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ARTICLES

Osx Transcriptional Regulation Is Mediated by Additional Pathways to BMP2/Smad Signaling

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Abstract Bone morphogenetic protein (BMP)-2 induces Osterix (*Osx*) in mouse C2C12 cells and chondrocytes. Genetic studies place *Osx* downstream to the BMP-2/Smad/Runx2 signaling pathway; however, limited information is available on the mediators of *Osx* expression in osteoblast lineage commitment. Several lines of research implicate the presence of Runx2-independent ossification. Therefore, the purpose of this study was to identify possible mediators of *Osx* expression beyond the BMP-2/Smad pathway. Using real-time RT-PCR, we showed upregulation of *Osx* in response to BMP-2 in human mesenchymal stem cells (hMSC). Insulin-like growth factor (IGF)-I upregulated *Osx*, but not *Runx2*. Further, IGF-I in combination with BMP-2 was synergistic for *Osx*, suggesting a pathway beyond Smad signaling. MAPK was tested as a common mediator across BMP-2 and IGF-I signaling pathways. Inhibition of MAPK component ERK1/2 did not affect *Runx2* gene expression, but inhibited *Osx* expression and matrix mineralization. BMP-2-mediated *Osx* expression was downregulated in response to p38 inhibition. We therefore conclude that during osteogenic lineage progression, in addition to the BMP-2/Smad pathway, IGF-I and MAPK signaling may mediate *Osx*. J. Cell. Biochem. 95: 518–528, 2005.

Key words: BMP-2; IGF-I; human mesenchymal stem cells; MAPK; Osx; Runx2; real-time RT-PCR

Osterix (Osx) is a transcription factor that was identified in a subtractive hybridization screen in extracts of C2C12 cells treated with bone morphogenetic protein-2 (BMP-2) [Nakashima et al., 2002]. The *Osx* mouse homologue is a 428 amino acid polypeptide with a molecular weight of 46 kDa [Nakashima et al., 2002]. Milona et al. [2003] recently isolated two cDNAs for human homologues of *Osx*, referred to as Sp7 alpha and beta isoforms. Sp7 is a member of the Sp subgroup of the Kruppel-like family of transcription factors characterized by three zinc-finger DNA binding domain at the carboxy-terminus of the protein. The SP proteins have no significant homology outside the zinc-finger DNA-binding domain and may contain glutamine-rich or serine/threonine-rich central activation domains [Gollner et al., 2001].

Osx-deficient mice do not form a bony skeleton, forming only cartilage [Nakashima et al., 2002] similar to the phenotype observed in *Runx2*-deficient mice [Komori et al., 1997]. There are important differences in *Runx2*- and *Osx*-null mutants that may be due to inhibition of hypertrophic chondrocyte differentiation in *Runx2* mutants [Komori et al., 1997; Otto et al., 1997]. *Runx2*-null mice display a marked decrease in vascular endothelial growth factor (VEGF) expression, which may prevent blood vessel invasion in the cartilage matrix [Zelzer et al., 2001]. In *Osx*-null mutant mice, the transcripts of the osteoblast markers, osteonectin, osteopontin, osteocalcin, and bone sialoprotein are not expressed, but *Runx2* is expressed

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at wild-type osteoblast levels [Nakashima et al., 2002]. However, *Runx2*-knockout mice lack *Osx* expression [Nakashima et al., 2002], suggesting that *Osx* lies downstream of *Runx2* in the pathway of osteoblast differentiation. *Osx*-null pre-osteoblasts that lack osteoblast markers, express marker genes characteristic of chondrocytes, implicating pre-osteoblasts may be capable of differentiation into either osteoblasts or chondrocytes. Therefore, a hypothetical role for *Osx* may involve segregation of osteoblast and chondrocyte lineages where *Runx2* is expressed by bi-potential precursor cells which express *Osx* to suppress chondrocyte lineage [Nakashima and de Crombrugghe, 2003].

Recently, several lines of research suggested the possibility of a *Runx2*-independent ossification program. First, the Wnt signaling pathway, proceeding via the low density lipoprotein receptor-related protein (LRP)-5 receptor, may control ossification independent of *Runx2* [Kato et al., 2002]. Second, *Msx2*-dependent vascular ossification may proceed via a pathway distinct from *Runx2* [Cheng et al., 2003]. Finally, *Dlx5*, a homeo-box transcription factor, acts downstream of BMP-2 and induces *Osx* expression independent of *Runx2* [Lee et al., 2003]. These studies suggest additional pathways may act in parallel to, or independent of *Runx2* to regulate *Osx* expression during osteogenic lineage progression.

BMPs (BMP-2, 4, 7) have been implicated in osteoblast differentiation and bone formation [Bitgood and McMahon, 1995; Rosen et al., 1996; Issack and DiCesare, 2003; Chen et al., 2004]. BMPs are members of the TGF-β superfamily and are highly conserved with sequence homology across species [Von Bubnoff and Cho, 2001]. BMPs play critical roles in embryonic development, including mesoderm patterning, craniofacial development, osteogenesis, chondrogenesis, and organogenesis [Hogan, 1996]. Only BMP-2 has been reported to upregulate *Osx* expression [Nakashima et al., 2002; Yagi et al., 2003]; however, other BMP members have not been evaluated.

At the cellular level, BMPs bind to serine-threonine transmembrane receptors and initiate a cascade of phosphorylation events that transduce the signal to downstream genes [Dijke et al., 2003]. BMPs signal by phosphorylation of Smad molecules that translocate to the nucleus when activated and regulate the transcription of specific target genes. BMP-2/Smad signaling is a known mediator of *Runx2* expression [Lee et al., 2000, 2002] is likely a target gene of BMP-2/Smad signaling. However, alternate pathways may exist for eliciting the BMP-2 signal. Binding of the ligand to a preformed receptor complex initiates the Smad pathway. A second pathway, which is Smad-independent, is activated via recruitment of the type II BMP receptor by the type I receptor [Hassel et al., 2003]. This mode of signaling involves p38, a component of mitogen activated protein kinase (MAPK).

