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SOX9 Directly Binds CREB as a Novel Synergism With the PKA Pathway in BMP-2–Induced Osteochondrogenic Differentiation

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ABSTRACT: SOX9 acts as a master transcription factor in osteochondrogenesis, and the phosphorylation by protein kinase A (PKA) has been shown to increase its DNA binding and transactivation activity. The PKA pathway is involved in the complex downstream signaling underlying the BMP-2–mediated osteochondrogenesis. This study therefore aimed at further analyzing the possible cross-talk between the SOX9 and the PKA regulation on the background of BMP-2 stimulation. It was first shown that the removal of the residues serine 64 and 211 of SOX9 diminished, but did not completely deplete, its stimulatory effect on the expression of both osteo- and chondrogenic markers. PKA activators and inhibitors increased and decreased the action of wildtype and mutated SOX9, respectively. Interestingly, the interplay of the SOX9 action with the PKA pathway was further shown to occur through direct physical association between SOX9 and CREB, a prototypical PKA downstream transcription factor. Moreover, the binding was shown to be an active biological event happening on BMP-2 stimulation. The C-terminal domain of SOX9 and amino acid residue serine at position 133 of CREB were identified to be involved in the interaction. The action of SOX9 was enhanced by overexpressing CREB. These results suggest that PKA signaling synergizes with SOX9 at the nuclear and cytoplasmic levels to promote BMP-2–induced osteochondrogenic differentiation.
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Key words: SOX9, protein kinase A, osteochondrogenic differentiation

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INTRODUCTION

SOX9 belongs to the SOX (SRY-type HMG box) transcription factor family and has been known to play essential roles in multiple aspects of the embryogenesis and postnatal development.[1] The mutations of SOX9 have been identified to be responsible for Campomelic dysplasia (CD) in human, a disease featured with severe hypoplasia of almost all cartilages and bones and sexual reversal.[2,3] During skeletal development, SOX9 is particularly known to regulate mesenchymal cells before and after condensation,[4–7] and the two main skeletal cell lineages, chondrocytes and osteoblasts, arise from *Sox9*-expressing cranial neural crest cells.[8] SOX9 shows strong binding and transactivation activity on corresponding enhancer or promoter elements existing in many chondrocyte-specific genes such as *Col2αI*,[1] *Col9αI*,[9] *Col11αII*,[10] *aggrecan*,[11] and *CD-RAP*.[12] Heterozygous *Sox9* mutant mice phenocopy the skeletal deformations of CD patients and exhibit defective cartilaginous condensations and severe skeletal hypoplasia.[6] The role of SOX9 in regulating chondrogenesis often seems to be predominant, as shown in many in vivo and in vitro systems. However, SOX9 is not limited to chondrogenic lineage cells and is also present in several other cell types, including osteogenic cells.[4,6,13] For example, Sox9 has been found in developing craniofacial bones, suggesting its involvement in ossification.[14] Moreover, SOX9-driven chondrocytes can also promote osteogenesis by secreting several soluble stimulatory factors.[15]

The activity of SOX9 is regulated by a number of intracellular factors.[16–18] Its multifaceted roles are therefore integrated with several major signaling pathways and involved in different physiological functions. In the growth plate, the PTH-related protein (PTHrP) signaling can activate cAMP-dependent protein kinase A (PKA), which in turn phosphorylates SOX9 and increases its DNA binding and transcriptional activities.[19,20] In SOX9-dependent gonadal cells, the phosphorylation by PKA has been further shown to induce the nuclear localization of SOX9 by enhancing its binding to the nucleo-cytoplasmic transport protein.[21] Furthermore, it has recently been shown that SOX9 is functionally associated with cAMP-response element-binding protein (CREB)-binding protein (CBP) and the paralog p300, the transcriptional co-regulators that participate in the activities of many different transcription

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factors, including chondrogenic factors.[17,22] Whether SOX9 synergizes with the PKA pathway also through interacting with other molecules involved in the SOX9–CBP complex network remains to be clarified.

Bone morphogenesis protein-2 (BMP-2), a member of the BMP family, is one of the best-characterized inducers of osteochondrogenesis and can increase the expression of hallmarks of both osteo- and chondrogenic lineage differentiations of progenitor cells, such as mesenchymal stem cells (MSCs)[23–25] and even pre-myoblast C2C12 cells.[26] The BMP-2 signaling pathway is known to be mainly mediated through Smad proteins and mitogen activation protein kinases.[27,28] We have recently shown that the PKA activation also forms an important part of the mechanisms by which BMP-2 mediates MSC differentiation.[29] Once activated, PKA usually phosphorylates its prototypical downstream transcription factor, CREB, which is associated with a number of important physiological functions including osteochondrogenic differentiation.[30–32] However, how BMP-2 signaling-related molecules regulates SOX9 function remains to be analyzed in greater details.

Accordingly, the aim of this study was to analyze the roles of the PKA signaling in regulating the promoting function of SOX9 in BMP-2–stimulated differentiation of progenitor cells. Particularly, the physical and functional interaction between SOX9 and CREB on the BMP-2–mediated differentiation background was shown.

MATERIALS AND METHODS

SOX9 expression constructs and mutagenesis

Substitution mutations were introduced into the expression plasmid containing a full-length murine SOX9 cDNA[33] using the Quickchange Multi Site-Directed Mutagenesis Kit (Stratagene). Constructs expressing FLAG-tagged and truncated SOX9 constructs were generated by inserting the *Eco*RI/*Xho*I DNA fragments, which were PCR-amplified using FLAG-tagged forward primers and reverse primers as shown in Table 1, into the pcDNA3 vector (Invitrogen).

Cell culture and transfection experiments

Murine mesenchymal stem cell line C3H10T1/2, murine myogenic progenitor line C2C12, and human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% FBS. For differentiation induction of C3H10T1/2 and C2C12 cells, rhBMP-2 at indicated concentrations was added to cells cultured with a low serum concentration (2%). Cell transfection was performed using Lipofectamine 2000 (Invitrogen). For the selection of stable transfectant clones, 5×10^5 transfectant cells were replated into T25 flasks and cultured in selective media containing 1 mg/ml of the drug G418 (Invitrogen) over a period of 3–4 wk.

