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Calmodulin-dependent kinase II regulates osteoblast differentiation through regulation of Osterix

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ABSTRACT

Osterix (Osx), a zinc-finger transcription factor, is required for osteoblast differentiation and new bone formation during embryonic development. Calmodulin-dependent kinase II (CaMKII) acts as a key regulator of osteoblast differentiation. However, the precise molecular signaling mechanisms between Osterix and CaMKII are not known. In this study, we focused on the relationship between Osterix and CaMKII during osteoblast differentiation. We examined the role of the CaMKII pathway in the regulation of protein levels and its transcriptional activity on Osterix. We showed that CaMKII interacts with Osterix by increasing the protein levels and enhancing the transcriptional activity of Osterix. Conversely, CaMKII inhibitor KN-93 decreases the protein levels and increases the stability of Osterix. The siRNA-mediated knockdown of CaMKII decreased the protein levels and transcriptional activity of Osterix. These results suggest that Osterix is a novel target of CaMKII and the activity of Osterix can be modulated by a novel mechanism involving CaMKII during osteoblast differentiation.

1. Introduction

Bone is a dynamic tissue that undergoes continuous remodeling throughout the course of its lifetime. Bone remodeling and homeostasis are largely the result of a coordinated action of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation while osteoclasts are responsible for bone absorption. A proper balance between osteoblasts and osteoclasts is essential for maintaining proper bone function. The activities of osteoclasts and osteoblasts can be regulated at the level of differentiation by various regulatory signals. Also, bone remodeling is regulated by various anabolic factors including Wnt, insulin, bone morphogenetic proteins (BMPs), insulin growth factor-I, members of the TGF-β family and kinases such as Akt. Bone formation is a complex developmental process involving differentiation of mesenchymal stem cells into osteoblasts. The regulation of osteoblast differentiation is mediated by BMPs and various transcription factors including Runx2, Osterix and several homeodomain (HD) proteins [1–5]. Osteoblasts are differentiated from mesenchymal stem cells [6]. Several transcription factors including homeodomain-containing Dlx proteins, Runx2 (Cbfa1/AML3) and Osterix regulate the differentiation of osteoblasts [7–10].

The expression of many osteogenic homeodomain transcription factors, including Osterix genes, is induced by BMPs [3–5] during early embryogenesis and adult skeletogenesis. Osterix (also known as Sp7) is a novel zinc finger-containing osteoblast-specific transcription factor essential for osteoblast differentiation, proliferation and bone formation [11–13]. The DNA-binding domain of Osterix is located at its C-terminus and contains three C2H2-type zinc finger domains that share a high degree of identity with similar motifs to Sp1, Sp3 and Sp4. In addition, a proline-rich region (RRR) is situated close to the N-terminus. The subcellular localization of Osterix is restricted to the nucleus. The RRR region is responsible for the inhibitory effect of Osterix on the Wnt signaling pathway. Osterix is necessary for osteoblast lineage. Osterix proteins regulate the expression of many osteogenic factors including Runx2, osteonectin, osteopontin, osteocalcin and alkaline phosphatase (ALP).

Ca^{2+} is one of the critical second messengers that regulate a variety of cellular responses including osteoblast differentiation [14,15]. Ca^{2+} signaling is mediated mainly by the Ca^{2+} binding protein calmodulin (CaM). Upon binding to Ca^{2+}, CaM interacts and activates various target proteins including calmodulin-dependent protein kinases (CaMKs), the major targets of CaM. CaMKs are multifunctional serine/threonine kinases, and the CaMK family includes CaMK I, II and IV. Among them, CaMKII plays important roles in the regulation of osteoblast differentiation [16]. CaMKII also regulates the growth of osteosarcoma cells by controlling the progression of cell cycle and by modulating the expression of

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collagenases [17,18]. Although these results indicate that CaMKII is involved in many aspects of bone development, its exact functions in osteoblastogenesis are still unclear.

In this study, we examined whether CaMKII regulates osteoblastogenesis in BMP4-stimulated osteoblast differentiation through regulation of Osterix. We found that CaMKII increases the protein levels and the protein stability of Osterix. Furthermore, we provided evidence that the transcriptional activity of Osterix is enhanced by CaMKII and repressed by the CaMKII inhibitor KN-93. Also, CaMKII interacts with exogenous and endogenous Osterix and phosphorylates Osterix. Our results indicate that CaMKII regulates osteoblast differentiation, at least in part, by up-regulating the function of Osterix.

2. Materials and methods

2.1. Cell culture

The C2C12 mouse pre-myoblast cell line, Bosc23 human kidney cell line and 293 human embryonic kidney epithelial cell line were maintained at 37 °C, 5% or 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic. DMEM, FBS and antibiotic–antimycotic were purchased from Gibco Life Technologies, Invitrogen.

