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ORIGINAL ARTICLE

Connective tissue growth factor is a downstream mediator for preptin-induced proliferation and differentiation in human osteoblasts

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Abstract Preptin, a newly isolated 34-amino-acid peptide hormone that is cosecreted with insulin and amylin from pancreatic beta-cells, has emerged as a regulatory element in bone metabolism, but its mechanism remains unclear. We assessed the effects of preptin on proliferation and differentiation of human osteoblasts and investigated the mechanism involved. Our results demonstrated that preptin promoted human osteoblasts proliferation and alkaline phosphatase activity. Suppression of connective tissue growth factor (CTGF), which was upregulated by preptin in a dose- and time-dependent manner, with small interfering RNA (siRNA) abolished the preptin-induced human osteoblasts proliferation and differentiation. Preptin induced activation of ERK mitogen-activated protein kinase (MAPK), but not p38 or JNK in human osteoblasts. Furthermore, pretreatment of human osteoblasts with the ERK inhibitor PD98059 abolished the preptin-induced CTGF secretion and blocked the promoting effect of preptin on osteoblasts proliferation and differentiation. These data demonstrated that preptin is involved in bone anabolism mediated by ERK/CTGF in human osteoblasts and may contribute to the preservation of bone mass observed in hyperinsulinemic states, such as obesity.

Keywords Preptin · Connective tissue growth factor · Osteoblasts · Extracellular signal regulated kinase

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Introduction

Extensive epidemiological data have shown that high body weight or BMI is correlated with high bone mass and that reductions in body weight may cause bone loss (Wardlaw 1996; Guney et al. 2003; Radak 2004). Although mechanical loading may contribute to this relationship, other factors are also involved (Hla et al. 1996). Obesity is associated with hyperinsulinemia, arising from resistance to the hypoglycemic effects of insulin. At least two other peptides, preptin and amylin, are cosecreted with insulin from β-cells (Cooper et al. 1987; Buchanan et al. 2001) and would be expected to circulate in increased concentrations during obesity.

Preptin is a 34-amino-acid peptide, which corresponds to Asp^{69}-Leu^{102} of the proinsulin-like growth factor II E-peptide (pro-IGF-IIE), purified from secretory granules isolated from cultured murine β-cells (Buchanan et al. 2001). It has been suggested that preptin is a physiological amplifier of glucose-mediated insulin secretion. A recent study showed that preptin stimulates osteoblasts proliferation and reduces osteoblasts apoptosis. Administration of preptin increased bone area and mineralizing surface in adult mice (Cornish et al. 2007). Therefore, preptin may be involved in anabolism in bone and may contribute to the preservation of bone mass observed in hyperinsulinemic states, such as obesity (Cornish et al. 2007). However, its mechanism remains unclear.

Connective tissue growth factor (CTGF/IGFBP-rP2), a secreted, extracellular matrix-associated protein, regulates diverse cellular functions in different cell types and belongs to a larger CCN gene family (CTGF, Cyr61 and nephroblastoma overexpressed). CTGF has special importance in skeletal development through acting as an anabolic growth factor to regulate osteoblasts proliferation, differentiation and function (Takigawa et al. 2003; Kubota and Takigawa 2007).

Since CTGF/IGFBP-rP2 binds IGF-I and IGF-II, and preptin belongs to IGF-IIE, we hypothesize that CTGF may mediate the effect of preptin on osteoblasts. In the present study, we determine the proliferation and differentiation effects of preptin on human osteoblasts and investigate how the ERK/CTGF signaling pathway is involved.

Materials and methods

Reagents

Recombinant human preptin was purchased from American Peptide Company Inc. (Sunnyvale, CA, USA). Anti-extracellular signal-regulated kinase (ERK), p-ERK, p38, p-p38, c-jun N-terminal Kinase (JNK) and p-JNK antibodies, anti-goat CTGF polyclonal antibody and anti-mouse and anti-rabbit IgG peroxidase conjugated antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). PD98059, SP600125 and SB203580 were purchased from Calbiochem (San Diego, CA, USA).

