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# Expression of core binding factor α1 up-regulated by IGF-I, GM-CSF, and EGF through MAPK pathway in MC3T3-E1 and C2C12 cells[1]

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**KEY WORDS** transcription factors; osteoblast; insulin-like growth factor I; granulocyte-macrophage colony-stimulating factor; epidermal growth factor; mitogen-activated protein kinases

## ABSTRACT

**AIM:** To study the regulating function and mechanism of insulin-like growth factor-I (IGF-I), granulocyte-macrophage colony-stimulating factor (GM-CSF), and epidermal growth factor (EGF) on murine core binding factor α1 (*Cbfa1*) gene expression. **METHODS:** Luciferase reporter gene method and RT-PCR technique were used to examine the effects of these growth factors on the promoter activity and mRNA expression of *Cbfa1* gene in MC3T3-E1 and C2C12 cells. **RESULTS:** IGF-I (from 1 nmol/L to 1 μmol/L), GM-CSF (100 nmol/L), and EGF (1 μmol/L) increased the luciferase expression in MC3T3-E1 cells ($P<0.05$). And mitogen-activated protein kinase (MAPK) inhibitor, PD 98059 (10 μmol/L), completely blocked IGF-1, GM-CSF, and EGF-induced expression of *Cbfa1* promoter activity ($P<0.01$). In C2C12 cells, IGF-I (from 1 nmol/L to 10 μmol/L), GM-CSF (100 nmol/L and 1 μmol/L), and EGF (100 nmol/L) enhanced the expression of luciferase reporter plasmid driven by *mCbfa1* promoter ($P<0.05$). Addition of PD 98059 also blocked the stimulatory effects of these growth factors on *Cbfa1* promoter activity ($P<0.01$). Moreover, *Cbfa1* mRNA expression was significantly increased after treatment with IGF-I (1 nmol/L, 100 nmol/L), GM-CSF (100 nmol/L, 1 μmol/L), and EGF (1 μmol/L, 100 nmol/L) in MC3T3-E1 and C2C12 cells, respectively ($P<0.05$). These stimulatory effects of IGF-I, GM-CSF, and EGF on *Cbfa1* mRNA expression were abolished by PD 98059. **CONCLUSION:** IGF-I, GM-CSF, and EGF could increase the promoter activity and the mRNA expression of murine *Cbfa1* gene in MC3T3-E1 and C2C12 cells. These stimulatory effects might be mediated by activating the intracellular MAPK-dependent signaling pathway.

## INTRODUCTION

Core binding factor α1 (*Cbfa1*), also known as Runx2, is an osteoblast-related transcription factor that is essential for bone formation[1]. In 1995, Ducy and Karsenty[2] characterized osteoblast-specific element 2 (OSE2), a *cis*-acting sequence in the promoter of the murine osteocalcin gene 2 (mOG2), which was required for osteocalcin gene expression in osteoblastic cells[3]. The factor binding OSE2, initially termed osteoblast-specific factor 2 (Osf2), was subsequently identified

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as *Cbfa1*, a member of the Cbf/Runt family of transcription factors, which share a DNA binding motif that is homologous to the *Drosophila* protein, Runt[1]. *Cbfa1* is essential for the differentiation of osteoblasts from mesenchymal precursors, since homozygous *Cbfa1-1-* mice show a complete lack of functional osteoblasts[4,5]. Moreover, this transcription factor is required for bone matrix synthesis by differentiated osteoblasts[6], indicating that it regulates osteoblast gene expression at multiple levels.

*Cbfa1* is an essential transcription factor for osteoblast differentiation and bone formation, however, the signaling pathways regulating *Cbfa1* have not been clarified. *Cbfa1* was phosphorylated and activated by the mitogen-activated protein kinases (MAPK) pathway[7]. MAPK pathway is a major point of convergence for a variety of intracellular signals initiated by ECM-interin interaction, mechanical stimulation and many growth/ differentiation factors binding to receptor tyrosine kinases[8]. These studies led us to speculate that the growth factors such as IGF-I, GM-CSF, and EGF maybe regulate *Cbfa1* gene expression through a pathway requiring MAPK activity. To test this hypothesis, the actions of IGF-I, GM-CSF, and EGF on *Cbfa1* gene were examined in murine MC3T3-E1 preosteoblast cells and C2C12 myoblast cells.

## MATERIALS AND METHODS

**Reagents** Recombinant murine IGF-I, GM-CSF, and EGF were purchased from R&D Systems (Inc Abingdon, UK). PD 98059, a specific inhibitor of MAPK pathway, was purchased from Sigma (St Louis, MO, USA). LipofectAMINE 2000 transfection reagent was obtained from Invitrogen (Life Technologies, Carlsbad, California).

**Cell culture** Murine preosteoblastic cell line MC3T3-E1 cells and myoblastic cell line C2C12 cells, purchased from Riken cell bank (Japan), were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Life Technologies, Grand island, NY), 10 % fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), and supplemented with antibiotics (benzylpenicillin 100 kU/L and streptomycin 100 kU/L) under a 5 % $CO_2$-95 % air atmosphere at 37 ℃.

**Plasmid construction** Mouse *Cbfa1* promoter luciferase reporter plasmid (named mP696-Luc) contained 696 bp (-641–+55) murine *Cbfa1* promoter region[9] and this region was cloned in PGL3-basic vector (Promega Corp, Madison, WI). To generate a targeting vector, genomic clones containing *Cbfa1* promoter (AB013129, nucleotides +6018/6713) region were isolated from the genome of C57BJ mouse white blood cells (Qiagen, Valencia, CA) by PCR. PCR reaction was amplified with the following primers: sense, 5'-AGCACTGTTGCTCAGAACGCCACACACCT-3'; antisense, 5'-TCCTGGAGAAAGTTTGCACCGCACTTG-3'. The gel-purified blunt-ending PCR fragment was ligated into T easy Vector (Promega Corp, Madison, WI), named mP696-Teasy. The PCR product and plasmid mentioned above were confirmed by sequencing. The fragment of *Cbfa1* promoter region was then generated by PCR from mP696-Teasy using a 5' primer with *Mlu*I site (5'-ATAGacgcgtAGCACTGTTGCTCAGA 3') and a 3' primer with *Xho*I site (5'GTACctcgag-TCCTGGAGAAAGTTTGC-3'). After digesting with *Mlu*I and *Xho*I, the fragment was ligated into the *Mlu*I/ *Xho*I sites of PGL3 Basic vector. The resulting product, mP696-Luc, contains nucleotides -641–+55 (696 bp) of the reported *Cbfa1* promoter.

