



Molecular Mechanisms Underlying the Short-Term Intervention of Forskolin-Mediated Bone Regeneration

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Abstract

Purpose Researchers have actively investigated different treatment strategies to heal bone diseases and injuries. Small molecule-based compounds enhancing osteogenesis have been adapted as one of the promising approaches to treat various bone disorders. Recent studies have demonstrated several small molecule-based compounds were able to induce long-lasting osteogenic effects (i.e., up to 21 days) following a relatively short 24-h exposure. For instance, previous work from our group revealed that 24-h forskolin exposure induced *in vitro* osteoblastic differentiation of adipose-derived stem cells (ADSCs) and bone marrow-derived stem cells (BMSCs). However, the molecular link underlying the aforementioned studies has not been described.

Methods Osteoprogenitor MC3T3-E1 cells were used to study the molecular mechanisms by which the short intervention of forskolin could lead to long-lasting osteogenesis.

Results We report here the observed effects were associated with upregulation of phosphorylated cAMP-response element binding protein (pCREB) and β -catenin. We also found a number of osteogenic genes were upregulated via PKA-dependent and PKA-independent pathways. We also discovered that the 24-h forskolin treatment scheme could induce osteoprogenitor MC3T3-E1 cells to endogenously produce their own osteogenic and angiogenic growth factors such as insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF).

Conclusion We propose that an early specific signal mechanism (i.e., cAMP signaling in this study) is important for triggering a bone-regenerating program. In addition, the small molecule forskolin may be capable of inducing cell differentiation towards osteogenic lineage utilizing growth factor-based inductive paracrine and autocrine loops. Thus, these two mechanisms may contribute to the aforementioned observation of the short-term forskolin-mediated bone regeneration.

Lay Summary Small molecule-based therapeutics have emerged as an alternative to traditional growth factor treatments such as recombinant human bone morphogenetic proteins (rhBMPs). In order to reduce the side effects of these treatments, researchers have evaluated approaches focusing on reducing the dosage and frequency of administration. This study describes the underlying behavior of this small molecule treatment approach in promoting bone formation within cells.

Future Work Future studies will focus on investigating the instructive set of signals (e.g., using RNA-seq or DNA microarray) that specifically trigger of MSCs upon short-term treatment of forskolin.

Keywords Growth factors · Signal transduction · Small molecules · Bone regeneration · Forskolin

Introduction

Bone fractures can affect people at all ages, often too severe to heal by itself [1, 2]. A few protein-based therapeutics are currently used to treat bone injuries and disorders; however, these approaches are often associated with many undesirable

side effects [3–5]. Small molecule-based bone regenerative engineering has been proposed as a promising strategy to repair various musculoskeletal tissues [6–8]. Advanced materials coupled with small molecule technologies, stem cell sciences, and controlled drug-delivery approaches can be utilized to facilitate bone regeneration [9]. However, large dosages and prolonged administration of small molecules can result in undesired adverse effects [10]. A new concept has been postulated, in which a short-term treatment (i.e., less than 24 h of exposure) of cells with osteogenic small

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molecules was recently envisioned [11]. The findings suggest that early, targeted interventions may overcome the need for ongoing therapies. Recent work has demonstrated the capability of regenerating an amputated adult African clawed frog hind limb using a five-small molecule drug cocktail after just 24 h of exposure [12]. This short-term treatment scheme enhanced the endogenous regenerative capability of the frogs over an 18-month time period, resulting in the regrowth of a functional limb.