Cell culture

Bone samples were obtained from donors with informed consent and after approval by the Local Research Ethics Committee. Primary cultures of normal human osteoblasts were prepared from trabecular bone obtained during surgery following traffic accidents. None of the bone donors had clinical symptoms or histories of bone metabolic disorders. Human osteoblasts were isolated from trabecular bone as previously described (Yuan et al. 2006; Xie et al. 2007). Briefly, samples were rinsed extensively with serum-free α-MEM (Sigma Chemical Corp., St. Louis, MO, USA) and digested with type IV collagenase (Sigma). The digested chips were cultured in phenol red-free α-MEM containing 10% fetal bovine serum (FBS, Gibco-BRL Corp. Grand Island, NY, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbic acid (Sigma) at 37°C. After 15 days, cells had migrated from within the bone particles and reached confluence after 25 days. Cells were passaged and subcultured in α-MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbic acid. The phenotypes of cells were characterized based on the expression levels of ALP, collagen type 1 and osteocalcin and the formation of mineralization nodules as previously described (Luo et al. 2006; Xie et al. 2007; Yuan et al. 2007).

For preptin treatment, human osteoblasts were plated in 25 cm^2 flasks in α-MEM containing 10% FBS. After 4 days, cells were subsequently treated with vehicle (serum-free DMEM) or 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M preptin for 24 h in serum-free α-MEM. To study the effects of inhibitors, cells were pretreated with PD98059, SP600125 or SB203580 for 2 h prior to treatment with 10^{-9} M preptin. Conditioned human osteoblasts culture media and the cell monolayers were collected, frozen and stored at 70°C until total protein determination and immunoblot analysis. The cell layers were also harvested for total protein determination and ALP activity assay.

Assessment of cell proliferation

Human osteoblasts proliferation was assessed by measuring [^{3}H]thymidine (2 μCi/ml) incorporation into trichloroacetic acid (TCA)-insoluble material. Briefly, cells were plated at a density of 2×10^4 cells/well in 24-well plates and treated with 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M recombinant preptin for 24 h as described above, in the presence of [^{3}H]thymidine. After 24 h, the plates were washed with PBS and 10% TCA solution was added to the wells. Incorporated [^{3}H]thymidine was released through washing with 0.2 N of NaOH and radioactivity was measured using a β-scintillation counter. Results are expressed as counts per minute.

Alkaline phosphatase (ALP) activity assay

ALP activity assays were performed as previously described (Luo et al. 2006). Briefly, human osteoblasts were grown to confluence in 24-well plates. The cells were then 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 μg/ml aprotinin. The lysates were homogenized and then ALP activity was assayed by spectrophotometric measurement of *p*-nitrophenol release at 37°C. To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay.

Western blot analysis

Medium aliquots from human osteoblasts cultures were precipitated with 10% trichloroacetic acid. The pellet was suspended in Laemmli sample buffer to give a final concentration of 2% SDS and fractionated on a 12% denaturing gel in the absence of reducing agents. Proteins were transferred to nitrocellulose membrane (Amersham Pharmacia

Biotech), blocked with 2% BSA and probed with a 1:2,000 dilution of Affigel affinity purified goat antibody raised against human CTGF in 1% BSA overnight. Blots were probed with a rabbit anti-goat IgG antiserum conjugated to horseradish peroxidase and developed with a horseradish peroxidase chemiluminescent detection reagent. Western blots are representative of three or more cultures.

Detection of MAPK activation by Western blot analysis

Human osteoblasts were first treated with 10^{-9} M preptin for 5–60 min. Then, cell monolayers were washed quickly with cold PBS containing 5 mM EDTA and 0.1 mM Na_3VO_4 and lysed with a lysis buffer consisting of 20 mM of Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4, 10% glycerol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM ABSF, 10 mg/ml leupeptin and 10 mg/ml aprotinin. Western blot analysis was performed as above. Proteins were then transferred to a nitrocellulose membrane. The membrane was incubated with anti-p-ERK1/2 and ERK1/2 primary antibodies diluted 1:500 in PBS for 2 h. The membrane was then incubated with 1:1,000 goat anti-mouse IgG conjugated with horseradish peroxidase in PBS for 1 h. Blots were processed using an ECL Kit (Santa Cruz) and exposed to X-ray film.

