Published in final edited form as:

Calcif Tissue Int. 2010 December ; 87(6): 533–540. doi:10.1007/s00223-010-9419-3.

Integration of Phosphatidylinositol 3-Kinase, Akt Kinase, and Smad Signaling Pathway in BMP-2-Induced Osterix Expression

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Abstract

Osterix (Osx), a BMP-2-regulated transcription factor, controls expression of genes essential for osteoblast differentiation. Using progressive deletion of the Osx promoter, we characterized a Smad binding element (SBE) between –552 and –839 bp from its transcription start site. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay showed binding and in vivo recruitment of Smads 1 and 5 to the Osx SBE. Inactivation of PI 3-kinase by the pharmacologic inhibitor Ly294002 or by dominant negative (DN) enzyme significantly blocked BMP-2-induced Osx protein and mRNA expression and Osx transcription. Finally, both DN PI 3-kinase and DN Akt significantly attenuated Smad 5-dependent transcription of Osx, demonstrating the first evidence for a concerted action of PI 3-kinase/Akt signaling with BMP-specific Smads for expression of Osx.

Keywords

Bone morphogenetic protein; Osterix; PI 3-kinase; Smad; Gene transcription regulation; Osteoblast

Osteoblast differentiation, an integral part of bone formation and maintenance, is regulated by unique transcription factors like osterix (Osx) and Runx2. Osx, discovered as a bone

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The authors have stated that they have no conflict of interest.

Electronic supplementary material The online version of this article (doi:10.1007/s00223-010-9419-3) contains supplementary material, which is available to authorized users.

morphogenetic protein-2 (BMP-2)—inducible gene, contains three C2H2-type Zn fingers at its C terminus for DNA binding, essential for its role as a transcriptional regulator [1]. It is expressed in differentiating osteoblasts and in developing bones [1]. Osteoblast differentiation is arrested in *Osx* null mice, resulting in absence of bone formation [1]. *Osx* expression is upregulated by BMP-2 through the involvement of the transcription factors *Dlx5*, *Runx2*, and *Msx2* [2–6].

BMP-2 binds to its serine-threonine kinase receptor type II (BMPRII), which recruits and activates BMPRI type I (BMPRI) by phosphorylating at the Gly-Ser (GS) domain [7]. Primed BMPRI complex recruits BMP-specific receptor-activated Smads (BR-Smads)—namely, Smads 1, 5, and 8—and phosphorylates them. Phosphorylated BR-Smads form complexes with the common-partner Smad (co-Smad), Smad4, and translocates to the nucleus to regulate gene transcription in coordination with other transcription factors and transcriptional coactivators [7,8]. The inhibitory Smad, Smad6, interacts with BMPRI or with the BR-Smads to block their nuclear localization and thus block Smad-induced gene transcription [8,9].

Recent reports indicate the importance of phosphatidylinositol 3 kinase (PI 3-kinase) signaling during BMP-2-mediated osteoblast differentiation and survival [10]. The PI 3-kinases are a group of enzymes that phosphorylate the 3-position hydroxyl group of the phosphatidylinositols (PIs). Class I PI 3-kinases preferentially phosphorylate PI 4,5-bisphosphates (PIP₂) to generate PI 3,4,5-trisphosphate (PIP₃). This second messenger initiates the signaling cascade involving the downstream target Akt kinase. The phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a phosphatase capable of dephosphorylating PIP₃, thus attenuating PI 3-kinase signaling. The role of PI 3-kinase signal transduction in osteoclast differentiation is well established [11,12]. More recently, the importance of PI 3-kinase and Akt signaling in osteoblast differentiation has been elucidated [10,13–16]. Progressive increase in bone mineral density and increased osteoblast differentiation in osteoblast-specific PTEN null mice confirmed a critical role of PI 3-kinase signaling in osteoblasts and in bone remodeling [16]. In this study we show direct interaction of Smads with the *Osx* promoter as a mechanism to induce its expression upon BMP-2 stimulation. Furthermore, we identify a signaling crosstalk between PI 3-kinase and Smads, which essentially regulates BMP-2-induced *Osx* gene expression.

