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ABSTRACT

Recent studies suggest that the elevation of intracellular cyclic adenosine monophosphate (cAMP) and the activation of the protein kinase A regulate BMP-induced osteogenesis. However, the precise mechanisms underlying the enhancing effect of cAMP on BMP2 signaling were not completely revealed. In this study we investigated the effect of elevated cAMP level and PKA activation on the BMP2-induced osteoblastic differentiation in pluripotent C2C12 cells. Alkaline phosphatase activity and its mRNA were consistently induced by BMP2 treatment. The pretreatment of C2C12 cells with Forskolin, a cAMP generating agent, dbcAMP, an analogue of cAMP, or IBMX (3-isobutyl 1-methyl xanthine), and a nonspecific inhibitor of phosphodiesterases elicited further activation of alkaline phosphatase. Furthermore, elevated intracellular cAMP level increased BMP2-induced MKP1. On the other hand, BMP2-induced Erk phosphorylation (p44/p42) and cell proliferation were suppressed in the presence of cAMP. Thus, cAMP might enhance BMP2-induced osteoblastic differentiation by a MKP1-Erk-dependent mechanism.

Bone morphogenetic proteins (BMPs) superfamily regulates the proliferation, differentiation, and apoptosis of various types of cells and organs not only in embryonic development but also in postnatal physiological function [1]. Genetic disruptions of BMPs have resulted in various skeletal and extraskeletal developmental abnormalities [2]. At the cellular level, BMPs bind to two major types of membrane-bound serine/threonine kinase receptors, type-I and type-II receptors. The classic BMP signaling pathway operates by activation of the Smad family of transcription factors, and there is evidence that it can also act through a Smad-independent p38 MAPK signaling pathway [3]. There have also been reports suggesting that other pathways, such as ERK, c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K) and Wnt may substitute for, activate, or modulate BMP signaling [4; 5]. Various BMPs, including BMP2, BMP4, and BMP7, induce the differentiation of multipotential mesenchymal cells (e.g., C3H10T1/2 cells) into both osteochondrogenic lineage cells and osteoblast precursor cells. BMP2 specifically converts the differentiation pathway of C2C12 myoblasts into that of osteoblast lineage cells [6].

Several evidences indicate that osteoblasts, chondrocytes, myocytes, and adipocytes are all derived from a common progenitor cells called undifferentiated mesenchymal cells [7; 8; 9]. During the process of their differentiation, progenitor cells acquire specific phenotypes depending upon the differentiated cell types under the control of respective regulatory factors. The differentiation process of osteoblasts can be divided into at least two stages. One is the commitment of undifferentiated mesenchymal cells into osteoblast progenitors. The other is the maturation of osteoblast progenitors into osteoblasts which express the various phenotypes of bone-forming cells; production of a large amount of extracellular matrix proteins

including type I collagen and osteocalcin, high levels of alkaline phosphatase (ALP) activity, and responsiveness to calcitropic hormones such as parathyroid hormone (PTH) (for reviews see [10; 11]).

The cyclic monophosphate nucleotides (cAMP and cGMP) are found ubiquitously in mammalian cells and act as second messenger transducers to effect the intracellular action of a variety of hormones, cytokines, and neurotransmitters. In turn, these nucleotides also modulate the signal transduction processes regulated by a range of cytokines and growth factors. The study reported by Isogai et al. [12] regarding the effects of PTH on osteogenic differentiation showed that continuous exposure to PTH stimulated osteogenic differentiation in immature osteoblasts and that the cAMP pathway was the key element of this mechanism. In another report, Tintut et al. [13] studied the role of the cAMP-signaling pathway in vascular calcification and showed that the cAMP-enhancing agents stimulated osteoblast-like differentiation of calcifying vascular cells. These findings substantiate the notion that the cAMP-signaling pathway is important in osteoblast recruitment from osteoprogenitor cells.

Recent studies demonstrate that the inhibition of phosphodiesterase (PDE), enzymes involved in the degradation of cAMP, by pentoxifyline and rolipram enhance BMP4-induced osteogenic differentiation of mesenchymal cells [14]. Although these effects might be linked to the increase in cAMP levels induced by PDE inhibitors, little is known about the precise mechanisms by which cAMP signaling contribute to the osteoblastic cells differentiation.