MAPK signaling acts as a common component in mediating the effects of insulin-like growth factor-I (IGF-I) [Bliziotes et al., 2000; Fruchtman et al., 2001] and BMP-2 [Nohe et al., 2002], as well as extracellular matrix (ECM) components [Xiao et al., 2002]. Smad signaling is restricted to TGF-β superfamily members [Massague, 1998; Wrana, 2000]. MAPK also mediates the effects of fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and BMP signals during vertebrate development [Kretzschmar et al., 1997; Pera et al., 2003]. Therefore, it is possible that BMP-2 signaling for *Osx* may involve components of the MAPK signaling cascade.

In this study, we address the hypothesis that alternate signaling pathways mediate *Osx* expression in addition to the BMP-2/Smad signaling. Current literature reports *Osx* as an osteoblast marker gene, but limited information is available on *Osx* regulation. Our studies in human adult mesenchymal stem cells (hMSC) indicate that BMP-2 and IGF-I synergistically induce *Osx*. This study is the first report that MAPK signaling components are required for *Osx* expression as hMSC progress into the osteoblast lineage.

MATERIALS AND METHODS

Cell Cultures

Bone marrow derived hMSC were obtained from BioWhittaker, Inc. (Walkersville, MD) and maintained according to manufacturer's instructions. hMSC were certified positive for adipogenic, chondrogenic, and osteogenic potential assays by Biowhittaker, Inc. hMSC were treated with recombinant human (rh)BMP-2 (100 ng/ml) (Wyeth, Madison, NJ), FGF-2 (50 ng/ml) (Peprotech, Rockyhill, NJ), IGF-I (200 ng/ml), and platelet derived-growth factor (PGDF)-BB (100 ng/ml) (Chiron, Emeryville,

CA), transforming growth factor (TGF)-β1 (2 ng/ml) (RDI, Flanders, NJ) for 48 h. Growth factors doses reported here were optimal for signal transduction in hMSC based on manufacturer's recommendations, unpublished data from the authors and published literature [Chaudhary and Avioli, 1997; Gori et al., 1999; Thomas et al., 1999; Lee et al., 2000].

Constructs

Adenoviral constructs for dominant negative ras and p38 were kindly provided by Dr. Ronald Kahn [Ueki et al., 1998]. Adenoviral dominant negative erk 2 construct was a kind gift from Dr. Louis Parada [Klesse et al., 1999] and adenoviral lacZ was a gift from Dr. Paul D. Robbins [Nishida et al., 1998].

Adenoviral Delivery of LacZ, DNErk, DNp38, and DNRas

hMSC were seeded at a density of 300,000 cells/cm^2 and allowed to attach overnight. Cells were washed three times with sterile 1× PBS. Following washes, cells were transduced with adenoviral vectors at MOI 100. Cells were exposed to the virus for 4 h, at 37°C, 5% CO_2 and humidity. Transductions were terminated by addition of complete osteogenic stem cell medium containing mesenchymal cell growth supplement, L-glutamine and penicillin/streptomycin (Biowhittaker, Inc.) was supplemented with 50 μg/ml L-ascorbic acid phosphate, 10 mM β-glycerol phosphate, and 100 nM dexamethasone (Biowhittaker, Inc.) for histochemical staining experiments. For gene expression studies, dexamethasone was excluded and the osteogenic supplement contained 10 nM 1,25-$(OH)_2$Vitamin D_3 (Biomol, Plymouth Meeting, PA) with or without 100 ng/ml rhBMP-2 for the final 48 h of culture.

RNA Extraction and Quantification

Total RNA was extracted using the RNeasy Kit and DNase I treatment according to the manufacturer's protocol (Qiagen, Valencia, CA). The amount of extracted RNA was quantified using the RiboGreen RNA Quantification Kit (Molecular Probes, Eugene, OR), a fluorescence-based solution assay. After addition of working reagent to RNA samples, total RNA content was photometrically analyzed with a Tecan Spectrafluor platereader (Research Triangle Park, NC) with excitation at 485 nm and emission at 595 nm. RNA concentrations were calculated based on a standard curve of ribosomal RNA (rRNA).