Alkaline phosphatase activity assay

Cytochemical staining for alkaline phosphatase (ALP) activity was performed using an ALP staining kit (Sigma). C2C12 cell clones transfected with empty vector or wild-type or mutated SOX9 expression plasmids were plated into 24-well plates and subjected to the treatment with rhBMP-2 in the presence or absence of the PKA activator 8-Br-cAMP and inhibitor H-89 (Sigma), respectively. In some experiments, stable cell clones were further transfected with pcDNA6B, pcDNA6B-PKIγ,[29] pCMV-CREB, or pCMV-CREBS133A (Clontech) before the stimulation with rhBMP-2. For the ALP assay, cells were washed twice with PBS and lysed with 0.1% Triton X-100 in PBS and by several cycles of freezing and thawing. The ALP activity in cell lysates was measured using an ALP assay kit (Sigma) and normalized by the protein concentration. ALP activities were reported as the average of triplicate cultures in one experiment representative for three to four similar ones.

TABLE 1. Nucleotide Sequences for PCR Primers Used in This Study

Primers for generating FLAG constructs
FLAG-tagged SOX9 (1–423)
5′-GAATTCATGGACTACAAGGACGACGATGACAAAA TGAATCTCCTGGACCCCTTC-3′ (F)
5′-CTCGAGTCAAGGAAGGTTGAAGGGGCTGTAGG-3′ (R)
FLAG-tagged SOX9 (mu5, 1–327)
5′-GAATTCATGGACTACAAGGACGACGATGACAAAA TGAATCTCCTGGACCCCTTC-3′ (F)
5′-CTCGAGTCAGGGTCTGGTGAGCTGTGTGTAGACT GG-3′ (R)
FLAG-tagged SOX9 (328–507)
5′-GAATTCATGGACTACAAGGACGACGATGACAAAcc TGCGACCGCGGGC CACGTGTGG-3′ (F)
5′-CTCGAGTCAGGGTCTGGTGAGCTGTGTGTAGACT GG-3′ (R)

Luciferase activity assay

The luciferase reporter plasmid pKN159B × 6luc[34] containing six tandem copies of the murine Col2aI chondrocyte-specific enhancer sequences, PG70[35] containing the 2.4-kb promoter of the murine *Col1aI* gene, or pCRE-luc (Stratagene) containing the CREB responsible element was transiently transfected into cells, with plasmid pGL3 as a negative and pRL-SV40 (Promega) an internal control for transfection efficiency normalization. In some cases as indicated, reporter constructs were co-transfected with wildtype or mutated SOX9 expression plasmids and with pcDNA6B, pcDNA6B-PKIγ, pCMV-CREB, or pCMV-CREBS133A, respectively. In some experiments, the specific PKA activator 8-Br-cAMP or inhibitor H-89 was added to cultured cells 6 h after co-transfection. Cells were harvested 48 h after co-transfection and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

PKA activity assay

The PepTag non-radioactive cAMP-dependent protein kinase assay kit (Promega) was used to measure the PKA activity in C3H10T1/2 cells. Cells were cultured in 10-cm dishes with or without 200 ng/ml rhBMP-2 for 3 days before

lysis in PKA extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM-mercaptoethanol, 1 g/ml leupeptin, 1 g/ml aprotinin). Cell lysates were subjected to the PKA assay according to manufacturer's recommendations.

Western blotting analysis

C2C12 and HEK293T cells were lysed 48 h after co-transfection of full-length or FLAG-tagged truncated SOX9, with either pCMV-CREB or pCMV-CREBS133A. C3H10T1/2 cells were lysed after induced by 500 ng/ml rhBMP-2 for 0, 6, 9, and 12 days. After washing twice with ice-cold PBS, cells were lysed for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate) containing protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The lysates were further clarified by centrifugation at 14,000*g* at 4°C for 20 min. Proteins were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted using antibodies specific to SOX9 or CREB (Santa Cruz). Because the epitope for the anti-SOX9 antibody locates in the C-terminal domain, which was absent in truncated SOX9 molecules, an anti-FLAG antibody, M2 (Sigma), was used. The signal was developed using the enhanced chemiluminescence (ECL) kit according to the manufacturer's recommendations (Amersham Biosciences).

Co-immunoprecipitation

C3H10T1/2 cells incubated with BMP-2 for different days or HEK293T cells co-transfected with wildtype, mutated, or FLAG-tagged truncated SOX9 and CREB or mutant CREBS133A were lysed as mentioned above. Cell lysates were harvested in the same way as for Western blotting (WB), and protein supernatants were pre-cleared with protein A-Sepharose (Amersham Biosciences) at 4°C for 1 h. Then 5 μg of antibody specific for each target protein was added into a 1.0-ml reaction containing 200 μg of total proteins. Immune complexes were precipitated by protein A-Sepharose overnight at 4°C and washed five times with lysis buffer. The immune complexes were boiled for 10 min in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromphenol blue, 20% glycerol, and 4% SDS) containing 10 mM dithiothreitol and analyzed by 10% SDS-PAGE. Western blotting analysis was performed after immunoprecipitation (IP).

GST pull-down assay

The full length of wildtype CREB or mutant CREBS133A protein was generated by in vitro transcription translation with the TNT protein system (Promega). GST-truncated SOX9 containing the amino acids 316–510 was obtained from Thermo Fisher Scientific. One microgram of GST-truncated SOX9 or the purified GST control was immobilized on 25-μl glutathione-Sepharose beads (Pharmacia) that had been pre-washed four times with the pull-down buffer containing 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsufonyl fluoride, and 0.5 mM dithiothreitol by rotating at 4°C for 1 h. Six microliters of wildtype CREB or mutant CREBS133A was added in a total volume of 500 μl and incubated at 4°C for another 1 h. The beads were washed three times with 500 μl of the pull-down buffer, boiled in 25 μl of the SDS-PAGE loading buffer, run on a 10% SDS-PAGE, and subjected to WB with anti-CREB antibody.

Statistical analysis

Quantitative data are presented as means ± SD. Data comparisons were made using the Student's *t*-test or one-way ANOVA (Super-ANOVA Software; Abacus Concepts). Two-way ANOVA was applied to analyze the difference of transfectant cells that were further stimulated with PKA regulators (see Fig. 2 below). The difference was considered significant when $p < 0.05$. All experiments were repeated at least three times, and representative experiments were shown in the figures.

