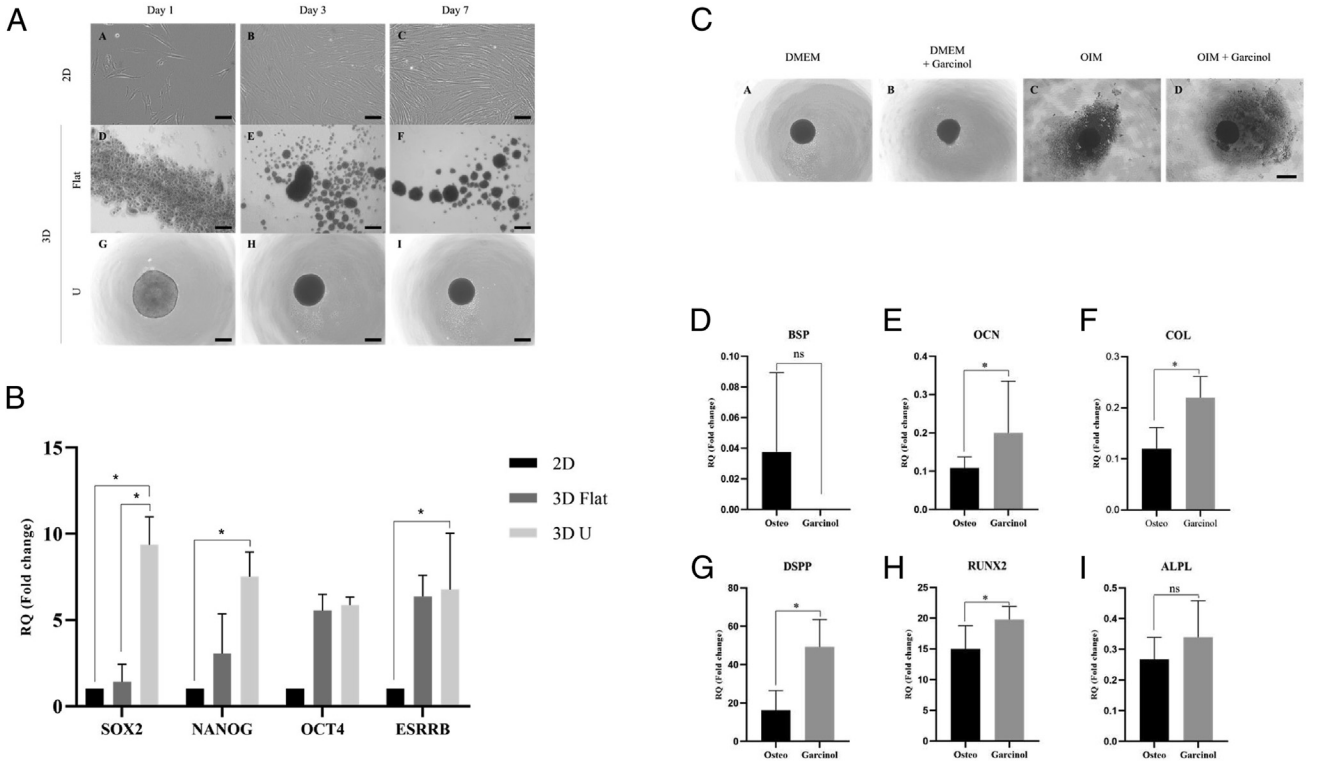


**FIGURE 2 –** Characteristics of garcinol-treated 3D hDPSCs. (*A*) Comparison of the morphology of hDPSCs cultured in (a-c) 2D and (d-f) 3D flat-shaped-bottom plate and (g-i) 3D U-shaped bottom plate. The scale bar is 50 μm for a 2D plate and 200 μm for a 3D plate. (*B*) The expression of lineage-specific genes and transcription factors with respect of the culture method of hDPSCs on day 7. The *P* values of all comparisons indicate significant differences between groups ($n \geq 3$). *$P < .05$. (*C*) Morphology of 3D-cultured hDPSCs under a microscope. DMEM, Dulbecco's modified Eagle's medium; OIM, osteogenic induction medium. The scale bar is 200 μm. (*D-I*) The expression of lineage-specific genes and transcription factors in 3D-cultured hDPSCs on day 7. The *P* values of all comparisons indicate significant differences between groups ($n \geq 4$). *$P < .05$, ns; not significant. 2D, two-dimensional; 3D, three-dimensional; hDPSC, human dental pulp stem cells.