RNA interference for CTGF

CTGF-siRNA plasmid vectors were constructed as previously reported. Two pairs of small interfering RNAs (siRNAs) and scrambled control siRNAs were synthesized by Genesil Biotechnology Co. (Wuhan, China). The targeted 21-nucleotide (nt) sequences derived from the human CTGF mRNA (Genbank No. NM_001901; 762–781 bp) were selected. The scrambled control siRNA with the same nucleotide composition as the CTGF siRNA but lacking significant sequence homology to the human genome was also constructed. For gene knockdown experiments, human osteoblasts were plated in a 60-mm diameter dish and cultured for 24 h in medium without antibiotics. Cells were transfected with siRNAs (1 nmol/well) using Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h of culture, cells were retransfected with siRNAs or controls and then recultured for another 48 h. Protein expression was analyzed by Western blot.

Statistical analyses

All experiments were repeated at least three times and representative experiments are shown. The results of the experiments were normalized relative to total protein levels as determined by Bradford's method. The data are expressed as means ± SD. Comparisons among values of more than two groups were performed by analysis of variance (one-way ANOVA). *P* values of less than 0.05 were considered statistically significant in all cases.

Results

Preptin stimulates proliferation and differentiation of human osteoblasts

First, we study the effect of preptin on the proliferation of human osteoblasts. As shown in Fig. 1a, at concentrations $>10^{-10}$ M, preptin stimulates the proliferation of osteoblasts at 24 h, which reaches a maximum effect at 10^{-9} M. Second,

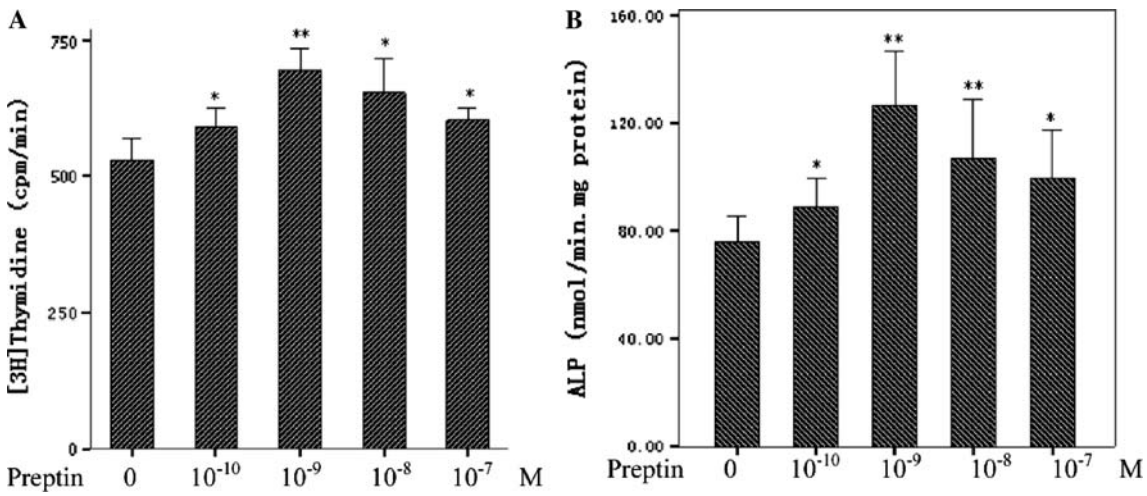

Fig. 1 Effects of preptin at various concentrations on [^{3}H]thymidine incorporation and ALP activity in cultured osteoblasts. **a** Cells were exposed to 0–10^{-7} M preptin for 24 h. Cell proliferation was determined by measuring [3H]thymidine incorporation. Results are expressed as counts per minute. **b** Cells were exposed to 0–10^{-7} M preptin for 24 h. The cells are homogenized for the ALP activity assay. The *bars* represent the mean ± SD ($n = 5$). *$P < 0.05$ vs. control, **$P < 0.01$ vs. control

