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Protein Kinase A Regulates the Osteogenic Activity of Osterix

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ABSTRACT

Osterix belongs to the SP gene family and is a core transcription factor responsible for osteoblast differentiation and bone formation. Activation of protein kinase A (PKA), a serine/threonine kinase, is essential for controlling bone formation and BMP-induced osteoblast differentiation. However, the relationship between Osterix and PKA is still unclear. In this report, we investigated the precise role of the PKA pathway in regulating Osterix during osteoblast differentiation. We found that PKA increased the protein level of Osterix; PKA phosphorylated Osterix, increased protein stability, and enhanced the transcriptional activity of Osterix. These results suggest that Osterix is a novel target of PKA, and PKA modulates osteoblast differentiation partially through the regulation of Osterix. J. Cell. Biochem. 115: 1808–1815, 2014.

KEY WORDS: PROTEIN KINASE A; OSTERIX; OSTEOBLAST DIFFERENTIATION; PROTEIN STABILITY; TRANSCRIPTIONAL ACTIVITY

Skeletal remodeling involves the timely formation and resorption of bone, accomplished by specialized osteoblasts and osteoclasts in a quantitative manner. The balance between osteoblasts and osteoclasts maintains bone volume and calcium homeostasis in vertebrates throughout their lives [Zhao, 2012]. Many anabolic factors, including Wnt, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factor 1, and kinases, such as protein kinase A (PKA), play important roles in the skeletal remodeling process [Swarthout et al., 2002]. Bone formation is a complex event involving the differentiation of mesenchymal stem cells into osteoblasts. Osteoblasts are responsible for the production of bone matrix proteins. Osteoblast differentiation can be regulated by several transcriptional factors and different signaling proteins such as Runx2 and Osterix [Komori, 2006; Caetano-Lopes et al., 2007].

Osterix is a C_2H_2-type zinc finger protein expressed in all developing bones and is required for the differentiation of preosteoblasts into functional osteoblasts and osteocytes [Fu et al., 2007]. A complete absence of intramembranous and endochondral bone formation is observed in Osterix-null mice [Nakashima et al., 2002]. Osterix is hardly detected in Runx2-null mice [Nakashima et al., 2002], indicating that Osterix acts downstream of Runx2. During the osteoblast differentiation, Osterix regulates the expression of several osteogenic factors, including osteonectin, osteopontin, osteocalcin (OC), and alkaline phosphatase (ALP) [Zhang, 2010]. During the osteoblastogenesis, both the activity and expression of Osterix are regulated by transcription factors. For example, X-box- binding protein 1 (XBP1) and Runx2 control positively the transcription of Osterix, while Runx3 regulates negatively the transcription of Osterix [Matsubara et al., 2008; Tohmonda et al., 2011]. Although these findings indicate significant progresses, the exact regulation of Osterix is still not completely understood.

PKA, also known as cyclic AMP (cAMP)-dependent protein kinase, regulates many cellular functions such as the modulation of

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the immune response, the participation in the DNA damage process, the effects on the cardiovascular system, and the regulation of lipid metabolism [Pasqualucci et al., 2006; Tseng et al., 2010]. PKA consists of two regulatory subunits and two catalytic subunits, which play an important role when PKA exerts its cellular functions. The catalytic subunits can be released when cAMP binds to the regulatory subunits, and the free catalytic subunits can then catalyze the transfer of ATP terminal phosphates to protein substrates at serine or threonine residues. In our study, we used a protein kinase inhibitor, PKIG, which acts as a pseudo-substrate for PKA, binding and inactivating its catalytic subunit.

PKA acts downstream of BMPs and enhances the osteogenic differentiation. In the previous study, it showed that the down-regulation of PKA inhibitor γ is needed for a BMP-2-induced osteoblastic differentiation [Zhao et al., 2006]. Several osteoblast-related proteins, including Runx2 and OC, have been shown to be regulated by PKA [Lo et al., 2012]. There is also an evidence that Runx2 can be phosphorylated by PKA at a PKA phosphorylation site [Selvamurugan et al., 2000]. Our previous studies also showed that PKA can phosphorylate Dlx5 and enhance its osteogenic function [Han et al., 2011].

Osterix has been shown to be activated and phosphorylated by Akt or glycogen synthase kinase 3 alpha (GSK3α) [Choi et al., 2011; Li et al., 2013], suggesting that the function of Osterix may be modulated by protein kinases in a post-translational manner. Thus, we considered whether PKA might regulate the function of Osterix. Here, we report that Osterix was phosphorylated by PKA. Additionally, Osterix protein expression, protein stability, and transcriptional activity were enhanced by the PKA activation.