RESULTS

Importance of the PKA phosphorylation sites of SOX9 in promoting osteochondrogenic markers

BMP-2 is the best characterized factor to induce chondrogenesis and/or osteogenesis of primary progenitor cells or established progenitor cell lines, depending on cell types and the presence of co-factors.[23–26] Particular to C3H10/1/2 cells, the BMP treatment has been shown to induce both chondrogenesis and osteogenesis. In our experiments, although expression of multiple osteogenic and chondrogenic markers were examined in cells stimulated with BMP-2 (Figs. 1A and 1B), the ALP expression was found to be a reliable and common marker for the osteochondrogenic differentiation (Figs. 1C and 1D) and therefore used in most subsequent experiments.

The two PKA phosphorylation sites, Ser64 and Ser211, in SOX9, have been previously shown to play an essential role in binding and transactivating the chondrocyte-specific Col2αI enhancer and promoter.[19] In this study, the SOX9 mutant mu1+2, with the conversion of both serine 64 and 211 into alanine (Fig. 1E), was generated. Wildtype SOX9, mu1+2 expression plasmids, or an empty control vector was introduced into C2C12 cells, respectively, and the constitutive overexpression of SOX9 or mu1+2 was confirmed by WB (Fig. 1F). The expressed protein of mu1+2 appeared slightly smaller in size compared with wildtype SOX9, probably because of the low level of phosphorylation. Mu1+2 not only showed remarkably impaired transactivation activity on the Col2αI enhancer, which is consistent with a previous report,[18] but also lost the activation function on the Col1αI promoter (Figs. 1G and 1H). Furthermore, G418-selected cells, C2C12(△wt), C2C12(△mu1+2), and C2C12(△ev), were exposed to different concentrations of rhBMP-2 for 3 days, and ALP activities were measured. C2C12(△wt) cells exhibited markedly higher ALP activities than C2C12(△ev) cells, although BMP-2 treatment increased ALP activities in both cell groups in a dose-dependent manner (Fig. 1I). However, the enhancing effect of SOX9 on the ALP

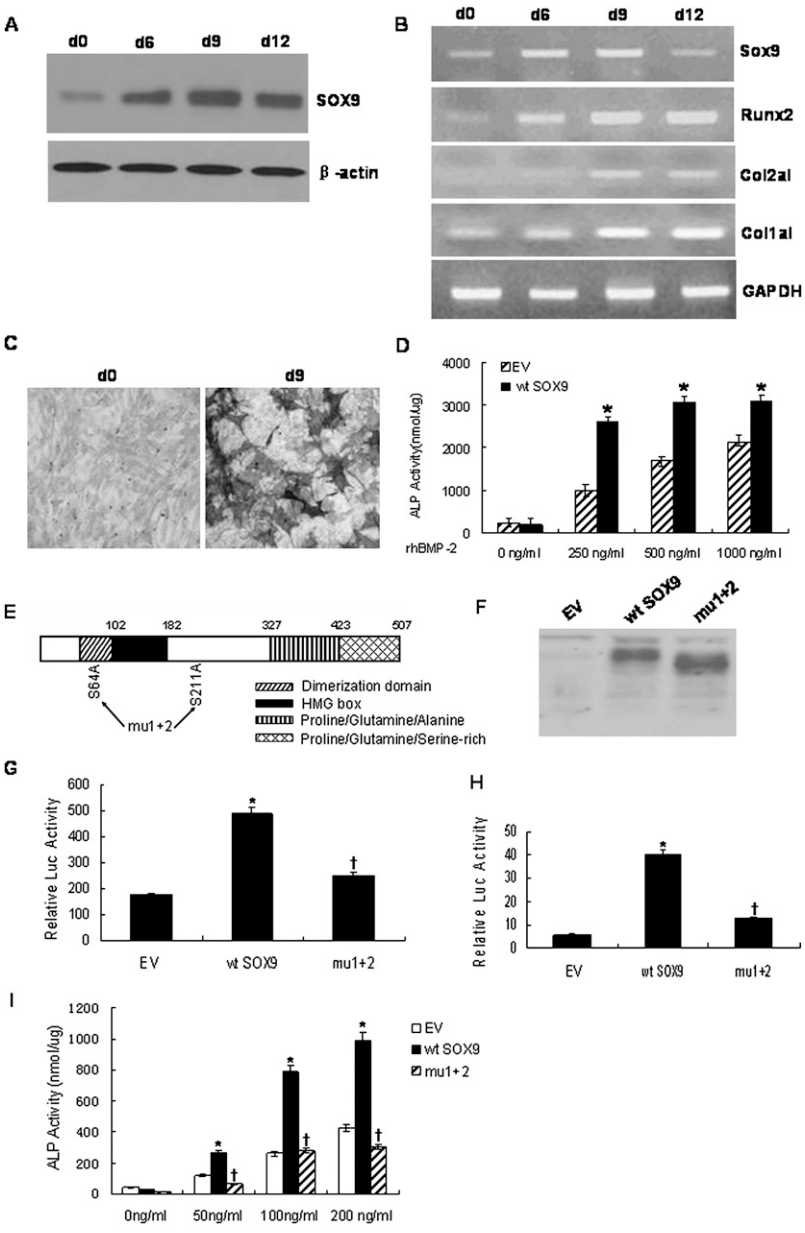

FIG. 1. Expression of osteochondrogenic markers mediated by BMP-2 and effects of PKA phosphorylation sites. (A) Upregulated SOX9 protein expression in C3H10T1/2 cells on BMP-2 treatment. (B) Upregulated mRNA expression of osteochondrogenic markers on BMP-2 treatment. (C) Enhanced ALP activity in C3H10T1/2 cells on BMP-2 treatment. (D) Dose-effect enhancement of ALP activity by BMP-2 treatment and further promotion by SOX9 overexpression in C3H10T1/2 cells. (E) A schematic illustration of SOX9 functional domains and mutation positions, with the serine-alanine substitution of the two PKA phosphorylation sites as indicated being termed mu1+2. (F) Lysates from C2C12 cells transfected with the empty vector (EV) control, wildtype SOX9 (wtSOX9), and mu1+2-expression plasmids, respectively, were blotted with the anti-SOX9 antibody. (G and H) Luciferase activities in C2C12 cells co-transfected with pKN159Bx6luc (G) or PG70 (H) and the empty vector, wildtype, or mu1+2 SOX9. (I) ALP activities in C2C12(△ev), C2C12(△wt), and C2C12(△mu1+2) cells treated with rhBMP-2 for 3 days at the indicated concentrations. *Significant differences from C2C12(△ev) cells ($p < 0.05$). †Significant differences from C2C12(△wt) cells ($p < 0.05$). Experiments were repeated at least three times independently.

activity was dramatically diminished in C2C12(△mu1+2), which appeared to be virtually the same as that in C2C12(△ev) cells. These data thus suggest that the phosphorylation of SOX9 by PKA is important for promoting osteochondrogenic marker expression.

