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# Mitogen Activated Protein Kinase-Dependent Inhibition of Osteocalcin Gene Expression by Transforming Growth Factor-β1

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## ABSTRACT

TGF-β (transforming growth factor-beta) plays a key role in osteoblast differentiation and bone development. While the ability of TGF-β to inhibit the expression of osteoblast differentiation genes has been well documented, the mechanism of this inhibition is not yet completely characterized. Runx2, a transcription factor necessary for expression of osteoblast differentiation genes is a central target of inhibition by TGF-β. In this study, we found that TGF-β1 inhibits expression of osteoblast differentiation genes without altering expression of Runx2. Transient transfection experiments determined that TGF-β1 inhibited osteocalcin promoter activity and this effect is mediated through Runx2. We further identified that there was no change in protein expression, cellular localization, or DNA binding affinity of Runx2 after TGF-β1-treatment of osteoblasts, suggesting that Runx2 undergoes post-translational modifications following TGF-β1 treatment. Co-immunoprecipitation experiments identified increased phosphorylation of Runx2 when differentiating osteoblasts were treated with TGF-β1. Mitogen activated protein kinase (MAPK) inhibitors relieved the TGF-β1-inhibitory effect of Runx2-mediated osteocalcin expression. Thus, our results suggest that TGF-β1-inhibition of osteoblast differentiation is dependent on the MAPK pathway and this effect is most likely mediated by post-translational modification of Runx2 such as phosphorylation rather than other regulatory mechanisms. J. Cell. Biochem. 106: 161–169, 2009. 

**KEY WORDS:** TGF-β; Runx2; OSTEOBLAST; OSTEOCALCIN

Signals that determine skeletal cell fate, function and apoptosis are critical to normal bone remodeling. Skeletal cells synthesize growth factors; some regulate cell replication, others regulate osteoblastic differentiation. Bone morphogenetic proteins (BMPs) and Wnts induce the differentiation of mesenchymal cells toward mature osteoblasts [Canalis et al., 2005]. TGF-β belongs to the BMP superfamily and is highly abundant in bone. In mammals, there are three isoforms, TGF-β1, TGF-β2, and TGF-β3, all of which are expressed by bone cells and interact with the known TGF-β receptor types I, II, and III (betaglycan). Both osteoblasts and osteoclasts are able to synthesize TGF-β [Centrella and Canalis, 1985; Oursler, 1994]. The two major functions of TGF-β in bone are to regulate cell proliferation and matrix formation [Centrella et al., 1994]. TGF-β1 knock-out mice display about 30% decrease in tibial

Abbreviations used: BMP, bone morphogenetic protein; TGF-β, transforming growth factor-beta; VDRE, vitamin D response element; IP: immunoprecipitation; CAT, chloramphenicol acetyl transferase; ChIP, chromatin immunoprecipitation; ALP, alkaline phosphatase; GAPDH, glyceraldehyde phosphate dehydrogenase; HDAC; histone deacetylase; DMSO, dimethyl sulfoxide; MAPK, mitogen activated protein kinase.

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length and reduction in bone mineral content [Geiser et al., 1998], consistent with the idea that TGF-β functions as a bone-forming agent. Transgenic mice where endogenous TGF-β signaling is inhibited in bone by over-expression of the dominant negative TGF-β type II receptor have increased trabecular bone mass due to decreased bone resorption by osteoclasts [Filvaroff et al., 1999]. Both increases and decreases in osteoclast formation, bone resorption, osteoblast proliferation, and osteoblast differentiation have been reported [Centrella et al., 1994; Rydziel et al., 1997].

Transcription factors, Runx2 and Osterix, are essential molecules for inducing osteoblast differentiation [Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002]. Induction of Runx2 expression is critical in osteoblast differentiation, as it is required for the expression of osteocalcin and other proteins that allow osteoblasts to mineralize collagen I-rich matrix to form bone. For example, Runx2 regulates expression of genes including alkaline phosphatase (ALP), types I and II collagen, RANKL, TGF-β type I receptor, C/EBPdelta, nuclear matrix associated proteins, and TWIST, a basic helix–loop–helix transcription factor [Lian et al., 2004]. Matrix metalloproteinase-13 (collagenase-3) is also a target of the transcriptional activator Runx2 in vivo [Jimenez et al., 1999]. Runx2 appears to be involved in the balance between bone formation and bone resorption [Lian et al., 2004] and participates in several bone and bone related diseases [Westendorf and Hiebert, 1999; Galindo et al., 2005; Javed et al., 2005; Pratap et al., 2005].