MATERIALS AND METHODS

PLASMIDS, ANTIBODIES AND CHEMICALS

6Myc-tagged Osterix and cAMP-dependent protein kinase (PKA) inhibitor G (PKIG) were constructed using a CMV promoter-derived mammalian expression vector (pCS4+). Antibodies against Myc (9E10, Roche Applied Science, Madison, WI), Osterix (sc-22536-R, Santa Cruz Biotechnology), α-tubulin (B-5-1-2, Sigma–Aldrich), and phospho-PKA substrate (100G7E, Cell Signaling) were used. The PKA activator forskolin (3442470) and inhibitor H89 (371963) were purchased from EMD Chemicals, Inc. (San Diego). Alkaline phosphatase (P0114) was purchased from Sigma–Aldrich.

CELL CULTURE AND DNA TRANSFECTION

The C2C12 mouse pre-myoblast cell line and the HEK 293 (human embryonic kidney) epithelial cell line were maintained at 37 °C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mix. DMEM, FBS, and antibiotic-antimycotic were purchased from Gibco Life Technologies, Invitrogen. Transient transfection was performed using the polyethylenimine (PEI; Polysciences, Inc.) method. Total amounts of transfected plasmids in each group were equalized by adding an empty vector.

IMMUNOBLOTTING (IB) AND IMMUNOPRECIPITATION (IP)

For IB, at 48 h after transfection, C2C12 cells or HEK 293 cells were lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). After centrifugation, supernatants were subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane and visualized using appropriate primary antibodies, HRP-conjugated secondary antibodies, and ECL reagents. For IP, the lysates were cleared by centrifugation and the supernatants were subjected to immunoprecipitation using appropriate antibodies and protein A- or G-Sepharose beads. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by immunoblotting.

PROTEIN STABILITY ASSAY

HEK 293 cells and C2C12 cells were transfected with 6Myc-tagged Osterix. On the second day, the cells were exposed to fresh media and then treated with forskolin or DMSO. Transfected cells were incubated for the indicated times, then treated with 40 μM cycloheximide (CHX), and harvested with lysis buffer as described above. Protein levels were analyzed by immunoblotting using an anti-Myc antibody.

LUCIFERASE REPORTER ASSAY

C2C12 cells were transfected with osteocalcin (OC-Luc), bone sialoprotein (BSP-Luc) reporter plasmids, pCMV-β-gal, and combinations of 6Myc-Osterix. After 24 h, the cells were treated with the indicated concentration of forskolin or H89. The cells were lysed at 36 h after transfection. Luciferase activities were measured using a Luciferase Reporter Assay Kit (Promega) and normalized to the corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicate and repeated at least three times.

TOTAL RNA EXTRACTION AND RT-PCR ANALYSIS

Total cellular RNA was prepared using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Randomly primed cDNAs were synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The following PCR conditions were used for amplification: initial denaturation at 94 °C for 1 min, followed by 28–30 cycles of denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The following PCR primers were used: ALP forward 5′-GAT CAT TCC CACGTT TTC AC-3′ and reverse 5′-TGCGGG CTT GTG GGA CCT GC-3′, ColIα1 forward 5′-TCT CCA CTC TTC TAG GTT CCT-3′ and reverse 5′-TTG GGT CAT TTC CAC ATG C-3′, BSP forward 5′-ACA CTT ACC GAG CTT ATG AGG-3′ and reverse 5′-TTG CGC AGT TAG CAA TAG CAC-3′, GAPDH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′.

ALKALINE PHOSPHATASE (ALP) STAINING

C2C12 cells were stimulated with BMP4. The cells were pretreated with BMP4 for 3 days. Transfected C2C12 cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS and stained with 300 μg/mL BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) solution (Sigma–Aldrich) for 20 min at RT. The alkaline phosphatase-positive cells were stained blue/purple.

STATISTICAL ANALYSIS

All experiments were performed with triplicate independent samples and were repeated at least twice, giving qualitatively identical results. Results are expressed as mean ± standard error of the mean. Data were analyzed using Student's t-test, with $P < 0.05$ indicating significance.

