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Lanthanum Enhances In Vitro Osteoblast Differentiation via Pertussis Toxin–Sensitive Gi Protein and ERK Signaling Pathway

Xi Wang,[1] Lan Yuan,[2] Jian Huang,[1*] Tian-Lan Zhang,[1*] and Kui Wang[1]

[1]*Department of Chemical Biology, Peking University School of Pharmaceutical Sciences, 38 Xueyuan Road, Beijing 100191, PR China*

[2]*Peking University Medical and Health Analysis Center, 38 Xueyuan Road, Beijing 100191, PR China*

ABSTRACT

Converging lines of evidence suggest that lanthanum tends to deposit in bone. The influence of lanthanum ion (La^{3+}) on osteoblast differentiation and the related mechanism are essential to understanding its effect on bone metabolism. In this study, La^{3+} treatment enhanced in vitro osteoblast differentiation as evidenced by promoting alkaline phosphatase (ALP) activity, osteocalcin (OC) secretion, and matrix mineralization. The expressions of osteoblast-specific genes of Cbfa-1, osteopontin (OPN), and bone sialoprotein (BSP) were all increased in the presence of La^{3+}, but no change was observed in that of type I collagen (COL-I). Further studies demonstrated that La^{3+} treatment enhanced phosphorylation of extracellular signal-regulated kinase (ERK). Inhibition of ERK activation by U0126 suppressed the effects of La^{3+} on osteoblast activity. Moreover, pretreatment of the cells with pertussis toxin (PTx), a Gi protein inhibitor, suppressed the La^{3+}-enhanced ERK phosphorylation and osteoblast differentiation. These findings suggest that La^{3+} exposure enhances in vitro osteoblast differentiation and the effect depends on ERK phosphorylation via PTx-sensitive Gi protein signaling. J. Cell. Biochem. 105: 1307–1315, 2008.

KEY WORDS: LA^{3+}; OSTEOBLAST; DIFFERENTIATION; GI PROTEIN; ERK PATHWAY

In recent years, lanthanide compounds have attracted intensive research interest in medical applications [Thompson and Orvig, 2006]. Lanthanum carbonate, as a phosphate binder, is effective in the treatment of hyperphosphatemia of chronic renal failure (CRF) [Berns and Kobrin, 2006]. Gadolinium (Gd) compounds have widely been used as a diagnostic contrast medium [Caravan, 2006]. In addition, a gadolinium complex is under clinical trial as a new anticancer agent [Magda and Miller, 2006]. In spite of these perspectives, the safety considerations of lanthanide compounds have been frequently issued.

Lanthanum (La) has been recognized as a "bone-seeking" element [Jarup, 2002] due to a marked bioinorganic similarity to Ca^{2+} [Wang, 1997] in ionic radii [Hardie et al., 2001] and coordination properties [Jalilehvand et al., 2001]. Converging lines of evidence suggest that lanthanum tends to deposit in bone in vivo, but its impact on bone formation remains uncertain. In most patients with some form of renal osteodystrophy at baseline, lanthanum carbonate is more likely to be associated with improvement in various measures of bone [Lacour et al., 2005]. Oral administration of lanthanum carbonate does not cause noticeable effect on bone of normal animals [Behets et al., 2004a]. However, at higher doses it could cause an impairment of bone mineralization in rats with CRF [Behets et al., 2004b]. Our previous study shows that low-dose, long-term treatment with $La(NO_3)_3$ retards bone mineral maturation in normal animals [Huang et al., 2006].

The conflicting results of in vivo studies emphasize the need to address the effect of La^{3+} on bone cell in vitro. Bone is a metabolically active tissue undergoing continuous remodeling which consists of osteoblastic bone formation and osteoclastic resorption. Whether La^{3+} may affect bone remodeling through interfering with cellular activities has not been fully understood. Osteoblasts play a crucial role in the formation phase of bone remodeling by laying down the structural components of bone (matrix and mineral) and secreting various cytokines and growth

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*Correspondence to: Jian Huang, PhD, and Tian-Lan Zhang, PhD, Department of Chemical Biology, Peking University School of Pharmaceutical Sciences, 38 Xueyuan Road, Beijing 100191, PR China. E-mail: hj_by2005@yahoo.com.cn; tlzhang@hsc.pku.edu.cn

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factors that influence both bone formation and resorption. Therefore, the direct effect of La^{3+} on osteoblasts and the related molecular mechanism need to be investigated.

Guanine nucleotide regulatory proteins (G proteins) are a family of GTP-binding proteins that mediate signal transduction and are involved in regulating the functional activity of osteoblasts. However, little is known about the role of the Gi class of G proteins in the intracellular signaling of osteoblasts. Previous studies have demonstrated that Gi proteins are associated with anabolic actions of trivalent aluminum ion and bivalent strontium ion in vivo [Quarles et al., 1994; Pi et al., 2000; Pi and Quarles, 2004]. These phenomena suggest that certain extracellular cations can exert net anabolic effects on bone, possibly through common mechanisms mediated by Gi protein signaling.

In the present work, we investigated the effect of La^{3+} on osteoblast differentiation and the related signaling pathways using an in vitro cell model, trying to find whether the effect of La^{3+} on osteoblast differentiation is linked to Gi protein and ERK signaling.