Involvement of the PKA pathway in BMP-2–stimulated osteochondrogenic differentiation by synergizing with SOX9

In our previous study,[36] it has been shown that BMP treatment could increase PKA activity in C2C12 cells. In this study, PKA activity was also found to be increased in C3H10T1/2 cells treated with BMP-2 in a dose-dependent manner (Fig. 2A). Along with this, CRE-driven luciferase activity increased (Fig. 2B). Furthermore, CRE-luciferase activity was significantly affected by PKA regulators. To show whether there exists an authentic synergism between SOX9 and PKA signaling in osteochondrogenic differentiation, PKA regulators were applied into the experimental systems. When BMP-2–stimulated C2C12(△ev) and C2C12(△wt) cells were incubated with the PKA activator 8-Br-cAMP, their ALP activities increased. Interestingly, the addition of 8-Br-cAMP to C2C12(△wt) cells led to an effect greater than the simple additive enhancement of the two single factors: 8-Br-cAMP and SOX9 (Fig. 2C). On the contrary, H-89 completely inhibited ALP activities in both the C2C12(△ev) and C2C12(△wt) cells (Fig. 2D). A similar effect was observed in the presence of the specific and potent cognate PKA inhibitor, PKIγ. When C2C12(△wt) cells were further transfected with pcDNA6B-PKIγ, the stimulatory effect of SOX9 on ALP activities was severely diminished (Fig. 2E).

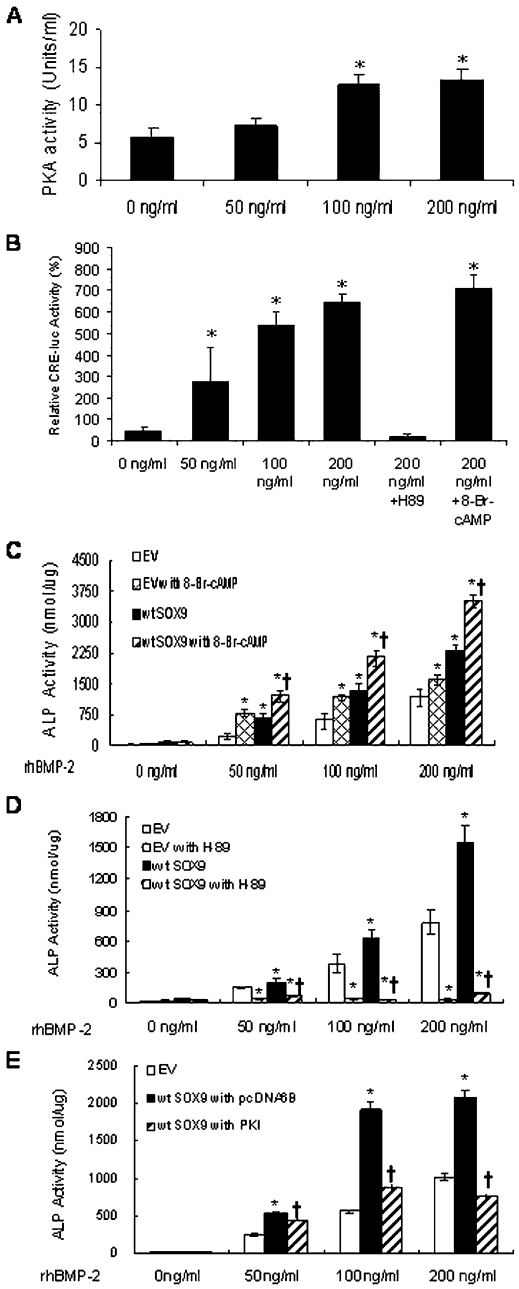

FIG. 2. Enhanced PKA signaling and functional synergy with SOX9. (A) PKA activity in cells treated with BMP-2 at different concentrations. PKA activity was measured after being treated with or without rhBMP-2 for 3 days. *$p < 0.05$ compared with cells without BMP-2. Experiments were repeated three times. (B) Luciferase activity was measured and was presented as percentage of vector control value (pGL3) (mean ± SD; $n = 3$). *$p < 0.005$ compared with the cell control without BMP-2 treatment. (C) ALP activities in C2C12(△ev) and C2C12(△wt) cells treated with rhBMP-2 at indicated concentrations in the presence or absence of 200 μM 8-Br-cAMP for 3 days. (D) ALP activities in C2C12(△ev) and C2C12(△wt) cells treated with rhBMP-2 at indicated concentrations in the presence or absence of 10 μM H-89 for 3 days. (E) ALP activities in C2C12(△ev) and C2C12(△wt) cells further transfected with pcDNA6B or pcDNA6B-PKIγ, which were all incubated with rhBMP-2 at indicated concentrations for 3 days. *Significant differences from C2C12(△ev) cells ($p < 0.05$). †Significant differences from C2C12(△wt) cells transfected with pcDNA6B ($p < 0.05$). Experiments were repeated at least three times independently.