In this study, we have investigated the effect of different cAMP generating agents on osteoblastic cells differentiation using C2C12 cells, a murine pluripotent mesenchymal cell line that is able to differentiate in osteoblast when treated with BMP2. C2C12 myoblast/osteoblast transdifferentiation induced by BMP2, in response to intracellular elevation of cAMP, was assessed by measuring the alkaline

phosphatase (ALP) activity, Osteocalcin, Osterix and Runx2 mRNA. In addition, we evaluated the signaling pathways involved in this effect.

Materials and methods

Reagents and antibodies. Forskolin, dibutyryl cyclic adenosine monophosphate (dbcAMP), IBMX, H89 and GF-109203X (GFX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126, PD-98059 and SB203580 were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). MKP-1 (C-19) Polyclonal antibody was obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Polyclonal anti-p38, anti-ERK1/2, anti-pERK1/2, anti-pERK5, anti-pp38, anti-pSmad1/5/8 were obtained from Cell signaling (Cell signaling Technology, MA, USA).

Cell cultures. C2C12, C3H10T1/2 and MC3T3-E1 clone 4 were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (C2C12) or alpha-minimal essential medium (MC3T3 and C3H10T1/2) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin). The cultures were never allowed to become confluent. The cells were trypsinized and passaged every 2–3 days. All cells were grown at 37°C in humidified air mixed with 5% CO_2. For the experiments, the cells were plated one day before treatment and treated with BMP2 in the presence or absence of different agents.

Assay of ALP activity and ALP staining. Alkaline phosphatase activity was measured as a marker of osteoblastic differentiation. Cells were seeded at a density of 5×10^4 cells/cm^2 in 24-well plates (n = 3 per group). One day later, cells were pre-

incubated 30 minutes with forskolin (F), dbcAMP or IBMX. BMP2 was then added to the cultures, and incubation was continued for 5 more days. After 5 days of incubation, medium was removed, and cells were washed with PBS and then scrapped in buffer A (0.56M 2-amino-2-methyl-1-propanol). The pellets were then homogenized for 10 seconds. After centrifugation, supernatant was collected and used for ALP assay using p-nitrophenylphosphate (Sigma) as a substrate. The protein content of the lysates was measured using Bradford protein assay reagent (Bio-Rad). Experiments were performed independently in triplicate. To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were stained as described in [6]. Image of stained cells were captured with a CDD camera.

Western blot analysis. Cells treated with different factors or compounds were rapidly frozen in liquid nitrogen and stored at −80°C until used for analysis. Cells were lysed at 4°C in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, supplemented with a protease inhibitor (Protease Inhibitor Cocktail Set III, Calbiochem), 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS for 10 min. Lysates were then cleared by centrifugation at 6000 × g for 30 min. Proteins were separated on a 12% SDS-PAGE gel, and transferred to Immobilon P membranes (Millipore, Bedford, MA). The phosphorylation of Smad1/5/8 and MAPK components was detected by using the appropriate primary antibodies followed by Peroxidase-coupled secondary antibody. The membranes were washed, treated with the ECL reagent and exposed to X-ray films. Filters that were reprobed were stripped according to the manufacturer's protocol.

RNA extraction and quantification by real-time PCR. Total RNA was extracted from cells at specific time points using RNAeasy kit with DNase I treatment as directed by the manufacturer. The total RNA in each sample was quantified by

absorbance readings at 260 nm. Quantitative PCR was carried out using the QuantiTectTM SYBR Green PCR kit (Qiagen) on a MJ Research PTC 100 Thermal Cycler (MJ Research Inc.). 18 S was used as an internal reference for each sample. The relative change in mRNA levels was normalized against the 18 S mRNA levels.