2.2. Plasmids, antibody and chemical

6Myc-tagged Osterix and 3Myc-tagged CaMKII were constructed in a CMV promoter-derived mammalian expression vector (pCS4+). The GFP-tagged PP2A was generously provided by Dr. Kyeong-Man Kim (Chonnam National University). For knockdown of CaMKII, oligonucleotides targeting following sequences were synthesized: sense-(CaMKII) 5′-GAT CCC CTG ATC GAA GCC ATA AGC AAT TCA AGA GAT TGC TTA TGG CTT CGA TCA TTT TTG GAA A-3′; and antisense-(CaMKII) 5′-AGC TTT TCC AAA AAT GAT CGA AGC CAT AAG CAA TCT CTT GAA TTG CTT ATG GCT TCG ATC AGG G-3′. Transient transfection was performed by the calcium phosphate-mediated method or the polyethyleneimine (PEI) (Polysciences, Inc.) mediated method. Total amounts of transfected plasmids in each group were equalized by adding an empty vector. Antibodies against Myc (9E10, Roche Applied Science, USA), Osterix (A-13, Santa Cruz Biotechnology, USA), CaMKII (H-300, Santa Cruz Biotechnology, USA), GFP (Santa Cruz Biotechnology, USA), α-tubulin (B-5-1-2, Sigma–Aldrich), CaMKII inhibitor KN-93 (422708, Calbiochem, USA) or inactive analog KN-92 (422709, Calbiochem, USA) were used.

2.3. Immunoblotting and immunoprecipitation

For immunoblotting, 48 h after transfection, HEK293 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 containing 30 μg of total protein were subjected to SDS–PAGE. Proteins were transferred to a PVDF membrane and visualized using appropriate primary antibodies, HRP-conjugated secondary antibodies and ECL reagent. For immunoprecipitation, 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.

2.4. Protein stability assay

HEK 293 cells were co-transfected with 6Myc-tagged Osterix or 3Myc-tagged CaMKII expression vectors. After 24 h, the cells were exposed to fresh media. Transfected cells were incubated for the indicated times and were treated with 40 μM of cycloheximide (CHX) and harvested with lysis buffer as described above. Protein levels were analyzed by immunoblotting using the anti-Myc antibody.

2.5. Luciferase reporter assay

Cells were seeded on 24-well plates the day before transfection. HEK293 cells were transfected with ALP (ALP-Luc), Bone sialoprotein (BSP-Luc) reporter plasmid, pCMV-β-gal, and combinations of Osterix, CaMKII expression vectors and KN-93 or KN-92 on 24-well plates and lysed for 36 h after transfection. Luciferase activities were measured using Luciferase Reporter Assay Kit (Promega) and normalized with corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicate and were repeated at least three times.

2.6. Total RNA extraction, reverse transcribed RT-PCR analysis

Total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Random-primed cDNAs were synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). The following conditions were used for amplification by PCR: 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, and then extension at 72 °C for 30 s; final extension at72 °C for 5 min. The following PCR primers were used: ALP forward 5′-GAT CAT TCC CAC GTT TTC AC-3′ and reverse 5′-TGC GGG CTT GTG GGA CCT GC-3′; ColIa1 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′; Osterix forward 5′-GGG TTA AGG GGA GCA AAG TCA GAT-3′ and reverse 5′-CTG GGG AAA GGA GGC ACA AAG AAG-3′, GAPDH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′.

2.7. Alkaline phosphatase (ALP) staining

C2C12 cells in 24-well plates were transfected using PEI. C2C12 cells were induced by stimulating the cells with BMP4. The cells were pretreated with BMP4 for 3 days. These cells were cultured at 5% CO_2, 37 °C. Transfected C2C12 cells were fixed in 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.

3. Results

3.1. Activation of CaMKII is involved in BMP4-stimulated osteoblast differentiation

In previous studies, regulation of osteoblast differentiation was found to be mediated by bone morphogenetic proteins, hedgehogs and Runx2 [2]. In the present study, we confirmed that BMP4 stimulation induces osteoblast differentiation in C2C12 myoblast cells. Osteoblast differentiation was measured by ALP staining. First, we