**Transfections and luciferase analysis** All cell lines were plated in 24-well plates at a density of $1\times10^5$ cells/cm$^2$. After 24 h, cells were transiently transfected with 0.5 μg mP696-Luc plasmid using the DNA-lipid complex, LipofectAMINE 2000, according to the manufacture's protocol. To assess transfection efficacy, and to normalize the firefly luciferase signal expressed by mP696-Luc plasmid, 20 ng of PRL SV40 (Promega Corp, Madison, WI), which encodes a Renilla luciferase gene downstream of a minimal SV40 promoter, was systematically added to the transfection mix. In the positive and negative experiments, *Cbfa1* constructs were replaced by pGL3-control plasmid (Promega Corp, Madison, WI) and empty pGL3-basic vector. Sixteen hours after transfection, cells were washed and culture medium was changed to serum free medium, HyQ-CCM5 (Hyclone, Logan, Utah), then cells were treated with growth factors and/or PD 98059 and cultured for an additional 24 h. Luciferase assays were performed with the Dual Luciferase Assay Kit (Promega Corp, Madison, WI), according to the manufacturer's instructions. A 50 μL quantity of cell lysate was assayed first for firefly luciferase and then for Renilla luciferase using a luminometer (Analytical luminoscencelab corp, USA). Firefly luciferase activity was normalized to Renilla luciferase activity. The mean corrected luciferase activity of the control group was defined as 1, and the relative luciferase activity (RLA) of the treat-

ment groups was obtained as compared to the control group.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)** MC3T3-E1 and C2C12 cells were plated in 6-well plates at a density of $1\times10^5$ cells/cm$^2$. After treatment with different growth factors and/or PD 98059 for 1-6 d, total RNA was isolated using Trizol reagent (Gibco-BRL, Life Technologies, Grand island, NY) following the manufacturer's instructions. For RT, a 25-μL reaction contained total RNA 2 μg, oligod$T_{(15)}$ primer 12.5 mg/L, each dNTP 500 μmol/L, 25 units of rRNasin ribonuclease inhibitor, and 200 units of M-MLV reverse transcriptase (Promega Corp, Madison, WI).

For PCR amplification, each 50-μL reaction contained 4 μL of RT aliquot, each dNTP 200 μmol/L, each primer 0.4 μmol/L, $MgCl_2$ 1.5 mmol/L, and 2.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co, Ltd Dalian). The PCR conditions were as follows. After 5 min of preincubation at 95 ºC, amplification was performed for 35 cycles consisting of 45 s of denaturing at 95 ºC, 45 s of annealing at 60 ºC, and 2 min of extension at 72 ºC. The primers used for murine *Cbfa1* were as follows. Sense: 5'-TTTAGGGCGCATTCCTCATC-3' (NM009820, nucleotides +969/988); antisense: 5'-TGTCCTTGTGGATTAAAAGGACTTG-3 (NM009820, nucleotides +1046/1070). As an internal control, the PCR analysis was also performed with the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) specific primers. Sense: 5'-TCCACTCACGGCAAATTCA-ACG-3' (M32599, nucleotides, +191/213); antisense: 5'-TAGACTCCACGACATACTCAGC-3' (M32599, nucleotides, +314/335). The RT-PCR products were separated by electrophoresis on 3 % agarose gels and the products of the expected size (102 bp of *mCbfa1* and 145 bp of mGAPDH) were obtained and sequenced. The *mCbfa1* signals were normalized to the GAPDH signals in the same reaction.

**Statistical analysis** All data were expressed as mean±SD of three wells per group. Each experiment was repeated at least three times. Statistical analyses were determined by Student's *t*-test.

## RESULTS

**IGF-I, GM-CSF, and EGF stimulate *Cbfa1* promoter activity and the MAPK pathway is involved** The effects of IGF-I, GM-CSF, and EGF on the promoter activity of murine *Cbfa1* in MC3T3-E1 and C2C12 cell lines were examined using a *Cbfa1* promoter-luciferase reporter vector (mP696-Luc) by transient transfection assay (Fig 1, $n$=3).

Treatment of MC3T3-E1 cells with IGF-I (1 nmol/L-1 μmol/L) resulted in 83 % ($P$<0.01), 59 % ($P$<0.05), 48 % ($P$<0.01), and 43 % ($P$<0.01) increases in *Cbfa1* promoter activity over that found in control cells (Fig 1A). Compared with control group, the RLA of GM-CSF (100 nmol/L, Fig 1B) group increased by 31 % ($P$<0.05). Treatment of MC3T3-E1 cells with 1 μmol/L EGF resulted in a 51 % ($P$<0.05) increase in *Cbfa1* promoter activity (Fig 1C).

In C2C12 cells, these growth factors had similar effects with MC3T3-E1 on the expression of luciferase reporter plasmid driven by the mp696-Luc. IGF-I (1 nmol/L-10 μmol/L) enhanced the RLA by 48 % ($P$<0.05), 68 % ($P$<0.01), 166 % ($P$<0.01), 55 % ($P$<0.05), and 46 % ($P$<0.01) compared with that of control group(Fig 1D). Treatment with GM-CSF (100 nmol/L and 1 μmol/L) resulted in 34 % ($P$<0.01) and 29 % ($P$<0.01) increases in Cbfa1 promoter activity over that found in control cells (Fig 1E). Compared with control group, the RLA of EGF (100 nmol/L) enhanced by 65 % ($P$<0.05, Fig 1F).