Activation of the cyclic AMP pathway with various cyclic AMP (cAMP) analogues or cAMP stimulators has been shown to enhance osteogenic differentiation both in vitro and in vivo [13, 14]. However, the major limitation of using these compounds is their potential cytotoxicity, as well as inhibitory effect, on the proliferation of stem cells [15]. Thus, there is a strong need to identify an improved approach that can augment bone-healing efficacy while minimizing the adverse effects and costs. Previous studies from our group have investigated whether fine-tuning the timing of cAMP signaling activation could enhance bone formation with minimal cytotoxicity effects in cells [16–18]. Interestingly, recent studies revealed that the exposure of forskolin, an inducer of intracellular cAMP, for 1 day promoted a long-lasting osteogenic effect on rabbit MSCs in vitro [19]. It is known that forskolin increases the intracellular concentration of cAMP by activating the enzyme, adenylate cyclase [20]. Forskolin has also been used as a natural remedy for treatment in obesity, cancer, glaucoma, asthma, and allergies [21, 22]. However, the underlying mechanisms responsible for the aforementioned observations towards osteogenic differentiation remain unclear. In this paper, we investigate the mechanisms by which the brief exposure of forskolin could lead to long-term in vitro bone regeneration and growth.

Materials and Methods

Reagents

Forskolin and dimethyl sulfoxide (DMSO) were purchased from MilliporeSigma (St. Louis, MO). H-89 dihydrochloride was purchased from Fisher Scientific (Pittsburgh, PA). Phospho-CREB (Ser133), CREB (48H2), phospho- β -catenin (Ser552), and β -catenin (6B3) were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-rabbit IgG H&L (HRP) (ab6721) and goat anti-mouse IgG H&L (HRP) (ab6789) were purchased from Abcam (Cambridge, UK). Phosphate-buffered saline (PBS), alpha minimum essential medium (α -MEM), fetal bovine serum (FBS), penicillin–streptomycin (P/S), and 0.25% trypsin–EDTA solution were purchased from Gibco (Grand Island, NY). Chemiluminescent substrate for the detection of HRP and

X-ray films was purchased from Thermo Fisher Scientific (Waltham, MA).

Cell Culture

Osteoblast-like MC3T3-E1 cells (passages 22–32; ATCC, Manassas, VA) were used for the in vitro cell studies. The cells were cultured in α -MEM supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin), and maintained in a humidified incubator at 37 °C containing 5% CO₂. Forskolin at 100 μ M was added to the culture medium at the time of cell seeding. For the intracellular cAMP and western blotting studies, the cells were treated with forskolin for 30 min before cell lysis. For the qRT-PCR and ELISA studies, the cells were treated with forskolin for 24 h and the medium was replaced with fresh culture medium without the drug supplement. The medium was changed 3–4 days until each experimental time point was reached. In the PKA inhibition experiments, the cells were pre-treated with H-89 (30 μ M) for 30 min before supplementation with forskolin. Forskolin was dissolved with DMSO; therefore, DMSO treatment was used as the negative control group.

Measurement of Intracellular cAMP Levels

The levels of intracellular cAMP produced by the MC3T3-E1 cells after treatment with forskolin were measured through a cAMP Parameter Assay Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. After completion of the assay, the plates were read at an absorbance of 450 nm using a plate reader (BioTek, Winooski, VT). The concentration of the produced cAMP (pmol/ml) was determined from a standard curve.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

PAGE and western blotting was performed as previously described [23].

Quantitative Real-Time PCR (qRT-PCR)

The gene expression of runt-related transcription factor (runx2), alkaline phosphatase (ALP), collagen I (col1a1), and osteopontin (spp1) of MC3T3-E1 cells after short-term treatment with forskolin was measured using the quantitative reverse transcription polymerase chain reaction (qRT-PCR). The cells were washed once with 1 \times PBS and the total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. After isolation, the complementary DNA was synthesized using the RNA to cDNA EcoDry Premix (Clontech, Mountain

View, CA). Real-time PCR was performed using a light cycler instrument (Bio-Rad iCycler iQ System, Hercules, CA) along with Taqman Gene Expression Assays (Applied Biosystems, Guilford, CT) and SuperMix Premix (Biorad, Hercules, CA). The relative fold expression of the genes of interest normalized to the housekeeping gene, GAPDH, was calculated using the $\Delta\Delta CT$ method.