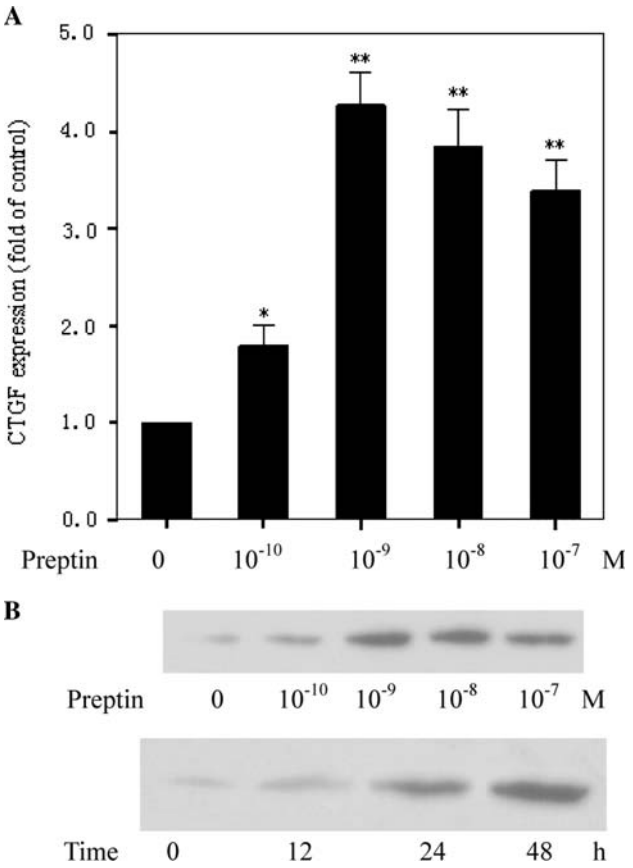

Fig. 2 Effect of preptin on CTGF protein secretion in cultured osteoblasts. **a** Preptin at 10^{-9} M has maximal effects on CTGF secretion in osteoblasts. Cells were exposed to fresh serum-free medium without or with indicated concentrations of preptin for 24 h. Western blot analysis was performed using an anti-CTGF antibody as described in "Materials and methods". The *bar* represents the mean ± SD ($n = 3$; $^*P < 0.05$ vs. control). **b** Time course analysis for CTGF protein expression in response to 10^{-9} M preptin in cultured osteoblasts

we investigate the effects of preptin on the expression of differentiation markers of human osteoblasts. Figure 1b shows the dose response of effects of preptin on the ALP activity in cultured human osteoblasts. After 24 h of culture with 10^{-10} M of preptin, the ALP activity is greater than that of controls ($P < 0.05$). At 10^{-9} M preptin, the ALP activity increases ($P < 0.001$). The promoted proliferation and differentiation effects of preptin reach the maximum at 10^{-9} M preptin and then decrease, which indicate a bell-shaped response to preptin.

Preptin induces CTGF expression in human osteoblasts

Treatment of human osteoblasts with preptin causes a time- and dose-dependent increase in CTGF (Fig. 2). The release of CTGF into medium (as detected by blotting) also shows a maximal response to preptin at 10^{-9} M. (Fig. 2a). The time-dependent effect is initially observed after 12 h of exposure to preptin at 10^{-9} M and is sustained for 24–48 h (Fig. 2b).

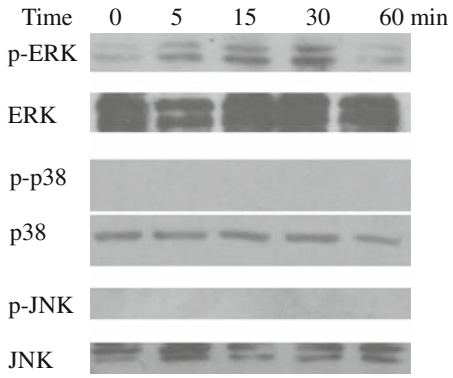

Fig. 3 Stimulation of ERK phosphorylation by preptin. Western blot analyses of phosphorylated ERK in osteoblasts cultures treated with 10^{-9} M preptin for the indicated time

Preptin induction of CTGF secretion in human osteoblasts involves ERK activation

Mitogen-activated protein kinase (MAPK) is well known to play an essential role in controlling cell proliferation and differentiation, and three major subfamilies of MAPKs have been identified, including extracellular signal-regulated kinases (ERK), c-jun N-terminal Kinases (JNK) and the p38 MAP kinases (p38). A previous study demonstrated that the ERK pathway was involved in the proliferation stimulating effect of preptin on primary osteoblasts (Cornish et al. 2007), and ERK was a key factor in CTGF expression (Yuan et al. 2007; Leivonen et al. 2005). Therefore, we examine MAPK signaling components induced by preptin. Our results show that preptin enhances the levels of phosphorylated ERK after 5 min of incubation as demonstrated by an increase in phosphorylated ERK (p-ERK) levels. Figure 3 shows the peak of ERK activation occurred after 30 min (no significant changes were noted for total ERK). In contrast, preptin has no effect on the activities of p38 or JNK and their phosphorylated forms are not detected (these results are not shown). These data demonstrate that preptin activated the ERK signaling pathways in primary human osteoblasts. We then continue our examination of ERK signaling cascades in CTGF gene regulation. We find that 10 μM of the ERK inhibitor PD98059 reduces the effect of preptin on CTGF secretion in human osteoblasts (Fig. 4).

