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Elevation of cAMP upregulates Cav1.2 expression and promotes odontogenic differentiation of dental pulp stem cells

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Abstract

Human dental pulp stem cells (hDPSCs) are promising cellular sources in dental tissue engineering. Although studies have reported that cyclic adenosine monophosphate (cAMP) and Cav1.2 played important roles in the differentiation of stem cells, the relation between cAMP and Cav1.2 in the odontogenic differentiation of hDPSCs still remained unclear. This study hypothesized that elevating cAMP contributed to the odontogenic differentiation of hDPSCs by regulating *Cav1.2* expression. Here, Forskolin was used to activate cAMP and Nimodipine was used to inhibit Cav1.2. This study firstly screened out the safety concentrations of Forskolin and Nimodipine by CCK-8 proliferation assay. Following, Forskolin was used to elevate cAMP during odontogeinic differentiation of hDPSCs. qPCR was performed to compare the odontogenic differentiation-related gene expression between groups. The odontogenic mineralization was evaluated by Alizarin Red Staining. Subsequently, in order to investigate the relation between cAMP and Cav1.2, hDPSCs was treated with Nimodipine in one hour before Forskolin adding. Finally, Alizarin Red Staining and qPCR were performed to observe mineralized deposit formation and the *Cav1.2* together with odongenic related gene expression in each group. Results showed that Forskolin promoted the formation of mineralized nodules in hDPSCs. The expression of odontogenic related genes (*ALP, RUNX2, OSX, BMP2, DSPP*) and *Cav1.2* were also upregulated after 14 days of odontogenic induction. Nimodipine inhibited the odontogenic differentiation and attenuated the promoting effect of Forskolin during the odontogenic differentiation of hDPSCs. The above results suggested that the elevation of cAMP could upregulate *Cav1.2* expression and significantly promote odongenic differentiation of hDPSCs.

Introduction

The development of stem cell transplantation technology provides a new way for tissue repair and regeneration in the therapy of pulp disease and periapical disease. Human dental pulp stem cells (hDPSCs) were firstly isolated and cultured in 2000 by Gronthos *et al.*[1]. Because of their self-renewal ability, multi-directional differentiation potential, easy acquisition and preservation, hDPSCs have been the most widely studied and applied cells in the field of pulp regeneration[2]. Both *in vitro* and *in vivo* experiments demonstrated that odontoblast-like cells and dentin-like tissues could be generated when DPSCs were seeded on the surface of dentin or implanted into root canals[3].However, the mechanism of odontogenic differentiation of hDPSCs still remains unclear.

Cell behavior and cellular metabolism are influenced by extracellular signals. cyclic adenosine monophosphate (cAMP) is a hydrophilic small molecule nucleotide that expresses in various cells and participates in extracellular signal transduction as a second messenger[4]. Intracellular cAMP levels are regulated by adenylate cyclase (AC)[5]. The use of siRNA to interfere the expression of AC could inhibit the production of cAMP in osteoblasts, and attenuate osteogenesis[6]. Bone marrow mesenchymal stem cells (BMSCs) treated with cAMP analogs CW008 enhanced osteogenic differentiation and mineralization capacity *in vitro* and *in vivo*, promoting bone formation in mouse osteoporosis model[7]. Previous study

used gelatin and alginate polyelectrolyte to deliver cAMP and found that it promoted the odontogenic differentiation of apical papillary stem cells[8]. These studies showed that cAMP has an important role in regulating cell differentiation and promoting hard tissue formation.

The L-type calcium channel Cav1.2 is a heteromeric transmembrane protein that can selectively transport extracellular calcium ions into cells[9]. In normal dental pulp tissue, Cav1.2 is higher expressed in the odontoblast layer and nerve fibers than other areas[10]. However, after acute pulp injury, Cav1.2 was upregulated immediately and involved in pulp repair and dentinogenesis[11]. The mutations of *CACNA1C*, encoding Cav1.2 caused Timothy syndrome, which leads to cardiac arrhythmias, autism disorders, abnormal bone and tooth development[12]. Another study found Cav1.2 was downregulated in an aging mouse model. Bayk8644 was used to activate Cav1.2 and promoted calcium influx and improved osteoporosis symptoms[13]. Our previous studies demonstrated that knockdown of *Cav1.2* in rat DPSCs and apical papilla stem cells downregulated *Dspp* and *Alp* expression, thereby inhibiting mineralized deposit formation [14, 15].