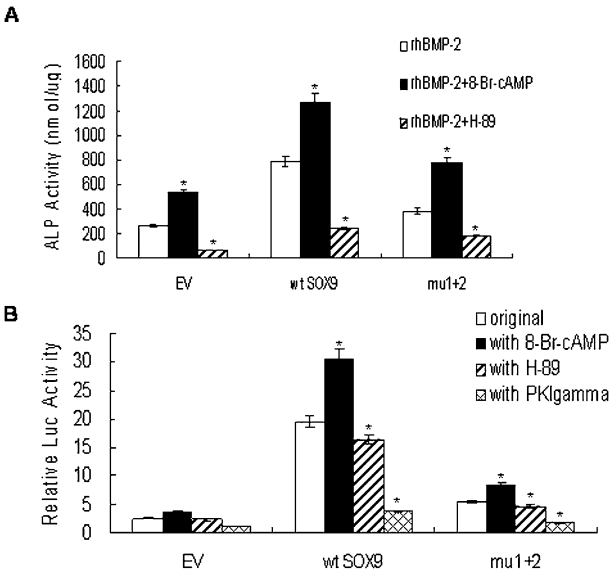

FIG. 3. The synergism between PKA signaling and SOX9 missing PKA phosphorylation sites. (A) ALP activities in C2C12(△ev), C2C12(△wt), and C2C12(△mu1+2) cells incubated with 100 ng/ml rhBMP-2 alone or together with 200 μM 8-Br-cAMP or 10 μM H-89 for 3 days. (B) Luciferase activities in C2C12 cells co-transfected with PG70 and empty vector (EV), wild-type SOX9 (wtSOX9), or mu1+2 (mu1+2), in the presence or absence of 200 μM 8-Br-cAMP or 10 μM H-89 or including pcDNA6B-PKIγ in the co-transfection. *Significant differences from cells treated with rhBMP-2 alone ($p < 0.05$). Experiments were repeated at least three times independently.

Synergism between SOX9 and PKA signaling is not merely mediated by the PKA phosphorylation

To further verify whether the two PKA phosphorylation sites of SOX9 are the sole ground of the synergism between the SOX9 function and PKA signaling, effects of the PKA activity alteration on the mu1+2 function were further examined by using PKA activator/inhibitors. C2C12(△ev), C2C12(△wt), and C2C12(△mu1+2) were exposed to rhBMP-2 together with either 8-Br-cAMP or H-89. It was shown that C2C12(△mu1+2) also displayed an enhancement in ALP activities, although to a lesser extent compared with C2C12(△wt), and could still be affected by 8-Br-cAMP and H-89 (Fig. 3A). Similar results were obtained from the assay for the Col1αI promoter activity. Moreover, PKIγ showed an inhibitory effect on the action of both mutated and the wildtype SOX9 proteins (Fig. 3B). It may therefore be speculated that, in addition to the phosphorylation of SOX9 by PKA through the two known sites, a new level of the functional interplay between SOX9 and the PKA signaling pathway may also occur.

SOX9 associates with the PKA downstream transcription factor CREB

To further explore the mechanism underlying the functional synergism of SOX9 with the PKA pathway, the possible interaction between SOX9 and CREB, one of the most representative downstream transcription factors

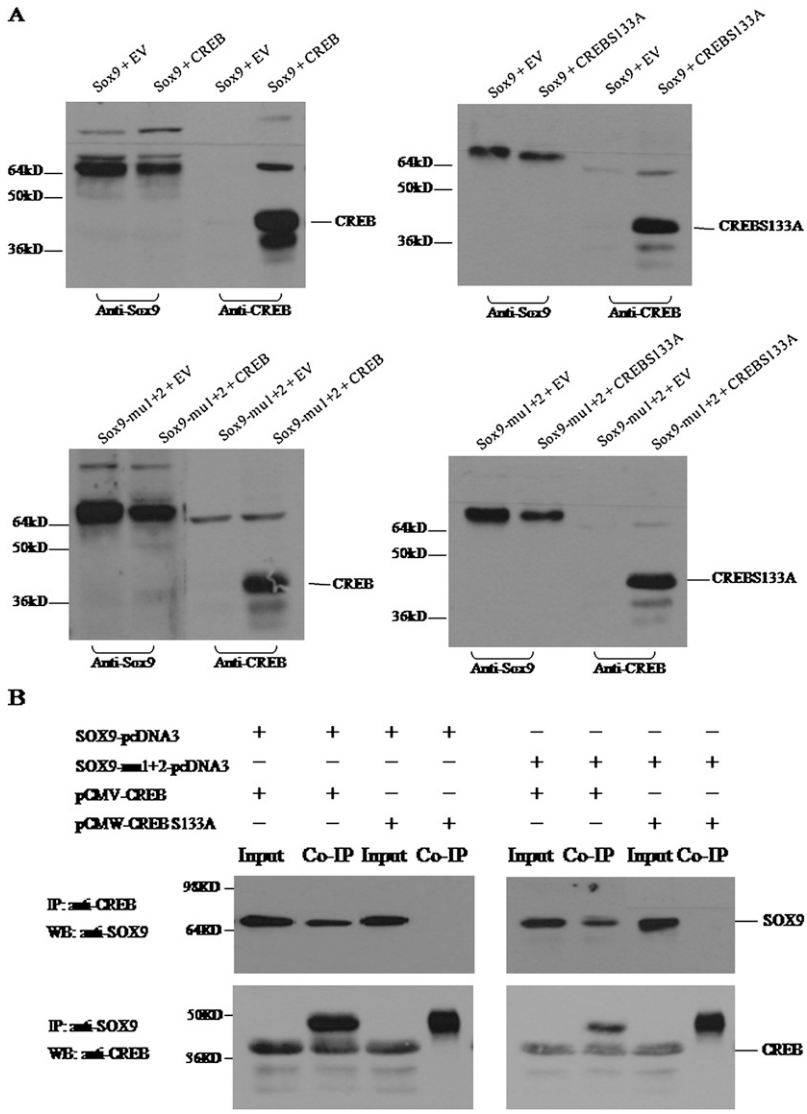

FIG. 4. The physical interaction between SOX9 and CREB in HEK293T cells. (A) Co-expression of wildtype and mutant SOX9 and CREB proteins in transfected HEK293T cells. Lysates from various co-transfectants, as indicated, were blotted with anti-SOX9 or anti-CREB antibodies, respectively. (B) Lysates of HEK293T cells co-transfected with wildtype SOX9 or mu1+2 SOX9 constructs and pCMV-CREB or CREBS133A were immunoprecipitated with the anti-CREB (top panel) or anti-SOX9 (bottom panel) antibody, respectively. The precipitated proteins (Co-IP) were subjected to Western blotting with the anti-SOX9 (top panel) or anti-CREB antibody (bottom panel), respectively. Ten percent of inputs (Input) were used as controls.

