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 Gs α (caGs α). 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 caGq α -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.

Journal of Molecular Endocrinology (2009) **43**, 197–207

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- α 1 (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-1 α 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 D₃, 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- α (TNF- α) 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 (D-Trp¹² and Tyr³⁴) 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 α (caGs α ; Q227L) and caGq α (Q209L) were purchased from Missouri S&T cDNA Resource Center (University of Missouri at Rolla, Rolla, MO, USA).

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 (Erlik & Mitchell 2002). CD1 mice with

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 treatment 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 α (Q227L) or Gq α (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 μ 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 μ 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 μ 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 μ l of the RT product of calvaria samples or 0.3 μ 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 μ l using standard cycling conditions (50 °C for 2 min, 95 °C for 10 min, 40 cycles

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 GCA GCA AGT TTG G 3'; reverse: 5' TTT TCC CAG GGC TGT TGA GT 3'; mouse *Osx* forward: 5' GGT CCC CAG CTC GAG GAT 3'; reverse: 5' CTA GAG CCG CCA AAT TTG CT 3'; rat *Runx2* forward: 5' CAG GTT CAA CGA TCT GAG ATT TGT 3'; reverse: 5' TGA AGA CCG TTA TGG TCA AAG TGA 3'; mouse *Runx2* forward 5' ACT GGC GGT GCA ACA AGA C 3'; reverse: 5' ACC ACA GTC CCA TCT GGT ACC T 3'; *GAPDH* forward: 5' CAT GGC CTT CCG TGT TCC TA 3'; reverse: 5' GCG GCA 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 ($RQ = 2^{-\Delta\Delta C_t}$) calculation, where C_t 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 5.4×10^5 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 (ΔNFκB), 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 10^5 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 beta-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 10^6 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 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 $\pm$ 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 (Erlik & 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 $74 \pm 7\%$ 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.

Figure 1 PTH regulation of *Osx* mRNA in osteoblasts. UMR-106-01 cells, E18.5, and PN4–5 calvaria were treated with PTH (1–34) for various times before quantification of *Osx* mRNA levels by relative real-time PCR standardized with GAPDH. (A) UMR-106-01 cells were stimulated with the indicated concentrations of PTH (1–34) for 3 h. PTH (1–34) significantly inhibited *Osx* mRNA levels at concentrations of 1 nM or higher. (B) Time course of PTH (1–34; 10 nM) stimulation showed that *Osx* was inhibited between 3 and 24 h of PTH treatment. Similar PTH-mediated suppression of *Osx* mRNA levels was observed in calvaria extracted from E18.5 (C) and PN4–5 (D) mice treated with 100 nM PTH (1–34) for the indicated time periods. Average *Osx* mRNA levels obtained from at least three independent experiments were plotted with error bars representing mean $\pm$ S.E.M. Statistical significance was represented by * $P < 0.05$, compared with cells incubated with PTH-free medium.

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

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 $\pm$ 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).

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), 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 RpcAMP, 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 caGα had ~50% of the Osx mRNA levels found in cells expressing a GFP control (Fig. 4C). Interestingly, those cells expressing caGqα expressed significantly more Osx than the control cells (Fig. 4C). Together, these data suggest that PTH inhibits Osx expression through activation of Gα and stimulation of adenylyl cyclase, and that the weak regulation of phospholipase C

Figure 3 PTH regulation of Runx2 in osteoblasts. UMR-106-01 cells and calvaria (E18.5 and PN4–5) were treated with 10 and 100 nM of PTH (1–34) respectively for the indicated time periods before RNA extraction. Osx mRNA levels were quantified by relative real-time PCR standardized with GAPDH. Time course of PTH showed no effect on Runx2 mRNA levels in UMR-106-01 cells (A) and calvaria extracted from E18.5 (C) and PN4–5 (D) mice. To study Runx2 activity, UMR-106-01 cells were transiently transfected with OSE2 reporter and treated with 10 nM PTH (1–34) or signaling mediators (10 μM Fsk and 1 mM 8-Br-cAMP) for 6 h (B). Average Runx2 mRNA levels and luciferase activities standardized with β-gal activities from at least three independent experiments were plotted with error bars representing mean $\pm$ S.E.M. Statistical significance was represented by * $P < 0.05$ compared with cells incubated with PTH-free medium.

Figure 4 PTH regulates Osx through a cAMP-dependent pathway. (A) UMR-106-01 cells were treated with PTH analogs (10 nM (1–34), 10 nM (1–31), 100 nM (3–34), and 100 nM (7–34)) for 3 h before RNA extraction. (B) UMR-106-01 cells were treated with 10 nM of PTH (1–34) or with cAMP-signaling mediators of 10 μ M forskolin (Fsk), 1 mM 8-Br-cAMP, 10 μ M RpcAMP, and 50 μ M 8CPT-2Me-cAMP (8CPT) for 3 h before RNA extraction. (C) UMR-106-01 cells were transfected with GFP, constitutively active Gs α (Q227L; caGs α), or constitutively active Gq α (Q209L; caGq α) and transfectants were selected in medium containing G418 for 4 days prior to RNA extraction. 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 $\pm$ s.e.m. Statistical significance was represented by * P < 0.05, compared with cells incubated with PTH-free medium. #Represents statistical significance compared with cells incubated with PTH (1–34) alone, P < 0.05.

(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 NF κ B, and so we examined whether this NF κ B could mediate part of the PTH inhibition of the Osx promoter. A reporter construct containing four NF κ B-binding sites was significantly stimulated by PTH (1–34), indicating that PTH (1–34) stimulated, rather than suppressed, NF κ B activity in the UMR cells (Fig. 6C). However, this stimulation of NF κ B activity had no effect on Osx expression since promoter activity of the full-length –1269/+91 Osx promoter with the NF κ B-binding site sequence deleted (Δ NF κ B) or mutated (m217/214) was suppressed by PTH (1–34) treatment (Fig. 6C).

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 μ M cycloheximide (CHX) for 3 h (A). Cells were treated with DRB (50 μ g/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 $\pm$ S.E.M. Statistical significance were represented by $*P < 0.05$, compared with cells incubated with PTH-free medium.

Discussion

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 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 $\pm$ 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 exposure on *Osx* expression *in vivo* 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 (Jouishomme *et al.* 1994), $\text{CaG}\alpha$, 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. Rp-cAMP, 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 $\text{CaG}\alpha$, 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 adenylyl 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 NF κ B, VDR, Dlx-5, NF-Y, and a GC-rich box that binds Sp transcription factors (Lu *et al.* 2006, Nishio *et al.* 2006).

NF κ B can be stimulated by PTH as shown here and by others (Ali *et al.* 1999), and NF κ B has been reported to regulate the proximal *Osx* promoter (Lu *et al.* 2006). However, mutation or deletion of the NF κ B site within the promoter construct had no effect on the ability of PTH to inhibit their activity in our assays, indicating that NF κ B 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 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.

Funding

This work was supported by a grant from the Canadian Institutes for Health Research (MOP15696).

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Received in final form 7 May 2009

Accepted 8 June 2009

Made available online as an Accepted Preprint 8 June 2009