Real-Time RT-PCR Analysis

After extraction and quantification of RNA, real-time RT-PCR reactions were performed using One-Step Taqman® RT-PCR Master Mix, primers and probes (Applied Biosystems, Foster City, CA). Eighty nanograms of total RNA were used per 10 μl reaction with Taqman® One-step RT-PCR Master Mix, sequence specific primers (50–200 nM), and Taqman® probes (100 nM). Real-time RT-PCR assays were carried out in triplicate using an ABI Prism 7900 Sequence Detection System. The human primer sequences were as follows: osteocalcin (Genebank #NM 000711) forward-AGCAAAGGTGCAGCCTTTGT, reverse-GCGCCTGGGTCTCTTCACT and Taqman® probe-CCTCGCTGCCCTCCTGCTTGG; *Runx2* (Genebank #NM 004348) forward-AACCCACGAATGCACTATCCA, reverse-CGGACATACCGAG GGACATG and Taqman® probe-CCTTTACTTACACCCCGCCAGTCACCTC; *Osx* (Genebank #AF477981) forward-CCCCACCTCTTG- CAACCA, reverse-CCTTCT AGCTGCCCACTATTTCC and Taqman® probe-CCAGCATGTCTTGCCCCAA GATGTCTA; bone/liver/kidney *Alp* (Genebank #XM 001826) forward-CCGTGGC AACTCTATCTTTGG, reverse-GC- CATACAGGATGGCAGTGA and Taqman® probe CATGCTGAGTGACACAGACAAGAAG- CCC. The mouse primer sequences were as follows: osteocalcin (Genebank #NM 007541) forward-CCGGGAGCAG TGTGAGCTTA, reverse-AGGCGGTCTTCAAGCCATACT and Taqman® probe-CCCTGCTTGTGACGAGCTATCAG; *Runx2* (Genebank #NM 009820) forward-AAATGCCTCCGCTGTTATGAA, reverse-GCTCCGGCCCACAAATCT and Taqman® probe-AACCAAGTAGCCAGGTTCAACGATCT; *Osx* (Genebank #NM 130458) forward-CCCTTCTCAAGCACCAATGG, reverse-AGGGTGGGTAGTCATT TGCATAG and Taqman® probe-CAGGCAGTCCTCCGGCCCC. Osteocalcin, *Runx2*, *Alp*, and *Osx* probes were labeled with a 5′ FAM reporter dye and 3′ TAMRA quencher dye. *18S* primers and probes were designed by Applied Biosystems. *18S* probe was labeled with a 5′ VIC reporter dye and 3′ TAMRA quencher dye.

Thermocycling conditions were as follows: 48°C for 30 min (reverse transcription), 95°C for 10 min (initial denaturation) followed by 40 cycles at 95°C for 15 s (denaturation) and 60°C

for 45 s (annealing and extension). Following completion of the real-time RT-PCR reactions, the threshold was set above the non-template control within the linear phase of target gene amplification to calculate the cycle number at which the transcript is detected (denoted C_T).

Target gene expression were normalized to the reference housekeeping gene *18S*. Validation experiments conducted previously demonstrated that efficiencies of target and reference gene were approximately equal with the absolute value of the slope of log input amount versus $\Delta C_T < 0.1$. Gene expression changes within the control groups were calculated by the ΔC_T method to determine total RNA signal detected. To calculate fold difference from controls, the comparative $\Delta\Delta C_T$ method was used as detailed in Applied Biosystems Bulletin #2 [Biosystems, 2001]. Fold differences were calculated for each treatment group using normalized C_T values of control as the calibrator. For real-time RT-PCR analysis, a C_T value above 36 was considered to be non-specific and given a zero value for statistical analysis.

Alizarin Red Staining

hMSC were seeded at a density of 300,000 cells/cm^2 and treated with MAPK inhibitors or adenoviral MAPK constructs in complete osteogenic stem cell medium supplemented with L-ascorbic acid phosphate, β-glycerol phosphate, and dexamethasone (Biowhittaker, Inc.) for 6 days. The cells were then treated with MAPK inhibitors U0126, U0124 (U0126 analog), SB203580, SB202474 (SB203580 analog) (Calbiochem, San Diego, CA) for an additional 15 days. The analogs and the control (DMSO) treatment gave the same results. Consequently, results are reported in comparison to the DMSO (control) treatment. To stain for calcium deposition, cells were fixed with 70% ethanol, rinsed five times with deionized water, treated 10 min with Alizarin red stain at pH 4.2 (Sigma, St. Louis, MO) and then washed with 1× PBS for 15 min with gentle agitation.

Alkaline Phosphatase (ALP) Activity

Cell lysates were centrifuged at 5,000*g* for 5 min, collected in 1× PBS and freeze-thawed once. Ten microliters of cell lysate was subjected to an ALP activity assay at 25°C. ALP activity was calculated according to the manufacturer's specifications (Sigma, Inc.). Units of ALP activity were normalized to milligrams of total protein content with BCA assay (Pierce, Rockford, IL) of the cell lysate.

Western Blot Analysis

Cell extracts (25 μg of protein) were subjected to SDS–PAGE and transblotted to Immobilon P membranes (Millipore, MA). The membranes were incubated with the following primary antibodies: phospho-p38 (1:200), p38 (1:500) (Santa Cruz, Santa Cruz, CA), Erk (1:1,000), phospho-Erk (1:2,000) (New England Biolabs, Beverly, MA) in dry milk containing 0.01% Tween-20-TBS. Membranes were washed and incubated with horse-radish peroxidase-conjugated secondary antibodies anti-mouse IgM (1:5,000) (Pierce), anti-rabbit IgG (1:10,000) (Sigma) for 1 h. Following washes in Tween-TBS, the membranes were developed with ECL Plus reagents according to manufacturer's instructions (Amersham, Sunnyvale, CA) and exposed to Kodak Biomax film (Kodak, New Haven, CT).

Statistical Analysis

For real-time RT-PCR assays, the coefficient of variation was calculated from three assay replicates and did not exceed 3% for all treatment groups. Intra-day variation did not exceed 5%. Treatment groups within experiments were performed in triplicate and reported as mean ± standard error of the mean (SEM). Each experiment was repeated a total of three times. Statistical analysis was performed using Statview software (Cary, NC) to determine significance among treatment groups. ANOVA followed by Tukey–Kramer post hoc test was performed for the experiments unless otherwise indicated. Student's *t*-test (two-tailed) was utilized for pair-wise comparisons. A *P*-value of ≤ 0.05 was considered significant.