Thymidine Incorporation. [3H]Thymidine incorporation was used to determine BMP2 proliferative effects on the control and forskolin pre-treated cells. The C2C12 cells were plated in 24-well tissue culture plates (5x104) in complete medium and grown for 24 h. Cells were serum starved for 24 h before stimulation. The cells were pre-treated with or without forskolin and then stimulated for 24 with BMP2 (500 ng/ml). The cells were labeled with [3H]thymidine (0.5 mCi, 2200 Ci/mmol; Dupont NEN) for the last 4 h of incubation. After two washes with cold phosphate-buffered saline (PBS), DNA was precipitated with 5% trichloroacetic acid (TCA) for 1 h at 4°C. The cell layers were then washed twice with TCA solution and solubilized in 0.1 M NaOH for 30 min. The amount of [3H] thymidine incorporated was analyzed by liquid scintillation counting in a Packard 1600 TR scintillation counter. All experiments were performed in triplicate.

Results and Discussion

Despite recent successes with drugs that inhibit bone resorption, the development of modalities to promote local or systemic bone formation is an important issue in the treatment of fractures or metabolic bone diseases such as osteoporosis. To date, however, the agents known to exhibit an anabolic action in bone are limited to parathyroid hormone (PTH), bone morphogenetic proteins (BMPs), and, potentially, statins (for reviews see [15; 16; 17]). A few recent studies suggest that cAMP enhance BMP signaling but the cross-talk between the BMP-signaling pathway and cAMP-signaling pathway has not been clarified so far. In this

study we investigated the molecular mechanisms by which the increases in cAMP level enhance the BMP2 signaling and osteoblastic differentiation of C2C12 cells.

cAMP enhances BMP2-induced ALP.

The osteogenic effects of forskolin and BMP2 on C2C12 cells were examined by ALP activity and ALP staining (Fig. 1A). These results confirmed the previous finding [6]: BMP2 redirects the differentiation of C2C12 myoblasts into that of the osteoblastic lineage. Forskolin, a cAMP generating agent, had no effect on ALP activity. In contrast, forskolin enhanced BMP2-induced ALP activity in a dose-dependent manner. As shown in Fig.1B, the effect of forskolin was mimicked by dbcAMP and IBMX, both leading to increased intracellular level of cAMP. Fig. 1C shows time-dependent changes in ALP mRNA expression following BMP2 treatment. The pretreatment with forskolin before BMP2 stimulation significantly enhance BMP2-induced ALP expression. These results show that cAMP increasing agents are capable to enhance the BMP2-induced differentiation toward osteoblasts.

cAMP enhances BMP2-stimulated expression of bone matrix proteins.

Next, we studied the effect of forskolin pretreatment in BMP2-induced transcription factors and bone matrix proteins. As shown by real-time PCR, 24h and 48h after BMP2 stimulation, the expression of osteocalcin and alkaline phosphatase was higher in the group with forskolin pretreatment compared to BMP2 alone (Fig. 2A). The expression of the osteogenic master transcription factors, Osterix and Runx2, was also increased in forskolin pretreatment group compared to BMP2 group (Fig. 2B), suggesting that elevation of intracellular cAMP levels subsequent to the forskolin treatment enhance the BMP2-induced osteoblastic differentiation.

The cAMP-PKA pathway is known to be a major intracellular pathway to regulate PTH-mediated expression of several osteoblastic genes, such as Runx2, type I collagen and osteocalcin (for review see [18]). It is therefore interesting to note that in MC3T3-E1 clone 4, which is sensitive to PTH and cAMP, and in C3H10T1/2 cells forskolin is able to enhance BMP2-induced ALP, but not in MC3T3-E1 clone 24 (data not shown). These results suggest that the enhancement of BMP2 action by forskolin is linked to responsiveness to cAMP

Mechanism of cAMP-increased BMP2 signaling.

The ability of BMP2 to induce the phosphorylation of Smad1/5/8, protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) Jnk, p38 and Erk has been shown previously [19; 20]. Thus, we analyzed the respective role of MAPK pathways for cAMP-induced changes in C2C12 cells. We performed ALP activity experiments with different inhibitors, and as presented in figure 3A the inhibition of PKC or JNK pathways did not exhibit significant effects on ALP activity. Results presented in figures 3B and 3C showed that pretreatment with PKA inhibitor (H89) abrogated the ALP activity induced by the combination of forskolin and BMP2. In contrast, MEK/Erk inhibitor (PD098059) pretreatment showed an enhancement in the ALP activity. These results suggest that cAMP increased BMP2-induced ALP activity by a mechanism involving PKA activation and probably MEK/Erk inhibition. In order to assess the direct correlation of MEK/Erk pathway in BMP2-induced ALP, C2C12 were treated 1 h with either their inhibitors (10 µM U0126 or 10 µM PD98059) before BMP2 stimulation. As presented in figure 3D, MEK/Erk inhibition enhances BMP2-induced ALP activity. These results argue that in C2C12 cells, lower levels of activated ERK are associated with improved BMP2 stimulation of ALP. This is consistent with findings that BMP-induced osteogenesis in poorly responsive human