Materials and Methods

Materials

Tissue culture and transfection reagents were from Invitrogen (Carlsbad, CA) and Roche Molecular Biology (Indianapolis, IN), respectively. The luciferase assay kit was from Promega (Madison, WI). The nuclear extraction kit was from Pierce Thermo Scientific (Rockford, IL). Antibodies were from Sigma (actin; St. Louis, MO), Santa Cruz Biotechnology (Smad6 and Smad1/5; Santa Cruz, CA), and Abcam (*Osx*; Cambridge, MA). TRI reagent for RNA isolation was purchased from Sigma. PVDF membrane was from Perkin Elmer (Waltham, MA). Recombinant BMP-2 was provided by Wyeth (Cambridge, MA).

Cells, Plasmids, and Adenoviral Vectors

C2C12 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. To drive osteoblastic differentiation, cells are routinely grown to 70–80% confluence and treated with 300 ng/ml recombinant BMP-2 in serum-free DMEM. The plasmids and adenoviral expression vectors used in this report have been described previously [10,15,17,18].

RNA Analysis

RNA isolation and RT-PCR were done as described earlier [10,15,17,18]. The primers used are described in the supplementary materials.

Transfection Assay

Cells were transfected using FuGENE HD according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN), and luciferase activity was quantified as described before [8,10,15,17,18].

Immunoblotting

Cells were lysed using a radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 0.1% protease inhibitor cocktail, and 1% NP-40), and equal amounts of protein were analyzed by immunoblotting [8,10,15,18].

Electrophoretic Mobility Shift Assay

The oligonucleotide probe (GAA GCT GCC GCG CCG CTG AGT AG, CTA CTC AGC GGC GCG GCA GCT TC) spanning the Smad binding element (SBE) in the *Osx* promoter was used in an electrophoretic mobility shift assay (EMSA) essentially as described previously [8,18].

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed using a kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions. A brief description of the method is provided in the supplementary materials.

Statistical Analysis

The significance of the data was determined by ANOVA followed by Student-Newman-Keuls analysis as described previously [10,17,18].

Results

Involvement of Smad Signaling in *Osx* Expression and Identification of a Functional Smad Interacting Region in the *Osx* Promoter

Osx was identified as a BMP-2-inducible gene in C2C12 cells [1]. Using C2C12 cells, we confirmed that expression of *Osx* protein and mRNA was regulated by BMP-2 (Supplementary Fig. S1a and b, compare lanes 2 with lanes 1). We also showed *Osx* transcriptional regulation by BMP-2 using a luciferase reporter plasmid driven by -987 bp *Osx* promoter (987-Luc) upstream of the transcription start site in C2C12 cells (Supplementary Fig. S1c). To identify the BMP-2-responsive region in the *Osx* promoter, we used progressive 5' deletion of 987-Luc reporter construct (Fig. 1a). These plasmids were transfected into C2C12 cells, followed by treatment with BMP-2. BMP-2 significantly increased luciferase activity from 987-Luc and 839-Luc (Fig. 1b). BMP-2 did not have any effect with 552-Luc (Fig. 1b). These results indicate the presence of a BMP-response region in the *Osx* promoter between -552 and -839 bp. To test the involvement of Smad signaling in *Osx* gene expression, we used plasmids expressing BR-Smads in combination with 987-Luc in transient transfection assays. Expression of Smad 1 as well as Smad 5 significantly increased *Osx* transcription from 987-bp *Osx* promoter (Fig. 1c and d, respectively). Expression of the inhibitory Smad 6 using an adenoviral expression plasmid (Supplementary Fig. S1d) suppressed expression of BMP-2-induced *Osx* protein and mRNA as well as *Osx*

