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Original Article

Forskolin enhanced the osteogenic differentiation of human dental pulp stem cells in vitro and in vivo

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KEYWORDS
Forskolin;
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Abstract *Background/purpose:* Human dental pulp stem cells (hDPSCs) are multipotent adult stem cells that can differentiate into various lineages such as odontoblasts, osteoblasts, and chondrocytes. Regulation of hDPSCs differentiation with small-molecule compounds can be a useful tool for tissue engineering and regenerative therapy. Forskolin is an agonist of adenylate cyclase that promotes cyclic adenosine monophosphate production. However, the role of Forskolin in regulating the osteogenic differentiation of hDPSCs is still unknown.
Materials and methods: A cell counting kit-8 (CCK-8) assay was performed to screen out the safety concentrations of Forskolin. Following, quantitative polymerase chain reaction (qPCR) and alizarin red staining were performed to detect bone-related gene expression and mineralized deposit formation. Furthermore, we prepared cell sheets which were followed by a 3D culture for cell pellet formation. Finally, the hDPSC cell pellets were transplanted into immunodeficient mice.
Results: CCK-8 assay showed 5 μM and 10 μM Forskolin had no significant inhibition on the proliferation of hDPSCs. The qPCR indicated Forskolin (5, 10 μM) enhanced osteogenic differentiation of hDPSCs by upregulating bone-related genes. Alizarin red staining and its quantification analysis demonstrated Forskolin in 5 μM and 10 μM similarly enhanced the mineralized deposit formation of hDPSCs in vitro. After six weeks of transplantation, immunohistochemical stains showed that osteopontin expression and bone formation were significantly boosted in the Forskolin-treated group than in the normal osteogenic inducing group.
Conclusion: Our results indicate Forskolin enhances osteogenic differentiation of hDPSCs in vitro and boosts bone formation in vivo.

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Introduction

In 2000, dental pulp stem cells (DPSCs) were discovered with the ability of self-renewal, high proliferation, and potential for multilineage differentiation such as adipogenic, neurogenic, and osteogenic phenotypes.[1—3] Compared with bone marrow mesenchymal stem cells (BMSCs), DPSCs have more advantages in bone tissue engineering. Studies have confirmed that DPSCs exhibited a higher activity of proliferation and osteogenic potential than BMSCs.[4,5] Pierdomenico et al. also demonstrated that DPSCs had lower immunoactivity when compared to BMSCs.[6] In addition, human DPSCs (hDPSCs) can be obtained clinically from routine extraction of deciduous or third molars. Therefore, obtaining the hDPSCs was much less invasive with lower ethical problems than BMSCs.[7] In the maxillofacial defect model, the implanted hDPSCs generated well-differentiated bone, while less bone formation was observed in the group without hDPSCs.[8]

Many studies have supported that the technology for stem cell sheet culture is a promising strategy in regenerative medicine, as this strategy provides a 3D culture microenvironment that is a benefit for cellular differentiation and tissue regeneration.[9] Yasuyuki et al. transplanted hDPSC sheets treated with helioxanthin into mouse calvaria defects and harvested bone formation without using any scaffolds or growth factors.[10] Therefore, the approaches or protocols aiming to enhance the osteogenic differentiation of hDPSCs will increase the benefits of bone tissue engineering, and offer new hope for patients with bone defects.

Cyclic adenosine monophosphate (cAMP) is a key second messenger that regulates a chain of important biochemical events, such as proliferation and differentiation.[11] Forskolin was discovered and used since ancient Ayurvedic medicine. At present, Forskolin has been used as the most common medicine for active cAMP. It has been applied to some diseases including heart disease, convulsions, spasms, and painful urination.[12] Recently, Chen et al. demonstrated that Forskolin can activate cAMP to promote proliferation and osteogenic differentiation of BMSCs by upregulating bone-related markers Runt-related transcription factor 2 (RUNX2) and Osterix (OSX).[13] Zhang et al. treated the stem cells of the apical papilla with Forskolin, revealing that Forskolin enhanced calcium deposition and stimulated the expression of the bone-related genes, such as *RUNX2*, alkaline (*ALP*), collagen type I (*COLI*), and osteocalcin (*OCN*).[14]

Although some previous studies have shown the positive effect of Forskolin in enhancing osteogenic differentiation of mesenchymal stem cells,[11,14] it is still unknown the boosting effect of inducing osteogenic differentiation of hDPSCs and the ability in bone formation in vivo. In this study, we applied Forskolin to boost cAMP levels during osteogenic differentiation of hDPSCs and screened out an appropriate working concentration for Forskolin to induce mineralization of hDPSC pellets in vitro, which were followed by transplanted subcutaneously into immunodeficient mice in vivo. After six weeks, samples were harvested and evaluated by immunochemical staining. We aimed to explore whether Forskolin could be developed as an activator to promote bone regeneration in future clinical treatment for bone defects.

Materials and methods

Isolation and culture of hDPSCs

Wisdom teeth were extracted from Chinese young adults (18—25 years of age) with informed consent and approval of the Ethical Committee of Huashan Hospital, Fudan University. The hDPSCs were isolated and proliferated as previously described.[15] Briefly, the pulp tissues were enzymatically dissociated for 30 min in Dulbecco's modified Eagle medium (DMEM) containing collagenase type I (3 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The digested solution was filtered through a 70 μm filter (BD Biosciences, Franklin Lakes, NJ, USA). After centrifugation at 1000*g* for 5 min, the cell suspension was plated in DMEM supplemented with 10% fetal bovine serum (Sciencell, San Diego, CA, USA) and 1% penicillin-streptomycin (HyClone, Logan, UT, USA) at 37 °C and 5% CO_2. All primary hDPSCs used in the current study were in passages 3—5. The hDPSCs osteogenic medium was prepared with 10% FBS (Sciencell), 1% penicillin/streptomycin, 10 nM dexamethasone (Sigma), 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid (Solarbio, Shanghai, China).