MSC requires modulation of ERK[21]. In figure 3 (A-D), effects of protein kinase inhibitors of PKC, PKA, and mitogen-activated protein kinase (MAPK) showed that the anabolic effect of forskolin might be mediated via the activation of PKA signaling pathway. Recently, Siddappa et al. [22] showed that the PKA activation results in robust in vivo bone formation by human mesenchymal stem cells (hMSCs). Interlacing of the BMP2 and PKA pathways is not surprising but has been demonstrated in chondrogenesis and kidney development during embryogenesis [23; 24].

Down-regulation of Erk1/2 phosphorylation and proliferation in cAMP treated cells.

To address the role of the ERK1/2 cascade in the cAMP-enhanced BMP2-mediated induction of osteoblast phenotype, C2C12 cells were pretreated 1 h with 10 µM of H89, then treated with 10µM of forskolin prior to the 1h exposure of the C2C12 cells to 150 ng/ml of BMP2. As shown in figure 3E ERK1/2 phosphorylation could be detected under BMP2 stimulation. The BMP2-induced Erk1/2 phosphorylation is completely repressed by pretreatment with forskolin. When the cells were incubated with PKA inhibitor, BMP2 still induced phosphorylation of Erk1/2 but the forskolin effect was reduced, suggesting that forskolin blocked BMP2-induced Erk1/2 phosphorylation by a PKA dependent-mechanism. The total amounts of ERK1/2 were unchanged during the time frame of these experiments. In parallel, we measured cell proliferation in BMP2-treated cells with and without forskolin pretreatment. As shown in Figure 3F, BMP2 significantly increased [3H]-thymidine incorporation (about 50%). Forskolin considerably inhibited the BMP2-induced [3H]-thymidine incorporation. These results suggest that elevation of intracellular level of cAMP induced by forskolin blocked Erk1/2 phosphorylation and proliferation induced by BMP2.

The role of MKP1 (MAP kinase phosphatase 1).

Phosphatases play an important role as negative regulators of MAPK activity (for reviews see [25; 26; 27]). We focused on MKP1 for the following reasons: 1) MKP1 was originally identified as an ERK-specific phosphatase and may be an important target gene in the anabolic effect of PTH on osteoblasts; and 2) constitutive MKP1 expression blocks G1-specific gene expression, suggesting a role in cell cycle control [28]. The results presented in figure 4A show that BMP2 induce MKP1 expression and forskolin pretreatment elicited a further activation of MKP1 expression. The increase in MKP1 protein and Erk1/2 dephosphorylation suggests that cAMP increased the BMP2-induced MKP1 expression which is involved in the inhibition of cell proliferation observed in forskolin + BMP2 treated cells (Fig. 3F).

This result implies that the induction of MKP-1 may be responsible for the decrease in ERK1/2 phosphorylation and subsequent suppresses proliferation and promotes differentiation. Accordingly, a model is proposed to explain how forskolin arrests osteoblastic cells proliferation and stimulates differentiation (Fig.4B). Forskolin increases intracellular level of cAMP and activates the PKA pathway. Modification of some transcription factors, such as cAMP-response element-binding protein, induces the expression of MKP-1. The increase in MKP-1 protein results in dephosphorylation of its substrate, phosphorylated ERKs, and leads to decreased proliferation and enhanced differentiation.

In summary, findings of this study provide new evidence that PKA pathway activation synergistically participates in the BMP2-induced osteoblast differentiation, possibly by MKP-1-Erk-dependent mechanisms.