The mutual relation between cAMP and Cav1.2 has been reported that they both regulated cellular functions in various cells. It has been confirmed that in cardiomyocytes, Cav1.2 combined with G protein-coupled receptors and AC to form a signaling complex through the carboxy terminus of Cav1.2, and the activity of this complex was regulated by the cAMP/PKA phosphorylation pathway, leading the changes in calcium flux and cell excitability[16]. In INS-1 cells, blocking Cav1.2 with nicardipine significantly inhibited the accumulation of cAMP, delaying or reducing glucose-stimulated ERK phosphorylation[17]. In addition, pre-treated with Forskolin (a cAMP activator) increased intracellular cAMP levels in osteoblasts, which subsequently regulated the combination of Actin4 and L-type calcium channels, affecting the physiological functions of osteoblasts[18]. Therefore, there is a relationship between the regulation of cAMP signal and the function of Cav1.2 mediated L type calcium channel. Nonetheless, the relationship between the cAMP and Cav1.2 in the odontogenic differentiation of hDPSCs has not yet been clarified.

This study aimed to investigate the potential relationship between Cav1.2 and cAMP in the odontogenic differentiation of hDPSCs, which might provide the deeper understanding in the mechanism of odontogenic differentiation in hDPSCs and develop a potential way for future dental pulp regeneration therapy.

MATERIALS AND METHODS

1.1 Isolation and culture of hDPSCs

Cell isolation and culture were performed as previously described[19]. Briefly, the pulp tissues were digested in α-minimal essential medium(α-MEM) containing 3mg/mL collagenase type I for 30min at 37℃ and 5% CO_2. After centrifugation at 1000g for 5min, dental pulp cells were seeded into a 10cm Petri dish in α-MEM supplemented with 10% fetal bovine serum (FBS; Sciencell,USA) and 1% penicillin-

streptomycin (HyClone, USA) at 37°C in a humidified atmosphere with 5% CO_2. Cells between 2–6 passages were used in this study.

1.2 Flow cytometric analysis

To identify the characteristics of hDPSCs, the culture cells were digested by 0.25% trypsin when the cells reached 90% confluence. Cells were washed with PBS (HyClone, USA) and incubated with monoclonal antibodies from Huma Mesenchymal Analysis Kit in dark for 30min at room temperature according to the manufacturer's instructions (BD Biosciences, USA). Then all samples were washed twice with BD Pharmingen™ Stain Buffer (BD, USA) and subjected to flow cytometer (BD Biosciences, USA). Finally, the data were analyzed with FlowJo software.

1.3 Oil Red O Staining

hDPSCs ($5x10^4$cells/well) were seeded into 6-well plate. When the cells reached 100% confluence, the culture medium was replaced with adipogenic induction medium supplemented with 200μM indomethacin, 10μg/mL insulin, 0.5mM IBMX and 1μM dexamethasone (Sigma, USA). After 28 days, Oil Red O Staining was used to detect lipid drops. The cells were fixed using 4% paraformaldehyde for 1 h at 37°C, washed with double-distilled water, and stained using Oil Red O Stain kit (Solarbio, China).

1.4 Cell Proliferation Assay

hDPSCs ($3x10^3$cells/well) were seeded into a 96-well plate. After cellular attachment for 24 hours, cells were treated with different concentrations of Forskolin (0, 1, 10, 100μM) (Topscience, Shanghai, China) or Nimodipine (0, 1, 50, 100μM) (Topscience, Shanghai, China) every 2 days. The culture medium from the 96-well plate was removed, and then added with 100uL 10% CCK-8 solution (Topscience, Shanghai, China) to each well. After incubation at 37℃ for 3 hours, the absorbance of the liquid was spectrophotometrically determined at 450 nm (Bio-tech, USA). CCK-8 assay was performed on 0, 1, 3, 5, 7 days. Based on data analysis, the non-cytotoxic concentration was screened out for the later study.