Treatment of PD 98059 (10 μmol/L), a specific inhibitor of MAPK, resulted in 20 %-30 % decreases in C*bfa1* promoter activity compared with that of control group ($P$<0.05). The RLA levels of growth factor+ PD 98059 groups were significantly lower than those of growth factor groups, respectively. These results indicated that PD 98059 suppressed basal and completely blocked IGF-I, GM-CSF, and EGF-induced expression of *Cbfa1* promoter activity not only in MC3T3-E1 cells but also in C2C12 cells.

**Induction of *Cbfa1* gene expression by IGF-I, GM-CSF, and EGF and the MAPK pathway is involved** To assess the expression of murine *Cbfa1* gene in response to growth factors, MC3T3-E1 and C2C12 cells were treated with IGF-I (1 nmol/L, 100 nmol/L), GM-CSF (100 nmol/L, 1 μmol/L), EGF (1 μmol/L, 100 nmol/L) respectively, and/or PD 98059 (10 μmol/L) from 1 to 6 d, then the mRNA levels of *Cbfa1* gene were detected by RT-PCR at different time points (Fig 2, $n$=3). There were no significant differences in basal mRNA expression of *Cbfa1* gene from 1 to 6 d both in MC3T3-E1 and C2C12 cells.

In MC3T3-E1 cells, *Cbfa1* expression was induced significantly by IGF-I treatment from 1 to 6 d (d 1: 101±3; d 2: 176±14; d 3: 217±29; d 4: 191±19; d 5: 120±6; d 6: 112±12, $P$<0.01 *vs* d 0: 69±0.4, respec-

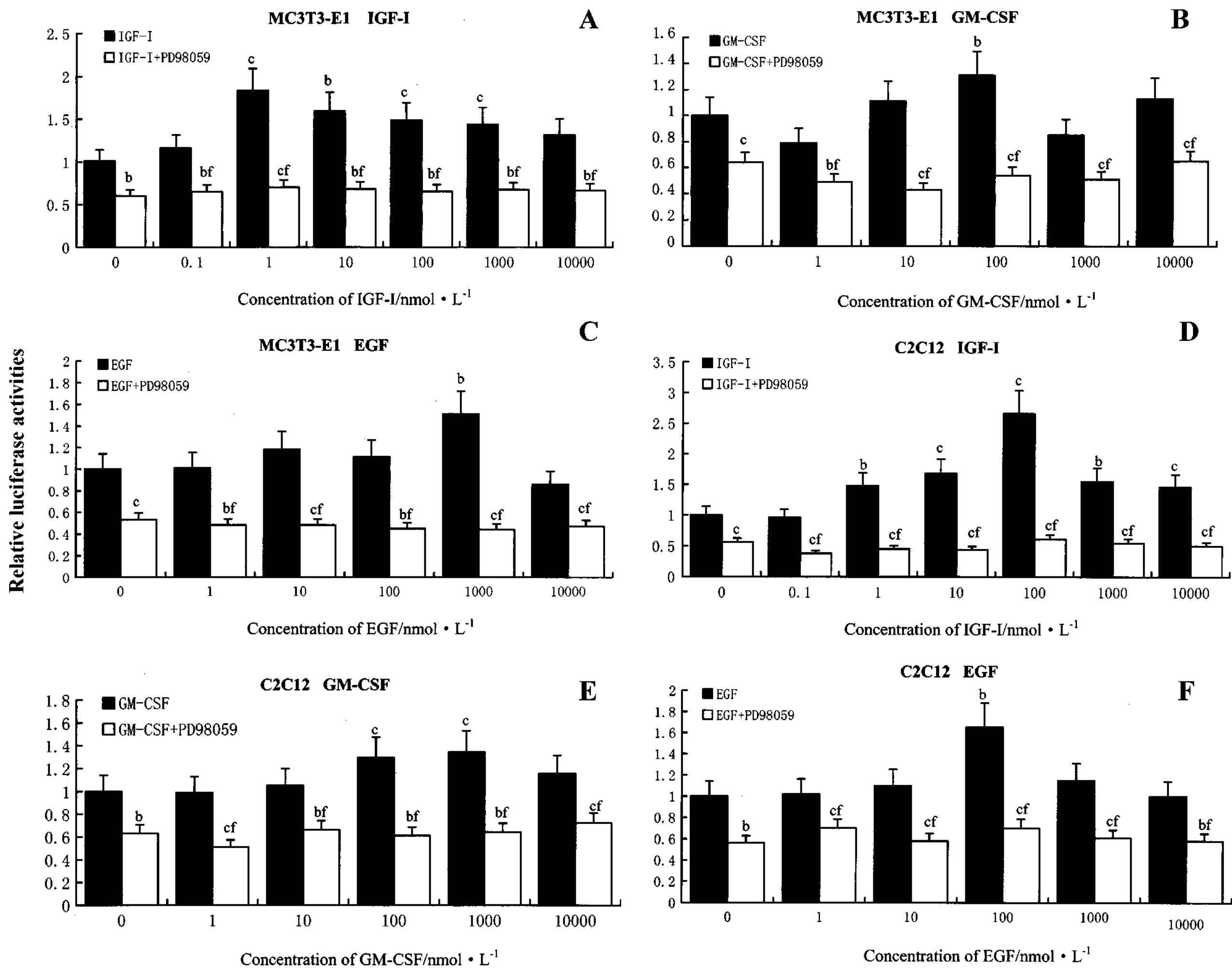


**Fig 1. Relative luciferase activities (RLA) of *Cbfal* pomoter (mP696-Luc) in MC3T3-E1 and C2C12 cells treated with different concentrations of growth factors (GF) and/or PD 98059 (10 μmol/L). MC3T3-E1 (A, B, C) or C2C12 (D, E, F) cells were transfected with the mP696-Luc reported plasmid and PRL-SV40 expression plasmid. Sixteen hours after transfection, cells were cultured in various concentrations of IGF-I, GM-CSF, and EGF with or without PD 98059 for additional 24 h. Forty hours after transfection, cells were harvested and reported activities were measured. Values normalized for transfection efficiency are shown as fold induction relative to basal promoter activity as described in materials and methods. *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group (the cells without any treatment). $^{f}P<0.01$ GF+PD 98059 groups *vs* GF groups.**

tively). GM-CSF caused increases of *Cbfa1* mRNA at d 4 and d 5 compared with d 0 (d 4: 87±7, *P*<0.01; d 5: 88±5, *P*<0.01 *vs* d 0: 68±2). Treatment with EGF resulted in increase of *Cbfa1* gene expression at d 2 (d 2: 96±8 *vs* d 0: 70±2, *P*<0.01). These significant differences at each time points of those groups mentioned above were found not compared with d 0, but also with the control group (Fig 2 A, B, C, and Fig 3).