RESULTS

PKA REGULATES OSTERIX-INDUCED OSTEOBLAST DIFFERENTIATION AND ENHANCES THE PROTEIN EXPRESSION OF OSTERIX

Osterix is a core transcription factor that has been shown to induce osteoblast differentiation [Huang et al., 2007]. The effect of PKA activation on Osterix-induced osteoblast differentiation was assessed by ALP staining. The results showed that Osterix enhanced BMP4-induced ALP activity, and forskolin further increased osteoblast differentiation, while H89 attenuated osteoblast differentiation (Fig. 1A). Protein levels of Osterix were evaluated in the C2C12 cell line and the HEK 293 cell line. C2C12 cells were transfected with 6Myc-Osterix, and then treated with combinations of BMP4 and forskolin or H89, and protein levels were compared by immunoblotting. BMP4 stimulated both exogenous and endogenous expression of Osterix, and forskolin further increased the expression of Osterix. However, H89 reduced both exogenous and endogenous expression of Osterix (Fig. 1B). Consistently, in the absence of BMP4 signaling, the exogenous and endogenous expression of Osterix in HEK 293 cells was increased by forskolin treatment, whereas H89 and PKIG attenuated the stimulation of Osterix expression (Fig. 1C, D). These results suggest that the PKA activation regulated positively the Osterix-induced osteoblast differentiation and increased the protein level of Osterix.

PKA INCREASES THE PROTEIN STABILITY OF OSTERIX

To identify the mechanism by which the PKA activation increased the expression of Osterix protein, we tested whether the PKA could affect the protein stability of Osterix using a translation inhibitor, CHX. HEK 293 and C2C12 cells were transfected with 6Myc-Osterix and then treated with forskolin or DMSO. To assess the protein stability of Osterix, the transfected cells were treated with 40 μM CHX for the indicated times and then harvested. According to the immunoblotting results, both exogenous and endogenous Osterix proteins were degraded and their half-life was ~4 h. However, the forskolin blocked Osterix protein degradation markedly and increased the half-life of exogenous and endogenous Osterix (Fig. 2A, B). These results illustrate that the PKA activation enhanced the protein level of Osterix via increasing its protein stability.

PKA REGULATES THE PHOSPHORYLATION OF OSTERIX

From the results above, we considered whether the PKA could phosphorylate Osterix in addition to increasing its protein stability. To investigate this, HEK 293 cells were transfected with 6Myc-Osterix or PKIG and then treated with forskolin or vehicle (DMSO). The phosphorylation of Osterix was examined by immunoprecipitation with a phospho-PKA substrate antibody followed by the detection of 6Myc-Osterix (Fig. 3A). The results showed that the phosphorylation of Osterix was induced by PKA, while PKIG