1.5 Odontogenic Differentiation

hDPSCs ($5x10^4$cells/well) were seeded into 6-well plate. When the cells reached 70% confluence, the culture medium was replaced with odontogenic differentiation medium (OM), supplemented with 10% FBS (Sciencell, USA), 10 nmol/L dexamethasone (Sigma, USA), 10 mmol/L β-glycerophosphate, and 50 μg/mL ascorbic acid (Solarbio, China). To investigate whether cAMP had an effect on Cav1.2, hDPSCs were pretreated with or without Nimodipine for one hour before adding Forskolin.

1.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

After the treatment with all the different odontogenic condition medium for 14 days, qPCR was performed to evaluate the related gene expression. Briefly, total RNA was extracted by using RNA-Quick Purification Kit (Yishan Biotechnology, Shanghai, China) according to the manufacturer's protocol. 1ug total RNA was

reverse transcribed to DNA using PrimeScriptTM RT reagent Kit (Takara, Japan). Real-time quantitative PCR (qPCR) was performed with TB Green Premix Ex Taq II Kit (Takara, Japan) and Lightcycler 480 system (Roche, USA).The expression level of GAPDH was used as an internal control and the fold change of target genes were calculated by $2^{-\triangle\triangle ct}$ .The primer used in study were purchased from Genscript (Nanjing, China) listed in Table 1.

Table 1
Primer sequences

Genes	Forward primer 5'→3'	Reverse primer 5'→3'
GAPDH	CCAGAACATCATCCCTGCCTCT	GACGCCTGCTTCACCACCTT
ALP	GAGATGTTGTCCTGACACTTGTG	AGGCTTCCTCCTTGTTGGGT
RUNX2	TCCAGACCAGCAGCACTCCATA	TCCATCAGCGTCAACACCATCA
OSX	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG
BMP2	TTCGGCCTGAAACAGAGACC	CCTGAGTGCCTGCGATACAG
DSPP	GGAGCCACAAACAGAAGCAACA	TGGACAACAGCGACATCCTCAT
Cav1.2	TGATTCCAACGCCACCAATTC	GAGGAGTCCATAGGCGATTACT

1.7 Alizarin Red Staining and Quantification

Following the previous qPCR assay, cells were treated with different inducing medium. After 21 days, mineralized deposit formation was assessed with Alizarin Red Staining. The cells were fixed by 4% paraformaldehyde for 20 min at room temperature, followed by 30 min incubation in Alizarin Red solution (1%, pH4.2) (Solarbio, China). The mineralized deposit was treated with 10% Cetylpyridinium chloride (CPC) solution (sigma, USA) at room temperature for 15min and the absorbance was measured by microplate reader (Bio-tech, USA) at a wavelength of 562 nm to harvest the quantitative comparation. Negative controls were supernatant from devoid of cells.

1.8 Data Analysis

All experiments were performed at least three times. The significance of differences between two groups was determined using Student t-tests. Differences were considered significant at $p < 0.05$.

Results

2.1 Characteristics of hDPSCs

hDPSCs were spindle-shaped showing holoclone-forming capabilities (Fig. 1A). Flow cytometry analysis showed that the cultured cells positively expressed stem cell surface markers (CD90, CD44, CD73 and

CD105), but negatively expressed the hematopoietic markers (CD34, CD11b, CD19, CD45, and HLA-D) (Fig. 1B). Alizarin Red Staining showed mineralized deposit formation after mineralized induction of hDPSCs, and Oil Red O Staining showed lipid droplets formation after adipogenic induction, which proved that hDPSCs isolated and cultured had the ability of multi-directional differentiation (Fig. 1C and D).

2.2 Elevation of cAMP promoted odontogenic differentiation of hDPSCs

To assess the role of cAMP in odontogenic differentiation of hDPSCs, cells were treated with or without Forskolin. Low concentration of Forskolin (1μM and 10μM) had no significant effect on the proliferation of hDPSCs, while the high concentrations of Forskolin (100μM) significantly inhibited the proliferation of hDPSCs (Fig. 2A). After 21 days of odontogenic induction with Forskolin (0,1,10,100μM), the positive rate of Alizarin Red Staining in all Forskolin groups was increased compared to OM and non-inducing group (DMEM) (Fig. 2B-C). The same trends were observed in quantitative analysis based on 10% CPC method. The absorbance values showed 10μM Forskolin significantly promoted mineralized deposit formation compared with 1μM and 100μM Forskolin groups. After 14 days of odontogenic induction with Forskolin (10μM), qPCR results showed the expression of *ALP*, *RUNX2*, *OSX*, *BMP2*, *DSPP* and *Cav1.2* were upregulated compared to OM group (Fig. 2D).