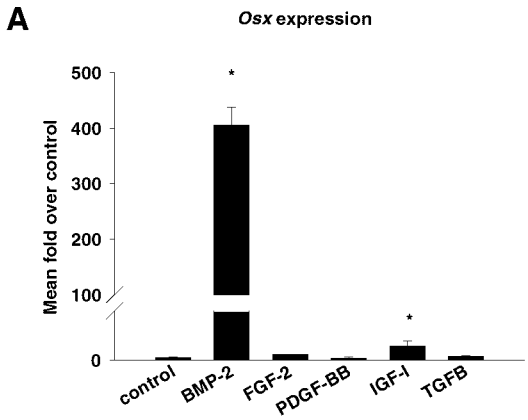
RESULTS

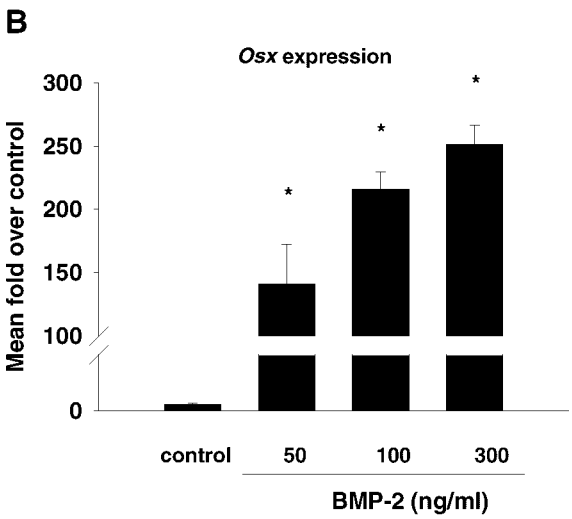
Effect of Bone-Related Growth Factors on *Osx* Expression

BMP-2 promoted a 400-fold increase in *Osx* expression ($P < 0.0001$) 72 h post-treatment (Fig. 1A) and the effect was dose-responsive (Fig. 1B) and time-dependent (Fig. 1C). Treatment with IGF-I induced a 2.5-fold increase in *Osx* expression over the control treatment ($P = 0.0431$). Further, BMP-2 and IGF-I had a synergistic effect on the induction of *Osx* at 277-fold over control ($P < 0.0001$), with the BMP-2

induction at 180-fold 48 h post-treatment (Fig. 2). IGF-I alone did not have an effect on *Runx2* ($P = 0.5074$), also reported by Thomas et al. [1999]. Whether IGF-I signal for *Osx* requires *Runx2* will need to be addressed in future studies.

Growth factor synergy has been reported for BMP-7 and IGF-I [Yeh et al., 1997; Shoba and Lee, 2003], as well as BMP-2 and IGF-I [Raiche and Puleo, 2004] for ALP activity and calcium deposition. BMP-2 and IGF-I synergy may be due to the convergence of downstream signaling components such as MAPK.





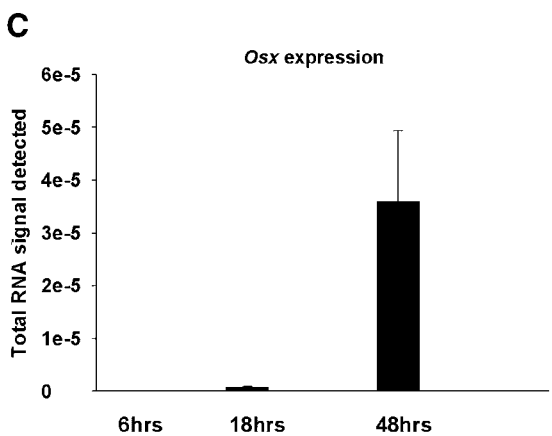


Fig. 1. Osterix (*Osx*) gene expression in response to various growth factors. hMSC were treated with 100 ng/ml rhBMP-2, 50 ng/ml rhFGF-2, 2 ng/ml rhTGF-β1, 200 ng/ml rhIGF-I, and 100 ng/ml rhPDGF-BB for 72 h in basal stem cell media. Total RNA was extracted and real-time RT-PCR analysis for *Osx* gene expression performed. **A**: *Osx* is a specific target of BMP-2 and IGF-I. **B**: The effect of BMP-2 was dose-dependent (48 h). **C**: BMP-2 induced *Osx* expression in hMSC as early as 18 h post-treatment and there was a 44-fold increase over the next 30 h. *Osx* expression is reported as total RNA signal detected. ND, gene expression not detected, a zero value was assigned for statistical analysis (see ''Materials and Methods''). Pairwise comparisons with the control treatment were conducted using Student's *t*-test. Mean of three independent cultures ± SEM. *Significant from control, $P < 0.05$.

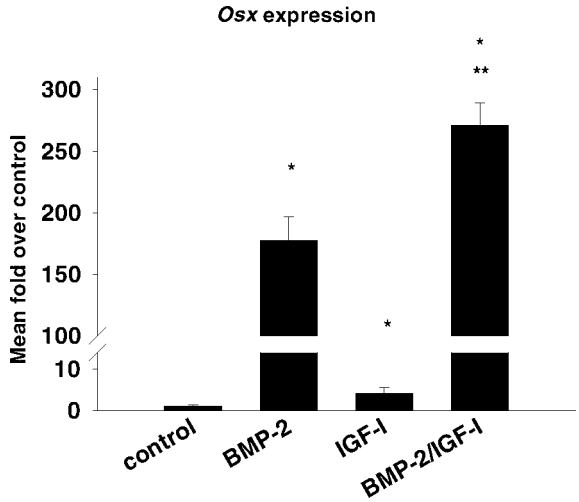


Fig. 2. BMP-2 and IGF-I synergistically interact to induce *Osx* gene expression. hMSC were treated with 100 ng/ml rhBMP-2 alone, 200 ng/ml rhIGF-I alone or 100 ng/ml rhBMP-2 and 200 ng/ml rhIGF-I for 48 h. *Osx* expression was analyzed. Pairwise comparisons with the control treatment were conducted with Student's *t*-test. *Significant from control, $P < 0.05$. Mean of three independent cultures ±SEM. **Significant from the additive individual values of BMP-2 and IGF-I, $P < 0.05$.