## RESULTS

### Evaluation of the Cellular Characteristics of Garcinol-treated hDPSCs

Cell viability did not decrease in a concentration-dependent manner during any treatment periods (Fig. 1*A*). The garcinol concentration of 15 μM or higher reduced cell viability significantly after 7 days and 14 days. These results showed that garcinol decreased the viability of hDPSCs over a concentration of 10 μM. Hence, we used a 10 μM concentration of garcinol for the following experiments. The 2D-cultured hDPSCs maintained a long and thin stellate shape regardless of garcinol treatment and the type of medium (Fig. 1B). The expression of CD90, CD105, and CD73 was higher than 98% and that of CD45, CD34, CD19, and HLA-DR was 0.18% in both groups (Fig. 1*C*). Adipogenic and chondrogenic differentiation were induced regardless of the garcinol treatment (Fig. 1*D*). However, osteogenic differentiation was induced to a lesser degree in the garcinol-treated group. These results showed that garcinol did not change the intrinsic properties of hDPSCs; however, it reduced osteogenic differentiation. To confirm these changes at the gene level, the expression of related genes was measured (Fig. 1*E-J*). Among these genes, the expression of *BSP, OCN*, and *COL* was lower and that of *DSPP, RUNX2*, and *ALPL* was higher in the garcinol-treated group than in the osteogenic differentiation group.

### Comparison of Stemness and Differentiation Capacity Between 2D and 3D Cultured hDPSCs

In the 3D culture method, many spheroids were observed in 1 well of the flat-shaped bottom plate, and only 1 spheroid was observed in the center of the well of the U-shaped bottom plate (Fig. 2*A*). The spheroids were approximately 20 μm in diameter and became more compact and smaller, and hDPSCs were separated from the boundary and scattered with the progression of incubation, maintaining a compact core and a regular margin (Fig. 2*B*). The expression of stemness-related gene markers was higher in 3D-cultured hDPSCs than in 2D-cultured hDPSCs and in the U-shaped bottom than in the flat-shaped bottom (Fig. 2*C*). Among the osteogenesis-related genes, only the expression of *BSP* showed a score close to zero in the garcinol-treated hDPSCs (Fig. 2*D-I*). The expression pattern of *DSPP, RUNX2*, and *ALPL* was the same as that observed in 2D-cultured hDPSCs. However, the expression of *OCN* and *COL* was higher in the garcinol-treated group, which was inconsistent with the results for 2D-cultured hDPSCs.

### Effects of Garcinol on the Expression of Osteogenic Differentiation Genes in hDPSCs

Based on the single-cell RNA sequencing data, the hDPSCs were separated into two populations—an osteogenic differentiation cluster and a garcinol-treated cluster (Fig. 3*A*)—that showed different gene expression profiles in an analysis of differentially expressed genes (Fig. 3*B*). Garcinol-treated hDPSCs showed lower expression of stanniocalcin 1, fibroblast growth factor 7, insulin like growth factor binding protein 5, collagen type III alpha 1, and collagen type I alpha 2, which are genes related to the regulation and stimulation of bone

