p38 REGULATES EXPRESSION OF OSTEObLAST-SPECIFIC GENES BY PHOSPHORYLATION OF OSTERIX

Mª José Ortúñ, Silvia Ruiz-Gaspà, Edgardo Rodríguez-Carballo, Antonio R.G. Susperregui, Ramon Bartrons, José Luis Rosa and Francesc Ventura

Departament de Ciències Fisiològiques II, Universitat de Barcelona, IDIBELL, L’Hospitalet de Llobregat, Spain.

Running title: Phosphorylation and activation of Osterix by p38

* Address correspondence to : Francesc Ventura. Departament de Ciències Fisiològiques II, Universitat de Barcelona, IDIBELL. C/ Feixa Llarg 69-75 E-08907 L’Hospitalet de Llobregat, SPAIN. phone 34-934024281. FAX 34-934024268. Email fventura@ub.edu

SUMMARY
Osterix, a zinc-finger transcription factor, is specifically expressed in osteoblasts and osteocytes of all developing bones. Since no bone formation occurs in osteix-null mice, Osterix is thought to be an essential regulator of osteoblast differentiation. We report that, in several mesenchymal and osteoblastic cell types, BMP-2 induces an increase in expression of the two isoforms of Osterix arising from two alternative promoters. We identified a consensus Sp1 sequence (GGGCGG) as Osterix binding regions in the fibromodulin and the bone sialoprotein promoters in vitro and in vivo. Furthermore, we show that Osterix is a novel substrate for p38 MAPK in vitro and in vivo, and that Ser73 and Ser77 are the regulatory sites phosphorylated by p38. Our data also demonstrate that Osterix is able to increase recruitment of p300 and Brg1 to the promoters of its target genes fibromodulin and bone sialoprotein in vivo and that it directly associates with these cofactors through protein-protein interactions. Phosphorylation of Osterix at Ser 73/77 increases its ability to recruit p300 and SWI/SNF to either fibromodulin or bone sialoprotein promoters. We therefore propose that Osterix binds to Sp1 sequences on target gene promoters and that its phosphorylation by p38 enhances recruitment of coactivators to form transcriptionally active complexes.

INTRODUCTION
Bone is a highly dynamic tissue that is constantly remodeled throughout life. Bone remodeling activity is dependent on a delicate balance between osteoclast resorption and osteoblast new bone formation. Deregulation of these two activities unleashes pathological states such as osteoporosis and osteosclerosis. Both endochondral and intramembranous ossification depends on osteoblasts that derive from pluripotent mesenchymal stem cells which, in response to various cellular and environmental signals, commit to the osteoblast phenotype. Among them, BMPs are essential for commitment and differentiation to the osteoblast lineage; they promote osteoblast differentiation in vitro and in vivo, bone regeneration and ectopic bone formation in vivo (1-3). The BMP signal is transduced through the binding to their heteromeric cell-membrane receptors (4, 5). BMP binding to receptors results in the activation of the Smad family of transcription factors which directly regulates target gene expression (6).

BMP target genes include a growing number of osteoblast-determining transcription factors. For instance, in vivo genetic evidence as well as osteogenic induction of bone marrow mesenchymal stem cells in vitro has identified several types of transcription factors such as Id1, homeodomain proteins such as Dlx3 and Dlx5, ATF4, Runx2, and Osterix (Osx) (7-9). Runx2 and Osx have been widely accepted as master osteogenic factors since neither Runx2 nor Osx-null mice form mature osteoblasts (10, 11). Osx contains a proline and serine-rich transactivation domain located in...
Osx-binding regions in the consensus Sp1 sequence (GGGCGG) as the expression. In this study, we identified a Osx promotes osteoblast-specific gene about the transcriptional mechanisms by which BMP-induced transcription factors and p38 are involved in these data suggest that osteoblast-specific osteogenic effects (20-25). Thus, although shown to be relevant in the induction of their BMP-2, IGF-I or mechanical stress has been MAPK-mediated phosphorylation (17, 20).

transcriptional activation of Dlx5 by p38-BMP-2 induction of Msx2 (17, 19). These studies also showed that independent mechanisms involving Dlx5 and Osterix indicate that BMP-2 activates expression of Runx2-deficient cells (18). Recent data with Osx and regulate its transcriptional NFATc or NO66 have been shown to interact addition, transcriptional regulators such as osteocalcin, collagen type I. In osteoblasts from osteochondroprogenitors appear, whereas Osx may have a role in the segregation of osteoblasts from osteochondroprogenitors (8). In addition, it has been shown that genetic polymorphisms in the Osx gene locus are associated with low bone mineral density (12, 13). This essential role of Osx relies on its ability to regulate the expression of a number of osteoblast markers such as osteopontin, osteocalcin, Dkk1 and collagen type I. In addition, transcriptional regulators such as NFAc or NO66 have been shown to interact with Osx and regulate its transcriptional responses (14, 15).

Runx2 and Osx transcription is stimulated by BMP treatment in vitro (11, 16, 17). Pretreatment with cycloheximide blocks Osx induction, suggesting that Osx is not a direct target of the BMP signaling cascade but requires the expression of newly synthesized intermediates (18). Interestingly, although expression of Osx in vivo requires Runx2, BMP-2 is still able to stimulate alkaline phosphatase activity and Osx expression in Runx2-deficient cells (18). Recent data indicate that BMP-2 activates expression of Osterix through Runx2-dependent as well as independent mechanisms involving Dlx5 and Msx2 (17, 19). These studies also showed that BMP-2 induction of Osx required the transcriptional activation of Dlx5 by p38-MAPK-mediated phosphorylation (17, 20).

Activation of p38 MAPK signaling by BMP-2, IGF-I or mechanical stress has been shown to be relevant in the induction of their osteogenic effects (20-25). Thus, although these data suggest that osteoblast-specific transcription factors and p38 are involved in BMP-induced Osx expression, little is known about the transcriptional mechanisms by which Osx promotes osteoblast-specific gene expression. In this study, we identified a consensus Sp1 sequence (GGGCGG) as the Osx-binding regions in the fibromodulin (Fmod) and the bone sialoprotein (Ibsp) promoters in vitro and in vivo. We also show that Osx is a novel substrate for p38 MAPK in vitro and in vivo, and that Ser73 and Ser77 are the regulatory sites phosphorylated by p38. The transactivation potential of Osx was increased by p38 phosphorylation, at least in part through increased interaction of Osx with the transcriptional cofactors p300 and Brg1. We propose a regulatory network in which BMP-2 activates expression of Osx and enhances its transcriptional activation by p38-mediated phosphorylation.

**EXPERIMENTAL PROCEDURES**

Plasmids, reagents and antibodies
cDNA fragments encoding both long and short Osterix-pCDNA3 expression vectors were kindly provided by Dr. K. Watanabe and Dr. B. de Crombrugghe respectively. Osx cDNA was used as a parental plasmid to generate the phosphorylation mutants using PCR approaches, or subcloned into pGEX vector to generate bacterial GST-Osx fusion constructs. Activated MKK6 expression vector (MKK6EE) and recombinant p38 were provided by Dr. P. Muñoz-Canoves. Fmod promoter-reporter construct from -2032 to +100 (2-kb pFmod-Luc) was kindly provided by Dr. F. Cimino. The pFmod300-Luc promoter-reporter was generated by PCR. The Ibsp promoter-reporter (pIbsp-Luc) construction encoding a conserved 300 bp enhancer of human Ibsp gene, located at -12400 from the transcriptional start site, was cloned in the minimal promoter of c-fos gene.