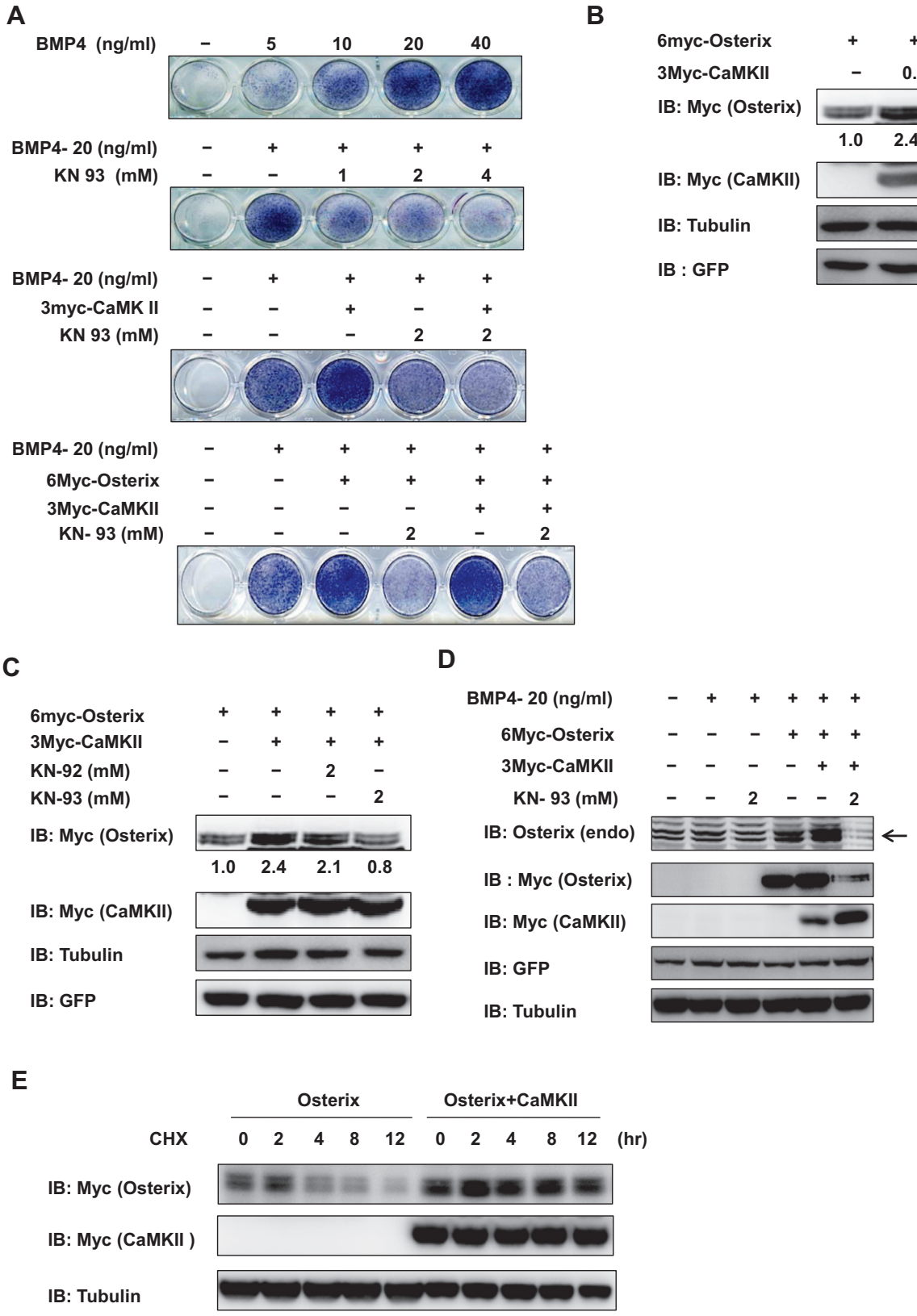

Fig. 1. CaMKII activation is induced in BMP4-stimulated osteoblast differentiation and increases the protein levels of Osterix. Cells were transfected with 3Myc-tagged CaMKII, 6Myc-tagged Osterix and treated with KN93, at the indicated concentrations in the absence or presence of BMP4. After 3 days, the extents of osteoblast differentiation were compared by ALP staining. ALP positive cells stained blue/purple. Similar results were obtained from three independent experiments. (B) HEK 293 cells were transfected with 6Myc-tagged Osterix and 3Myc-tagged CaMKII (0.5–1.0 μg) or a control vector along with GFP. GFP was used as a transfection control and tubulin was used as a loading control. (C) HEK 293 cells were transfected with 6Myc-tagged Osterix, 3Myc-tagged CaMKII and treated with KN-93 or untreated vehicle as a control (DMSO). (D) BMP4 induced osteoblast differentiation in C2C12 cells and C2C12 cells were transfected with 6Myc-tagged Osterix, 3Myc-tagged CaMKII and treated with KN-93 (2 mM) or untreated vehicle as a control (DMSO). Protein levels were determined by immunoblotting using anti-Osterix and anti-Myc antibody. (E) HEK 293 cells were co-transfected with Myc-tagged Osterix, CaMKII and treated with cycloheximide (40 μM). After 24 h, transfected cells were harvested at the indicated times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