In C2C12 cells, *Cbfa1* expression was increased significantly by IGF-I treatment from 1 to 6 d (d 1: 121±6; d 2: 194±7; d 3: 110±5; d 4: 108±5; d 5: 105±2; d 6: 106±5, *P*<0.01 *vs* d 0: 30±3, repectively). GM-CSF enhanced the *Cbfa1* mRNA level from 1 to 6 d (d 1: 85±5; d 2: 73±5; d 3: 71±7; d 4: 67±4; d 5: 69±6; d 6: 69±9, *P*<0.01 *vs* d 0: 30±4, respectively). Treatment of EGF resulted in increases in *Cbfa1* gene expression at d 2 and d 3 (d 2: 40±2; d 3: 39±1, *P*<0.05 *vs* d 0: 30±5). The *Cbfa1* mRNA levels of these groups mentioned above were significantly higher than those of control group (Fig 2 D, E, F, and Fig 4).

Treatment with PD 98059 alone resulted in decreases of murine *Cbfa1* gene expression both in MC3T3-E1 cells (d 1: 44±5; d 2: 50±8; d 3: 49±10; d 4: 47±9; d 5: 46±7; d 6: 46±10, *P*<0.05 or *P*<0.01 *vs* d 0: 69±0.4) and in C2C12 cells (d 1: 19±2; d 2: 22±3; d 3: 22±5; d 4: 21±4; d 5: 20±3; d 6: 20±4, *P*<0.05 or *P*<0.01