BMP-2 was generously provided by Wyeth and was used at a final concentration of 2nM. SB203580 (Calbiochem) was used at a final concentration of 10 µM. Antibodies against Osx (Abcam), phospho-serine (Abcam), SB203580 (Calbiochem) was used at a final concentration of 10 µM. Antibodies against Osx (Abcam), phospho-serine (Abcam), MKK6 (Abcam), Brg1 (Upstate), p300 (SantaCruz), α-tubulin (Sigma) were used 1:1000.

Cell culture and transfection
C2C12, Saos-2 and MEFs cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco). MC3T3-E1 cell line was maintained in the α-Modified MEM (LabClinics), supplemented with 10% FBS Human primary osteoblasts were obtained from Lonza and cultured in DMEM/F-12, supplemented with 15% Foetal Bovine Serum (FBS), 10 µg/ml ascorbic acid and 1.25 µg/ml amphotericin B. Bone marrow mesenchymal
stem cells (BM-MSCs) were maintained in DMEM, supplemented with 15% FBS. All media were supplemented with 0.2mM glutamine, 0.1mM pyruvate, and 100 U/ml penicillin-streptomycin. C2C12, MC3T3-E1, MEFs and Saos cell differenciation was induced with media lacking serum and BMP-2 at a final concentration of 2nM. For human primary osteoblasts, differenciation was induced with medium lacking serum, 50 μg/ml ascorbic acid and 2nM BMP-2, and for BM-MSCs with 10% FBS, 50 μM ascorbic acid 5mM β-glycerophosphate and 2nM BMP-2. C2C12, MC3T3 and MEFs cells were transiently transfected using Lipofectamine LTX (Invitrogen).

**Western blot analysis**

Cells were washed twice in cold PBS and lysed with 50 mM Tris pH 7.5, 150 mM NaCl, 0.2% Igepal, 10% glycerol supplemented with protease and phosphatase inhibitors. Protein extracts were resolved on 10% SDS-polyacrylamide gels, transferred to nitro-cellulose membrane (Millipore) and subjected to Western blot using the above indicated antibodies at 1:1000 dilution. Immunocomplexes were visualized with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (1:10000) followed by incubation with ECL-Western-blot reagent (GE Healthcare).

**Immunofluorescence assay**

Twenty-four hours after either transfection with Osx or treatment with BMP-2, C2C12 cells were fixed as described previously (17). Cells were stained with rabbit anti-Osx (Abcam) at 1:500, followed by goat anti-rabbit IgG conjugated with Alexa 488, at 1:400. Labeling was detected using a Leica TCS SL inverted laser scanning confocal microscope.

**RT-qPCR analysis**

Total RNA was isolated from C2C12, MC3T3, Saos-2 and BM-MS cells using the Ultraspec RNA Isolation System (Biotecx). 5 μg of total RNA were reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCRs were carried out using the ABI Prism 7900 HT Fast Real-Time PCR System and a Taqman 5'-nuclease probe method (Applied Biosystems). All transcripts were normalized to GAPDH and transfection efficiency was assessed by GFP expression. Designed human Taqman assays (Applied Biosystems) were used to quantify gene expression of Osx (Mm00504574_m1), Fmod (Mm00491215_m1), Ibsp (Mm00492555_m1) and GAPDH (Mm99999915_g1).

**Reporter assays**

Luciferase activities were quantified using the Luciferase Assay System (Promega). Luciferase values were normalized using β-Galactosidase activity measured with the Luminescent β-Galactosidase Detection Kit II (Clontech).

**Biotinylated Oligonucleotide Precipitation Assays**

C2C12 cells were lysed as described previously (26). Biotinylated oligonucleotides were incubated overnight with cell extracts in presence of 1μg poly(dI/dC) (GE Healthcare) and collected with streptavidin-agarose beads (GE Healthcare). Bound Osx was detected by immunoblotting.

**Chromatin Immunoprecipitation**

ChIP assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer’s instructions but repeating all the washes twice. Antibodies used were anti-Osx (Abcam), anti-p300 (N-15, Santa Cruz Biotechnology), Brg1 (Upstate), anti-RNA Pol-II and anti-IgG (Upstate). Primer sequences used for the PCR were: for human primary osteoblasts and Saos-2 cells, HsIbspF 5’-cttctttctcatgtggccaacactcg; HsIbspR 5´-tggcatcaggagatgtcctctctt; HsFmodF5´-ggacccagggctgccaat and HsFmodR 5’-cgtccctctgtctggcctccttgggtt. For C2C12 cells, MmIbspF 5’-ttcaggctgccaatgctccagggaa; MmFmodR 5’-tgaccacgtccttctgtctggtct; MmIbspF 5’-ccagtttcaacatccaatcataggg; MmIbspR 5’-ttggcactggagagagatcctcctt. Immunoprecipitation Cells were lysed as above. The supernatant fraction was incubated with 1μg of anti Flag-M2 antibody (Sigma) overnight, followed by incubation with Protein G-Sepharose beads for 1 hour. Bound proteins were washed four times in lysis buffer. Bound proteins were detected by immunoblotting using the antibodies described above.

**In vitro phosphorylation by p38 MAPK**

GST-p38 MAPK was activated by MBP-MKK6-DD (5:1 ratio) in 50mM Tris-HCl pH 7.5, 10mM MgCl2, 2mM DTT and ATP 200μM. Activated p38 (200ng for each condition) was incubated with recombinant
Osx overexpression increased the expression of the gene whose expression has been shown to be abolished in Osx deficient cells. We also focused on fibromodulin (Fmod) which increased its expression 2-fold in response to Osx. We also focused on bone sialoprotein (Ibsp) as a late osteoblast marker, whose expression has been shown to be abolished in Osx deficient cells. Quantitative PCR (RT-qPCR) confirmed that Osx overexpression increased the expression levels of both Fmod and Ibsp.

RESULTS

Expression of two isoforms of Osterix is stimulated by BMP-2 in mesenchymal and osteogenic cells. Previous studies showed that Osx mRNA levels increase after BMP-2 addition in mesenchymal cell types, including primary osteoblasts, mesenchymal stem cells, mesenchymal C2C12 and osteoblast MC3T3-E1 cells (11, 17, 20, 27). Analysis of protein expression by immunoblotting indicated that Osx was not significantly expressed in undifferentiated C2C12 cells or MC3T3-E1 cells, and showed some expression in the osteoblastic cell line Saos-2 cells (Fig. 1A). However, Osx protein levels were strongly increased in all cell types at 24 hours after addition of 2nM BMP-2. In all cell types, Osx appeared as multiple bands of different mobility (Fig. 1A). Previous work showed that both the human and mouse Osx mRNA are expressed as two isoforms arising from two alternative promoters (28-30). RT-PCR using unique 5’ primers and a common 3’primer yielded to different species which corresponded to the long (MASSLL) and the short isoforms (MLTAAC) of Osx. These results indicated that both isoforms are induced at significant levels by BMP-2 in these cell lines (Fig. 1B). We also analyzed the subcellular localization of Osx after BMP-2 induction. Immunofluorescence analysis of C2C12 cells, either transfected with an Osx expression vector or 24 hours after BMP-2 addition showed that Osx is a constitutively nuclear transcription factor (Fig. 1C).

Osx-responsive regions in the promoters of fibromodulin and bone sialoprotein. In order to analyze the mechanisms of transcriptional activation by Osx, we used microarrays to search for Osx target genes by comparing RNA expression of C2C12 cells mock or transfected with an Osx expression vector. Among the differentially expressed genes, we focused on fibromodulin (Fmod) which increased its expression 2-fold in response to Osx. We also focused on bone sialoprotein (Ibsp) as a late osteoblast marker, whose expression has been shown to be abolished in Osx deficient cells (11, 31). Quantitative PCR (RT-qPCR) confirmed that Osx overexpression increased the expression levels of both Fmod and Ibsp in C2C12. Moreover we analyzed the expression levels of Fmod and Ibsp in MC3T3-E1 cells. Similarly to C2C12 cells, Osx induces expression of both genes (Fig. 2A).

