Interactions Between Extracellular Signal-Regulated Kinase 1/2 and P38 Map Kinase Pathways in the Control of Runx2 Phosphorylation and Transcriptional Activity

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ABSTRACT

RUNX2, a key transcription factor for osteoblast differentiation, is regulated by ERK1/2 and p38 MAP kinase-mediated phosphorylation. However, the specific contribution of each kinase to RUNX2-dependent transcription is not known. Here we investigate ERK and p38 regulation of RUNX2 using a unique P-RUNX2-specific antibody. Both MAP kinases stimulated RUNX2 Ser319 phosphorylation and transcriptional activity. However, a clear preference for ERK1 versus p38α/β was found when the ability of these MAPKs to phosphorylate and activate RUNX2 was compared. Similarly, ERK1 preferentially bound to a consensus MAPK binding site on RUNX2 that was essential for the activity of either kinase. To assess the relative contribution of ERK1/2 and p38 to osteoblast gene expression, MC3T3-E1 preosteoblast cells were grown in control or ascorbic acid (AA)-containing medium $\pm$ BMP2/7. AA-induced gene expression, which requires collagen matrix synthesis, was associated with parallel increases in P-ERK and RUNX2-S319-P in the absence of any changes in P-p38. This response was blocked by ERK, but not p38, inhibition. Significantly, in the presence of AA, BMP2/7 synergistically stimulated RUNX2 S319 phosphorylation and transcriptional activity without affecting total RUNX2 and this response was totally dependent on ERK/MAPK activity. In contrast, although p38 inhibition partially blocked BMP-dependent transcription, it did not affect RUNX2 S319 phosphorylation, suggesting the involvement of other phosphorylation sites and/or transcription factors in this response. Based on this work, we conclude that extracellular matrix and BMP regulation of RUNX2 phosphorylation and transcriptional activity in osteoblasts is predominantly mediated by ERK rather than p38 MAPKs. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: OSTEOBLAST; TRANSCRIPTION; PHOSPHORYLATION; MAPK; RUNX2

Introduction

Several transcription factors including DLX5/6, TWIST1/2, ATF4, RUNX2, and Osterix control bone cell lineages.[1–6] Of these, RUNX2 and Osterix (OSX) have the most selective roles in osteogenesis. Deletion of either factor in mice results in severe defects in skeletal development. *Runx2*-null mice have a cartilaginous skeleton that contains no bone mineral or hypertrophic cartilage. In contrast, *Osx*, which is genetically downstream of *Runx2*, has a more selective role in osteoblast formation and skeletal mineralization.[3–5] In addition to its essential role in bone development, *Runx2* is necessary throughout life to promote the differentiation of new osteoblasts during bone remodeling.[2]

Consistent with its fundamental role in bone formation, RUNX2 is tightly regulated. In addition to transcriptional control by factors such as bone morphogenetic proteins,[7] RUNX2 activity is regulated both by its interaction with a number of accessory nuclear factors and by posttranslational modifications, including phosphorylation. We have been particularly interested in this latter control mechanism and showed that ERK1/2 MAPK-dependent phosphorylation of RUNX2 is critical for osteoblast-specific gene expression and differentiation.[8,9] This pathway mediates the response of bone cells to a variety of signals including hormone/growth factor stimulation,[10,11] extracellular matrix binding/matrix tension,[12–15] and mechanical loading.[16,17] ERK1/2 phosphorylates four serine residues on RUNX2 (S43, S301, S319, and S510, using the amino acid residue numbering for murine Type II RUNX2 isoform having N-terminal sequence MASN).[18] Of these, S301 and S319 are required for transcriptional activity because S to A mutations at these sites greatly reduces the ability of RUNX2 to stimulate osteoblast gene

expression. ERK1 and ERK2 directly bind to RUNX2 via a consensus MAPK docking or "D" site in its C-terminal region distal to the runt domain. This RUNX2–ERK interaction also occurs on the chromatin of target genes in vivo and is necessary for activation of RUNX2 by the ERK/MAPK pathway.[18,19]

Further evidence for the crucial role of ERK/MAPK signaling in osteogenesis is provided by transgenic mouse studies. Specifically, overexpression of constitutively active or dominant-negative forms of the MAPK intermediate, MEK1, in osteoblasts, respectively stimulated or inhibited bone development, as well as RUNX2 phosphorylation.[20] Effects of MAPK on development are at least in part mediated by RUNX2, because the cleidocranial dysplasia phenotype of *Runx2* heterozygous null mice can be partially rescued by crossing these animals with mice expressing constitutively active MEK1. In related studies, *Prx1-cre*-mediated inactivation of *Erk2* in osteochondroprogenitor cells of developing *Erk1*-null mice was shown to block osteoblast differentiation leading to ectopic cartilage formation in regions of the perichondrium that normally form bone. Furthermore, increased ERK/MAPK signaling in the same cell population increased osteoblast differentiation and inhibited chondrogenesis.[21]

Recent evidence also supports a role for the p38 MAPK pathway in osteoblast differentiation as part of downstream signaling from the transforming growth factor (TGF)-β/bone morphogenetic protein (BMP)-responsive kinase, TAK1.[22] Specifically, conditional deletion of TAK1 in osteoblasts using *Osx-Cre* results in a severe skeletal phenotype that includes low cortical and trabecular bone mass, clavicular hypoplasia, and delayed fontanelle fusion. Although TAK1 stimulates ERK, JNK, and p38 MAP kinases,[23] its actions in osteoblasts were attributed to activation of p38 and subsequent RUNX2 phosphorylation. Consistent with this model, mice harboring deletions in the p38 MAPK intermediates, *Mkk3, Mkk6, p38α,* or *p38β,* all had decreased bone mass.[22] Three p38 phosphorylation sites on RUNX2 were identified (S31, S254, and S319) and their combined mutation was shown to reduce RUNX2 transcriptional activity.

Interestingly, one of the p38 phosphorylation sites on RUNX2 (S319) had previously been identified as a substrate for ERK1/2(18). This finding raises the intriguing possibility that ERK and p38 MAPKs have overlapping functional roles in the control of osteoblast gene expression and differentiation. In the present study, we explore this concept as well as compare the relative importance of these two MAPKs in directly controlling RUNX2 phosphorylation and transcriptional activity.

Experimental Procedures

Reagents

The reagents used in this study were obtained from the following sources: tissue culture medium and fetal bovine serum from Hyclone (Logan, UT, USA), and Invitrogen (Carlsbad, CA, USA); recombinant BMP2/7 from R&D (Minneapolis, MN); ITS, U0126, and SB203580 from Sigma (St. Louis, MO, USA); phospho-ERK, total-ERK, phospho-p38, total p38, and total-SMAD1/5/8 antibodies from Cell Signaling (Danvers, MA, USA); phospho-SMAD1/

5/8 antibody from Santa Cruz (Santa Cruz, CA, USA). and RUNX2 antibody from MBL (Japan).

Generation of a phospho-RUNX2-specific antibody

Convance (Princeton, NJ, USA) using the following peptide as immunogen, produced an antibody that specifically detects RUNX2 phosphorylated at S319: YPSYLSQIMTS(P)PSIHSTTPL. The peptide was conjugated to Keyhole limpet hemocyanin (KLH) and injected into rabbits at 4-week intervals for a total of three injections. After 12 weeks, the serum was harvested and affinity purified using a column containing nonphosphorylated peptide to remove antibody against total RUNX2 followed by a second affinity step using a phosphopeptide-containing column.

DNA constructs and viral expression vectors

The 6OSE2-luc RUNX2 reporter and pCMV-RUNX2 expression vector were previously described.[24,25] Constitutively active MEK1 (Meksp) and dominant negative MEK1 (MEKDN and kinase dead ERK1 (ERK1DN) expression vectors were obtained from Dr Kun Liang Guan (Univ. of California San Diego).[26,27] Constitutively active MKK6, dominant negative MKK6, and dominant negative p38 expression vectors were described previously.[28] Plasmids expressing constitutively active MEK1, wild-type RUNX2, S301A,S319A mutant RUNX2, and RUNX2 containing a deletion of the MAPK-binding D site (Δ200–215) were developed in the project laboratory.[18,29]

Cell cultures and animal studies

COS7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Delbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics. MC3T3-E1 clone 42 cells (MC42 cells), which contain stably integrated copies of 1.3 kilobytes (kb) murine mOG2 promoter driving luciferase, were previous developed in this laboratory.[30] To induce differentiation, cells were plated at a density of 5×10^4 cells/cm^2 and cultured in α-Minimum Essential Medium (MEM), 10% FBS containing 50 µg/mL ascorbic acid (AA) for up to 14 days. Primary calvarial cells were isolated from newborn transgenic mice previously developed in this laboratory that use a 0.6 kb mOG2 promoter to drive dominant negative (TgMekdn) or constitutively active (TgMeksp) MEK1 in osteoblasts[20] and differentiation/mineralization was induced by growth in α-MEM, 10% FBS, 50 µg/mL AA and 3 mM inorganic phosphate for 14 days. For calvarial bone organ cultures, calvaria were isolated from E18.5 C57BL6 mice and cultured in α-MEM with 1% ITS, 50 µg/mL AA, and 3 mM inorganic phosphate for up to 6 days. Medium was changed daily. Mineral was visualized using the either Alizarin Red or von Kossa staining as indicated. All studies with mice were performed in compliance with the University of Michigan Committee for the Use and Care of Animals.

Transfections

COS7 cells were plate at a density of 5×10^4 cells/cm^2 and transfected with the indicated plasmids using Lipofectamine (Invitrogen). For each dish, 0.05 µg of pRL-SV40 containing a

cDNA for *renilla reformis* luciferase was used as a control for transfection efficiency. Cells were harvested 48 hours after transfection and assayed using a dual luciferase kit (Promega, Madison, WI, USA) with a Monolight 2010 luminometer (Pharmingen, San Diego, CA, USA).

