Cyclic adenosine monophosphate promotes odonto/osteogenic differentiation of stem cells from the apical papilla via suppression of transforming growth factor beta 1 signaling

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Abstract

Aim: To investigate the underlining interplay of cyclic adenosine monophosphate (cAMP) and transforming growth factor-β1 (TGF-β1) on the odonto/osteogenic differentiation of stem cells from apical papilla (SCAPs).

Methodology: SCAPs were stimulated with an activator of cAMP (Forskolin), in the presence of either TGF-β1 or TGF-β1 inhibitor. The amounts of calcium mineral deposition and alkaline phosphatase activity were determined. Quantitative real-time polymerase chain reaction was performed to elucidate cAMP on the TGF-β1-mediated odonto/osteogenic differentiation of SCAPs. The effect of cAMP on the phosphorylation of Smad2/Smad3 (p-Smad2/Smad3) and extra-cellular-regulated kinase/P38 (p-ERK/P38) in the presence or absence of TGF-β1 was analyzed by Western blotting.

Results: Co-treatment with Forskolin and TGF-β1 inhibitor enhanced the alkaline phosphatase activity and deposition of calcium minerals in SCAPs. Moreover, TGF-β1 inhibitor synergized the effect of Forskolin on the expression of alkaline phosphatase and runt-related transcription factor 2. Western blotting revealed that Forskolin attenuated the unregulated expression of p-Smad3 and p-ERK induced by TGF-β1, and a cAMP inhibitor (H89) antagonized this effect.

Conclusion: This study demonstrated that cAMP signaling exerts its upregulating effects on the odonto/osteogenic differentiation of SCAPs by interfering with TGF-β1 signaling via inhibiting Smad3 and ERK phosphorylation.

INTRODUCTION

Stem cell-based tissue engineering is a promising approach for regenerating the lost tissues and restoring their physiological functions [1]. Stem cells from apical papilla (SCAPs) are a type of dental mesenchymal stem cell that resides in the root apex of a developing permanent tooth, and these cells contribute to the formation of radicular pulp and root dentin [2]. *In vitro* and *in vivo* studies have shown that SCAPs are a potential resource for dental tissue regeneration, especially pulp/dentin complex regeneration [3,4]. However, limited information is available regarding the molecular mechanism that directs the odontogenic differentiation of SCAPs.

Cyclic adenosine monophosphate (cAMP) plays a key role in regulating osteogenic differentiation and extracellular matrix production in various cell types [5, 6]. cAMP activates protein kinase A (PKA), inducing osteoblastic differentiation in bone that has sustained the secretion of bone-related cytokines, such as bone morphogenetic protein 2 (BMP-2) [7]. Previous study demonstrated that overexpression of cAMP-response element-binding protein (CREB), a downstream signaling molecule of the cAMP pathway, promotes odonto/osteogenic differentiation of SCAPs *in vitro* [8]. Similarly, activation of cAMP/PKA/CREB cascade pathway promotes osteogenesis of human mesenchymal stem cells *in vivo* [5]. Apart from cAMP signaling, the TGF-β superfamily was also reported to modulate a wide range of biological processes, including embryonic development, cell growth, differentiation, tissue repair and extracellular matrix production [9-11].

TGF-β initiates its actions by binding to TGF-receptors I and II (TβR I and II), which triggers Smad-dependent and Smad-independent pathways. When Smad3 is phosphorylated after TGF-β1 stimulation, it will then translocate into the nucleus and modulates the transcription of target genes [10]. Besides the canonical pathway, TGF-β activates mitogen-activated protein kinase signaling pathways, including c-Jun N-terminal kinase, extra-cellular-regulated kinase (ERK) and p38 independent of Smads. Recent studies suggested that TGF-β1 signaling inhibits the odontogenic differentiation of SCAPs through the activation of Smad3

[12, 13].

A study reported the interactions between the cAMP/PKA and TGF-β signaling pathways were reported [14]. One potentially important interaction is the biphasic role of cAMP in TGF-β1-induced fibrosis in Madin-Darby canine kidney (MDCK) cells [14]. Another study found that increasing cAMP reduced α-SMA expression and myofibroblast trans-differentiation that induced by TGF-β1 in rabbit keratocytes [15]. Investigations into the underlying molecular mechanism further suggested the involvement of TGF-β1 in the cAMP signaling pathway. Nonetheless, the underlying crosstalk between the TGF-β1 and cAMP signaling pathways in SCAPs remains largely unknown. Hence, this study aimed to investigate the interplay between cAMP and TGF-β1 signaling in the odonto/osteogenic differentiation of SCAPs.

MATERIALS & METHODS

Culture of stem cells from the apical papilla

Human SCAPs were cultured in six-well dishes containing the Alpha Modification of Eagle's Medium (α-MEM, HyClone, Logan, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100μg/ml streptomycin and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was changed every 3 days. After reaching 80%-90% confluence, the cells were digested with 0.25% trypsin (Gibco), passaged at a ratio of 1:3 and cultured in α-MEM containing 10% FBS. SCAPs at passage numbers from 3-6 were used for all experiments.