We also analyzed the temporal expression pattern of both Fmod and Ibsp in response to BMP-2 compared to the induction of Osx. As shown in Figure 2B, induction of Fmod and Ibsp gene expression in either C2C12 or MC3T3-E1 cells strongly correlated with that of Osx. All genes required at least 8 hours to increase their mRNA levels significantly after BMP-2 addition.

Because these findings suggested that either Osx or BMP-2 is able to increase Fmod expression, we decided to determine whether Osx and BMP-2 stimulation was transcriptional by analysis of Fmod promoter activity. Using a 2-kb upstream regulatory sequence of the murine Fmod gene (2-kb pFmod-Luc) in C2C12 cells, we observed a 2.3-fold increase in activation in cells overexpressing Osx and a 1.7-fold increase in activation after treatment with 2nM BMP-2 (Fig. 3A). More interestingly, BMP-2 promoted an additive effect to Osx overexpression in C2C12 cells. We then used reporter assays to analyze deletions of the 2-kb promoter. The deletion of the sequence from -2032 to -192 provoked only slight decreases in Osx or BMP-2 reporter induction (Fig. 3B). Homology analysis using the ECR browser (http://ecrbrowser.dcode.org) showed that the -297/+100 region on the Fmod promoter is highly conserved among mammalian species and detected two putative Sp1 transcription factor binding sites (Suppl. Fig. 1 and 32). Similar analysis on Ibsp promoter region detected a highly conserved 300bp enhancer located at -12400bp from the Ibsp transcription start site that contains a Runx2 binding site, a palindromic homebox binding site (TAATTA) and a Sp1 binding site (Suppl. Fig. 1). To test whether this region alone has the ability to render a minimal promoter responsive to Osx and/or BMP-2, we further assayed the responses of a reporter construct containing this region upstream of a heterologous c-fos minimal promoter. Whereas the minimal c-fos promoter showed no response at all to Osx or BMP-2, the Ibsp enhancer region was increased 2-fold and 3-fold by overexpression of Osx and addition of 2 nM BMP-2 respectively (Fig. 3C).
to Fmod reporter assays, BMP-2 promoted an additive effect to Osx overexpression on this enhancer in C2C12 cells. The reporter assays of Fmod and Ibsp indicate that, in addition to the induction of Osx expression, BMP-2 activates alternative pathways that result in further Fmod and Ibsp transcriptional activation by Osx.

**Osx interacts with Sp1 consensus regions in regulatory sites of Fmod and Ibsp.** It has been shown that Osx interacts with the promoters of osteogenic genes such as Colla1, Dkk1 or Bglap in vivo (14, 15, 31). In all cases, a GC-rich canonical Sp1 sequence has been shown to be the specific Osx binding site. Therefore, we analyzed whether Osx bound to the Sp1 sequence of Fmod (GGGGCGG) and studied the Osx binding preferences by comparing them to Sp1 sequences located in the bone-enhancer of the Colla1 promoter (AGGGCGG or GGGGAGG, respectively) which have been shown to be bound by Osx (Fig. 4A)(33). We performed oligonucleotide pull-down assays from C2C12 cells either overexpressing Osx or cells after induction of endogenous Osx by BMP-2 addition. Osx required a functional Sp1 site, since no binding at all to the DNA was observed when extracts were incubated with the Sp1-mutated oligonucleotide (Fig. 4A). The results also suggest that the sequence GGGCGG is preferred for Osx binding, at least in vitro, since it bound better than the sequence GGGAGG.

To investigate binding of Osterix in both Fmod promoter and Ibsp enhancer in vivo, we performed chromatin immunoprecipitation assays (ChIP) in Saos-2 cells or human primary osteoblasts treated with 2nM BMP-2 for 24 hours. As shown in Figure 4B, binding of Osx to the proximal Fmod promoter or Ibsp enhancer in vivo was increased after treatment with BMP-2. The increased binding of Osx to these promoter regions correlated with increased recruitment of RNA Polymerase II to the start site of Fmod gene. It has been previously reported that p300 HAT is required for the expression of several osteogenic genes in response to BMP-2 (17, 34-36). Results of the ChIP assay indicated that p300 was recruited onto both Fmod proximal promoter and Ibsp enhancer sequences in response to BMP-2. Similarly, binding of the SWI/SNF subunit Brg1 was also increased in human primary osteoblasts after BMP-2 addition (Fig. 4B). Altogether, these findings suggest that Osx and transcriptional coactivators are recruited onto these promoter regions and involved in the transcriptional program of induction of both Fmod and Ibsp expression by BMP-2.

**Osx is phosphorylated by p38.** The fact that addition of BMP-2 was able to further increase the responsiveness to Osx of both the proximal Fmod and the Ibsp enhancer regions led us to investigate the additional transcriptional effects of BMP-2 in the expression of Fmod and Ibsp target genes. Interestingly, SDS-PAGE analysis of cell extracts showed that both long and short forms of Osx appeared as two species. To determine whether the respective retarded bands were phosphorylated forms, cell extracts from MC3T3-E1 cells after BMP-2 induction were treated with alkaline phosphatase. Phosphatase treatment abrogated slow-migrating forms, indicating that those were due to phosphorylation events (Fig. 5A). Similar results were obtained by overexpressing exogenously either long or short forms of Osx, indicating that both forms were substrates of cellular kinase/s (Fig. 5A).

Activation of p38 MAPK by BMP-2 has been shown to play a relevant role in the osteogenic effects of this cytokine (21-23). More specifically, the p38 pathway has been directly implicated in the BMP-induced sequential recruitment of the transcriptional coactivators p300, and the SWI/SNF complex onto osteogenic genes (34, 36). We therefore analyzed whether Osx could be a target of the p38 pathway. In agreement with previous reports (17), treatment of C2C12, MC3T3-E1 or human primary osteoblasts with the specific p38 inhibitor SB203580 inhibits the BMP-2 induced expression of Osx (Fig. 5B). In addition to the decrease in total expression, SB203580 reduced the phosphorylated Osx bands. To further confirm the ability of p38 to phosphorylate Osx, we used a constitutive active form of MKK6 (M KK6EE), which phosphorylates and activates p38, and analyzed cell extracts from C2C12 cells expressing either short or long forms of Osx. The amount of the upper band corresponding to phosphorylated Osx appeared as two species. To determine whether the respective retarded bands were phosphorylated forms, cell extracts from MC3T3-E1 cells after BMP-2 induction were treated with alkaline phosphatase. Phosphatase treatment abrogated slow-migrating forms, indicating that those were due to phosphorylation events (Fig. 5A). Similar results were obtained by overexpressing exogenously either long or short forms of Osx, indicating that both forms were substrates of cellular kinase/s (Fig. 5A).
isoforms for Osx phosphorylation, we analyzed MKK6EE-induced Osx phosphorylation in p38α-/- mouse embryonic fibroblasts (MEFs). MKK6EE induced phosphorylation of Osx in wild type but not in p38α-/- MEFs (Figure 5D). Taken together, these results demonstrate that p38 MAPK is able to phosphorylate Osx.