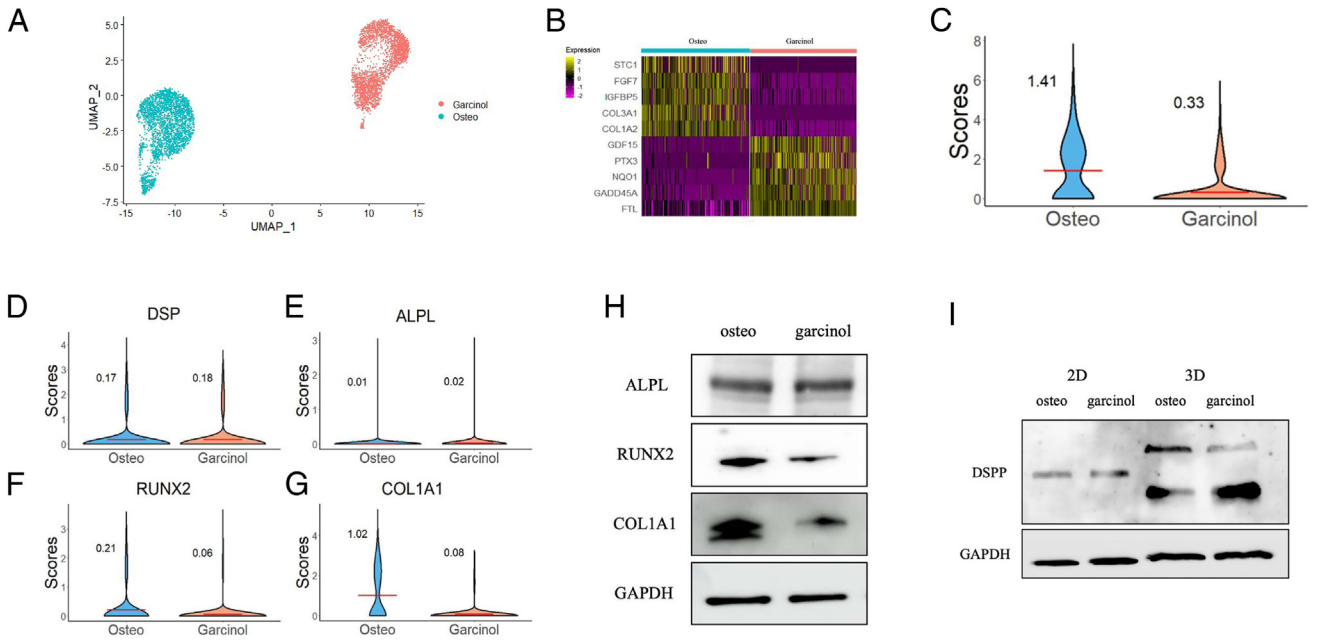


**FIGURE 3 –** Heterogeneity and representative genes in osteogenic differentiated hDPSCs and garcinol-treated hDPSCs. (*A*) The osteogenic differentiation group (left) and the garcinol-treated group (right) were separated in the UMAP. (*B*) The expression of genes associated with the regulation and stimulation of bone mineralization of osteogenic differentiation group and garcinol-treated group are shown in the heat map. Gene markers with the largest difference in expression between the two groups are listed on the y-axis. Their expression is colored in yellow and purple. If the expression of that gene is relatively high, it is colored yellow. STC1, stanniocalcin 1; FGF7, fibroblast growth factor 7; IGFBP5, insulin like growth factor binding protein 5; COL3A1, collagen type III alpha 1; COL1A2, collagen type I alpha 2; GDF15, growth differentiation factor 15; PTX3, pentraxin 3; NQO1, NAD(P)H quinone dehydrogenase 1; GADD45 A, growth arrest and DNA damage inducible alpha; FTL, ferritin light chain. (*C*) Scores of total lineage-specific genes of two groups and (*D*) DSP; (E) ALPL; (*F*) RUNX2; and (*G*) COL1A1 of two groups in violin plot. (*H*) Protein expression of ALPL, RUNX2, and COL1A1 in osteogenic differentiation and garcinol treated groups. (*I*) Protein expression of DSPP in osteogenic differentiation and garcinol treated groups cultured in 2D and 3D methods. 2D, two-dimensional; 3D, three-dimensional; ALPL, alkaline phosphatase; COL1A1, collagen type I alpha; DSP, dentin sialoprotein; DSPP, dentin sialophosphoprotein; hDPSC, human dental pulp stem cells; RUNX2, runt-related transcription factor 2; UMAP, uniform manifold approximation and projection.

mineralization, than those in the osteogenic differentiation group. The differentiation potency of the groups was calculated as a score representing the sum of log count per million across gene markers in each category. The scores showed that garcinol-treated hDPSCs demonstrated lower osteogenic differentiation (Fig. 3*C*). The scores of *DSP* and *ALPL* were slightly increased and those of *RUNX2* and *COL1A1* were remarkably reduced in garcinol-treated hDPSCs (Fig. 3*D-G*). Protein expression corresponding to these genes displayed a similar pattern as observed in the single-cell RNA sequencing data (Fig. 3*H*). The expression of DSPP was increased in garcinol-treated hDPSCs in both the 2D and 3D culture methods (Fig. 3*I*). In the 3D culture method, the cleavage of DSPP was shown separately, but their sum showed higher expression in garcinol-treated hDPSCs.