Immunofluorescence microcopy

MC3T3-E1 clone 42 and transfected COS7 cells were grown on glass cover slips, fixed with 4% formaldehyde, and incubated overnight at 4°C with primary antibodies to total and phosphorylated RUNX2 (1:500 dilution). Alexa Fluor 488 conjugated donkey antimouse and Alexa Fluor 555 conjugated donkey antirabbit (Invitrogen) antibodies were used to visualize the localization of total and S319-P RUNX2. Fluorescence detection was accomplished using Nikon Eclipse 50i microscope and imaging system.

Western blots and immunoprecipitation

For Western blots, cell extracts were prepared by directly dissolving cells in $2 \times$ SDS sample buffer (Invitrogen). For immunoprecipitations, cells were harvested in immunoprecipitation (IP) buffer containing 20 mM Tris, pH 7.6, 500 mM NaCl, 0.25% NP40, 5 mM NaF, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% proteinase inhibitor, and 1% phosphatase inhibitor (Sigma). The indicated antibodies were added and incubated overnight at 4°C with gentle rocking. Immunocomplexes were then collected by protein A/G agarose beads and released by boiling with SDS sample buffer. Samples were fractionated by SDS-PAGE using 6% to 20% gradient gels (Invitrogen). After a transfer to nitrocellulose membranes, samples were incubated with the indicated antibody (1:500 dilution) overnight at 4°C. Sheep antimouse or donkey antirabbit second antibody conjugated with horseradish peroxidase (GE Healthcare, UK) was used at a 1:10,000 dilution. Horseradish peroxidase activity was detected by ECL (Amersham, Arlington Heights, IL, USA).

Statistical analysis

All statistical analyses were performed using SPSS 16.0 Software. Unless indicated otherwise, each reported value is the mean ± SD of triplicate independent samples. Statistical significance was assessed using a one-way analysis of variance.

Results

Development of a phospho-RUNX2-specific antibody

The ERK/MAPK pathway plays an important role in the control of osteoblast gene expression and differentiation by stimulating RUNX2 phosphorylation.[8,14,18,20] S301 and S319 are of particular importance in that their mutation to alanine greatly reduces the ability of RUNX2 to stimulate osteoblast gene expression. P38 MAPK can also phosphorylate RUNX2 in response to TGF-β/BMP activation of TAK1.[22] Interestingly, one of the ERK1/2 sites we described, S319, is also phosphorylated by p38α. Because it is a substrate for both kinases, we considered it likely that S319-P would be indicative of overall RUNX2 activation state and a good candidate for antibody production. The peptide containing

S319-PO$_4$, which was used to immunize rabbits, is conserved across mouse, rat, and human species. A site homologous to S319 is also present in RUNX1 (AML1), although the flanking sequence is different from RUNX2 (Fig. 1A). A polyclonal antibody was generated to the RUNX2 phosphopeptide and affinity purified using positive and negative selection (see Experimental Procedures). As shown in Figure 1B, the P-RUNX2 antibody selectively detects wild-type RUNX2, but not RUNX2 containing S301A,S319A mutations. Furthermore, staining intensity increased when COS7 cells were transfected with a constitutively active form of MEK1 (Meksp). In contrast, total RUNX2, measured with a commercially available antibody, remained unchanged under these conditions. The phospho-RUNX2 antibody did not crossreact with phosphorylated RUNX1 (result not shown).

The S-319-P antibody also selectively detects phospho-Runx2 in intact cells when examined by immunofluorescence microscopy (Fig. 1C). Antibody clearly stains phosphorylated RUNX2 in COS7 cells transfected with wild-type RUNX2, but gave no signal with the S301A,S319A mutant. Staining for both wild-type and mutant RUNX2 revealed a predominantly nuclear localization. In summary, the RUNX2-S319-P antibody is able to discriminate between RUNX2 in different phosphorylation states and should be a powerful tool for monitoring activation of this transcription factor.

RUNX2 is phosphorylated and activated by both ERK1/2 and p38 MAP kinases; preferential activation by ERK1/2

To compare the ability of ERK1/2 and p38 to stimulate RUNX2 phosphorylation and transcriptional activity, COS7 cells were transfected with a RUNX2 expression vector and increasing amounts (0.05–1.0 μg plasmid DNA) of constitutively active forms of MEK1 (Meksp; activates ERK1/2) or MKK6 (Mkk6sp; activates p38). RUNX2-dependent transcriptional activity was measured using a 6OSE2-luc-reporter gene (contains six copies of the RUNX2-specific enhancer, OSE2, driving luciferase; Fig. 2, top) and changes in RUNX2 phosphorylation and MAP kinase activation were measured on Western blots (Fig. 2, bottom). Meksp or Mkk6sp each dose-dependently increased P-ERK or P-p38, respectively, and stimulated RUNX2-S319 phosphorylation and transcriptional activity. Maximum stimulation by each MAP kinase kinase was achieved with 0.5 μg plasmid DNA, with no further increases noted at higher concentrations. This indicates that Meksp or Mkk6sp plasmids were in excess relative to their respective MAPK substrate (ERK1/2 or p38) at or above this level. However, large differences were observed in the magnitude of responses. Saturating amounts of Meksp stimulated transcriptional activity approximately 12-fold and strongly induced RUNX2 phosphorylation while Mkk6sp only stimulated transcription 2.9-fold and induced a barely detectable increase in RUNX2-P. Similarly, both kinases increased levels of nuclear RUNX2-S319-P in osteoblasts as measured by immunofluorescence, but the amount of the stimulation was much greater in the Meksp-treated group.

To exclude the possibility that endogenous levels of ERK and p38 were limiting the magnitude of Meksp and Mkk6sp stimulation in the experiment shown in Figure 2, a second
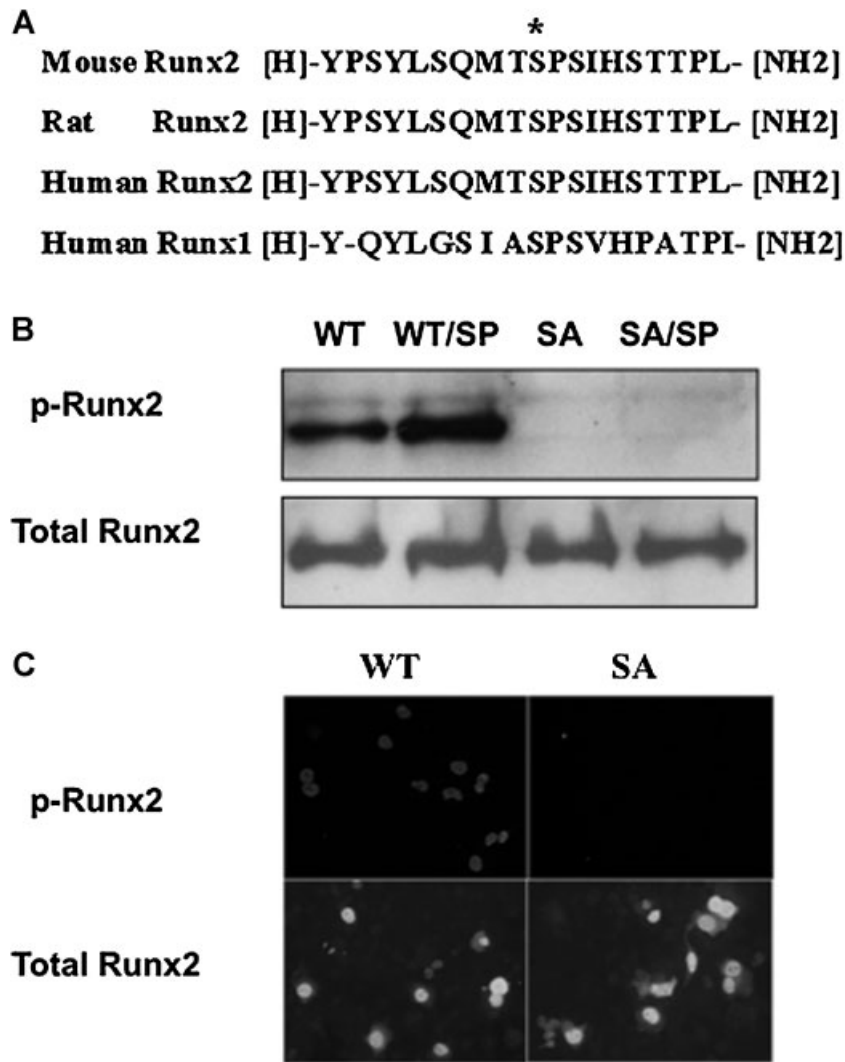
Fig. 1. Characterization of RUNX2-S319-PO$_4$-specific antibody. (*A*) Sequence alignment of mouse, rat, and human RUNX2 and human RUNX1. The conserved phosphoserine site is indicated (*). (*B*) P-RUNX2 antibody specifically detects RUNX2-S319 phosphorylation. COS7 cells were transfected with wild-type (WT) or S301A,S319A mutant RUNX2 (SA) expression vectors in the presence or absence of a constitutively active MEK1 (SP). Western blots were probed with P-RUNX2 or total RUNX2 antibody. (*C*) Immunofluorescence detection of P-RUNX2. COS7 cells were transfected with wild-type (WT) or S301A,S319A mutant RUNX2 expression vectors and stained with the indicated antibodies. *Red*, phospho-RUNX2; *green*, total RUNX2. Bar = 20 μm.

study was carried out where cells were transfected with increasing amounts of ERK1, p38α, or p38β in the presence of excess Meksp or Mkk6sp plasmids, respectively (Fig. 3A, B). The amount of Meksp or Mkk6sp expression plasmid used (0.5 μg DNA each) was based on results in Figure 2 that showed maximal stimulation of ERK or p38 phosphorylation at or above this amount of plasmid. Studies focused on p38α and β because these isoforms were previously reported to phosphorylate RUNX2.[22] In the absence of upstream activation, ERK1 or p38α/β overexpression minimally affected RUNX2-dependent transcriptional activity (Fig. 3A). However, a clear dose-dependence was found for all three MAPKs when cells were also transfected with excess Meksp or Mkk6sp. Peak activation of RUNX2 phosphorylation and transcriptional activity was found with 0.5 μg of each MAPK expression vector with a partial drop found with 0.75 μg. However, large differences were found in the magnitude of 6OSE2-luc reporter activation with ERK1 having

the highest activity (Meksp/control = 16) followed by p38β (Mkk6sp/control = 6.9) and p38α (MKK6/control = 4.3). Large differences in peak luciferase activities were also found. Normalized luciferase activities were as follows: ERK1, 6.0; p38β, 1.5; p38α, 0.65 (ratios: ERK1:p38β:p38α = 9.1:2.3:1.0). Similar differences were found in the ability of each MAPK to stimulate RUNX2 phosphorylation with RUNX2 being more extensively phosphorylated by the ERK1/Meksp combination than by p38β/Mkk6sp or p38α/Mkk6sp (Fig. 3B).