MAPK Pathway may be Essential for hMSC Differentiation Into Osteoblast Lineage and the Induction of *Osx*

MAPK signaling is involved in cell proliferation and differentiation [Cobb, 1999; Widmann et al., 1999; Chang and Karin, 2001]. MAPK regulates the expression of alkaline phosphatase and the deposition of bone matrix proteins

[Chaudhary and Avioli, 1997; Cobb, 1999; Lai et al., 2001]. MAPK signaling components p38 and Erk1/2 were activated in response to BMP-2 treatment in Western blot analysis (Fig. 3A,B).

Chemical inhibitors U0126 and SB203580 were used to block specific MAPK components, ERK1/2 and p38, respectively. Treatment with Erk1/2 inhibitor abolished matrix mineralization, while p38 inhibitor did not have an effect

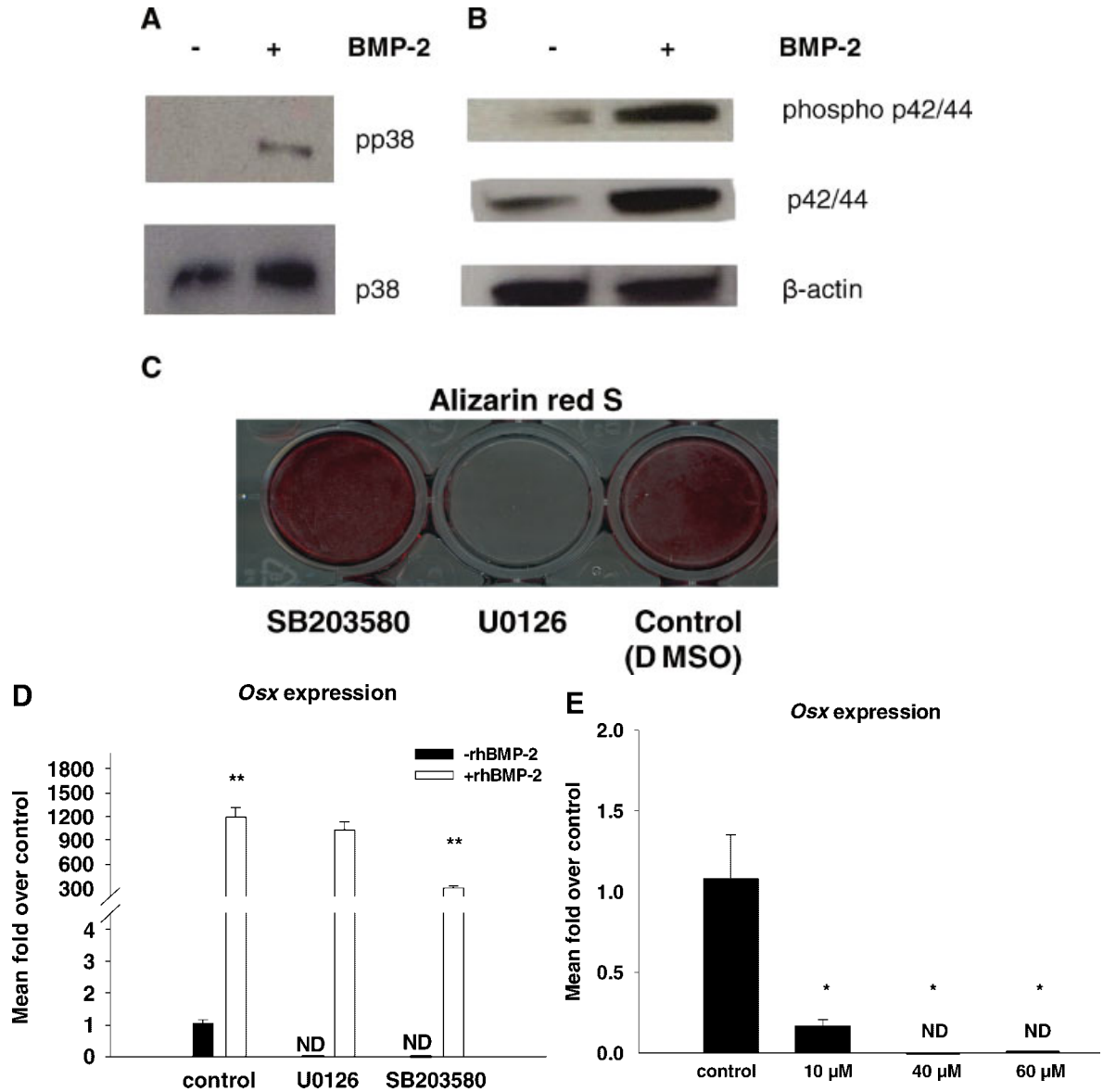


Fig. 3. Response to MAPK inhibition in hMSC. hMSC were treated with or without BMP-2 (100 ng/ml) for 1 h. Cell lysates were subjected to Western blot analysis and (**A**) probed for the active p38 using antibody against phosphorylated p38 (p-p38) or for total p38 with total-p38 (p38) antibody (**B**) probed for the active Erk1/2 using antibody against phosphorylated Erk1/2 (p-p42/p-p44) or for total Erk1/2 with total-Erk1/2 (p42/p44) antibody. Membranes were stripped and reprobed with anti-actin to confirm the nearly equal loading of the proteins. **C**: Scanned images of fixed cells stained with Alizarin red S for calcium deposition. hMSC were treated with 10 μM SB203580 or 40 μM U0126 or control (DMSO) and cultured in osteogenic stem cell media supplemented with L-ascorbic acid-phosphate, β-glycerophosphate and dexamethasone for 21 days. **D**: hMSC were cultured under osteogenic conditions for 6 days prior to treatment with MAPK inhibitors for 3 days. *Osx* gene expression analyzed. ND, gene expression not detected, a zero value was assigned for statistical analysis. *Significant from control (DMSO) without BMP-2 treatment, $P < 0.05$. **Significant from control with BMP-2 treatment, $P < 0.05$. Mean of three independent cultures ±SEM. **E**: hMSC were treated for 3 days with different doses of U0126 in osteogenic stem cell media without BMP-2. ND, gene expression not detected, a zero value was assigned for statistical analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

