Regulation of osterix (Osx, Sp7) and the Osx promoter by parathyroid hormone in osteoblasts

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Abstract

Parathyroid hormone (PTH) binds to its receptor on osteoblasts to regulate gene transcription primarily through the elevation of the second messenger cAMP. A number of genes regulated by PTH in osteoblasts contain GC-rich and Sp-binding sites. Osterix (Osx, Sp7) is a transcription factor required for the differentiation of osteoblasts that can bind to Sp-binding sites on gene promoters and regulate their expression. Here, we report the effect of PTH (1–34) on Osx expression in osteoblastic UMR-106-01 cells and murine calvaria. PTH (1–34) and PTH (1–31) inhibited Osx mRNA and protein expression, and this effect could be mimicked by forskolin, 8-bromo-cAMP, or expression of constitutively active Gsα (caGsα). Treatment of the cells with PTH (3–34) or the EPAC-selective agonist 8CPT-2Me-cAMP had no effect on Osx mRNA, whereas PTH (7–34) or expression of caGsα-stimulated Osx mRNA levels. PTH (1–34) treatment did not require new protein synthesis and did not involve changes in Osx mRNA stability. Osx promoter fragments coupled to a luciferase reporter were inhibited by PTH (1–34) treatment in a similar manner to the inhibition of Osx mRNA and protein. Deletion analysis localized PTH inhibition to two regions flanking the Osx1 start site; −304/−119 and −71/+91. These results demonstrate that prolonged exposure to PTH inhibits Osx expression in osteoblasts through sites on its proximal promoter and this suppression occurs through PTH stimulation of cellular cAMP.

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Introduction

Osterix (Osx, Sp7) is a zinc finger-containing transcription factor belonging to the Sp subgroup of the Krüppel-like family. It was first identified as a bone morphogenic protein-2 (BMP-2)-induced gene in osteoprogenitor cells (Nakashima et al. 2002). The mouse Osx homolog is a 428 amino acid polypeptide with a molecular mass of ~45 kDa. More recently, three alternatively spliced variants of the murine Osx mRNA have been identified by 5' RACE experiments (Nishio et al. 2006), with the most abundant isoform being the transcript originally reported by Nakashima et al. (2002). In humans, two isoforms have been identified and named as Sp7-α and -β isoforms, sharing 95% sequence homology with murine Osx (Milona et al. 2003, Gao et al. 2004).

Osx is expressed specifically in osteoblasts and transiently in differentiating chondrocytes (Nakashima et al. 2002). Osx null mice lack bone formation with a phenotype similar to that of runt-related transcription factor 2 (Runx2, also known as core-binding factor-α1 (Cbfa1)) null mice (Komori et al. 1997). In both Runx2- and Osx-deficient mice, osteoblast differentiation is arrested and expression of a number of osteoblast markers, osteonectin, osteopontin, osteocalcin, and bone sialoprotein is absent (Komori et al. 1997, Nakashima et al. 2002). Runx2 null mice do express Osx while Osx null mice lack Runx2 expression, suggesting that Osx is either acting downstream of Runx2 or expressed later in the osteoblast differentiation pathway.

Functional roles for Osx in bone and cartilage have only recently been investigated. Osx is thought to regulate transcription of many osteoblast marker genes containing GC-rich and Sp-binding sites on their promoters. Forced expression of Osx in vitro has been reported to induce expression of osteocalcin and collagen type 1A1 (Nakashima et al. 2002), collagen type 11A2 (Goto et al. 2006), and osteopontin (Kim et al. 2006). Osx may also inhibit osteoblast proliferation through the inhibition of the Wnt-signaling pathway (Zhang et al. 2008) and act as a negative regulator of chondrogenesis (Tominaga et al. 2009). Osx expression has also been suggested to play a role in tumor growth and metastasis, and osteolytic activity of tumor cells may be regulated by Osx via down-regulation of interleukin-1α gene transcription (Cao et al. 2008). Therefore, understanding the regulation of Osx gene expression could be beneficial in modulating the osteoblast differentiation, bone formation, and tumor activity.
Several factors that can modulate Osx expression have been identified. Both BMP-2 and insulin-like growth factor-1 (IGF-1) can induce Osx expression in undifferentiated mesenchymal stem cells through Runx2-dependent (Celil & Campbell 2005) and Runx2-independent (Lee et al. 2003) pathways. Ascorbic acid and 1,25 (OH)_{2} vitamin D_{3}, which have positive roles in osteoblast function, have also been shown to up-regulate Osx expression (Maehata et al. 2006, Xing et al. 2007). Negative regulators of osteoblastogenesis, such as tumor necrosis factor-\alpha (TNF-\alpha) and p53, can inhibit Osx expression (Lu et al. 2006, Wang et al. 2006a, b, Zambetti et al. 2006).