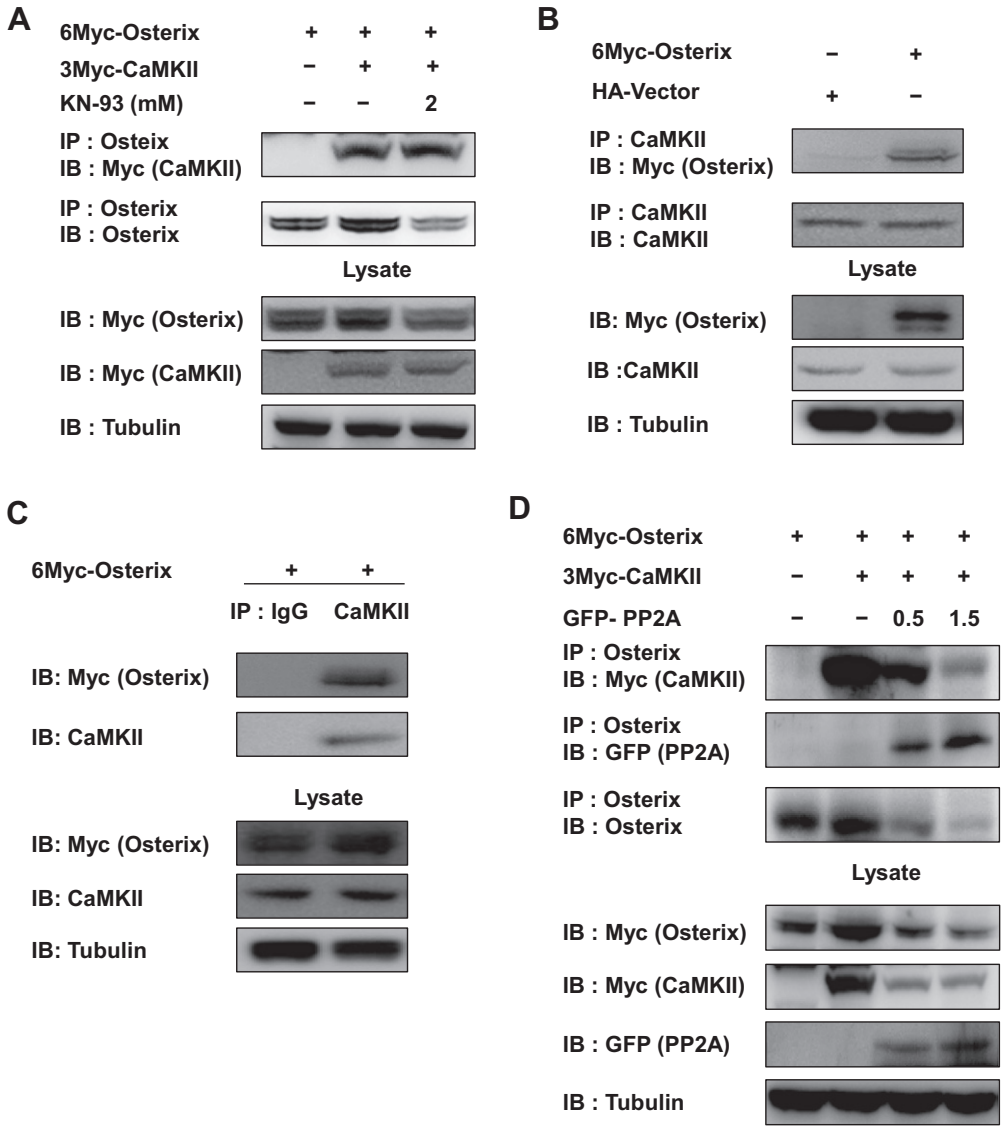

Fig. 2. Osterix interact with CaMKII and protein phosphatase PP2A. (A) HEK 293 cells were transfected with Myc-tagged Osterix, Myc-tagged CaMKII and treated with the CaMKII inhibitor, KN-93. To examine the binding of Osterix and CaMKII, we performed immunoprecipitation (IP) using anti-Osterix antibody followed by Western blot analysis using anti-Myc antibody. (B) HEK 293 cells were transfected with Myc-tagged Osterix and HA-tagged vector. For the endogenous interaction of Osterix and CaMKII, we performed IP using CaMKII Ab and Western blotting with Myc Ab. (C) Myc-tagged Osterix was transfected to examine whether the binding of Osterix and CaMKII is endogenous. The binding of Osterix in HEK 293 cells was performed IP using IgG or CaMKII Ab and Western blotting with Myc Ab. (D) Myc-tagged Osterix, Myc-tagged CaMKII and GFP-tagged PP2A were transfected to examine the dephosphorylation of Osterix in HEK 293 cells. The dephosphorylation of Osterix was determined by IP using an anti-Osterix antibody followed by Western blot analysis using anti-Myc antibody.

examined whether BMP4 induced CaMKII activation in C2C12 cells. When we pretreated with BMP4 alone or with BMP4 and increasing amounts of the KN-93 at the indicated concentrations, BMP4 increased ALP activity in a dose-dependent manner. ALP staining showed that blocking CaMKII activation with KN-93 strongly suppressed ALP (Fig. 1A). Second, we examined whether CaMKII affected the protein levels of Osterix. HEK 293 cells were transfected with combinations of Osterix and CaMKII. The levels of Osterix were increased by CaMKII in a dependent manner (Fig. 1B). However, inhibition of CaMKII by KN-93 decreased the protein levels of Osterix, but not the inactive analog KN-92 (Fig. 1C). These results suggest that CaMKII regulates Osterix-induced osteoblastogenesis.