2.3 Inhibiting Cav1.2 attenuated the promoting effect of cAMP in odontogenic differentiation of hDPSCs

To choose the non-cytotoxic concentration for not inhibiting cell proliferation, concentrations of Nimodipine (0-100μM) were tested by CCK-8 assay (Fig. 3A). CCK-8 result demonstrated 1μM Nimodipine is a non-cytotoxic concentration for hDPSCs proliferation rather than 50μM and 100μM groups. To assess the role of the Cav1.2 in cAMP-mediated odontogenic differentiation of hDPSCs, cells were pretreated with Nimodipine (1μM) for one hour before adding Forskolin (10μM) during the process of odontogenic differentiation. Firstly, Nimodipine attenuated mineralized deposit formation and some odonogenic related gene expression in *RUNX2*, *BMP2*, *DSPP* and *Cav1.2*, compared to OM group. Thereafter, in the combination group of Nimodipine and Forskolin, both mineralized deposit and odontogenic related gene expression together with *Cav1.2* were increased compared to Nimodipine group. In spite of the Forskolin afterwards adding significantly rescued the odontogenic differentiation of hDPSCs, the reversal trends showed in both mineralized deposit and gene expression of *DSPP* and *Cav1.2* were still lower than Forskolin only (Fig. 3B-D). These results indicated inhibiting Cav1.2 attenuated cAMP stimulation on odontogenic differentiation of hDPSCs.

Discussion

Previous studies have shown cAMP actively regulated osteogenic/odontogenic differentiation of different types of stem cells[8, 20]. Whereas, the active role of cAMP has not been unidentified in hDPSCs. In this study, a small-molecule compound Forskolin was applied to clarify the effect of cAMP on odontogenic differentiation of hDPSCs. After 21 days of Forskolin treatment, Alizarin Red Staining showed all the

concentrations of Forskolin increased mineralized deposit formation. However, the amount of mineralized deposit was different depends on the Forskolin concentrations. The Forskolin higher concentration with 100μM significantly inhibited the proliferation of hDPSCs, and mineralized deposit formation was also less compared with the other lower concentration groups. This trend was consistent with a previous study from Doorn's group. They found that Forskolin increased gene expression in a dose-dependent manner and high concentration of Forskolin might impair cAMP-mediated cell differentiation by inhibiting cell proliferation[20]. In our study, 10μM Forskolin harvested the best effect in promoting mineralized deposit formation. Therefore, 10μM Forskolin was selected as working concentration of Forskolin in the following experiments. cAMP activated by Forskolin increased the gene expression of *ALP*, *RUNX2*, *OSX*, *BMP2* and *DSPP*. It further indicated that cAMP positively regulated hDPSCs odontogenic differentiation.

Cav1.2 was reported to be upregulated after acute pulp injury and involved in pulp repair and dentinogenesis[11]. Our previous study has confirmed that knockdown of Cav1.2 or treatment with the dihydropyridine compound Nimodipine generated the same effects in inhibiting odontogenic differentiation of rat dental pulp stem cells[15]. In this study, Nimodipine also significantly reduced the gene expression of *RUNX2*, *BMP2*, *DSPP*, *Cav1.2* and attenuated the ability of mineralization in hDPSCs compared to OM group. However, the changes of *ALP* and *OSX* was opposite, showing an increasing trend in the presence of Nimodipine after 14 days. The similar circumstance was also observed in the osteogenic differentiation of BMSCs which have similar biological properties to DPSCs. Study found the calcium channel inhibitor benidipine upregulated *ALP* expression in mice BMSCs[21]. Controversially, other studies reported the different trend that calcium channel inhibitor nifedipine downregulated or had no effect on *ALP* expression in rat and human BMSCs respectively[22, 23]. The reason for these controversial results still remains unclear. It might be related with the different species, different types of calcium channel inhibitors and complex regulatory mechanisms.