Parathyroid hormone (PTH) is known to have both anabolic and catabolic effects on bone depending on the duration of exposure (Frolik et al. 2003). Many osteoblast marker genes including type I collagen (Kream et al. 1980), osteopontin (Noda & Rodan 1989), osteonectin (Termine et al. 1981), and osteoprotegerin (Lee & Lorenzo 1999) are regulated by PTH. Some gene regulation by PTH may be mediated by PTH stimulation of the activity of the skeletal-specific transcription factor Runx2 (Ducy et al. 1997). The best characterized role of PTH stimulation of Runx2 is the stimulation of matrix metalloproteinase-13 (MMP-13) in UMR-106-01 and other osteoblast cell lines (Selvamurugan et al. 2000). The aim of this study was to examine whether PTH regulated Osx in osteoblasts and whether this was downstream of Runx2 activation.

Materials and methods

Materials

Rat PTH (1–34), human PTH (1–31), bovine PTH (3–34), and bovine (\alpha-Trp^{12} and Tyr^{31}) PTH (7–34) amide were purchased from Bachem Bioscience, Inc. (King of Prussia, PA, USA). H89, 8-bromo-cAMP (8-Br-cAMP), forskolin (Fsk), and 5,6-dichlorobenzimidazole riboside (DRB) were purchased from EMD Biosciences, Inc. (San Diego, CA, USA). Cycloheximide (CHX) was purchased from Sigma. 8CPT-2Me-cAMP was purchased from Cedarlane Laboratories (Burlington, Ontario, Canada). cDNAs encoding constitutively active Gs\alpha (Gs\alpha (Q227L) and Gq\alpha (Q209L) or a control vector expressing green fluorescent protein (GFP) were generated by transfection of cDNAs in pcDNA3.1 vectors using Lipofectamine (Life Technologies). Two days after transfection, transfected cells were selected by incubating the cells in medium containing 400 \mu g/ml G418 (Sigma) for 7 days. Cells were subcultured into media containing G418 and grown for 3 days prior to RNA extraction as outlined above.

Real-time PCR

Before reverse transcriptase (RT) reaction, 1 \mu g of total RNA was digested with 1 U of DNase I (Fermentas, Burlington, Ontario, Canada) to remove any contaminating genomic DNA. Each RNA sample was then reverse transcribed using 200 U of MMLV reverse transcriptase (Invitrogen) in a total volume of 20 \mu l with standard RT procedure. The RT products were amplified by real-time PCR amplification using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). For each reaction, 0-8 \mu l of the RT product of calvaria samples or 0-3 \mu l of RT product of UMR samples was amplified in a cocktail containing Power SYBR Green PCR Master Mix (Applied Biosystems), 0-3 mM forward and reverse primers in sterile distilled water in a total volume of 20 \mu l using standard cycling conditions (50 °C for 2 min, 95 °C for 10 min, 40 cycles timed pregnancy were purchased from Charles River Laboratories (St Constant, Quebec, Canada). Animals were treated according to the guidelines of the University of Toronto animal care committee. Frontal and parietal sections of calvaria were dissected from E18.5 and PN4–5 mice. Periosteal membranes were removed and calvaria were hemisected. Calvaria were cultured in DMEM (high glucose) containing antibiotic-antimycotic and 10% fetal bovine serum with two randomly selected calvaria halves/well in 24-well culture plates containing 1 ml of medium/well.

Cell culture and RNA extraction

UMR cells were plated at a density of 5·4×10^{5} cells/well in six-well plates on day 1 and treated with PTH on day 2 and day 3 for up to 24 h. On day 3, total RNA was extracted using TRIzol (Gibco BRL, Life Technologies) according to the manufacturer’s instructions. For experiments using CHX, DRB, or signaling activators, the same protocol was followed as for PTH. In experiments using signaling inhibitors, cells were pretreated with specific inhibitors for 30 min before the addition of PTH. For calvaria, four halves of calvaria were collected in 1 ml of TRIzol and homogenized in a dounce tissue homogenizer (Wheaton, Millville, NJ, USA) before RNA extraction.