**Osx is phosphorylated by p38 at Ser73 and Ser77.** Sequence analysis of Osx revealed four distinct serines corresponding to consensus sites for MAPKs conserved in Osx orthologs, and one of these was only present in the long form of Osx. We generated site-specific mutants corresponding to the two sites present in both long and short forms of Osx where Ser was changed to Ala. Expression of MKK6EE induced the appearance of retarded phosphorylated bands when co-transfected with either the short or long forms of Osx (Fig. 6A). However, either single point mutants S73A, S77A as well as double mutant S73/77A lost p38-dependent phosphorylation and were detected exclusively as a single fast-migrating band irrespective of expression of MKK6EE (Figure 6A). The fact that neither single mutant were phosphorylated by p38 may indicate that both of them are phosphorylated or, since both sites are located very close to each other in a region of Osx with a predicted coiled structure, Ser to Ala mutation in one site might also hinder phosphorylation at the other site.

We then examined whether Osx was a direct substrate of p38, by *in vitro* phosphorylation assays using recombinant proteins. Recombinant wild type Osx was phosphorylated *in vitro* by activated recombinant p38, whereas the double mutant S73/77A showed impaired phosphorylation by p38 (Fig. 6B). Analysis of the efficiency of this phosphorylation indicates that addition of recombinant p38 induces almost a complete phosphorylation of wild type Osx, whereas the double mutant S73/77A only showed minor effects on either phosphorylation on serines or in its electrophoretic mobility (Fig. 6B).

In order to examine the functional consequences of Osx phosphorylation, we first analyzed whether phosphorylation changed the Osx protein turnover. *Wild type* and *p38α-/-* defective MEFs were transfected with Osx and MKK6EE and treated with the protein synthesis inhibitor cycloheximide for different periods of time. Quantification of the kinetics of Osx disappearance displayed not significant differences between the phosphorylated and unphosphorylated forms of Osx. Similarly, the profile of degradation of Osx was similar irrespective of the presence or absence of MKK6EE or co-transfection of wild type Osx with MKK6EE in p38α-/- MEFs (Fig. 6C).

**p38 phosphorylation enhances Osx transcriptional activity and increases its interaction with co-activators.** To investigate whether phosphorylation by p38 has a functional effect on Osx transcriptional activity, C2C12 cells were transfected with either Fmod or Ibsp promoter constructs, and cells were treated with BMP-2 in presence or absence of the p38 inhibitor SB203580. Increases in reporter activity induced by BMP-2 were significantly inhibited by addition of SB203580 in both cases, suggesting a functional role for p38-induced Osx phosphorylation (Fig. 7A). We further analyzed the endogenous expression of these target genes by RT-qPCR upon differentiation of bone-marrow mesenchymal stem cell cultures. Induction of *Fmod* and *Ibsp* gene expression by BMP-2 was reduced in presence of the p38 inhibitor SB203580 (Fig 7B). In view of these results, we assessed the relevance of Ser73 and Ser77 phosphorylation to the transcriptional activity of Osx. Whereas overexpression of Osx wild type led to a 2 to 3-fold induction of the activity of *Fmod* and *Ibsp* promoter constructs, induction of promoter activity by the S73/77A mutant construct was significantly lower (Fig. 7C). Similarly, addition of BMP-2 further increased the transcriptional activity of wild type Osx on both reporters, whereas the mutant form showed impaired responses to the cytokine addition (Fig. 7C). Likewise, we further analyzed the endogenous expression of these target genes by RT-qPCR. As expected, induction of *Fmod* and *Ibsp* endogenous genes by S73/77A Osx was reduced in comparison with induction by wild type Osx, in both C2C12 and MC3T3-E1 cells (Fig 7D).

To investigate the relevance of the phosphorylation of Osx to its binding and transcriptional activation of the *Fmod* and *Ibsp* genes *in vivo*, we performed ChIP assays in C2C12 cells transfected with wild type or double mutant Osx. As shown in Fig. 8A, Osx bound to *Fmod* promoter and *Ibsp* enhancer. Increased Osx binding was sufficient to recruit p300 and the RNA Polymerase II to the *Fmod* promoter and p300 to the *Ibsp* enhancer. More importantly, although transfection efficiency
and expression levels of either wild type or mutant Osx were similar, binding of mutant Osx and its ability to recruit RNA Polymerase II, p300 or Brg1 to the Fmod or Ibsp regulatory regions was lower, suggesting that phosphorylation of Osx enhances assembly of transcriptionally active complexes in Osx target genes.

Since overexpression of Osx is sufficient to induce recruitment of p300 and Brg1 to the responsive promoters, we analyzed whether these factors might interact with each other and studied the role of Osx phosphorylation in this interaction. We analyzed the ability of Osx to interact with endogenous p300 and Brg1. Thus, we expressed Flag-tagged wild type and the S73/77A forms of Osx in C2C12 cells either alone or together with MKK6EE. After immunoprecipitation of Osx complexes, we found p300 and Brg1 to be associated to Osx by Western blot analysis (Fig. 8B). In addition, overexpression of MKK6EE, which led to phosphorylated Osx, increased the amounts of interacting p300 and Brg1, whereas addition of p38 inhibitor SB203580 decreased the binding of both transcriptional coactivators. These interactions were mostly abrogated when the Osx form mutated at S73/77A was analyzed. Moreover, addition of BMP-2 to cells expressing Flag-tagged wild type Osx also increased interaction of Osx with p300 and Brg1. These BMP-induced effects were partially abolished by addition of SB203580 (Fig. 8C). Taken together, our results strongly suggest that Osx binds to Sp1 sequences on target gene promoters and that phosphorylation of Osx by p38 may enhance recruitment of coactivators.

**DISCUSSION**

Osterix and Runx2 are widely considered to be master osteogenic factors since neither Runx2 nor Osx-null mice form mature osteoblasts (11). Here, we show that exposure of distinct mesenchymal and osteoblastic cells to BMP-2 induces the appearance of both the long and short Osx forms, which also become phosphorylated by p38 MAPK. Our results also emphasize the essential role of p38 and chromatin remodeling in the control of Osx expression and function. Osx, by virtue of its binding to Sp1 sequences, activates transcription of several target genes in osteoblasts and mesenchymal cell lines, such as Fmod and Ibsp. More importantly, we identified that phosphorylation of Osx by p38 at Ser 73/77 promotes assembly of stable, transcriptionally active complexes containing Osx, p300 and Brg1.

The essential role of Osx in osteogenesis relies on its ability to regulate the expression of a number of osteoblast markers, such as Osteopontin, Dkk1 and Collagen type I. We identified Fmod and Ibsp as novel osteoblastic genes regulated by Osx and characterized Sp1 binding regions in their promoters which are able to mediate BMP-2 and Osx activation. Our data constitute the first evidence of the mechanisms of osteogenic regulation of Fmod expression by Osx and BMP-2. In the case of Ibsp, functional cooperativity was previously shown between the osteogenic factors Dlx5 and Runx2 in the proximal promoter of Ibsp, where Runx2 and Dlx5 bind to adjacent sites and physically interact to drive promoter activation (37). There is some evidence that Osx binding activity also plays an essential role in mediating these effects on Ibsp expression. Previous studies using 2.5- and 2.7-kb Ibsp promoters failed to show absolute tissue-specific expression in transgenic mice (38) and required, at least, a 9 kb promoter to show osteoblast-specific expression (39). We identified an Ibsp enhancer region with an 80% sequence homology across human, mouse and dog genes (Suppl. Fig. 1). This enhancer contains fully conserved binding sites for Osx, Runx2 and homeodomain transcription factors, suggesting that it could be critical for osteoblast-specific expression. Induction of Ibsp and Fmod by BMP-2 correlates with that of Osx and requires at least 16-24 hours to reach maximal induction, while the induction of Runx2 and Dlx5 by BMP-2 requires much less time (17, 40). Similarly, the temporal expression pattern in other models of differentiation of osteoblast precursors indicates that the expression of Osx always precedes that of Fmod, Ibsp and other markers of terminal differentiation such as osteocalcin or alkaline phosphatase. Finally, blocking the Osx function either in mice or cell cultures leads to loss of expression of both Ibsp and Fmod (11, 15, 31, 41, 42). Altogether, our findings are consistent with the idea that BMP-dependent induction of expression of late osteogenic markers depends on Osx and its cooperative action with other osteogenic master genes.
Osx belong to the Sp-1/Kruppel family of transcription factors which presents a marked preference for binding to GC-rich motifs (43). However, since only a few mammalian Osx-responsive promoters have been characterized, there is no evidence for preferences to bind to specific motifs. Our results suggest that Osx displays the ability to bind either the GGGCGG or the GGGAGG sequences with, at least in vitro, a higher DNA-binding affinity to GGGCGG compared to GGGAGG. Interestingly, our sequence analysis of the Osx-responsive Sp1 boxes in both Fmod and Ibsp promoters is consistent with other Osx-responsive genes characterized like Colla1, Colla2, Dkk1. In all cases the responsive motifs in vitro and in vivo contain one of these two types of sequences (14, 31, 41).