Acknowledgement

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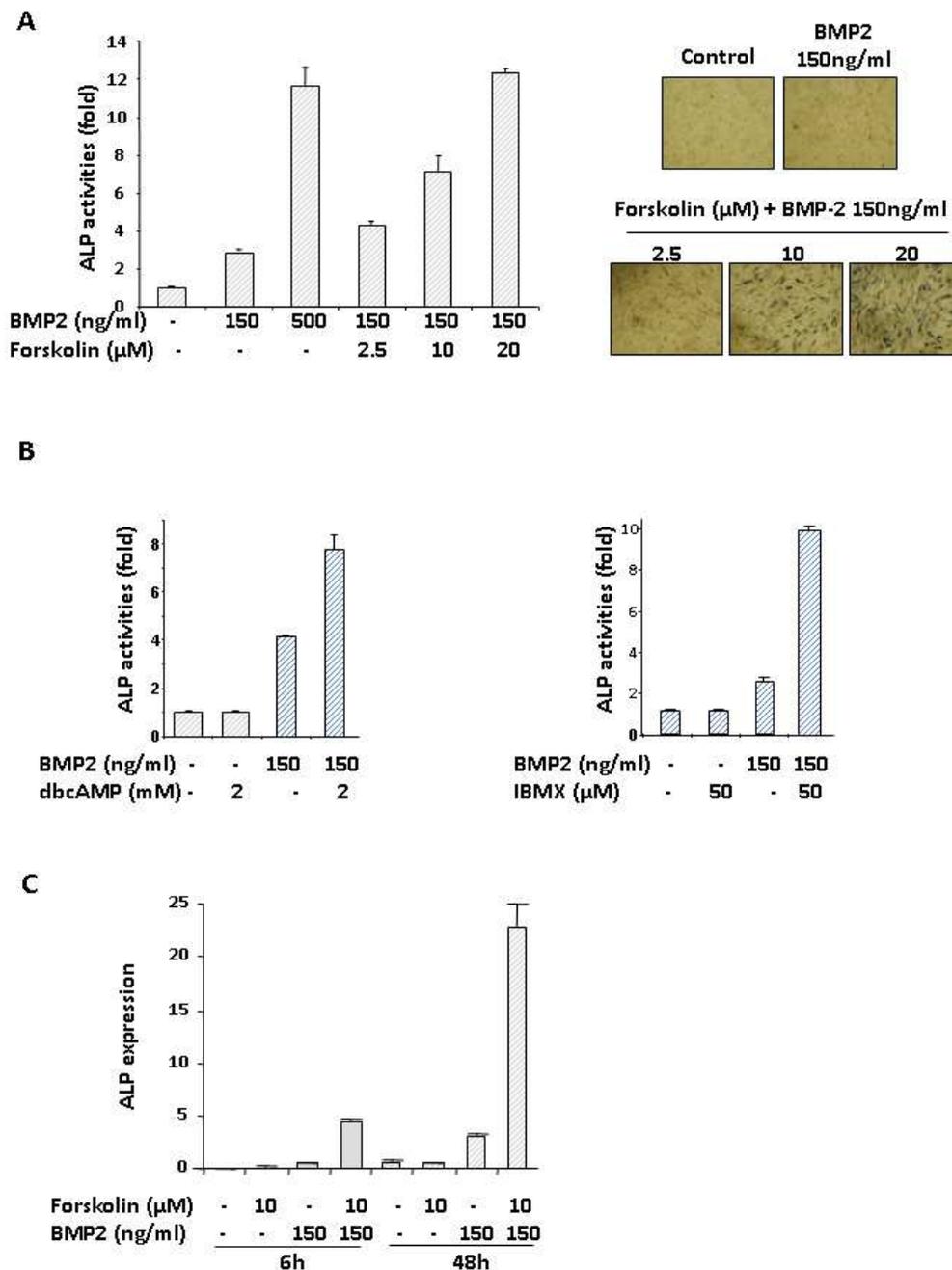
Fig. 1. Effects of cAMP on BMP-induced osteoblast differentiation in C2C12 cells. (A) Cells were treated with BMP2, or BMP2 after 1h pretreatment with forskolin as indicated. After 5days, the cells were used to measure ALP activities or fixed and stained for ALP as described in Experimental Procedures. (B) Cells were pretreated with BMP2 alone or pretreated with dbcAMP or IBMX before BMP2 stimulation. After 5 days, the cells were used to measure ALP activities. (C) C2C12 cells were stimulated for 6h and 48h as indicated, total RNA was extracted and expression of Alkaline phosphatase (ALP) was analyzed using real-time RT-PCR, normalized to S18 expression and presented as the level relative to unstimulated cells (-).
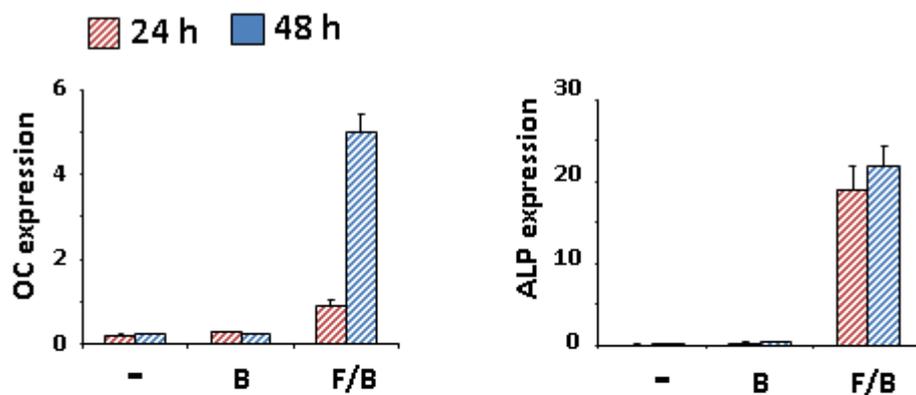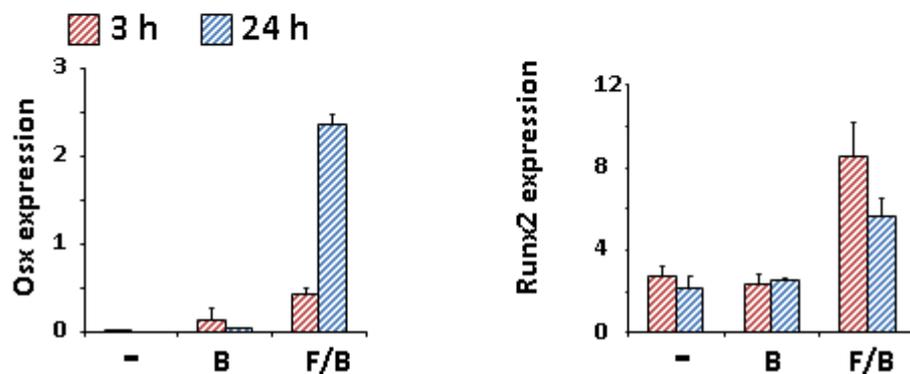