UMR cell lines expressing Gs\alpha (Q227L) or Gq\alpha (Q209L) or a control vector expressing green fluorescent protein (GFP) were generated by transfection of cDNAs in pcDNA3.1 vectors using Lipofectamine (Life Technologies). Two days after transfection, transfected cells were selected by incubating the cells in medium containing 400 \mu g/ml G418 (Sigma) for 7 days. Cells were subcultured into media containing G418 and grown for 3 days prior to RNA extraction as outlined above.

Cell culture and mouse calvaria preparation

UMR-106-01 osteosarcoma cells (UMR; a gift from Dr N Partridge, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA) were grown as previously described (Erclik & Mitchell 2002). CD1 mice with
of 95 °C for 15 s, and 60 °C for 1 min). Primers for amplifying mouse and rat Osx, Runx2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using the Primer express software (v.3; Applied Biosystems). The primers used were: rat Osx forward: 5’ CAG CCT GGA AGT TTG G 3’; reverse: 5’ TTT TCC CAG GCC TGT TGA GT 3’; mouse Osx forward: 5’ GGT CCC CAG GTC GAG GAT 3’; reverse: 5’ CTA GAG CCG CCA AAT TTG CT 3’; rat Runx2 forward: 5’ CAG GTT GAT CCA CGA TCT GAT TGT 3’; reverse: 5’ TGA AGA CGG TTA TGG TGA AAG TGA 3’; mouse Runx2 forward 5’ ACT GCC GGT GCA ACA AGA C 3’; reverse: 5’ ACC ACA GTC CCA TCT GGT ACC T 3’; GAPDH forward: 5’ CAT GCC CCT CCG TGT TCC TA 3’; reverse: 5’ GGG CCA CGT CAG ATC CA 3’. GAPDH was used as an internal control for the quality and quantity of the cDNA products as well as a normalization standard for relative quantification analysis. All real-time PCR results were analyzed by relative quantification (RQ = 2–ΔΔCt) calculation, where Ct is the cycle number required to reach the threshold minus the cycle number of the baseline.

**Protein extraction and western blotting**

UMR cells were plated at a density of 5–4×10⁵ cells/well in six-well plates on day 1 and treated with PTH on day 2 and day 3. On day 3, cells were extracted in a buffer containing 50 mM Tris pH 7.5, 16 mM EDTA, 1 mM dithiothreitol, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM sodium fluoride, 1.5 mM sodium orthovanadate, 1% NP40, 0.2% deoxycholate, and 10 μg/ml trypsin inhibitor as previously described (Lai et al. 2005). Protein samples were separated by electrophoresis on 11% SDS–polyacrylamide gels, transferred to nitrocellulose membranes overnight at 4 °C, and blocked with 5% milk, 0.1% Tween, Tris-based buffer (TBST) for 2 h. To determine specific protein expression, nitrocellulose membranes were probed with specific antibodies diluted in TBST containing 1% BSA overnight at 4 °C. To determine Osx protein expression, nitrocellulose membranes were probed with Osx polyclonal antibody (Abcam, Cambridge, MA, USA) diluted (1:500) in TBST containing 1% BSA overnight at 4 °C and probed with HRP-conjugated anti-rabbit IgG antibody (GE Healthcare, Baie d’Urfe, Quebec, Canada) diluted (1:10 000) in TBST with 5% milk for 1 h. To determine Erk1/2 protein expression, nitrocellulose membranes were probed with polyclonal Erk1/2 antibody (Cell Signaling Technology, Danvers, MA, USA) diluted (1:1000) and probed with HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology) diluted (1:5000) with the same conditions. Nitrocellulose membranes were then incubated with ECL-enhanced chemiluminescence solution (GE Healthcare) and exposed to X-ray films. Protein bands were quantitated by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