of PKA signaling, was further studied using several approaches. First, HEK293T cells were co-transfected with the SOX9 and CREB or CREBS133A-expressing plasmids, followed by co-immunoprecipitation experiments. The expression levels of SOX9 and CREB/CREBS133A in transfected cells were confirmed through WB (Fig. 4A). When cell lysates were immunoprecipitated with an anti-CREB antibody, SOX9 was detected by WB using an anti-SOX9 antibody (Fig. 4B). Consistent results were obtained when the co-immunoprecipitation and immunoblotting antibodies were switched with each other. However, SOX9 was not co-immunoprecipitated with the mutant CREBS133A, which could not be phosphorylated by PKA and has thus lost its transcriptional function. Interestingly, despite the lack of the two PKA phosphorylation sites, SOX9 mu1+2 still showed an ability to associate with wildtype CREB (Fig. 4B).

To determine the region of SOX9 responsible for the interaction, several FLAG-tagged SOX9 truncations were generated (Fig. 5A). Like the full-length protein, the N-terminal truncate of SOX9 (containing amino acids 328–507), consisting of only the PQA and the PQS transactivation domains, was found to possess the capability of interacting with CREB (Fig. 5A). However, the two C-terminal truncates (containing amino acids 1–423 and 1–327) of the SOX9 molecule showed no binding at all. Therefore, the results clearly indicated that SOX9 associates with CREB through its C-terminal transactivation domain.

To further clarify whether the observed SOX9–CREB association is through a direct binding or the participation of a third partner molecule, a GST pull-down experiment was performed. For this purpose, bacterially expressed GST-truncated SOX9 containing amino acid residues 316–510 and in vitro translated CREB and CREBS133A were used. Whereas the purified GST control did not pull down either wildtype CREB or mutant CREBS133A, GST-SOX9 was found to be able to pull down phosphorylated wildtype CREB efficiently (Fig. 5B). Consistent with the co-immunoprecipitation results mentioned above, mutant

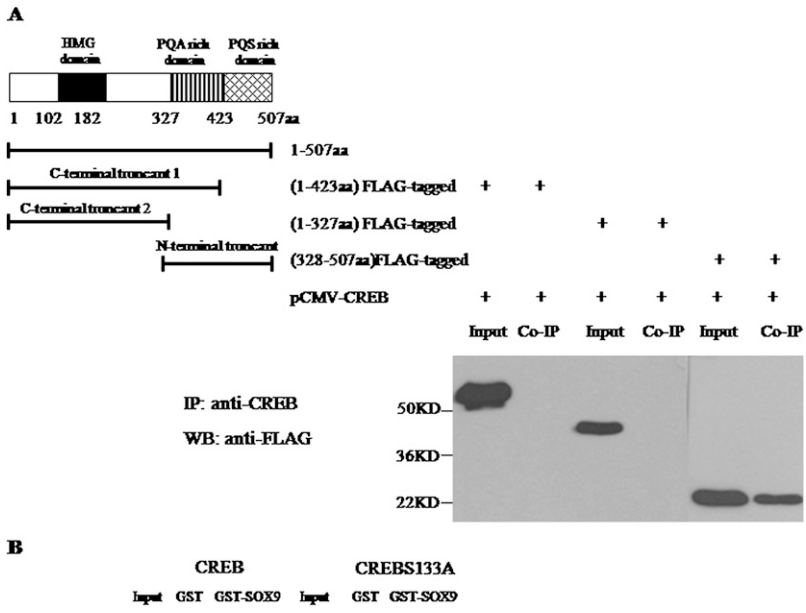

FIG. 5. Localization of the SOX9 domain responsible for CREB binding. (A) Constructs expressing FLAG-tagged truncated SOX9, as shown in the schematic illustration, were co-transfected with pCMV-CREB into HEK293T cells. The cell lysates were blotted with the anti-FLAG, or anti-CREB antibody. The same cell lysates were immunoprecipitated with the anti-CREB antibody. The precipitated proteins (Co-IP) were subjected to Western blotting with the anti-FLAG antibody. (B) *In vitro* translated wild type CREB or mutant CREBS133A was incubated with either GST alone or GST-truncated SOX9 (GST-SOX9) containing the C-terminal domain of SOX9. The pull-down products were subjected to Western blotting with the anti-CREB antibody.

CREBS133A could not bind with SOX9 and thus gave rise to no band in the GST pull-down assay. Importantly, the results indicated that SOX9 directly interact with CREB through its C-terminal domain, with the critical participation of serine 133 of CREB.

Functional interaction between SOX9 and CREB occurs in BMP-2–induced osteochondrogenesis

The functional implication of the SOX9–CREB interaction was further explored by detecting the association between endogenous SOX9 and CREB in progenitor cells induced by BMP-2. The precipitation was performed with an anti-CREB antibody followed by WB with an anti-SOX9 antibody. It was shown that the two endogenous molecules could indeed interact within osteochondrogenic-differentiating C3H10T1/2 cells. Interestingly, the interaction appeared increasingly active with ongoing BMP-2 induction, because the amount of precipitated molecules became larger, especially on the late days of BMP-2 treatment (Figs. 6A and 6B). It was shown that the expression level of SOX9 in the same cells was increased by BMP-2 induction (Fig. 6C), but there was no significant change on CREB expression (Fig. 6D). Therefore, the increased affinity between SOX9 and CREB, together with the elevated SOX9 expression, probably contributed to the increased association of SOX9 with CREB as detected in immunoprecipitation.

The functional significance of the interaction between CREB and SOX9 was further studied. It was found that transfection of pCMV-CREB into C2C12(△wt) enhanced ALP activities (Fig. 6E). On the contrary, CREBS133A was unable to enhance ALP activities of C2C12(△wt) and even showed inhibitory effects to a certain extent. CREB also exhibited a transactivation effect on the promoter of *Col1αI*. In this regard, it was shown that the synergetic enhancement by the co-presence of CREB and SOX9 seemed greater than the simple addition of the effects contributed by the two factors separately (Fig. 6F).