Fig. 2. Role of cAMP in BMP2-stimulated expression of bone matrix proteins and transcription factors. C2C12 cells were treated with BMP2 alone at 150 ng/ml for 3h, 24h and 48h. In parallel, cells were pretreated with forskolin (10µM) for 1 h at 37 °C before BMP2 stimulation. As controls, cells were maintained in medium only (-). Real time PCR was performed to quantify the expression of (A) osteocalcin (OC), Alkaline phosphatase (ALP), (B) osterix (Osx) and Runx2 during the time course. All expression of target genes was normalized to 18S.
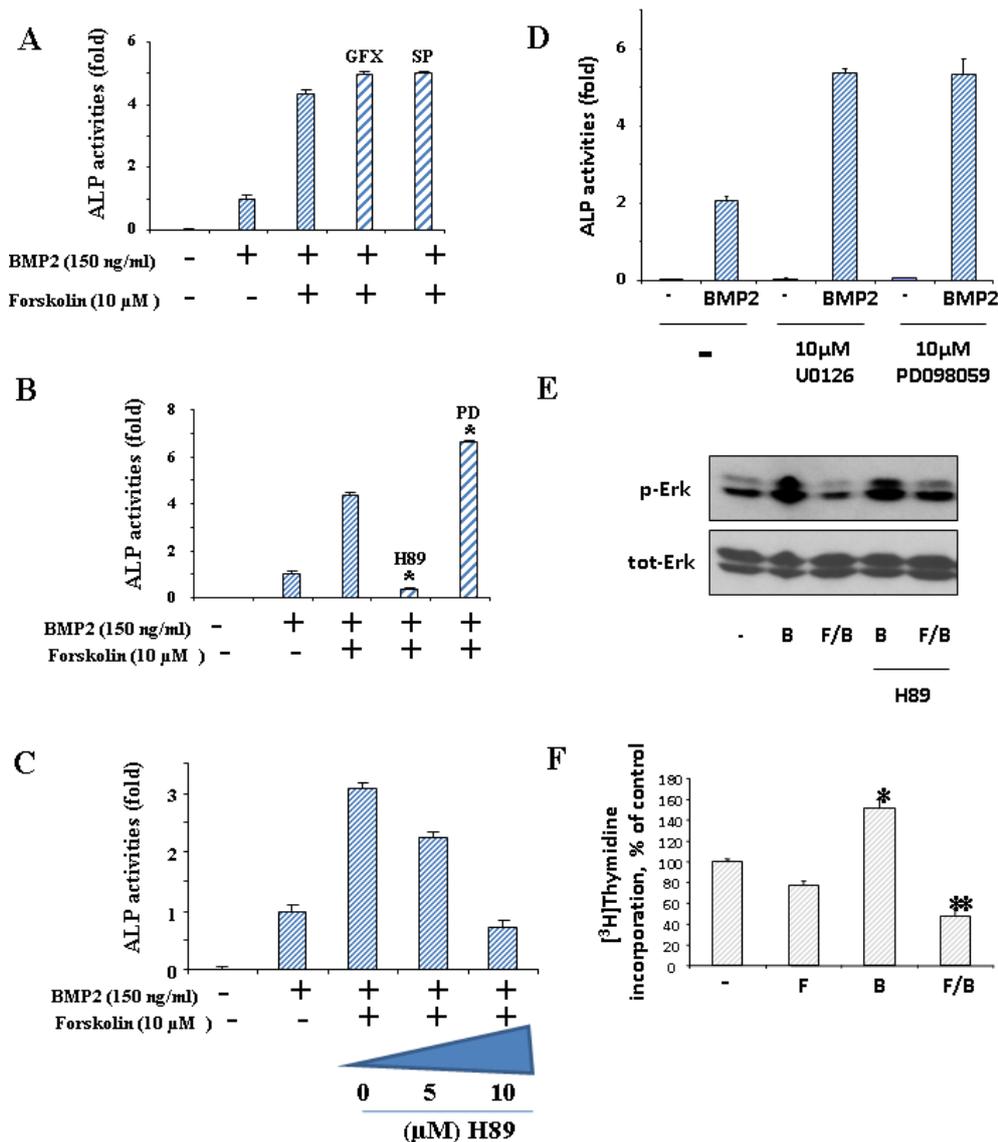
Fig. 3. Forskolin increased BMP-2-induced ALP by PKA dependent mechanisms and blocked Erk phosphorylation and proliferation induced by BMP2. (A) Cells were incubated 1h with 10 μM of different inhibitors GFX (GFX109203X, PKC inhibitor), SP (SP600125, JNK inhibitor). (B) PD(PD98059, MAPK inhibitor) and H89 (PKA inhibitor), (C) dose-response to H89 and then pretreated with 10μM of forskolin before 150 ng/ml of BMP2 was added. After 5 days stimulation, ALP activity was measured and presented as the fold relative to BMP2-stimulated cells. (D) Erk/MEK Inhibitors and BMP2-induced ALP activity in C2C12 cells. Alkaline phosphatase (ALP) was