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PTH Regulates Myeloid ELF-1-Like Factor (MEF)-Induced MAB-21-Like-1 (MAB21L1) Expression Through the JNK1 Pathway

Byung-Gyu Kim,[1] Youn-Je Park,[2] Towia A. Libermann,[3*] and Je-Yoel Cho[1*]

[1]*Department of Biochemistry, School of Dentistry, Kyungpook National University, and 2nd BK21 program 700-422, Korea*

[2]*Department of Applied Bioscience, CHA University, Seoul, 135-081, Korea*

[3]*BIDMC Genomics Center, Harvard Medical School, Boston, MA*

ABSTRACT

Continuous treatment with parathyroid hormone (PTH) or excess endogenous PTH due to primary hyperparathyroidism causes increased bone resorption and, subsequently, decreased bone volume. Our previous studies showed that myeloid Elf-1-like factor (MEF) not only suppresses osteoblast differentiation through inhibition of Runx2 activity and other osteogenesis-related genes but also specifically increases the expression of Mab21, a potential transcriptional repressor of osteoblast differentiation. Here we show that the JNK1 pathway is involved in the MEF-mediated up-regulation of Mab21 expression due to PTH stimulation. PTH increased the transcription level of Mab21 in MG63 human osteoblastic cells, in contrast to the suppressive effect of TGFβ1. PTH phosphorylates serine residues of MEF as well as c-Jun, a known substrate of JNK1. By *in vitro* kinase assay, we confirmed that MEF is phosphorylated by JNK1, but not by ERK. Co-transfection of MEF with both MKK4 and JNK1 increased the promoter activity of Mab21 in CV1 cells significantly more than MEF alone. We also identified the phosphorylation of MEF serine 641 by *in vitro* and *in vivo* JNK1 kinase assays combined with a proteomics approach. In conclusion, our findings indicate that MEF is involved in PTH suppression of osteoblasts through activating the MKK4/JNK1 pathway and subsequently up-regulating Mab21 expression. J. Cell. Biochem. 112: 2051–2061, 2011.

KEY WORDS: ETS TRANSCRIPTION FACTOR; OSTEOBLAST; PARATHYROID HORMONE; MASS SPECTROMETRY; MAB21; JNK

The *ETS* family is one of the largest transcription factor families, containing more than 30 members, and has become increasingly recognized as a critical regulator in such diverse biological processes as hematopoiesis, the immune response, homeostasis, spermatogenesis, cancer progression and bone development [Vary et al., 2000; Chen et al., 2005; Gallant and Gilkeson, 2006; Dwyer et al., 2007; Gutierrez-Hartmann et al., 2007]. Recently, ETS-1 was reported to be involved in the transcriptional regulation of extracellular matrix enzymes such as collagenase and MMP-13 in PTH-stimulated osteoblasts [Charoonpatrapong-Panyayong et al., 2007; Quinn et al., 2000]. However, the functions of other member of the *ETS* family, including MEF (myeloid Elf-1-like factor, also known as ELF4), in PTH-stimulated osteoblasts and the molecular mechanism by which those genes are targeted has not been elucidated. MEF, a member of the elf-1/e74 family of ETS proteins, is known to regulate the basal expression of anti-microbial

Abbreviations: MEF, myeloid elf-1-like factor; MAB21L1, MAB-21-like 1; PTH, parathyroid hormone; MAPK, mitogen-activated protein kinase; MKK4, mitogen-activated protein kinase 4; JNK1, c-Jun N-terminal kinase 1; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight.

Byung-Gyu Kim and Youn-Je Park contributed equally to this study.

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*Correspondence to: Je-Yoel Cho, DVM, PhD, Department of Biochemistry, School of Dentistry, Kyungpook National University, 101 Dong In-Dong, Jung-Gu, Daegu 700-422, Korea.
E-mail: jeycho@knu.ac.kr

*Correspondence to: Towia A. Libermann, PhD, BIDMC Genomics Center, Harvard Medical School, Boston, MA.
E-mail: tliberma@bidmc.harvard.edu

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peptides in epithelial cells, to be required for normal NK cell and NK T-cell development, and to activate the expression of various ligands in hematopoietic stem cells [Miyazaki et al., 1996; Kai et al., 1999; Suico et al., 2002; Lacorazza and Nimer, 2003; Suico et al., 2004; Gallant and Gilkeson, 2006].

In our previous study, we showed that the expression of MEF suppressed BMP2 action, which up-regulated Runx2 activity and the expression of its target genes in preosteoblast MC3T3E1 cells [Kim et al., 2007]. Our recent study also demonstrated that osteoblast-specific expression of MEF induces osteopenia by down-regulating osteogenesis and up-regulating osteoclastogenesis in transgenic mice [Seul et al., 2011]. Conversely, MEF is down-regulated during BMP2-induced osteoblast differentiation. Thus, the up-regulation of BMP2-induced osteogenic factors could be achieved by attenuating the transcription of MEF (which suppresses Runx2), resulting in increased Runx2 activity. These results strongly suggest that MEF has a suppressive effect on osteoblast differentiation.

Our group showed that BMP2 also suppresses the transcription of Mab21L1, which is a transcriptional repressor and is required for embryonic development and sensory organ development [Mariani et al., 1998; Wong and Chow, 2002; Yamada et al., 2003; Merello et al., 2004; Kim et al., 2007]. Recently, it has been reported that the suppressive function of Mab21L1 is mediated by its physical interaction with Sin3, a known co-repressor [Choy et al., 2007].

PTH, an 84-amino acid polypeptide, is secreted by the parathyroid glands and increases the concentration of calcium in the blood by acting on parathyroid hormone receptors in the bone, kidney and intestine [Neer et al., 2001; Kaji, 2007]. Although exogenous PTH is an anabolic agent when administered intermittently [Goltzman, 2008], endogenous PTH is considered to be a catabolic agent for bone in both physiological [Krieger and Tashjian, 1980; Poole and Reeve, 2005] and pathophysiological conditions (such as hyperparathyroidism) [Marx, 2000]. The pathologic conditions of primary hyperparathyroidism is characterized by an abnormally high level of PTH in blood which is caused by the direct disturbances of the parathyroid gland, such as parathyroid adenoma, parathyroid hyperplasia and parathyroid cancer. Increased levels of PTH can also lead to progressive bone diseases, such as secondary osteoporosis, by increasing bone resorption rates such that they exceed bone formation rates [Marx, 2000; Poole and Reeve, 2005]. PTH is largely mediated via the cAMP/PKA and PKC pathway [Partridge et al., 1994; Swarthout et al., 2002], but some of its actions are regulated via the ERK [Gesty-Palmer et al., 2006; Rey et al., 2007] or PI3K pathway. However, the molecular mechanism of the direct effect of PTH on osteoblast differentiation is not clear [Ihara-Watanabe et al., 2004; Yamamoto et al., 2007; Lowry et al., 2008].