After Forskolin treatment, qPCR exhibited that *Cav1.2* expression was increased and showed the same trend observed in other odontogenic-related genes. This result suggested *Cav1.2* yield to cAMP regulation. In the combination groups, Forskolin was secondly added after Nimodipine, the odontogenesis-related genes reduced by Nimodipine treatment were reversely promoted. We speculate the reasons might be the following two possibilities. Firstly, Nimodipine may not have a completely inhibitory effect on Cav1.2 in the presence of Forskolin. In spite of the confirmed consensus that Nimodipine blocks calcium influx by competitively binding to the dihydropyridine site in Cav1.2[24], the increased intracellular cAMP levels could activate PKA and EPAC to phosphorylate the Cav1.2 α1c and β subunit's multiple sites to open Cav1.2 channel[25, 26]. Secondly, Cav1.2 may not be the key downstream of cAMP promoting the differentiation of hDPSCs. It has been reported that cAMP also regulated the odontogenic-related gene expression through other paths. For example, in BMSCs, cAMP could highly induce *OSX* expression and enhance its activity, thereby promoting mineralization[27]. In addition, cAMP was reported to enhanced odontogenic differentiation of apical papilla stem cells through inhibiting TGF-βI signaling[28]. In this study, it was found that Forskolin also upregulated the expression of *OSX* and

BMP2, suggesting that cAMP may affect odontogenic differentiation of hDPSCs through other signaling pathways.

It is remarkable that in our study some genes (*DSPP* and *Cav1.2*) expression were not returned to the same high level compared to Forskolin only. It is suggested inhibiting Cav1.2 could attenuate the process of odontogenic differentiation of hDPSCs regulated by cAMP. We speculated that incubation time may be a reason. In future studies, it is necessary to identify whether the gene expression levels are increasing as time going in the combination group of Nimodipine and Forskolin.

In conclusion, cAMP and Cav1.2 both participate in the odontogenic differentiation of hDPSCs, and activation of cAMP can positively regulate the expression of *Cav1.2* in odontogenic differentiation. Inhibition of Cav1.2 could not completely block the positive effect of cAMP on odontogenic differentiation of hDPSCs. The activation of cAMP could upregulate the expression of *Cav1.2* and odontogenic related genes. Therefore, *Cav1.2* was regulated by cAMP and participated in the regulation of odontogenic differentiation of hDPSCs.

Declarations

Ethical Approval and Consent to participate

The normal third molars were collected from patients (aged 18-28 years) with informed consents and approval of the Ethical Committee of Huashan Hospital, Fudan University.

Consent for publication

No applicable.

Availability of supporting data

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Changlong Jin: Methodology; Data curation; Writing-original draft; Yanqing Ju: Validation; Supervision; Shouliang Zhao: Validation; Supervision; Han Xie: Validation; Supervision; Writing-review & editing.