Characterization of SCAPs

Flow cytometric analysis were performed to identify the specific surface antigens of the cultured cells. Briefly, SCAPs were harvested with 0.25% trypsin, and approximately 1.0×10^6 cells were incubated with monoclonal antibodies specific for CD44, CD45, CD90 and CD105 (Becton & Dickinson, CA, USA) at room temperature for one hour in the dark. Then, the stained cells were washed twice with ice-cold phosphate-buffered saline (PBS) and

centrifuged at 1000 rpm for 5 min. The samples were analyzed using a Beckman Coulter Epics XL flow cytometer, and the data were analyzed with the FlowJo 7.6.5 analysis program (FlowJo, Ashland, OR, USA). To identify the multi-lineage differentiation capacity of the stem cells *in vitro*, the cells were cultured in mineralization-inducing medium, which contained 50μg/ml ascorbic acid, 10mM β-glycerophosphate, and 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS. For a period of 21 days, the cultures were stained with Alizarin Red S (Sigma) (pH 4.2) to detect mineralization. Cells were incubated in an adipogenic differentiation kit (Cyagen Biosciences, Guangzhou, China) for 28 days. The presence of lipid droplets was identified by fixing the cells in 75% ethanol and staining with 0.3% (w/v) Oil-Red O (Sigma). To induce neurogenic differentiation, the cells were cultured in Neurobasal A medium (Technologies Inc., Carlsbad, CA, USA) containing B27 supplement, 20ng/ml epidermal growth factor (EGF) (Pepro Tech, NJ, USA), 40ng/ml basic fibroblast growth factor (b-FGF) (Wako Pure Chemical, Richmond, VA, USA) for 7 days.

Immunofluorescence

Cells were fixed with 4% polyoxymethylene for 15 min, rinsed with PBS and were then permeabilized using 0.1% Triton-X100 for 10min. Primary antibodies (anti-βIII-tubulin (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added at 4℃ overnight. The samples were incubated with secondary antibody for 30 min in the dark (Alexa Fluor 488) (Abcam, Hong Kong, China).

Alizarin Red staining and alkaline phosphatase staining

SCAPs were cultured in a mineralization-inducing medium containing 10% FBS supplemented with Forskolin (1μM) in the absence or presence of TGF-β1(5ng/ml) (R&D Systems, Minneapolis, MN, USA) or specific TGF-β1 receptor Ⅰ inhibitor (SB431542) (2μM) (InvivoGen, San Diego, CA, USA). After 21 days of mineralization induction, the cells were rinsed twice with PBS and fixed with 4% polyoxymethylene for 15 min. Then, the cells were stained with 2% Alizarin Red in the dark for 45 min. Photomicrographs of the mineralized nodules were captured. To determine quantitatively the calcium content, 500μl 10% cetylpyridinium chloride (Aladdin, Shanghai, China) was added to each well to dissolve the

nodules. Aliquots (100μl) of the supernatant were measured at an absorbance of 562nm on a multiplate reader (μQuant MQX200, Bio-Tek). For alkaline phosphatase staining, SCAPs were fixed with 4%(w/v) paraformaldehyde for 15 min, rinsed with deionized water and stained with a BCIP (5-bromo-4-chloro-3-indolylphosphate)-NBT (nitrobluetetrazolium) solution (Sigma-Aldrich) according to the manufacturer's instructions for 15 min. After being washed with deionized water, the cells were observed under an inverted light microscope (Nikon). The quantification of ALP staining intensity was achieved using Quantity One 4.6.9 software (BioRad, Hercules, CA, USA).

Quantitative reverse-transcription polymerase chain reaction

The treated cells were cultured in six-well plates in a mineralization-inducing medium for the times indicated, and total ribonucleic acid (RNA) was extracted from the cells in each group using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For each sample, 500ng of total RNA was subjected to reverse transcription using the Prime Script II System (Takara, Tokyo, Japan). Quantitative real-time PCR gene expression analyses were performed with the SYBR Premix Ex Taq kit (Takara, Tokyo, Japan) using the Mx3000P Real-Time Quantitative Polymerase Chain Reaction (QPCR) System (Applied Biosystems, Grand Island, NY). The primers used for the QPCR were purchased from Sangon (Shanghai, China) and were as follows:

β-actin (Forward: GCCAAGTGGGTGGTATAGAGG, Reverse: GTGGGATGGTGGGTGTAAGAG);

Runx2 (Forward: CGCCTCACAAACAACCACAG, Reverse: ACTGCTTGCAGCCTTAAATGAC); and

ALP (Forward: CCACGTCTTCACATTTGGTG, Reverse: AGACTGCGCCTGGTAGTTGT).

The expression level of β-actin was used as an internal control. The relative gene expression values were calculated via the $2^{-\Delta\Delta Ct}$ method.