Enzyme-Linked Immunosorbent Assay (ELISA)

The secretion of VEGF and IGF-1 from the MC3T3-E1 cells after 24 h treatment with forskolin was quantified using a mouse VEGF and human IGF-I/IGF-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The absorbance of the assay product solution was measured using a plate reader (BioTek, Winoski, VT) at a wavelength of 450 nm. The resulting absorbance was converted to VEGF or IGF-1 concentration (pg/ml) using the provided standards in the kits.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software; San Diego, CA) was used for statistical analysis and graph design. Sample sizes of at least $n=3$ were used per study. Quantitative data was reported as mean \pm standard deviation. Comparisons between two groups was determined using the unpaired Student's *t*-test. Comparisons between more than two groups were conducted using one-way ANOVA with Tukey post hoc test. Statistical significance was evaluated at $*p < 0.05$, $**p < 0.01$; $***p < 0.001$, and $****p < 0.0001$.

Results

Short-Term Treatment of Forskolin Increases Intracellular cAMP Production and Induces Phosphorylation of CREB and β -Catenin Through PKA-Dependent Signaling

Forskolin has been shown to increase intracellular cAMP through the activation of the catalytic enzyme, adenylyl cyclase, in mammalian cells [20]. To confirm the production of intracellular cAMP within MC3T3-E1 osteoblast-like cells after short-term exposure of forskolin, a cAMP parameter assay of the collected cell lysate was conducted (Fig. 1). After 30 min of forskolin treatment, there was a statistically significant increase in intracellular cAMP concentration compared to the DMSO control. Approximately 93.60 pmol/ml of cAMP was produced in the forskolin-treated cells, while approximately 5.09 pmol/ml of cAMP was observed in the DMSO group, representing the basal levels of cAMP within the cell.

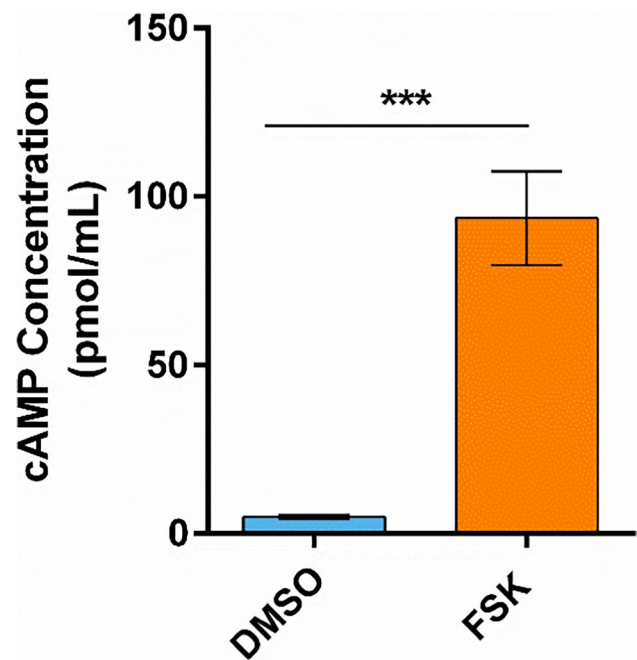


Fig. 1 Short-term treatment of forskolin increased intracellular cAMP production in MC3T3-E1 cells. The cells were treated with DMSO or forskolin (100 μ M) for 30 min. The cells were lysed and the concentration of cAMP produced within the cells was measured through ELISA ($n=3$). Bars are sample means, and error bars denote standard deviation. Comparison between the two means was determined using the unpaired Student's *t*-test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