Runx2 activity can be modulated in several ways, including direct regulation of its gene expression (transcriptional), and post-transcriptional and post-translational modifications. In vitro and in vivo studies have reported that multiple integrated complex path ways (such as Wnt/LRP5/β-catenin, BMP/Smads, 1, 25-$(OH)_2$-vitamin$D_3$/VDR/VDRE pathway, etc.) and several regulatory proteins (such as Msx2, Dlx5, Twists, etc.) play critical roles in modulating Runx2 gene expression, activity, and subsequent bone formation. Runx2 activity is tightly regulated at different levels in response to various treatments [Franceschi et al., 2003] but the molecular mechanisms for TGF-β-inhibition of Runx2 activity to control osteoblast function have not yet been completely investigated.

It has been shown that TGF-β inhibits Runx2 function through direct interaction of Smad3 with Runx2 at Runx2-binding DNA sequences of osteoblast differentiation genes such as osteocalcin [Kang et al., 2005]. Even though TGF-β1 inhibits osteocalcin expression by repressing Runx2 activity through Smad3-dependent recruitment of co-repressors (HDACs), the molecular mechanisms of TGF-β1's recruitment of co-repressors through Smad3 to Runx2 are not completely known. This study delineates the molecular mechanisms conveying TGF-β1 action on osteoblasts via Runx2 for inhibition of differentiation. Here we demonstrate that TGF-β1 inhibited expression of osteoblast differentiation genes such as ALP, type I collagen, and osteocalcin but without altering expression, localization, or DNA binding affinity of Runx2. Runx proteins are subject to numerous covalent modifications that affect their activity. We report here that TGF-β1 stimulated phosphorylation of Runx2, and inhibition of MAPK phosphorylation abolished the TGF-β1-inhibitory effect on osteoblast differentiation. Hence, MAPK activation appears to be necessary for TGF-β1 to inhibit expression of osteoblast differentiation genes via Runx2.

## MATERIALS AND METHODS

### MATERIALS

Human TGF-β1 was purchased from R & D systems, MN. Tissue culture media and reagents were obtained from Invitrogen. The Runx2, α-tubulin, phosphothreonine, and phosphotyrosine antibodies were purchased from Santa Cruz Biotechnology (CA). The phosphoserine antibody was obtained from Sigma, MO. The kinase inhibitors were purchased from Santa Cruz Biotechnology. Other chemicals were obtained from Sigma (MO).

### CELL CULTURE

Rat primary osteoblasts were isolated as follows: Osteoblasts were derived from postnatal day 1 rat calvariae by sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A, 0.25% trypsin. Cells from digests one and two were discarded. Cells from the third digest were plated at $6.4 \times 10^3$ cells/$cm^2$ and grown in minimal essential medium (MEM) supplemented with 10% FBS. After reaching confluence (day 7), the medium was switched to $BGJ_b$ with 10% FBS containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to allow for initiation of differentiation (9–13 days) and mineralization (14–21 days) [Shalhoub et al., 1992; Kwok et al., 2005]. ROS17/2.8 and C3H10T1/2 cells were maintained in 10% FBS containing DMEM-F-12 medium at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### TRANSIENT TRANSFECTIONS

Plasmid DNAs were transiently transfected into cells using GeneJammer (Stratagene). Briefly, cells were plated at $2–4 \times 10^5$/well in 6-well plates in 10% FBS containing medium. The following day, the cells were transfected with 1 μg DNA and 5 μl GeneJammer per well in 1 ml of serum-free medium. After 3 h, 1 ml of 10% FBS-containing medium was added. After 24 h, the cells were treated with either control or TGF-β1 (1 ng/ml; R&D system)-containing media for 24 h. CAT activity was measured by reacting 50 μl of cell lysate in duplicate in a 100 μl reaction volume consisting of final concentrations of 250 μM *n*-butyryl-coenzyme A and 23 mM [$^{14}$C]-chloramphenicol (0.125 μCi/assay). The values were normalized to protein as determined by the Bradford dye binding (BioRad, Hercules, CA) method. A standard curve using purified CAT was performed in every experiment to determine the linear range of the enzyme assay. The Renilla luciferase construct was co-transfected to normalize the transfection efficiency. The firefly and Renilla luciferase assays were carried out using the dual luciferase assay kit from Promega [Selvamurugan et al., 1998, 2004].