ERK1 and p38β share and compete for a common docking site on RUNX2

To better compare ERK1/Meksp and p38β/Mkk6sp stimulation of RUNX2 activity, COS7 cells were transfected with optimal amounts of each expression vector in the combinations indicated. Results are presented both in terms of absolute
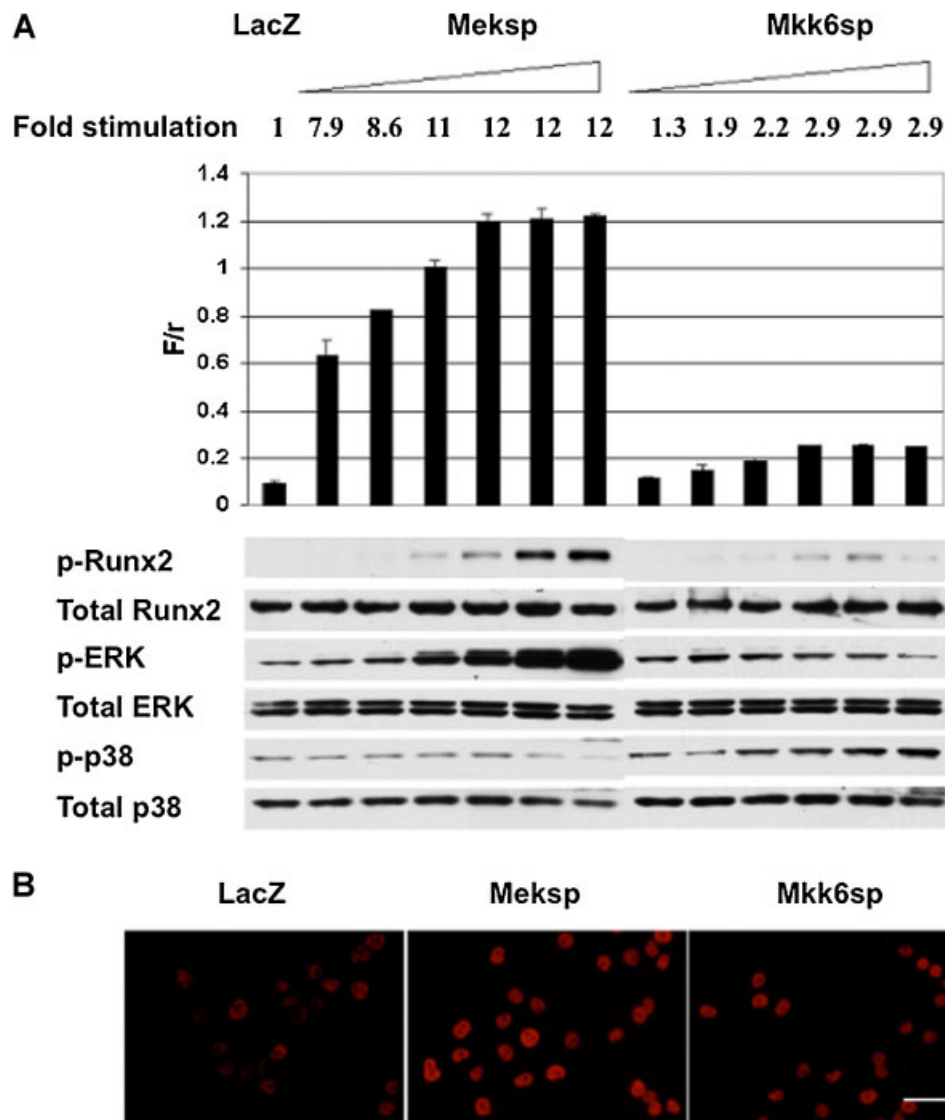
Fig. 2. ERK and p38 MAP kinase stimulation of RUNX2 phosphorylation and transcriptional activity. (*A*) MEK1 and MKK6 dose–response. COS7 cells were transfected with wild-type RUNX2 expression vector, 6OSE2-luc reporter, and increasing amounts of either constitutively active MEK1 (Meksp) or MKK6 (Mkk6sp) expression vectors (0.05, 0.1, 0.25, 0.5, 0.75, 1.0 µg/dish) (*upper panel*). Firefly luciferase activities were normalized to *renilla pyriformis* luciferase plasmid. Results are presented as normalized luciferase activity (F/r) and fold-stimulation relative to a LacZ control transfection. RUNX2-S319-P, P-ERK1/2, and P-p38 were detected by Western blotting using the indicated antibodies (*lower panel*). (*B*) Immunofluorescence detection of endogenous RUNX2 phosphorylation in MC3T3E1 preosteoblast cells. MC3T3-E1 clone 4 cells were transfected with control (LacZ), Meksp, or Mkk6sp expression vectors and stained with P-RUNX2 antibody as indicated. Bar = 20 µm.

activity and fold-stimulation relative to control cells transfected with 6OSE2-luc and RUNX2 only (Fig. 3*C*). Because of the increased availability of endogenous ERK1 in COS7 cells, overexpression of Meksp alone induced a 12-fold stimulation of luciferase activity. This was further increased to 16-fold with ERK1 transfection. In contrast, MKK6 increased activity 2.9-fold, and this was further increased to only 6.9-fold with p38β. Surprisingly, when optimal amounts of ERK1/Meksp and p38β/Mkk6sp were combined, neither an additive nor synergistic stimulation of transcriptional activity or RUNX2 phosphorylation was observed. Instead, RUNX2 phosphorylation and activity were actually lower than the ERK1/Meksp group ($p < 0.01$).

Competition between ERK1 and p38β for a common docking site on RUNX2 is a possible explanation for this result. Our