### Evaluation of the Capacity of Garcinol to Reduce Osteogenic Differentiation of hDPSCs *In Vivo*

In the single-cell analysis results, five clusters were observed: fibroblasts (39.7%), immune cells (37.9%), endothelial cells (7%), monocytes (4%), and neuronal cells (2.5%) (Fig. 4*A*). These clusters were identified based on the expression of related genes on the heat map (Fig. 4*B*). Based on these results, fibroblast pellets treated with 10 μM garcinol, endothelial cell pellets, and neuronal cell pellets were implanted in mouse kidneys at a ratio of 16:3:1. The garcinol-treated group showed lower hard tissue volume than osteogenic differentiation group (Fig. 4*C* and D), which was consistent with the results of the single-cell analysis of hDPSCs. Hematoxylin and eosin staining showed the formation of island-like hard tissues in the kidneys of mice in each group (Fig. 4*E* and F). Immunostaining showed higher expression of dentin matrix protein, platelet and endothelial cell adhesion molecule, vascular endothelial growth factor 2, neural cell adhesion molecule, and S100 calcium binding protein B and lower expression of BSP in the garcinol-treated group. Based on these *in vivo* results, garcinol-treated hDPSCs displayed a downregulated osteogenic differentiation capacity and upregulated odontogenic differentiation capacity. Garcinol also promoted the endothelial and neuronal differentiation of hDPSCs.

## DISCUSSION

It is important to evaluate the appropriate concentration of garcinol before application. Treating cells with a lower concentration of the substance could minimize its harmful effects, but also narrows the range of drug applications. Therefore, the highest applicable concentration of garcinol helps to broaden the range of choices for *in vivo* experiments or clinical use. We used a 10 μM concentration of garcinol in this study, which was identified as the optimal concentration that did not affect cell viability. This is a higher concentration than used in previous studies[18], which might be attributed to differences in cell types and measurement methods.

Because the spontaneous spheroid formation method, also known as the nonadherent surface method, has a coated bottom for cell flotation and does not require external equipment or scaffolds, spheroids form easily without any inflammatory