**Reporter constructs and luciferase assays**

The Runx2 reporter plasmid containing six copies of the Runx2-binding site (OSE2) in a p4Luc promoter-less luciferase expression plasmid (Ducy & Karsenty 1995) was used to assess Runx2 activity. The Osx promoter plasmid (pGL3−1269/+91 bp) containing 5'-flanking region −1269 bp upstream from the initiation methionine start site to +91 bp of downstream sequence attached to a pGL3-basic luciferase reporter plasmid (Promega) and deletion constructs have been previously described (Lu et al. 2006). Mutation construct pGL3−1269/+91 (m214/217), deletion construct pGL3−1269/+91 (ΔNfKb), and a plasmid containing four repeats of NFκB-binding site (−228/−208 bp Osx promoter sequence) attached to the promoter SV40-luciferase pGL-promoter (pGL−NFκB×4) were also previously described (Lu et al. 2006). For luciferase assays, UMR cells were plated at a density of 10⁵ cells/well in 24-well plates on day 1. Transfections were performed on day 2 using lipofectamine (Invitrogen). About 0.2 μg of reporter constructs and 0.2 μg of pSV-β-gal cDNA were transfected with 3 μl of lipofectamine in DMEM:F12 serum-free, antibiotic–antimycotic-free medium for 6 h. pSV-β-gal, a β-galactosidase (β-gal) expression plasmid under the control of a simian virus 40 promoter (Promega), was used to correct for transfection efficiency between wells. After transfection, cell media were replaced with fresh DMEM:F12 medium containing 5% serum and antibiotic–antimycotic. On day 3, cells were treated with PTH for up to 24 h, and then lysed with 100 μl cell lysis buffer/well (Promega). Luciferase activity was assessed using a commercial kit (Promega). All luciferase results were corrected for transfection efficiency using β-gal activity. In a subset of experiments, UMR cells were plated at a density of 10⁶ cells/well in 100 mm plates on day 1. On day 2, pFLAG-cmv2–Sp7, an Osx expression plasmid containing a full-length cDNA for the mouse Sp7/Osx and N-terminal FLAG epitope tag, and p-cmv2 were obtained from Toshihisa Komori (Division of Oral Cytology and Cell Biology, Department of Developmental and Reconstructive Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; Goto et al. 2006) were transfected with a 1:4 ratio of DNA to lipofectamine 2000 in DMEM:F12 serum-free, antibiotic–antimycotic-free medium for 5 h followed by a change of medium to fresh DMEM:F12 medium containing 5% serum and antibiotic–antimycotic. On day 3, proteins were extracted and immunoblotted for Osx expression.
Data presentation and statistical analysis

Data from at least three independent experiments were expressed with error bars indicating ± S.E.M. All statistical analyses were performed with GraphPad Prism software (San Diego, CA, USA). One-way ANOVA, where \( P < 0.05 \) was considered statistically significant, was used to determine the effect of treatment on gene expression and protein expression followed by Bonferroni post hoc analysis. Unpaired \( t \) tests were performed to determine the effect of PTH on Osx mRNA stability and the effect of PTH on reporter constructs in luciferase assays.

Results

PTH suppresses Osx mRNA levels

To study the effect of PTH on Osx mRNA levels, UMR cells were treated with increasing concentrations of PTH (1–34) for 3 h. As shown in Fig. 1A, PTH (1–34) inhibits Osx mRNA in a concentration-dependent manner. Treatment of UMR cells with 10 nM PTH (1–34) has been reported to strongly activate PTH1R signal transduction pathways to regulate a number of genes involved in bone formation and bone degradation (Erclik & Mitchell 2002, Qin et al. 2003). Treatment of UMR cells with this concentration inhibited Osx mRNA level by 27–47% over a time course of 3–24 h (Fig. 1B). To determine whether PTH had similar effects on primary mouse osteoblasts, calvaria were dissected from mice at either E18.5 or PN4–5 and cultured with 100 nM PTH (1–34) for various times. Higher concentrations of PTH were used with the calvaria, as primary osteoblasts have previously been shown by others to have lower PTH1R receptor density than UMR cells (Forrest et al. 1985, Bos et al. 1996). In calvaria where bone matrix was intact and other cell types were present, 4–24 h exposure to 100 nM PTH (1–34) significantly suppressed Osx mRNA levels indicating consistency with the effect observed in UMR cells (Fig. 1C and D). Over the time course examined PTH (1–34) treatment suppressed Osx mRNA levels by 70–62% in E18.5 and 59–49% in PN4–5 calvaria.