transcription (Supplementary Fig. S1a–c). Our results indicate involvement of Smads in BMP-2-induced *Osx* expression. Upon analysis of the sequence in this region, a putative SBE was identified at –577 bp of the *Osx* promoter [4]. Using a radioactive probe spanning the SBE in EMSA, we detected increased DNA–protein complex formation upon BMP-2 stimulation (Supplementary Fig. S2 and Fig. 1e–g, compare lanes 2 with lanes 1). Use of unlabeled SBE in EMSA specifically blocked formation of the DNA–protein complex (Fig. 1e, compare lane 3 with lane 2, indicated by top arrow). Unlabeled oligonucleotide specific for binding AP2, used as control, failed to compete for binding of the radioactive SBE (Fig. 1e, compare lane 4 with lane 2). To test the involvement of BMP-specific Smad in forming this protein–DNA complex, we used an antibody which recognizes both Smads 1 and 5 (anti-Smad1/5). The Smad 1/5 antibody specifically inhibited BMP-induced DNA–protein complex formation, confirming interaction of these Smads with *Osx* SBE (Fig. 1f, compare lane 3 with lane 2). Nonimmune IgG did not block SBE binding to Smads (Fig. 1f, compare lane 4 with lane 2). Furthermore, expression of the inhibitory Smad 6 significantly blocked BMP-2-induced protein–DNA complex formation (Fig. 1g, compare lane 3 with lane 2). To confirm in vivo the interaction of Smads 1 and 5 with the *Osx* SBE, we performed a ChIP assay using Smad 1/5 antibody. Figure 1h shows specific recruitment of Smads 1 and 5 onto the *Osx* SBE. These results provide the first evidence identifying a functional SBE regulating transcription of *Osx* in response to BMP-2.

BMP-2-Induced PI 3-Kinase and Akt Signaling Regulates *Osx* Expression

Osx protein expression is regulated by BMP-2 in C2C12 cells (Supplementary Fig. S1a). We have shown the involvement of PI 3-kinase/Akt signaling in the regulation of osteoblast differentiation and osteoblastic gene expression [10, 18]. The involvement of PI 3-kinase signaling in BMP-2-induced *Osx* expression was tested. Treatment of C2C12 cells with the PI 3-kinase inhibitor Ly 294002 completely blocked BMP-2-induced *Osx* protein expression (Fig. 2a, compare lanes 2 and 4), suggesting that PI 3-kinase plays a role in *Osx* protein expression. To confirm this unique observation, we blocked PI 3-kinase signaling by adenoviral vector–mediated expression of a deletion mutant of p85 regulatory subunit of PI 3-kinase in C2C12 cells, which we have shown previously to inhibit the catalytic activity of this enzyme (Ad DNPI3K) [18, 19]. We also used an adenoviral vector expressing wild-type PTEN, which blocks PI 3-kinase signaling [18]. BMP-2-stimulated *Osx* protein expression was significantly inhibited upon expression of dominant negative (DN) PI 3-kinase or PTEN in these cells (Fig. 2b, c). PI 3-kinase signal activates Akt kinase, which in turn activates gene transcription [10, 20, 21]. We tested the requirement of Akt kinase activation for BMP-2-induced *Osx* protein expression by employing an adenoviral vector expressing DN Akt (Ad DNAkt) [10, 15, 18]. Expression of DN Akt abrogated BMP-2-induced *Osx* protein expression (Fig. 2d). Next, we tested the involvement of the PI 3-kinase/Akt-signaling pathway in *Osx* mRNA expression in response to BMP-2. RNA isolated from C2C12 cells treated with BMP-2 in the presence of Ly 294002 or from cells expressing DN PI 3-kinase, PTEN, or DN Akt was analyzed for *Osx* mRNA expression using semiquantitative RT-PCR. BMP-2-induced *Osx* mRNA expression was significantly inhibited in cells where PI 3-kinase/Akt signaling was abrogated using Ly 294002, DN PI 3-kinase, PTEN, or DNAkt (Fig. 2e–h).