### IMMUNOPRECIPITATION

Cells were lysed in immunoprecipitation buffer containing protease inhibitors and phenylmethylsulfonyl fluoride (PMSF) [Selvamurugan et al., 2000, 2004]. Protein A/G beads were added to the extracts and allowed to pre-absorb for 1 h, after which primary antibody and protein A/G bead mixture were added. Incubation was continued at 4°C with agitation for 2 h or overnight.

After washing four times with 1 ml IP buffer, the beads were suspended in 2× SDS sample buffer, boiled for 5 min and centrifuged. The proteins in the resulting supernatant were separated by SDS–PAGE and subjected to Western blotting [Selvamurugan et al., 2000, 2004].

### CHROMATIN IMMUNOPRECIPITATION (CHIP)

ChIP assay was carried out using the EZ-ChIP kit from Upstate Biotechnology. Cells were incubated for 10 min at room temperature with medium containing 1% formaldehyde. Cells were then washed in ice-cold PBS containing protease inhibitors and 1 mM PMSF, and resuspended in SDS lysis buffer containing protease inhibitors for 10 min on ice. Samples were sonicated to reduce the DNA length to 0.1–0.8 kb, and diluted to 10-fold in dilution buffer supplemented with protease inhibitors. Protein concentrations were determined using BioRad reagent. Prior to chromatin immunoprecipitations, the samples were pre-cleared with 100 μl of a 25% (v/v) suspension of DNA-coated protein A/G-agarose. The supernatants were recovered. An aliquot (1/100) of total chromatin DNA before immunoprecipitation was saved (input) for PCR reaction. The supernatant (soluble chromatin) was used directly for immunoprecipitation experiments with appropriate antibody overnight at 4°C. Immune complexes were mixed with 100 μl of a 25% precoated (DNA) protein A/G-agarose suspension followed by incubation for 1 h at 4°C. Beads were washed and the protein-DNA complexes were eluted two times by adding a 250 μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$. The samples had added to then 20 μl of 5 M NaCl and the cross-linking reaction was reversed by 6 h incubation at 65°C. Further, the samples were digested with RNase and proteinase K. The DNA was recovered by DNA-mini columns provided with the kit and used for semi quantitative PCR [Kang et al., 2005].

### REAL-TIME QUANTITATIVE PCR

PCR reactions were performed [Kwok et al., 2005] using a real-time PCR DNA Opticon Engine (MJ Research, Inc., Watertown, MA) according to the manufacturer's instructions, which allows real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCRs was purchased from PerkinElmer Life Sciences (Boston, MA). Primers were designed using Primer Express software (PerkinElmer Life Sciences). For PCR amplification, the following sets of rat specific primers were used:

ALP: 5′ AGGCAGGATTGACCACGG 3′ and 5′ TGTAGTTCTGCTCATGGA 3′;
$\alpha_1$(I) procollagen: 5′ AGATTGAGAACATCCGCAGCC 3′ and 5′ TCCAGTACTCTCCGCTCTTCCA 3′;
Osteocalcin: 5′ AAGCAGGAGGGCAATAAGGT 3′ and 5′ AGCTGCTGTGACATCCCATAC 3′;
GAPDH: 5′ AACCCATCACCATCTTCCAGG 3′ and 5′ GCCTTCTCCATGGTGGTGAA 3′.

### OSTEOBLAST DIFFERENTIATION AND MINERALIZATION (VON KOSSA STAINING)

Cells were fixed with 10% formaldehyde in phosphate buffered saline (PBS) for 15 min. The cells were then rinsed with water, dried and incubated with 5% silver nitrate solution for 1 h. The cells were rinsed with water, dried and photographed [Selvamurugan et al., 2007].

## RESULTS

To study the effect of TGF-β1 on expression of osteoblast differentiation marker genes, osteoblasts from rat calvariae were isolated and characterized by the method of Shalhoub et al. [1992]. Differentiating osteoblasts (day 13) were treated with TGF-β1. Total RNA was isolated and subjected to real-time quantitative RT-PCR. TGF-β1 inhibited mRNA expression of ALP, collagen, and osteocalcin in rat differentiating osteoblasts; whereas expression of Runx2 mRNA was not altered in these cells (Fig. 1A). When cells were treated with BMP-2, expression of both Runx2 and osteocalcin mRNAs was increased (Fig. 1B). Thus, inhibition of osteocalcin expression and no change in Runx2 mRNA expression appears to be specific for TGF-β1 action in rat differentiating osteoblastic cells.