Flow cytometric analysis

Cells were trypsinized and resuspended at a density of 1×10^6 cells/mL in staining buffer (BD Biosciences). 100 μL of prepared cell suspension was added to the tubes containing different monoclonal antibodies according to the manufacturer's instructions (BD Biosciences). Tubes were incubated in dark for 30 min at room temperature. Then cells were washed twice with staining buffer and analyzed on a flow cytometer (BD Biosciences). The data were processed using FlowJo v10 software (BD Biosciences).

Proliferation

hDPSCs were seeded at a density of 3×10^3 cells/well into 96-well plates (Corning, Inc., Corning, NY, USA). 24 h later, cells were treated with DMEM containing different concentrations of Forskolin (5, 10, 25, and 50 μM) as experimental groups. Forskolin (Topscience, Shanghai, China) powder was firstly dissolved in dimethyl sulfoxide (DMSO) (Sigma) to prepare different concentrations of stock solutions (5, 10, 25, and 50 mM), and then diluted as 1:1000 in DMEM to obtain working solutions (5, 10, 25, and 50 μM). The solvent of Forskolin was 0.1% DMSO (Sigma) as a control group. 10% cell counting kit-8 (CCK-8) solution was added to each well according to the manufacturer's instructions (Topscience, Shanghai, China). The 96-well plates were incubated in dark for 3 h at 37 °C. The absorbance of the solution in each well was measured at 450 nm by a microplate reader (BioTek Instruments, Winooski, VT, USA). Cell proliferation assay was performed at 0, 1, 2, 3, 4, and 5 days.

Quantitative polymerase chain reaction (qPCR)

hDPSCs were seeded at a density of 5×10^4 cells/well into 96-well plates. When the cells reached 70% confluence, the culture medium DMEM was replaced with osteogenic medium (OM) containing different concentrations of Forskolin (0, 5, 10 μM). After 14 days, RNA was extracted using RNAQuick Purification Kit (Yishan Biotechnology, Shanghai, China) according to the manufacturer's protocol. RNA concentrations were measured by NanoDrop 2000 Microspectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse transcribed to DNA using PrimeScriptTM RT reagent Kit (Takara Bio Inc, Shiga, Japan). A master mix containing DNA, primer (Genscript, Nanjing, China), TB Green was prepared according to the manufacturer's instructions (Takara). And qPCR was performed on Lightcycler 480 system (Roche, Basel, Switzerland). The fold changes of target genes were calculated by $2^{-\triangle\triangle ct}$. Primer sequences are listed in Table 1.

Alizarin red staining and quantification

The process of cell treatment was the same as the previous qPCR assay. After 21 days, the cells were fixed with 4% paraformaldehyde (Servicebio, Wuhan, China) for 20 min in dark. Then the cells were washed three times with PBS and stained with alizarin red solution (Solarbio) at room temperature for 15 min. The cells and calcium nodules formation were imaged under a microscope (Nikon, Tokyo, Japan). The mineralized deposits were dissolved in 10% Cetylpyridinium chloride (CPC) solution (Sigma). And the absorbance of the solution in each well was measured by a microplate reader (BioTek) at 562 nm.

Dentin chamber preparation

Noncarious human wisdom teeth were cut into cuboids of about $5 \times 5 \times 3$ mm^3 by using a diamond blade at a low speed with cooling saline. Next, a small chamber was reshaped in the middle part of the dentin cuboids. The dentin chambers were immersed into distilled saline containing 1% penicillin/streptomycin and cleaned with the ultrasonic cleaner (100 W, 40 kHz, Jiekang, Dongguan, China) for 30 min, followed by washing twice with PBS and stored in PBS containing 1% penicillin/streptomycin at 4 °C. The dentin chamber was used as a cellular sample holder for the following mouse transplantation.

3D culture of cell pellet

hDPSCs were seeded at a density of 2.5×10^4 cells/mL and cultured in DMEM supplemented with 10% FBS with 50 μg/mL ascorbic acid (Solarbio). When the cells reached 100% confluence and the edge of the cell sheet was wrapped by a blunt tip. The cell sheet was scraped off gently and transferred into Eppendorf (EP) tube for 3D culture with 10 μM Forskolin at 37 °C and 5% CO_2. The culture medium was replaced with or without the osteogenic medium. After 7 days, spherical cell pellets were seeded inside the dentin chambers for mouse transplantation.

Subcutaneous transplantation of the cell—dentin complex

All animal experiments were approved by the Ethical Committee of Huashan Hospital, Fudan University, and all procedures were carried out following the relevant guidelines and regulations. The complex of 3D pellets combined with dentin chambers ($n = 8$) was implanted into the subcutaneous space of immunodeficient mice ($n = 4$) (Nod SCID, Shanghai, China). The anesthesia was pentobarbital (30 mg/kg). The mice were held in a Specific Pathogen Free animal laboratory house. Six weeks later, the implants were retrieved and fixed in 4% paraformaldehyde for immunohistochemical stains.

Immunohistochemical stains

Paraffin blocks were cut into samples of 5 μm thickness. Each sample was placed on a glass slide (Citotest, Haimen, China). Osteopontin (OPN) antibody (Abcam, Cambridge, MA, USA) was used at 1:1000 dilution for 12 h at 4 °C. The biotinylated rabbit anti-mouse secondary antibodies conjugated to streptavidin-horseradish peroxidase and DAB solution were

Table 1 Primer sequences.