MATERIALS AND METHODS

MATERIALS

Dulbeccol's Modified Eagle's Medium (DMEM) was obtained from GibcoBRL; fetal bovine serum (FBS) was purchased from Hyclone. Anti-extracellular signal-regulated kinase1/2 (ERK1/2), phosphorylated ERK, and the corresponding secondary antibodies were from Cell Signaling (Beverly, MA). Pertussis toxin (PTx) was from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The membrane-permeable calcium probe, 1-[2-amino-5-(2, 7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-[2-amino-5-methylphenoxy]ethane-*N*,*N*,*N*^{9},*N*^{9}-tetraacetic acetoxymethyl ester (Fluo-3/AM), was purchased from Molecular Probes. Unless otherwise indicated, all other reagents, including the MAPK inhibitor U0126, were from Sigma (St. Louis, MO). Lanthanum chloride solution was prepared from lanthanum oxide (purity $>$ 99.9%).

OSTEOBLAST CULTURES AND TREATMENT

Osteoblasts were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats (1% trypsin in PBS for 20 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 90 min), as previously described [Robey and Termine, 1985]. The first two digests were discarded. Cells from the third digest were collected by centrifugation and resuspended in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in 95% humidified air containing 5% CO_2. The cells between passages three and seven were used for all experiments.

Cells were treated with various concentrations of La^{3+} and specific inhibitors, including PTx (2 ng/ml) to inhibit PTx-sensitive Gi pathway and U0126 (10 M) to inhibit ERK pathway in normal or differentiation medium (DMEM containing 10 mM sodium -glycerophosphate and 50 g/ml ascorbic acid).

CELL VIABILITY ASSAY

Eighty percent of confluence osteoblasts in exponential growth were incubated at 37°C with various concentrations of La^{3+} and inhibitors in DMEM containing 5% FBS for the indicated time intervals. MTT in PBS solution (5 mg/ml) was added to the culture medium to reach a final concentration of 0.5 mg/ml. After the cells were left at 37°C for 4 h, the supernatant was removed and the formazan dye dissolved in 200 l of DMSO. Absorbance was measured on a microplate reader at 570 nm with a reference wavelength of 650 nm (TECAN SUNRISE, Switzerland).

MEASUREMENT OF OSTEOBLAST DIFFERENTIATION MARKERS

Osteoblasts were plated at a density of 2×10^4 cells/well into 24-well plates and incubated in medium containing 5% FBS with or without La^{3+} and inhibitors under study. At various intervals, cells were washed with PBS, and then the cell layers were scraped into solution containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3, and 1 g/ml aprotinin. The lysates were homogenized and alkaline phosphatase (ALP) activity was determined with *p*-nitrophenyl phosphate as the substrate.

To determine osteocalcin (OC) secretion, the cell-conditioned media were collected at day 14 of the culture and OC secretion was measured by radioimmunoassay (RIA) employing [^{125}I] RIA kit (East Asia Institute of Immune Technology, Beijing, China). The OC expression was normalized to total protein content of the cell layer, which was determined with the Lowry method [Lowry et al., 1951].

Osteoblasts in 24-well plates were cultured in differentiation medium containing 10% FBS, 50 g/ml ascorbic acid, and 10 mM sodium -glycerophosphate in the absence or presence of La^{3+} and inhibitors for 14 and 21 days. Then, calcium deposition in the matrix was assessed by a modified Wada procedure [Wada et al., 1999]. Alternatively, matrix mineralization was detected by Alizarin Red S staining [Bodine et al., 1996]. The stained matrix was assessed using a Nikon Diaphot inverted microscope and was photographed using a Nikon 35-mm camera (Nikon, Tokyo, Japan).

QUANTITATIVE REAL-TIME RT-PCR

Osteoblasts were harvested at a density of 8×10^5 cells in circular dishes (100 mm in diameter), and then incubated with or without La^{3+} for 4 days in 5% FBS DMEM. Total RNA was prepared using the Trizol reagent (Invitrogen) according to the manufacturer's specifications and quantified spectrophotometrically by measuring absorbance at 260 nm. Before first-strand complementary DNA (cDNA) synthesis, extracted RNA was treated with RNase-free DNase I (35 U/ml) (Invitrogen, Life Technologies) for 30 min at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 min. Oligo-dT was used to prime reverse transcription reactions and the synthesis was carried out by Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Promega). SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa Bio Inc., Japan) was used to detect accumulation of PCR products during cycling with the DNA Engine Opticon (MJ Research, CA). The thermocycling conditions were as follows: predenaturation at 95°C for 10 min, amplification with use of three-step cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min, for a duration of 30 cycles, with the final dissociation cycle at 95°C for 15 s, 60°C for

1 min and 95°C for 15 s. Real-time RT-PCR were carried out in triplicate with three independent experiments (n = 3). The rat-specific primer sequences for real-time RT-PCR were designed to produce product sizes under 200 bp as follows:

COL (Genebank#Z78279) forward-CAA GGT GAC AGA GGC ATA AAG G, reverse-AGG GAG ACC GTT GAG TCC AT;
Cbfa-1 (Genebank#AB1145746) forward-ACA ACC ACA GAA CCA CAA G, reverse-TCT CGG TGG CTG GTA GTG;
OPN (Genbank#AB001382) forward-AGA CCA TGC AGA GAG CGA G, reverse-ACG TCT GCT TGT GTG CTG G;
BSP (Genbank#AB001383) forward-CCG GCCA CGC TAC TTT CTT, reverse-TGG ACT GGA AAC CGT TTC AGA;
-actin (Genebank#BC063166) forward-CTT TCT ACA ATG AGC TGC GTG, reverse-ATG GCT GGG GTG TTG AAG G.

The -actin gene was used as an internal standard to normalize the amount of total RNA present in each reaction and the comparative C_t (threshold cycle number) (C_t) method were calculated to compare the expression levels among different treatment groups [Livak and Schmittgen, 2001].