3.2. CaMKII affects Osterix in BMP4-induced osteoblast differentiation

We confirmed that CaMKII activation is involved in BMP4-induced osteoblast differentiation (Fig. 1). In order to understand the mechanism of relationship the major transcription factor Osterix and CaMKII in BMP4-induced osteoblast differentiation. First, we examined whether CaMKII affects Osterix-induced osteoblast differentiation. C2C12 cells were transfected with Osterix, CaMKII and treated with KN-93. Osterix directly induced the expression of ALP. In the presence of BMP4 stimulation, CaMKII inhibitor KN-93 significantly decreased ALP staining (Fig. 1A). Second, the protein level of endogenous Osterix was increased by BMP4 and decreased by KN-93 (Fig. 1D). The protein levels of Osterix were increased by CaMKII (Fig. 1B–D). These results suggest that CaMKII affects Osterix in BMP4-induced osteoblast differentiation and increases the protein levels of Osterix.

3.3. CaMKII regulates the protein stability of Osterix

CaMKII may regulate the expression of Osterix at the level of transcription, translation or protein stability. The exogenous and endogenous protein levels of Osterix were increased by CaMKII

(Fig. 1B and D). To identify the molecular mechanism for the CaMKII-induced increase of Osterix protein levels, we examined whether CaMKII affected the protein stability of Osterix using cycloheximide (CHX). To estimate the stability of Osterix by CaMKII, the transfected cells were treated with 40 μM CHX for the indicated times and then harvested. The protein levels of Osterix were determined by Western blotting. Osterix protein was degraded in the absence of CaMKII with a half-life of about 4 h (Fig. 1E). However, CaMKII significantly blocked Osterix degradation and prolonged the half-life of the Osterix protein (Fig. 1E). CaMKII clearly extended the half-life of the Osterix protein. These results suggest that CaMKII signaling is critical for the maintenance of Osterix stability.

3.4. CaMKII interacts with and phosphorylates Osterix

Given the results above, we questioned whether Osterix might interact with CaMKII. To confirm this idea, HEK 293 cells were transfected with Myc-Osterix and Myc-CaMKII and then performed immunoprecipitation (IP). We found that Osterix was bound to exogenous CaMKII (Fig. 2A). Furthermore, IP was performed again for the interaction between Osterix and endogenous CaMKII. We determined that Osterix was bound to endogenous CaMKII (Fig. 2B and C). In a previous study, the CaMKIV regulated by protein serine/threonine phosphatase 2A (PP2A). The CaMKIV-associated PP2A and then dephosphorylated CaMKIV T200, thereby terminating the autonomous activity and CaMKIV-mediated gene transcription [19]. To investigate the phosphorylation of Osterix by CaMKII, we next examined whether PP2A can dephosphorylate the phosphorylated Osterix by CaMKII. HEK 293 cells were co-transfected with Myc-Osterix, CaMKII and GFP-PP2A. The protein level of Osterix revealed that phosphatase PP2A decreased the CaMKII activation induced protein level of Osterix (Fig. 2D). These results indicate that CaMKII interacts with Osterix and may phosphorylate Osterix.

3.5. CaMKII enhances the transcriptional activity of Osterix

To analyze whether CaMKII can modulate the transcriptional activity of Osterix, C2C12 cells were transfected with ALP-Luc and BSP-Luc osteoblast reporter genes, which are known to be markers of osteoblast differentiation. C2C12 cells were transfected with ALP-Luc or BSP-Luc along with combinations of Osterix and CaMKII or were treated with KN-93 using the indicated concentrations. Osterix or CaMKII by itself increased the reporter expression above the basal level, and co-transfection of them further increased the reporter expression (Fig. 3A and B). However, transfection of Osterix with KN-93 treatment failed to increase the reporter expression. We then examined the effect of CaMKII activity on the BMP4 and Osterix-induced expression of osteoblast marker genes. C2C12 cells were transfected with combinations of Osterix and CaMKII, and then treated with KN-93 and BMP4. The transcriptional levels of the following osteoblast markers were examined: ALP, BSP, collagen type I α (Collα) and Osterix. Osterix was increased by the BMP4-induced expression of the osteoblast markers (Fig. 3C). CaMKII further increased the expression of the markers.