Western blot

SCAPs were serum starved for 24 hours and then pretreated with Forskolin for 1 hour in the presence or absence of cAMP inhibitor (H89, 10μmol/L, sigma). To investigate whether cAMP had an effect on the Smad-dependent and Smad-independent pathways that TGF-β1 induced, SCAPs were treated with or without Forskolin for 1 hour before TGF-β1 activated

them for another 1 hour in the presence or absence of H89. Treated cells were lysed in radio-immuno-precipitation assay lysis buffer on ice for 15 min. The denatured proteins (25μg) from each sample were separated via sodium dodecylsulfate-polyacrylamide gel electrophoresis and were then transferred onto a 0.22-μm polyvinylidene fluoride membrane (Millipore, Bedford, MA) at 200 mA for 60 min. After blocking in 5% (w/v) non-fat dried milk dissolved in Tris-buffered Saline with Tween at room temperature for 1 hour, the membrane was incubated overnight at 4°C with a primary antibody (Smad3, p-Smad3, Smad2, p-Smad2, ERK1/2, p-ERK1/2, P38 and p-P38, 1:1000, Abcam). Finally, the membrane was washed three times with Tris-buffered Saline with Tween for 10 min each before incubation in horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz) for 60 min at room temperature. The protein bands were visualized using WesternBright Quantum Western blotting detection kit (Advansta, Menlo Park, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000, Abcam) served as an internal control in these experiments.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences 24.0 (SPSS Inc., Chicago, US). One-way analysis of variance was used to compare the difference between experimental groups. The level of statistical significance for all tests was set at 0.05.

RESULTS

Identification of the SCAPs

Flow cytometry indicated that the SCAPs expressed the mesenchymal stem cell surface markers of CD90, CD44 and CD105 but were negative for the hematopoietic marker of CD45 (Fig. 1A-D). Furthermore, Alizarin Red staining revealed that the cells formed mineralized nodules after 21 days of culture in a mineralization-inducing medium (Fig. 1E). Moreover, oil red O was detected after 28 days of culture in an adipogenic medium (Fig. 1F). After inducing in neurogenic medium for 7 days, cells were positively stained with neurogenic marker (βIII-tubulin) (Fig.1G-I), indicating that the isolated cells possessed the capability of differentiating into multiple lineages.

TGF-β1pathway in the cAMP-induced odonto/osteogenic differentiation of SCAPs

To assess the role of the TGF-β1 pathway in the cAMP-mediated odonto/osteogenic differentiation of SCAPs, cells were pretreated with Forskolin in the presence or absence of TGF-β or TGF-β receptor I inhibitor SB431542, which both inhibit the activation of p-smad3 and p-ERK1/2 that TGF-β1 induced. Compared with Forskolin incubation alone, ALP activity was significantly increased in the treatment group featuring the combination of Forskolin and SB431542 (Fig. 2A, B). Consistently, the Alizarin Red staining showed that the Forskolin-treated SCAPs presented more mineralized nodules (Fig.3A). TGF-β1 impaired the cAMP-induced calcium deposition *in vitro* (Fig.3A). When the treatment was combined with SB431542, it notably enhanced the effect of Forskolin on SCAPs mineralization. The quantitative calcium measurements were consistent with the Alizarin Red staining (Fig.3B). Moreover, the positive effect of cAMP signaling on osteogenic genes was further confirmed with RT-PCR. Forskolin and TGF-β1 together inhibited the mRNA expression of the osteogenic markers, ALP and Runx2, and compared with Forskolin stimulation only (Fig.3C-D). In contrast, SB431542 significantly enhanced the promotion effect of Forskolin on ALP and Runx2 mRNA levels (Fig.3C-D). These above results indicated that the inhibition of the TGF-β1pathway largely enhanced cAMP-stimulation on differentiation in SCAPs.

Effect of cAMP on TGF-β1-induced Smad-dependent and Smad-independent pathways

Two major pathways are involved in TGF-β1-induced gene expression: the Smad-dependent pathway (Smad2 and Smad3) and Smad-independent pathway (ERK and P38). We first sought to elucidate whether cAMP signaling modulates the Smad-dependent pathway in SCAPs. As shown in Fig.4A, Forskolin alone had no effect on the phosphorylation of Smad2 and Smad3, indicating that the activation of cAMP alone did not affect Smad activity. Similarly, no significant change in the level of p-P38 was observed in response to Forskolin (Fig. 4B). However, Forskolin induced the rapid increase of the phosphorylation of ERK at 5 min, but it returned to baseline at 60 min (Fig. 4B). H89, a specific PKA inhibitor, exerted an effect against Forskolin on the expression of p-ERK (Fig. 5B). The cAMP pathway could antagonize the TGF-β-induced pathway in different cell types. Thus, whether cAMP signaling affects the