WESTERN BLOTTING ANALYSIS OF ERK1/2 PHOSPHORYLATION

Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 50 mM Tris–Cl pH 7.4, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Lysates were sonicated for 20 min on ice and centrifuged at 10,000*g* for 10 min. The protein concentrations of the supernatants were determined with the Lowry method [Lowry et al., 1951]. SDS–polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gels and the resolved proteins were transferred onto PVDF membrane. The membrane was blocked with 5% BSA solution in Tris balanced saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 1 h, and then blotted with appropriate antibodies in 5% BSA/TBS-T (0.1% Tween-20 in TBS) at 4 °C overnight. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody for 1 h. After extensive washing, bands were visualized by chemiluminescent method according to the manufacturer's instructions. The optical densities of bands were quantified by a Scion Image software.

DETERMINATION OF INTRACELLULAR CA^{2+} LEVEL

A Ca^{2+}-sensitive fluorescent probe compatible with laser excitation, Fluo-3/AM, was used to monitor changes in intracellular Ca^{2+}. Osteoblasts grown in petri dishes were cultured with 10^{-7}–10^{-5} M La^{3+} in serum-free DMEM for 2 h, and loaded with Fluo-3/AM (2 mg/ml) at room temperature for 30 min to 1 h in Kreb's buffer (concentration in mM: 133 NaCl, 4.7 KCl, 16.3 $NaHCO_3$, 1.35 NaH_2PO_4, 0.6 $MgSO_4$, 2.5 $CaCl_2$, 7.8 glucose, final pH 7.2). After washing, cells were incubated in 0.5 ml Kreb's buffer and the changes in intracellular Ca^{2+} were measured using a confocal laser-scanning fluorescence microscope (excitation wavelength, 488 nm; emission wavelength, above 510 nm. Leica, TCS SP2, Germany). Fluorescence images were collected at room temperature.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviation (SD). Single factor analysis of variance (ANOVA) was used to assess statistical significance of results. Scheffe's method was employed for multiple comparison tests at levels of 95% and 99%. All experiments were repeated at least three times, and representative experiments are shown.

RESULTS

LA^{3+} AFFECTS OSTEOBLAST VIABILITY

The effect of La^{3+} on cell viability of osteoblasts was both concentration- and time-dependent (Fig. 1). The viability was enhanced after incubation with La^{3+} at lower concentrations for 24 h, but became suppressed at higher concentrations and after prolonged treatment.

LA^{3+} ENHANCES OSTEOBLAST DIFFERENTIATION

ALP is one of the early marker enzymes for functional osteoblasts. Figure 2A shows the concentration- and time-dependent effect of La^{3+} on ALP activity of cultured osteoblasts. On day 2, in the range of 10^{-8}–10^{-5} M, La^{3+} enhanced ALP activity with a maximum at 10^{-6} M. On day 4, the increase in ALP activity became significant at lower concentration (10^{-8}–10^{-7} M); but the activity decreased at the higher concentrations (10^{-5}–10^{-4} M). On day 7, the inhibitive effect was observed in the whole range of concentrations.

As a marker of the terminal differentiation of osteoblasts, OC secretion in the culture medium was increased significantly in the presence of La^{3+} in the range of 10^{-8}–10^{-6} M (Fig. 2B).

The effect of La^{3+} on matrix mineralization was displayed by the changes in calcium deposition in cell cultures, as shown in Figure 2C. La^{3+} treatment promoted matrix calcification on day 14 and 21, with a maximum at 10^{-6}–10^{-5} M. By means of Alizarin Red S staining, larger mineralized nodules were observed on day 21

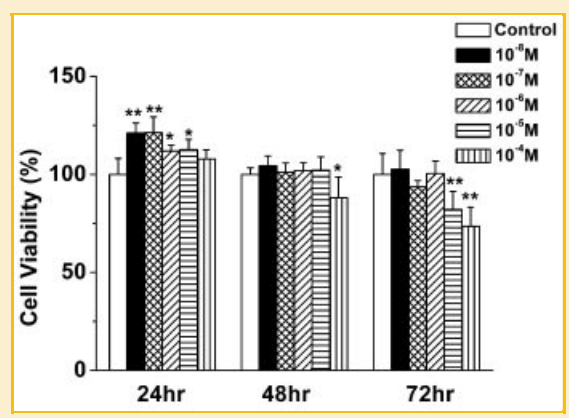

Fig. 1. Concentration- and time-dependent effects of La^{3+} on cell viability. Cells in exponential growth were treated with various concentrations of La^{3+} for different time intervals, and then analyzed by MTT assay. Data are mean ± SD of four independent experiments performed in duplicate ($^{*}P < 0.05$, $^{**}P < 0.001$, vs. control).