previous work showed that RUNX2 contains a conserved ERK1/2 docking motif or "D" site between amino acid residues 200 and 215.[18] Because this motif is also recognized by JNK/SAPKs and p38 MAPKs when present in other transcription factors such as SAP-1/2, ATF-2, or ELK-1,[31,32] it is possible that it could also serve as binding site for p38. In fact, p38 was previously shown to bind RUNX2 in coimmunoprecipitation assays, although the actual binding region was not defined.[22] In the study shown in Figure 4*A*, coimmunoprecipitation assays were used to demonstrate competition between P-ERK1/2 and P-p38 for binding sites on RUNX2 under conditions where either ERK or p38 was activated by increasing amounts of Meksp or Mkk6sp. For all groups, COS7 cells were transfected with FLAG-tagged RUNX2 and optimal amounts of either Mkk6sp or Meksp. M2 antibody
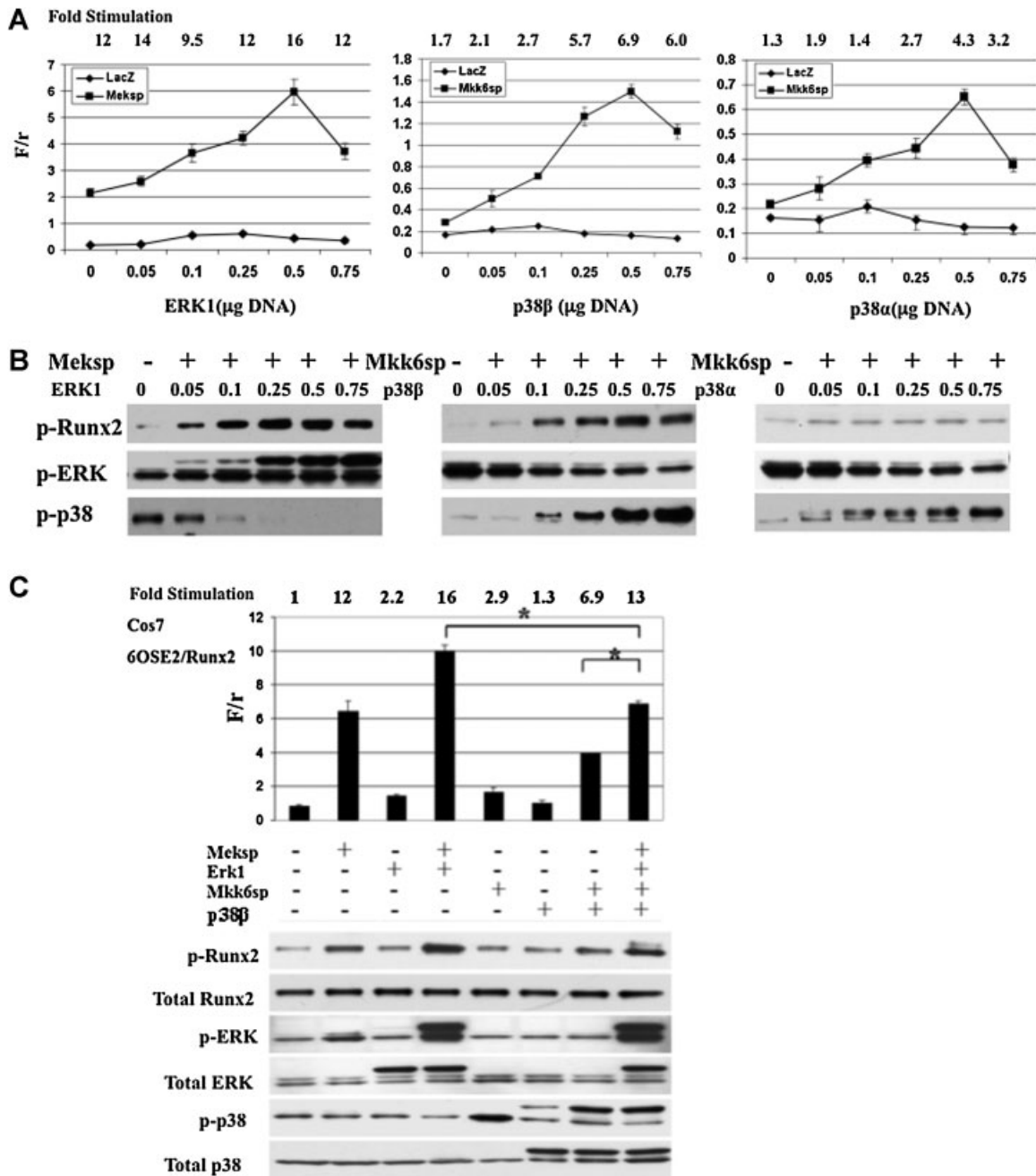
Fig. 3. Preferential stimulation of RUNX2 phosphorylation and transcriptional activity by ERK1 versus p38α and p38β. COS7 cells were transfected with wild-type RUNX2 expression vector, 6OSE2-luc reporter, and the indicated amounts of ERK1, p38α, or p38β with or without excess Meksp or Mkk6sp (0.5 μg each). (A) RUNX2 transcriptional activity. Results are expressed in terms as normalized luciferase activity and fold-stimulation (Meksp/LacZ or Mkk6sp/LacZ). (B) RUNX2, ERK, and p38 phosphorylation. (C) Combined activities of ERK1/Meksp and p38β/Mkk6sp. COS7 cells were transfected with optimal amounts of ERK1/Meksp or p38β/Mkk6sp combinations (0.5 μg of each plasmid) as determined in (A). RUNX2-dependent transcriptional activity (*upper panel*) and phosphorylation of RUNX2, ERK1/2, and p38 (*lower panel*) are shown. Note that ERK1/Meksp plus p38β/Mkk6sp group has less activity than ERK1/Meksp alone. (*) Statistically significant differences are indicated, $p < 0.01$.

(anti-FLAG) was then used to immunoprecipitate total RUNX2 and associated P-ERK or P-p38. As cells were transfected with increasing amounts of Meksp in the presence of optimal Mkk6sp, a concomitant increase in RUNX2-associated P-ERK1/2 was observed while the amount of bound P-p38 decreased. Similarly, when cells were transfected with increasing Mkk6sp, bound P-p38 increased at the expense of P-ERK.

To determine whether competition between ERK and p38 affected RUNX2 transcriptional activity and phosphorylation, COS7 cells were transfected with optimal amounts (based on results shown in Fig. 3) of either Meksp/ERK1 (Fig. 4B, D) or Mkk6sp/p38β (Fig. 4C, E) expression plasmids and increasing amounts of dominant-negative ERK1 or p38. Cells were then assayed for RUNX2-dependent transcriptional activity (Fig. 4B, C)
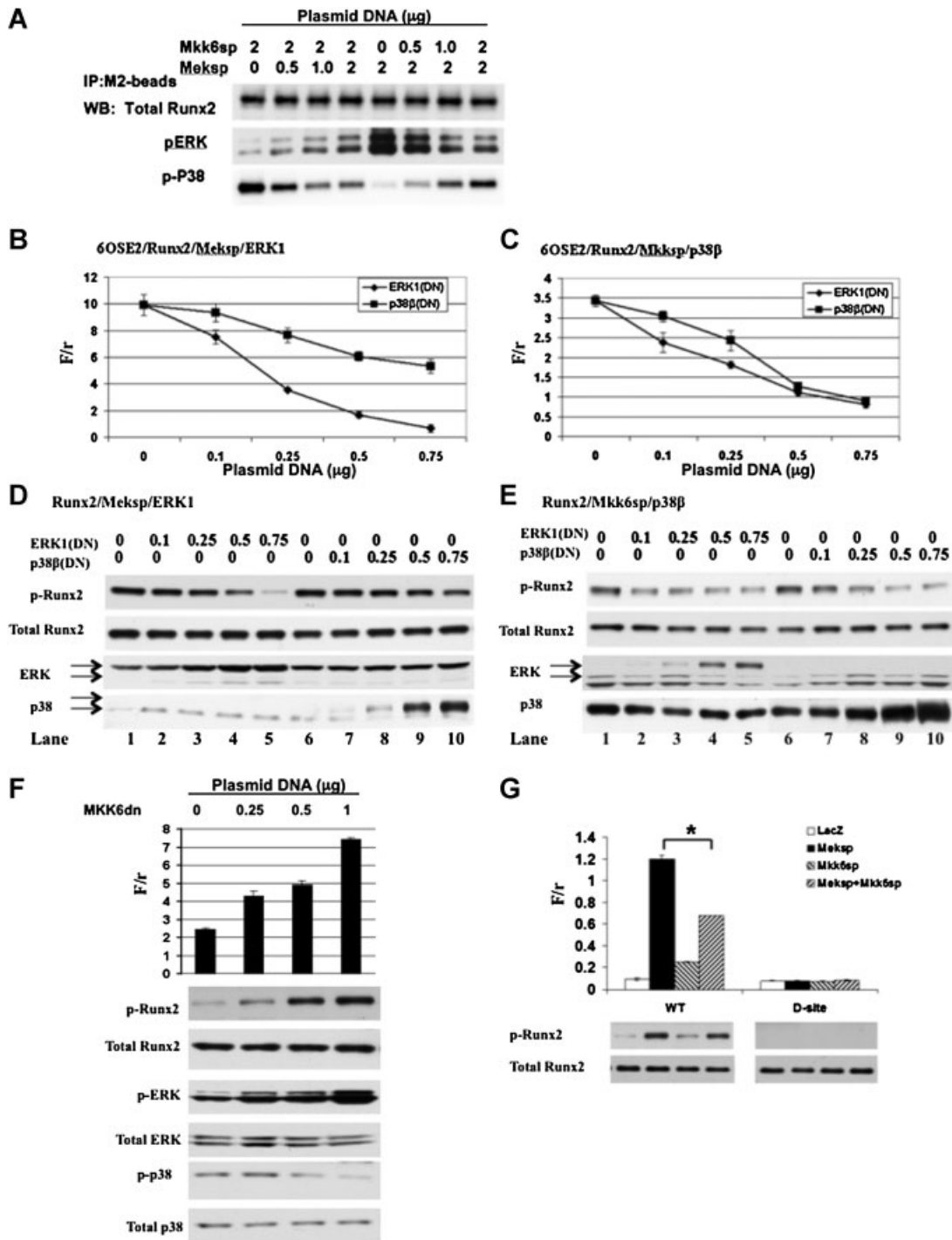
Fig. 4. ERK and p38 compete for a common docking site on RUNX2. (*A*) Coimmunoprecipitation studies. COS7 cells were transfected with a FLAG-tagged RUNX2 expression vector and the indicated amounts of Mkk6sp or Meksp vectors. Nuclear extracts were immunoprecipitated with M2 (anti-FLAG) antibody and blots probed with anti-RUNX2, anti-P-ERK, and anti-P-p38 antibodies as indicated. Note the reciprocal relationship between bound P-ERK and P-p38. (*B–E*) Dominant-negative ERK1 or p38β inhibit both ERK and p38 stimulation of RUNX2 transcriptional activity. COS7 cells were transfected with RUNX2 expression vector, 6OSE2-Luc, and optimal amounts (0.5 μg DNA each) of Meksp/ERK1 (*B,D*) or Mkk6sp/p38β (*C,E*) and the indicated amounts of ERK1(DN) or p38β(DN). Samples were then assayed for luciferase activity (*B,C*) and Western blots were probed with antibodies against total or P-RUNX2, total ERK1/2 and total p38 (*D,E*). The ERK blot detected both endogenous (*lower arrow*) and transfected ERK/ERK(DN) (*upper arrow*), while the p38 blot detected endogenous p38 (*lower arrow*) and transfected p38/p38(DN) (*upper arrow*). (*F*) Dominant-negative MKK6 (Mkk6dn) stimulates RUNX2 transcriptional activity and phosphorylation. COS7 cells were transfected with a RUNX2 expression vector and 6OSE2-Luc with the indicated amounts of Mkk6dn vector. Shown are normalized luciferase activity (*upper panel*) and RUNX2, ERK, and p38 phosphorylation (*lower panel*). (*G*) Ability of ERK and p38 to stimulate RUNX2-dependent transcription requires a MAPK docking site on RUNX2. COS7 cells were transfected with wild-type RUNX2 or RUNX2 containing a MAPK docking site deletion (D-site), 6OSE2-luc reporter ± Meksp, Mkk6sp, or both expression vectors. Cells were assayed for luciferase activity (*upper panel*) or total/phosphorylated RUNX2 (*lower panel*). (*) Statistically significant difference is indicated, $p < 0.01$.

and levels of RUNX2-S319-P, total ERK, and total p38 protein (Western blots; Fig. 4D, E). Because they were epitope-tagged, transfected ERK1, ERK1(DN), p38β, and p38β(DN) could be distinguished from endogenous kinases by their reduced electrophoretic mobility (see arrows, Fig. 4D, E, bottom 2 rows). For example, in the row probed for total ERK in Panel D, the upper band in lane 1 reflects levels of wild-type ERK1 transfected into cells. Because transfected wild-type and mutant ERK have the same mobility, the increased intensity of this band in the lanes 2–5 reflects increasing amounts of transfected ERK(DN). Similarly, the upper band in lanes 6–10 of the p38 row measures p38(DN). The same analysis applies to the results shown in Panel E, except in this case all cells were transfected with p38β instead of ERK1, so the ERK(DN) band is clearly visible (lanes 1–5), whereas p38(DN) is only distinguished from transfected wild-type p38β by the increased intensity of the total p38 band in samples transfected with increasing amounts p38(DN) (lanes 6–10). As expected, ERK1(DN) strongly inhibited ERK1/Meksp-stimulated RUNX2 activity and phosphorylation. Under this condition, p38(DN) was only weakly inhibitory. On the other hand, when Mkk6sp/p38β activity was examined, ERK1(DN) and p38(DN) were both strongly inhibitory although ERK1(DN) was still somewhat more active. These are the results that would be expected if ERK1 had a higher affinity for the RUNX2 D site than p38.