PTH down-regulates Osx protein

Western blotting was performed to investigate whether the effect of PTH (1–34) on Osx mRNA resulted in a change of Osx protein level. Osx was expressed as two isoforms in the UMR cells where the identity of Osx protein isoforms was confirmed with transient transfection of exogenous Osx expression plasmid (Fig. 2C). A 46 kDa Osx protein originally reported by Nakashima et al. (2002) was detected by the Osx polyclonal antibody (Fig. 2A), and an additional, higher molecular mass of 56 kDa form was also detected and appeared to be regulated by PTH in the same manner (Fig. 2A). In UMR cells, Osx protein levels were significantly suppressed after 6–24 h of 10 nM PTH (1–34) treatment with maximal 74 ± 7% suppression seen after 24 h (Fig. 2B). Thus, our experiments indicated that both Osx mRNA and protein are regulated by PTH (1–34) in osteoblasts with the change in mRNA preceding the change in protein level.

PTH inhibition of Osx is not mediated by Runx2

Runx2 is a transcription factor in osteoblasts whose expression has been shown to precede Osx expression during osteoblastic differentiation. Since Runx2 has been shown to stimulate Osx expression in osteoblasts and PTH has been shown to regulate Runx2, we examined whether the loss of Runx2 could mediate the inhibitory effect of PTH on Osx. In the UMR cells, PTH (1–34) treatments from 3 to 24 h had no effect on Runx2 mRNA levels in UMR cells (Fig. 3A). However, PTH (1–34) could stimulate Runx2 activity...
Involvement of cAMP in PTH-mediated effect on Osx

To investigate the signaling pathway involved in PTH-mediated suppression of Osx, several PTH analogs and selective signaling activators were utilized. PTH (1–31), which induces cAMP as strongly as PTH (1–34) (Jouishomme et al. 1994), also inhibited Osx expression (Fig. 4A). PTH (3–34), which does not induce cAMP (Erclik & Mitchell 2002), had no effect (Fig. 4A), whereas PTH (7–34), which does not activate PTH1R, can act as an antagonist stimulated Osx expression (Fig. 4A).

8-Br-cAMP and Fsk treatments mimicked the effect of PTH in suppressing Osx mRNA levels, suggesting a cAMP-dependent pathway (Fig. 4B). Addition of Rp-cAMP, a competitive PKA inhibitor, was able to partially abrogate PTH (1–34) inhibition of Osx mRNA, whereas 8CPT-2Me-cAMP, a selective stimulator of EPAC (Kang et al. 2003), had no effect on Osx mRNA levels. Furthermore, UMR cells expressing caGsa had ∼50% of the Osx mRNA levels found in cells expressing a GFP control (Fig. 4C). Interestingly, those cells expressing caGqa expressed significantly more Osx than the control cells (Fig. 4C). Together, these data suggest that PTH inhibits Osx expression through activation of Gsa and stimulation of adenylyl cyclase, and that the weak regulation of phospholipase C

Figure 2 PTH regulation of Osx proteins in UMR-106-01. UMR cells were treated with PTH (1–34) for the indicated time periods before protein extraction. Total cell lysates (25 μg) were separated by SDS-PAGE, blotted into nitrocellulose, and probed with specific polyclonal Osx antibody. Two isoforms of Osx were suppressed by PTH (1–34) in a concentration-dependent (A) and time-dependent (B) manner. The 45 kDa Osx was quantified from at least three blots where the averages were plotted with error bars representing mean ± S.E.M. Statistical significance was represented by *P<0.05, compared with cells incubated with PTH-free medium. (C) UMR-106-01 cells were transfected with the indicated amounts of cDNA encoding pFLAG-Sp7 or control p-cmv2 vector before protein extraction. Total cell lysates (25 μg) were separated by SDS-PAGE, transferred onto nitrocellulose, and probed with Osx-specific antibody.

as demonstrated by a construct containing six OSE2 Runx2-binding elements fused to luciferase (Fig. 3B). The use of the cAMP analog 8-Br-cAMP and the adenylyl cyclase activator Fsk showed that PTH-stimulated Runx2 activity via a cAMP pathway (Fig. 3B), which is consistent with previous reports by others (Selvamurugan et al. 2000). PTH (1–34) also had no significant effect on Runx2 mRNA levels in calvaria harvested from either E18.5 or PN4–5 mice (Fig. 3C and D).
PLC) by PTH in these cells (Mitchell & Bansal 1997, Cheung et al. 2005) is not likely to contribute to this inhibition.