phosphorylation of Smad2, Smad3, ERK and P38 that TGF-β1 elicits were explored in SCAPs. Forskolin was added 60 min before incubation with TGF-β1 (5ng/ml). The results shown in Fig.5A indicated that the exposure of TGF-β1 resulted in the rapid increase of the phosphorylation of Smad3 and Smad2 in SCAPs, whereas cAMP directly attenuated the TGF-β1-induced activation of phosphorylation of Smad3, not Smad2. H89 completely reversed the inhibitory effect of Forskolin on the phosphorylation of Smad3 that TGF-β1 induced. In addition, TGF-β1 induced p-ERK, whereas Forskolin suppressed p-ERK in the presence of TGF-β1, and this effect was abrogated via pretreatment with H89 (Fig. 5B). Similar to p-Smad2, TGF-β1 rapidly activated p-P38, but Forskolin had no effect in the presence or absence of H89 (Fig. 5B). Collectively, these data indicated that cAMP signaling suppressed the TGF-β1pathway via Smad3 and ERK, not Smad2 and P38.

DISCUSSION

Signaling pathways have been reported to intricately regulate the process of stem cells' differentiation [16, 17]. cAMP, a second messenger is implicated as a modulator of cell growth and differentiation in several cell types [18, 19]. Activation of cAMP pathway could enhance the osteoblastic differentiation of precursor cells and promote human mesenchymal stem cells (hMSCs) to form robust bone *in vivo* [7]. In the present study, elevating cAMP levels through Forskolin treatment consistently increased the calcified nodule formation. In addition, it also increased the mRNA expression of ALP and Runx2. ALP is an early marker of osteogenic differentiation and hard tissue formation [20]. Runx2 is one of the most important transcription factors involved in osteogenic differentiation and plays a crucial role in early osteogenic differentiation [21]. Thus, the results suggested that cAMP contributes to the odonto/osteogenic differentiation of SCAPs.

Studies reported that TGF-β1 inhibits the differentiation of SCAPs in a Smad3-dependent manner [12, 13]. Besides, TGF-β1 and cAMP signaling pathways exerted mutual inhibition in murine $CD4^+$ T cells and human dermal fibroblasts [22, 23]. Therefore, it is essential to study the exact role of cAMP signaling in the TGF-β1-mediated odonto/osteogenic

differentiation of SCAPs. Using Forskolin, the activation of cAMP signaling attenuated the inhibitory effect of TGF-β1 on Runx2 and ALP mRNA expression and matrix mineralization were found, which was further confirmed via Alizarin red staining. However, inhibition of TGF-β1 signaling with SB431542 enhanced the increasing effect of cAMP on SCAPs. The results suggested that cAMP signaling may promote the odonto/osteogenic differentiation of SCAPs via the inhibition of the TGF-β1 pathway. Additionally, the cAMP activation failed to regulate the phosphorylation of Smad2, Smad3 and P38. Strikingly, after stimulation with Forskolin for 5 min, cAMP significantly induced the phosphorylation of ERK. Then, the effect of cAMP activation on the Smad-dependent and Smad-independent pathways induced by TGF-β1 was investigated. The data showed that Forskolin repressed the TGF-β1-induced phosphorylation of Smad3 and ERK, which was reversed by H89. These results indicated that cAMP co-treatment significantly attenuated the TGF-induced phosphorylation of Smad3 and ERK through cAMP-dependent PKA activation. Thus, these results suggest a proposed model of the effects of inhibiting TGF-β1 promotes cAMP signaling in odonto/osteogenic differentiation in SCAPs via Smad3 and ERK (Fig. 6).

Studies indicated that a complex interaction takes place between the TGF-β1 and cAMP/PKA signaling pathways during a variety of physiological and pathological processes in different cell types [22-25]. Most of the investigations have suggested an inhibitory relationship between the cAMP and TGF-β1signaling pathways [22-24]. Forskolin inhibits the profibrotic effects of TGF-β1in cardiac fibroblasts largely through inhibiting ERK phosphorylation but also by reducing the Smad-mediated recruitment of transcriptional co-activators [24]. Increased intracellular cAMP prevented the TGF-β1-induced interaction of Smad3 with its transcriptional co-activator, cAMP-response CREB-binding protein (CBP)/p300 [24]. Therefore, the cAMP pathway is thought to be a potent but differential and promoter-specific regulator of the TGF-β-mediated effects involved in extracellular matrix homeostasis [23,24]. Additionally, activation of cAMP pathway increased proliferation of retinal pigment epithelium, partly due to the inhibition of TGF-β1 signaling. It was possible that increasing cAMP antagonized the MAPK/ERK signaling cascade, leading to blocking the TGF-β1's effect [25]. Besides, the activation of cAMP decreased Smad3 mRNA and protein

levels via PKA, leading to resistance to TGF-β1-induced apoptosis in adrenocortical cells [26]. In contract, a recent study suggested that the cAMP signaling pathway directly accelerates the production of TGF-β1 in MDCK cells, but when TGF-β1 and its downstream pathways are highly expressed, cAMP negatively regulates TGF-β1-induced p-ERK [14]. The apparent discrepancies of these observations can be related to cell type, culture micro-environment and the sensitivity and complexity of signaling pathways. Meanwhile, further studies are essential to investigate the detailed molecular mechanisms underlying the interactions between TGF-β1and cAMP/PKA signaling.