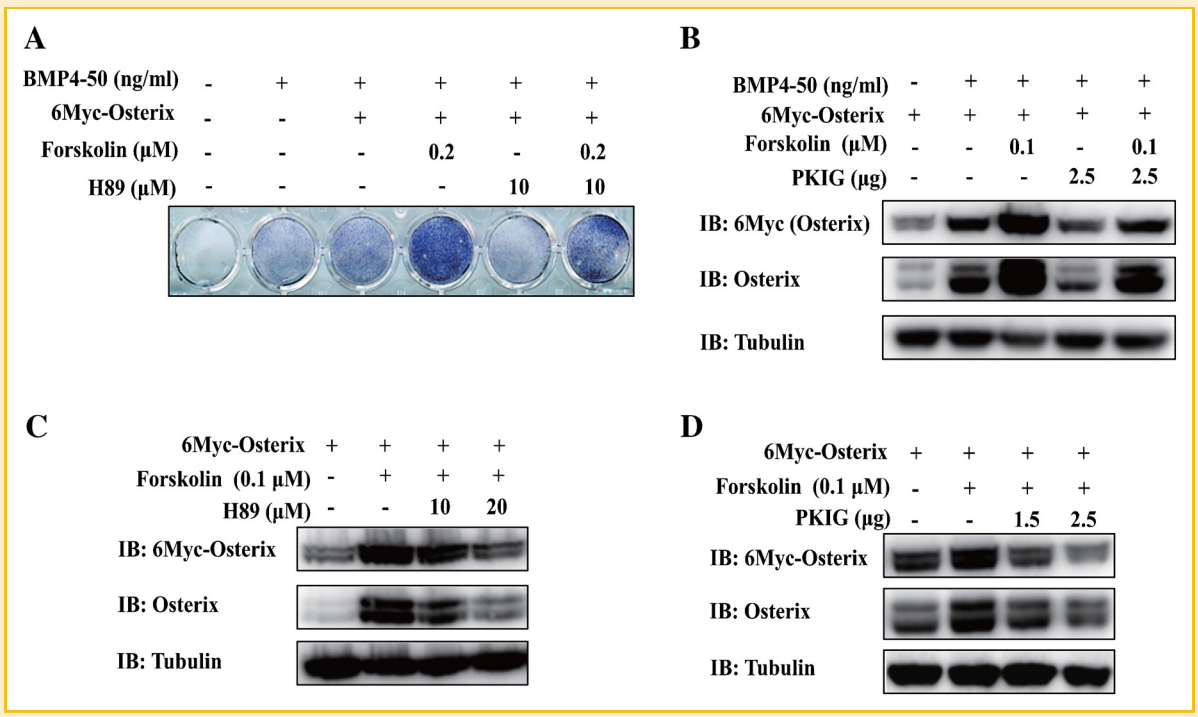

Fig. 1. PKA regulated the protein levels of Osterix during osteoblast differentiation. (A) C2C12 cells were transfected with/without 6Myc-Osterix. On the second day, the medium was changed and the cells were then treated with DMSO or forskolin or H89, at the concentrations indicated in the absence or presence of BMP4. (B) C2C12 cells and (C and D) HEK 293 cells were transfected with 6Myc-Osterix, PKIG (PKA inhibitor) for 24 h and then treated with forskolin or H89 in the absence or presence of BMP4.

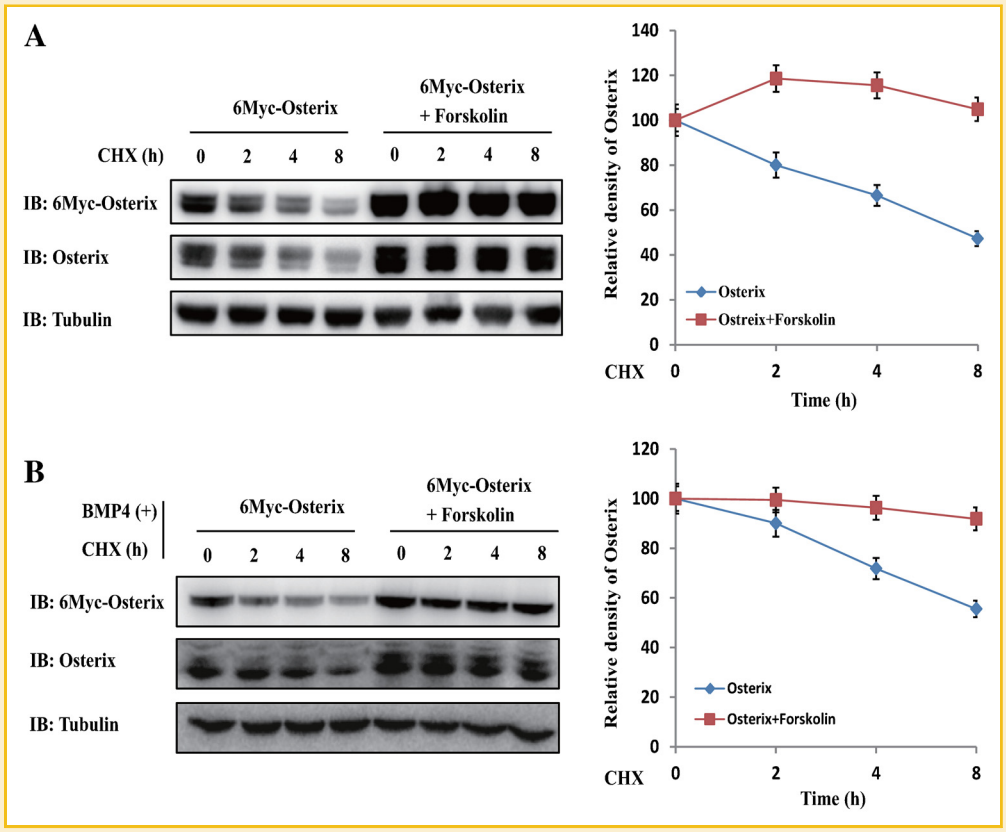

Fig. 2. PKA increased the protein stability of Osterix. HEK 293 (A) and C2C12 (B) cells were transfected with 6Myc-tagged Osterix and then treated with DMSO (control) or forskolin (0.1 μM). For C2C12 cells, BMP4 was used to induce osteoblast differentiation. Cells were then treated with a translation inhibitor, cycloheximide (CHX, 40 μM) for the times indicated. The protein levels of Osterix were detected by immunoblotting using anti-Myc or anti-Osterix. Tubulin was used as a loading control.