In the present study, we demonstrated that treatment of low molar (*M*) concentrations of PTH up-regulates MEF activity via the JNK/MAPK pathway and consequently increases the transcription of Mab21L1 in MG63 cells. We also identified a JNK-activated MEF phosphorylation site by proteomics techniques. In addition, we found that both MEF and Mab21L1 are related to the survival mechanism of human osteoblast cells. These findings could provide an understanding of human bony defect diseases caused by PTH dysfunction.

MATERIALS AND METHODS

MATERIALS

Bioactive recombinant human parathyroid hormone (PTH 1–34) protein and recombinant human TGFβ1 were obtained from Sigma (St. Louis, MO). LipofectAMINE Plus reagents were purchased from Invitrogen (Grand Island, NY). The Bradford protein assay kit and the Coomassie G250 stain reagents were obtained from Bio-Rad (Hercules, CA). The anti-FLAG agarose affinity gel, 3 × FLAG peptide and anti-FLAG M2 monoclonal antibody were purchased from Sigma (St. Louis, MO). The phospho-Jun antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

CELL CULTURE AND TREATMENT REAGENTS

MG63 a human immature osteoblastic cell line, CV-1 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal bovine serum (FBS) and antibiotic-antimycotic liquid (1006, GIBCO) at 37°C in a 5% CO_2 incubator. To examine the effects of PTH and TGFβ1 on *Mab21L1* levels in the presence or absence of MG63, the cells were cultured for the indicated periods with or without treatment with the indicated amounts of the reagents. All cells were harvested in phosphate-buffered saline (PBS) at 4°C by scraping with a rubber policeman.

MICROARRAY ANALYSIS

To explore the gene expression changes associated with over-expression of *ETS* transcription factors, MG 63 cells were transfected with expression vectors for Nerf-1a, Nerf-1b, Nerf-2, Elf-1 and MEF as well as with a control vector, followed by collection of RNAs from the cells. The cRNAs were prepared from two biologically independent experiments and were hybridized with human oligo HU95Av2 arrays (Affymetrix, Santa Clara, CA). All the GeneChip analyses, including cRNA probe synthesis and scanning, were done as suggested in the protocol provided by the manufacturer (Affymetrix, Santa Clara, CA). All Affymetrix data was screened for those genes that showed a large change (more than 2 fold) in gene expression in the *ETS* family gene transfected cells compared to the control vector (pCi) cells.

REAL-TIME PCR

Quantitative real-time PCR (RT-PCR) analysis was used to confirm the microarray results for the *Mab21L1* gene in MG63 cells with overexpression vectors of MEF, ELF, NERF2, NERF1B, NERF1A or pCi. GAPDH was used as an internal control in all mRNA expression analyses, and fluorescence-based real-time PCR was performed with the DNA Engine OPTICON® 2 system (MJ Research, Waltham, MA). SYBR green I Dye (Molecular Probes, Eugene, OR) and Go Taq® Flexi DNA polymerase (Promega, Madison, WI) were used for the PCR reactions. Primers used for the real-time PCR for *Mab21L1* expression were Mab21L1-F1, 5′-CACCAGGGAAGTGAAACTGA-3′ and Mab21L1-R1, 5′-TTGAGGATGGAGAGGCACTT-3′.

LUCIFERASE ASSAY

Cos-7 cells, a transformed monkey kidney cell line, or CV-1 cells, an African green monkey kidney cell line, were cultured in 96-well plates at a density of 1.5×10^4 cells/well for 16–24 h and transfected

with the expression and luciferase vectors using the LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA). A total of 250 ng of DNA was used per well in each transfection of the expression vectors (pCi, NERF1A, NERF1B, NERF2, ELF and MEF) and the *Mab21L1*-pGL2B reporter (2.0 kb, 1.5 kb or 0.5 kb). To measure the effect of MEF on Mab21L1 promoter activity via the MKK4/JNK1 pathway, CV-1 cells were used. After 3 h, the media was replaced by media containing 10% FBS, and cells were cultured overnight. Cell lysates were prepared, and luciferase activity was determined with the Luciferin-Glo Assay System kit (Promega).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) ANALYSIS

The sense-strand sequence of the double-strand oligonucleotides (from probe E) are as follows: sense primer, 5′-AGCTGCCTTTA-TAATTTCCACACGAG-3′; and anti-sense primer, 5′-TCGACTCG-TGTGGAAATTATAAAGGC-3′ (underlined nucleotides indicate the Ets transcription factor consensus binding site). *In vitro* transcription, translation and EMSA were done as previously described [Chae et al., 2009]. Double-stranded DNA probes (Probe E) were end-labeled with α-^{32}P-dCTP using the Klenow enzyme. The N1a, N1b, N2, ELF-1 and MEF proteins were produced by *in vitro* transcription and translation using TNT Coupled Reticulocyte lysate (Promega). The proteins were then bound to the labeled, double-stranded DNA probes in the presence or absence of a 50-fold or 100-fold molar excess of unlabeled competitor (for MEF only) for 20 min at room temperature.

IN VITRO KINASE REACTION

For the [γ–^{32}P] ATP *in vitro* kinase reactions of MEF, the substrate MEF proteins were first purified from the MG63 osteoblastic cells transfected with Flag-MEF-pCDNA3 expression construct or control Flag-pCDNA3 construct. For the JNK or ERK kinase reaction, anti-FLAG mAb-conjugated agarose beads were used to immunoprecipitate Flag-MEF proteins. Then, the anti-Flag immune complexes were washed five times with dilution buffer and twice with cold PBS and then incubated with kinase buffer (20 mM HEPES, pH 7.4, 0.03% Triton X-100, 75 mM $MgCl_2$, 500 mM cold ATP and 10 mCi [γ–^{32}P]ATP) for 1 h at room temperature. The JNK kinase reactions were stopped by adding protein gel loading dye and boiling for 5 min. The products of the kinase reactions were resolved by SDS–PAGE. Labeled protein bands were visualized by autoradiography.

For the identification of MEF phosphorylation sites by JNK kinase, the JNK kinase reaction was done. The substrate MEF proteins were purified by anti-Flag mAb conjugated agarose beads from the HEK293T cells transfected with Flag-MEF-pCDNA3 expression construct. The immunoprecipitated proteins were washed three times with cold PBS and once with kinase buffer (20 mM HEPES, pH 7.4, and 0.03% Triton X-100). Reactions were carried out in 200 μL kinase buffer supplemented with 0.2 mM ATP and 50–100 ng JNK (Cell Signaling, Beverly MA, USA) for 1 h at 30°C. Assayed proteins were washed twice with cold PBS and once with cold PBST (PBS with 0.1% Tween 20), and 50 mL of non-reducing SDS sample buffer was added. The proteins were boiled for 3 min and centrifuged at 2500*g* for 1 min. The supernatant was transferred to an Eppendorf tube, and β-mercaptoethanol was added to a final concentration of 1:20 (v/v). The reduced proteins were resolved by SDS-PAGE and stained with Coomassie Blue reagents.