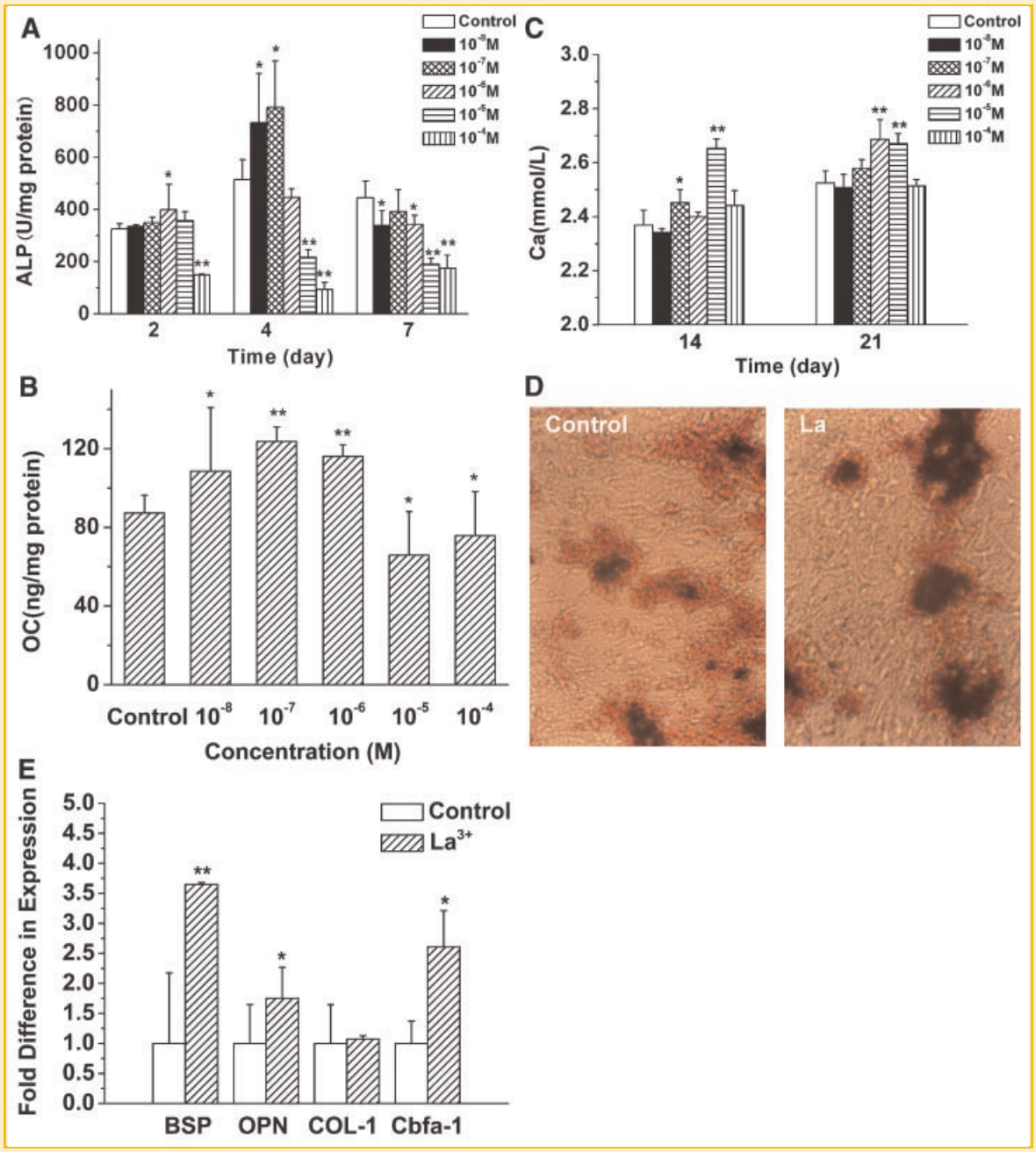

Fig. 2. Effects of La^{3+} on osteoblast differentiation. A: ALP activity was assayed at day 2, 4, and 7 of culture after the cells were exposed to various doses of La^{3+}. B: OC secretion was measured at day 14 of culture after the cells were exposed to various doses of La^{3+}. C: Calcium deposition was determined at day 14 and 21 of culture after the cells were exposed to various doses of La^{3+}. D: Confluent osteoblasts were treated without or with 10^{-6} M La^{3+} in DMEM (containing 50 g/ml ascorbic acid) supplemented with 10% FBS and 10 mM -glycerophosphate for 21 days. The mineralized matrix was stained with Alizarin red-S (×200). Data are mean ± SD obtained in three independent experiments. E: Gene expression profiles of bone-specific markers of osteoblast differentiation as determined by qRT-PCR. Data are representative of three separate trials and presented as fold difference in mRNA expressions of COL-I, BSP, OPN, and Cbfa-1 normalized against -actin ($^{*}P < 0.05$, $^{**}P < 0.001$, vs. control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

under a microscope in cultures containing 10^{-6} M La^{3+}, compared to the control (Fig. 2D).

The level of gene expression associated with osteoblast differentiation also altered after La^{3+} treatment. As depicted in Figure 2E, La^{3+} treatment upregulated the gene expressions of bone sialoprotein (BSP) and osteopontin (OPN), but had no significant effect on that of COL-1. Furthermore, the expression of Cbfa-1, one of the essential transcription factor for osteoblast differentiation and bone formation [Nakashima and de Crombrugghe, 2003], was also upregulated by La^{3+} treatment.

These data indicated that exposing the cells to La^{3+} enhanced in vitro osteoblast differentiation by stimulating the expression of the osteoblast-specific genes and increasing ALP activity, osteocalcin secretion, and matrix mineralization.

LA^{3+} ACTIVATES ERK SIGNALING PATHWAY IN OSTEOBLASTS

As shown in Figure 3, exposing the cells to 10^{-6} M La^{3+} caused the expression of phosphorylated ERK1/2 in a time-dependent manner. The phosphorylated ERK level increased rapidly within 30 min, and remained in a high level after 3 h, while that of the vehicle group decreased. In addition, phosphorylated ERK1/2 increased with the increasing concentration of La^{3+} in culturing medium after 3 h incubation.