abolished the phosphorylation of Osterix. It was also examined using C2C12 cells (Fig. 3B). The osteogenic effect of BMP4 increased the phosphorylation of Osterix while H89 attenuated it. Our hypothesis was further confirmed by the dephosphorylation of Osterix using alkaline phosphatase (ALP) [Labugger et al., 2000]. HEK 293 cells were transfected with 6Myc-Osterix and then treated with forskolin or vehicle (DMSO). Osterix was purified by immunoprecipitation. Pretreatment with alkaline phosphatase before electrophoresis induced the dephosphorylation of Osterix and changed the gel shift of 6Myc-Osterix (Fig. 3C). Dephosphorylation was also demonstrated with the C2C12 cell line. Alkaline phosphatase abolished the phosphorylation effect, which was enhanced by forskolin treatment (Fig. 3D), and overexpressed phosphatase PP2A severely attenuated the expression of Osterix (Fig. 3E). We also checked the endogenous expression of Osterix. Consistent with previous data, endogenous Osterix expression could be up- or downregulated by forskolin or H89 treatment (Fig. 3F, G). However, alkaline phosphatase totally abolished the endo-Osterix bands that were enhanced by the forskolin treatment (Fig. 3 H, I). These results demonstrate that the PKA phosphorylates both exogenous and endogenous Osterix and that the phosphorylation was necessary for the Osterix expression.

PKA AFFECTS THE TRANSCRIPTIONAL ACTIVITY OF OSTERIX

We questioned whether PKA could modulate the transcriptional activity of Osterix, because Osterix is a pivotal transcription factor during osteoblast differentiation. Osteocalcin (OC)-Luc and bone sialoprotein (BSP)-Luc osteoblast reporter genes were used. C2C12 cells were transfected with OC-Luc, BSP-Luc, and with 6Myc-Osterix. Luciferase reporter gene expression was induced by Osterix, and it was further increased by the forskolin treatment, whereas H89 treatment decreased the transcriptional activities of BSP and OC, which were induced by Osterix (Fig. 4A, B). Furthermore, the combination treatment with BMP4 and forskolin showed a synergistic effect on the transcriptional activity of OC (Fig. 4C). The mRNA expression of osteogenic differentiation marker genes such as Collα1, BSP, and ALP, was analyzed by using semi-quantitative RT-PCR. Osterix increased the mRNA levels of Collα1, BSP, and ALP. The treatment with forskolin further enhanced the expression of these marker genes, while the treatment with H89 decreased their expression levels (Fig. 4D). These results show that the PKA activation regulates the transcriptional activity of Osterix, and is important for the Osterix-induced expression of at least a subset of osteoblast marker genes.

DISCUSSION

Osterix belongs to the Sp-1/Krüppel family of transcription factors that promote osteoblast differentiation [Fu et al., 2007]. Osterix regulates the expression of several osteogenic factors by binding to specific GC-rich sequences [Zhang, 2010]. Osterix-deficient