The protein kinase A (PKA) and wingless-related integration site (Wnt) signaling cascades are fundamental pathways that are important in the production of mineralized bone. To determine whether the phosphorylation of CREB and β -catenin in MC3T3-E1 cells was mediated through the PKA-dependent signaling pathway as evidence for PKA and Wnt signaling crosstalk has been reported both during osteogenesis, western blot analysis with the PKA inhibitor, H-89, was performed (Fig. 2). H-89 is a competitive antagonist of the ATP binding sites on the catalytic subunits of PKA, which prevents the subsequent phosphorylation of PKA substrate targets [24]. When compared to the DMSO-treated control, a higher amount of pCREB was detected in the MC3T3-E1 cell when treated with forskolin for 30 min (Fig. 2a). However, when the cells were pre-treated with H-89, and then subsequently treated with forskolin, the amount of pCREB returned to the DMSO control levels. A similar trend was observed in the phosphorylation of β -catenin (Fig. 2b). Upon exposure to forskolin, a significant upregulation of phospho- β -catenin was observed compared to the DMSO-treated cells. In contrast, when the PKA activity of the cells was inhibited with H-89, there was no significant difference in the levels of phospho- β -catenin compared to the control group.

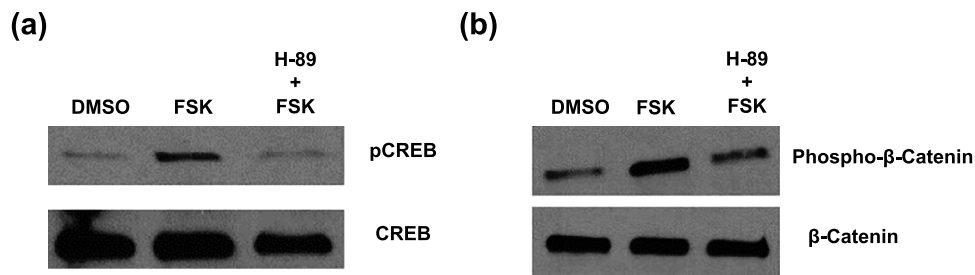


Fig. 2 Short-term treatment of forskolin induces the phosphorylation of **a** CREB and **b** β -catenin in osteoblast-like cells through the PKA signaling pathway. Western blot analysis was used to detect the levels

of pCREB (Ser133), phospho- β -catenin (Ser552), CREB (48H2), and β -catenin (6B3) in the cell lysates. The total CREB and β -catenin levels were used to confirm equal protein loading

Forskolin Modulates Osteogenic Gene Expression of MC3T3-E1 Cells Through Short-Term Administration

To investigate whether the short-term treatment of forskolin modulates osteogenic gene expression in MC3T3-E1 cells through PKA-dependent signaling, qRT-PCR of the collected cell lysates was conducted (Fig. 3). ALP is one of the earliest markers of osteogenic differentiation [25]. At day 3, a statistically significant increase in ALP gene expression was observed in the cells treated with forskolin for 24 h compared to the DMSO control group (Fig. 3a). However, upon the addition of H-89 to inhibit PKA activity, a statistically significant decrease in ALP gene expression was observed at day 3. At day 7, there was also a significant reduction in ALP gene expression in the H-89 and forskolin-treated cells compared to the cells treated with forskolin alone. This data suggested that the gene expression of ALP is PKA dependent. Runt-related transcription factor (*runx2*) is another early-stage marker that is expressed during the onset of osteogenesis [26]. At day 3, a significant decrease in *runx2* expression was observed in the forskolin-treated cells compared to the DMSO control, followed by a more pronounced decrease upon the addition of H-89 (Fig. 3b). At day 7, the reverse trend in *runx2* expression was detected, with a statistically significant increase in the cells treated with forskolin alone, as well as with H-89, compared to the DMSO-treated cells. In addition to *runx2*, collagen type I alpha 1 (*col1a1*) is an early-stage marker of osteoprogenitor cells [27]. At day 3, there was a statistically significant decrease in *col1a1* expression when the cells were treated with H-89 and forskolin compared to the DMSO or forskolin alone treatment groups, respectively (Fig. 3c). At day 7, there was a significant increase in *col1a1* gene expression in both the forskolin groups with and without H-89 treatment compared to the DMSO control. Osteopontin (OPN) is a late-stage marker of osteogenic differentiation [28]. At both day 3 and day 7, there was a statistically significant increase in OPN expression in the MC3T3-E1 cells upon exposure to forskolin for 24 h (Fig. 3d). After the addition

of H-89, there was also a significant increase detected in the OPN expression compared to the DMSO control. Interestingly, osteocalcin (OCN) was not upregulated at days 3 and 7 in the presence of forskolin in the groups both with and without H-89 treatment compared to the DMSO control (Fig. 3e). Taken together, these data suggest that the expressions of *runx2*, *col1a1*, and OPN are modulated through a PKA-independent pathway.