Involvement of CTGF and ERK signaling in preptin regulation of human osteoblasts proliferation and differentiation

In order to illuminate whether and how CTGF and MAPK signaling involved in the regulation of human osteoblasts,

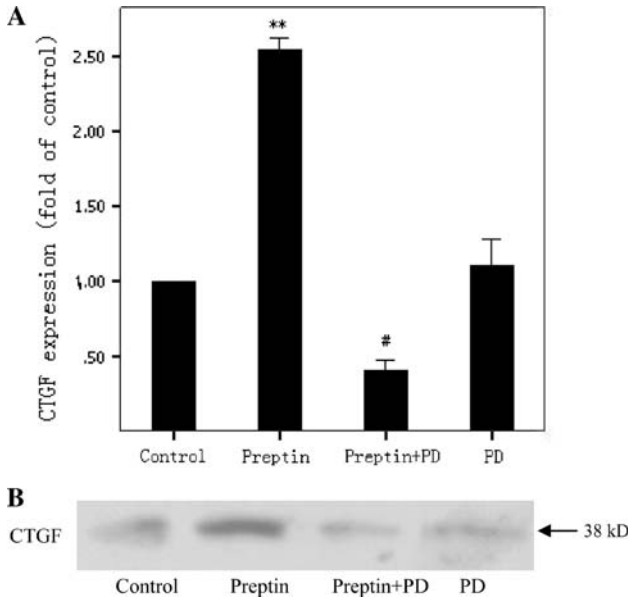

Fig. 4 ERK inhibitor PD98059 blocks the increase of CTGF produced by preptin. **a** The levels of CTGF were quantitated by densitometric analysis of the three autoradiographs. Pooled data from three separate experiments are presented as fold change over control values. The *bar* represents the mean ± SD ($n = 3$, $^{**}P < 0.01$ vs. control, $\#P < 0.01$ vs. control). **b** A representative western blot from three independent experiments is shown

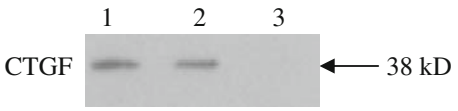

Fig. 5 Inhibition of CTGF expression in human osteoblasts using CTGF siRNA. Conditioned medium was subjected to Western blot analysis with anti-CTGF antibody. *Lane 1* conditioned medium from human osteoblasts; *lane 2* conditioned medium from human osteoblasts treated with control siRNA; *lane 3* conditioned medium from human osteoblasts treated with CTGF siRNA

we use SiRNA and signal inhibitors for blocking CTGF and MAPK signal pathway, respectively, and then observe the expression of proliferation and differentiation markers.

The CTGF knockdown efficiency by RNA interference in human osteoblasts is shown in Fig. 5. As seen in the figure, treatment with siRNA-CTGF, but not siRNA control, blocks the expression of CTGF protein secreted in human osteoblasts successfully. Figure 6a shows that pretreatment of cells with the ERK inhibitor PD98059, but not the JNK inhibitor SP600125 or p38 inhibitor SB203580, blocks the proliferation stimulation on human osteoblasts induced by preptin. Suppression of CTGF with CTGF siRNA, but not control siRNA, inhibits the proliferation stimulation of human osteoblasts induced by preptin. These data indicate that preptin-induced human osteoblasts proliferation is mediated by the ERK/CTGF pathway. Pretreatment of cells with the ERK inhibitor PD98059, but not the JNK inhibitor SP600125 or p38 inhibitor SB203580, blocks the increase in ALP activity by preptin (Fig. 6b). Suppression of CTGF with siRNA also inhibits the increase in ALP activity by preptin. These data indicates that the preptin induction of ALP activity is mediated by the ERK/CTGF pathway.

Discussion

A previous study reported that preptin directly stimulated proliferation and reduced apoptosis in osteoblasts, but had no effect on bone resorption (Cornish et al. 2007). However, the mechanism of preptin in osteoblasts was not elucidated. The present study demonstrates that preptin promotes proliferation and differentiation of human osteoblasts and the response is mediated by the ERK/CTGF pathway. Our results also add to characterization of the physiological function of preptin in osteoblasts.