CONCLUSION

The present study demonstrated that the inhibition of TGF-β1 promotes cAMP signaling in odonto/osteogenic differentiation in SCAPs via Smad3 and ERK. Regulating cAMP and/or TGF-β1 signaling in SCAPs could be a potential strategy for enhancing dentin regeneration for dental tissue engineering.

ACKNOWLEDGEMENT

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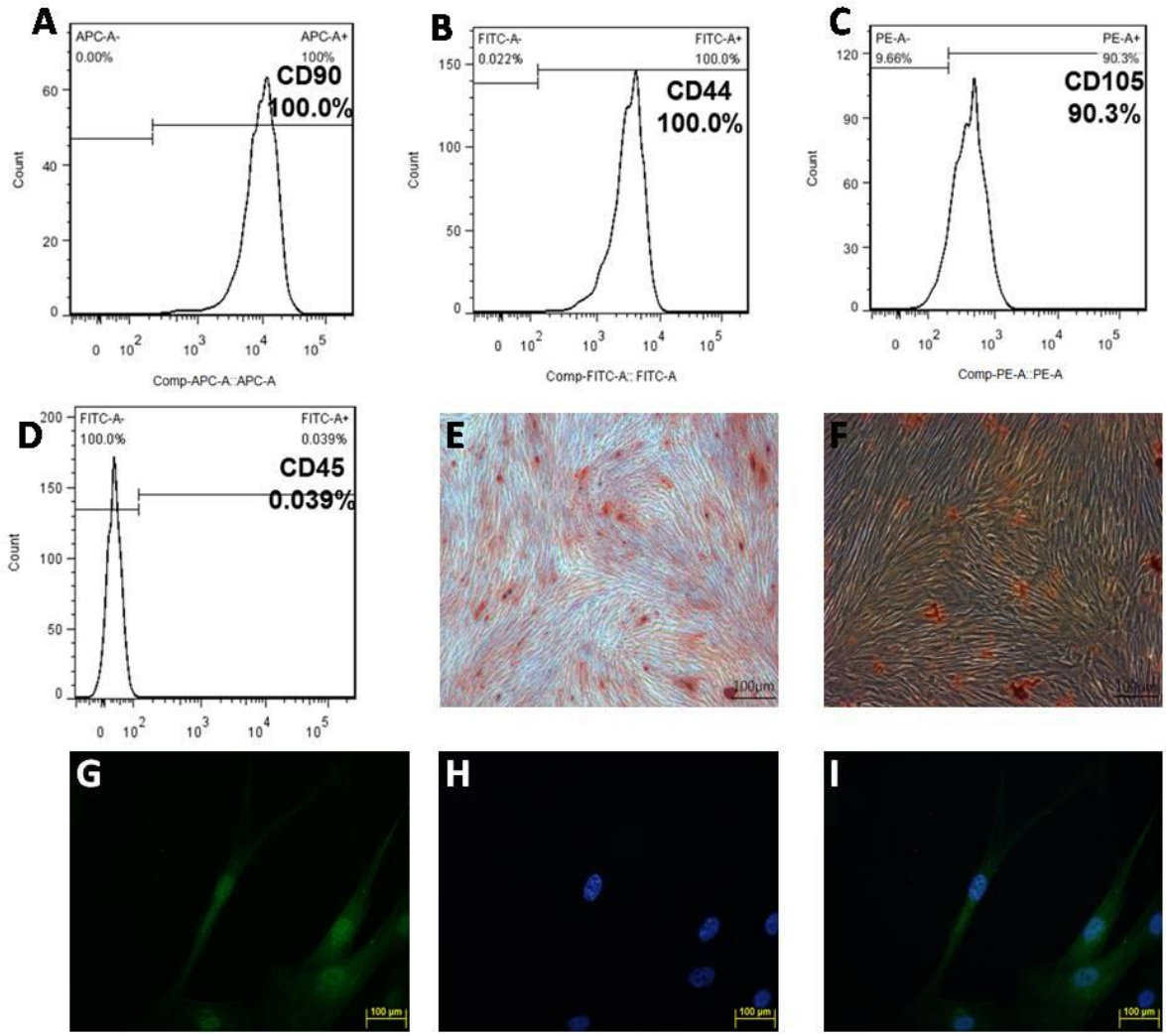
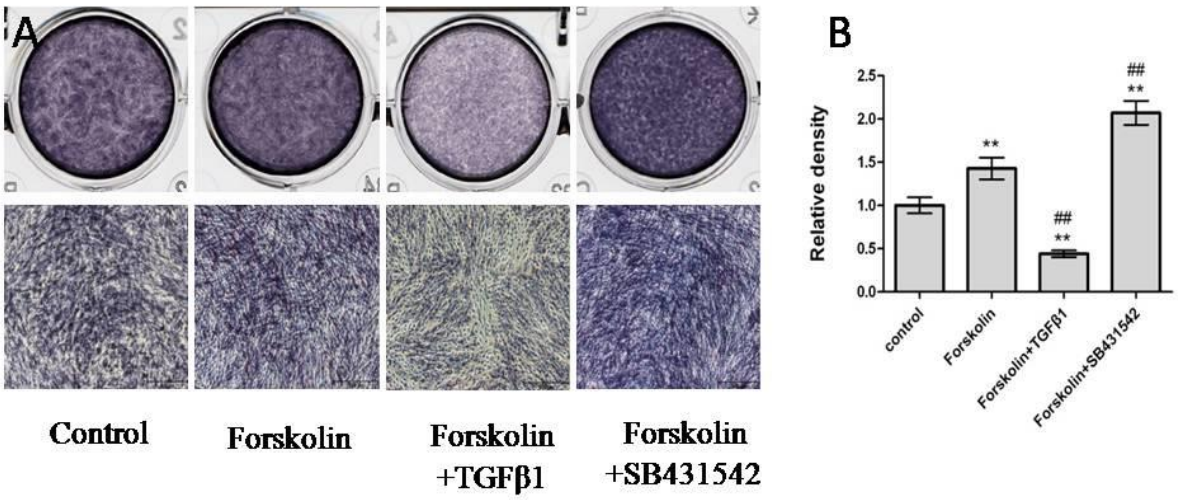
Figure 1 Characterization of the isolated SCAPs