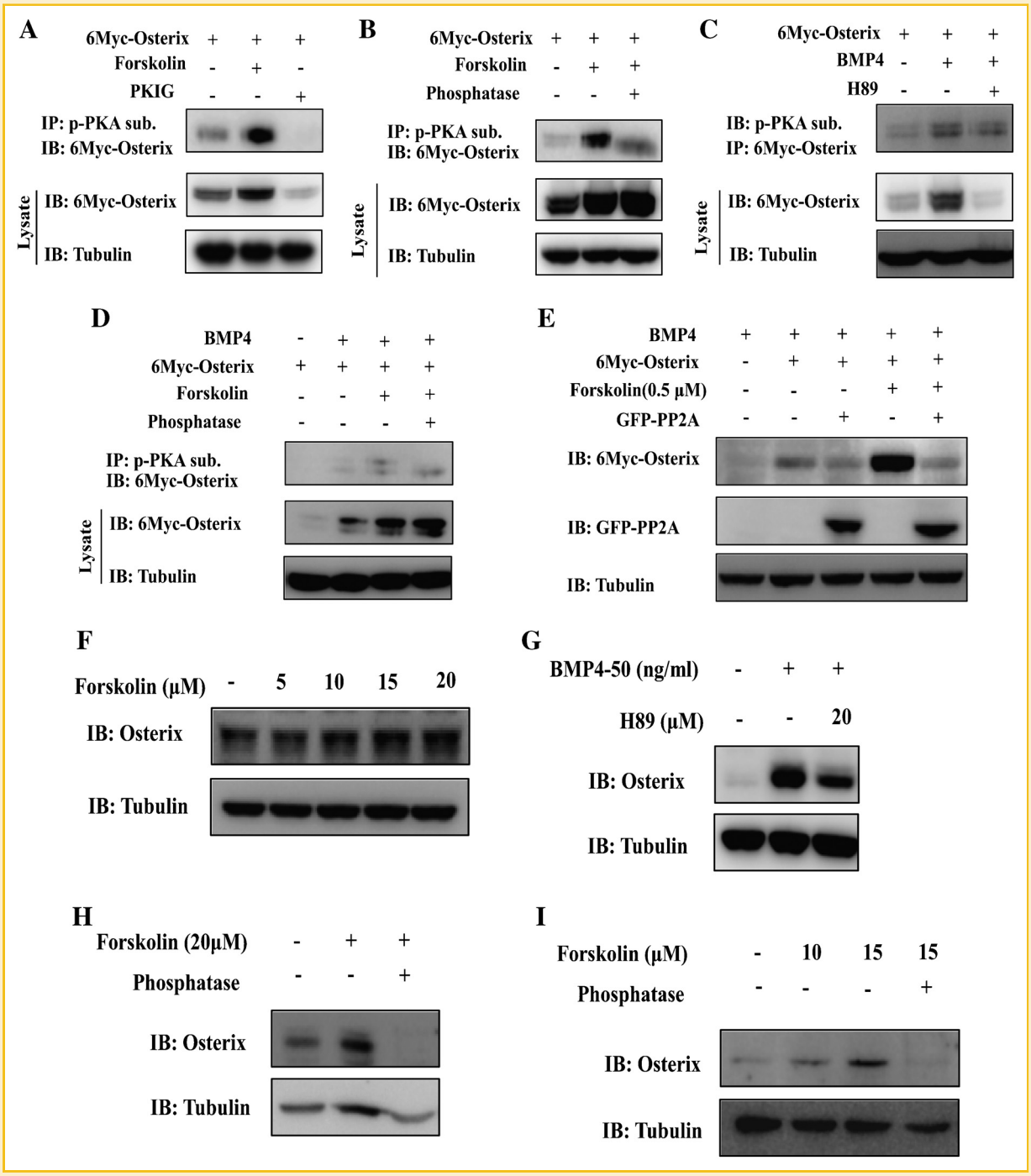

Fig. 3. PKA phosphorylated Osterix. (A) HEK 293 cells were transfected with 6Myc-Osterix, PKIG. On the second day, the cells were treated with DMSO or the indicated dose of forskolin in absence of BMP4 for 24 h. Cell lysates were subjected to immunoprecipitation using anti-phospho-PKA substrate (RRXS/T) antibody (IP: p-PKA sub.). Immunoprecipitates were analyzed by immunoblotting using an antibody against Myc. (B) C2C12 cells were transfected with 6Myc-Osterix and were pretreated with forskolin, alkaline phosphatase in absence of BMP4 for 24 h. Cell lysates were subjected to immunoprecipitation using anti-phospho-PKA substrate (RRXS/T) antibody (IP: p-PKA sub.). Immunoprecipitates were analyzed by immunoblotting using an antibody against Myc. (C) C2C12 cells were transfected with 6Myc-Osterix and were pretreated with H89, alkaline phosphatase in present of BMP4 for 24 h. Cell lysates were subjected to immunoprecipitation using anti-phospho-PKA substrate (RRXS/T) antibody (IP: p-PKA sub.). Immunoprecipitates were analyzed by immunoblotting using an antibody against Myc. (D) C2C12 cells were transfected with 6Myc-Osterix and were pretreated with forskolin, alkaline phosphatase in present of BMP4 for 24 h. Cell lysates were subjected to immunoprecipitation using anti-phospho-PKA substrate (RRXS/T) antibody (IP: p-PKA sub.). Immunoprecipitates were analyzed by immunoblotting using an antibody against Myc. (E) C2C12 cells co-expressed GFP-PP2A and underwent forskolin treatment in presence of BMP4 for 24 h. Cell lysates were examined by immunoblotting. (F) HEK 293 cells were cultured in forskolin or DMSO-treated medium. (G) C2C12 cells were cultured in H89 or DMSO-treated medium in the presence of BMP4. (H and I) HEK 293 and C2C12 cells were cultured in forskolin, alkaline phosphatase or DMSO-treated medium. Cell lysates were analyzed by immunoblotting directly or pretreated with alkaline phosphatase before electrophoresis.