Previous study demonstrated an 18-kDa fragment of IGF-IIE containing the sequence of intact preptin that has been implicated in hepatitis C-associated osteosclerosis, an acquired disorder characterized by a marked increase in biochemical and histological indices of bone formation and bone mass (Khosla et al. 1998). Preptin is a novel hormone that is cosecreted with insulin and amylin from pancreatic beta-cells (Buchanan et al. 2001). Insulin and amylin are already known to have effects on bone cells in vivo, ex vivo and in cell lines (Cornish et al. 1995, 1996, 1998). Both have anabolic functions in osteoblasts. Amylin, which belongs to the calcitonin family, also inhibits osteoclastic bone resorption. The present experiments show that preptin promotes human osteoblasts proliferation in a dose-dependent manner. This is consistent with a recent report by Cornish et al. (2007) that preptin stimulates proliferation in primary fetal rat osteoblasts and osteoblast-like cell lines. Stimulation of alkaline phosphatase activity by preptin that we observed corresponds with increases induced by CTGF (e.g. Nishida et al. 2000; Safadi et al. 2003) and obviously connects to augmentation of this growth factor by preptin. This suggests that the effects of preptin on bone cells observed in the present study may be associated with anabolic effects on bone mass in normal human physiology. The bell-shaped response of cell proliferation and especially of alkaline phosphatase to preptin maybe due to the high content of polar and ionic side chains as well as of the aromatic pi electron structures that preptin has. These special structures may relate to multiple targets preptin could act on. Higher concentrations of preptin could be increasingly inhibitory, by mass action, to more sensitive targets.