(Figure 1E – I scale bars: 100 μm)

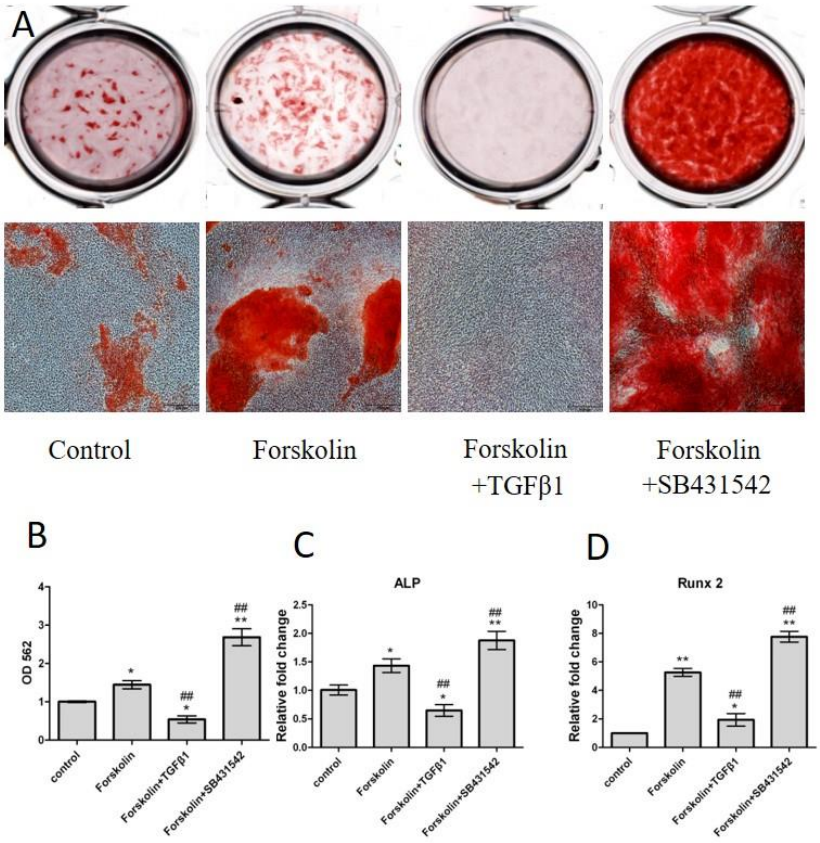
(A-D) The flow cytometric analysis revealed that the cultured SCAPs were positive for CD90 (100.0%) (Figure 1A), CD44 (100.0%) (Figure 1B) and CD105 (90.3%) (Figure 1C) but were negative for CD45 (0.039%) (Figure 1D). (Figure 1E showed Alizarin Red S staining for mineralized nodules after the SCAPs were cultured in osteogenic-inducing medium for two weeks. Figure 1F showed that oil red O staining revealed lipid droplets in the SCAPs after adipogenic induction for four weeks). Figure G-I: Immunofluorescence staining of neurogenic marker (βIII-tubulin) (green). The cells nuclei were stained blue with 4', 6-diamidino-2-phenylindole (DAPI).

Figure 2 Effect of cAMP on ALP activity



(Figure 2A Scale bars: 100 μm). The cells were cultured in a mineralization-inducing medium containing Forskolin (1μM) in the absence or presence of TGF-β1 (5ng/ml) or SB431542 (2μM). Figure 2A showed that ALP activity was stained with BCIP-NBT solution. Figure 2B is a quantitative analysis of the ALP staining. $^{**}p<0.01$ when compared with the control; $^{\#\#}p<0.01$ when compared with the Forskolin group.