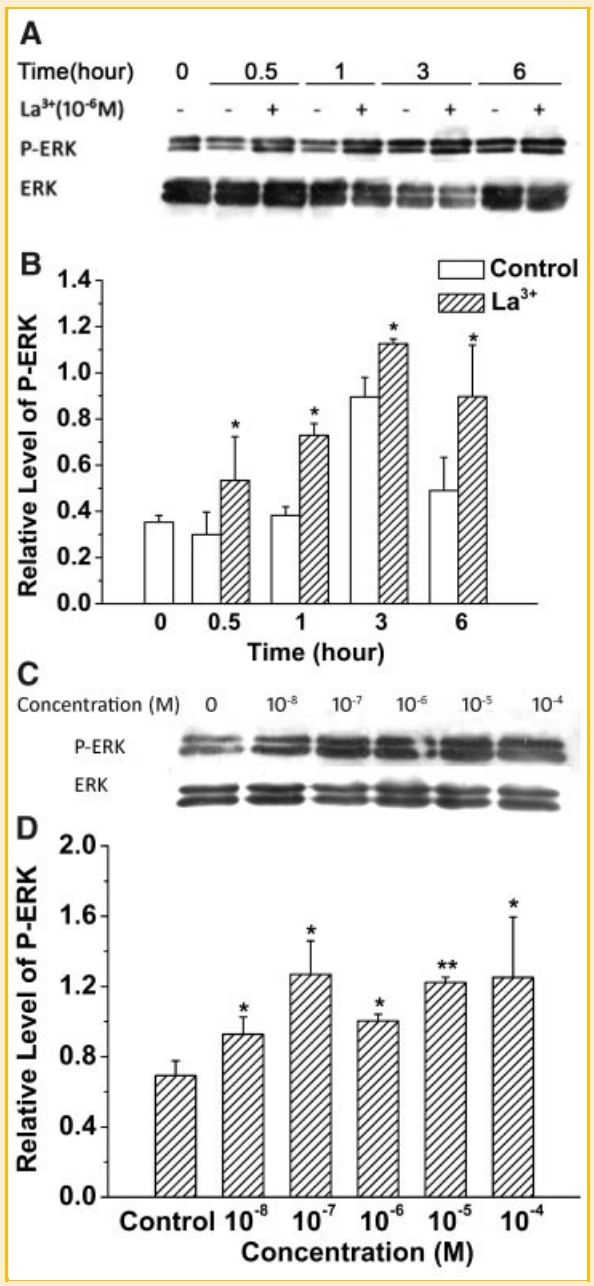

Fig. 3. Effect of La^{3+} on ERK1/2 phosphorylation in osteoblasts. A: Time dependency of ERK1/2 phosphorylation induced by La^{3+}. Cells were cultured in serum-free DMEM for 24 h, followed by incubation with 10^{-6} M La^{3+} or its vehicle for the indicated time. Cells were then lysed and the phosphorylated ERK1/2 was detected. B: The blots were quantified by a Scion Image software and plotted against La^{3+} incubation time. C: Concentration dependency of ERK1/2 phosphorylation induced by La^{3+}. Cells were serum-starved for 24 h, followed by incubation with various concentrations of La^{3+} for 3 h. Then, the phosphorylated ERK was detected. D: The blots were quantified by a Scion Image software and plotted against La^{3+} concentration. Data are representative of three separate trials ($^{*}P < 0.05$, $^{**}P < 0.001$, vs. control).

To further evaluate the involvement of the ERK signaling pathway in the La^{3+}-induced osteoblast differentiation, cells were pretreated with U0126, an ERK inhibitor, for 2 h before the addition of 10^{-6} mol/L La^{3+}. As presented in Figure 4A,B, the inhibitor not only inhibited the stimulation of cell viability at 24 h, but also suppressed the La^{3+}-induced elevation in ALP activity at 4 days. Further, U0126 completely inhibited La^{3+}-induced upregulation of BSP and Cbfa-1 mRNA expressions (Fig. 4C). These findings indicate that the La^{3+}-enhanced osteoblast differentiation was associated with ERK signaling pathway.

PTX SUPPRESSES LA^{3+}-INDUCED INCREASE IN INTRACELLULAR CA^{2+}, ERK1/2 PHOSPHORYLATION AND OSTEOBLAST DIFFERENTIATION

The effects of La^{3+} on intracellular Ca^{2+} level were analyzed on a confocal laser-scanning fluorescence microscope. La^{3+} in the range of 10^{-7}–10^{-5} M induced a sustaining increase in intracellular Ca^{2+} level (Fig. 5A). Among the tested groups, 10^{-6} M La^{3+} exhibited the strongest effect.

To investigate whether the effect of intracellular Ca^{2+} was mediated via a PTx-sensitive Gi protein signaling pathway, osteoblasts were pretreated with the Gi protein inhibitor PTx. As shown in Figure 5B, PTx significantly suppressed La^{3+}-induced increase in Ca^{2+} level in osteoblasts, suggesting that PTx-sensitive Gi protein was involved in mediating the effect of La^{3+} on intracellular Ca^{2+} level. There is no statistical difference between La–PTx and PTx control. Besides, PTx pretreatment for 24 h reduced the La^{3+}-promoted cell viability, ALP activity, and OC secretion (Fig. 6); while PTx alone (without La) caused no significant difference compared with the control-vehicle. In addition, the La^{3+}-enhanced ERK1/2 phosphorylation was also partially inhibited by PTx (Fig. 7).

DISCUSSION

The present study shows that La^{3+} treatment enhances in vitro osteoblast differentiation, and this effect is mediated by Cbfa-1 that depends upon the activation of ERK via the PTx-sensitive Gi protein signaling.