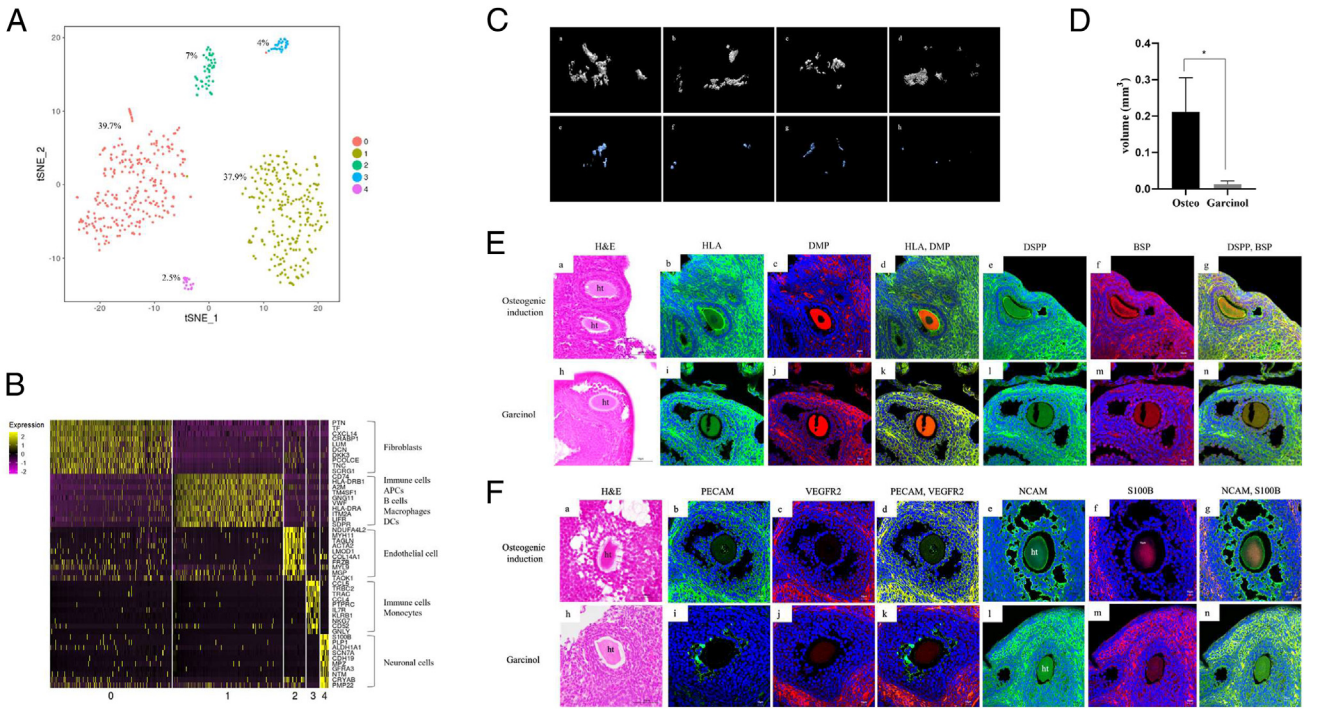


**FIGURE 4 –** hDPSCs treated with 10 μM demonstrate the potential to promote dentin formation and to reduce bone formation *in vivo*. (*A*) Five clusters including fibroblasts, immune cells, muscle cells, monocytes, and nerve cells. 0; fibroblasts, 1; immune cells, antigen presenting cells (APC), B cells, macrophages, dendritic cells (DCs), 2; endothelial cells, 3; monocytes, 4; neuronal cells in the UMAP of human pulp tissues. (*B*) Five clusters with differentially expressed genes. Zero to 4 have the same meaning as in (*A*). Their expression is colored in yellow and purple. If the expression of that gene is relatively high, it is colored yellow. APC, antigen presenting cell; DC, dendritic cell. (*C*) Hard tissue formation of hDPSCs demonstrating osteogenic differentiation and garcinol-treated hDPSCs on micro-CT on day 7. (*D*) Volume of hard tissue of both groups. The *P* values of all comparisons indicated significant differences between groups ($n = 4$). $^*P < .05$. (*E*) Hematoxylin-eosin (H&E) staining and immunostaining of hard tissue: (a, h) H&E staining of the mouse kidneys in each group. (b-d, i-k) Immunostaining of the mouse kidneys with HLA (green), DMP (red), and DAPI (blue) in the mouse kidney of each group. The garcinol-treated group showed a relatively higher expression of DMP than the osteogenic differentiation group, which contributed to a higher color change observed upon merging of HLA and DMP. (e-g, l-n) Immunostaining of the mouse kidneys with DSPP (green), BSP (red), and DAPI (blue) in each group. The osteogenic differentiation group showed a relatively higher expression of BSP than the garcinol-treated group, which contributed to a higher color change observed upon merging of DSPP and BSP. ht, hard tissue. (F) H&E staining and immunostaining of hard tissue: (a, h) H&E staining of the mouse kidneys in each group. (b-d, i-k) Immunostaining of the mouse kidneys with PECAM (green), VEGFR2 (red), and DAPI (blue) in each group. (e-g, l-n) Immunostaining of the mouse kidneys with NCAM (green), S100 B (red), and DAPI (blue) in each group. PECAM and VEGFR2 related with endothelial differentiation and NCAM and S100 B related with neuronal differentiation were expressed higher in garcinol-treated group, which contributed to a higher color change observed upon merging of them. BSP, bone sialoprotein; CT, computed tomography; DMP, dentin matrix protein; DSPP, dentin sialophosphoprotein; hDPSC, human dental pulp stem cells; ht, hard tissue; NCAM, neural cell adhesion molecule; PECAM, platelet and endothelial cell adhesion molecule; S100B, S100 calcium binding protein B; VEGFR2, vascular endothelial growth factor 2; UMAP, uniform manifold approximation and projection.

reaction. However, they are not formed in a constant or uniform size and shape as intended. Bu et al reported that medium replacement was difficult with U-shaped bottom plates, and the increased number of dead cells in the spheroid core led to a decrease in cell viability[12]. As shown in our microscopic results, 1 spheroid centered on the well in a U-shaped bottom plate made it easier to avoid cell loss during medium replacement and showed higher stemness. Unlike the previous study involving a density of $1.2 \times 10^6$ cells, we used a density of $5.0 \times 10^4$ hDPSCs, as determined by a pilot study that investigated the effect of cell density on cell viability in the spheroid core. Therefore, it is important to recognize that experimental conditions, convenience, and proficiency can bias the results and to determine how these factors can be adjusted to avoid bias.