Genes	Forward primer 5′→3′	Reverse primer 5′→3′
GAPDH[a]	CCAGAACATCATCCCTGCCTCT	GACGCCTGCTTCACCACCTT
ALP[b]	GAGATGTTGTCCTGACACTTGTG	AGGCTTCCTCCTTGTTGGGT
RUNX2[c]	TCCAGACCAGCAGCACTCCATA	TCCATCAGCGTCAACACCATCA
OSX[d]	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG
OPN[e]	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT
BMP2[f]	TTCGGCCTGAAACAGAGACC	CCTGAGTGCCTGCGATACAG

[a] GAPDH: glyceraldehyde-3-phosphatedehydrogenase.
[b] ALP: Alkaline phosphatase.
[c] RUNX2: Runt-related transcription 2.
[d] OSX: Osterix.
[e] OPN: Osteopontin.
[f] BMP2: Bone morphogenetic protein 2.

used for the visualization of immunoreactivity (Abcam). All the sections were observed and photographed with a light microscope (Olympus, Tokyo, Japan).

Statistics

All experiments were performed in triplicate. Data were analyzed in SPSS 20 software (IBM, Armonk, NY, USA). The significance of differences between the two groups was determined using Student t-tests. The comparison of multiple groups was analyzed using one-way ANOVA. Differences were considered significant at $P < 0.05$.

Results

Characterization of hDPSCs

The mesenchymal stem cell surface markers were analyzed by flow cytometry. The isolated hDPSCs positively expressed stem cell markers of CD90, CD44, CD73, and CD105 and negatively expressed hematopoietic markers of CD11, CD34, CD19, CD45, and HLA-DR (Fig. 1).

Optimization of Forskolin concentrations

To assess the effect of Forskolin-induced cAMP on osteogenic differentiation of hDPSCs, the concentration of Forskolin working solution was optimized by CCK-8 assay before inducting study. Data showed concentrations of Forskolin in 5 μM and 10 μM did not inhibit hDPSC proliferation compared to the 0.1% DMSO and DMEM (0 μM) controls after 5 days. Whereas, the concentrations in 25 μM and 50 μM significantly decrease cellular proliferation compared with other groups. There is no obvious difference in proliferation between hDPSCs cultured in DMEM alone and cells cultured in DMEM with 0.1% DMSO, which is a solvent of Forskolin (Fig. 2). Therefore, Forskolin in 5 μM and 10 μM were selected for the following osteogenic inducing study.

Forskolin promoted osteogenic differentiation of hDPSCs in vitro

hDPSCs were cultured in osteogenic inducing media with or without Forskolin. We performed qPCR after 14 days of inducing media treatment and observed bone-related genes

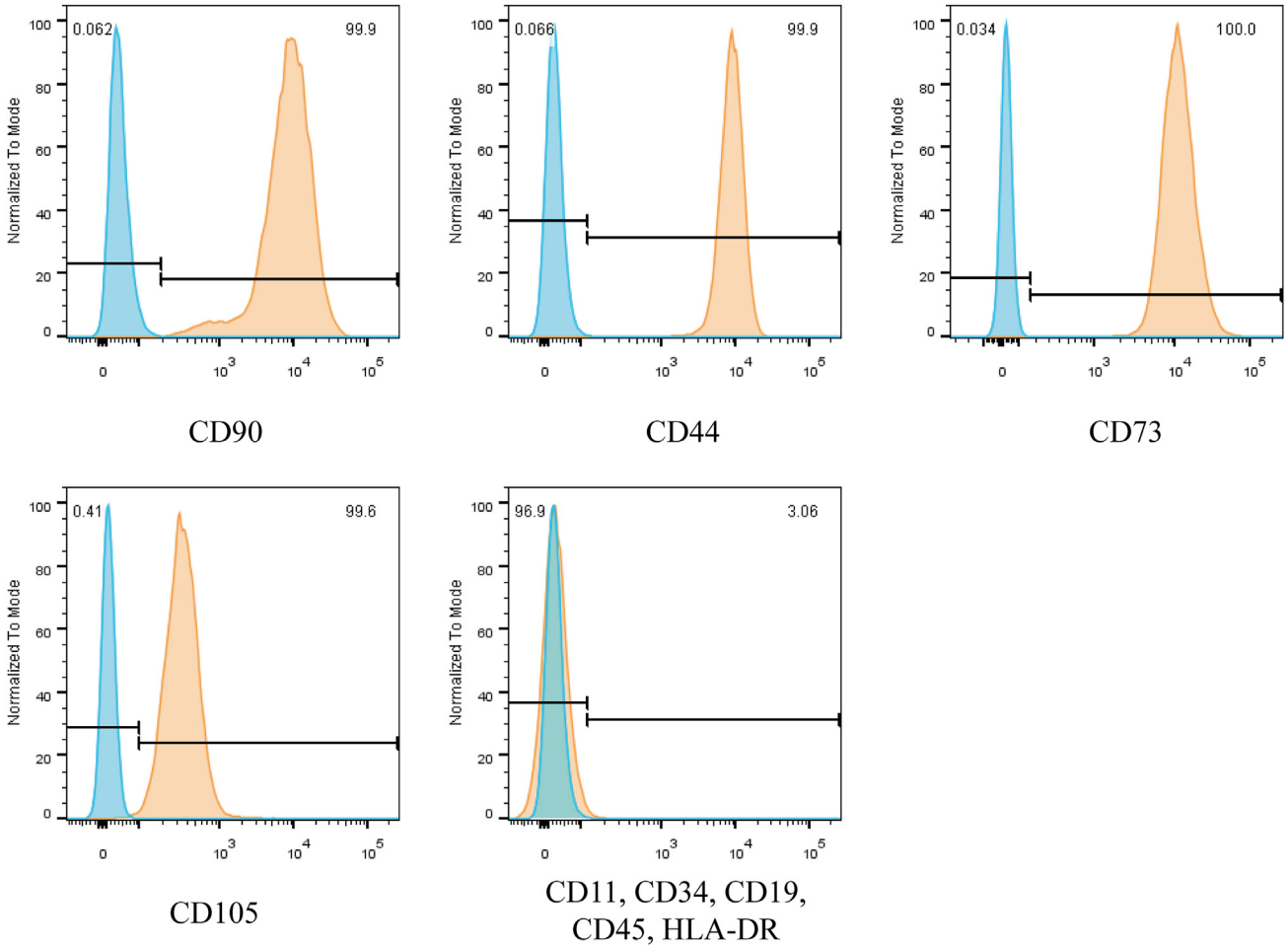

Fig. 1 Characterization of human dental pulp stem cells (hDPSCs). The hDPSCs were characterized by a flow cytometry test. Data showed the isolated hDPSCs highly expressed stem cell markers of CD90, CD44, CD73, and CD105, while negatively expressed the hematopoietic markers of CD11, CD34, CD19, CD45, and HLA-DR.