MALDI-TOF/TOF MASS SPECTROMETRY ANALYSIS

In-gel trypsin digestion of the bands from the Coomassie stained gel was performed as previously reported [Park et al., 2009]. Digested peptides were directly spotted onto a MALDI target plate (polished steel 96 target plate, Bruker), and the matrix solution (5 mg/mL of alpha-cyano-4-hydroxycinnamic acid, HCCA, Bruker and 1 mg/mL of 2,5-dihydroxybenzoic acid (DHB) Bruker in 50% ACN containing 0.1% TFA) was added to each spot according to the dried droplet method (Bruker manufacturer's instructions). The tryptic peptide mixtures were analyzed using a MALDI-TOF/TOF instrument (Ultraflex I, Bruker).

Peptides of interest were identified by peptide mass fingerprints (PMF) and by comparison with the MASCOT 2.0 database (Matrix Science, UK). The peak mass list was created using Flex Analysis 2.4 with the following parameters: a signal-to-noise ratio of 3, a quality factor of 50, a window of analysis between 600 to 4000 m/z, an exclusion of classic contaminants (keratin, matrix cluster ions, sodium adducts, and trypsin autolysis peaks), and a maximum number of selected peaks of 200. The mass list obtained was used to query the IPI Human 3.29 database (69,965 entries) with the following parameters: charge state of +1, mass tolerance 150 ppm, variable modifications including phosphorylation on S, T, and Y, and tolerance for one missed cleavage.

For the MEF peptides, the peak list for the MS/MS data analysis for amino acid residues 641–657, (containing the residue S641) was generated using Flex Analysis 2.4 (signal-to-noise ratio of 12 and a relative intensity threshold of 0%). The MS/MS database search (BioTools 3.0) was performed with a tolerance of three missed cleavages in the IPI Human 3.29 database. The mass tolerance for precursor ions was set at 0.2 and 0.5 Da for fragment ions, and the cutoff score was set to the default MASCOT threshold of 5%.

TRANSIENT TRANSFECTION AND IMMUNOPRECIPITATION

Cells were transfected with Lipofectamine Plus reagent according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA). After HEK 293T cells were cultured in 150-mm dishes for 24 h, the medium was replaced with EMEM media containing charcoal-stripped serum, and the cells were transfected with either the CMV-5′Flag-MEF-pCDNA3.1, the CMV-JNK1-pCi construct, CMV-MKK4-pCi, or empty vector. The cells were then transferred to fresh growth medium for 24–36 h and collected in lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 1:30 diluted protease inhibitor mixture, Roche Applied Science, and 1:100 diluted phosphatase inhibitor cocktail, Sigma-Aldrich). The insoluble cell debris was removed by centrifugation at 14,000*g* for 20 min at 4°C, and the supernatants were transferred to new tubes. Total cell lysates were diluted at a 4:6 ratio with immunoprecipitation dilution buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1:30 diluted protease inhibitor mixture). Twenty microliters of anti-FLAG mAb-conjugated agarose beads (M2-agarose, Sigma) were added to the cell lysates, and immunoprecipitations were carried out overnight at 4°C with slow rotation. Immunoprecipitated complexes were

washed five times with 25 mM Tris, 2.7 mM KCl, and 137 mM NaCl (pH 7.4). After the final wash, the bound proteins were eluted in non-reducing SDS sample buffer (63 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 0.005% bromophenol blue) or with 3x FLAG peptide solution (150 ng/mL final concentration; Sigma). The samples were boiled for 3 min in SDS sample buffer and loaded onto an acrylamide gel.

WESTERN BLOT ANALYSIS

Western blot analysis was done as previously described [Kim et al., 2009]. Briefly, PTH at 10^{-10} M, 10^{-9} M, 10^{-8} M and 10^{-9} M was treated for 5 min before cell collection to detect phospho-c-jun levels. To determine the effect of PTH on the phosphorylation state of MEF, the MG63 cells were treated with PTH under the indicated conditions for 0 min, 10 min, 30 min, 120 min, and Flag-MEF proteins were immunoprecipitated (using an Anti-Flag M2 bead solution). Protein samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho-c-Jun (Ser-63) rabbit antibody or anti-phospho-serine antibody (Zymed, Carlsbad, CA). The blots were then reacted with horseradish peroxidase-coupled goat secondary antibody. Signals were developed with the ECL-PLUS detection reagent, and the membranes were developed by exposure to x-ray film for an appropriate length of time.

RESULTS

MAB21L1 IS A POTENTIAL TARGET OF THE MEF TRANSCRIPTION FACTOR

To obtain information on the target genes of the selected *ELF* family *ETS* genes in osteoblastic cells, we performed oligo-based microarray analysis using the RNAs from MG63 cells transiently transfected with pCi (control vector), NERF-1a, -1b, -2, ELF-1 or MEF for 24 h. The microarray analysis data showed that among all the regulated genes, *MAB21L1* was highly up-regulated in MG63 human osteoblastic cells when MEF was over-expressed (Fig. 1) (complete data will be published at a later date). All of these ELF family genes were expressed endogenously in MG63 cells when analyzed by RT-PCR. The endogenous expression levels of the ELF1 and MEF tend to be higher than NERF1a, 1b and 2 in the cells (Supplementary Figure 1). Real-time PCR data of the mRNA expression of *MAB21L1* in the MG63 cells transiently expressing the ELF family genes showed consistent results with the microarray data (Fig. 2A).

MEF TRANSACTIVATES MAB21L1 BY BINDING ON ITS PROMOTER

To study whether MEF has a direct transactivity on the *MAB21L1* promoter, we first cloned the −2.0 kb upstream promoter region into the pGL2B vector. The results of the *MAB21L1* −2.0 kb promoter assay in MEF-transfected cells were generally consistent with the microarray data, although both ELF-1 and MEF up-regulated *MAB21L1* levels in a similar manner (Fig. 2B). The deletion of the promoter down to −0.5 kb resulted in a similar level of activity as the promoter in both the ELF-1- and MEF-expressing Cos-7 cells, although only a slight decrease was observed in the −1.6 kb promoter constructs (Fig. 2C). Then we synthesized 9 different EMSA probes with a potential MEF binding sites with core motif of either GGAA or TTCC (Fig. 2D). Our EMSA data showed that probe E had strongest binding activity (data not shown). When using probe E, the EMSA showed that the DNA binding affinity of MEF to the promoter proximal probe E of *MAB21L1* was greater than that of ELF (Fig. 2D). Thus, we focused on the regulatory mechanisms of MEF on *MAB21L1* transcription in osteoblast cells. Because MEF appeared to be a negative regulator of osteoblast differentiation in our previous studies [Kim et al., 2007; Seul et al., 2010], we speculated that *MAB21L1*, which is up-regulated by MEF, could also be involved in suppressing several catabolic osteogenic factors in the osteoblast differentiation process.