on mineralization (Fig. 3C). Mineralization requires the control of osteoblast gene expression. Consequently, U0126 and SB203580 were used to determine the effect of MAPK inhibition on osteoblast gene markers. Treatment with the analogs of the MAPK-specific inhibitors was equivalent to DMSO treatment.

For gene expression studies, hMSC were maintained in osteogenic stem cell medium for 6 days to promote extracellular matrix synthesis and osteoblastic lineage progression. The cells were treated with MAPK inhibitors U0126 (40 μM) and SB203580 (10 μM) for an additional 72 h. *Osx* expression was abolished in response to a block in p38 and Erk1/2 signaling ($P < 0.0001$) (Fig. 3D). BMP-2 mediated induction of *Osx* was significantly downregulated due to inhibition of p38 signaling ($P < 0.0001$) (Fig. 3D). The inhibitory effect of U0126 on *Osx* was dose-responsive (Fig. 3E).

The results from the MAPK inhibition experiments with chemical inhibitors were further confirmed via a genetic approach. A dominant negative adenoviral construct for p38 (DNp38) blocked p38 activation and inhibited *Osx* expression ($P < 0.0001$), mineralization still occurred (Fig. 4A,B). Blocking Erk1/2 or Ras signaling with dominant negative Erk (DNErk) or Ras (DNRas) inhibited *Osx* expression and blocked hMSC mineralization (Fig. 4A,B). Ras can be activated in response to growth factors such as BMP-2 and in turn can activate MAPK signaling [Lai and Cheng, 2002]. The results of the adenoviral MAPK delivery correlate with the chemical inhibitor results.

BMP-2 signaling and MAPK may be associated with osteoblast marker gene expression upstream of, or in parallel to *Osx* expression, therefore, we studied *Alp* and *Runx2* expression in response to MAPK inhibition. BMP-2 induced *Alp* expression was inhibited by SB203580 treatment ($P < 0.0001$) (Fig. 5A). ALP enzymatic activity was also inhibited due to SB203580 (Fig. 5B), similar to the results of the gene expression analysis (Fig. 5A). BMP-2 mediated induction of *Runx2* was blocked in response to p38 inhibition (Fig. 5C). These observations agree with a previous report that MAPK activates Runx2 at the protein level [Xiao et al., 2000]. The MAPK inhibitor SB203580 did not have an inhibitory effect on osteocalcin (*Ocn*), a late osteoblast marker gene (Fig. 5D) either with or without BMP-2 in the osteogenic media. *Ocn* was induced by SB203580 treatment. This

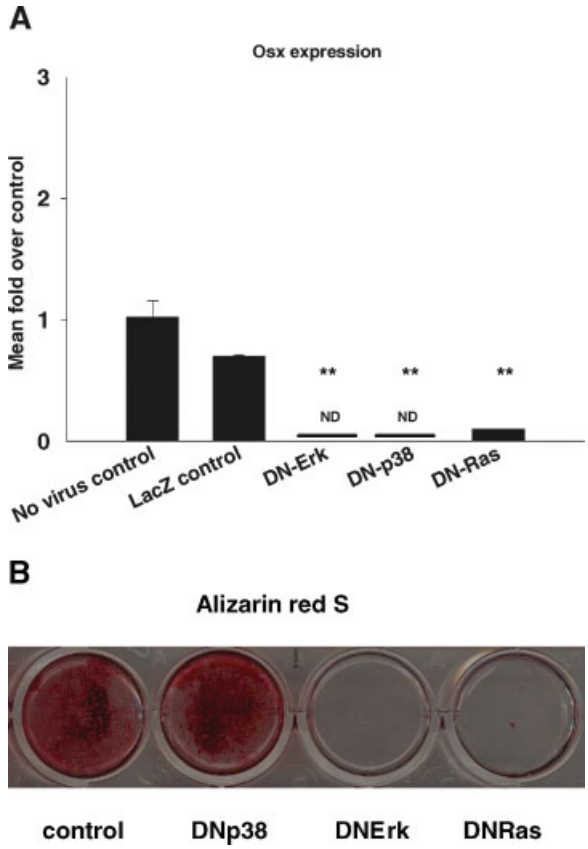


Fig. 4. Adenoviral DNErk, DNp38, and DNRas block *Osx* expression. **A**: hMSC were transduced with Ad-LacZ, Ad-DNErk, Ad-DNp38, or Ad-DNRas for 4 h and maintained in osteogenic stem cell media for 5 days prior to gene analysis. ND, gene expression not detected, a zero value was assigned for statistical analysis. **Significant from virus control, $P < 0.05$. Mean of three independent cultures ±SEM. **B**: Transduced cells were maintained in osteogenic stem cell media for 21 days prior to Alizarin red stain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

outcome could be explained by the differentiation state of the hMSC at the time gene expression analysis was performed. We and other research groups have also observed the BMP-2 mediated inhibition of *Ocn* [Gori et al., 1999; Jadlowiec et al., 2004] (Fig. 5D).