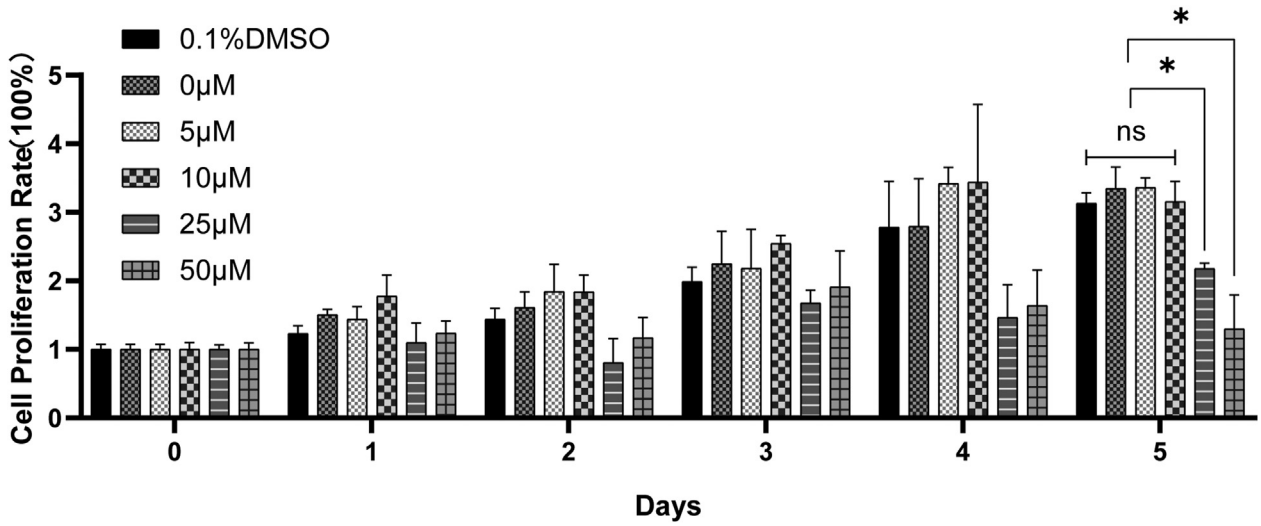

Fig. 2 Effect of Forskolin on cell proliferation of human dental pulp stem cells (hDPSCs) evaluated using CCK-8 assay. The concentrations of Forskolin were optimized by using the CCK-8 assay that showed the lower concentration of 5 μM and 10 μM did not inhibit hDPSCs proliferation compared to the 0.1% dimethyl sulfoxide and 0 μM on the day 5, while the higher concentrations in 25 μM and 50 μM significantly reduced the hDPSC proliferation compared with other groups. DMSO: dimethyl sulfoxide. *$P < 0.05$.

were significantly elevated in 10 μM Forskolin groups. Forskolin in 5 μM and 10 μM similarly boosted the gene expression of *RUNX2*, *OSX*, *OPN*, and bone morphogenetic protein 2 (*BMP2*) (Fig. 3). Aiming to compare the ability of mineralization in hDPSCs after 21 days of treatment with or without Forskolin, alizarin red staining was performed and quantitatively analyzed by using a 10% CPC solution. The quantitative data indicated Forskolin in 5 μM and 10 μM did not show a statistical difference in inducing mineral deposit formation in hDPSCs (Fig. 4).

Forskolin enhanced bone formation by hDPSCs in vivo

To investigate the boosting effect of Forskolin on bone formation, hPDSCs (Fig. 5A) were cultured in DMEM (50 μg/mL ascorbic acid) for 7 days to form a cell sheet (Fig. 5B and C), which was detached from a well plate and transferred into EP tube for another 7 days of 3D culture treated by 10 μM Forskolin to product pellet (Fig. 5D). Furthermore, hDPSC cell pellets were fixed into a designed dentin chamber and transplanted into immunodeficient mice for six weeks. Immunochemical stains demonstrated the Forskolin-treated hDPSC pellet (experimental group: OM + Forskolin) generated stronger bone tissue and highly expressed OPN compared to the control group without Forskolin treatment (control group: OM) (Fig. 5E and F).

Discussion

Most published studies indicated that the activation of cAMP by Forskolin can stimulate osteogenic differentiation of bone marrow stem cells,[11] adipose-derived mesenchymal stem cells,[16] and stem cells of apical papilla[14] by inducing the bone-related gene expression. It still has not been reported the effect Forskolin has on inducing osteogenic differentiation of hDPSCs. In the present study, we evaluated the osteogenic effect of Forskolin on hDPSCs and the in vivo potential of bone formation using cell-sheet technology. We first demonstrated that the optimal concentration of Forskolin on the osteogenic differentiation of hDPSCs is 10 μM. Then, hDPSC sheets were prepared and followed by a short time treatment with Forskolin. Furthermore, we demonstrated that the bone formation of Forskolin-induced hDPSCs was increased compared with the normal inducing group.