**PTH-mediated suppression of Osx does not require protein synthesis and is not mediated by changing Osx mRNA stability**

The protein synthesis inhibitor CHX was used to determine whether de novo protein synthesis was required for the effect of PTH (1–34) on Osx. Three hours of CHX treatment alone did not significantly change the Osx mRNA levels (Fig. 5A). Co-treatment of CHX and PTH (1–34) significantly suppressed Osx mRNA levels, suggesting that de novo protein synthesis was not required for PTH-mediated Osx suppression (Fig. 5A). To determine the effect of PTH (1–34) on Osx mRNA stability, UMR cells were treated with the RNA polymerase II inhibitor, DRB. In the absence of PTH (1–34), Osx mRNA half-life was 0.55 h; while in the presence of PTH (1–34) this was 0.74 h, which was not significantly different, indicating that PTH treatment did not inhibit Osx mRNA levels by decreasing its stability (Fig. 5B).

**PTH regulates Osx transcription**

We next examined whether PTH regulated Osx transcription using an Osx promoter coupled to luciferase in reporter assays. As shown in Fig. 6A, PTH (1–34) significantly suppressed the activity of the −1269 bp Osx promoter construct in a time-dependent manner, which was similar to the effects seen on Osx mRNA. To localize PTH-responsive region(s), Osx promoter fragments with serial deletions from the 5’ end were used. After truncation of the promoter, about half of the PTH-inhibitory effect on Osx promoter activity was localized to −304/−119 and the remaining inhibitory effect to the more proximal promoter between −71 and +91 (Fig. 6B). It is possible that a protein–DNA interaction involving more than one discrete promoter region confers PTH inhibition. The −304 to −119 bp sequence contained a putative binding site for NFkB, and so we examined whether this NFkB could mediate part of the PTH inhibition of the Osx promoter. A reporter construct containing four NFkB-binding sites was significantly stimulated by PTH (1–34), indicating that PTH (1–34) stimulated, rather than suppressed, NFkB activity in the UMR cells (Fig. 6C). However, this stimulation of NFkB activity had no effect on Osx expression since promoter activity of the full-length −1269/+91 Osx promoter with the NFkB-binding site sequence deleted (ΔNFkB) or mutated (m217/214) was suppressed by PTH (1–34) treatment (Fig. 6C).
Our results demonstrate that PTH is a major regulator of Osx expression in osteoblasts. Significant suppression of Osx mRNA was seen within 3–4 h of PTH (1–34) application to the UMR cells or calvaria, and this was quickly followed by detectable loss of Osx protein. The suppression of both mRNA and protein was sustained over a 24-h period, and Osx proteins were barely detectable during the last 8 h of this treatment. Our results agree with previous studies in which the PTH-related peptide, PTHrP (1–34), which also interacts with the PTH1R, caused inhibition of Osx mRNA in differentiated KS483 cells with a similar time course to that seen in our study (van der Horst et al. 2005).

Our studies disagree with a previous report by Wang et al. (2006a,b) in which they demonstrated that a low concentration of PTH (1–34)-stimulated Runx2 and Osx mRNA levels in UMR-106 cells. Their results are clearly different from ours as well as studies from the Partridge laboratory which showed that PTH did not increase Runx2 mRNA or protein, but rather activates Runx2 by PKA-mediated phosphorylation (Selvamurugan et al. 2000, 2009). It is possible that

**Figure 5** PTH regulation of Osx is independent of de novo protein synthesis and Osx mRNA stability. UMR-106-01 cells were treated with 10 nM PTH (1–34) and/or 1 μM cycloheximide (CHX) for 3 h (A). Cells were treated with DRB (50 μg/ml) or co-treated with PTH (1–34; 10 nM) for the indicated time periods to measure the half-life of Osx mRNA (B). Osx mRNA levels were quantified by relative real-time PCR standardized with GAPDH. Average Osx mRNA levels obtained from at least three independent experiments were plotted with error bars representing mean ± S.E.M. Statistical significance were represented by *P<0.05, compared with cells incubated with PTH-free medium.