DISCUSSION

Osx was originally described by Nakashima et al. [2002]. While *Osx*-knockout mice expressed wild-type osteoblast levels of *Runx2*, *Osx* is not expressed in *Runx2*-knockout mice, suggesting that *Osx* lies downstream to *Runx2* [Nakashima et al., 2002]. However, there may be additional transcription factors involved in the signaling events [Yagi et al., 2003]. We

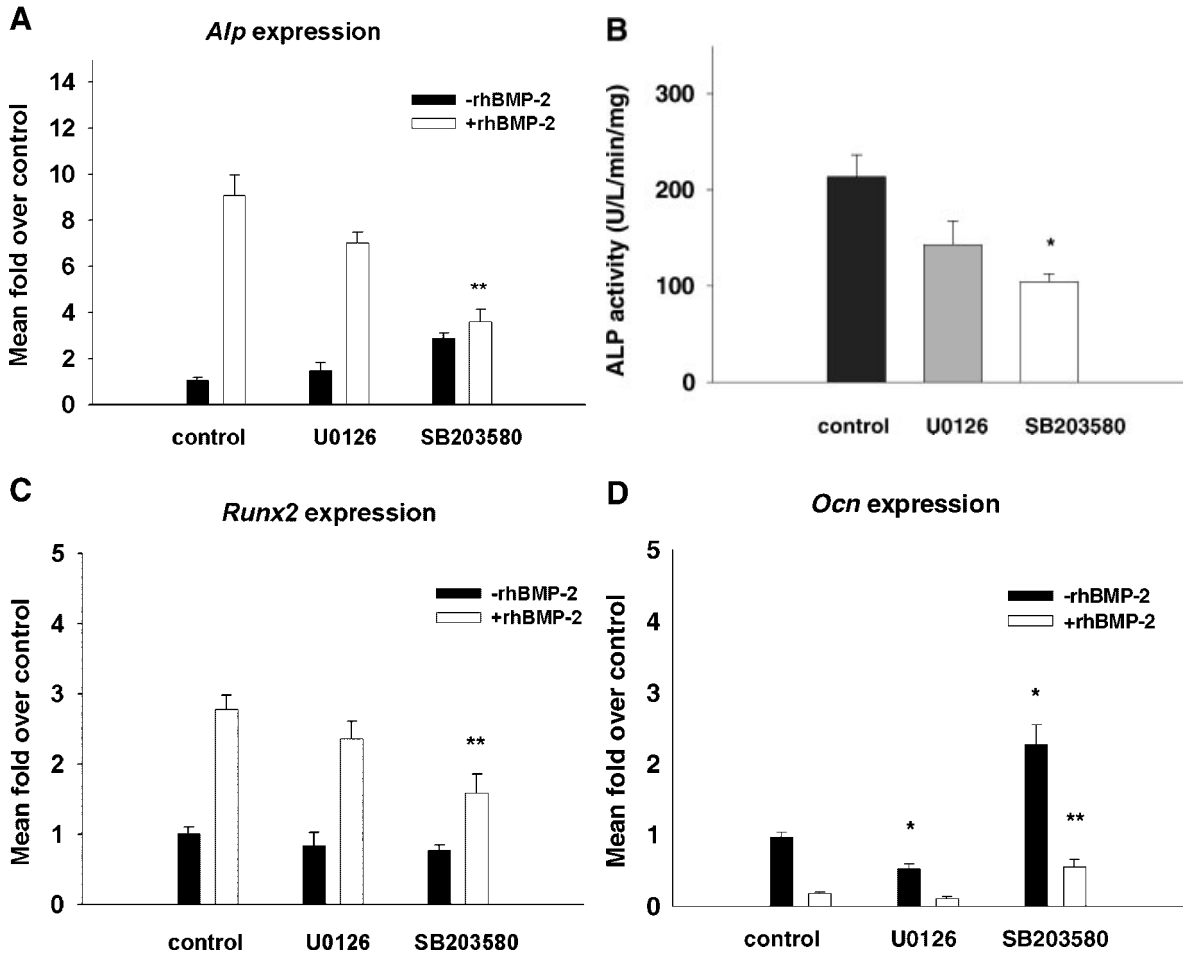


Fig. 5. **A**: p38 inhibitor (SB203580) blocks BMP-2 mediated bone/liver/kidney *Alp* gene expression. hMSC treated with 10 μM SB203580 or 40 μM U0126 or control (DMSO) in osteogenic stem cell media with or without BMP-2. **Significant from control in osteogenic stem cell media with BMP-2, $P < 0.05$ (white bars). Mean of three independent cultures ±SEM. **B**: Alkaline phosphatase enzymatic activity measured with the rate of hydrolysis of *p*-nitrophenyl-phosphate to *p*-nitrophenol by absorbance at 405 nm and normalized to total protein. hMSC were treated with the inhibitors for 3 days prior to cell lysate collection. Inhibition of p38 blocks ALP enzymatic activity. *Significant from control in osteogenic stem cell media without BMP-2, $P < 0.05$. **C**: Inhibition of p38 blocks BMP-2 mediated increase in *Runx2* expression. **D**: Inhibition of p38 induces *Ocn* expression in osteogenic stem cell media with BMP-2. *Significant from control in osteogenic stem cell media without BMP-2, $P < 0.05$. **Significant from control in osteogenic stem cell media with BMP-2, $P < 0.05$.

examined the possible involvement of additional pathways to BMP-2/Smad in *Osx* upregulation. Uncommitted hMSC were used to determine the effects of the *Osx*-associated signaling pathways to promote osteoblastic lineage progression.