p38 MAPK is known to play an important role in several steps of the osteoblast lineage progression, and is necessary but not sufficient for the BMP-induced acquisition of the osteoblast phenotype (21, 44). Several evidence also indicates that p38 activity modulates Osx expression induced by BMP in calvarial as well as bone-marrow-derived mesenchymal stem cells (17, 20, 24, 27). Similarly, other osteogenic stimuli such as mechanical stress, drugs, or cytokines exert their osteogenic effects through activation of the p38, followed by an increase in Osx expression and function (17, 45-47). Our results provide a mechanism by which BMP-2-activated p38 could enhance transcriptional activity of Osx through direct phosphorylation in Ser73 and Ser77. First, activated p38 phosphorylates Osx in Ser73 and Ser77. Second, phosphorylation of Osx is induced by the expression of constitutive active MKK6, while it is blocked by the pharmacological inhibition of p38. Finally, p38-mediated phosphorylation of Osx appears to increase its interaction with the transcriptional coactivators p300 and Brg1. Indeed, the p38 inhibitor SB203580 inhibits BMP-2 and Osx-driven Fmod or Ibsp promoter activation and the mutant Osx S73/77A displays reduced transcriptional activity and BMP-2 responsiveness. The existence of regulatory phosphorylations affecting the activity of Sp1 is well known (32), and some reports indicate that this could be also the case for Osx, since it has been shown to interact and become dephosphorylated by calcineurin (48).

Regulatory effects of p38 phosphorylation were also observed previously in Dlx5 transcriptional activity onto the promoter of Osx itself (17). We therefore propose a positive osteogenic regulatory network where p38 activation regulates the expression of Osx through Dlx5 phosphorylation and further transcriptional activation of Osx through its phosphorylation in Ser73/77.

Ser 73 and 77 are located in a solvent-exposed coiled region of the proline and serine-rich transactivation domain of Osx. This region has been shown to interact with cofactors such as TF-IIB, a Junonji family histone demethylase (NO66), or Brg1 through its zinc-finger domain (15, 49). Functionally equivalent regions of Sp1 have been shown to be phosphorylated by distinct kinases, resulting in activity changes based on their ability to recruit transcriptional cofactors (reviewed in 32). Thus, it is likely that phosphorylation of Osx in the transactivation domain might modulate its ability to recruit transcriptional cofactors.

Acetylation and/or methylation at specific sites in histones in chromatin is commonly associated with transcriptionally active genes, and p300, histone deacetylases (HDACs) and the NO66 methylase have been shown to bind and regulate the acetylation or methylation of the core histones H3 and H4 in several osteogenic gene promoters. For instance, p300 is recruited to the osteocalcin promoter in response to osteogenic signals (35, 50), or JunB recruits p300 to activate dentin matrix protein 1 (Dmp1) (34) whereas HDAC4 inhibits Runx2 activity and HDAC4 null mice exhibit strong skeletal defects (51). BMPs have been shown to switch recruitment from HDAC1 to p300 in osteocalcin, Osx or Fgfr3 gene promoters and to promote hypomethylation of CpG sites in Dlx5 and Osx promoter regions (35-36, 52). Interestingly, BMP-2 has been shown to increase binding of Osx to Ibsp or osteocalcin promoters with a concomitant decrease of NO66 binding and activity (15). Similarly, BMPs increase the expression and function of the ATP-dependent chromatin remodeling complex SWI/SNF, which has been shown to be absolutely essential for osteogenesis, especially the complexes containing Brg1 as a catalytic core (53-55). Our study demonstrates that Osx is able to increase recruitment of p300 and Brg1 to the promoters of its target genes Fmod and
Ibsp in vivo and that Osx directly associates to these cofactors through protein-protein interactions which are further enhanced by BMP signaling. Furthermore, phosphorylation of Osx at Ser 73/77 increases its ability to recruit p300 and SWI/SNF to Fmod or Ibsp promoters, as has been shown for p38-dependent recruitment of p300 by JunB on Dmp1 promoter or by Sp1 to Fgfr3 promoter (34, 36). Recruitment of both cofactors is likely to be cooperative since, in addition to its HAT activity, the p300 protein may act as a bridging factor to connect sequence-specific transcription factors to other cofactors and to the basal transcriptional machinery, and acetylated chromatin is the preferred substrate for SWI/SNF recruitment (56). In conclusion, Osx is able to upregulate Fmod and Ibsp expression levels through its ability to recruit p300 and Brg1. Furthermore, phosphorylation of Osx by p38 is able to enhance the recruitment of these transcriptional cofactors. Formation of this complex may initiate chromatin remodeling, responsible for the initiation and maintenance of high transcription rates of their target genes.
References