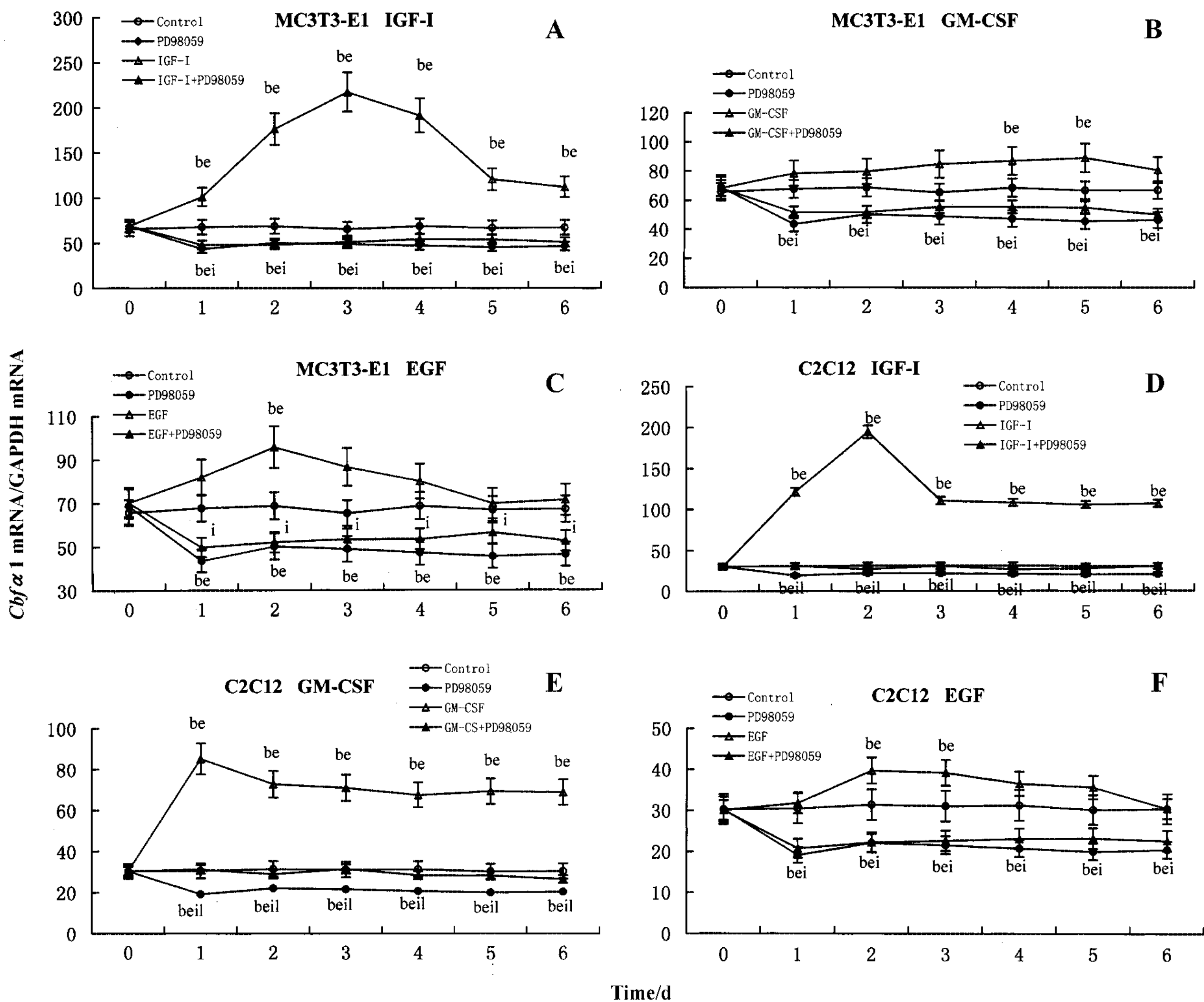


**Fig 2. The mRNA expression of *Cbfa1* gene in MC3T3-E1 and C2C12 cells treated with growth factors (GF) and/or PD 98059 (10 μmol/L). MC3T3-E1 (A, B, C) or C2C12 (D, E, F) cells were cultured in DMEM (2 % FBS) with or without IGF-I, GM-CSF, EGF, and PD 98059 for 0-6 d. Then total RNA was isolated using Trizol reagent and the mRNA expression levels of *Cbfa1* gene were detected by RT-PCR. The RT-PCR products were separated by electrophoresis on 3 % agarose gels and mCbfa1 signals (102 bp) were normalized to the GAPDH signals (146 bp) in the same reaction. $^{b}P<0.05$ *vs* d0 of GF or PD 98059 groups. $^{e}P<0.05$ *vs* control groups. $^{i}P<0.05$ *vs* GF groups. $^{l}P<0.05$ *vs* PD 98059 groups.**

*vs* d 0: 30±3) compared with d 0. Moreover, The *Cbfa1* mRNA levels of growth factors plus PD 98059 groups were significantly lower than those of growth factors groups. These results indicated that PD 98059 blocked IGF-I, GM-CSF, and EGF-induced *Cbfa1* gene expression not only in MC3T3-E1 cells but also in C2C12 cells.

## DISCUSSION

Osteoblasts originate from common progenitors, which are capable of differentiating into other mesenchymal cell lineages such as chondrocytes, myoblasts, bone marrow stromal cells, *etc*[10-13]. And various hormones and cytokines participate this process[14-18]. Among those, IGF-I, GM-CSF, and EGF are the most potent inducers and stimulators, since they are synthesized by osteoblast lineage and present in substantial concentration in bone tissue[19-21]. Because none of these growth factors is specific for cells of the osteoblast lineage. Therefore, mechanisms to induce skeletal tissue specificity might be mediated by some specific intracellular molecules.

During the past several years, our molecular understanding of osteoblast biology has made rapid progress due to the characterization of the function of

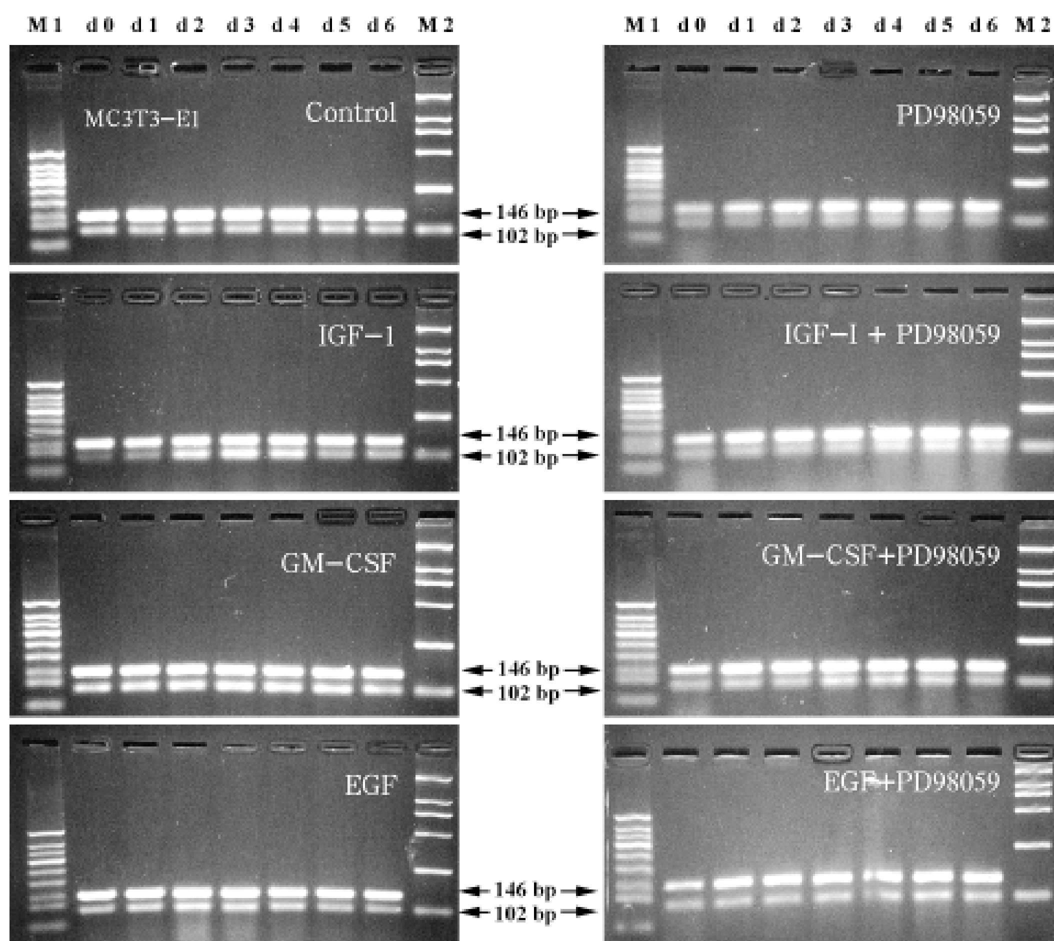


**Fig 3. mRNA Expression of *Cbfa1* gene in MC3T3-E1 cells treated with growth factors (GF) and/or PD 98059 (10 μmol/L). MC3T3-E1 cells were cultured in DMEM (2 % FBS) with or without IGF-I, GM-CSF, EGF, and PD 98059 for 0-6 d. Then total RNA was isolated using TriZol reagent and the mRNA expression of *Cbfa1* gene was detected by RT-PCR. The RT-PCR products were separated by electrophoresis on 3 % agarose gels and *mCbfa1* signals (102 bp) were normalized to the GAPDH signals (146 bp) in the same reaction. M: marker.**