Figure 3 Involvement of TGF-β1 signaling in cAMP-inducedodonto/osteogenic differentiation of SCAPs. (Figure 3A Scale bars: 100 μm).



The cells were cultured in a mineralization-inducing medium containing Forskolin (1μM) in the absence or presence of TGF-β1 (5ng/ml) or SB431542 (2μM). Figure 3A demonstrated that calcium depositions were assayed via Alizarin Red staining. Figure 3B is a quantitative analysis of the Alizarin red staining. Figure 3C-D: Gene levels of ALP and Runx2 were assayed via RT-PCR. β-actin was used as an internal control. * $p<0.05$, ** $p<0.01$ when compared with the control; $^{\#\#}p<0.01$ when compared with the Forskolin group.

Figure 4 Effects of activation of cAMP signaling on the expression of Smad-dependent and Smad-independent pathway

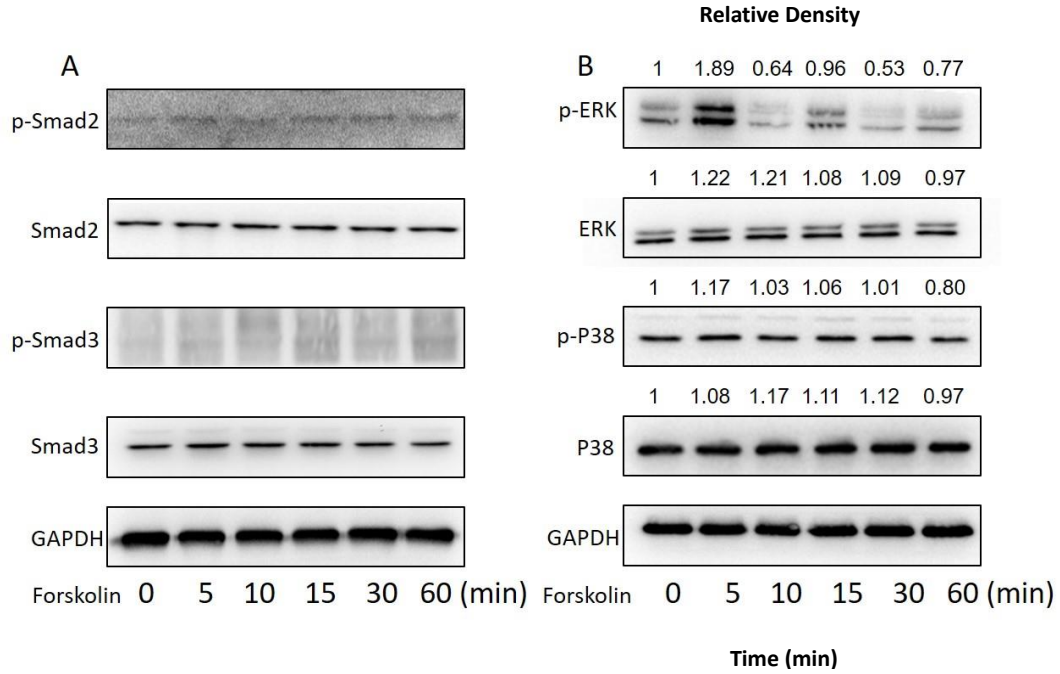


Figure 4A: Protein expression levels of Smad2 and Smad3 were determined by Western blotting. Figure 4B: Protein expression levels of ERK and p38 were determined by Western blotting. GAPDH was used as a control. The numbers above the bands denote the relative density values.

Figure 5. Inhibitory effect of cAMP on TGF β1-induced Smad-dependent and Smad-independent pathways