PKA-Dependent Secretion of VEGF and IGF-1 from Forskolin-Induced MC3T3-E1 Cells

To assess the role of PKA-dependent signaling on VEGF and IGF-1 production in MC3T3-E1 cells, we treated the cells with forskolin for 24 h both in the presence and absence of H-89 pre-treatment (Fig. 4). The activation of cAMP signaling has been shown to promote VEGF and IGF-1 secretion from osteoprogenitor cells in vitro [15, 18, 29, 30]. ELISA data from the collected cell medium revealed that forskolin at a concentration of 100 μ M significantly promoted both VEGF and IGF-1 secretion compared to the DMSO-treated cells. Yet, when forskolin was administered to the H-89 pre-treated cells, there was a statistically significant decrease in VEGF and IGF-1 secretion comparable to the DMSO control levels.

Discussion

The goal of our research is to develop a simple, safe, and cost-effective method to heal bone disorders and injuries including bone fractures. We have first proposed that short-term treatment (less than 24 h) of a number of selected small molecule drugs induces long-lasting osteogenic effects of osteoprogenitor MC3T3-E1 cells [16, 18, 31, 32]. This finding may provide a very promising approach to mitigate risks associated with small molecule-based drugs, such as non-specificity and toxicity [33, 34]. However, the underlying molecular mechanism responsible for these observations remains unclear. Our study examined the potential molecular