one molecule, *Cbfa1*. This member of the runt/*Cbfa* family of transcription factors was first identified as the nuclear protein binding to an osteoblast-specific *cis*-acting element activating the expression of osteocalcin, the most osteoblast-specific gene[2]. *Cbfa1* was then shown to regulate the expression of all the major genes expressed by osteoblasts, such as osteocalcin, alkaline phosphatase (ALP)[22], α1 and α2(I) collagen[23], osteopontin[1], and osteoprotegerin ligand[24,25]. Indeed, *Cbfa1* is a critical molecule not only for osteoblast differentiation but also for osteoblast function. However, The regulation of *Cbfa1* gene expression and *Cbfa1* protein activity by systemic hormones and local factors are just beginning to be understood. It has been shown that *Cbfa1* expression and activity are regulated by PTH[26], 1,25-$(OH)_2$-vitamin $D_3$[27], dexamethasone[28], bone morphogenetic protein (BMP)[29], transforming growth factor-beta (TGF-β)[30], and inflammatory cytokine tumor necrosis factor alpha (TNFα)[31], *etc*.

Considering of the stimulatory effects of IGF-I, GM-CSF, and EGF on osteoblast differentiation, we speculated that *Cbfa1* might play an important role in osteoblast differentiation regulated by these growth factors. So *Cbfa1* promoter activity and mRNA expression induced by IGF-I, GM-CSF, and EGF were determined in the present study.

To investigate whether IGF-I, GM-CSF, and EGF regulate the promoter activity of *Cbfa1* gene, we firstly cloned a mouse *Cbfa1* promoter-luciferase reporter vector, mP696-Luc. Using the mP696-Luc vector, we provided evidence that *Cbfa1* promoter activity was stimulated by IGF-I, GM-CSF, and EGF in both pre-osteoblastic (MC3T3-E1) and myoblastic (C2C12) cells by transient transfection assay.

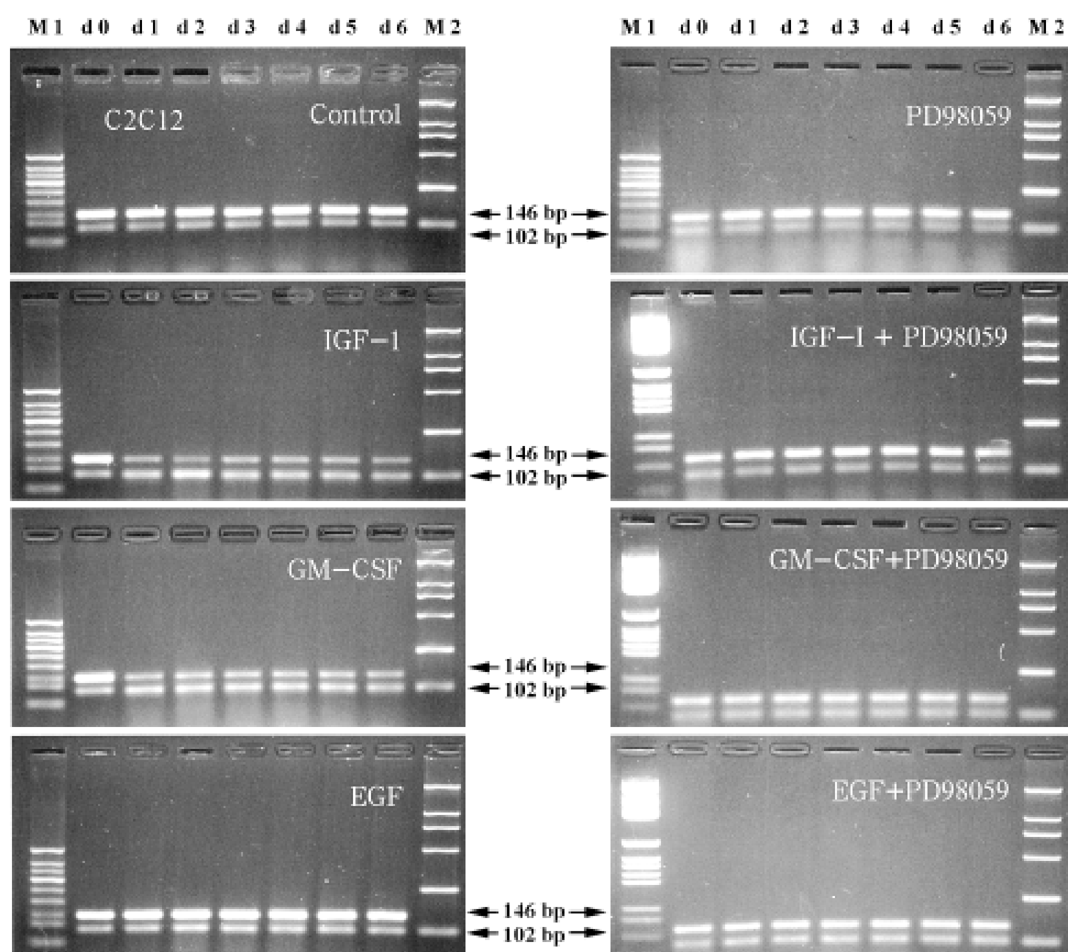


**Fig 4. mRNA expression of *Cbfa1* gene in MC3T3-E1 cells treated with growth factors (GFs) and/or PD 98059 (10 μmol/L). C2C12 cells were cultured in DMEM (2 % FBS) with or without IGF-I, GM-CSF, EGF, and PD 98059 for 0-6 d. Then total RNA was isolated using Trizol reagent and the mRNA expression of *Cbfa1* gene was detected by RT-PCR. The RT-PCR products were separated by electrophoresis on 3 % agarose gels and *mCbfa1* signals (102 bp) were normalized to the GAPDH signals (146 bp) in the same reaction. M: marker.**

To confirm the stimulatory effects of IGF-I, GM-CSF, and EGF on *Cbfa1* gene expression, we next examined the mRNA expression of murine *Cbfa1* gene in response to these growth factors. RT-PCR analysis indicated that these three growth factors could increase the levels of *Cbfa1* mRNA respectively.