As an essential part of bone formation, osteoblast differentiation can be affected by La^{3+}. We found that La^{3+} enhanced osteoblast differentiation in vitro, as indicated by the upregulation of ALP activity and OC expression, as well as the increased matrix calcification. In addition, La^{3+} exposure stimulated the gene expression for matrix proteins including OPN and BSP, but not type I collagen (COL-I). Synthesis and secretion of these matrix proteins are necessary to bone nodule formation [Lynch et al., 1995; Harada and Rodan, 2003]. A few lines of evidence suggest that osteoblast differentiation depends, at least in part, upon the expression of cell-specific transcription factor Cbfa-1 [Komori, 2003; Nakashima and de Crombrugghe, 2003]. In this work,

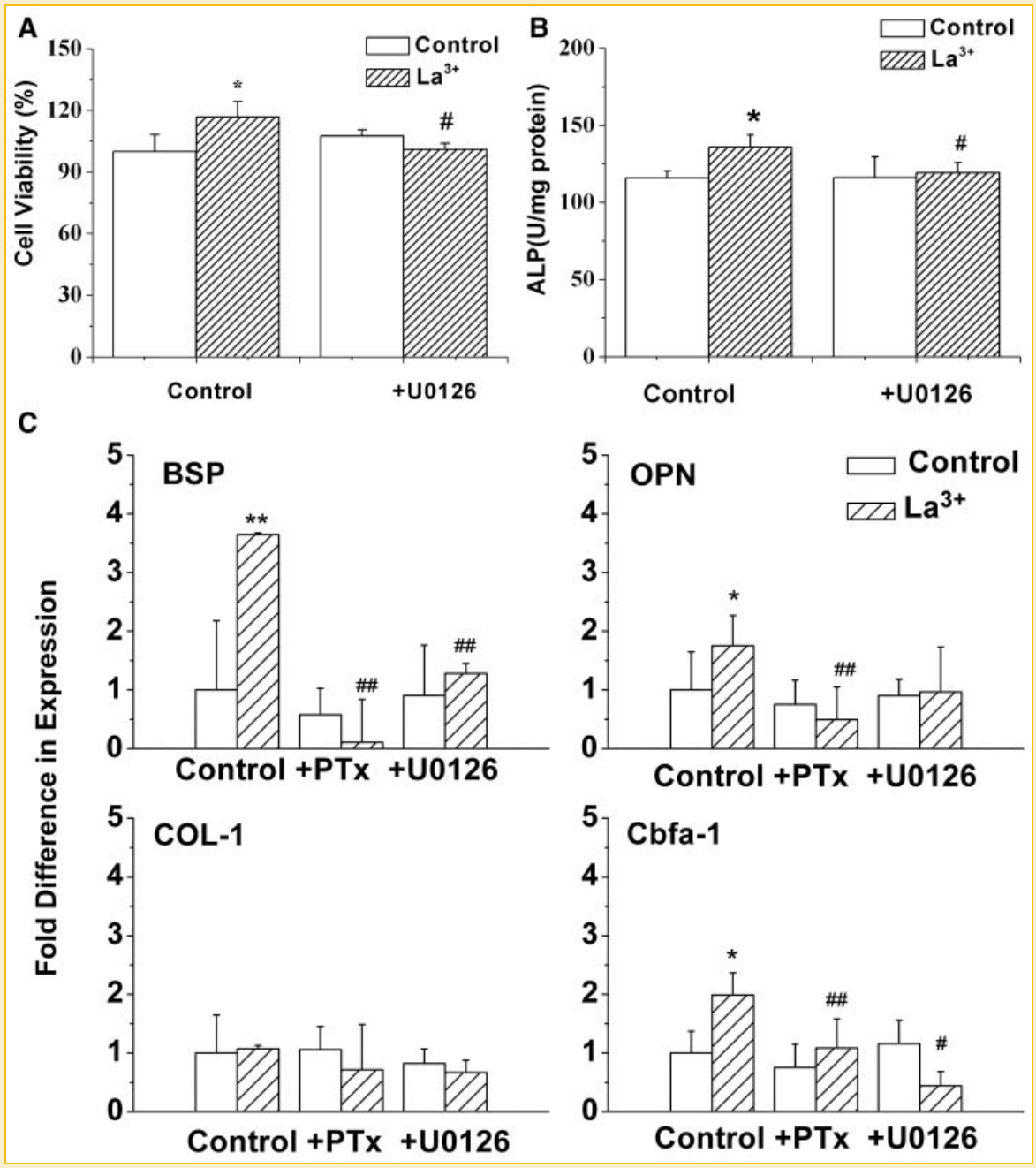

Fig. 4. Effects of ERK inhibitors on La^{3+}-induced increases in osteoblast proliferation, ALP activity. Cells were pretreated with 10 M U0126 for 2 h prior incubation with or without 10^{-6} M La^{3+}. A: Cell viability was analyzed by MTT assay after 24 h. B: ALP activity was determined on day 4. C: Gene expression of COL-I, BSP, OPN, and Cbfa-1. Data are mean ± SD of 4 determinations from a representative experiment ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. La^{3+}).

treatment of the cells with La^{3+} resulted in a significant upregulation of mRNA levels of Cbfa-1, a fact that may account for the potent effect of La^{3+} on differentiation.

The effect of La^{3+} is associated with Gi protein signaling. La^{3+} has been used extensively as a calcium channel blocker in cellular or other biological systems [Miledi, 1971; Block et al., 1998]. However, in the present study, La^{3+} induced an increase in intracellular Ca^{2+} (Fig. 5). A similar phenomenon has been reported by Yu et al. [2006] in NIH 3T3 cells. In addition, the Ca^{2+} elevation could be suppressed by pretreatment with the Gi protein inhibitor PTx. Gi proteins have been demonstrated to be potent mediators of cell proliferation and ALP activity of osteoblast-like cells in response to factors acting through G protein-coupled receptors (GPCRs) [Suzuki et al., 1998; Chen et al., 2003]. PTx can inhibit GPCRs-mediated activation of these Gi proteins, and therefore, is frequently used as a tool to identify Gi protein-mediated effecter responses [Peng et al., 2008]. The in vitro actions of fluoride, catecholamine, and strontium to promote proliferation and differentiation of osteoblasts are all