To further study the effect of TGF-β1 on expression of Runx2 and osteocalcin genes, rat osteoblastic cells from differentiating (days 9, 11, and 13) and mineralizing (days 15, 17, and 19) phases were treated with TGF-β1 on each of these days. Total RNA was isolated and subjected to real-time quantitative RT-PCR. TGF-β1 inhibited mRNA expression of osteocalcin during differentiation of the osteoblasts; whereas expression of Runx2 mRNA was not altered in these cells (Fig. 1C). A similar effect has been observed during mineralization (data not shown).

To further examine the TGF-β1-inhibition of genes expressed during osteoblast differentiation, we used the rat osteocalcin promoter in our studies. The rat osteocalcin promoter containing 208 nucleotides upstream of the transcription start site has a functional Runx binding site [Zaidi et al., 2004]. This promoter (rOC) construct containing chloramphenicol acetyl transferase (CAT) as a reporter gene was transiently transfected into ROS17/2.8 cells and treated with TGF-β1. The results indicate that TGF-β1 significantly inhibited activity of this osteocalcin promoter in ROS17/2.8 cells (Fig. 2A). Since Runx2 has been shown to be an inducer of expression of osteoblast differentiation genes [Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997], we wanted to identify whether Runx2 mediates the TGF-β1-inhibitory effect. To do this, we used murine multipotent mesenchymal stem cells (C3H10T1/2) where there is no expression of Runx2. C3H10T1/2 cells transfected with Runx2 strongly expressed osteoblast markers, such as ALP and osteocalcin [Yang et al., 2003]. Here, the rat osteocalcin promoter was transiently transfected along with either the pCMV vector or pCMV-Runx2 into C3H10T1/2 cells, followed by TGF-β1 treatment. The CAT activity was assayed. The results indicate that Runx2 increased osteocalcin promoter activity; whereas TGF-β1 significantly decreased its activity in the presence of Runx2 (Fig. 2B). Thus, TGF-β1-inhibition of osteocalcin promoter activity appears to be mediated by Runx2 in C3H10T1/2 cells.

Since there was no significant change in Runx2 mRNA level after TGF-β1 treatment in rat differentiating osteoblastic cells (Fig. 1A,B), we determined whether there is any change in Runx2 protein expression. Rat differentiating osteoblasts were treated with TGF-β1