BMP-2-Activated PI 3-Kinase/Akt Signaling Regulates *Osx* Transcription

Since PI 3-kinase/Akt signal transduction regulates *Osx* mRNA expression in response to BMP-2, we examined the role of this lipid kinase cascade in *Osx* transcription. C2C12 cells transiently transfected with 987-Luc were treated with the PI 3-kinase inhibitor Ly294002 prior to incubation with BMP-2. Ly294002 significantly inhibited BMP-2-induced transcription of *Osx* (Fig. 3a). To confirm this observation, we cotransfected plasmid vector expressing either a deletion mutant of p85 regulatory subunit of PI 3-kinase (Δ p85) that

elicits a DN effect [10,18] or PTEN along with the 987-Luc-reporter plasmid. The results showed that expression of DN PI 3-kinase or PTEN inhibited BMP-2-induced Osx transcription (Fig. 3b and c, respectively). Furthermore, cotransfection of DN Akt with 987-Luc significantly prevented BMP-2-stimulated transcription of Osx (Fig. 3d). Use of adenoviral vectors to express DN PI 3-kinase or DN Akt also blocked Osx transcription by BMP-2 (Supplementary Fig. S3a, b). These results indicate that PI 3-kinase/Akt signaling regulates BMP-2-induced Osx transcription.

PI 3-Kinase and Akt Modulate BMP-2-Induced Smad-Mediated Osx Expression

We have shown above that Smads 1 and 5 form complexes with SBE present in the Osx promoter (Fig. 1f, g). Additionally, we showed recruitment of Smads 1 and 5 onto the SBE in the Osx promoter in vivo (Fig. 1h), indicating a direct role of BMP-specific Smads in Osx transcription. Additionally, expression of Osx mRNA and protein and its transcriptional activation essentially require activation of PI 3-kinase and its downstream target Akt (Figs. 2, 3). We examined whether the lipid kinase signals converge onto Smad5-induced transcription of Osx. 987-Luc was transfected with Smad5 and the Δ p85 subunit of PI 3-kinase expression vectors in C2C12 cells. Smad5 expression increased Osx transcription (Fig. 4a). BMP-2 treatment increased Osx transcription to a level similar to Smad5, indicating the effectiveness of Smad5 expression (Fig. 4a). Treatment of Smad5-transfected cells with BMP-2 further increased Osx transcription (Fig. 4a). Expression of Δ p85 significantly inhibited BMP-2 as well as Smad5-mediated Osx transcription (Fig. 4a), suggesting a functional crosstalk between PI 3-kinase and Smad signaling for Osx expression. Using a similar approach, we tested the involvement of Akt kinase in Smad- and BMP-2-directed Osx transcription. Expression of DN Akt abrogated transcription of Osx by Smad5 and BMP-2 (Fig. 4b). To delineate the underlying mechanism, we tested the role of PI 3-kinase and Akt kinase on the interaction of Smads with Osx SBE (as identified in Fig. 1). EMSA was performed in cells expressing DN PI 3-kinase or DN Akt using the synthetic radiolabeled Osx SBE as probe. Expression of DN PI 3-kinase or DN Akt inhibited BMP-2-induced Smad binding to Osx SBE (Fig. 4c and d, compare lanes 4 with lanes 2). These results indicate that PI 3-kinase and its downstream target Akt are critical in converging the Smad signal onto the Osx promoter for augmenting its expression in response to BMP-2.

Discussion

In the present study, we demonstrate an essential role of PI 3-kinase signaling in BMP-2-induced Osx protein and mRNA expression. PI 3-kinase controls Osx transcriptional activation. We also identified a functional SBE in the Osx promoter, which binds the Smads that in turn regulate Osx transcription in response to BMP-2. Furthermore, our results provide the first evidence for a crosstalk between BMP-specific Smads and PI 3-kinase/Akt signaling for induction of Osx transcription.