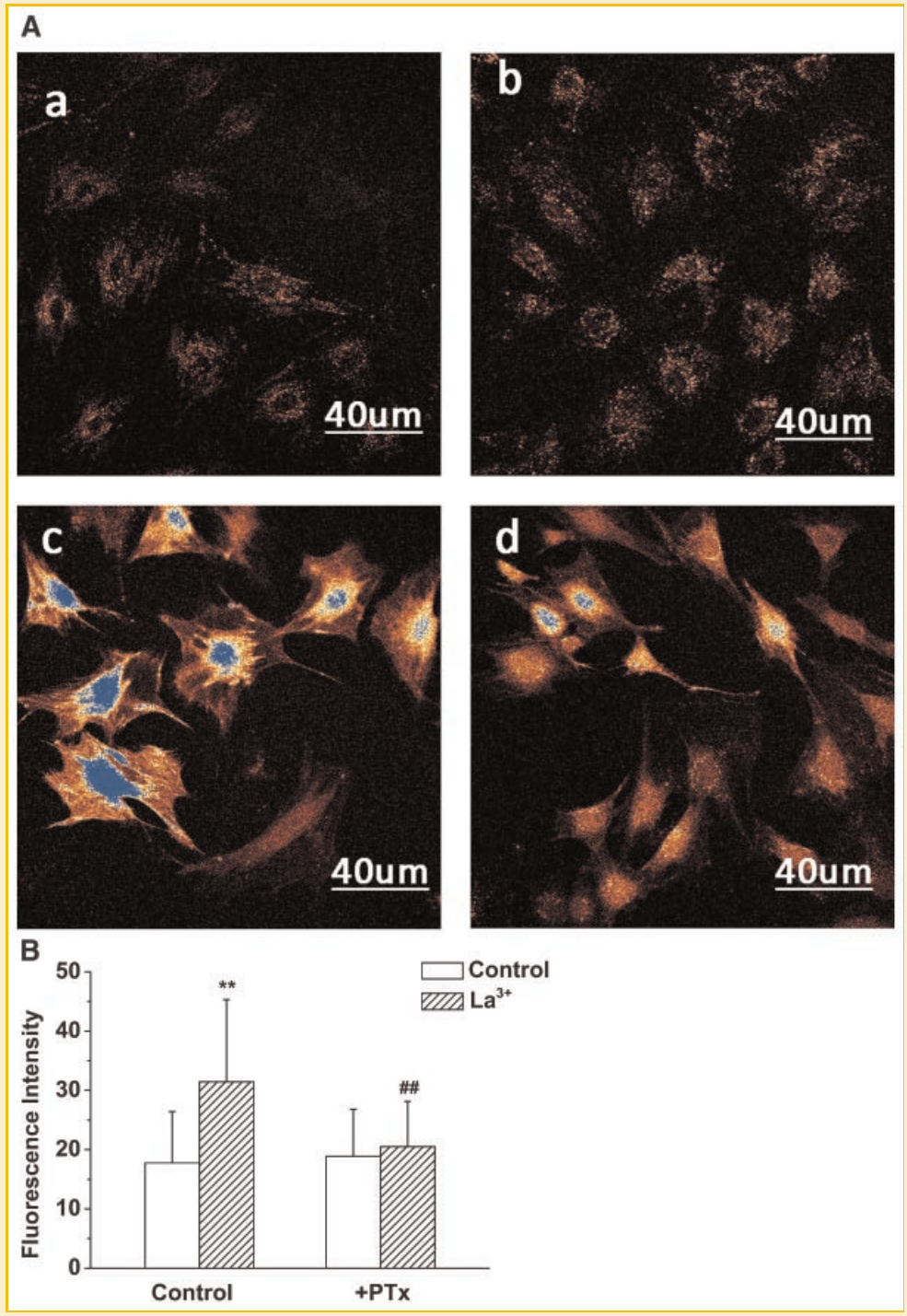

Fig. 5. Effects of La^{3+} on intracellular Ca^{2+} level monitored by a confocal laser-scanning fluorescence microscope. A: Representative images of intracellular Ca^{2+} level were shown. (a) control; (b) 10^{-5} M La^{3+}; (c) 10^{-6} M La^{3+}; (d) 10^{-7} M La^{3+}. B: Effects of PTx pretreatment on La^{3+}-induced increases in intracellular Ca^{2+} level. The fluorescent intensity was measured after loading of Fluo-3/AM. Cells were treated in the absence or presence of 10^{-6} M La^{3+} without or with PTx pretreatment for 24 h. Results are shown as mean ± SD of different cells ($n = 50$; $^{**}P < 0.001$ vs. control; $^{\#\#}P < 0.001$ vs. La^{3+}). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

inhibited by PTx [Susa et al., 1997; Lau and Baylink, 1998; Suzuki et al., 1998; Pi et al., 2005]. The in vivo anabolic actions of these agents could, at least in part, be mediated by stimulation of Gi signaling. Our results demonstrated that PTx effectively suppressed La^{3+}-induced ERK phosphorylation and changes in osteoblast viability and differentiation, implying that PTx sensitive Gi proteins may act as an important signal in mediating the effect of La^{3+}.