1. Chen, D., Zhao, M., and Mundy, G. R. (2004) *Growth Factors* **22**, 233-241
2. Tsumaki, N., and Yoshikawa, H. (2005) *Cytokine Growth Factor Rev.* **16**, 279-285
3. Wan, M., and Cao, X. (2005) *Biochem. Biophys. Res. Commun.* **328**, 651-657
4. Moustakas, A., and Heldin, C. H. (2009) *Development* **136**, 3699-3714
5. Miyazono, K., Maeda, S., and Imamura, T. (2005) *Cytokine Growth Factor Rev.* **16**, 251-263
6. Massague, J., Seoane, J., and Wotton, D. (2005) *Genes Dev.* **19**, 2783-2810
7. Karsenty, G. (2008) *Annu. Rev. Genomics Hum. Genet.* **9**, 183-196
8. Nakashima, K., and de Crombrugghe, B. (2003) *Trends Genet.* **19**, 458-466
9. Lian, J. B., Stein, G. S., Javed, A., van Wijnen, A. J., Stein, J. L., Montecino, M., Hassan, M. Q., Gaur, T., Lengner, C. J., and Young, D. W. (2006) *Rev. Endocr Metab. Disord.* **7**, 1-16
10. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) *Cell* **89**, 747-754
11. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002) *Cell* **108**, 17-29
12. Styrkarsdottir, U., Halldorsson, B. V., Gretarsdottir, S., Gudbjartsson, D. F., Walters, G. B., Ingvarsson, T., Jonsdottir, T., Saemundsdottir, J., Center, J. R., Nguyen, T. V., Bagger, Y., Gulcher, J. R., Eisman, J. A., Christiansen, C., Sigurdsson, G., Kong, A., Thorsteinsdottir, U., and Stefansson, K. (2008) *N. Engl. J. Med.* **358**, 2355-2365
13. Timpson, N. J., Tobias, J. H., Richards, J. B., Soranzo, N., Duncan, E. L., Sims, A. M., Whittaker, P., Kumanduri, V., Zhai, G., Glaser, B., Eisman, J., Jones, G., Nicholson, G., Prince, R., Seeman, E., Spector, T. D., Brown, M. A., Peltonen, L., Smith, G. D., Deloukas, F., and Evans, D. M. (2009) *Hum. Mol. Genet.* **18**, 1510-1517
14. Koga, T., Matsu, Y., Asagiri, M., Kodama, T., de Crombrugghe, B., Nakashima, K., and Takayanagi, H. (2005) *Nat. Med.* **11**, 880-885
15. Sinha, K. M., Yasuda, H., Coombes, M. M., Dent, S. Y., and de Crombrugghe, B. (2010) *EMBO J.* **29**, 68-79
16. Lee, M. H., Javed, A., Kim, H. J., Shin, H. I., Gutierrez, S., Choi, J. Y., Rosen, V., Stein, J. L., van Wijnen, A. J., Stein, G. S., Lian, J. B., and Ryoo, H. M. (1999) *J. Cell. Biochem.* **73**, 114-125
17. Ulsamer, A., Ortuno, M. J., Ruiz, S., Susperregui, A. R., Osses, N., Rosa, J. L., and Ventura, F. (2008) *J. Biol. Chem.* **283**, 3816-3826
18. Lee, M. H., Kwon, T. G., Park, H. S., Wozney, J. M., and Ryoo, H. M. (2003) *Biochem. Biophys. Res. Commun.* **309**, 689-694
19. Matsubara, T., Kida, K., Yamaguchi, A., Hata, K., Ichida, F., Meguro, H., Aburatani, H., Nishimura, R., and Yoneda, T. (2008) *J. Biol. Chem.* **283**, 29119-29125
20. Wang, X., Goh, C. H., and Li, B. (2007) *Endocrinology* **148**, 1629-1637

21. Guicheux, J., Lemonnier, J., Ghayor, C., Suzuki, A., Palmer, G., and Caverzasio, J. (2003) *J. Bone Miner. Res.* **18**, 2060-2068

22. Noth, U., Tuli, R., Seghatoleslami, R., Howard, M., Shah, A., Hall, D. J., Hickok, N. J., and Tuan, R. S. (2003) *Exp. Cell Res.* **291**, 201-211

23. Vinals, F., Lopez-Rovira, T., Rosa, J. L., and Ventura, F. (2002) *FEBS Lett.* **510**, 99-104

24. Celil, A. B., Hollinger, J. O., and Campbell, P. G. (2005) *J. Cell. Biochem.* **95**, 518-528

25. Fan, D., Chen, Z., Wang, D., Guo, Z., Qiang, Q., and Shang, Y. (2007) *J. Cell. Physiol.* **211**, 577-584

26. Lopez-Rovira, T., Chalaux, E., Massague, J., Rosa, J. L., and Ventura, F. (2002) *J. Biol. Chem.* **277**, 3176-3185

27. Celil, A. B., and Campbell, P. G. (2005) *J. Biol. Chem.* **280**, 31353-31359

28. Lu, X., Gilbert, L., He, X., Rubin, J., and Nanes, M. S. (2006) *J. Biol. Chem.* **281**, 6297-6306

29. Milona, M. A., Gough, J. E., and Edgar, A. J. (2003) *BMC Genomics* **4**, 43

30. Nishio, Y., Dong, Y., Paris, M., O'Keefe, R. J., Schwarz, E. M., and Drissi, H. (2006) *Gene* **372**, 62-70

31. Zhang, C., Cho, K., Huang, Y., Lyons, J. P., Zhou, X., Sinha, K., McCrea, P. D., and de Crombrugghe, B. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 6936-6941

32. Tan, N. Y., and Khachigian, L. M. (2009) *Mol. Cell. Biol.* **29**, 2483-2488

33. Jin, H., van't Hof, R. J., Albagha, O. M., and Ralston, S. H. (2009) *Hum. Mol. Genet.* **18**, 2729-2738

34. Narayanan, K., Srinivas, R., Peterson, M. C., Ramachandran, A., Hao, J., Thimmapaya, B., Scherer, P. E., and George, A. (2004) *J. Biol. Chem.* **279**, 44294-44302

35. Lee, H. W., Suh, J. H., Kim, A. Y., Lee, Y. S., Park, S. Y., and Kim, J. B. (2006) *Mol. Endocrinol.* **20**, 2432-2443

36. Sun, F., Chen, Q., Yang, S., Pan, Q., Ma, J., Wan, Y., Chang, C. H., and Hong, A. (2009) *Nucleic Acids Res.* **37**, 3897-3911

37. Roca, H., Phimphilai, M., Gopalakrishnan, R., Xiao, G., and Franceschi, R. T. (2005) *J. Biol. Chem.* **280**, 30845-30855

38. Chen, J., Thomas, H. F., Jin, H., Jiang, H., and Sodek, J. (1996) *J. Bone Miner. Res.* **11**, 654-664

39. Paz, J., Wade, K., Kiyoshima, T., Sodek, J., Tang, J., Tu, Q., Yamauchi, M., and Chen, J. (2005) *Matrix Biol.* **24**, 341-352
40. Hassan, M. Q., Tare, R. S., Lee, S. H., Mandeville, M., Morasso, M. I., Javed, A., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2006) *J. Biol. Chem.* **281**, 40515-40526

41. Goto, T., Matsui, Y., Fernandes, R. J., Hanson, D. A., Kubo, T., Yukata, K., Michigami, T., Komori, T., Fujita, T., Yang, L., Eyre, D. R., and Yasui, N. (2006) *J. Bone Miner. Res.* **21**, 661-673

42. Baek, W. Y., de Crombrugghe, B., and Kim, J. E. (2010) *Bone* **46**, 920-928

43. Wierstra, I. (2008) *Biochem. Biophys. Res. Commun.* **372**, 1-13

44. Caverzasio, J., Higgins, L., and Ammann, P. (2008) *J. Bone Miner. Res.* **23**, 1389-1397

45. Chang, J., Sonoyama, W., Wang, Z., Jin, Q., Zhang, C., Krebsbach, P. H., Giannobile, W., Shi, S., and Wang, C. Y. (2007) *J. Biol. Chem.* **282**, 30938-30948

46. Zhao, Y., Wang, C., Li, S., Song, H., Wei, F., Pan, K., Zhu, K., Yang, P., Tu, Q., and Chen, J. (2008) *Eur. J. Oral Sci.* **116**, 199-206

47. Lee, H. W., Suh, J. H., Kim, H. N., Kim, A. Y., Park, S. Y., Shin, C. S., Choi, J. Y., and Kim, J. B. (2008) *J. Bone Miner. Res.* **23**, 1227-1237

48. Okamura, H., Amorim, B. R., Wang, J., Yoshida, K., and Haneji, T. (2009) *Biochem. Biophys. Res. Commun.* **372**, 440-444

49. Hatta, M., Yoshimura, Y., Deyama, Y., Fukamizu, A., and Suzuki, K. (2006) *Int. J. Mol. Med.* **17**, 425-430

50. Sierra, J., Villagra, A., Paredes, R., Cruzat, F., Gutierrez, S., Javed, A., Arriagada, G., Olate, J., Imschenetzky, M., Van Wijnen, A. J., Lian, J. B., Stein, G. S., Stein, J. L., and Montecino, M. (2003) *Mol. Cell. Biol.* **23**, 3339-3351

51. Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A., Karsenty, G., and Olson, E. N. (2004) *Cell* **119**, 555-566

52. Lee, J. Y., Lee, Y. M., Kim, M. J., Choi, J. Y., Park, E. K., Kim, S. Y., Lee, S. P., Yang, J. S., and Kim, D. S. (2006) *Mol. Cells* **22**, 182-188

53. Young, D. W., Pratap, J., Javed, A., Weiner, B., Ohkawa, Y., van Wijnen, A., Montecino, M., Stein, G. S., Stein, J. L., Imbalzano, A. N., and Lian, J. B. (2005) *J. Cell. Biochem.* **94**, 720-730

54. Villagra, A., Cruzat, F., Carvallo, L., Paredes, R., Olate, J., van Wijnen, A. J., Stein, G. S., Lian, J. B., Stein, J. L., Imbalzano, A. N., and Montecino, M. (2006) *J. Biol. Chem.* **281**, 22695-22706

55. Flowers, S., Nagl, N. G., Jr, Beck, G. R., Jr, and Moran, E. (2009) *J. Biol. Chem.* **284**, 10067-10075

56. Mujtaba, S., Zeng, L., and Zhou, M. M. (2007) *Oncogene* **26**, 5521-5527
FOOTNOTES

We thank Wyeth for providing BMP-2 and Drs F. Cimino, B. de Crombrugghe, P. Muñoz-Cánoves and K. Watanabe for reagents. We also thank E. Adanero, E. Castaño and B. Torrejón for technical assistance. M.J. Ortuno, E. Rodriguez-Carballo and A. Garcia-Susperregui are recipients of fellowships from University of Barcelona, IDIBEL, and the MEC respectively. This research was supported by grants from the MEC (BFU2008-02010), ISCIII (RETIC RD06/0020).

FIGURE LEGENDS

Figure 1.- Expression of two isoforms of Osterix by BMP-2 in mesenchymal and osteogenic cells. (A & B) Cells were treated with BMP-2 in media without serum for 24h. (A) Osx and tubulin were detected by immunoblotting. (B) Both short (S) and long (L) Osx mRNA were detected by PCR from cells treated with 2 nM BMP-2 for 24 hours using specific forward primers (short: 5’-cacccattgccagtaatcttcaagcca; long: 5’-ctcggaggatggcgtcctctctgcttg) and a common reverse primer ( 5´-ggactgcctgcaggagagagga) (C) Subcellular localization of Osx in C2C12 cells was analyzed by immunofluorescence with anti-Osx antibody in cells either transiently transfected with short Osx construct (Osx) or incubated in media without serum and treated with BMP-2 for 24h. Bar, 10μm.

Figure 2.- Fmod and Ibsp expression is induced by Osx. (A) Cells were transiently transfected with mock or Osx construct overnight and incubated in media without serum for 24h. Fmod and Ibsp mRNA were measured by RT-qPCR, normalized to GAPDH and expressed as relative expression +/- S.E.M. of four independent experiments. Expression of the different constructs was analyzed by immunoblotting (data not shown). (B) Confluent cells were incubated in media without serum overnight and treated with BMP-2 for the indicated times. Osx, Fmod and Ibsp mRNA were measured by RT-qPCR, normalized to GAPDH and plotted as relative expression to time zero +/- S.E.M. of three independent experiments (***p<0.001 compared with mock condition, two-way ANOVA followed by Bonferroni’s test).

Figure 3.- Fmod and Ibsp transcriptional activation by Osx and BMP-2. (A) Cells were co-transfected with 2-kb pFmod-Luc reporter vector and either mock or Osx construct overnight and treated with or without BMP-2 in media without serum media for 24h. (B) Cells were transfected with 2-kb pFmod-Luc or pFmod300-Luc reporter vectors overnight and treated with BMP-2 in media without serum for 24h. (C) Cells were co-transfected with pIbsp-Luc reporter vector and either mock or Osx construct overnight and treated with or without BMP-2 in media without serum for 24h. (A, B & C) Luciferase activity was measured and normalized against β-galactosidase activity. Relative luciferase activities were expressed as mean ± S.E.M. for triplicates from five independent experiments (*p<0.05, **p<0.01, ***p<0.001; one-way ANOVA followed by Bonferroni’s multiple comparison test).

Figure 4.- Osx interaction with Sp1 regions in regulatory sites of Fmod and Ibsp genes. (A) (Upper panel) Sp1 regions analyzed by oligo pull-down. Biotinylated oligonucleotides sequences used: F-1 5’- taggaatttggggcgggaccctgt-biot; C-1 5’- ggaacagagggagggagggag-biot; C-2 5’-caggagtcgctctctctctcc-biot; C-2 mut 5’- caggagttatttgttctctctcc-biot. (Lower panel) The indicated double-stranded biotinylated oligonucleotides were incubated with equal aliquots of extracts from C2C12 cells either transfected with a Osx construct using mock as control or treated without (cto) or with BMP-2 in media without serum for 24h. Precipitated complexes were analyzed by immunoblotting using Osx antibody. (B) (Upper panel) Promoter regions analyzed by chromatin immunoprecipitation. (Lower panels) Saos-2 cells or human primary osteoblasts (HOB) were treated with BMP-2 for 24h in media without serum. Cells were fixed with formaldehyde and chromatin immunoprecipitation analysis was performed by incubating DNA-protein complexes using the indicated antibodies and IgG as a control. Quantification of the results of two independent experiments is shown below each panel as mean +/-S.E.M.
Figure 5.- Osx phosphorylation by p38. (A) MC3T3-E1 cells were treated with or without BMP-2 in media without serum. C2C12 cells were transfected with empty vector, short or long Osx constructs. After 24h, cells extracts were incubated with alkaline phosphatase for 1h. Osx forms were detected by immunoblotting (B) Cells were treated with or without BMP-2 in media without serum for 24h and pretreated with SB203580 (SB) for 30 min where indicated. Osx and tubulin were detected by immunoblotting. (C) C2C12 cells were co-transfected with constitutive active MKK6 (MKK6EE) and short or long Osx constructs. After 24h, cells were treated with SB for 3h. Osx, MKK6 and tubulin were detected by immunoblotting. (D) Wild type (wt) and p38α-/- MEF cells were co-transfected with MKK6 construct and the short Osx construct. After 24h, cells were treated with SB for 3h. Osx, MKK6 and tubulin were detected by immunoblotting. Arrows indicate phosphorylated forms.

Figure 6.- Phosphorylation of Osx at Ser 73 and Ser 77 by p38. (A) C2C12 cells were co-transfected with constitutive active MKK6 (MKK6EE) and the different Osx constructs as indicated using an empty vector as control. (B) 5μg of GST-Osx wild type or the S73/77A mutant were incubated with activated p38 MAPK (200ng for each condition) in presence of γ-[^32P]ATP, visualized by SDS-PAGE and autoradiography (left panel) or visualized by immunoblotting against Osx or phospho-serine (right panel). (C) Wild type (wt) and p38α-/- MEF cells were co-transfected with MKK6EE and Osx constructs as indicated. After 24h, cells were treated with cycloheximide for the indicated times. Osx was detected by immunoblotting. Quantification of the results of three independent experiments is shown below each panel as mean +/-S.E.M.