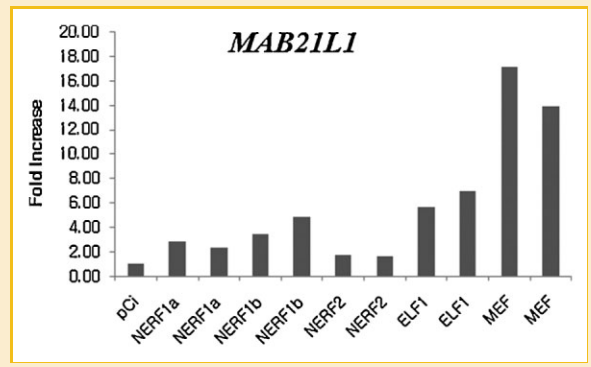

Fig. 1. MEF increases *MAB21L1* (a cell-fate determining factor) transcription levels. RNAs were isolated from MG63 osteoblastic cells 24 h after the transient transfection (in duplicate) of pCi, NERF-1a, -1b, -2, Elf-1 or MEF-expressing vectors. The mRNA expression levels were analyzed by an Affymetrix oligo-based microarray. The graph shows the changes in MAB21L1 mRNA expression in the cells after overexpression of ELF subfamily transcription factors. MEF overexpression produced the most significant increases in the levels of MAB21L1 (17.1- and 13.9-fold in two experiments).

PTH INCREASES MAB21L1 TRANSCRIPTION, BUT TGFβ DECREASES MAB21L1 TRANSCRIPTION

To examine whether *MAB21L1* is regulated by PTH, a representative suppressive factor, we tested the effect of PTH on the transcription level of *MAB21L1* in MG 63 cells at different time points and under different doses. Real-time PCR results showed that PTH (10^{-10} or 10^{-8} M) up-regulated *MAB21L1* levels at 30 min and 24 h after stimulation (Fig. 3A). TGFβ1 (1–3 ng/ml) stimulation, however, resulted in a significant reduction of *MAB21L1* mRNA levels at 4, 8 and 24 h of treatment; although smaller decreases in mRNA levels were observed at higher concentrations for the time period tested (Fig. 3B). These results suggest that MAB21L1 and MEF are involved in the negative regulation of PTH during osteoblast differentiation.

PTH ACTIVATES JNK1, WHICH PHOSPHORYLATES MEF AND INCREASES ITS TRANSACTIVITY

The cAMP-protein kinase A (PKA) pathway is well known as a major contributor to PTH signaling. However, there are several reports that MAPK (p38, JNK or ERK) signaling is also activated either through the PKA/PKC pathway or by direct activation by PTH receptor after PTH treatment [Buzzi et al., 2007; Liu et al., 2008; Rey et al., 2007];

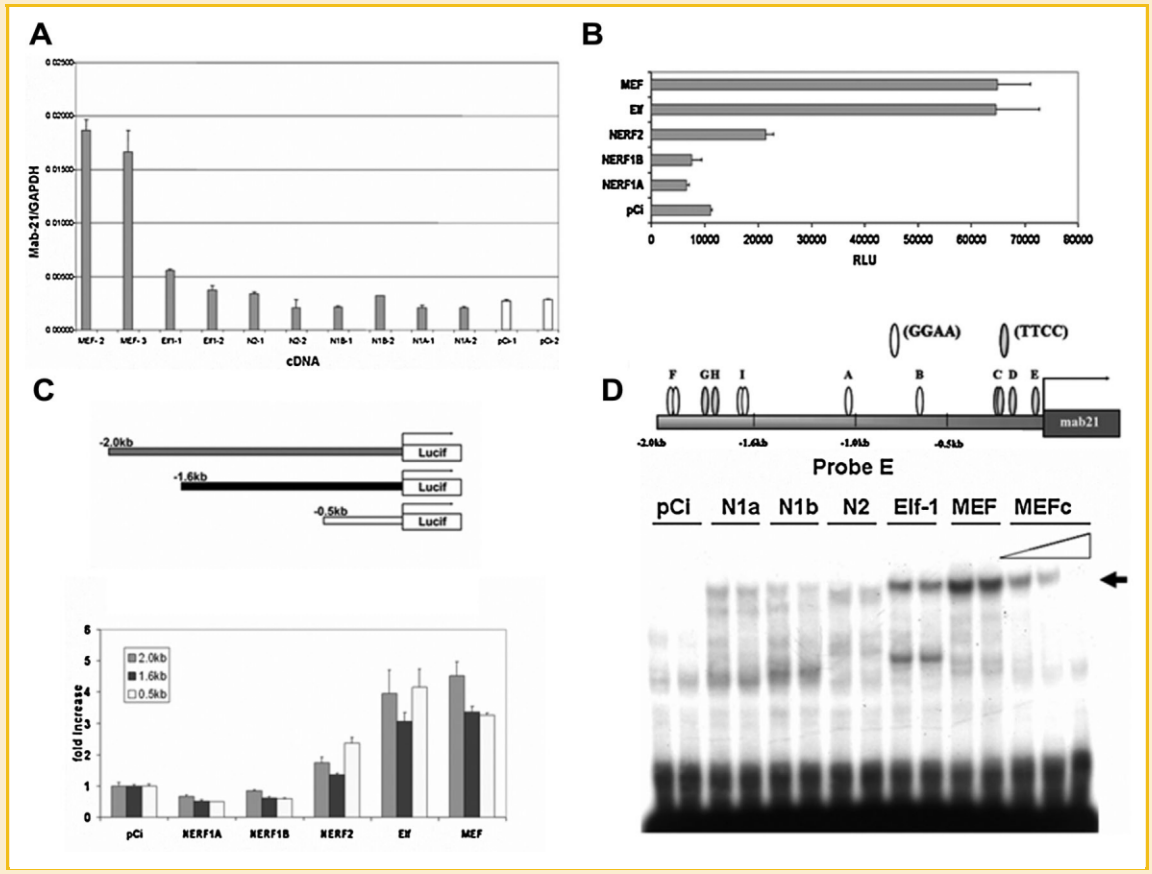

Fig. 2. MEF up-regulates *MAB21L1* promoter activity. **A:** Real-time PCR assay of *MAB21L1* showing that *MAB21L1* mRNA levels were increased by MEF. Duplicate experiments were done, with triplicate analysis for each. **B:** Luciferase assay was done using by transfecting the Cos-7 cells with the −2.0 kb MAB21L1-Luc and each ETS expression vector. The data showed that MEF and ELF-1 activate the −2.0 kb *MAB21L1* promoter. **C:** Three constructs of *MAB21L1* promoters (−2.0 kb, −1.6 kb and −0.5 kb) were cloned into pGL2B luciferase construct. The luciferase assay, with a 1:1 ratio of expression vector to reporter, showed that the −0.5 kb *MAB21L1* promoter was still activated by MEF and Elf-1. **D:** Schematic of potential MEF response elements (A to I) with the core motif of GGAA or TTCC within the *MAB21L1* −2.0Kb promoter regions. EMSA of ETS family using various probes (A to I) showed that MEF can directly bind to the potential ETS binding site within probe E (data not shown). The EMSA shows that probe E binds most strongly to MEF, and cold MEF (MEFc) competed off the binding of MEF to probe E.