used as a marker to evaluate the effects of Erk/MEK inhibitors on BMP2-induced osteoblastic differentiation. Cells were pre-treated with medium containing Erk/MEK inhibitors for 60 min before treatment with BMP2. After 5 days stimulation, ALP activity was measured. (E) C2C12 cells were stimulated for 60 min with BMP2 (250ng/ml), with or without pretreatment with forskolin:F (10 μM). The experiment was performed in presence or absence of 10μM of PKA inhibitor (H89). PhosphoERK1/2 (top panel) and total-ERK (lower panel) levels were determined by Western blot analysis. (F) C2C12 cells (2 × 104) were plated in 24-well plates and grown overnight. On the second day, medium was changed and cells were incubated in serum-free medium for 24 h. Cells were then incubated for 24 h in fresh medium with forskolin 10μM (F), BMP2 250ng/ml (B) or BMP2 250ng/ml after 1h pretreatment with 10μM forskolin (F+B). Subsequently, cells were pulse labeled with 1 μCi/ml [3H]-thymidine for 6 h. Cells were then washed twice with ice-cold phosphate buffered saline (PBS) and fixed with trichloroacetic acid. Precipitates were then dissolved in 0.1 M NaOH and the incorporated radioactivity was determined by liquid scintillation counting.
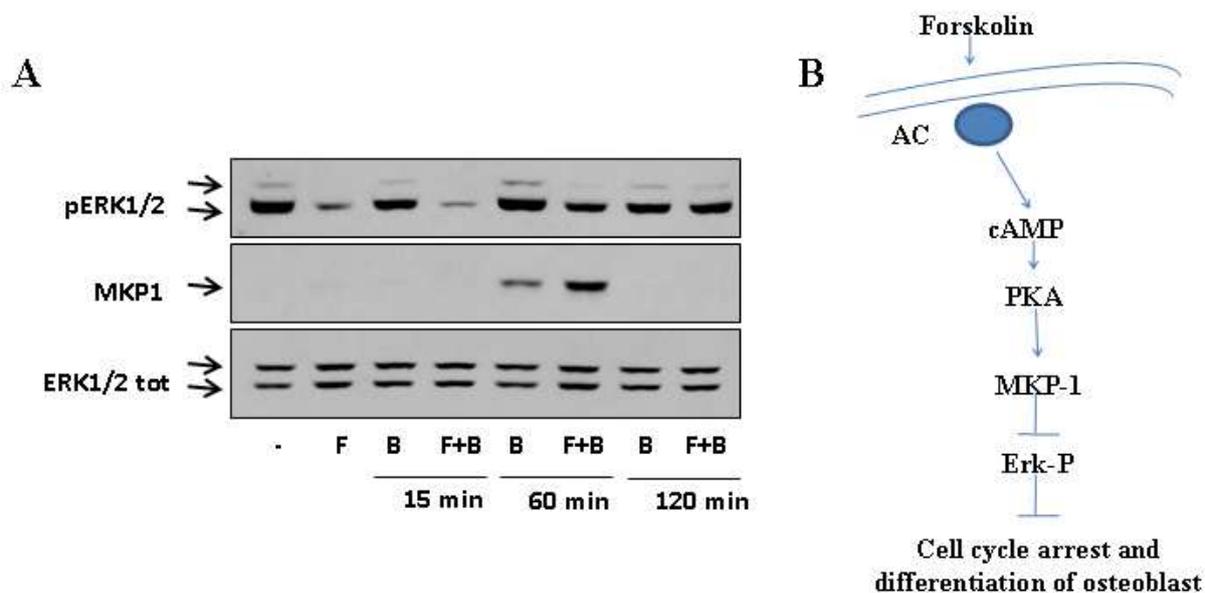
Fig. 4. Effect of Forskolin on BMP2-induced MKP-1 and a schematic drawing of the proposed signaling cascade. (A) C2C12 Cells were stimulated for the indicated times with 250ng/ml BMP2 with (F+B) or without (B) 1h pretreatment with 10µM forskolin. Proteins were extracted and analyzed by Western blot for phosphoErk1/2 and MKP-1. (B) Schematic drawing of the signaling pathway. The adenylate cyclase activator, forskolin induces a rapid and very pronounced intracellular accumulation of cyclic AMP in C2C12 cells. The cAMP accumulation caused by forskolin activates the PKA pathway. Modifications of some transcription factors, such as cAMP-response element-binding protein, induce the expression of MKP-1. The increase in MKP-1 protein results in dephosphorylation of its substrate, phosphorylated ERKs, and leads to a decrease in proliferation and a promotion of differentiation.