**Figure 6** PTH regulation of Osx promoter. UMR-106-01 cells were transiently transfected with the −1269/+91 Osx promoter reporter and treated with 10 nM of PTH (1–34) for the indicated time periods (A). To localize PTH-response region(s), cells were transiently transfected with Osx promoter fragments deleted at the 5‘ end and treated with 10 nM PTH (1–34) for 12 h (B). To determine the involvement of NFκB, cells were transiently transfected with NFκB reporter containing four NFκB-binding sites (NFκB×4) or −1269/+91 Osx promoter with NFκB-binding site deleted (ΔNFκB) or mutated (m217/214) and treated with 10 nM PTH (1–34) for 12 h (C). Luciferase activities standardized with β-gal activities were plotted as percentage of the basal activity of the specific construct without PTH treatment. Results from at least three independent experiments were plotted with error bars representing mean ± S.E.M. Statistical significance were represented by *P<0.05.
the UMR-106 cells used by Wang et al. are different from the UMR-106-01 clone used in our study, which was derived from the UMR-106-01 cell line first generated by Partridge’s group. In any event, the UMR cells used in our study showed the same effect of PTH (1–34) on Osx expression as seen in primary mouse calvaria, and therefore they are more likely to be mimicking effects that would be seen in vivo.

The inhibition of Osx by PTH may be part of the catabolic effects seen in response to continuous exposure to the hormone. Previous reports have shown that prolonged exposure of calvaria-derived osteoblasts to PTH results in decreased osteoblast differentiation with decreased expression of collagen 1a1, bone sialoprotein, and osteocalcin (Xing et al. 2007). Some of these genes could have been suppressed as a result of PTH-mediated Osx inhibition, as both osteocalcin and collagen 1a1 are regulated by Osx. The effect of prolonged PTH expression in UMR cells has not been tested; however, intermittent PTH administration to mice with bone fractures showed increased Osx expression in bone marrow-derived mesenchymal stem cells with increased osteoblast differentiation (Kaback et al. 2008). It will be interesting to examine whether continuous and intermittent treatment with PTH has opposite effects on Osx expression in vivo.

Two Osx isoforms of ~46 and 56 kDa were detected in our studies. The identity of the Osx protein isoforms was further confirmed by transient overexpression of a cDNA encoding Osx protein in UMR cells. Alternative splicing generates three Osx mRNAs resulting in two protein isoforms that are much closer in molecular weight than the two forms that we see in UMR cells and calvaria (Gao et al. 2004, Nishio et al. 2006). It is possible that post-translational modifications may be responsible for the higher molecular weight Osx isoform we observed. For example, Sp1 is extensively glycosylated with O-linked sugars (Jackson & Tjian 1988) and phosphorylated (Jackson et al. 1990). Osx, as a member of the Sp family, may also be glycosylated and phosphorylated accounting for the multiple molecular weight isoforms seen here; however, more investigations are necessary to understand post-translational modifications of Osx and the significance of these processes in transcriptional regulation.

Runx2 has been shown to transactivate the Osx promoter in chondroprogenitor cells (Nishio et al. 2006). We did not see any loss of Runx2 mRNA in our cells or calvaria following PTH (1–34) treatment for up to 24 h, although a trend toward suppression that was not statistically significant was observed, and PTH (1–34) treatment for 6 h was clearly capable of stimulating an OSE2 reporter construct that responds to stimulated Runx2, suggesting that within the time of onset of PTH inhibition of Osx, Runx2, which typically acts as an activator, is unlikely to mediate this process. PTH has been reported to deplete around 40% of Runx2 protein through proteasomal degradation in OB-6 cells (Bellido et al. 2003); however, the effect was transient with full recovery of Runx2 protein by 24 h with continuous PTH stimulation. We did not evaluate the amount of Runx2 protein in our samples following PTH (1–34) treatment; however, given our results showing no change in Runx2 mRNA and stimulation of Runx2 activity, we think it is unlikely to be a major factor in PTH-mediated inhibition of Osx.

Investigations into the mechanism by which PTH (1–34) inhibits Osx expression in our studies clearly showed a role for cAMP. PTH (1–31), a PTH fragment that specifically stimulates cAMP (Jouihomme et al. 1994), caGsz, Fsk, and 8-Br-cAMP all inhibited Osx mRNA levels. PTH (3–34), previously shown not to stimulate cAMP (Erclik & Mitchell 2002), had no effect on Osx mRNA levels. KpAMP, a competitive inhibitor of PKA, was able to partially block PTH (1–34) inhibition of Osx, whereas the EPAC-selective stimulator 8CPT-2Me-cAMP (Kang et al. 2003) had no effect. Thus, it seems likely that PKA rather than EPAC may mediate some of the effects of PTH on Osx; however, further studies are required to fully elucidate the signaling pathway between cAMP and Osx gene transcription.