although some reports have shown PTH-induced inhibition of JNK [Doggett et al., 2002]. Because many ETS transcription factors are activated by MAPK signaling pathways [Wasylyk et al., 1998], we hypothesized that the MAPK pathway was also involved in the signal transduction from PTH/PTHR to MEF activation. Thus, we next examined whether PTH regulates Mab21 transcription through the activation of MEF via the MAPK/JNK pathway. First, PTH was able to phosphorylate c-jun, a representative substrate of JNK (detailed procedures are described in Fig. 4A legend). However, PTH treatment did not significantly change the Erk1/2 kinase activities, as measured by the phospho-Elk-1 levels determined by the anti-p-Elk-1 antibody, after incubation with the purified Elk-1 and ATP on immunoprecipitated Erk1/2 (p44/42) from MG63 cells; although the kinase activity was slightly elevated 10 min after addition of PTH (Fig. 4B top panel). The kinase activity of p38α was also not significantly changed by PTH treatment (data not shown). Second, the serine residues of MEF were weakly phosphorylated by PTH treatment for 10 min, while the MEF phosphorylation was stronger by PTH treatment for 120 min (Fig. 4B bottom panels). Third, an *in vitro* JNK1 kinase assay confirmed the phosphorylation of MEF by JNK1 (Fig. 4C, details are in the legend and Methods). Finally, transfection of MEF combined with JNK and MKK4, which is known to be an up-stream kinase of JNK, increased the activity of the *MAB21L1* promoter more significantly than the transfection of MEF only (Fig. 4D). These results demonstrated that *MAB21L1* transcriptional up-regulation by PTH in human osteoblast cells could be partially achieved by activated MEF via the MKK4/JNK1 pathway.

JNK1 KINASE PHOSPHORYLATES MEF ON S641

Reversible protein phosphorylation plays a crucial role in the regulation of signaling pathways. We first tested whether JNK1α kinase could phosphorylate MEF *in vitro*. The Flag-MEF proteins over-expressed in HEK293T cells were immunoprecipitated, and then an *in vitro* kinase reaction with active JNK1 was performed. After the JNK1 kinase reaction, the MEF (which was also identified by mass spectrometry) band was shown to be retarded in a SDS-PAGE gel compared to the no-enzyme (JKN1) control, suggesting that MEF can be directly phosphorylated by JNK1 (Fig. 5A arrow). Slight MEF band retardation in SDS-PAGE was also observed after

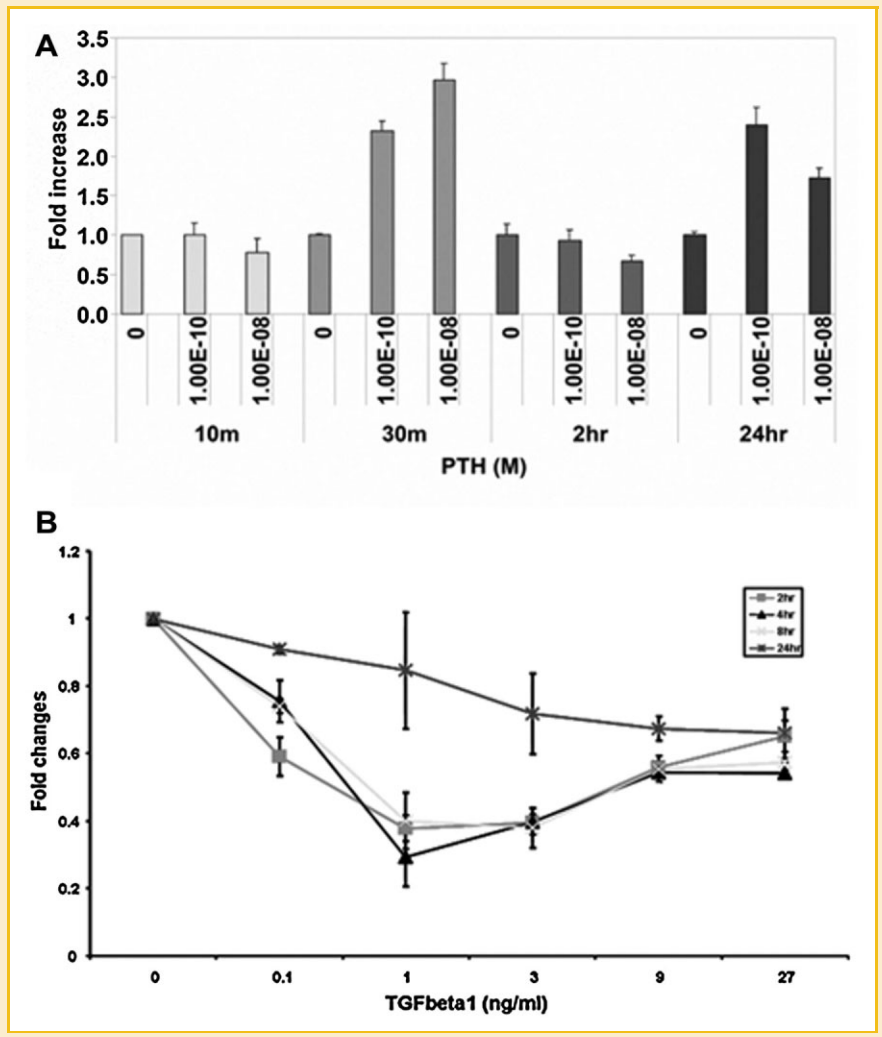

Fig. 3. PTH increases *MAB21L1* transcription. **A:** Real-time PCR results show that *MAB21L1* mRNA levels were increased by PTH addition at the concentrations of 10^{-10} and 10^{-8} M for both 30 min and 24 h. **B:** *MAB21L1* transcription was down-regulated by TGFβ1 addition at various time points and doses. The decrease in mRNA levels of *MAB21L1* was most significant at the concentration of 1 ng/ml TGFβ1 for 4 h to 24 h of treatment.

in vivo co-transfection of Flag-MEF along with JNK1α and MKK4 into HEK293T cells (Fig. 5B).

Recent advances in mass spectrometry allow the mapping of phosphorylation sites on specific target proteins, and MALDI-TOF/TOF analysis has been found to be useful in the identification of Serine/Threonine phosphorylation by neutral loss analysis [Prodhomme et al., 2010]. To examine whether JNK1 phosphorylates MEF *in vivo*, anti-FLAG M2 agarose beads were used to co-immunoprecipitate (IP) bound proteins from the transfected cell lysates, and the proteins were resolved by SDS-PAGE and stained with Coomassie blue reagents (Fig. 5B). The major bands were cut-out and analyzed in MALDI-TOF/TOF-MS analysis. Through MS analysis, we found that MKK4 and JNK1 physically interact with MEF, which was not detected in control lane (Fig. 5B).