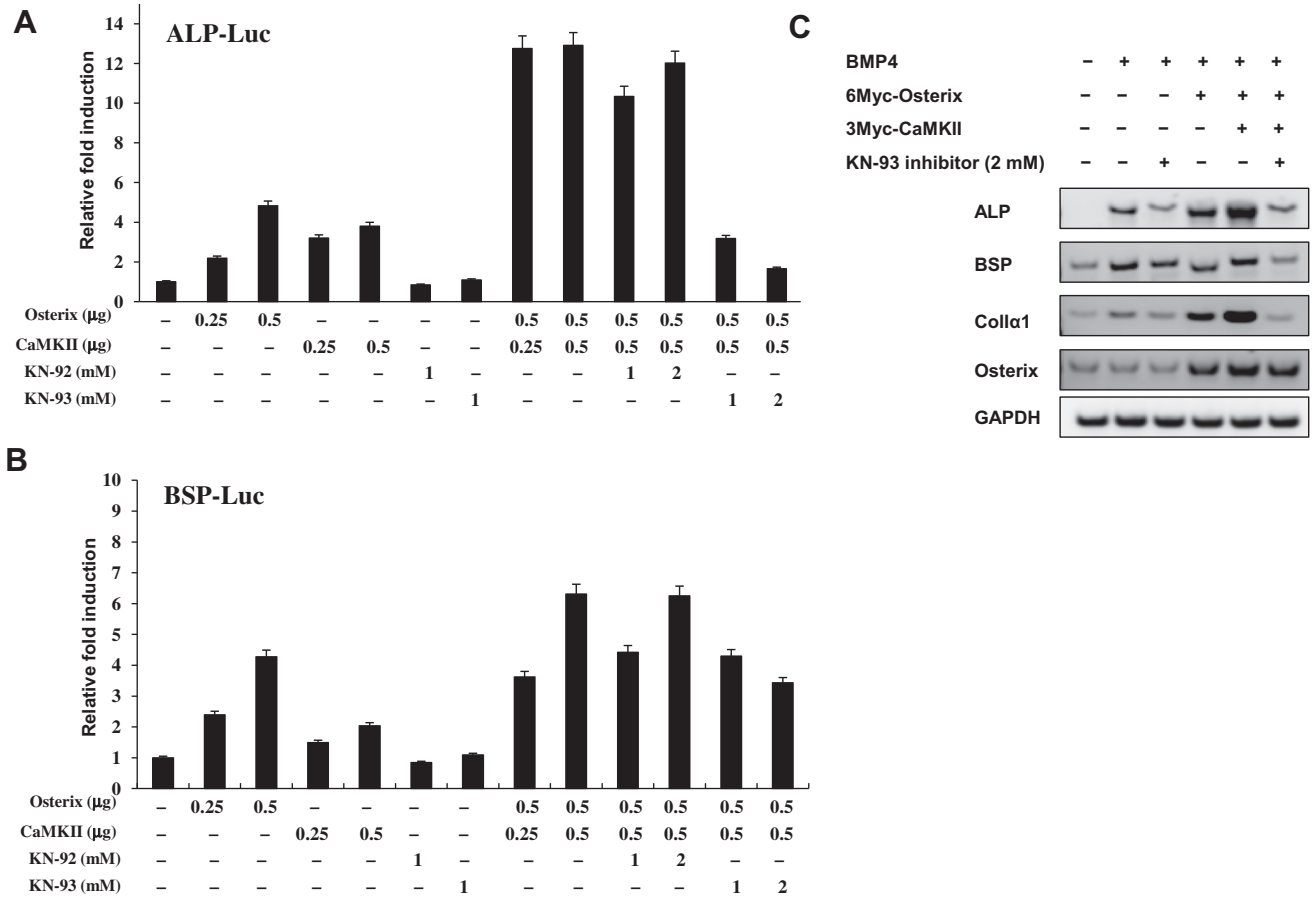

Fig. 3. CaMKII enhances the transcriptional activity of Osterix. (A and B) C2C12cells were transfected with pCMV-β-gal, BSP-Luc, ALP-Luc reporter vector along with the indicated combinations of Osterix and CaMKII expression vectors and were treated with KN-93 or KN-92. Reporter activity was then measured. Data are expressed as relative induction ratios to an internal control and relative luciferase activities with SD are shown. The experiment was performed in triplicate. (C) C2C12 cells were transfected for 24 h with the indicated combinations of Osterix and CaMKII or were treated with KN-93. Cells were then treated with BMP4 (5 ng/ml) for 24 h. The expression levels of bone sialoprotein (BSP), collagen type I α (Collα), alkaline phosphatase (ALP) and Osterix were compared by semi-quantitative RT-PCR. GAPDH was used as an internal control.