Forskolin is a lipid-soluble activator of cAMP. It has been extracted from the root plant of Coleus and produced as commercial medicine used for the treatment of cardiovascular diseases, asthma, and glaucoma in Japan.[17] In China, Forskolin, as one of the active components in Chinese medicine (MaoHouQiaoRuiHua), has been used for asthma treatment bronchitis (SFDA No. Z1096005, Z20113029).[18] The natural compound Forskolin has been used for centuries in traditional medicine and its safety has been confirmed in recent medicine.[19] In macrophages, Forskolin induced mitogenesis and increased cell proliferation by protein kinase A/cAMP response element-binding protein (PKA/CREB) and exchange protein directly activated by cAMP/Ras-related protein 1 signaling pathway.[20] However, different cell lines have been reported to have different cytotoxic responses to Forskolin. Kenichi et al. indicated that Forskolin enhanced the cAMP level and significantly increased the cytotoxicity when combined use with mitomycin C in AH66 cells (ascites hepatoma cell line). On the contrary, Forskolin generated the scarcity effect of cAMP in AH66F cells (a variant cell line obtained from AH66).[21] Therefore, Forskolin-induced cellular proliferation or cytotoxicity is varied between cell types. In our study, we optimized Forskolin concentration before the osteogenic inducing study. In the CCK-8 assay, the solvent

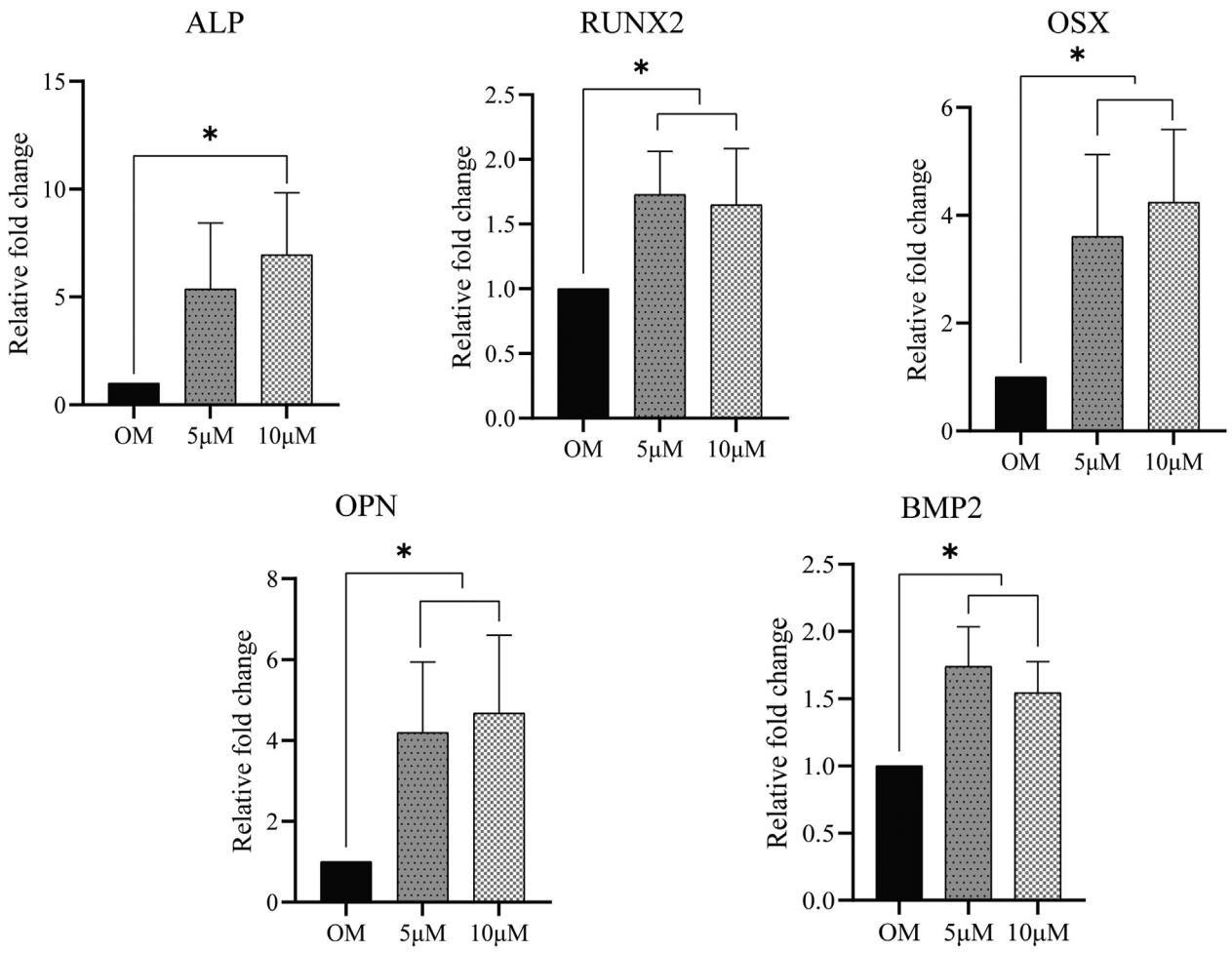

Fig. 3 Effect of Forskolin on the mRNA expression of osteogenic genes. Quantitative polymerase chain reaction was performed and analyzed after 14 days of osteogenic differentiation. Forskolin in 10 μM boosted the expression of bone-related genes in *ALP*, *RUNX2*, *OSX*, *OPN* and *BMP2*. Forskolin in 5 μM and 10 μM similarly enhanced the expression of *RUNX2*, *OSX*, *OPN*, and *BMP2*. OM: osteogenic medium. $^{*}P < 0.05$.

concentration of Forskolin, 0.1% DMSO was involved in CCK-8 groups as well since it might be cytotoxic for cellular proliferation that has been reported in other studies.[22,23] CCK-8 results showed 0.1% DMSO did not inhibit hDPSC proliferation in 5 days, while the Forskolin in higher concentrations of 25 μM and 50 μM generated cytotoxicity for hDPSCs. Therefore, cytotoxicity of Forskolin is dosage-dependent for hDPSCs. Based on the CCK-8 result, 10 μM Forskolin was selected as a safe concentration for the following inducing study.