Dlx5, a homeobox transcription factor, acts downstream of BMP-2 and induces *Osx* independent of *Runx2* [Lee et al., 2003]. Lee et al. further observed that *Runx2*-overexpression in C2C12 cells was not sufficient for the induction of *Osx*. Moreover, a combination of BMP-2 treatment and *Runx2* overexpression strongly enhanced *Osx* expression in comparison to the effect of BMP-2 treatment alone [Lee et al., 2003]. The expression of *Dlx5* alone induced *Osx* expression in *Runx2* (−/−) cells, thus implicating the presence of *Runx2*-independent mechanisms for *Osx*.

Recently, evidence was presented for an ossification program that proceeds independent of Runx2 activity [Kato et al., 2002]. Mice with a targeted disruption of *Lrp5* receptor of the Wnt signaling pathway developed low bone mass phenotype and decreased osteoblast proliferation and function in a Runx2-independent manner [Kato et al., 2002]. Further, Cheng et al. [2003] showed that *Msx2*-dependent

vascular ossification may take place independent of Runx2. The authors reported a 10-fold induction of *Osx* expression in response to *Msx2*, while *Runx2* levels remained unchanged [Cheng et al., 2003]. Consequently, there may be Runx2 independent pathways for osteoblast proliferation and function. Additional signaling pathways to *Runx2* may regulate *Osx* expression in osteogenic lineage progression in mesenchymal progenitor cells.

Uncommitted hMSC are specific targets of BMP-2 and IGF-I signaling for *Osx* expression (Fig. 1A). There was synergy between IGF-I and BMP-2 to induce *Osx* (Fig. 2). Recently, Raiche and Puleo [2004] reported that combined and sequential delivery of BMP-2 and IGF-I could target cell behavior and differentiation of mouse C3H10T1/2 into osteoblast lineage on a gelatin-based matrix. Shoba and Lee [2003] have demonstrated that BMP-7 (OP-1) may potentiate the response to IGF-I by regulating the expression or activation of specific components of the IGF-I signaling machinery. BMP-7 and IGF-I act synergistically to induce cell differentiation and proliferation possibly via down-regulation of the inhibitory IGF binding proteins [Yeh et al., 1997]. A similar scenario may also be valid for the BMP-2/IGF-I synergy (Fig. 2).

Another possible explanation for growth factor synergy is convergence of signaling pathways. Components of MAPK signaling act as a common component in mediating the effects of IGF-I [Bliziotes et al., 2000; Fruchtman et al., 2001] and BMP-2 [Nohe et al., 2002]. As described by Nohe et al. [2002], BMP-2 is known to have two modes of signaling via a preformed complex or recruitment of BMP type I receptor upon ligand binding. The latter mode is thought to induce *Alp* expression via the MAPK component p38 [Nohe et al., 2002]. Data presented from our studies confirmed that BMP-2 mediated *Alp* expression (Fig. 5A) is down-regulated due to inhibition of p38 signaling. However, *Runx2* levels remained unchanged in response to MAPK inhibition (Fig. 5C). An explanation for this outcome is that the effect of MAPK on Runx2 is post-translational (via phosphorylation), not transcriptional [Xiao et al., 2000].

The involvement of MAPK in osteoblast lineage progression was analyzed with osteoblast marker genes that may act downstream of *Osx*. We and others have observed negative regulation of *Ocn* by BMP-2 [Gori et al., 1999; Jadlowiec et al., 2004]. Further, *Ocn* is upregulated in response to a block in p38 inhibition (Fig. 5D). An explanation for this outcome may be the cell differentiation state. Mineralization of cells may be controlled by a fine-tuning of the MAPK signaling pathway [Higuchi et al., 2002]. For the MAPK inhibition experiments, the cells were cultured in osteogenic stem cell media for several days prior to treatment with the inhibitors. The p38 signaling may initially upregulate early markers such as *Alp* while directly or indirectly suppressing *Ocn*, a late osteoblast marker [Cowan et al., 2003]. Therefore, treatment of the cells with SB203580 may relieve the suppression on *Ocn* expression. The involvement of p38 with the upregulation of the early osteoblast markers also may explain the lack of a negative effect on matrix mineralization due to SB203580 treatment.

Erk1/2 seemed to be involved in *Osx* expression (Fig. 3D), but not BMP-2 mediated *Osx* expression in hMSC. Blocking Erk1/2 inhibited matrix mineralization (Fig. 3C), unlike the effect of p38 inhibition. One shortcoming of our studies is the lack of analysis of *Osx* at the protein level. A possible explanation for the different outcomes observed with p38 and Erk1/2 inhibitors on matrix mineralization may be the involvement of these MAPK components with post-translational regulation of *Osx*.

We propose that multiple signaling pathways mediate *Osx* expression in the commitment to the osteoblast lineage. IGF-I signaling upregulates *Osx* and this effect is synergistic with BMP-2. The significance of *Runx2* in bone formation is implicated by the genetic studies, but an unanswered question is whether *Runx2* is required and sufficient for *Osx* expression. To our knowledge, this paper is the first study to show the involvement of IGF-I and MAPK signaling in mediating *Osx*. Further elucidation of the involvement of Smad/Runx2 mediated signaling and the existence of alternative pathways in mediating *Osx* will address whether Smad signaling is required for *Osx* regulation. MAPK signaling may antagonize Smad signaling via receptor tyrosine kinase signaling [Kretzschmar et al., 1997; Wrana, 2000]. Further, IGF, FGF, and BMP have opposing activities during vertebral development, where Smad1 transcriptional activity is inhibited via the activation of receptor tyrosine kinases that

signal though MAPK [Pera et al., 2003]. It will be interesting to determine how osteogenic lineage cells fine-tune the BMP-2 or MAPK mediated effect on *Osx*. Future studies should also elucidate the translational regulation of *Osx*.

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