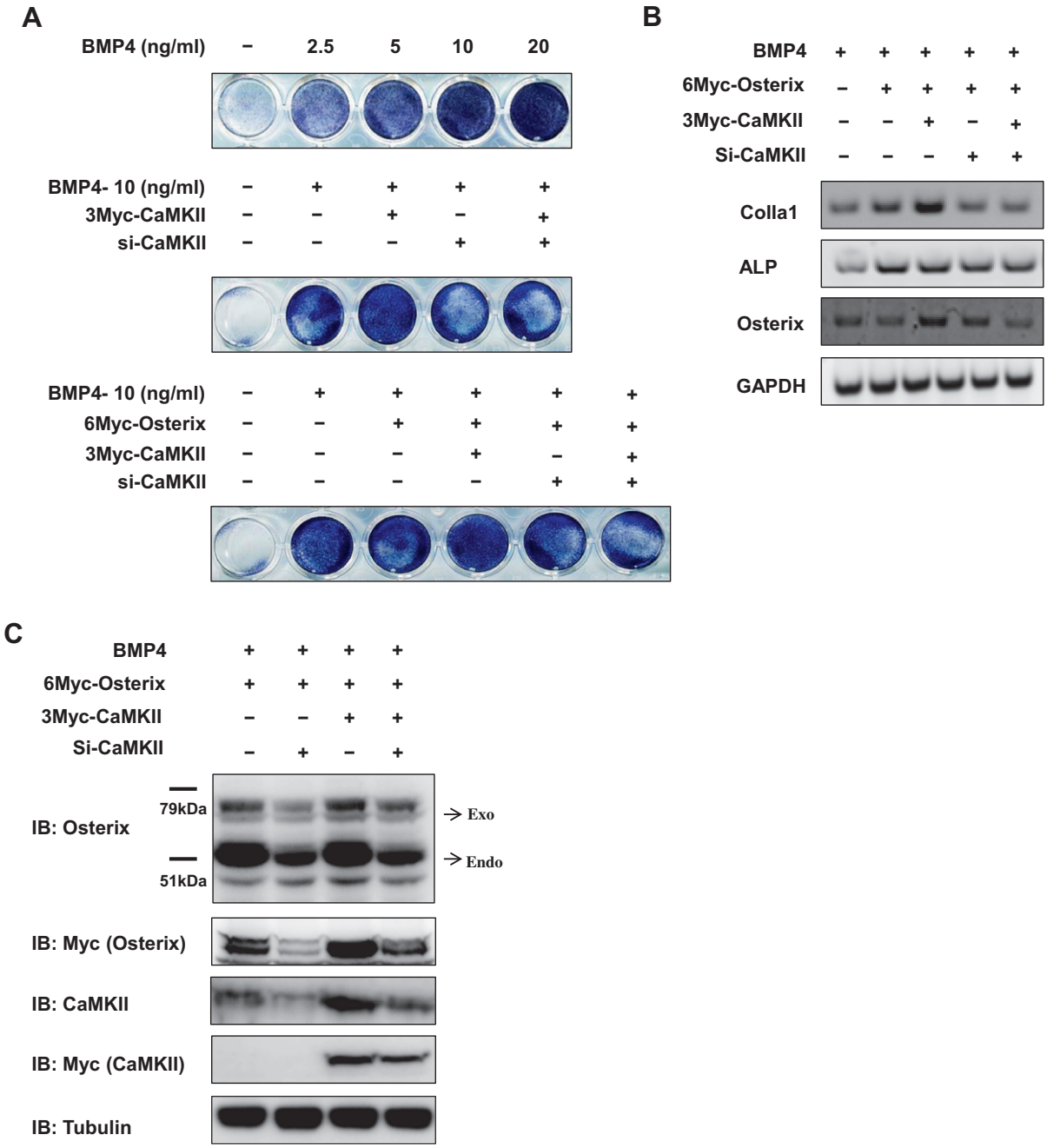

Fig. 4. Knockdown of CaMKII by the specific siRNA affects the mechanism of the major transcription factor of Osterix. (A) Cells were transfected with Myc-tagged CaMKII WT and siRNA-CaMKII at the indicated concentrations in the absence or presence of BMP4. After 3 days, the extent of osteoblast differentiation was compared by ALP staining. (B) C2C12 cells were transfected for 24 h with the indicated combinations of Osterix and CaMKII WT or siRNA-CaMKII. Cells were then treated with BMP4 (5 ng/ml) for 24 h. The expression levels of Collα, ALP and Osterix were compared by semi-quantitative RT-PCR. GAPDH was used as an internal control. (C) For endogenous protein levels by siRNA-CaMKII, C2C12 cells were transfected with Myc-tagged Osterix, Myc-tagged CaMKII WT and siRNA-CaMKII. GFP was used as a transfection control and tubulin was used as a loading control. Protein levels were determined by immunoblotting using anti-Osterix antibody.

Interestingly, KN-93 decreased the expression of BSP, ALP, Osterix and Collα below the levels seen with Osterix alone or with BMP4. These results indicate that CaMKII activity regulates the transcriptional activity of Osterix and is critical for the Osterix-induced expression of at least a subset of osteoblast markers.