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Figures

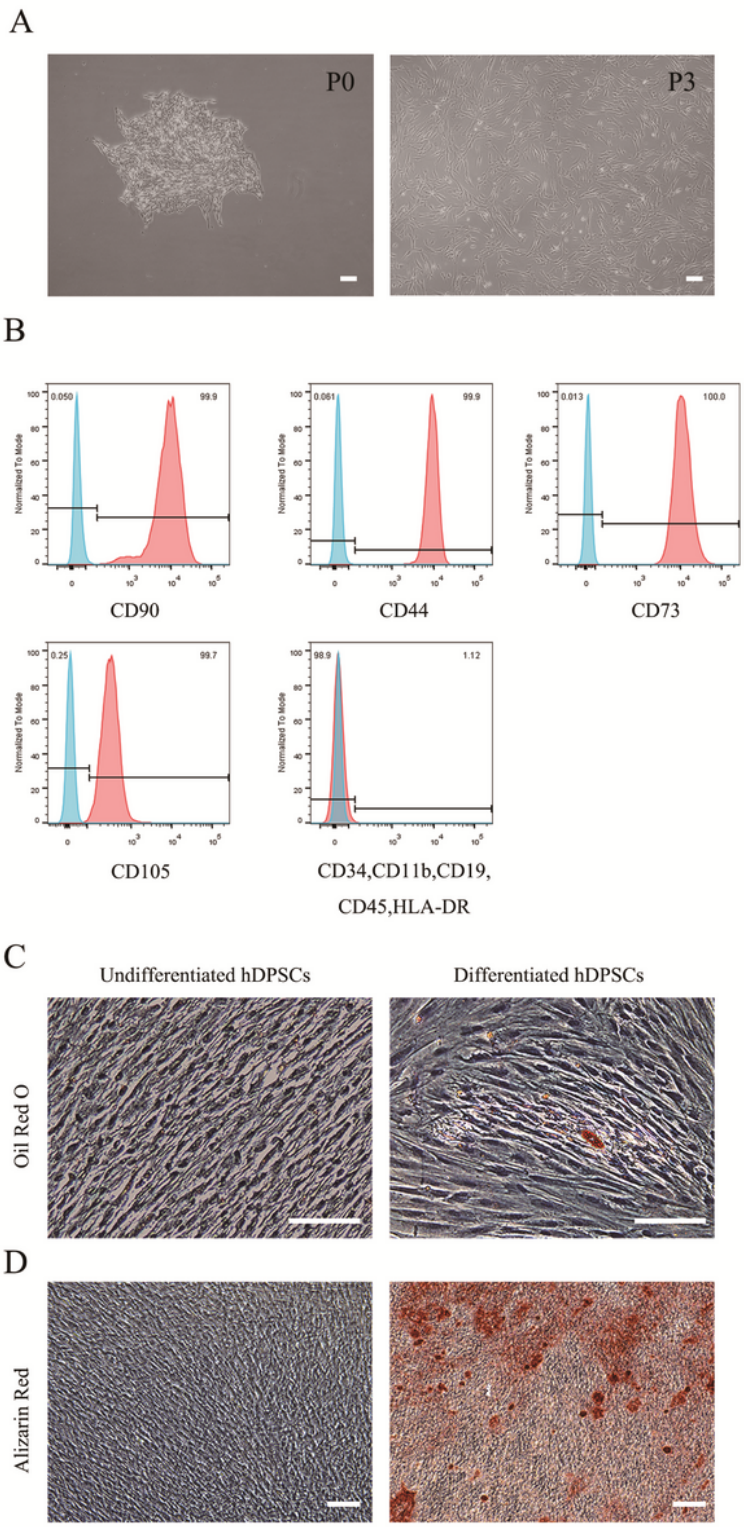

Figure 1

Characterization of hDPSCs. (A) Morphology of the hDPSCs from P0. (B) Flow cytometric analysis showed hDPSCs were positive for CD44, CD73, CD90 and CD105, and negtive for CD34, CD45, CD11b, CD19 and HLA-DR. Blue curves were control groups with only secondary antibodies, red curve indicated the experiment groups with both primary and secondary antibodies. (C) Oil Red O Staining showed lipid droplets formation after adipogenic induction of hDPSCs for 28 days. (D) Alizarin Red Staining showed mineralized deposit formation after osteogenic induction of hDPSCs for 21 days. P0, Passage 0; P3, Passage 3; Scale bar = 100μm.