The skeleton is both a source and a repository for many growth factors, including IGF-I, which plays a key role in skeletal growth and remodeling. IGF-I plays an essential role in longitudinal bone growth in response to growth hormone exposure[32,33]. Moreover, IGF-I is mitogenic for less differentiated bone cells, whereas it increases the synthesis of collagen and perhaps other matrix proteins by osteoblasts[34,35]. Our results showed that treatment with IGF-I resulted in increases of *Cbfa1* promoter activity and mRNA levels in both MC3T3-E1 and C2C12 cells. These findings suggest that the *Cbfa1* may be involved in the process of osteoblasts differentiation induced by IGF-I. In contrast to our results with MC3T3-E1 and C2C12 cells, Ducy *et al*[1] showed that IGF-I had no effect on *Cbfa1* gene expression in the rat osteoblast-like osteosarcoma cell line, ROS17/2.8. These inconsistent results can be attributed to the use of different osteogenic cell lines at different stages of osteoblast differentiation. ROS17/2.8 cells are phenotypically more mature than MC3T3-E1 and C2C12 cells, which both require inductive signals provided by ECM before they will express osteoblast marker genes.

The other two growth factors, GM-CSF and EGF had similar effects with IGF-I on the expression of luciferase reporter plasmid driven by the mP696-Luc and the levels of *Cbfa1* mRNA. Many studies have shown that GM-CSF is a stimulating factor of osteoclast[36,37]. However, the stimulatory effect of GM-CSF on osteo-

blast differentiation has not been widely accepted. Postiglione *et al*[38] found that SaOS-2 cells expressed GM-CSF receptor, *in vitro* treatment of SaOS-2 cells with recombinant human GM-CSF caused a decreased cell proliferation and an increased production of osteopontin, ALP and most but not all ECM components. But Modrowski *et al*[39] reported that GM-CSF promoted the proliferation of human osteoblastic cells. Our results showed that GM-CSF stimulated *Cbfa1* gene promoter activity and increased *Cbfa1* mRNA levels, indicating that GM-CSF maybe regulate osteoblast differentiation through induction of *Cbfa1* gene expression. The expression of EGF receptor on osteogenic cells indicates that EGF maybe regulate osteoblast differentiation directly. *In vitro* studies have demonstrated that treatment with EGF stimulates the proliferation of osteoprogenitor cells, inhibits collagen synthesis, downregulates ALP activity of matured osteoblasts[21]. In our study, two well-characterized cell lines of early differing stage of differentiation were chosen as experimental cell models. We found that EGF enhanced *Cbfa1* gene expression in MC3T3-E1 and C2C12 cells. However, additional experiments should be done to gain further insight into the role of EGF on fully differentiated osteoblastic cells.

The present study showed that IGF-I, GM-CSF, and EGF stimulated *Cbfa1* promoter activity and increased *Cbfa1* mRNA levels. But little is known about the mechanisms regulating *Cbfa1* gene expression by these growth factors. *Cbfa1* protein was phosphorylated and activated by MAPK pathway[7,40]. Since *Cbfa1* gene expression is up-regulated by *Cbfa1* itself[1]. And a major route for IGF-I, GM-CSF, and EGF receptor signaling involves activation of the MAPK ERK kinases (MEK)/ERK branch of the MAPK pathway. We speculated that MAPK pathway might be required for the stimulatory effects of IGF-I, GM-CSF, and EGF on *Cbfa1* gene. To test this hypothesis, PD 98059 was used in our experiment. PD 98059 is a potent, cell permeable and selective inhibitor of MAPK. It blocks the activation of MEK1, therefore inhibiting the subsequent phosphorylation and activation of MAPK. The present study demonstrated that the *cbfa1* promoter activity and mRNA expression were both completely abolished by supplement with PD 98059 both in basic or after stimulation with IGF-I, GM-CSF, and EGF. These results raise the possibility that MAPK pathway is necessary for stimulation of *Cbfa1* gene expression by IGF-I, GM-CSF, and EGF. But two questions still remain regarding the regulation mechanisms of MAPK on *Cbfa1* gene expression. Firstly, we do not yet know whether MAPK-dependent phosphorylation cascade regulates *Cbfa1* gene expression directly. Further experiments should be done for resolving this question. For example, MAPK stimulator may be used, or we can examine directly whether *Cbfa1* mRNA and promoter activity will be increased after transfection of cells with constitutively active MEK1, the kinase immediately before ERK1/2 in the MAPK cascade, thus more evidence can be obtained. Secondly, it is not currently clear whether the other subfamilies of MAPK are also involved in regulating *Cbfa1* gene. Multicellular organisms have three well-characterized subfamilies of MAPK that control a vast array of physiological processes[41]. These MAPK include the ERK, the c-Jun $NH_2$-terminal kinases (JNK), and the four p38 enzymes, p38$\alpha$, p38$\beta$, p38$\gamma$, and p38$\delta$. In the present study, only a specific MEK1/ERK inhibitor, PD98059, was used. So further studies should be conducted to clarify the role of JNK and P38 MAPK in regulating *Cbfa1* gene expression.

All the data together, our results indicated that IGF-I, GM-CSF, and EGF could increase the promoter activity and the mRNA expression of murine *Cbfa1* gene both in preosteoblastic cells (MC3T3-E1) and myoblastic cells (C2C12). And the MAPK pathway might be responsible for these growth factors' stimulatory effects.

## REFERENCES


1 Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 1997; 89: 747-54.

2 Ducy P, Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. Mol Cell Biol 1995; 15: 1858-69.

3 Frendo JL, Xiao G, Fuchs S, Franceschi RT, Karsenty G, Ducy P. Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression *in vivo*. J Biol Chem 1998; 273: 30509-16.

4 Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, *et al*. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997; 89: 755-64.

5 Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, *et al*. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997; 89: 765-71.

6 Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, *et al*. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 1999;

13: 1025-36.