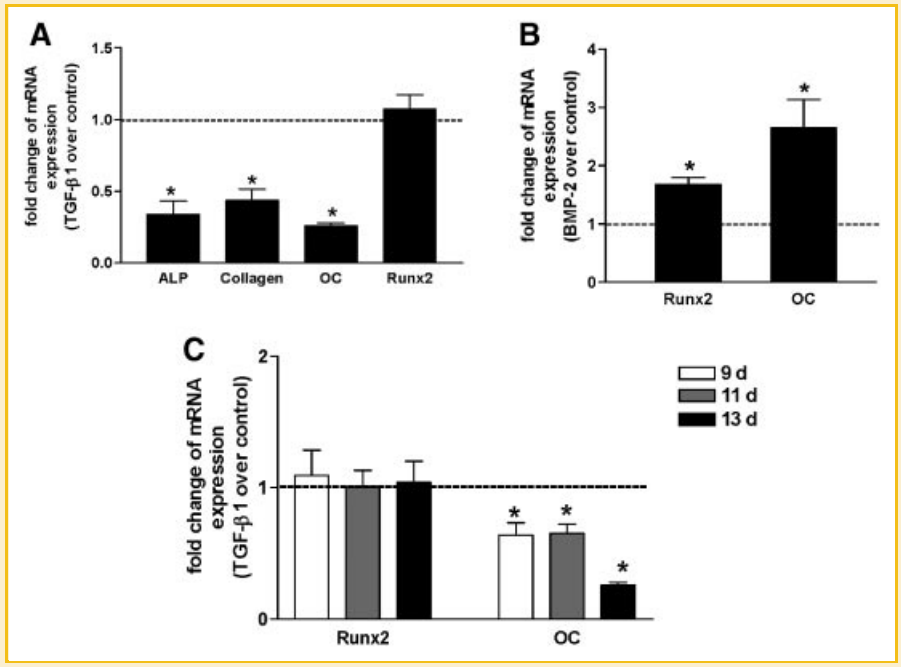


Fig. 1. A: Rat differentiating osteoblasts (day 13) were treated with control medium or medium containing TGF-β1 (1 ng/ml) for 24 h. B: Rat differentiating osteoblasts (day 13) were treated with control medium or medium containing BMP-2 (25 ng/ml) for 24 h. C: Rat differentiating osteoblasts (days 9, 11, and 13) were treated with TGF-β1 (1 ng/ml) for 24 h. Total RNA was isolated and subjected to real-time RT-PCR using specific primers for rat, as indicated. The relative levels of mRNAs were normalized to GAPDH, and the TGF-β1- and BMP-2-fold changes were calculated over controls. The asterisks represent $P < 0.05$ compared with control. Data represent means ± SE ($n = 3$).

for different times, whole cell extracts isolated, and subjected to Western blot analysis. The results indicate that both control and TGF-β1-treated extracts contained a major protein of ~60 kDa that was recognized by anti-Runx2 antibody and TGF-β1 did not change Runx2 protein levels in these cells (Fig. 3A). Thus, no apparent changes in the levels of Runx2 mRNA (Fig. 1A,B) or protein in rat differentiating osteoblasts (Fig. 3A) either under control conditions or after TGF-β1 treatment suggested that TGF-β1 may regulate Runx2 by post-translational events in these cells. It has been shown that inhibition of Runx2 activity could also involve sequestration of Runx2 in the cytoplasm [Kim et al., 2003b]. Hence, we determined whether TGF-β1 has any effect on Runx2 shuttling. ROS17/2.8 cells were treated with control or TGF-β1-containing media for 1 h. The cells were incubated with the primary Runx2 antibody followed by secondary Alexa fluor 488 antibody. The cells were then visualized by immunoflorescence microscopy. To visualize nuclei, cells were incubated with DAPI before mounting. The results clearly indicate that Runx2 expression was mostly in the nucleus, and TGF-β1 had no effect on nuclear export of Runx2 protein (Fig. 3B).

To determine whether TGF-β1 changes binding of Runx2 protein to the rat osteocalcin promoter DNA, ROS17/2.8 cells were treated with control or TGF-β1-containing media for 1 h and subjected to chromatin immunoprecipitation (ChIP) experiments. The result indicates that there was apparently no change in the level of Runx2 protein associated with the rat osteocalcin promoter in vivo under control and TGF-β1-conditions (Fig. 3C). Hence, we suggest that TGF-β1 regulates Runx2 at the post-translational level and this may be necessary for TGF-β1-inhibition of osteoblast differentiation.

There are several lines of evidence supporting phosphorylation as a determining factor for Runx activity [Selvamurugan et al., 2000; Xiao et al., 2000; Wee et al., 2002; Franceschi et al., 2003; Kim et al., 2003a]. In order to identify TGF-β1 stimulation of Runx2 phosphorylation, rat differentiating osteoblasts (day 13) were treated with control or TGF-β1-containing media. Whole cell lysates were prepared, and immunoprecipitated with Runx2 antibody. The immunoblot was analyzed with anti-phospho-serine, anti-phospho-threonine, anti-phospho-tyrosine or anti-Runx2 antibodies. As shown in Figure 4A, TGF-β1 stimulated Runx2 phosphorylation on threonine and tyrosine amino acids. Phosphorylated serine in Runx2 was found to be the same in both control and TGF-β1-treated cells. There was no change in immunoprecipitated and immunoblotted Runx2 protein under control or TGF-β1-treated conditions. These results suggest that Runx2 activity could be regulated by phosphorylation at different amino acids in rat differentiating osteoblasts.

Since TGF-β1 stimulated Runx2 phosphorylation at threonine and tyrosine amino acids in rat differentiating osteoblasts (Fig. 4A), we next investigated the functional correlation of phosphorylation and expression of the osteoblast differentiation gene, osteocalcin. ROS17/2.8 cells were pretreated with DMSO ($Me_2SO$) and MAPK inhibitors, such as PD98059 (MEK) and U0126 (ERK), or a tyrosine kinase inhibitor (PP2), followed by TGF-β1 treatment. Total RNA was isolated and subjected to real-time RT-PCR. The results indicate that TGF-β1 significantly decreased osteocalcin mRNA expression; whereas pretreatment with MAPK inhibitors, PD98059 and U0126 prevented the inhibitory effect of TGF-β on osteocalcin expression