Similarly, in the experiment shown in panel F, COS7 cells were transfected with increasing amounts of dominant-negative MKK6 (MKK6dn). This strongly inhibited basal P-p38 levels while dramatically increasing P-ERK, RUNX2 phosphorylation, and transcriptional activity. Again, this would be the result expected if suppression of the less active p38 were allowing more RUNX2 docking sites to be available for P-ERK, leading to increased RUNX2 phosphorylation and transcriptional activity.

A final experiment in this section examined the requirement for an intact RUNX2 D site for ERK and p38-mediated RUNX2 phosphorylation/transcriptional activity (Fig. 4G). COS7 cells were transfected with wild-type RUNX2 or RUNX2 containing a D site deletion (Δ200–215) and stimulated with Meksp, Mkk6sp, or both kinases. With wild-type RUNX2, Meksp preferentially stimulated transcriptional activity and RUNX2-S319-P versus Mkk6sp, and, as expected, the Meksp/Mkk6sp combination was less active than Meksp alone. In contrast, neither kinase had any activity with the D site deletion mutant.

Effect of ERK and p38 MAPK inhibitors; reciprocal activation of ERK1/2 and p38 pathways

Pharmacological inhibitors were used to further explore the role of ERK and p38 MAPKs in bone cells. Studies shown in Figure 5 examined effects of inhibitors on transfected COS7 cells, MC3T3-E1 clone 42 preosteoblast cells, and calvarial bone organ cultures. In all cases, cultures were titrated with the indicated inhibitor to give maximal suppression of p-ERK1/2 or P-p38, respectively (result not shown). Inhibitors were added 16 hours before harvest to minimize the nonspecific toxic effects associated with prolonged exposures.[14] In COS7 cells, the ERK/MAPK inhibitor, U0126, blocked RUNX2 activity ($p < 0.01$) and inhibited ERK and RUNX2 S319 phosphorylation. In contrast,

SB203580 (SB), a p38/MAPK inhibitor, while clearly blocking p38 phosphorylation, actually increased P-ERK, RUNX2 transcriptional activity, and phosphorylation (Fig. 5A). This may explain why there was no decrease in overall RUNX2-dependent transcription under this condition. Addition of both inhibitors decreased RUNX2 activity and phosphorylation below levels found with U0126 alone, which suggests that p38/MAPK also contributed to basal RUNX2 activity.

To assess the applicability of these findings to bone cells, inhibitor effects were also examined in MC42 preosteoblast cells (Fig. 5B) and calvarial organ cultures (Fig. 5C, D). MC42 cells, which contain stably integrated copies of a 1.3- kb mOG2-luc reporter,[30] were grown in AA-containing medium for 6 days to induce differentiation and mOG2-luc activity. In these cells, mOG2-luc activity is proportional to endogenous osteoblast gene expression and differentiation.[30] Cells were then treated with U0126 and/or SB203580 for 16 hours immediately before harvest. As previously reported,[14] U0126 dramatically inhibited mOG2-luc activity. This inhibitor also blocked ERK and endogenous RUNX2 phosphorylation while increasing P-p38. In contrast, SB actually mildly stimulated ERK and RUNX2 phosphorylation and transcription. However, when it was combined with U0126, SB further decreased mOG2-luc activity and RUNX2 phosphorylation.

For calvarial organ cultures, bones were isolated from E18.5 mouse embryos and cultured for 5 days with AA and 2 mM inorganic phosphate ± U0126 or/and SB203580 (Fig. 5C, D). Mineralization was measured using Alizarin red staining. During the 5-day culture period, sutures went from a patent state to nearly total fusion. U0126 or SB203580 each significantly inhibited mineralization although the ERK inhibitor was somewhat more active and an even greater inhibition was observed when both compounds were used. Similar to cell culture results, Western blot analysis of calvarial extracts showed that U0126 reduced P-ERK and RUNX2 S319 phosphorylation while mildly increasing P-p38. In contrast, P-p38 inhibition had no obvious effect on RUNX2 phosphorylation and increased P-ERK while combining U0126 and SB blocked both pathways and reduced RUNX2 phosphorylation below levels found with U0126 alone.