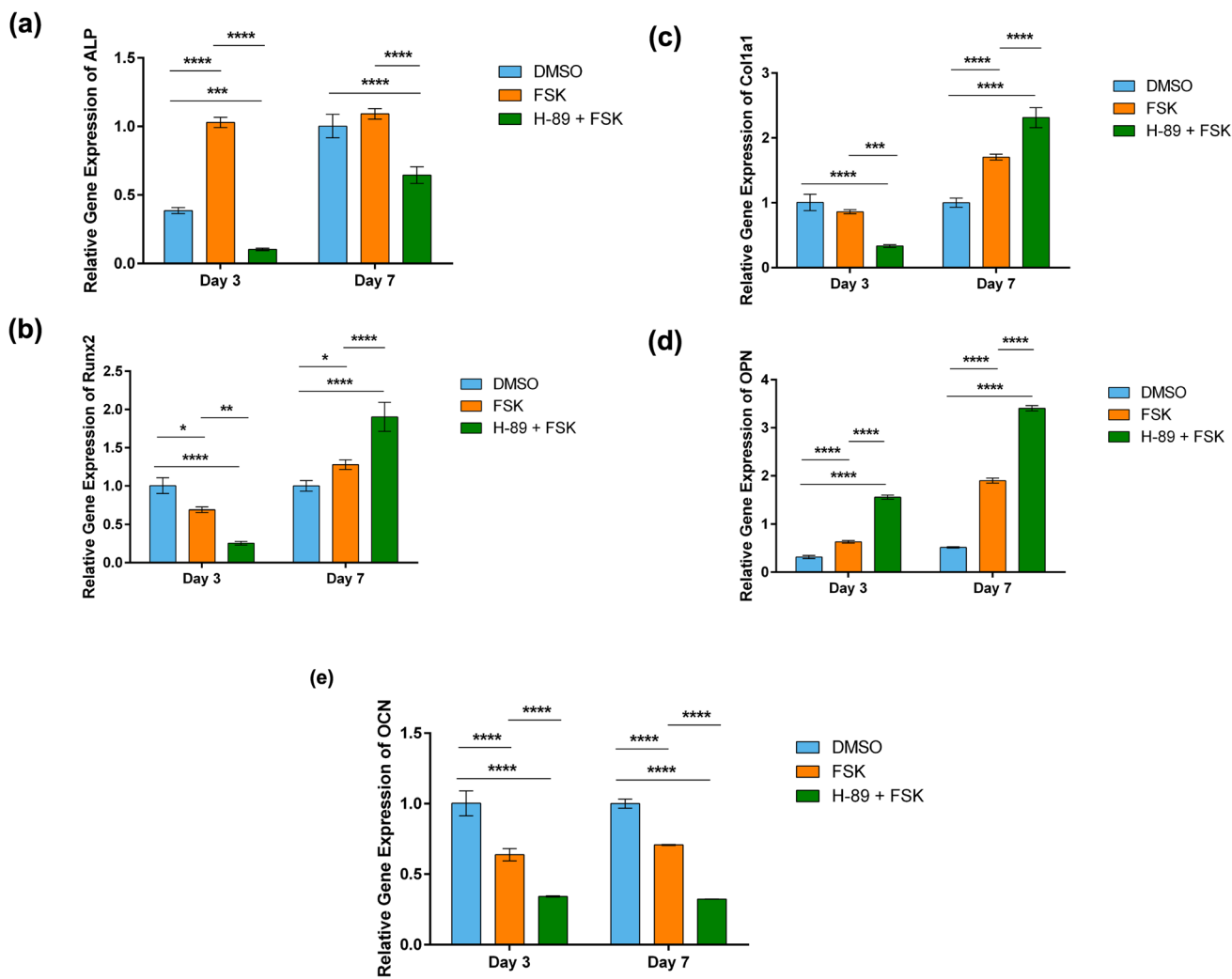
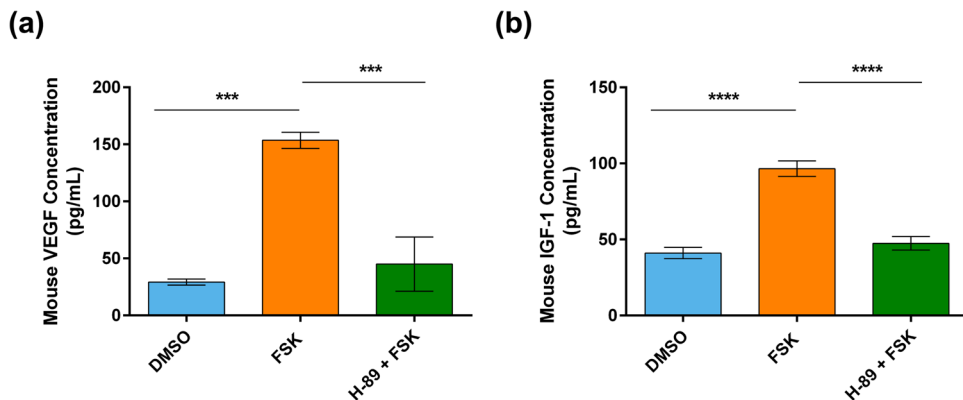


Fig. 3 Gene expression of **a** alkaline phosphatase (ALP), **b** runt-related transcription factor (runx2), **c** collagen I (coll1a1), **d** osteopontin (spp1), and **e** osteocalcin (bglap) of osteoblast-like MC3T3-E1 cells after short-term treatment with forskolin. Cells were treated with DMSO, forskolin (100 μ M), or forskolin (100 μ M)+H-89 (30 μ M)

for 24 h. After 24 h, the medium was replaced with fresh growth medium and the gene expression was measured at days 3 and 7 ($n=4$). Bars are sample means, and error bars denote standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Fig. 4 PKA-dependent secretion of **a** VEGF and **b** IGF-1 from osteoblast-like MC3T3-E1 cells after short-term treatment with forskolin. The cultured cells were stimulated with DMSO, forskolin (100 μ M), or forskolin (100 μ M)+H-89 (30 μ M) for 24 h. The media was collected and analyzed through ELISA ($n=3$). Bars are sample means, and error bars denote standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)