Despite studies that have demonstrated the active effect of Forskolin-induced cAMP on regulating osteogenic differentiation of mesenchymal stem cells,[11,13] it is still controversial among studies. We found Forskolin significantly enhanced osteogenic differentiation of hDPSCs by upregulating bone-related genes (*ALP*, *RUNX2*, *OSX*, *OPN*, *BMP2*), and boosted mineral deposit formation in vitro. Nevertheless, this positive effect was not observed in rodent cell types. cAMP/PKA signaling was demonstrated to inhibit osteogenic differentiation and bone formation in mouse and rat mesenchymal stem cells by downregulating ALP, OCN, and collagen type I. It was found cAMP even stimulated adipogenic differentiation in rat BMSCs.[24] Therefore, the cellular data obtained from animal models need further verification in human cells. The elevation of cAMP in promoting osteogenic differentiation is not only correlated with the concentrations of activator, but also with timing. For example, although it was proved that dibutyryl-cAMP as a cAMP/PKA activator enhanced bone formation by hBMSCs both in vitro and in vivo,[11] the intermittent exposure of dibutyryl-cAMP inhibited osteogenic differentiation of hBMSCs by suppressing the expression of ALP.[25] In our study, we continuously treated hDPSCs with 10 μM Forskolin for one week before implantation into mice. The duration of Forskolin treatment in bone formation will be needed for future verification.

In the present study, we applied cell-sheet technology in hDPSCs. hDPSC cell sheet was prepared and followed by a 3D cell-pellet culture in an EP tube maintained in Forskolin inducing media with 37 °C, 5% CO_2 for one week. After six-week transplantation in vivo, the level of mineral tissues was elevated by OPN expression, while OPN is an important

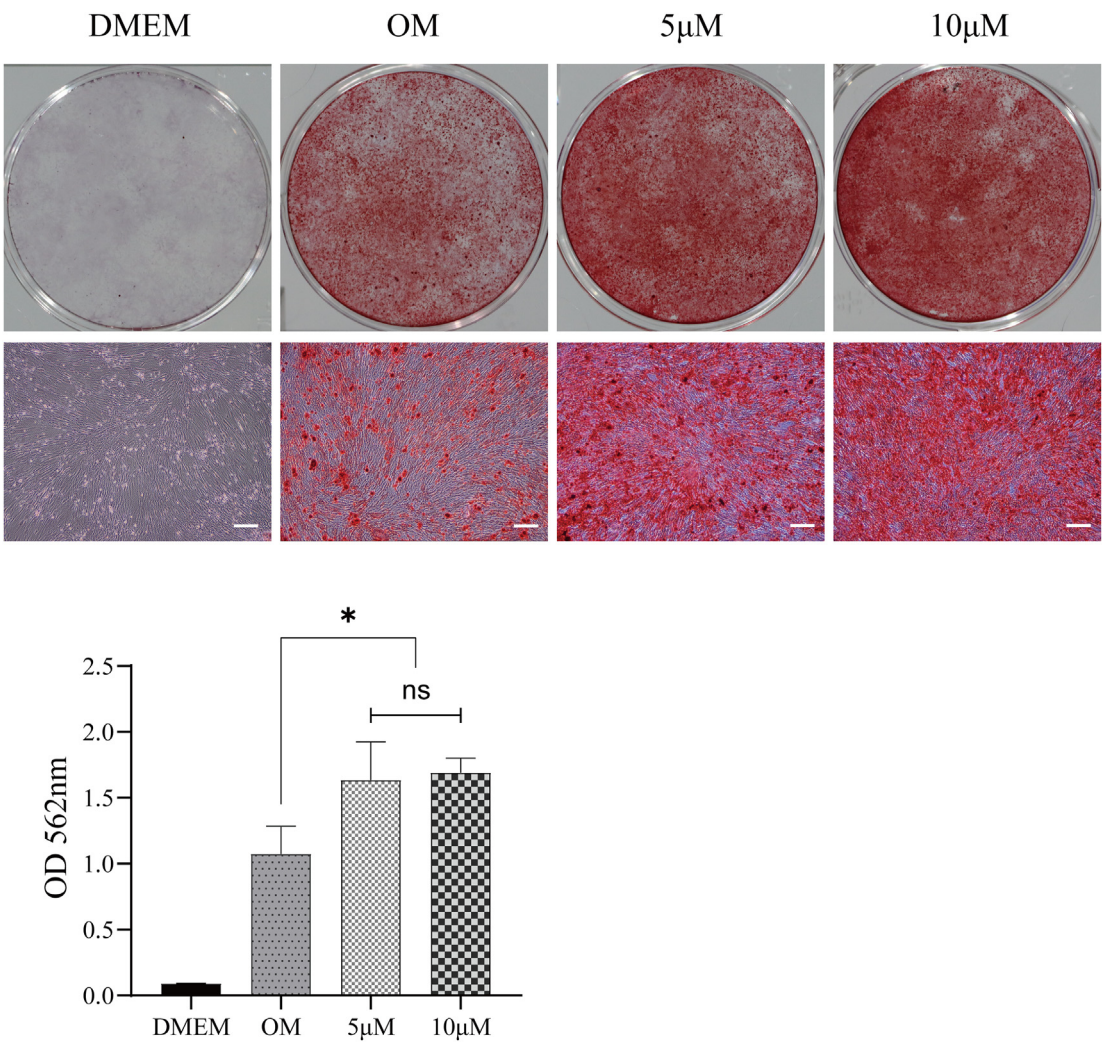

Fig. 4 Alizarin red staining and quantification. To evaluate the ability of Forskolin on inducing mineralized formation in human dental pulp stem cells (hDPSCs), alizarin red staining was performed after 21 days of osteogenic inducing study. Staining and quantified results demonstrated that Forskolin of 5 μM and 10 μM were equally able to enhance mineral deposits in hDPSCs compared to normal osteogenic medium treatment. OM: osteogenic medium. *$P < 0.05$. Scale bars: 200 μm.