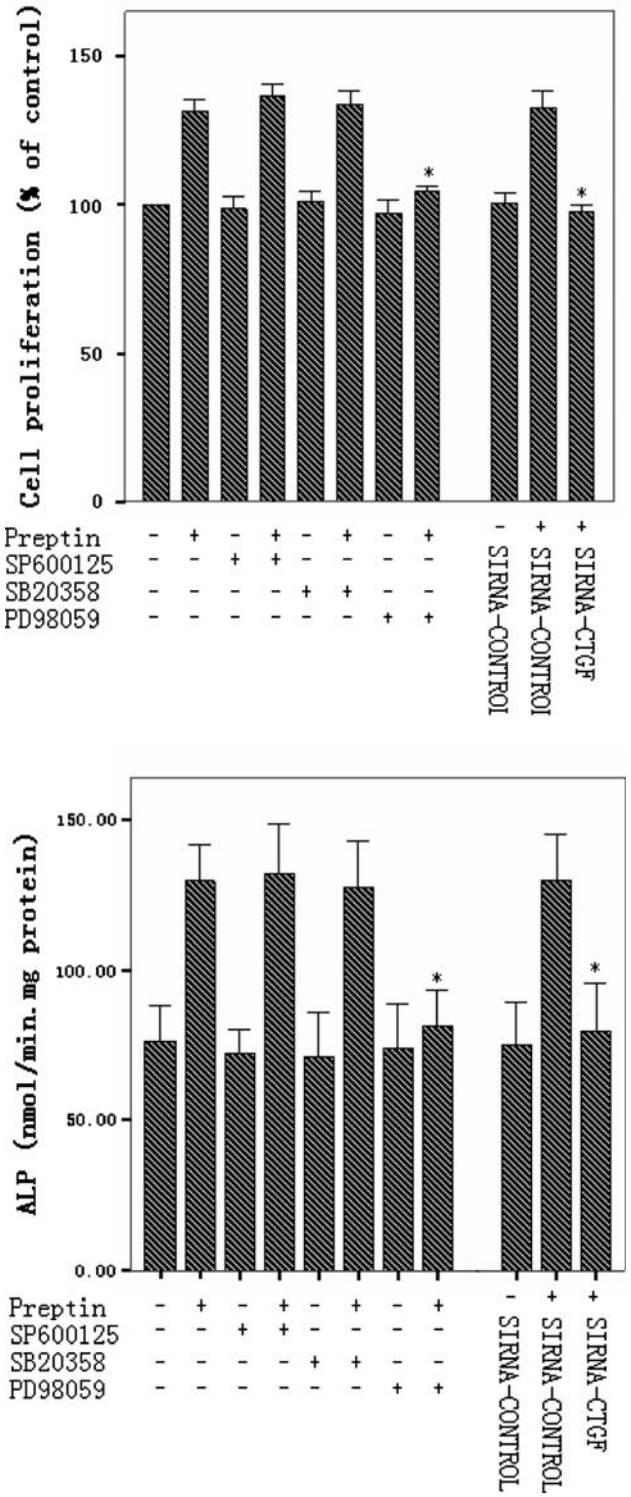

Fig. 6 ERK/CTGF pathways mediate preptin-induced osteoblasts proliferation and differentiation. **a** Cells were incubated with PD98059 (10 μM), SP600125 (10 μM) or SB203580 (10 μM) for 2 h prior to treatment with 10^{-9} M preptin for 24 h. Cells were also treated with control or CTGF siRNA in the presence of 10^{-9} M preptin. Cell proliferation was determined by measuring [^{3}H]thymidine incorporation. The *bars* represent the mean ± SD ($n = 5$; $^*P < 0.05$ vs. preptin-treated control). **b** Cells were incubated with PD98059 (10 μM), SP600125 (10 μM) or SB203580 (10 μM) for 2 h prior to treatment with 10^{-9} M preptin for 24 h. Cells were also treated with control or CTGF siRNA in the presence of 10^{-9} M preptin. Cell lysates were collected for ALP activity assays. The *bars* represent the mean ± SD ($n = 5$; $^*P < 0.05$ vs. preptin-treated control)

CTGF (also called CCN2/IGFBP-rP2), which is a classical member of the CCN family, is a conductor/modulator of bone growth. Many studies have confirmed that CTGF is expressed in osteoblasts in both organismic and cell-line settings (Nishida et al. 2000; Safadi et al. 2003; Parisi et al. 2006), and it plays an important role in bone metabolism. In vitro studies clearly indicated that CTGF promoted proliferation and differentiation of osteoblasts, as evaluated by the expression of a few marker genes, such as ALP and osteocalcin, and mineralization of osteoblastic cells (Parisi et al. 2006; Nishida et al. 2000; Smerdel-Ramoya et al. 2008). Moreover, CTGF plays a critical role in TGF-beta 1 induced osteoblasts differentiation (Arnott et al. 2007). Since CTGF/IGFBP-rP2 binds IGF-I and IGF-II, and preptin belongs to IGF-IIE, we hypothesized that preptin may perform an anabolic function in bone cells through regulating CTGF expression. Our data show that preptin upregulated CTGF in a dose- and time-dependent manner. Furthermore, suppression of CTGF with siRNA abolishes the induction of osteoblasts proliferation and ALP activity by preptin. This demonstrates that preptin regulates bone proliferation and differentiation through upregulation of CTGF in human osteoblasts.

To gain further insight into the mechanism by which preptin regulated CTGF expression, we evaluate the MAPK signaling pathways. MAPKs are well known to play an essential role in controlling cell proliferation, differentiation, and gene expression. We find that preptin induces activation of ERK in human osteoblasts, consistent with the report by Cornish et al. (2007), but has no effect on activation of p38 and JNK. Pretreatment of cells with the ERK inhibitor PD98059, but not p38 and JNK inhibitors, abolishes the preptin-induced proliferation and differentiation of human osteoblasts. Furthermore, we investigate the relationship between preptin-induced ERK activation and CTGF expression. Numerous studies have found that the ERK signaling pathway is involved in CTGF expression (Leivonen et al. 2005;Yuan et al. 2007; Smerdel-Ramoya et al. 2008). We confirm the role of this signaling pathway in mediating the effects of preptin on CTGF protein expression. The ERK inhibitor PD98059 abrogates the effects of preptin on CTGF gene expression. Using siRNA-mediated gene knockdown experiments, we find that knockdown of CTGF expression significantly diminishes preptin-induced human osteoblasts proliferation and differentiation. These results suggest that ERK/CTGF

may function as a critical mediator of preptin-induced osteoblasts signaling.

In conclusion, the present study provides evidence that preptin induces human osteoblasts proliferation and differentiation, which is mediated by the ERK/CTGF pathway. These findings suggest that osteoblasts cells are the direct targets of preptin, and the relationship between preptin and bone metabolism should be investigated further.

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