3.6. Knockdown of endogenous CaMKII by the specific siRNA affects the mechanism of the major transcription factor Osterix

The protein levels, stability and transcriptional activity of Osterix were regulated by CaMKII (Figs. 1 and 4). In order to understand clearly the mechanism of the major transcription factor Osterix, we examined whether knockdown of endogenous CaMKII by the specific siRNA affected osteoblast differentiation, the protein levels and transcriptional activity of Osterix. First, siRNA-CaMKII suppressed osteoblast differentiation in BMP4 signaling (Fig. 4A). Second, exogenous or endogenous protein levels of Osterix were significantly decreased by siRNA-CaMKII (Fig. 4C). Finally, the increased mRNA levels of osteoblast markers by CaMKII were significantly abolished by siRNA-CaMKII (Fig. 4B). These results suggest that the CaMKII signaling pathway may regulate the mechanism of the major transcription factor Osterix in osteoblast differentiation.

4. Discussion

In the present study, we investigated the potential regulation of Osterix by CaMKII. We examined the effects of CaMKII on the function of Osterix during osteoblast differentiation. The results evidently show a novel regulatory mechanism of CaMKII in the regulation of osteoblast differentiation. First, when assessed by ALP staining, Osterix/BMP4-induced osteoblast differentiation is inhibited by KN-93, but not by the inactive analog KN-92. Second, CaMKII increases the protein stability of Osterix. Third, Osterix interacts with and is dephosphorylated by PP2A through regulation of CaMKII. Fourth, CaMKII increases the transcriptional activity of Osterix while this increase is abolished by KN-93. Lastly, the siRNA-mediated knockdown of endogenous CaMKII suppressed osteoblast differentiation and decreased the protein levels, mRNA levels of Osterix.

In adults, bone is constantly removed by osteoclasts and replenished by osteoblasts. The coordinated action of osteoblasts and osteoclasts maintains the proper bone volume and calcium homeostasis. While Ca^{2+} homeostasis is under the control of osteoblasts and osteoclasts, Ca^{2+} also regulates bone remodeling as an important intracellular second messenger. An increase in the level of intracellular Ca^{2+} is considered to promote osteoblast proliferation and/or differentiation during bone remodeling [20,21]. Ca^{2+} signaling is mainly mediated by calmodulin (CaM) and calmodulin-dependent kinases (CaMKs) [14]. Several lines of evidence strongly suggest that CaMKII plays an essential role in osteoblast differentiation [15]. The role of CaMKII in osteoblast differentiation is an interesting subject as Ca^{2+} signaling is also important for bone homeostasis. In this aspect, regulation of Osterix function by CaMKII may play a significant role in osteoblast differentiation.

The activity of Osterix can be modulated by several kinases through post-translational modifications (PTM). Recent studies have shown that p38 MAPK, Akt1 and calcineurin phosphorylate increase the transcriptional activity of Osterix [22–24].

PP2A is a ubiquitous heterotrimeric serine/threonine phosphatase consisting of a dimeric core enzyme composed of structural A and catalytic C subunits and a regulatory B subunit. PP2A has diverse cellular functions in the regulation of protein dephosphorylation in oncogenic signaling cascades involving Raf, MEK and AKT [25–27]. PP2A also regulates GSK3 isoforms that play significant roles in WNT signaling [28]. In the present study, we used the dephosphorylation function of PP2A to study the CaMKII effect on Osterix. The results showed that over-expressed PP2A interacted with CaMKII and Osterix. Also, PP2A abrogated the CaMKII activation-induced protein level of Osterix (Fig. 2D).

BMP4 regulates the transcription of Osterix [14], while p38 regulates the transactivation activity of Osterix by phosphorylation [17]. The modulation of Osterix function by several kinases suggests that Osterix may serve as a point of signal integration for osteogenic signals and their intracellular effectors. Our results suggest that CaMKII also modulates the function of Osterix, possibly through direct phosphorylation. This suggests that CaMKII may partially function as a mediator of BMP4 signaling, which also provides an argument for the performance of CaMKII activation-enhanced cell osteoblast differentiation.

Mechanisms of how CaMKII regulates bone development are not well understood. Our results indicate that CaMKII regulates Osterix during osteoblast differentiation. Our work provides a basis for understanding the roles of CaMKII during osteoblast differentiation. We showed that CaMKII modulates Osterix function by suppressing protein degradation. Further studies are needed to understand the mechanism of Osterix protein degradation, and to understand the significance of such regulation in osteoblast differentiation. Ubiquitination and sumoylation are the two main pathways for protein degradation. Elucidation of upstream and downstream regulatory components involved in the regulation of Osterix by CaMKII will help to understand the significance of this novel interaction. Our work provides a basis for understanding the roles of CaMKII during osteoblast differentiation. CaMKII modulates Osterix function through regulation of protein stabilization, transcriptional activity and phosphorylation.

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