In the MALDI-TOF-MS analysis of the in-gel trypsin-digested MEF band from the JNK activated sample, we searched for peptide mass fingerprint peaks separated by 80 Da. The 1801.1-Da and 1881.1-Da peaks were selected using this criterion for MS/MS analysis (Fig. 6A). MS/MS analysis revealed that the 1801.1-Da peak is the unphosphorylated MEF 641–657 peptide, SPTPAPFSPFNPTSLIK (Fig. 6B), whereas the 1881.1-Da peak is the phosphorylated peptide, S*PTPAPFSPFNPTSLIK (Fig. 6C). Furthermore, among the possible MAPK (JNK) phosphorylation targets with S/TP motif) TS641, T643, and S648, only the y17–98 neutral loss peak was observed in the MS/MS scan (Fig. 6C), suggesting that the 1881.1 peptide is the resultant of S641 phosphorylation of the 1801.1-Da MEF peptide (Fig. 6D). These results show that both MKK4 and JNK1 physically interact with MEF, and JNK1 can directly activate MEF through the phosphorylation of Serine 641.

DISCUSSION

Evidence demonstrating the many crucial roles of the ETS transcription factor family in diverse biological processes, including bone development and remodeling, has been accumulating in the

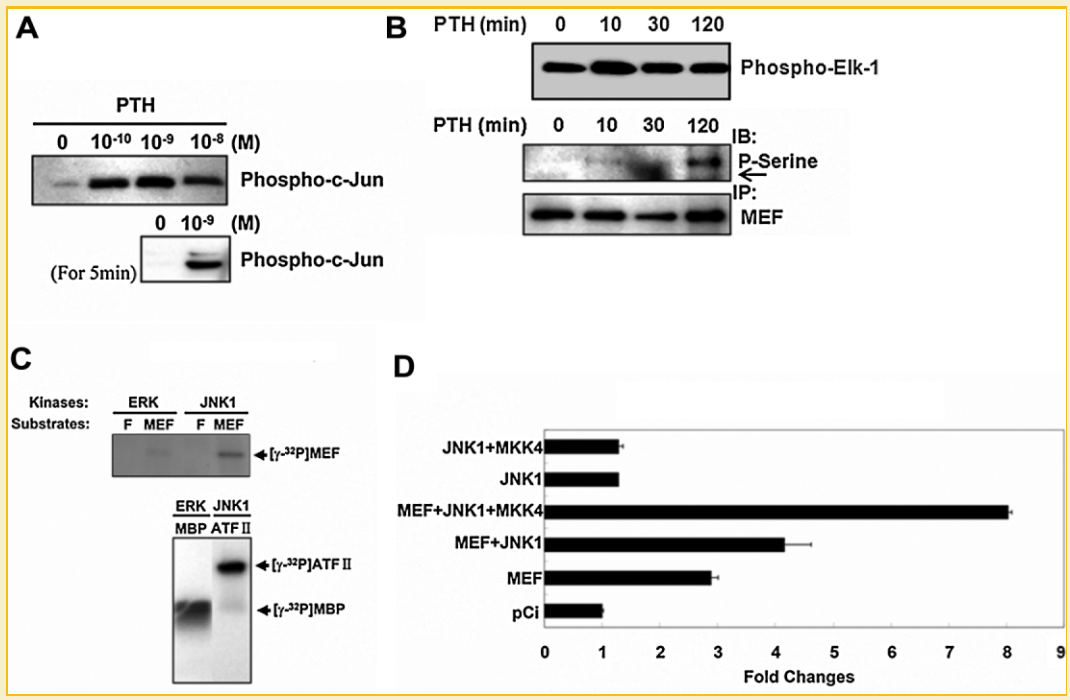

Fig. 4. PTH phosphorylates MEF via the JNK pathway. **A:** Phospho-c-Jun levels show that JNK kinase is activated in MG63 osteoblastic cells by a low concentration of PTH. PTH was added at 10^{-10} M, 10^{-9} M, or 10^{-8} M concentrations for 15 min or at 10^{-9} M for 5 min before cell collection. JNK was purified by immunoprecipitation with anti-JNK antibodies. To measure JNK kinase activity, purified c-Jun protein and ATP were incubated at 30°C for 30 min with the JNK-immunoprecipitated beads. A western blot using an anti-phospho-c-Jun (Ser-63) antibody was used on the supernatant of the JNK kinase activity reaction. **B:** PTH treatment results in increased phosphorylation of MEF. PTH (10^{-8} M) was treated to the MG63 osteoblastic cells at 10 min, 30 min and 120 min before cell collection. Erk1/2 was purified by immunoprecipitation with anti-p44/42 antibodies. To measure Erk1/2 kinase activity, 2 μg of purified Elk-1 protein and 250 μM ATP were incubated at 30°C for 30 min, and a western blot was performed using anti-phospho-Elk-1 (Ser-383) (Top panel). MEF protein was also purified from MG63 cells by immunoprecipitation with the anti-Flag-antibody, and a western blot was performed on the phosphorylated MEF using anti-phospho-serine antibody (bottom panels). Arrow indicates non-specific artifact upon film development. **C:** The in vitro MAP kinase assay was done using purified MEF substrate protein, and ERK or JNK1 enzymes along with ^{32}P-ATP as written in Materials and Methods. The data showed that JNK1 strongly phosphorylated MEF, but ERK did so very weakly. Phospho-MBP and phospho-ATFII were also measured to show the efficiency of ERK and JNK1 kinase activity, respectively as controls. F indicates the cell lysates from the cells transfected with Flag-pCDNA3 control vector. **D:** The luciferase assay showed that JNK1 increases and MKK4 with JNK1 further increases the transactivity of MEF on the *MAB21L1* promoter. CV1 cells were transfected with the expression vector and the MAB21L1p-luciferase reporter construct (E:R) at 1:3 ratio for the luciferase assay.

literature. We previously demonstrated an important role for MEF expressed in pre-osteoblast cells in the promotion of osteoblast proliferation at early time points, as well as down-regulation of MEF in BMP2-induced osteoblast differentiation conditions [Kim et al., 2007]. Over-expression of MEF suppresses the expression of osteogenic related genes, such as ALP, Dlx5, OP, and OC, which are required for the terminal differentiation of an osteoblast. Moreover, osteoblast-specific expression of MEF induced osteopenia through down-regulation of osteoblastogenesis and up-regulation of osteoclastogenesis [Seul et al., 2010]. Furthermore, our unpublished data also showed that overexpression of MEF or Mab21 could induce apoptosis of MG63 osteoblastic cells by increasing the level of cleaved caspase-3. This suppressive role of MEF might have an important role in osteoblast metabolism by balancing the number of osteoblasts in a specific region or environment.