Figure 7.- Induction of Osx transcriptional activity by p38 phosphorylation. (A) C2C12 cells were transfected with either 2-kb pFmod-Luc or plbsp-Luc reporter vectors overnight, treated with or without BMP-2 in media without serum for 24h and pretreated with SB203580 (SB) for 30 min when indicated. Luciferase activity was measured and normalized against β-galactosidase activity. Relative luciferase activities were expressed as mean ± S.E.M. for triplicates from four independent experiments (**p<0.01; one-way ANOVA followed by Bonferroni’s multiple comparison test). (B) BM-MSCs were cultured for 4 days and treated at a confluent state at days 0 and 3 with BMP2 (2 nM) and/or SB203580 during the last 24 hours in osteogenic differentiating medium. Fmod and Ibsp mRNA were measured by RT-qPCR, normalized to GAPDH and expressed as relative expression +/- S.E.M. of three independent experiments (**p<0.01; two-way ANOVA followed by Bonferroni’s multiple comparison test). (C) C2C12 cells were co-transfected with 2-kb pFmod-Luc or plbsp-Luc vectors and mock, wild type (wt) or double mutant (mut) Osx constructs and treated with or without BMP-2 in media without serum for 24h. Luciferase activity was measured and expressed as above. (*p<0.05, **p<0.01; two-way ANOVA followed by Bonferroni’s multiple comparison test). (D) Cells were transfected with mock, wild type (wt) or double mutant (mut) Osx constructs and treated with or without BMP-2 in media without serum for 24h. Luciferase activity was measured and expressed as above. (*p<0.05, **p<0.01; two-way ANOVA followed by Bonferroni’s multiple comparison test). (E) Cells were transfected with mock or Flag-tagged wild type Osx. After 24h, cells were treated with BMP and/or SB203580 (SB) for 3h as indicated. Expression of the constructs and anti-Flag co-immunoprecipitated proteins were analyzed by immunoblotting.

Figure 8.- p300 and Brg1 recruitment induced by Osx phosphorylation. (A) C2C12 cells were transfected with mock, wild type (wt) or double mutant (mut) Osx constructs for 24h. Cells were fixed with formaldehyde and chromatin immunoprecipitation analysis was performed by incubating DNA-protein complexes using the indicated antibodies. IgG was used as a control. Quantification of the results of three independent experiments is shown below each panel as mean +/-S.E.M. Expression of the constructs was analyzed by immunoblotting. (B) C2C12 cells were transfected with mock, Flag-tagged wild type (wt) or double mutant (mut) Osx constructs and constitutive active MKK6 (MKK6EE) as indicated. After 24h, cells were treated with SB203580 (SB) for 3h as indicated. Expression of the constructs and anti-Flag co-immunoprecipitated proteins were analyzed by immunoblotting. (C) C2C12 cells were transfected with mock or Flag-tagged wild type Osx. After 24h, cells were treated with BMP and/or SB203580 (SB) for 3h as indicated. Expression of the constructs and anti-Flag co-immunoprecipitated proteins were analyzed by immunoblotting.
Figure 2

A

C2C12 cells

MC3T3 cells

Relative mRNA expression

Fmod

Ibsp

***

***

B

C2C12 cells

MC3T3 cells

Relative mRNA expression

Osx

Fmod

Ibsp

hours

0 4 8 16 24

0 4 8 16 24
Figure 3

A

Fmod

Luciferase activity (fold induction)

Osx - + - + +
BMP-2 - - + + +

C

Ibsp

Luciferase activity (fold induction)

- + - + +

B

-2032

Sp1 Sp1

Fmod

Luciferase activity (fold induction)

-192

Sp1 Sp1
Figure 5

A

MC3T3 cells
BMP-2
ALP
anti-Osx
C2C12 cells
short
ALP
anti-Osx
C2C12 cells
long
ALP
anti-Osx

B

C2C12 cells
BMP-2
SB
anti-Osx
anti-tubulin
MC3T3 cells
BMP-2
SB
anti-Osx
anti-tubulin
HOB cells
BMP-2
SB
anti-Osx
anti-MKK6
anti-tubulin

C

mock       short Osx       M KK6       long Osx
-          -           -       -          -
-          -           -       -          -
-          -           +       +          +
-          -           +       +          +

D

wt MEF cells       p38α −/− MEF cells
Osx
MKK6
SB
anti-Osx
anti-MKK6
anti-tubulin
-          -           -          +
-          -           -          +
-          -           -          +
-          -           -          +

+          +           +          +
+          +           +          +
+          +           +          +
+          +           +          +
Figure 6

A

|        | mock | long | short | S7773A | S73A | S77A |
|--------|------|------|-------|--------|------|------|
| MKK6   |      |      | +     |        |      |      |
| anti-Osx |     |      |      | -      |      |      |

B

|        | GST | GST-Osx wt | GST-Osx mut |
|--------|-----|------------|-------------|
| p38*  | -   | +          |             |
| anti-Osx |     |            |             |
| anti-pSer |     |            |             |

C

| wt MEF cells | p38α−/− MEF cells |
|--------------|-------------------|
| hours        |                   |
| 0            |                   |
| 2            |                   |
| 4            |                   |
| 8            |                   |
| CHX          |                   |
| Osx          |                   |
| up           |                   |
| 100          |                   |
| 71 ± 5       |                   |
| 47 ± 7       |                   |
| 36 ± 4       |                   |
| low          |                   |
| 100          |                   |
| 70 ± 7       |                   |
| 56 ± 3       |                   |
| 36 ± 6       |                   |
| Osx/MKK6     |                   |
| up           |                   |
| 100          |                   |
| 66 ± 6       |                   |
| 52 ± 7       |                   |
| 33 ± 2       |                   |
| low          |                   |
| 100          |                   |
| 71 ± 12      |                   |
| 49 ± 13      |                   |
| 31 ± 9       |                   |
**Figure 8**

A

|       | Fmod          | lbsp          |
|-------|---------------|---------------|
|       | anti-RNA Pol II |               |
| Osx   | mock          | mock          |
|       | wt            | wt            |
|       | mut           | mut           |
|       | 1.8 ± 0.1     | 2.7 ± 0.3     |
|       | 1.1 ± 0.1     | 1.7 ± 0.3     |
| anti-Osx |               |               |
|       | 1.8 ± 0.3     | 1.3 ± 0.1     |
| anti-p300 |               |               |
|       | 1.4 ± 0.1     | 1.3 ± 0.1     |
|       | 1.3 ± 0.2     | 1.0 ± 0.1     |
| anti-Brg1 |               |               |
|       | 1.1 ± 0.1     | 1.2 ± 0.1     |
|       | 0.7 ± 0.1     | 0.6 ± 0.1     |
| anti-IgG |               |               |
| INPUT |               |               |
| mock  |               |               |
| wt    |               |               |
| mut   |               |               |
|       |               | anti-Osx      |

B

|       | mock | Flag-Osx wt | Flag-Osx mut |
|-------|------|-------------|--------------|
| MKK6  | -    | -           | +            |
| SB    | -    | -           | +            |
|       | -    | +           | +            |
| anti-Brg1 |     |              |              |
| anti-p300 |     |              |              |
| anti-Osx |     |              |              |
| anti-tubulin | |              |              |
|       |     | IP- Flag    |              |
|       |     | extracts    |              |

C

|       | mock | Flag-Osx |
|-------|------|----------|
| BMP2  | -    | +        |
| SB    | -    | +        |
| anti-Brg1 |     |          |
| anti-p300 |     |          |
| anti-pSer |     |          |
| anti-Osx |     |          |
| anti-tubulin | |          |
|       |     | IP- Flag |
|       |     | extracts |
p38 regulates expression of osteoblast-specific genes by phosphorylation of Osterix
Maria Jose Ortuno, Silvia Ruiz-Gaspa, Edgardo Rodriguez-Carballo, Antonio R. G. Susperregui, Ramon Bartrons, Jose Luis Rosa and Francesc Ventura

J. Biol. Chem. published online August 3, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.123612

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/08/03/M110.123612.DC1