bone matrix protein synthesized by osteoblasts.[26,27] Positive immunoreactivities of OPN demonstrated both experimental and control groups supported bone regeneration based on 3D pellet implants without scaffold embedded inside. Notably, the Forskolin inducing group exhibited the higher expression of OPN and significantly harvested stronger bone formation compared to the normal inducing group. The bone formation in the normal inducing group was accumulated in the central area by surrounded fiber-like tissue. In Forskolin inducing group, bone formation was distributed evenly with less fiber-like tissue compared to the normal inducing group. In regenerative medicine, the microenvironment for stem cells is crucial for cellular differentiation.[28] The natural cells have wide cross-linking connections in natural tissue, once isolated in vitro, cells in 2D culture lost natural interactions between the cellular and extracellular microenvironments, the way of division, cellular morphology, and polarity.[29,30] We generated hDPSC pellet based on cell sheet culture. The cell pellet has a compact profile with plenty of accumulated cells and is easier to be handled for cell implants. After six weeks, bone was successfully generated inside the cell pellet. Mari et al. found bone formation by human adipose stem cells in 3D culture with osteogenic inducing media was superior to the cells maintained in growth factors (BMP2 and vascular endothelial growth factor) culture media.[31] Therefore, 3D culture technology is a promising approach that is more closely able to mimic the natural condition.

In conclusion, we demonstrated that 10 μM Forskolin could increase the expression of bone-related genes in hDPSCs. Furthermore, bone formation was successfully generated in vivo. In the future, the regulation of hDPSCs with Forskolin might be a promising approach used in bone tissue engineering.

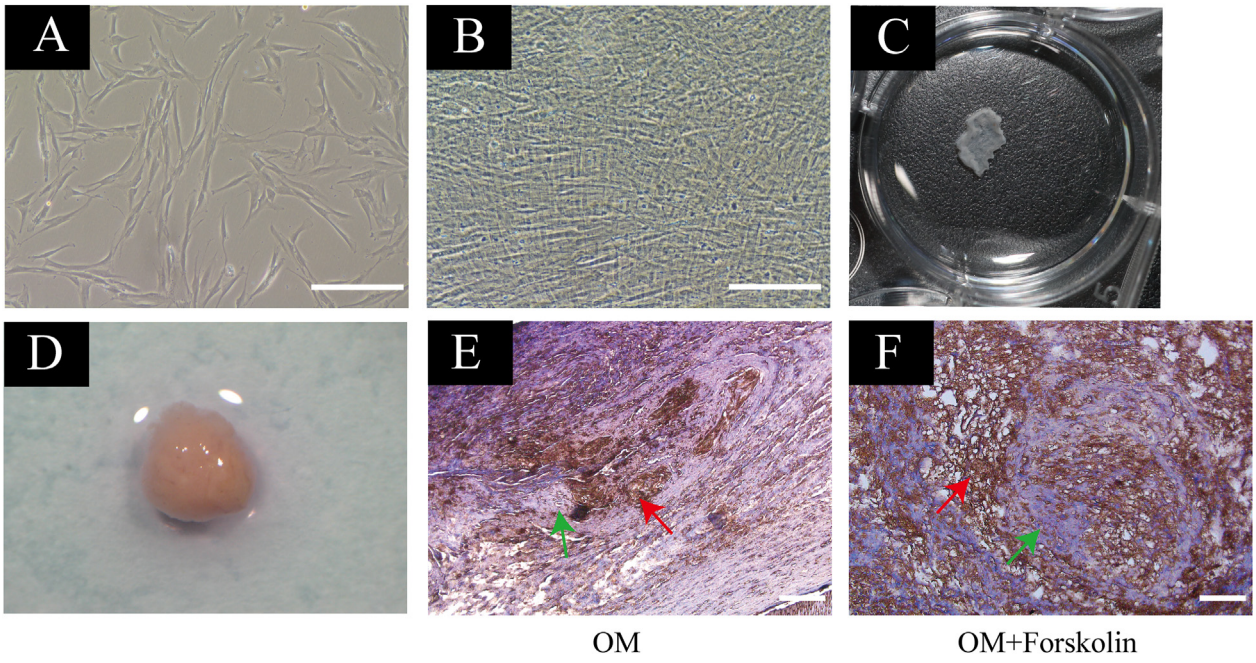

Fig. 5 Forskolin enhanced bone formation by human dental pulp stem cells (hDPSCs) in vivo (A) hDPSCs were seeded in a 6-well plate with the density of 2.5×10^4 cells/mL and maintained for 7 days to form hDPSC sheet (B—C), which was gently detached from the bottom of a plate and transferred into an EP tube for continuous 7 days of 3D culture with 10 μM Forskolin to obtain 3D hDPSC pellet (D), which was transplanted into subcutaneous pockets of immunodeficient mice. After six weeks, there was the formation of bone structure (E-F, green arrows) that presented positive expression for the marker of osteogenic differentiation of OPN (E-F, red arrows). Forskolin enhanced stronger bone formation compared to the osteogenic medium group. OM: osteogenic medium. Scale bars: 200 μm.