7 Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, *et al*. MAPK pathway activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem 2000; 275: 4453-9.

8 Takeuchi Y, Suzawa M, Kikuchi T, Nishida E, Fujita T, Matsumoto T. Differentiation and transforming growth factor-β receptor down-regulation by collagen-α2β1 integrin interaction is mediated by focal adhesion kinase and its downstream signals in murine osteoblastic cells. J Biol Chem 1997; 272: 29309-16.

9 Fujiwara M , Tagashira S , Harada H. Isolation and characterization of the distal promoter region of mouse Cbfa1. Biochim Biophys Acta 1999; 1446: 265-72.

10 Taylor SM, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 1979; 17: 771-9.

11 Grigoriadis AE, Heersche JNM, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol 1988; 106: 2139-51.

12 Yamaguchi A, Kahn AJ. Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tissue Int 1991; 49: 221-5.

13 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-7.

14 Aubin JE, Liu F. The osteoblast lineage. In: Bilezikian JP, Raisz LG, Rodan GA. (eds) Principles of Bone Biology. San Diego (CA): Academic Press; 1996. p 51-67.

15 Owen M. Marrow stromal stem cells. J Cell Sci 1988; Suppl 10: 63-76.

16 Caplan AI. Mesenchymal stem cells. J Orthop Res 1991; 9: 641-50.

17 Choy L, Skillington J, Derynck R. Roles of autocrine TGF-β receptor and Smad signaling in adipocyte differentiation. J Cell Biol 2000; 149: 667-82.

18 Centrella M, Horowitz MC, Wozney JM, McCarthy TL. Transforming growth factor-β gene family members and bone. Endocr Rev 1994; 15: 27-39.

19 Tumber A, Meikle MC, Hill PA. Autocrine signals promote osteoblast surival in culture. J Endocrinol 2000; 167: 383-90.

20 Modrowski D, Basle M, Lomri A, Marie PJ. Syndecan-2 is involved in the mitogenic activity and signaling of granulocyte-macrophage colony-stimulating factor in osteoblasts. J Biol Chem 2000; 275: 9178-85.

21 Chien HH, Lin WL, Cho MIl. Down-regulation of osteoblastic cell differentiation by epidermal growth factor receptor. Calcif Tissue Int 2000; 67: 141-50.

22 Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, *et al*. Cbfa1 isoforms exert functional differences in osteoblast differentiation. J Biol Chem 1999; 274: 6972-8.

23 Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem 2001; 276: 7101-7.

24 Gao YH, Shinki T, Yuasa T, Kataoka-Enomoto H, Komori T, Suda T, *et al*. Potential role of cbfa1, an essential transcriptional factor for osteoblast differentiation, in osteoclastogenesis: regulation of mRNA expression of osteoclast differentiation factor (ODF). Biochem Biophys Res Commun 1998; 252: 697-702.

25 Thirunavukkarasu K, Halladay DL, Miles RR, Yang X, Galvin RJ, Chandrasekhar S, *et al*. The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. J Biol Chem 2000; 275: 25163-72.

26 Selvamurugan N, Pulumati MR, Tyson DR, Partridge NC. Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. J Biol Chem 2000; 275: 5037-42.

27 Drissi H, Pouliot A, Koolloos C. 1,25-$(OH)_2$-vitamin $D_3$ suppresses the bone-related *Runx2/Cbfa1* gene promoter. Exp Cell Res 2002; 274: 323-33.

28 Prince M, Banerjee C, Javed A. Expression and regulation of Runx2/Cbfa1 and osteoblast phenotypic markers during the growth and differentiation of human osteoblasts. J Cell Biochem 2001; 80: 424-40.

29 Nishimura R, Hata K, Harris SE, Ikeda F, Yoneda T. Core-binding factor $\alpha_1$ (Cbfa1) induces osteoblastic differentiation of C2C12 cells without interactions with Smad1 and Smad5. Bone 2002; 31: 303-12.

30 Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, *et al*. Runx2 is a common target of transforming growth factor beta 1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 2000; 20: 8783-92.

31 Gilbert L, He X, Farmer P. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alphaA) is inhibited by tumor necrosis factor-alpha. J Biol Chem 2002; 277: 2695-701.

32 Van Den Brande JL. A personal view on the early history of the insulin-like growth factors (1). Horm Res 1999; 51 (Suppl S3): 149-75.

33 Sims NA, Clement-Lacroix P, Da Ponte F. Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. J Clin Invest 2000; 106: 1095-103.

34 Hock JM, Centrella M, Canalis E. Insulin-like growth factor I has independent effects on bone matrix formation and cell replication. Endocrinology 1988; 122: 254-60.

35 McCarthy TL, Centrella M, Canalis E. Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. Endocrinology 1989; 124: 301-9.

36 Liggett WJ, Shevde N, Anklesaria P, Sohoni S, Greenberger J, Glowacki J. Effects of macrophage colony stimulating factor and granulocyte-macrophage colony stimulating factor on osteoclastic differentiation of hematopoietic progenitor cells. Stem Cells. 1993; 11: 398-411.

37 Hattersley G, Chambers TJ. Effects of interleukin 3 and of granulocyte-macrophage and macrophage colony stimulating factors on osteoclast differentiation from mouse hemopoietic tissue. J Cell Physiol. 1990; 142:201-9.

38 Postiglione L, Domenico GD, Montagnani S, Spigna GD,

Salzano S, Castaldo C, *et al*. Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces the osteoblastic differentiation of the human osteosarcoma cell line SaOS-2. Calcif Tissue Int 2003; 72: 85-97.

39 Modrowski D, Lomri A, Marie PJ. Glycosaminoglycans bind granulocyte-macrophage colony-stimulating factor and modulate its mitogenic activity and signaling in human osteoblastic cells. J Cell Physiol 1998; 177: 187-95.

40 Ziros PG, Gil AP, Georgakopoulos T. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. J Biol Chem 2002; 277: 23934-41.

41 Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK and p38 protein kinases. Science 2002; 298: 1911-2.