Osteoblasts are derived from multipotent mesenchymal stem cells (MSCs), which can also differentiate to form chondrocytes, adipocytes, and myoblasts [22]. In addition to being the key transcription factor involved in osteoblastic gene expressions, Osx is shown to be inhibitory for chondrogenic differentiation of mesenchymal progenitor cells and, thus, might play an important role in driving MSCs to an osteoblastic lineage [1,23,24]. The expression of Osx by BMP-2 therefore must be tightly regulated and is conceivably controlled by multiple transcription factors that in turn are governed by more than one signaling pathway. Commitment of MSCs to the osteoblast lineage follows a sequence of events comprising lineage expansion, generation of committed osteoprogenitor, increased proliferation of preosteoblasts, and differentiation into mature osteoblasts that are responsible for laying down bone [22,25,26]. These steps are tightly controlled by critical signal-transduction pathways that transcriptionally regulate the expression of genes involved in driving the

MSCs to osteoblast differentiation. Among the signaling pathways involved in this process the most important are hedgehog (Hh), Wnt, and BMP. Hh and Wnts are involved in embryonic skeletal patterning, fetal skeletal development, and adult skeletal remodeling [27–29]. There are possible crosstalk and interdependence of the Hh and Wnt signaling pathways during skeletogenesis [29]. BMPs play an important role in directing MSCs to chondrogenic and osteogenic cell lineages [30]. In the bone microenvironment, BMPs act in coordination with IGF-I to mediate *Osx* expression in human MSCs involving protein kinase D and MAPK [2].

While the PI 3-kinase signaling pathway is essential for proliferation and maintenance of embryonic stem cells [31], it is also involved in regulating osteoblast differentiation, survival, and mechanotransduction [10,13,14,32]. However, coimmunoprecipitation experiments did not show any association between *Osx* and Akt kinase in response to BMP-2 (Supplementary Fig. S4). Our data show that inhibition of PI 3-kinase and Akt signaling significantly blocked *Osx* gene expression (Figs. 2,3), offering a mechanism for the essential requirement of this signaling pathway in BMP-2-induced osteoblast differentiation [10].

Recent reports indicate that BMP-2 integrates Smad and PI 3-kinase signaling to activate osteoblastic gene expression, leading to osteoblast and osteoclast differentiation [8,10,18]. Our data show that increased *Osx* transcription induced by Smads is abrogated upon inactivation of PI 3-kinase and its downstream target, Akt kinase, indicating that Smad-mediated *Osx* expression is controlled by these lipid kinase activities (Fig. 4a and b, respectively). As a mechanism for this novel observation, we show that activation of PI 3-kinase and Akt kinase is required for Smad binding to the *Osx* SBE (Fig. 4c, d). Thus, PI 3-kinase/Akt signaling is essential for Smad-induced *Osx* expression in response to BMP-2. Further investigations are required to understand the molecular mechanism by which Akt inactivation blocks the interaction of Smads with the *Osx* promoter region. Possible mechanisms may include direct association of activated Akt with the Smads or an Akt influence on the association of transcriptional coactivators with Smads; both mechanisms have been shown to be feasible [33,34]. In summary, we characterized a functional SBE in the *Osx* promoter, which binds BMP-specific Smads 1 and 5 for induction of its gene expression. Furthermore, we show that the non-Smad PI 3-kinase/Akt signal-transduction pathway converges onto the Smads to regulate the key osteoblastic transcription factor *Osx*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH (RO1 AR52425) and VA Medical Research Service Merit Review (to N. G.-C.) and grants from the NIH (RO1 DK50190), VA Merit Review, and Juvenile Diabetes Research Foundation (to G. G. C.). G. G. C. is a recipient of the Research Career Scientist Award from the Department of Veterans Affairs.

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Fig. 1.