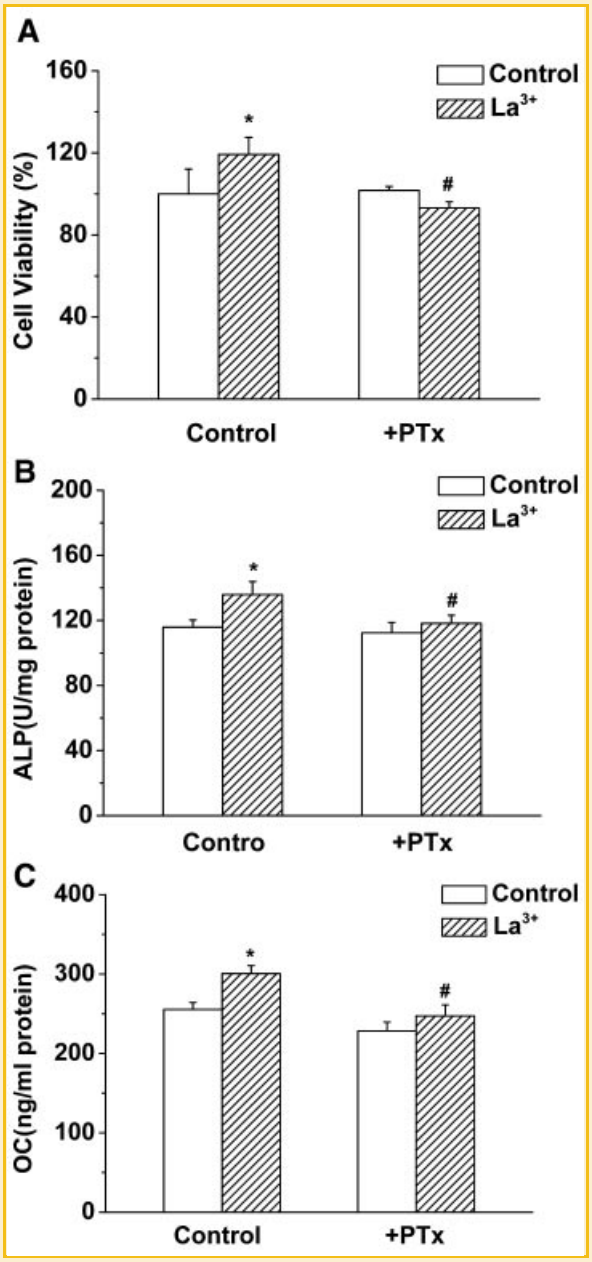

Fig. 6. Effects of PTx pretreatment on La^{3+}-induced increases in osteoblast viability, ALP activity, OC secretion, and gene expression of osteoblast differentiation markers. Cells were treated in the absence or presence of 10^{-6} M La^{3+} without or with PTx pretreatment for 24 h. A: Cell viability. B: ALP activity. C: OC secretion. Data are mean ± SD of 4 determinations from a representative experiment ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. La^{3+}).

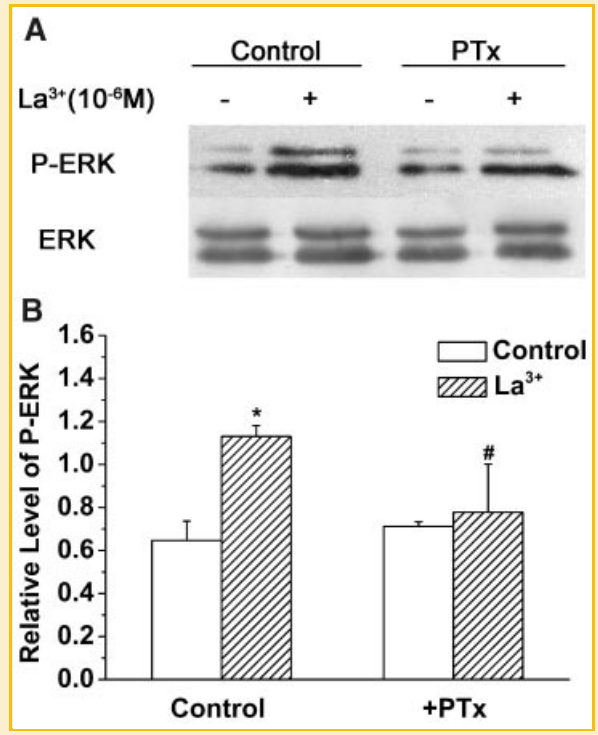

Fig. 7. Effects of PTx pretreatment on La^{3+}-induced ERK1/2 phosphorylation as detected (A) and quantified by a Scion Image software (B). Data are mean ± SD of two separate trials ($^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. La^{3+}).

The mechanism by which La^{3+} activates Gi proteins in osteoblasts remains unknown. Gadolinium cation (Gd^{3+}) is a mimic of extracellular Ca^{2+}. Recent evidence indicates that Gd^{3+} stimulates osteoblast differentiation most likely acting through the calcium-sensing receptor (CaR), a G protein-coupled receptor [Nemeth et al., 1998; Dvorak et al., 2004]. In addition to CaR, there is evidence for the presence of another cation-sensing G-protein coupled receptor in osteoblasts [Caverzasio et al., 1997; Spurney et al., 1999; Pi et al., 2005], which can be activated by both Ca^{2+} and Gd^{3+} [Abe et al., 2003]. Likewise, La^{3+} might act through CaR or other cation-sensing receptors coupled to Gi proteins, and then influence osteoblast differentiation. Further studies are required for the identification of the molecular target of La^{3+} in osteoblasts.

Gi protein signaling can activate ERK in growth factor-stimulated osteoblast differentiation [Caverzasio et al., 2000; Naor et al., 2000]. Previous studies have demonstrated that sustained activation of MAPK activity is a key mediator of cellular differentiation [Strack, 2002; Shin et al., 2008]. Consistently, we found that La^{3+} treatment promoted long-lasting activation of ERK, and pretreatment with ERK inhibitors abolished the La^{3+}-induced changes in ALP activity in osteoblasts, suggesting that the ERK activation is crucial for La^{3+}-induced osteoblast differentiation.

In summary, as demonstrated in the present study, La^{3+} acts as a positive regulator of osteoblast differentiation, and its effect is, at least partially, mediated by ERK activation via PTx-sensitive Gi protein signaling.

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