PTH (7–34), which does not activate PTH1R, actually stimulated the Osx mRNA level above basal. This PTH analog can induce internalization of the receptor in some cell types’ cell-specific manner (Sneddon et al. 2003, 2004); however, UMR-106-01 cells express NHERF1 that has been shown to inhibit PTH1R internalization (Wheeler et al. 2008). An alternative explanation for the ability of PTH (7–34) to stimulate Osx is that UMR cells express and secrete small amounts of PTHrP (Suda et al. 1996), which can also bind to the PTH1R receptor. Therefore, PTH (7–34) may have competed with endogenously expressed PTHrP for PTH1R occupancy, resulting in a net decrease in PTH1R-basal activity and higher Osx levels. Finally, Osx mRNA levels were stimulated in UMR cells expressing caGqz, suggesting that stimulators of PLC/PKC may positively regulate Osx expression in osteoblasts. PTH (1–34) stimulates PLC activity in UMR-106-01 cells but only weakly (Mitchell & Bansal 1997, Cheung et al. 2005), and therefore the cAMP pathway is dominant resulting in a net decrease in Osx expression. Further studies are required to determine whether PTH analogs reported to stimulate PLC and not adenyl cyclase (reviewed by Morley et al. 1999) can stimulate Osx levels in either osteoblast cell lines or in calvaria.

Our studies did demonstrate that PTH (1–34) inhibition of Osx is most likely to be at the transcriptional level since it had no effect on Osx mRNA stability and is independent of new protein synthesis in the cells. A similar pattern of direct inhibition of Osx was seen...
in MC3T3 cells with TNF-α (Lu et al. 2006). Using a 1292-bp fragment of the Osx promoter to drive a luciferase reporter, PTH (1–34) was found to inhibit the activity of the promoter with a similar time course as seen for the inhibition of Osx mRNA. The profound inhibition of the luciferase activity seen with longer PTH treatments may be due in part to the loss of autoregulation of the promoter by Osx itself. Although there are several binding sites for PTH-responsive transcription factors, such as Runx2 and CREB, within the longest promoter fragment used in our studies, none of these is likely to mediate PTH inhibition of the promoter activity as deletion constructs without these putative binding sites retained full responsiveness to PTH. Within the PTH-responsive region of the promoter, there are putative binding sites for NFκB, VDR, Dlx-5, NF-Y, and a GC-rich box that binds Sp transcription factors (Lu et al. 2006, Nishio et al. 2006).

NFκB can be stimulated by PTH as shown here and by others (Ali et al. 1999), and NFκB has been reported to regulate the proximal Osx promoter (Lu et al. 2006). However, mutation or deletion of the NFκB site within the promoter construct had no effect on the ability of PTH to inhibit their activity in our assays, indicating that NFκB is not involved in PTH inhibition of Osx. PTH has been reported to either up-regulate or down-regulate vitamin D receptors depending on the conditions. The only reported effect of vitamin D on Osx showed an increase in Osx mRNA levels in a human osteoblastic cell line, MG-63 cells (Maehata et al. 2006); thus, it is unlikely that the VDR response element in the Osx promoter mediates PTH inhibition of Osx transcription. Dlx-5 is a transcription factor required for the development of a number of calcified tissues, and it has recently been found to mediate BMP-2 induction of Osx (Ulsamer et al. 2008). Stimulation of Dlx-5 mRNA by PTH has been reported, and therefore it is again unlikely to mediate PTH inhibition of Osx.

A GC-rich sequence is within the shortest PTH-responsive region of the Osx promoter tested in our assays. A number of transcription factors belonging to the Sp family including Sp1, Sp3, and Sp7 itself can all combine with other members of the Sp family and bind to such GC-rich elements. Sp1 and Sp3 are ubiquitously expressed members of this family and can combine with other members of the Sp family to have both positive and negative effects on gene transcription (Goto et al. 2006). Further analysis of the response elements within the proximal 70 bp of the Osx promoter will be required to determine whether Sp proteins or other transcription factors are involved in PTH-mediated suppression of Osx transcription.

In summary, we have demonstrated for the first time that PTH down-regulates Osx expression in osteoblasts by a transcriptional mechanism mediated by cAMP signaling. The sites of inhibition within the Osx promoter have been shown to reside within the first 300 bp of the 5′-flanking region of the osx1 transcription start site. Further studies are required to identify the specific transcriptional repressors that mediate this effect, and studies in vivo are required to determine whether inhibition of Osx by PTH contributes to its catabolic effects of this hormone on bone.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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