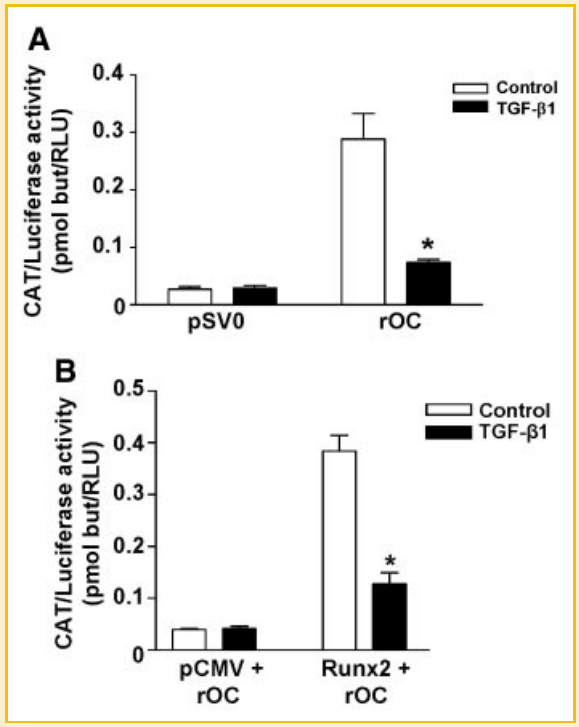


Fig. 2. A: The rat osteocalcin promoter (rOC) construct was transiently transfected into ROS17/2.8 cells. The cells were then treated with or without TGF-β1 (1 ng/ml) for 24 h and assayed for CAT activity. pSV0 represents promoterless vector. B: The rat osteocalcin promoter was transiently transfected into C3H10T1/2 cells along with either empty vector (pCMV) or Runx2 vector (pCMV-Runx2-type II) for 24 h. The cells were then treated with control or TGF-β1-(1 ng/ml)-containing media for 24 h and assayed for CAT activity. Data represent mean ± SE of three replicate plates. The transfection efficiency was normalized by co-transfection with Renilla luciferase construct. Asterisk (*) indicates significant difference compared with control ($P < 0.05$).

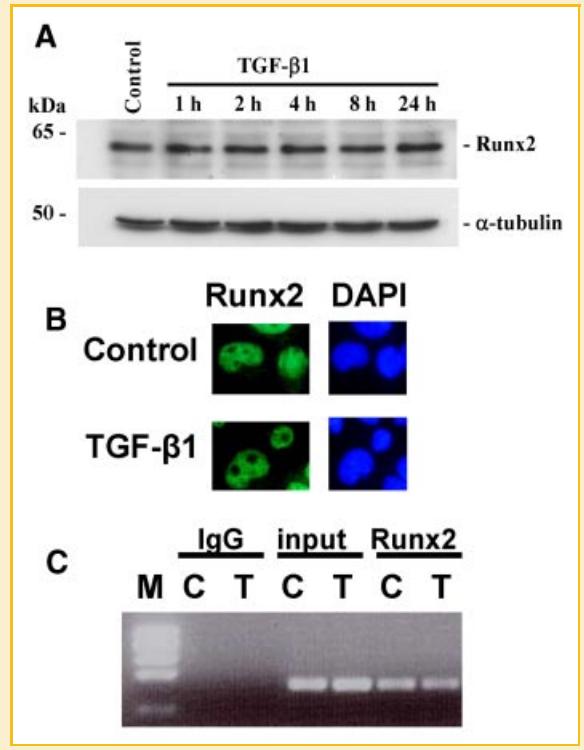


Fig. 3. A: Rat primary differentiating osteoblasts (day 13) were treated with control or TGF-β1 (1 ng/ml)-containing medium for the indicated times. Whole cell extracts were isolated and subjected to Western blot analysis using anti-Runx2 antibody. The membrane was stripped and reprobed with anti-α-tubulin antibody to show equal amounts of protein loaded on the membrane. B: ROS17/2.8 cells were treated with control or TGF-β1-(1 ng/ml)-containing media for 1 h. The cells were then fixed with 4% paraformadehyde, incubated with rabbit polyclonal Runx2 antibody, followed by incubation with rabbit Alexa fluor 488 antibody. The cells were visualized by immunoflorescence microscopy (40×). C: ROS17/2.8 cells were treated with control (C) or TGF-β1 (1 ng/ml)-containing media (T) for 1 h. Cells were fixed with formaldehyde and soluble chromatin was prepared, as described in methods section. After immunoprecipitation of the cross-linked lysates with either IgG or Runx2 antibody, the DNA was subjected to PCR with primers that contain the regions of the Runx binding sites or osteoblast specific element-2 (OSE2) of the rat osteocalcin promoter. Input DNA (1/100) is positive control for the assay. M: marker. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in these cells (Fig. 4B). The tyrosine kinase inhibitor had no effect on osteocalcin mRNA expression (Fig. 4B). These results suggest that TGF-β1-inhibition of osteocalcin expression could involve the MAPK/MEK/ERK signaling pathway in osteoblastic cells.