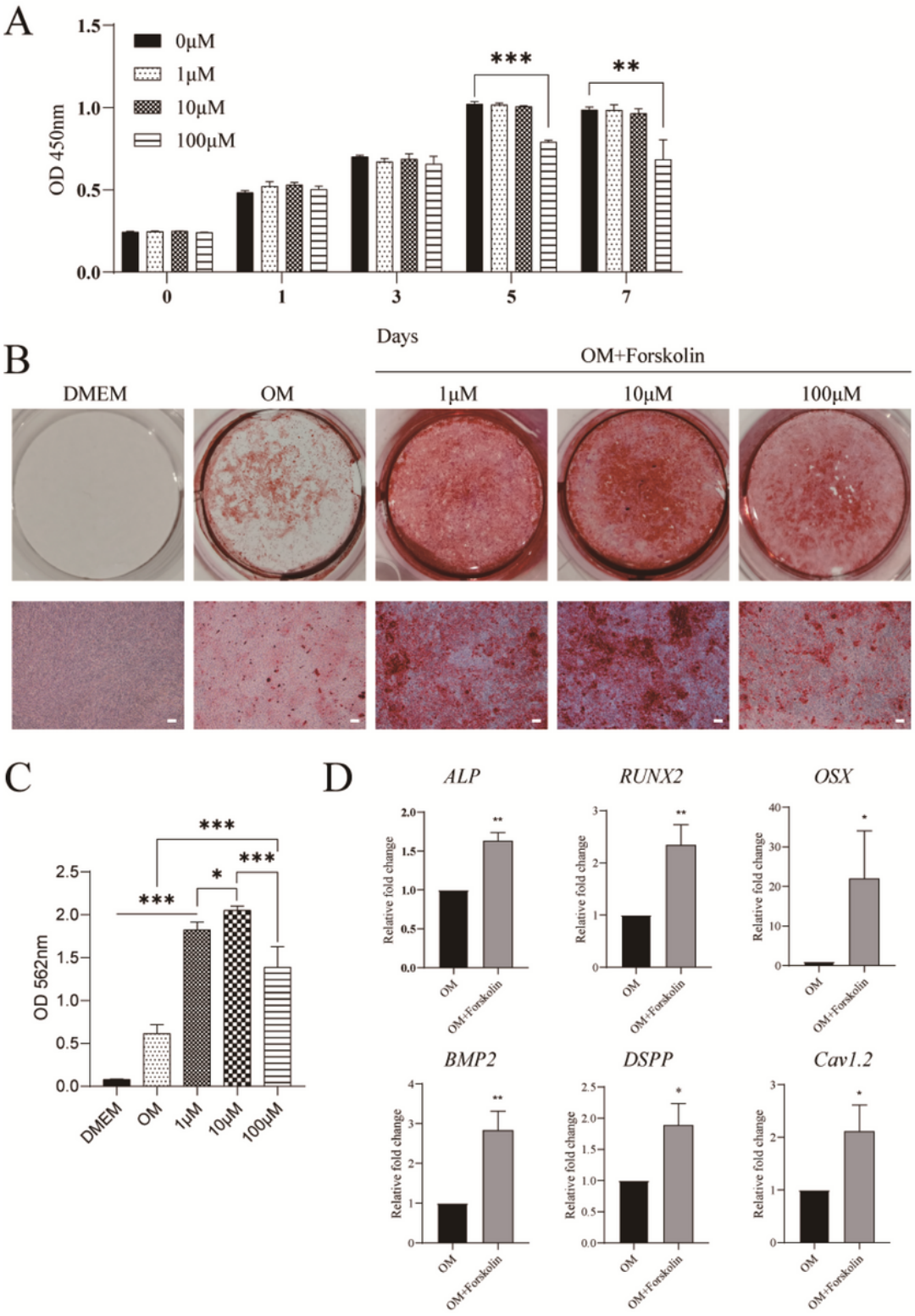

Figure 2

Forskolin promoted the odontogenic differentiation of hDPSCs. (A) CCK-8 assay showed low concentration of Forskolin (1μM and 10μM) had no inhibitory effect on hDPSCs proliferation in 7 days. (B) The mineralized deposits were stained by Alizarin Red Staining after 21 days of odontogenic induction. Mineralized deposit formation was increased in OM+Forskolin group compared to OM group (C) Quantification of Alizarin Red Staining after CPC treatment. (D) 10μM Forskolin significantly increased the expression of *ALP*, *RUNX2*, *OSX*, *BMP2*, *DSPP* and *Cav1.2* after 14 days of odontogenic induction. Scale bars = 100μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$.