Declaration of competing interest

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

Acknowledgments

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References

1. Gronthos S, Brahim J, Li W, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;81:531—5.
2. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *P Natl A Sci India B* 2000;97:13625—30.
3. Tamaki Y, Nakahara T, Ishikawa H, Sato S. In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. *Odontology* 2013;101:121—32.
4. Kunimatsu R, Nakajima K, Awada T, et al. Comparative characterization of stem cells from human exfoliated deciduous teeth, dental pulp, and bone marrow-derived mesenchymal stem cells. *Biochem Bioph Res Co* 2018;501:193—8.
5. Ito K, Yamada Y, Nakamura S, Ueda M. Osteogenic potential of effective bone engineering using dental pulp stem cells, bone marrow stem cells, and periosteal cells for osseointegration of dental implants. *Int J Oral Maxillofac Implants* 2011;26:947—54.
6. Pierdomenico L, Bonsi L, Calvitti M, et al. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 2005;80:836—42.
7. La Noce M, Paino F, Spina A, et al. Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. *J Dent* 2014;42:761—8.
8. Monti M, Graziano A, Rizzo S, et al. In vitro and in vivo differentiation of progenitor stem cells obtained after mechanical digestion of human dental pulp. *J Cell Physiol* 2017;232:548—55.
9. Li M, Ma JUN, Gao Y, Yang LEI. Cell sheet technology: a promising strategy in regenerative medicine. *Cytotherapy* 2019;21:3—16.
10. Fujii Y, Kawase-Koga Y, Hojo H, et al. Bone regeneration by human dental pulp stem cells using a helioxanthin derivative and cell-sheet technology. *Stem Cell Res Ther* 2018;9:1—12.
11. Doorn J, Siddappa R, Van Blitterswijk CA, De Boer J. Forskolin enhances in vivo bone formation by human mesenchymal stromal cells. *Tissue Eng* 2012;18:558—67.
12. Dubey MP, Srimal RC, Nityanand S, Dhawan BN. Pharmacological studies on coleonol, a hypotensive diterpene from coleus forskohlii. *J Ethnopharmacol* 1981;3:1—13.
13. Chen B, Lin T, Yang X, Li Y, Xie D, Cui H. Intermittent parathyroid hormone (1-34) application regulates cAMP-response element binding protein activity to promote the proliferation and osteogenic differentiation of bone mesenchymal stromal cells, via the cAMP/PKA signaling pathway. *Exp Ther Med* 2016;11:2399—406.
14. Zhang J, Zhao IS, Yu OY, et al. Layer-by-layer self-assembly polyelectrolytes loaded with cyclic adenosine monophosphate enhances the osteo/odontogenic differentiation of stem cells from apical papilla. *J Biomed Mater Res* 2021;109:207—18.

15. Liu L, Xie H, Zhao S, Huang X. The GLUT1—mTORC1 axis affects odontogenic differentiation of human dental pulp stem cells. *Tissue Cell* 2022;76:101766.
16. Rumiński S, Kalaszczyńska I, Lewandowska-Szumieł M. Effect of cAMP signaling regulation in osteogenic differentiation of adipose-derived mesenchymal stem cells. *Cells* 2020;9:1587.
17. Ju H, Zhang C, Lu W. Progress in heterologous biosynthesis of forskolin. *J Ind Microbiol Biotechnol* 2021;48:kuab009.
18. Schaneberg BT, Khan IA. Quantitative analysis of forskolin in coleus forskohlii (lamiaceae) by reversed-phase liquid chromatography. *J AOAC Int* 2003;86:467—70.
19. Loftus HL, Astell KJ, Mathai ML, Su XQ. Coleus forskohlii extract supplementation in conjunction with a hypocaloric diet reduces the risk factors of metabolic syndrome in overweight and obese subjects: a randomized controlled trial. *Nutrients* 2015;7:9508—22.
20. Misra UK, Pizzo SV. Coordinate regulation of forskolin-induced cellular proliferation in macrophages by protein kinase A/cAMP-response element-binding protein (CREB) and Epac1-Rap1 signaling: effects of silencing CREB gene expression on Akt activation. *J Biol Chem* 2005;280:38276—89.
21. Miyamoto K, Matsunaga T, Koshiura R, Takagi K, Satake T, Hasegawa T. Comparative studies on the combined cytotoxic effect of forskolin with mitomycin C and responsiveness to forskolin in rat ascites hepatoma AH66 cells and AH66F cells. *J Pharmacobio-Dyn* 1987;10:346—52.
22. Pal R, Mamidi MK, Das AK, Bhonde R. Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. *Arch Toxicol* 2012;86:651—61.
23. Hajighasemi F, Tajic S. Assessment of cytotoxicity of dimethyl sulfoxide in human hematopoietic tumor cell lines. *Iran J Blood Cancer* 2017;9:48—53.
24. Siddappa R, Mulder W, Steeghs I, et al. cAMP/PKA signaling inhibits osteogenic differentiation and bone formation in rodent models. *Tissue Eng* 2009;15:2135—43.
25. Siddappa R, Doorn J, Liu J, et al. Timing, rather than the concentration of cyclic AMP, correlates to osteogenic differentiation of human mesenchymal stem cells. *J Tissue Eng Regen Med* 2010;4:356—65.
26. Si J, Wang C, Zhang D, Wang B, Hou W, Zhou Y. Osteopontin in bone metabolism and bone diseases. *Med Sci Mon Int Med J Exp Clin Res* 2020;26:e919159-1. Available from: https://pubmed.ncbi.nlm.nih.gov/31996665/.
27. Makishi S, Yamazaki T, Ohshima H. Osteopontin on the dental implant surface promotes direct osteogenesis in osseointegration. *Int J Mol Sci* 2022;23:1039.
28. Metallo CM, Mohr JC, Detzel CJ, de Pablo JJ, Van Wie BJ, Palecek SP. Engineering the stem cell microenvironment. *Biotechnol Prog* 2007;23:18—23.
29. Kapałczyńska M, Kolenda T, Przybyła W, et al. 2D and 3D cell cultures-a comparison of different types of cancer cell cultures. *Arch Med Sci* 2018;14:910—9.
30. Duval K, Grover H, Han LH, et al. Modeling physiological events in 2D vs. 3D cell culture. *Physiology* 2017;32:266—77.
31. Tirkkonen L, Haimi S, Huttunen S, et al. Osteogenic medium is superior to growth factors in differentiation of human adipose stem cells towards bone-forming cells in 3D culture. *Eur Cell Mater* 2013;25:144—58.