The Mab21 gene family members are required for the differentiation of the eye, midbrain and neural tube, embryonic development and sensory organ formation in both invertebrates and vertebrates [Margolis et al., 1999; Yamada et al., 2003; Merello et al., 2004; Choy et al., 2007]. However, the expression and transcriptional regulation by this family was not evident in osteoblast cells. In this study, we found that PTH regulates the transcription of *MAB21L1* in human osteoblastic cells. Although PTH treatment at continuous manner has been shown to have a suppressive function on osteoblast cells [Kousteni and Bilezikian, 2008], in our study, the *MAB21L1* gene was up-regulated by PTH (Fig. 3), indicating that *MAB21L1* is positively regulated by PTH under these experimental conditions.

Continuous excesses of PTH due to primary hyperparathyroidism cause a decrease in bone volume and, to a lesser extent, bone formation, by up-regulating osteoclastogenesis through an indirect effect via the RANK-L/RANK/OPG pathway. These findings are consistent with our ideas on the function of MEF/MAB21L1 in the bone metabolism process because the secreted ligands from MEF-transfected stable cells promote osteoclastogenesis and the over-expression of either MEF or *MAB21L1* lead to apoptosis of MG63 osteoblastic cells (our unpublished data). Interestingly, it has also been reported that PTH inhibits the growth of osteoblastic cell lines, arresting them in G1 phase [Qin et al., 2005]. Thus, it is suggested

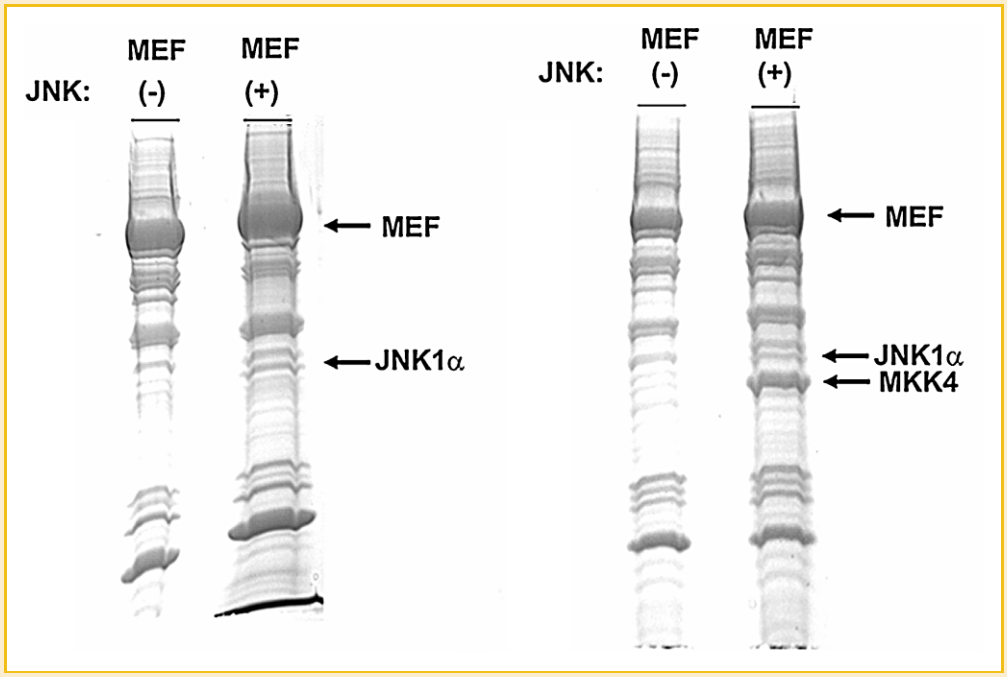

Fig. 5. *In vitro* and *in vivo* JNK kinase assays to identify the phosphorylation site on MEF using mass spectrometry. **A:** *In vitro* kinase reactions were performed by incubating MEF immunoprecipitated from HEK293T cells with or without activated JNK1α kinase and ATP, followed by SDS-PAGE and Coomassie staining. Arrows indicate the MEF and JNK1α protein bands. **B:** *In vivo* JNK kinase assay. The *MEF* construct was co-transfected into HEK293T cells with JNK1α and MKK4 expression vectors or empty vectors. Immunoprecipitated MEF and its interaction partners were separated by SDS-PAGE and stained with Coomassie blue. Arrows indicate MEF, JNK1α and MKK4 bands. These results also showed that MEF physically interacts with JNK1α and MKK4 *in vivo*. Distinctive bands were cut out, in-gel digested and analyzed by MALDI-TOF-TOF for the identification of proteins. The MEF bands were further analyzed by mass spectrometry to obtain data for the phosphorylation analysis shown in Fig. 6.

that PTH-induced up-regulation of *MAB21L1* expression could occur concurrently with RANKL production in osteoblasts, which is achieved by a different pathway. In the future study, it will be interesting to examine whether MAB21L1 is also involved in the positive activation of the RANKL promoter.

Though the cAMP/PKA pathway is a dominant route for RANKL production in osteoblast cells, emerging evidence indicates that the MAPK pathway is also activated by PTH stimulation in a context-dependent manner [Buzzi et al., 2007; Khundmiri et al., 2008; Khundmiri et al., 2004; Lederer et al., 2000; Ogita et al., 2008; Rey et al., 2007]. In general, JNK is activated by protein kinase MKK4, which is in turn activated by the phosphorylation of MEKK1. Our results indicate that JNK1 phosphorylates MEF and that the promoter activity of *MAB21L1* is significantly elevated by a combination of MEF with JNK1/MKK4; these are the first results to demonstrate that PTH can activate MEF through the MKK4/JNK pathway. In this model, phosphorylated MEF could translocate into nucleus and consequently up-regulate *MAB21L1* transcription levels.

Using MALDI-TOF-TOF-MS analysis, our study revealed that JNK1 phosphorylates the C-terminal Ser-641 of MEF. Because phosphorylated proteins exist as a small fraction of the total cellular proteins, the characterization of phosphorylation on a proteomic scale is difficult. Additionally, it is challenging to know which specific kinase phosphorylates a motif. To overcome these limitations, we used a combination of MEF over-expression and JNK1 and MKK4 kinases *in vivo* and a kinase reaction with only active JNK1 kinase *in vitro* to determine whether JNK1 phosphorylates MEF. We have also shown the feasibility of identifying a serine phosphorylation site by MALDI-TOF-TOF using a neutral loss scan in the MS^2 of phosphorylated peptides and a combined pseudo MS^3 spectrum and LID spectrum [Prodhomme et al., 2010].