mechanism responsible for short-term forskolin-mediated osteogenic differentiation of MC3T3-E1 cells. A plausible mechanism has been described consisting of the activation of PKA, a key modulator of the cAMP signaling, that upregulates the phosphorylation of CREB and results in the induction of osteoblast-associated gene transcription (e.g., ALP) downstream [35–39]. The cAMP/PKA/CREB is also known as a molecular signaling pathway that is actively involved in embryogenesis, which helps the body take shape [40–42]. Thus, activation of this signaling pathway could allow the burden of growth and differentiation to be handled by the cells themselves [43]. Intriguingly, it appeared that the produced intracellular cAMP from forskolin may regulate a number of osteogenic gene transcriptions (runx2, OPN, Col1) via other cAMP/PKA-independent pathway(s). At days 3 and 7, runx2 was both downregulated and upregulated upon treatment with forskolin after PKA knockdown with H-89. Thus, suggesting runx2 expression is modulated

by a PKA-independent mechanism. It is also interesting to note that OCN was not upregulated at both time points. This observation is consistent with the reported data that OCN is rarely expressed at the early stages of osteoblastic differentiation, and that OCN can be detected in the mineralization of bone matrix [44]. A single-dose, short-term treatment of forskolin and the PKA-independent targeting analogue, 8-Br-cAMP, have both been shown to promote *in vitro* mineralization of MC3T3-E1 osteoblastic-like cells [18, 19]. Therefore, it is expected at later stages of osteogenic differentiation, OCN may be detected. The growth factors, IGF-1 and VEGF, have been shown to be important for modulating the osteoblastic and endothelial commitment of MSCs [45]. The present study also examined how short-term forskolin treatment could affect IGF-1 and VEGF production and secretion, which would both critical for osteogenesis and efficient vascularization of engineered scaffolds for bone tissue engineering applications [46].