Since TGF-β1 stimulated Runx2 phosphorylation and MAPK inhibitors relieved the TGF-β1-inhibition of osteocalcin expression (Fig. 4), we next investigated the functional correlation of Runx2 phosphorylation and osteocalcin expression. The rat osteocalcin promoter was transiently transfected with vector or pCMV-Runx2 constructs into C3H10T1/2 cells. The cells were then pretreated with either DMSO or the MAPK inhibitor, PD98059 for 20 min and then treated with control or TGF-β1-containing media. The CAT activity was measured. The results indicate that Runx2 stimulated rat osteocalcin promoter activity, and Runx2-stimulated promoter activity was significantly decreased by TGF-β1 treatment (Fig. 5A); while pretreatment with a MAPK inhibitor prevented the Runx2-mediated inhibition of the osteocalcin promoter activity by TGF-β1 in these cells (Fig. 5A). To address whether MAPK inhibition alters mRNA expression, ROS17/2.8 cells were pretreated with either DMSO or the MAPK inhibitor, U0126, and treated with control or TGF-β1-containing media. Total RNA was isolated and subjected to real-time RT-PCR. The results indicate that neither TGF-β1 nor MAPK inhibitor treatment together with TGF-β1 had any effect on the expression of Runx2 mRNA (Fig. 5B). In contrast, TGF-β1 significantly decreased osteocalcin mRNA expression and inclusion of the MAPK inhibitor prevented the TGF-β1-inhibitory effect in these cells (Fig. 5B).

## DISCUSSION

TGF-β plays a key role in osteoblast differentiation and bone development and remodeling. TGF-β has been reported to inhibit expression of ALP and osteocalcin, among other markers of osteoblast differentiation and function [Centrella et al., 1994]. Our results are consistent with these observations since we showed that

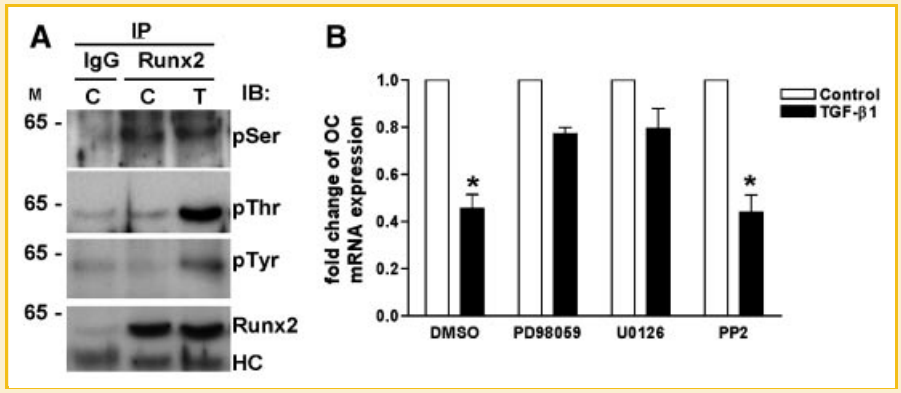


Fig. 4. A: Rat differentiating osteoblasts (day 13) were treated with control (C) or TGF-β1 (T) (1 ng/ml)-containing media for 5 min. Whole cell lysates were immunoprecipitated with either IgG or Runx2 antibody and immunoblotted with antibodies as indicated and followed by detection by ECL kit. B: ROS17/2.8 cells were pretreated with DMSO, PD98059 (25 μM), U0126 (10 μM) or PP2 (10 μM) for 20 min. The cells were then treated with control or TGF-β1-(1 ng/ml)-containing media for 24 h. Total RNA was isolated and subjected to real-time RT-PCR using specific primers for rat osteocalcin and GAPDH. The relative levels of mRNAs were normalized to GAPDH, and the TGF-β1-fold change was calculated over control. Asterisk (*) indicates significantly decreased compared with control ($P < 0.05$); Data represent means ± SE (n = 3). (IP: immunoprecipitation; IB: immunoblot; M: marker; HC: heavy chain; p: phosphorylated).