Identification of the SBE for the Osx promoter. **a** Sequential 5' deletion constructs of -987 bp Osx promoter-luciferase plasmid were generated containing -552 and -839 bp of the upstream sequences from the transcription start site. **b** C2C12 cells were transfected with these three promoter-luciferase constructs, followed by incubation with 300 ng/mL BMP-2. Luciferase activities were measured in cell lysates as described in Materials and Methods. Mean $\pm$ SE of triplicate determinations is shown. * P < 0.005 vs. control. **c, d** C2C12 cells were transfected with 987-Luc with Smad 1 (**c**), Smad 5 (**d**), or vector. Cell lysates were used for luciferase activity. Mean $\pm$ SE of triplicate determinations is shown. * P < 0.001 vs. control. **e, f** Nuclear extracts from C2C12 cells with or without BMP-2 treatment were incubated with cold SBE or cold AP2 oligonucleotides (**e**) or with anti-Smad1/5 antibody or control IgG (**f**) prior to incubation with 32 P-labeled SBE, and EMSA was performed as described in Materials and Methods. **g** EMSA was performed using nuclear extracts isolated from C2C12 cells expressing Ad Smad 6 with or without BMP-2 treatment and 32 P-labeled SBE probe. *Top arrow* indicates SBE complexed with Smads 1/5. *Middle and bottom panels* show Smad6 expression and corresponding loading control. The respective samples used in EMSA were immunoblotted with Smad6 (*middle panel*) or actin (*bottom panel*) antibody. **h** ChIP assay was performed in C2C12 cell lysates using PCR primers spanning Osx SBE following immunoprecipitation with anti-Smad1/5 antibody (*lane 3*) or control IgG (*lane 2*) as described in Materials and Methods. *Lane 1* shows input sample profile without immunoprecipitation

Fig. 2.

PI 3-kinase/Akt signaling regulates BMP-2-induced Osx protein and mRNA expression. Expression of Osx protein (**a–d**) or mRNA (**e–h**) was determined in C2C12 cells treated with BMP-2 with or without Ly294002 pretreatment (**a, e**) or in the presence or absence of DN PI 3-kinase (**b, f**), PTEN (**c, g**), or DN Akt (**d, h**) expression. Western blotting with the indicated antibodies (**a–d**) or semiquantitative RT-PCR (**e–h**) using synthetic oligonucleotide primers for Osx (*upper panels*) or GAPDH (*lower panels*) was used. Expression of p85, PTEN, or HA-tagged DN Akt was determined by immunoblotting with the respective antibodies (**b–d**, respectively)

Fig. 3.

Activation of PI 3-kinase and Akt is essential for Osx transcription. C2C12 cells were transfected with 987-Luc plasmids with or without Ly294002 pretreatment (a) or cotransfection of plasmids expressing HA-tagged $\Delta p85$ (b), HA-tagged PTEN (c), or HA-tagged DN Akt (d) with or without BMP-2 treatment. Mean $\pm$ SE of triplicate determinations is shown. * $P < 0.001$ vs. control, ** $P < 0.01$ vs. BMP-2-treated. Lower panels in b–d show expression of HA-tagged $\Delta p85$, PTEN, and DN Akt, respectively, from representative lysates used for luciferase activity

Fig. 4.

PI 3-kinase/Akt signaling controls Smad-induced Osx transcription and Smad binding to Osx promoter. **a, b** C2C12 cells were transfected with 987-Luc and Smad5 expression plasmids along with plasmids expressing Δp85 (**a**) or DN Akt (**b**) with or without BMP-2 treatment. Luciferase activities were determined as described in Figure 1. *White bars* represent vector-transfected cells; *gray bars* represent cells treated with BMP-2 alone; *black bars* represent cells transfected with Smad5; *shaded bars* represent cells transfected with Smad5 and treated with BMP-2 for additive effects. The second set of four bars in each panel represents cells transfected with Δp85. Mean $\pm$ SE of triplicate determinations is shown. $^{\#}P < 0.05$ vs. control; $^{*}P < 0.01$ vs. control; $^{@}P < 0.001$ vs. control; $^{##}P < 0.05$ vs. BMP-2; $^{**}P < 0.001$ vs. Smad5; $^{@@}P < 0.001$ vs. Smad5 + BMP-2. **c, d** EMSA was performed using nuclear extracts from C2C12 cells infected with Ad DN PI 3-kinase (**c**), Ad DN Akt (**d**), or Ad GFP (as control in both panels) in the presence or absence of BMP-2, using ^{32}P labeled Osx SBE probe. *Top arrow* indicates Smad interaction with SBE