Our result suggests that up-regulation of MEF activity via site-specific phosphorylation could play a role in the suppressive function of PTH in osteoblast cells. However, because our approach had limited sensitivity, JNK1-activated phosphorylation sites (other than MEF S641) were not detected. There could be other JNK-activated phosphorylation sites on MEF which can be found with other improved method. It was previously reported that Thr-643 and Ser-648 at the C terminus of MEF can be phosphorylated by CDK2 [Liu et al., 2006], leading to a decreased level of MEF proteins due to ubiquitination-proteasomal degradation in the G1/S transition phase. It has also been reported that MEF Ser-641 can be phosphorylated by CDK [Suico et al., 2004], although this site was less influential in decreasing MEF levels. In this study, we suggest that phosphorylation of Ser-641 could conversely participate in the up-regulation of MEF activity though the suppressive action of PTH (via MAB21L1) in osteoblast cells (Fig. 7). Research into pharmacological modulators of those phosphorylation reactions may provide novel therapeutics to overcome suppressive osteogenic diseases, such as primary hyperparathyroidism.

In general, PTH does not affect the replication of uncommitted osteoblast progenitors and instead suppresses the proliferation of committed osteoprogenitors [Kousteni and Bilezikian, 2008]. When

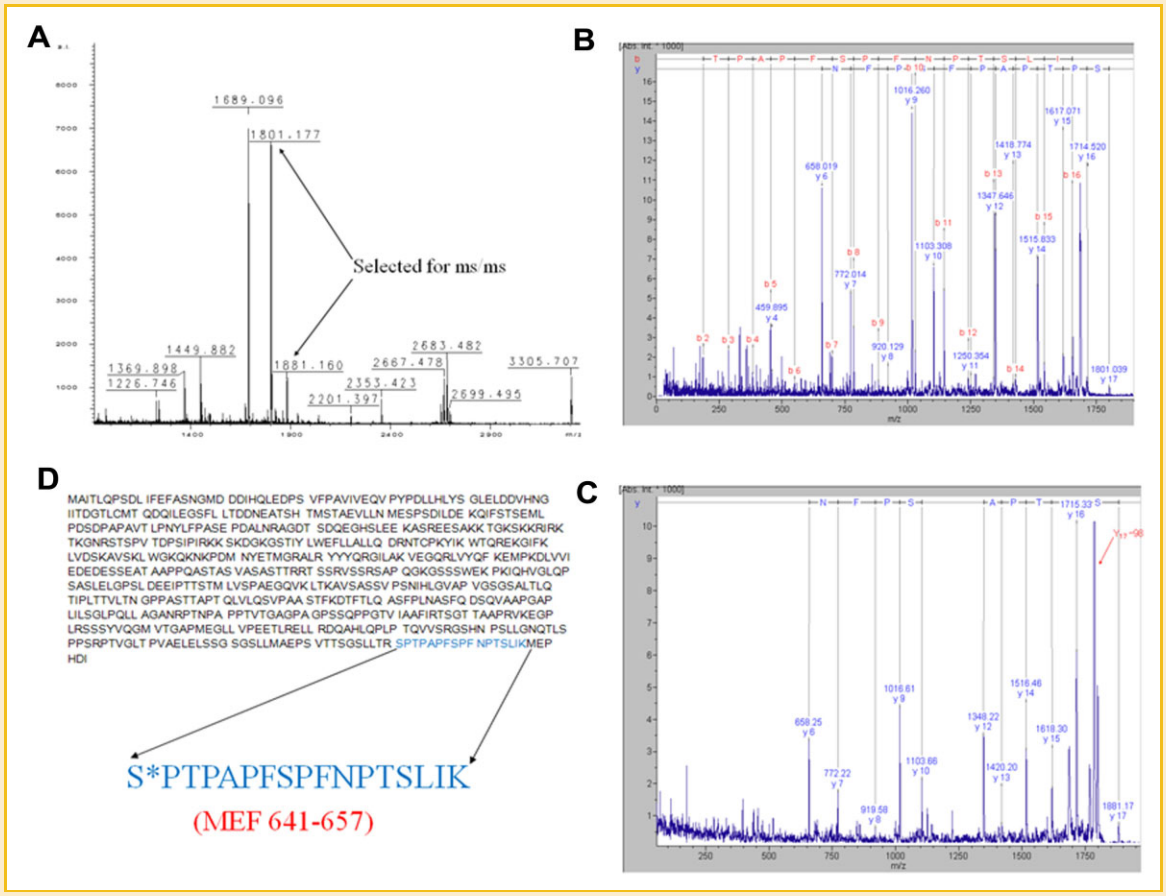

Fig. 6. JNK1 phosphorylates Serine-641 of MEF. **A:** Analysis of tryptic-digested peptides of MEF in MS mode by MALDI-TOF/TOF. Two peptides (1801.1 and 1881.1 Da) showing an 80-Da difference were selected for ms/ms analysis in (B) and (C). **B:** Ms/ms analysis of the 1801.1-Da peptide identified it as unphosphorylated MEF 641–657 (SPTPAPFSPF NPTSLIK). **C:** Ms/ms analysis of the 1881.1-Da peptide identified the peptide as phosphorylated MEF 641–657 with a neutral loss of Y17–98 Da, indicating phosphorylation at Serine 641. (D) The full amino acid sequence of human MEF and the 641–657 region magnified with a marker of S641 phosphorylation.

PTH binds to the PTHR on the osteoblast, it increases the expression of RANKL, promoting the resorption of bone by activating osteoclasts and consequently enhancing the release of calcium from bones into the blood [Filvaroff and Derynck, 1998].

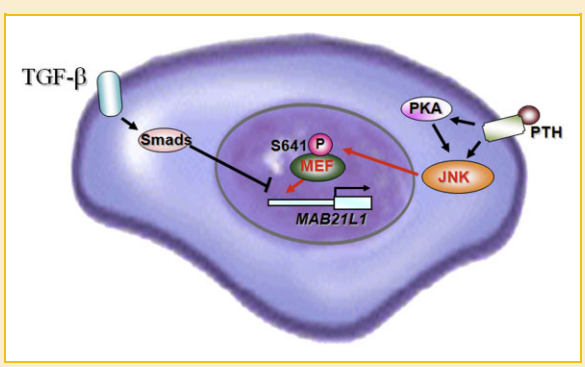

Fig. 7. A model for MEF regulation of *MAB21L1* transcription in osteoblasts. PTH activates JNK directly or indirectly (via PKA), and the activated JNK then phosphorylates MEF on Serine 641 (and possibly other sites), which increases transcription of *MAB21L1*. The TGFβ1/Smad pathway suppresses *MAB21L1* transcription. Arrows and bars represent promotion or inhibition, respectively.

Additionally, in response to PTH binding, the osteoblast decreases production collagen, ALP and OP expression and increases production of OC and cytokines [Partridge et al., 1994]. Interestingly, in our *in vitro* and *in vivo* transgenic mouse study, we observed that MEF increased RANKL cytokine levels but decreased OP and other osteogenic factors [Kim et al., 2007; Seul et al., 2011]. In this study, we showed that through the activation of MEF by PTH, MAB21L1 transcription increases, mediating the suppressive function of PTH on osteoblast cells.

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