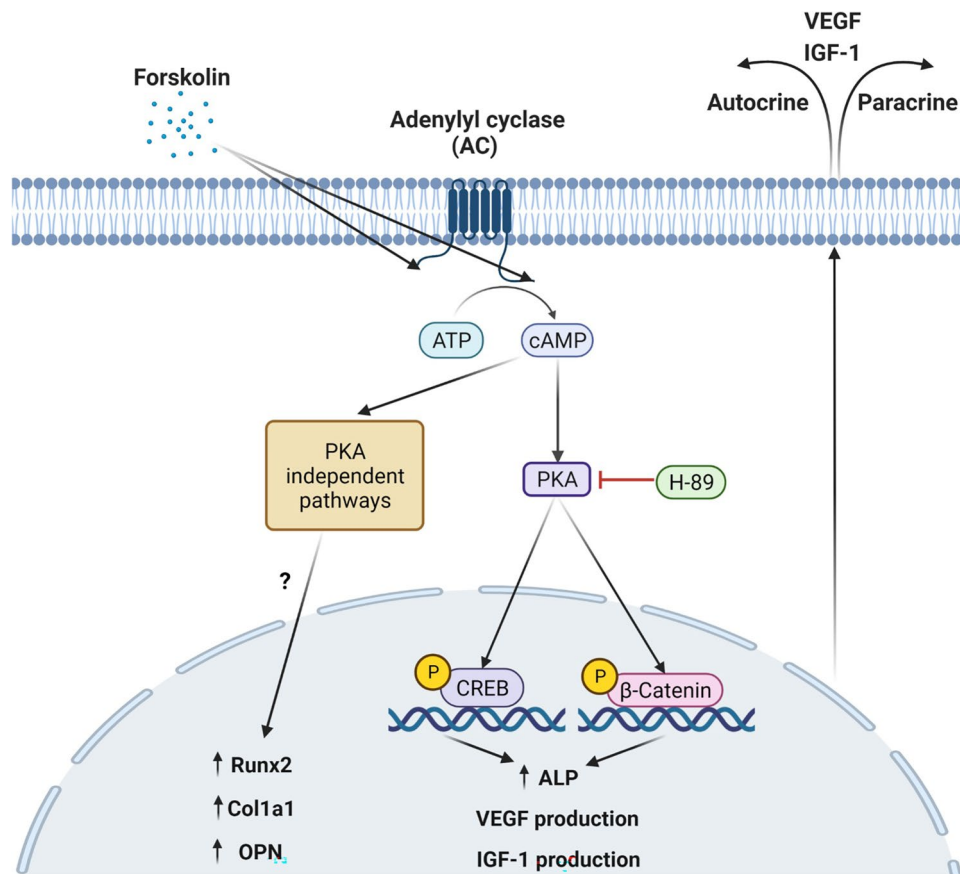


Fig. 5 Schematic representation of proposed short-term forskolin signaling pathway involved in osteogenesis of osteoblast-like MC3T3-E1 cells. The small molecule forskolin passes through the cell membrane and activates the enzyme, adenylyl cyclase, which catalyzes the conversion of ATP into cAMP. The intracellular cAMP activates PKA in the cytoplasm, which leads to the phosphorylation of CREB and β -catenin. pCREB and phospho- β -catenin will translocate from the cytoplasm to the nucleus. pCREB and phospho- β -

catenin will then directly modulate the transcription of ALP and induce production of VEGF and IGF-1. The secreted VEGF and IGF-1 can function within an autocrine/paracrine loop to interact with the host or surrounding cells. Alternatively, the produced intracellular cAMP from forskolin may also interact with other cAMP/PKA-independent pathways to upregulate or downregulate other osteogenic gene transcription within the cell's nucleus. Created with BioRender.com

The fact that it required only a short treatment of the small molecule forskolin to jumpstart a weeklong in vitro bone regenerative process suggests that mesenchymal stem cells may have latent regenerative capabilities that can be called up for action [19, 47, 48]. The results presented in this paper also suggested that small molecule forskolin could trigger long-lasting osteogenic effects on cells that is carried out by the induction of cell-based osteogenic and angiogenic growth factors production and secretion (Fig. 4). Specifically, small molecule forskolin is capable of inducing cell differentiation down to osteogenic lineages utilizing protein growth factor-based inductive paracrine and autocrine loops (Fig. 5). Thus, we speculate that a single dose of forskolin for 24 h is capable of inducing the cells to produce and secrete sufficient amounts of growth factors in the medium, towards inducing osteogenic differentiation via autocrine and paracrine loops [49]. Further studies will evaluate the dynamic of the secreted growth factors in the culture media. Taken together, we propose that 2-stage process may be required to jumpstart bone regeneration: (1) an early initiating signal mechanism (i.e., cAMP signaling) that specifically triggers a bone-regenerating program and (2) harnessing the repertoire of factors secreted by cells may provide necessary long-lasting autocrine and paracrine signaling for bone regeneration.

Conclusion

Over the past few decades, many natural small molecule-based compounds with the potential of regenerating bone tissue have been reported [32, 33]. To our knowledge, this paper provides the first evidence towards understanding the underlying mechanism of a short-term exposure of the natural small molecule forskolin towards osteoblastic differentiation of osteoprogenitor cells in vitro. Future studies will focus on investigating the instructive set of signals (e.g., using RNA-seq) that specifically trigger of cells upon short-term treatment of forskolin.

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Declarations

Conflict of Interests Dr. Cato T. Laurencin is the editor-in-chief and Dr. Kevin Lo is the assistant managing editor of Regenerative Engineering and Translational Medicine. Dr. Cato T. Laurencin has the following competing financial interests: Mimedx, Alkermes Company, Biobind, Soft Tissue Regeneration/Biorez, and Healing Orthopaedic Technologies-Bone. The authors have no non-financial competing interests.

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