DISCUSSION

It is already known that PKA signaling regulates several osteo- or chondrogenic transcriptional factors.[36,37] Runx2, a key osteogenic regulator, for instance, has been shown to be phosphorylated by PKA through a consensus phosphorylation site.[35] PKA can also increase transcription of another osteogenic marker, osteocalcin.[36] We have recently reported that PKA signaling is involved in BMP-2–mediated osteogenic differentiation, mainly by down-regulating the endogenous PKA inhibitor, PKIγ (named PKIG in humans).[29] On the other hand, SOX9 is a key factor in skeletal development, and the upregulation of the SOX9 expression is a common and important downstream event of BMP-2 signaling.[24,38] Based on these results, it is reasonable to speculate that SOX9 and the PKA pathway functionally synergize in physiology and development. Indeed, the phosphorylation of SOX9 by PKA through Ser64 and Ser211 has been shown to be needed for the SOX9 nuclear import implicated in its sex determination function.[21] The same phosphorylation is a downstream event of PTHrP signaling in the chondrogenic context and is able to enhance the transactivation activity of SOX9.[19,20] This study further proved that the PKA phosphorylation is necessary for SOX9 function in BMP-2–mediated cell differentiation. Therefore, substitutions of Ser64 and Ser211 with alanine (mu1+2) dramatically lost the ability to enhance the activities of ALP and the promoter region of *Col1αI and Col2aI* genes, which both contain specific SOX9-binding elements. On the other hand, the inclusion of the PKA-specific activator in the BMP-2–induced differentiation of C2C12(△wt) cells produced the ALP activity higher than the simple addition of SOX9 and the

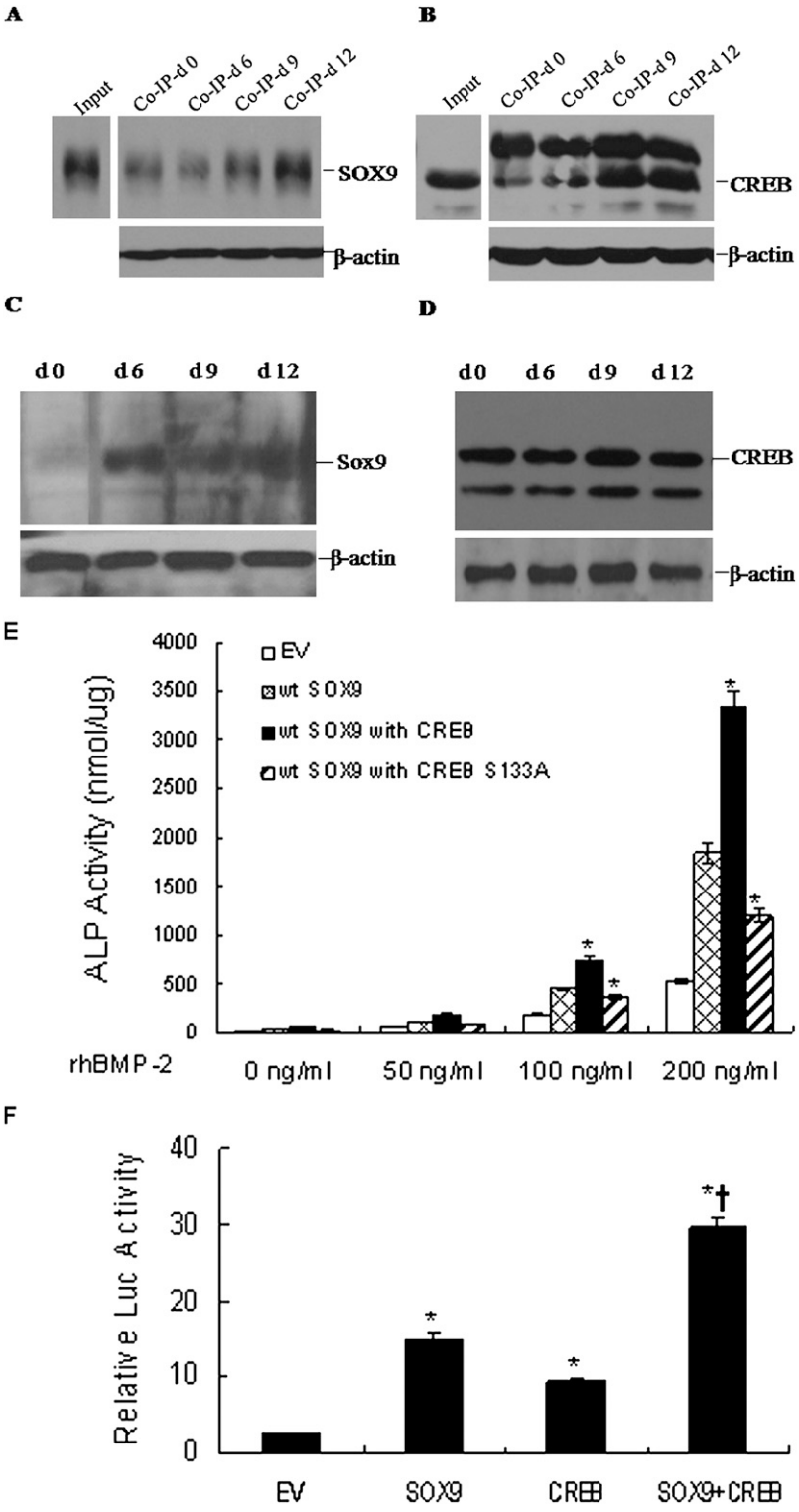

FIG. 6. The physical and functional synergy of SOX9 with CREB in BMP-2–stimulated cells. (A and B) Lysates from C3H10T1/2 cells incubated with 500 ng/ml rhBMP-2 for 0, 6, 9, and 12 days, respectively, were immunoprecipitated with the anti-CREB (A) or anti-SOX9 (B) antibody. The precipitated proteins (Co-IP) were subjected to Western blotting with the anti-SOX9 (A) or anti-CREB antibody (B), respectively. Ten percent of inputs (Input) from untreated C3H10T1/2 cells were used as positive controls, and β-actin was used as an internal control. (C) The protein expression of SOX9 in C3H10T1/2 cells treated with 500 ng/ml rhBMP-2 for 0, 6, 9, and 12 days, respectively, with β-actin as an internal control. (D) The protein expression of CREB in C3H10T1/2 cells treated with 500 ng/ml rhBMP-2 for 0, 6, 9, and 12 days, respectively, with β-actin as an internal control. (E) ALP activities in C2C12(△ev) and C2C12(△wt) cells transfected with pCMV-CREB or CREBS133A, followed by incubation with rhBMP-2 at indicated concentrations for 3 days. *Significant differences from C2C12(△wt) cells ($p < 0.05$). (F) The PG70-mediated luciferase activity in C2C12 cells co-transfected with the empty vector, SOX9 construct and pCMV-CREB, or SOX9 and pCMV-CREB, simultaneously. *Significant differences from cells co-transfected with PG70 and empty vector ($p < 0.05$). †Significant differences from cells co-transfected with PG70 and SOX9 constructs ($p < 0.05$). Experiments were repeated at least three times independently.