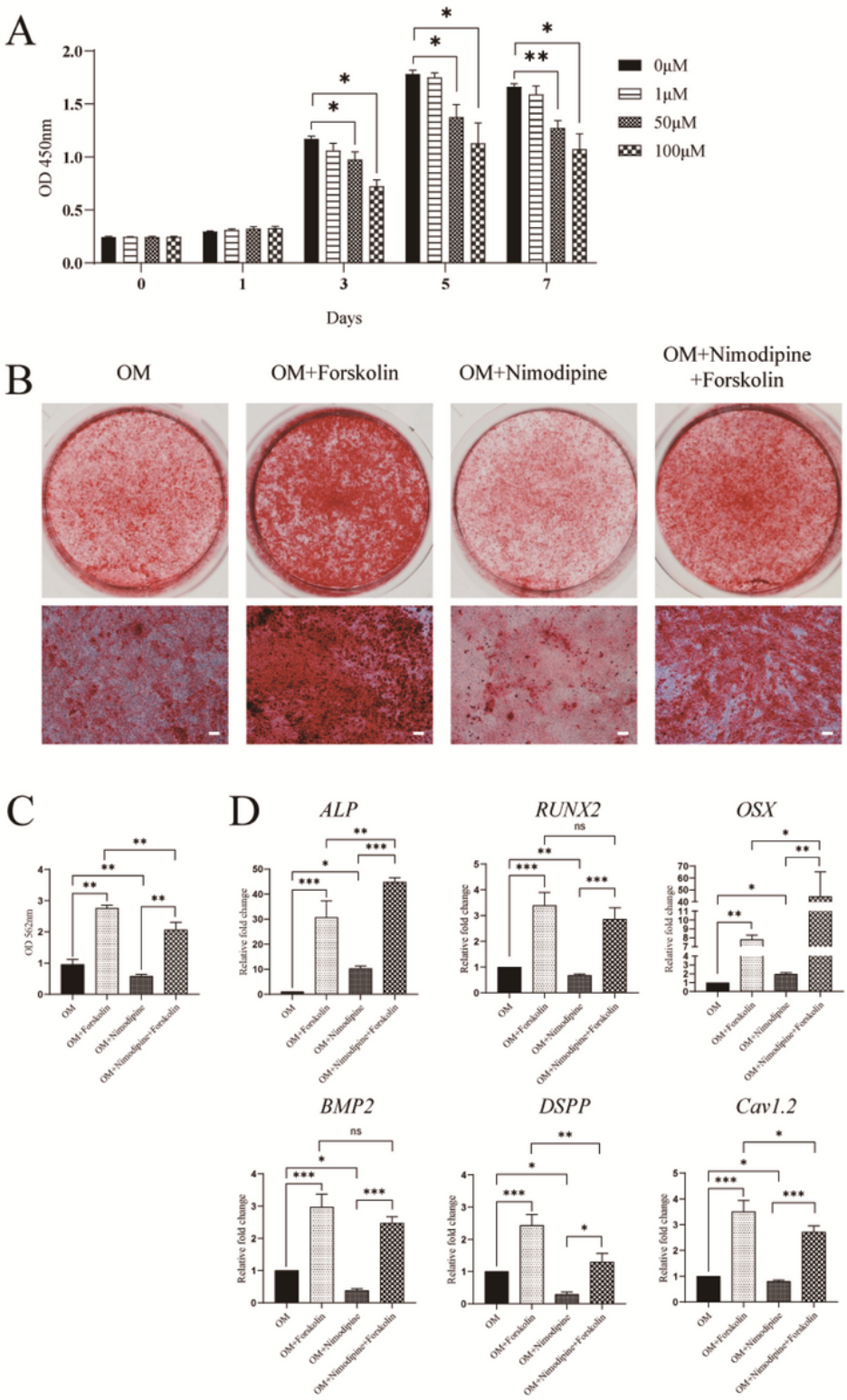

Figure 3

Inhibiting Cav1.2 attenuated the promoting effect of cAMP in odontogenic differentiation of hDPSCs. (A) CCK-8 assay demonstrated there was no significant difference in hDPSCs proliferation between 1μM Nimodipine and normal DMEM group. (B) Mineralized deposit formation was inhibited by Nimodipine, but rescued by Forskolin treatment in OM+Nimodipine+Forsklin group. However mineralized deposit still decreased in OM+Nimodipine+Forskoin compared to OM+Forskolin. (C) Quantification of Alizarin Red

Staining after CPC treatment. (D) Gene levels of *RUNX2*, *BMP2*, *DSPP* and *Cav1.2* were inhibited in OM+Nimodipine group and rescued by Forskolin treatment in OM+Nimodipine+Forskolin group. But *DSPP* and *Cav1.2* expression were still attenuated in OM+Nimodipine+Forskolin group compared to OM+Forskolin group. Scale bars = 100μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$.