A final example of this reciprocal regulation is shown in Figure 6. In this study, calvarial osteoblasts were isolated from previously generated transgenic mice in which a 0.6-kb mOG2 promoter was used to drive dominant negative (TgMekdn) or constitutively active (TgMeksp) MEK1 in osteoblasts (Fig. 6A, B). In these animals, Mekdn delayed embryonic bone development while Meksp accelerated osteogenesis.[20] Cells were prepared from newborn wild-type mice and cultured under differentiating conditions for 14 days. As previously reported, TgMeksp cells exhibited increased mineralization relative to wild-type while TgMekdn cells were under mineralized.[20] Western blot analysis (Fig. 6B) revealed that endogenous ERK and RUNX2-S319 phosphorylation was decreased in TgMekdn cells while P-p38 was increased. In contrast, ERK and RUNX2 phosphorylation were increased and P-p38 was inhibited in TgMeksp cells. U0126 inhibited ERK and RUNX2 phosphorylation in WT and TgMekdn groups, but not in cells from TgMeksp mice that were resistant because of the constitutively active Mek1 transgene. U0126 also
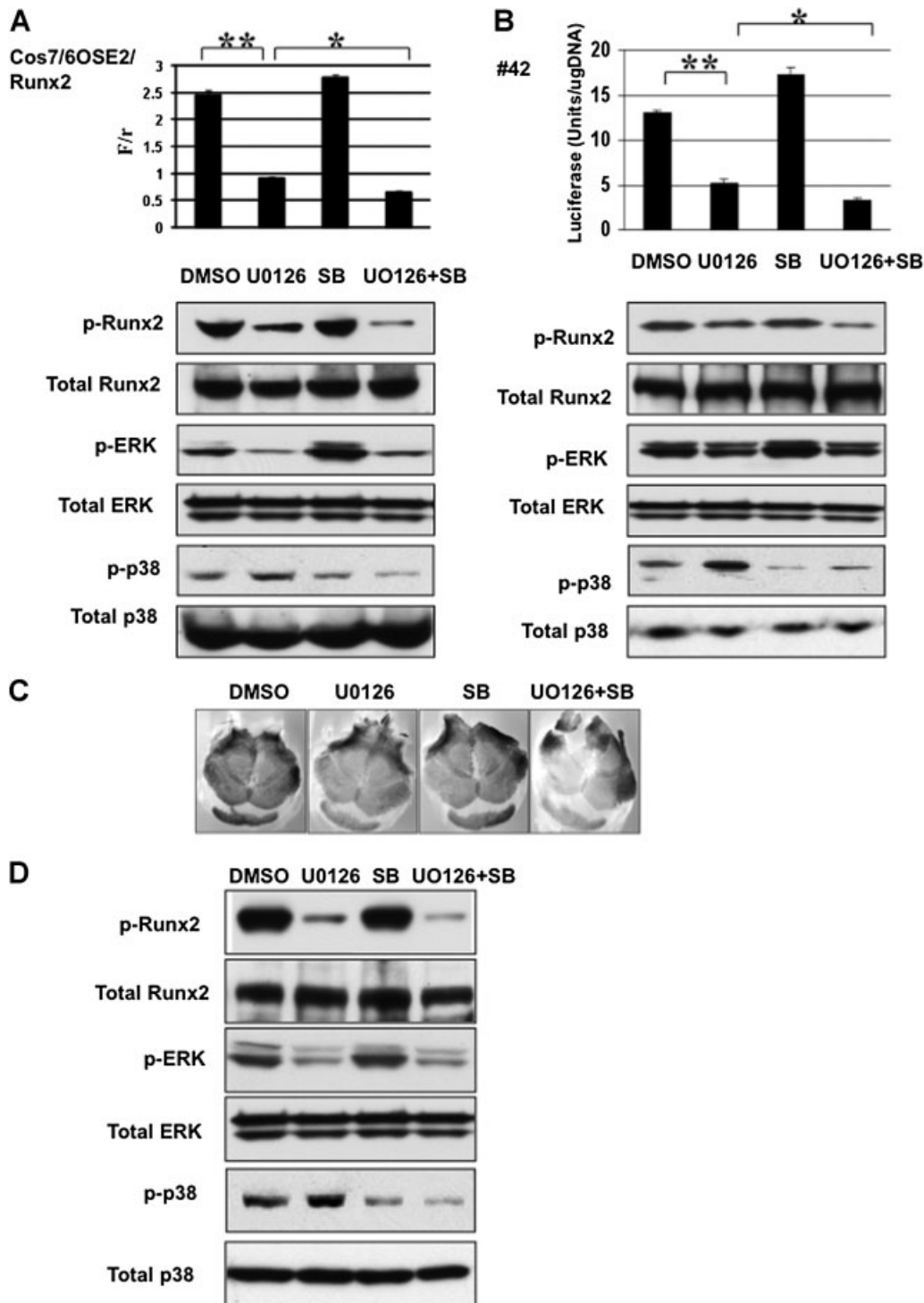
Fig. 5. Reciprocal relationship between ERK1/2 and p38 MAPK activities: effect of ERK1/2 and p38 MAPK inhibitors on RUNX2 transcriptional activity and phosphorylation. (*A*) COS7 cells. Cells were transfected with a wild-type RUNX2 expression vector and 6OSE2-Luc reporter and grown for 48 hours. Sixteen hours before harvest, cells were treated with 20 μM U0126 (ERK/MAPK inhibitor), 20 μM SB203580 (SB, a p38/MAPK inhibitor), or both compounds. Cells were assayed for luciferase activity (*upper panel*) and phosphorylation of RUNX2, ERK1/2, and p38 (*lower panel*). (*B*) MC3T3-E1 clone 42 preosteoblasts. These cells contain stably transfected copies of a 1.3-kb murine osteocalcin gene 2 (mOG2) promoter driving a firefly luciferase reporter. Cells were cultured using differentiation conditions described in Experimental Procedures and treated with U0126 or SB203580 for 16 hours before harvest. *Upper panel*: mOG2 promoter activity. *Lower panel*: RUNX2, ERK, and p38 phosphorylation. (*C,D*) Calvarial organ cultures. Calvaria were isolated from E18.5 day mouse embryos and cultured for 5 days as described in Experimental Procedures ± U0126 or/and SB203580. Cultures were stained for mineral using Alizarin red (*upper panel*). *Lower panel*: phosphorylation of RUNX2, ERK, and p38. Statistically significant differences are indicated: $^*p < 0.05$; $^{**}p < 0.01$.
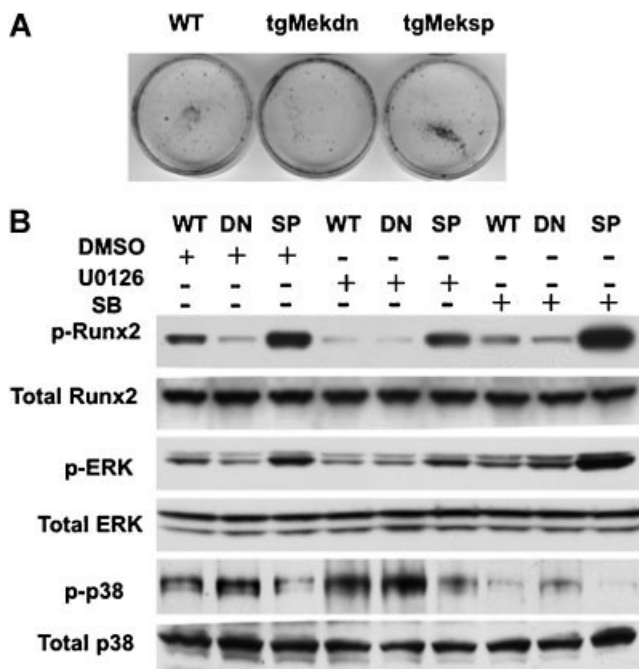
Fig. 6. Reciprocal relationship between ERK1/2 and p38 MAPK activities: Transgenic modification of ERK/MAPK signaling alters osteoblast differentiation, RUNX2 phosphorylation, ERK1/2, and p38 activities. Primary calvaria cells were isolated from newborn wild-type mice (WT), mice expressing dominant-negative (TgMekdn) or constitutively active (TgMeksp) MEK1 in osteoblasts and differentiation was induced as described in Experimental Procedures. Cells were grown for 14 days and treated with U0126 or SB203580 16 hours before harvest as indicated. (*A*) Mineralization. Cells were stained according to the method of von Kossa. (*B*) RUNX2, ERK, and p38 phosphorylation.

further increased the already high levels of phosphorylated p38 in TgMekdn cells. Inhibition of p38 lowered RUNX2 phosphorylation and mildly increased P-ERK in wild-type and TgMekdn cells, but led to a dramatic increase in P-ERK, and RUNX2 phosphorylation in TgMeksp cells.

Taken together, these studies show that there is a reciprocal relationship between ERK1/2 and p38 MAPK pathways; inhibition of one leads to activation of the other. Furthermore, because of competition between the two kinases for the RUNX2 docking site, inhibition of p38 actually makes RUNX2 more available for ERK phosphorylation and activation.

Role of ERK1/2 and p38 signaling in extracellular matrix and BMP-induced osteoblast differentiation

Extracellular matrix (ECM) and BMP signals synergistically interact to stimulate osteoblast gene expression and differentiation.[14] The basis for this interaction is not known. However, binding of type I collagen to $\alpha 2\beta 1$ integrins or the discoidin receptor type 2 (DDR2) stimulates ERK/MAPK, RUNX2 phosphorylation, and gene expression,[13,33,34] whereas BMP activation of type I and II BMP receptors activates receptor SMAD proteins as well as TAK1. Because this kinase also stimulates ERK and p38 MAP kinases,[22,23] the potential exists for extracellular matrix and BMP signals to interact at the level of kinase activation of RUNX2. To explore this possibility, we examined MAP kinase

activation and RUNX2 S319 phosphorylation in MC42 cells during AA-induced differentiation in the presence or absence of BMP2/7 heterodimer (Fig. 7). Previous worked showed that actions of this vitamin on differentiation require collagen synthesis and binding to $\alpha 2\beta 1$ integrins.[13,35]

To first evaluate the AA-induced component of kinase signaling, cells were grown in control or AA-containing medium for 3, 7, 10, and 14 days and gene expression (1.3 kb mOG2-luc activity), kinase activation and RUNX2 phosphorylation were measured (Fig. 7A, B). As previously reported, AA strongly induced osteocalcin promoter activity.[13,30] Furthermore, time-dependent increases were found in ERK1/2 and RUNX2 S319 phosphorylation only in cells grown in AA-containing medium. In contrast, little to no change was found in the level of p38 phosphorylation. This suggests that AA-induced differentiation is mainly associated with ERK/MAPK activation/phosphorylation of RUNX2.

Studies shown in Figure 7C and D examined combined effects of AA and BMP2/7 on transcriptional activity, kinase signaling, and RUNX2-S319 phosphorylation. As previously reported,[14] BMP treatment dose-dependently increased mOG2-luc activity and this response was synergistically stimulated when BMP2/7 was added in the presence of AA (Fig. 7C). In the absence of AA, BMP2/7 also weakly stimulated RUNX2, ERK, p38, and Smad 1/5/8 phosphorylation without affecting total RUNX levels (Fig. 7D). P-RUNX2 and P-ERK levels were reduced by U0126 while p38 inhibition did not affect promoter activity or Smad phosphorylation and slightly increased RUNX2 and ERK phosphorylation. Consistent with results shown in Figure 5B, treatment with AA alone increased promoter activity, RUNX2, and ERK phosphorylation, and all these responses were sensitive to ERK, but not p38 inhibition. Significantly, in the presence of AA, BMP2/7 dramatic stimulated mOG2-luc activity, RUNX2, ERK, and p38 phosphorylation and modestly increased Smad1/5/8 phosphorylation without affecting total RUNX2. ERK/MAPK inhibition reduced promoter activity and ERK/RUNX2 phosphorylation to basal levels without affecting SMAD phosphorylation. Under these conditions, p38 inhibition also partially inhibited mOG2-luc activity, but surprisingly, did not affect RUNX2 phosphorylation. Two important conclusions may be drawn from these studies: first, the BMP pathway can stimulate RUNX2 S319 phosphorylation and transcriptional activity, particularly in the presence of AA, and this stimulation is not associated with increased levels of RUNX2. Second, although BMPs via TAK1 are know to activate all three MAPKs, ERK1/2 is a major mediator of this response in cultured osteoblasts with p38 having a minor role via a mechanism unrelated to RUNX2 S319 phosphorylation.

Discussion

In this study, a variety of approaches were used to examine ERK1/2 and p38 signaling in osteoblasts and relate kinase activities to phosphorylation and activation of the RUNX2 transcription factor. Although RUNX2 was a substrate for both kinases, it was preferentially phosphorylated/activated by ERK1 relative to p38α or β. Additional novel findings include the discovery that both ERK and p38 MAPKs compete for a common docking site on

RUNX2, the observation that these two kinase classes are reciprocally regulated and the demonstration that RUNX2-S319 phosphorylation is strongly stimulated by BMP treatment, particularly in the presence of ascorbic acid. Last, ERK and p38 were shown to have separate but overlapping roles in mediating the response of bone cells to AA and BMP signals.

Transgenic studies clearly established the requirement for both ERK1/2 and p38 in osteoblast differentiation and bone development.[18,22] Furthermore, both pathways were shown to regulate osteoblast gene expression, at least in part, by controlling the level of RUNX2 phosphorylation at specific serine residues. However, the relative ability of ERK and p38 to phosphorylate/activate RUNX2 and potential interactions between these two kinases in osteoblasts had not previously been examined. To address this issue, we developed a unique antibody to monitor RUNX2 phosphorylation at serine 319, a site phosphorylated by both ERK1/2 and p38α.[18,22] Under a variety of conditions, a good correlation was observed between the level of RUNX2 S319 phosphorylation measured on Western blots and RUNX2 transcriptional activity measured using either a RUNX2-specific reporter (6OSE2-luc) or a 1.3-kb murine osteocalcin gene 2 promoter–luciferase construct (1.3mOG2-luc). Despite the fact that ERK1/2 and p38 can each phosphorylate RUNX2 at other sites, the level of S319 phosphorylation appears to be a good indicator of RUNX2 transcriptional activity (Figs. 5–7). For this reason, this antibody should be a useful reagent for monitoring RUNX2 activation under different physiological conditions.