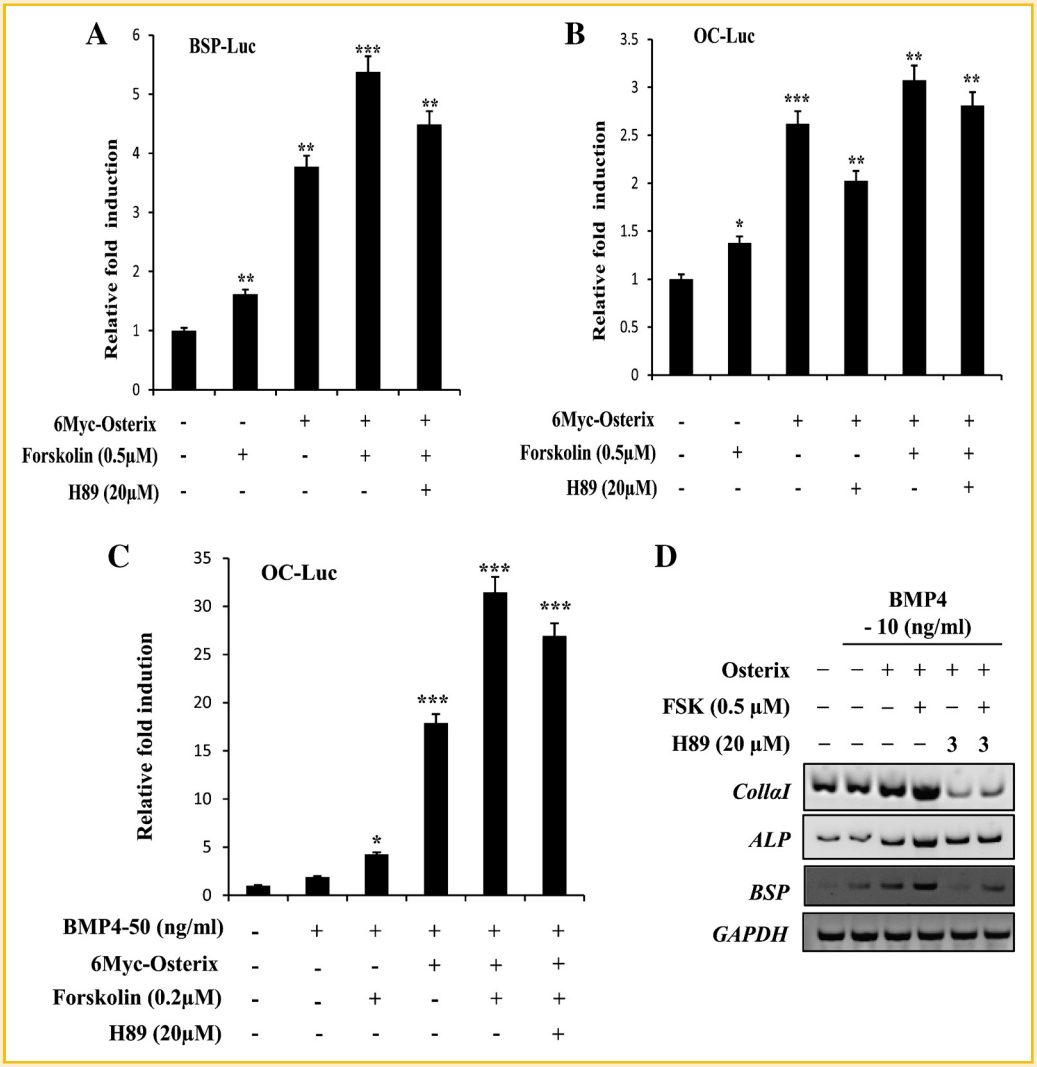

Fig. 4. Transcriptional activity of Osterix was upregulated by PKA. (A and B) C2C12 cells were transfected with p-CMV-β-gal, BSP-Luc, or OC-Luc luciferase reporter gene and 6Myc-Osterix, and the indicated concentration of forskolin or H89. Luciferase activities were measured and normalized to the corresponding β-galactosidase activities. The experiment was repeated three times, and the average and standard deviation of relative luciferase activities are shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus the corresponding control, and comparing Osterix versus Osterix +Forskolin. (C) C2C12 cells were transfected with p-CMV-β-gal, OC-Luc luciferase reporter gene and 6Myc-Osterix, and then treated with combinations of BMP4 (50 ng/mL) and the indicated concentration of forskolin or H89. * $P < 0.05$ and *** $P < 0.001$ versus the corresponding BMP4, and comparing Osterix versus Osterix +Forskolin. (D) The expression of Collα1, BSP, and ALP was compared using semi-quantitative RT-PCR. GAPDH was used as an internal control.

mesenchymal cells fail to differentiate into osteoblasts. In our previous study, it was shown that the function of Osterix can be regulated by the post-translational modification. Our team has also demonstrated that Osterix can be phosphorylated by calmodulin-dependent kinase II (CAMKII) and GSK3α [Choi et al., 2013; Li et al., 2013]. In this study, we focused on the relationship between Osterix and PKA. First, we found that the protein expression of Osterix was enhanced by the PKA activation, while both a chemical inhibitor and a pseudo-substrate of PKA blocked the increase in the protein level of Osterix (Fig. 1). Second, we demonstrated that the half-life of Osterix was prolonged by the PKA activation (Fig. 2). Third, we found that the endogenous and exogenous Osterix was phosphorylated by the PKA (Fig. 3). Finally, we showed that the PKA upregulated the transcriptional activity of Osterix and was important for the Osterix-induced expression of at least a subset of osteoblast marker genes (Fig. 4). Taken together, these findings indicate that the PKA regulates the osteogenic function of Osterix through the post-translational modification and further demonstrate that PKA plays a functional role in the osteoblast differentiation.

BMPs belong to the transforming growth factor beta (TGF-β) family. TGF-β/BMP signaling is involved in a wide variety of cellular processes. Among them, the BMP signaling pathway is widely recognized as a significant inducer of the osteoblast differentiation, committing the maturation of osteoblasts. BMPs function by activating multiple signaling pathways during the osteogenic differentiation. However, not every member of the BMP family is