In single-cell RNA-sequencing analysis, a switch in cellular composition in subpopulation clusters occurs depending on differences such as cells, tissues, and culture conditions[19]. Although the number of cells in this study was smaller than those examined in other studies, the fact that the fibroblasts accounted for the highest percentage was similar, reflecting their role in constituting the cell-rich zone of pulp and their important contribution to differentiation and proliferation into odontoblasts. This fibroblast pellet was formed with 10 μM of garcinol, and the volume of hard tissue and osteogenic differentiation-related gene expression were reduced in mice experiments. These results were consistent with 3D-cultured hDPSCs and single-cell RNA-sequencing data, which showed that *in vitro* experiments can lead to the same results as under *in vivo* conditions when stem cells are provided with an environment that closely mimics the actual environment through modified culture methods, and when using appropriate analytic methods. Among the *in vitro* results, RNA-sequencing analysis conducted with only 1 cell is considered to be a powerful and reliable method that mimics *in vivo* conditions most closely for identifying and quantifying gene expression and molecular activity[17]. Interestingly, the garcinol-treated group showed higher expression of *DSP*, a specific gene for odontoblast formation[20]

and higher cleavage of the *DSPP* gene[21], while the expression of the *BSP* gene, which is primarily associated with bone formation, decreased to near zero. Dentinogenesis is used to describe the formation of dentin, the structure of the tooth, and osteogenesis literally means the development and formation of bone. These two processes involve the interaction of stem cells that characterize multilineage differentiation and many related genes; therefore, many of them are used interchangeably to study these two processes[22,23]. The DSP and BSP genes also belong to the small integrin-binding ligand family and share many features, so they are used simultaneously for both studies. Nevertheless, they were not expressed simultaneously during mineralization *in vitro* and *in vivo* conditions. *BSP* was expressed highly in the cementum, osteoblasts of developing teeth, and alveolar bone, whereas *DSPP* was expressed highly in odontoblasts and dentin[24]. Based on this finding, Vijaykumar et al suggested that these genes can be used together to distinguish osteogenic cells from dentinogenic cells and to investigate mechanisms underlying the regulation of the differentiation of progenitors into odontoblasts versus osteoblasts. In fact, *DSPP* is a representative gene for odontoblast formation[20] and DSP initiates dentin mineralization via induction of DPSC differentiation into odontoblast-like cells and facilitates regeneration and repair in dental diseases[25].

A limitation of this study is that it did not analyze mechanisms; nonetheless, it performed a multifaceted analysis of the inhibitory effect of garcinol on the osteogenic differentiation of hDPSCs. We found that garcinol upregulated odontogenic differentiation in hDPSCs and downregulated osteogenic differentiation of hDPSCs according to both *in vitro* and *in vivo* experiments. We also found that garcinol treatment promoted endothelial differentiation and neurogenesis in hDPSCs, which is consistent with previous studies reporting that garcinol could promote neurogenesis[26] and induce the proliferation of human hematopoietic stem cells[6]. If the upregulated innervation of blood vessels and nerves and the downregulated differentiation of osteogenesis are achieved, true pulp regeneration would be possible. This, in turn, would lead to a long lifespan of natural teeth and combined with previous efforts to minimize calcification in RET, will lead to appropriate endodontic treatment being performed when it is necessary[27-29]. Therefore, this study is a crucial step toward distinguishing between odontogenic and osteogenic differentiation and analyzing the influence of garcinol on related genes, cell signals, and factors, considering their interactions, and the results bring garcinol closer to clinical applicability in the dental field, especially for RET.

## CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

**Sunmi Jang:** Writing – original draft, Investigation, Resources. **Uk-Seong Kim:** Writing – original draft, Data curation, Formal analysis. **Sukjoon Lee:** Methodology, Resources, Investigation. **Euiseong Kim:** Writing – review & editing, Conceptualization. **Han-Sung Jung:** Writing – review & editing, Supervision. **Su-Jung Shin:** Writing – review & editing. **Sumi Kang:** Writing – review & editing. **Chang, Insoon**: Writing – review & editing, Formal analysis. **Sunil Kim:** Writing – review & editing, Conceptualization, Supervision, Project administration, Funding acquisition.

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*Sunmi Jang and Uk-Seong Kim contributed equally to this work as first author.*

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*The authors deny any conflicts of interest related to this study.*

## SUPPLEMENTARY MATERIAL

*Supplementary material associated with this article can be found in the online version at www.jendodon.com (10.1016/j.joen.2023.08.016).*

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