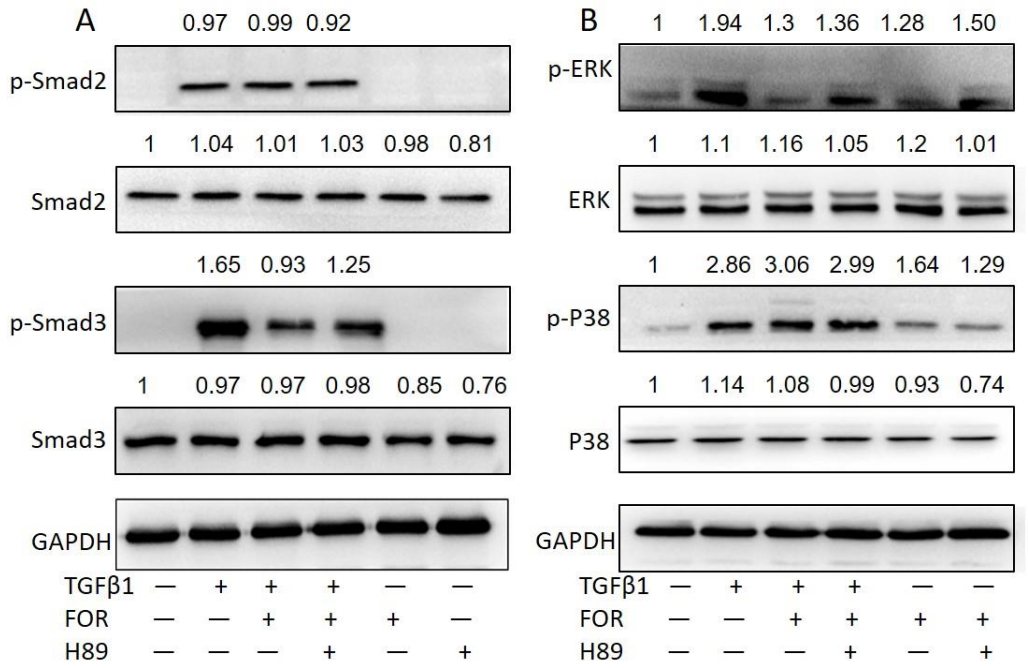
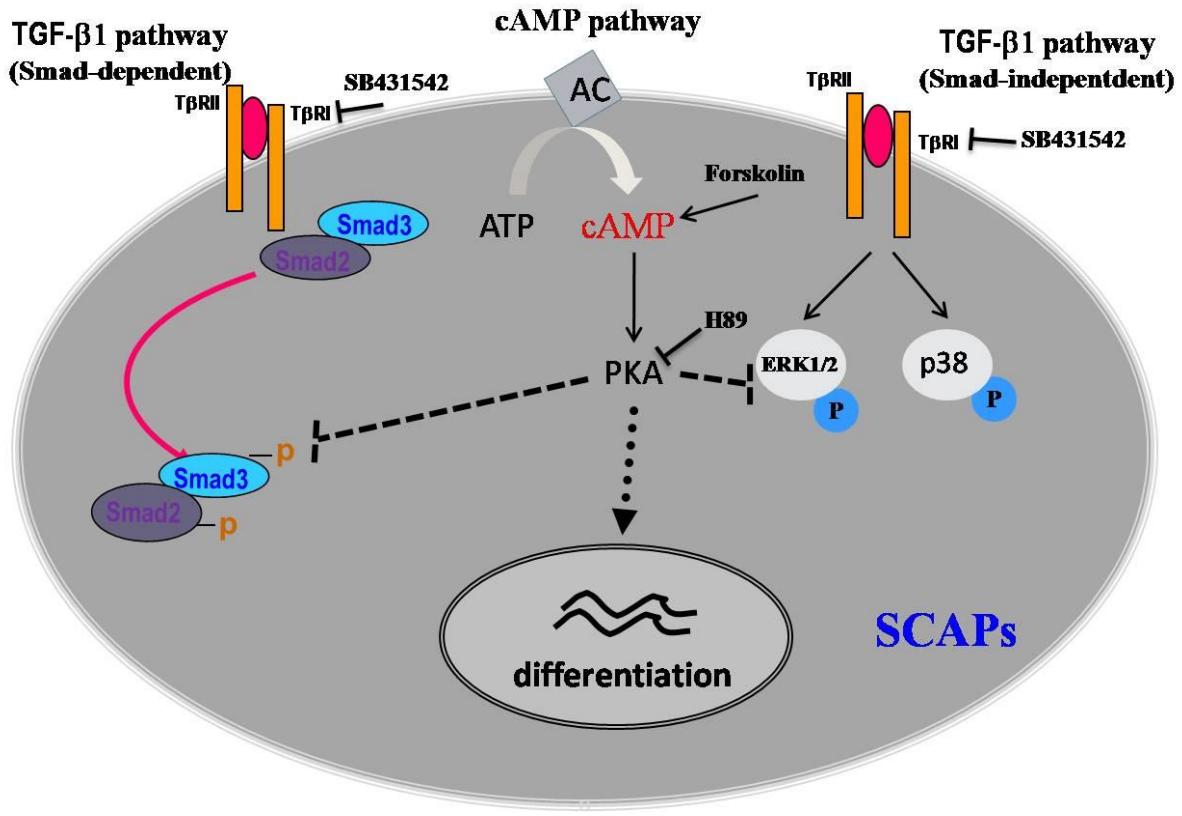


Figure 5A: Protein expression levels of Smad2 and Smad3 were determined by Western blotting. Figure 5B: Protein expression levels of ERK and P38 were determined by Western blotting. The numbers above the bands denote the relative density values. FOR:Forskolin.

Figure 6 Interplay of cyclic adenosine monophosphate (cAMP) and transforming growth factor-β1 (TGF-β1) in the differentiation of stem cells from apical papilla (SCAPs)



Legend: cAMP induced the odonto/osteogenic differentiation of SCAPs via suppression of ERK1/2 and Smad3 induced by TGF-β1.

AC: adenyly cyclase.

ATP: adenosine triphosphate

Forskolin: an activator of cAMP;

SB431542: TGF-β1 receptor inhibitor;

PKA: cAMP activates protein kinase A

H89: an inhibitor of PKA

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