Although ERK1/2, p38α and p38β can each phosphorylate and activate RUNX2, large differences were noted when kinases were compared. When assayed under conditions where each MAPK was optimally stimulated by its respective MAPKK (constitutively active MEK1 or MKK6), ERK1 was found to be four times more active than p38β and nine times more active than p38α in stimulating RUNX2-dependent transcription (Fig. 3). As expected, differences in transcriptional activation were paralleled by differences in the magnitude of RUNX2-S319 phosphorylation. The observation that ERK1/2 and p38 can both phosphorylate RUNX2 at the same site is consistent with findings in other systems. For example, T581 of MSK1 is phosphorylated by ERK and p38 in response to oxidative stress,[36,37] while both kinases phosphorylate the EGF receptor at T669, S1046, and S1047.[38]

Phosphorylation/activation by either class of MAPKs required a consensus MAPK docking D site in RUNX2 at residues 200–215 (RGKSTLTITVFTNPP; consensus sequence Rxxxxθxθ where θ is L/I/V).[39] There are several examples of other transcription factors with D sites that bind more than one MAPK. These include
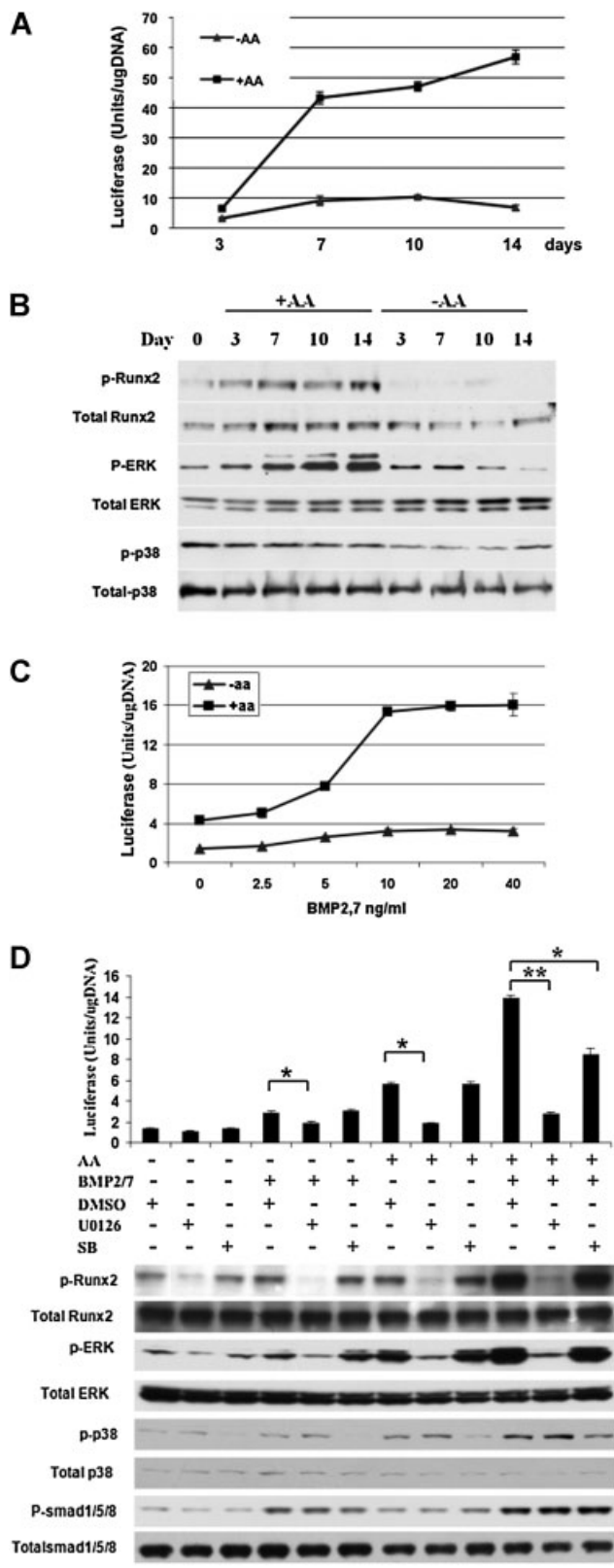
Fig. 7. Comparison of ERK1/2 and p38 signaling in extracellular matrix and BMP-induced osteoblast differentiation. (*A,B*) ECM-induced RUNX2 phosphorylation and differentiation is selectively associated with ERK1/2 activation. MC-42 cell were grown ± 50 μg/mL AA for the indicated times. Cells were harvested for measurement of luciferase (*A*) and Western blot measurement of RUNX2, ERK, and p38 phosphorylation (*B*). (*C,D*) Role of ERK1/2 and p38 signaling in ECM/BMP-induced differentiation. (*C*) Dose-dependent induction of osteoblast gene expression by BMP2/7. MC-42 cell were grown for 7 days ±50 μg AA and the indicated concentrations of BMP2/7 heterodimer and assayed for mOG2-luc activity. (*D*) Differential effects of ERK1/2 and p38 MAPK inhibitors on ECM and BMP-induced RUNX2 phosphorylation and gene expression. MC-42 cell were grown for 7 days in the presence or absence of AA and/or saturating BMP2/7 (50 μg/mL) and treated with U0126 and/or SB203580 16 hours before harvest as indicated. Cells were assayed for mOG2-luc activity (*upper panel*) or RUNX2, ERK, p38, and Smad1,5,8 phosphorylation (*lower panel*). Statistically significant differences are indicated: $^*p < 0.05$, $^{**}p < 0.01$.

Elk-1 (ERK and JNK), ATF-2 (p38 and JNK), and SAP-1/SAP-2 (ERK and p38).[31] The D site in RUNX2 has properties similar to those of other MAPK-responsive transcription factors in that it is immediately N-terminal to a transcription activation domain that contains MAPK phosphoacceptor sites necessary for transcriptional activation[31] (ERK sites at S301, S319, S510[18] and p38 sites at S254, S319).[22] Interestingly, ERK and p38 were shown to compete for binding to the RUNX2 D site (Fig. 4). This competition was demonstrated in two ways: (1) in coimmunoprecipitation experiments, increasing levels of P-ERK were able to disrupt RUNX2:P-p38 complexes and vice versa. (2) Expression of increasing amounts of DN ERK1 strongly inhibited ERK and p38-dependent RUNX2-S319 phosphorylation and transcriptional activity while DN p38β strongly inhibited p38 and partially inhibited ERK1. Results from both these studies support the conclusion that ERK1 has a relatively higher affinity for the D site when compared with p38. One consequence of this competition is that when constitutively active MKK6/p38β was expressed in the presence of MEK1/ERK1, RUNX2 phosphorylation and transcriptional activity was actually lower than that observed with constitutively active MEK1/ERK1 alone, a result likely because of displacement of ERK by the less active p38 (Fig. 3C). Also, overexpression of DN MKK6, which blocked p38 activation, actually increased P-ERK, RUNX2-S-319-P, and transcriptional activity, again consistent with the idea that endogenous p38 was initially competing with ERK on RUNX2 (Fig. 4F).

An additional important finding concerns the relationship between ERK and p38 activities. Regardless of the experimental system, we consistently found that suppression of one MAPK was associated with activation of the other. This was true regardless of whether we used pharmacological inhibitors to block ERK versus p38 signaling (Fig. 5) or transgenic modification of MEK1 activity (Fig. 6) and was found in transfected COS7 cells, MC3T3-E1 preosteoblasts, calvarial organ cultures, or primary osteoblasts from transgenic mice. This reciprocal regulation had some unexpected consequences. Although the ERK1/2 inhibitor, U0126, consistently blocked RUNX2 phosphorylation, transcriptional activity and calvarial mineralization (Fig. 5), p38 inhibition with SB203580 either had no effect or actually increased RUNX2 phosphorylation and transcriptional activity. Because p38 stimulated RUNX2 activity in the overexpression studies shown in Figures 2–4, one would have predicted its inhibition to reduce activity. This paradoxical result may be a consequence of the stimulation in ERK/MAPK activity found after SB203580 treatment combined with a reduction of p38 at the RUNX2 D site thereby allowing increased docking and phosphorylation by ERK1/2. On the other hand, when cells/organ cultures were treated with both ERK and p38 inhibitors, RUNX2 phosphorylation, transcriptional activity and mineralization (organ cultures only) were reduced to levels below those observed with the ERK inhibitor alone. This suggests that p38 is responsible for the residual RUNX2 activity found in U0126-treated samples, but does not have a major role in controlling RUNX2 activity under conditions where there is a strong ERK/MAPK signal. Reciprocal regulation of ERK and p38 MAPKs was previously reported in other systems such as osteoclasts where inhibition of one MAPK increases the other[40] and in osteoblasts from subchondral bone that exhibit increased ERK and reduced p38 activity.[41]