involved in bone formation. BMP 2, 4, 6, and 7 are osteoinductive. [Kamiya, 2012] Here, we used BMP4 as an osteogenesis inducer for C2C12 cells. C2C12 cells are myoblasts that can undergo the osteoblast differentiation with BMP stimulation [Katagiri et al., 1994].

In a previous study, it was shown that the PKA pathway was activated during the osteoblast differentiation induced by BMP2 [Siddappa et al., 2008]. Crosstalk between the BMP and PKA pathways is not surprising, but has been demonstrated in chondrogenesis and kidney development in embryogenesis [Lee and Chuong, 1997]. SOX9, a master transcription factor in osteochondrogenesis, is involved in the regulation between the PKA pathway and BMPs [Zhao et al., 2009]. Interlacing of BMP and PKA pathways has also been shown during the osteoblast differentiation [Zhao et al., 2006; Zhang et al., 2011; Chen et al., 2012]. These studies suggest that the PTH-cAMP- cAMP response element-binding protein (CREB) signaling pathway synergizes the anabolic signaling of BMP for osteoblast differentiation. PKA, cAMP, and CREB are the major downstream factors in the PTH signaling pathway that play important roles in bone formation. In osteoblasts, PTH binding to its receptor causes abundant releasing of cAMP, activating PKA, which, in turn, activates CREB. It is recognized that the transcription factor CREB mediates PTH signaling in osteoblasts, and BMP2 can act as a key transcriptional target of CREB. Here, we showed that the altered activity of PKA by forskolin or H89 markedly affected BMP4-induced ALP activity (Fig. 1). Because BMP signaling participates in the regulation of Osterix, this means that the BMP signaling pathway exerts its effect on Osterix synergistically with the PKA signaling pathway.

The function of Osterix can be controlled at several levels – at the levels of transcription, translation, and post-translational modification – by multiple signal transduction pathways. According to our data, Osterix was phosphorylated by PKA within a consensus target sequence of RRXS*/T*, suggesting that Osterix contains a phospho-Ser/Thr residue with arginine at the -3 and -2 positions. We did not attempt to determine the exact phosphorylation site(s). More studies are needed to fully determine the exact regulatory mechanism(s) of Osterix.

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