activator, indicating that PKA may not only enhance cell differentiation on its own but also function through further activating SOX9. Experiments with inhibitors further suggest that the PKA activation is actually a prerequisite for SOX9 function. Therefore, the synergism with the PKA pathway seems to be an important aspect for SOX9 function in promoting the BMP-2–mediated osteochondrogenic differentiation. However, the removal of the two PKA phosphorylation sites did not completely abolish the influence of the PKA pathway on the SOX9 function, because the PKA activator/inhibitor could still affect ALP and promoter activities mediated by mu1+2. Therefore, the

PKA phosphorylation should be only one of multiple points whereby Sox9 interplays with the PKA pathway.

CREB family members, including CREB and ATF4 (also termed CREB2), have been known to regulate osteochondrogenesis initiated by different factors through different signaling pathways.[30,39,40] In the chondrogenic context, CREB-binding protein (CBP) and its paralog, p300, has been shown to associate with SOX9 and activate its transcriptional activity.[17] CREB has also been shown to be involved in the transcriptional activation of osteocalcin, bone sialoprotein, type XXIV collagen, and SOX9.[30–32,41] In this study, we inferred that the SOX9 action and PKA signaling might also occur through a direct interaction between the two factors. Indeed, it was shown that SOX9 physically associated CREB in HEK293T cells, which were chosen because foreign genes could be easily expressed at a high level and the detection of protein–protein interactions could be facilitated. The direct association of bacterially recombinant SOX9 with in vitro transcribed CREB further clarified that there is no third molecule needed for the SOX9–CREB interaction. Moreover, the interaction between endogenous SOX9 and CREB in C3H10T1/2 cells, which were undergoing BMP-2–mediated differentiation, has also been detected. In this regard, it is interesting to note that the increasing amounts of the two proteins seemed to be associated with the time of the BMP-2 treatment. This may more strongly implicate the functional significance of the interaction in activating downstream genes. It is therefore possible that a transcription complex may be formed by SOX9, CREB, with or without the participation of other co-factors (e.g. CBP), on the promoter region of downstream genes, which may remodel the chromatin structure and facilitate the gene transcription.[42] In the promoter region of the *Col1aI* gene, for instance, the potential SOX9 binding site is only 290 bp upstream of the CREB binding site. Although assays such as chromatin immunoprecipitation will be needed to confirm this, this study has indeed shown that the association of activated CREB and SOX9 could promote expression of ALP and *Col1aI* in a synergic way.

Although HMG of SOX9 is known to bind to DNA, the C-terminal domain of SOX9 seems to be the important region for interacting with co-factors because it has been known to bind CBP/p300 and PGC-1[17,43] and now has been shown to be responsible for the association with CREB. On the CREB side, the phosphorylation of Ser133 is crucial for the interaction. To this end, it is worth noting that CREBS133A not only lost its synergic role but also exhibited an inhibitory effect on the SOX9 action, probably through exerting a dominant negative function when accessing to the SOX9–chromatin complex.

In summary, the synergism between SOX9 and the PKA pathway is complex and at many levels, including the PKA-mediated upregulation of the Sox9 gene transcription,[41] the cytoplasmic phosphorylation and subsequent nuclear translocation of the SOX9 protein,[18–20] and the functional and physical association of SOX9 with CREB and possibly CBP/p300.[17] In the meanwhile, PKA activation can also upregulate the transcription of osteochondrogenic-related genes.[36,37,44] The revelation

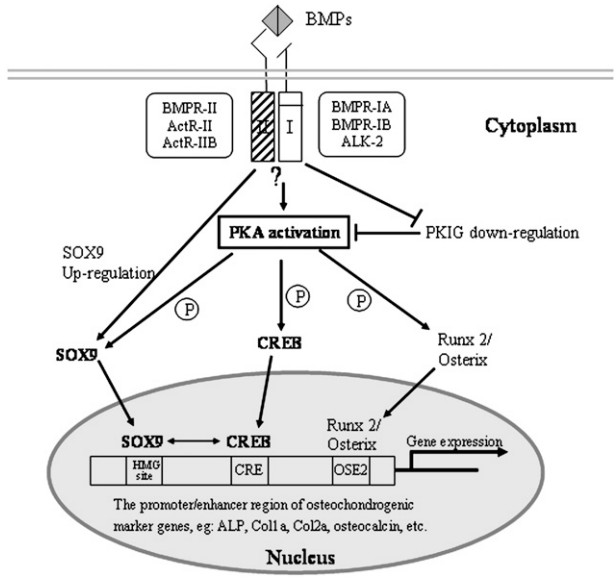

FIG. 7. A simplified model for the multilevel interplay between SOX9 and PKA signaling. BMP-2 induces the activation of PKA, mainly by downregulating PKIG[29] and upregulates mRNA expression of SOX9.[24,38,40] The activated PKA in cytoplasm phosphorylates and thus activates various downstream factors including SOX9, CREB, and other osteochondrogenic factors. The PKA activation in turn regulates transcription and function of osteochondrogenic factors.[36,37,43] In the cell nucleus, SOX9 and CREB interact together and bind to specific DNA elements within the promoter/enhancer regions of downstream genes and activate their expression. CRE, cAMP response element. Dashed line shows the unclarified mechanism.

of these interplaying pathways (Fig. 7) should provide new insights into our knowledge about their functional patterns in osteochondrogenic progenitor cells and open a new path to modulate in vitro, in vivo, and, ultimately, clinical osteogenesis processes.

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