Although in vivo transgenic studies clearly showed that ERK and p38 MAPK pathways are both necessary for bone formation,[20–22] based on our studies, it is unlikely that these two kinases function in a redundant manner to control RUNX2 activity and osteoblast differentiation. Some insight into their separate functions may be gleaned from the studies shown in Figure 7 that compared AA and BMP-induced osteoblast activities. Standard conditions for inducing osteoblast differentiation involve providing an environment conducive to collagen matrix secretion by inclusion of AA in tissue culture media. Numerous studies showed that the resulting ECM induces osteoblast differentiation, at least in part, by interacting with cells via collagen-binding integrins ($\alpha 2\beta 1$ and $\alpha 1\beta 1$) and the discoidin domain receptor 2 (DDR2), leading to activation of ERK/MAPK signaling.[13,33] Although AA may also regulate gene expression via activation antioxidant-responsive elements in target genes,[42] its long-term effects on osteoblast differentiation are most likely mediated by the ECM/integrin/MAPK pathway described above. The ability of AA to dramatically stimulate ERK/RUNX2 phosphorylation and osteoblast gene expression was shown in Figure 7B. Under these conditions, P-p38 levels remained essentially unchanged. AA-induced RUNX2 S319 phosphorylation and gene expression were highly sensitive to the ERK/MAPK inhibitor, but were refractory to p38 inhibition (Figs. 5, 7D). A different situation was encountered when the combined effects of AA and BMP treatment were examined. As previously reported,[14,43] induction of osteoblast gene expression/differentiation by BMPs is synergistically stimulated by AA (Fig. 7CD). The mild stimulation of gene expression by BMPs in the absence of AA was sensitive to ERK, but not p38 inhibition. In contrast, in the presence of AA, BMP dramatically stimulated P-ERK and RUNX2-S319 phosphorylation while synergistically stimulating mOG2-luc activity. This response was still completely blocked by ERK inhibition and partially reduced with the p38 inhibitor. However, although ERK inhibition completely blocked RUNX2 phosphorylation, p38 inhibition was without effect.

Several important conclusions may be drawn from these studies: first, the BMP pathway can stimulate RUNX2 S319 phosphorylation and transcriptional activity, particularly in the presence of AA, and this stimulation is not associated with increased levels of RUNX2. This provides an alternate route for BMP regulation of RUNX2 in addition to the previously described induction of *Runx2* expression.[44] Second, although BMPs via TAK1 are known to activate all 3 MAPKs, ERK1/2 is a major mediator of this response in osteoblasts with p38 having a minor role via a mechanism unrelated to RUNX2 S319 phosphorylation. Finally, the synergistic stimulation in gene expression observed with BMP and AA treatment was also partly dependent on p38. This p38 component distinguishes the AA/BMP response from the response in cells treated only with AA that were resistant to p38 inhibition. However, blocking p38 in this case did not noticeably affect RUNX2-S319-P. This suggests either that additional RUNX2 phosphorylation sites are involved or that p38 regulates osteoblast gene expression by controlling the activity of other transcription factors. In this regard, p38 is known to phosphorylate/activate OSX or DLX5 in response to BMP treatment.[45,46]

Disclosures

Acknowledgments

References

1. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, Takayanagi H. NFAT and Osterix cooperatively regulate bone formation. Nat Med. 2005;11(8):880–5.

2. Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev. 1999;13(8): 1025–36.

3. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89(5):755–64.

4. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell. 1997;89(5):765–71.

5. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002;108(1):17–29.

6. Bialek P, Kern B, Yang X, Schrock M, Sosic D, Hong N, Wu H, Yu K, Ornitz DM, Olson EN, Justice MJ, Karsenty G. A twist code determines the onset of osteoblast differentiation. Dev Cell. 2004;6(3):423–35.

7. Tou L, Quibria N, Alexander JM. Transcriptional regulation of the human Runx2/Cbfa1 gene promoter by bone morphogenetic protein-7. Mol Cell Endocrinol. 2003;205(1–2):121–9.

8. Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, Franceschi RT. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem. 2000; 275(6):4453–9.

9. Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. Transcriptional regulation of osteoblasts. Ann NY Acad Sci. 2007;1116:196–207.

10. Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT. Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem. 2002;277(39):36181–7.

11. Hurley MM, Marcello K, Abreu C, Kessler M. Signal transduction by basic fibroblast growth factor in rat osteoblastic Py1a cells. J Bone Miner Res. 1996;11(9):1256–63.

12. Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-beta receptor down-regulation by collagen-alpha2beta1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem. 1997;272(46):29309–16.

13. Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem. 1998;273(49): 32988–94.

14. Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res. 2002;17(1):101–10.

15. Khatiwala CB, Kim PD, Peyton SR, Putnam AJ. ECM compliance regulates osteogenesis by influencing MAPK signaling downstream of RhoA and ROCK. J Bone Miner Res. 2009;24(5):886–98.

16. You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem. 2001;276(16): 13365–71.

17. Boutahar N, Guignandon A, Vico L, Lafage-Proust MH. Mechanical strain on osteoblasts activates autophosphorylation of focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. J Biol Chem. 2004;279(29):30588–99.

18. Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, Franceschi RT. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. J Biol Chem. 2009;284(47):32533–43.

19. Li Y, Ge C, Franceschi RT. Differentiation-dependent association of phosphorylated extracellular signal-regulated kinase with the chromatin of osteoblast-related genes. J Bone Miner Res. 2010; 25(1):154–63.

20. Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol. 2007;176(5):709–18.

21. Matsushita T, Chan YY, Kawanami A, Balmes G, Landreth GE, Murakami S. Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. Mol Cell Biol. 2009;29(21):5843–57.

22. Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, Lotinun S, Sano Y, Baron R, Park JM, Arthur S, Xie M, Schneider MD, Zhai B, Gygi S, Davis R, Glimcher LH. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. J Clin Invest. 2010; 120(7):2457–73.

23. Shim JH, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, Gygi S, Glimcher LH. TAK1 is an essential regulator of BMP signalling in cartilage. EMBO J. 2009;28(14):2028–41.

24. Ducy P, Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol. 1995;15(4):1858–69.

25. Thirunavukkarasu K, Mahajan M, McLarren KW, Stifani S, Karsenty G. Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfbeta. Mol Cell Biol. 1998;18(7): 4197–208.

26. Stewart S, Sundaram M, Zhang Y, Lee J, Han M, Guan KL. Kinase suppressor of Ras forms a multiprotein signaling complex and modulates MEK localization. Mol Cell Biol. 1999;19(8): 5523–34.

27. Zheng CF, Guan KL. Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem. 1993;268(32):23933–9.

28. Huang S, Jiang Y, Li Z, Nishida E, Mathias P, Lin S, Ulevitch RJ, Nemerow GR, Han J. Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. Immunity. 1997;6(6):739–49.

29. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. J Virol. 1997; 71(3):1842–9.

30. Xiao G, Cui Y, Ducy P, Karsenty G, Franceschi RT. Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. Mol Endocrinol. 1997;11(8): 1103–13.

31. Sharrocks AD, Yang SH, Galanis A. Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem Sci. 2000; 25(9):448–53.

32. Yang SH, Whitmarsh AJ, Davis RJ, Sharrocks AD. Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. EMBO J. 1998;17(6):1740–9.

33. Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, Yao L. An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. J Bone Miner Res. 2010;26(3):604–17.

34. Suzawa M, Tamura Y, Fukumoto S, Miyazono K, Fujita T, Kato S, Takeuchi Y. Stimulation of Smad1 transcriptional activity by Ras-extracellular signal-regulated kinase pathway: a possible mechanism for collagen-dependent osteoblastic differentiation. J Bone Miner Res. 2002;17(2):240–8.

35. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res. 1992;7(2):235–46.

36. Kefaloyianni E, Gaitanaki C, Beis I. ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-kappaB transactivation during oxidative stress in skeletal myoblasts. Cell Signal. 2006; 18(12):2238–51.

37. Deak M, Clifton AD, Lucocq LM, Alessi DR. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J. 1998; 17(15):4426–41.

38. Nishimura M, Shin MS, Singhirunnusorn P, Suzuki S, Kawanishi M, Koizumi K, Saiki I, Sakurai H. TAK1-mediated serine/threonine phos-phorylation of epidermal growth factor receptor via p38/extracellular signal-regulated kinase: NF-{kappa}B-independent survival pathways in tumor necrosis factor alpha signaling. Mol Cell Biol. 2009;29(20): 5529–39.

39. Akella R, Moon TM, Goldsmith EJ. Unique MAP Kinase binding sites. Biochim Biophys Acta. 2008;1784(1):48–55.

40. Hotokezaka H, Sakai E, Kanaoka K, Saito K, Matsuo K, Kitaura H, Yoshida N, Nakayama K. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J Biol Chem. 2002;277(49):47366–72.

41. Prasadam I, van Gennip S, Friis T, Shi W, Crawford R, Xiao Y. ERK-1/2 and p38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteo-blasts. Arthritis Rheum. 2010;62(5):1349–60.

42. Xing W, Singgih A, Kapoor A, Alarcon CM, Baylink DJ, Mohan S. Nuclear factor-E2-related factor-1 mediates ascorbic acid induction of osterix expression via interaction with antioxidant-responsive element in bone cells. J Biol Chem. 2007;282(30): 22052–61.

43. Asahina I, Sampath TK, Nishimura I, Hauschka PV. Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. J Cell Biol. 1993;123(4):921–33.

44. Hassan MQ, Tare RS, Lee SH, Mandeville M, Morasso MI, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. BMP2 commitment to the osteogenic lineage involves activation of Runx2 by DLX3 and a homeodomain transcriptional network. J Biol Chem. 2006;281(52): 40515–26.

45. Ulsamer A, Ortuno MJ, Ruiz S, Susperregui AR, Osses N, Rosa JL, Ventura F. BMP-2 induces Osterix expression through up-regulation of Dlx5 and its phosphorylation by p38. J Biol Chem. 2008;283(7): 3816–26.

46. Ortuno MJ, Ruiz-Gaspa S, Rodriguez-Carballo E, Susperregui AR, Bartrons R, Rosa JL, Ventura F. p38 regulates expression of osteo-blast-specific genes by phosphorylation of osterix. J Biol Chem. 2010;285(42):31985–94.