TGF-β1 inhibited expression of endogenous osteoblast differentiation genes (Fig. 1A,C) and osteocalcin promoter activity (Fig. 2). In response to various growth factors, Runx2 can be regulated at different levels and that may result in either activation or inhibition of osteocalcin expression. For example, increased Runx2 expression along with increased osteocalcin was seen by BMP-2 treatment; whereas no change in Runx2 expression but down regulated osteocalcin expression by TGF-β1 was observed in rat differentiating osteoblastic cells (Fig. 1B).

Depending on particular experimental parameters, TGF-β modulates various bone cell activities in different ways via Runx2. Alliston et al. [2001] have shown a decreased Runx2 mRNA level from TGF-β1 treatment of osteoblastic cells. Hjelmeland et al. [2005] have reported that there was no change in Runx2 mRNA levels after TGF-β1 treatment in rat osteoblastic cells. We report here that there was no change in expression of Runx2 after TGF-β1 treatment in rat osteoblastic cells (Figs. 1A,C and 3A). The discrepancy in transcriptional regulation of Runx2 by TGF-β1 could be due to duration of the treatment time (continuous or intermittent), concentrations of TGF-β1 used (1–10 ng/ml), the species difference (rat or mouse), the differentiation stage (early or late), and the difference in the passage numbers of transformed cells.

Runx2 activity can be modulated at different levels, including transcriptional, post-transcriptional, translational, and post-translational modifications. The expression of the Runx2 gene is regulated by at least two distinct promoters that generate two mRNA transcripts, and additional Runx2 isoforms may arise as a result of differential use of transcriptional start sites or alternative splicing [Xiao et al., 2001]. Runx2 is also regulated by its 5′-UTRs that impart IRES (internal ribosome entry site) activity. Translational control by

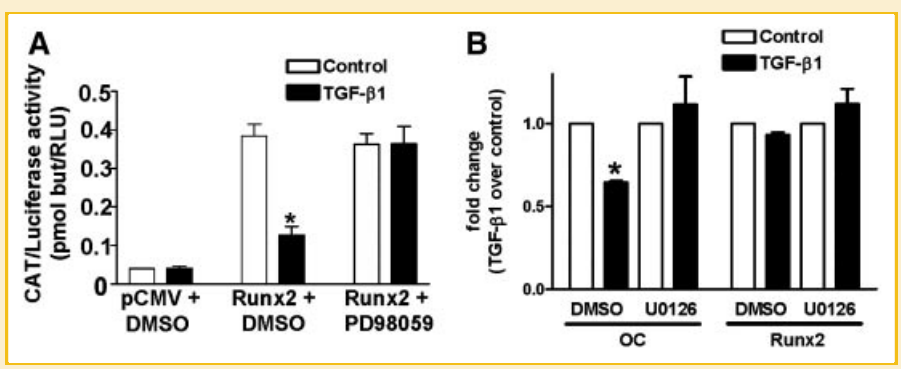


Fig. 5. A: Rat osteocalcin promoter was transiently transfected with either pCMV or pCMV-Runx2 (type II) into C3H10T1/2 cells for 24 h. The cells were pretreated with either DMSO or MAPK inhibitor, PD98059 (25 μM) for 20 min, followed by control or TGF-β1-(1 ng/ml)-treatment for 24 h and assayed for CAT activity. Data represent mean ± SE of three replicate plates. The transfection efficiency was normalized by co-transfection with Renilla luciferase construct. Asterisk (*) indicates significant difference compared with control ($P < 0.05$). B: ROS17/2.8 cells were pretreated with either DMSO or U0126 (10 μM) for 20 min. The cells were then treated with control or TGF-β1-(1 ng/ml)-containing media for 24 h. Total RNA was isolated and subjected to real-time RT-PCR using specific primers for osteocalcin and Runx2. The relative levels of mRNAs were normalized to GAPDH, and the TGF-β1-fold change was calculated over control. Data represent mean ± SE of three replicate plates. Asterisk (*) indicates significantly decreased compared with control ($P < 0.05$).

IRES-dependent activity provides a secondary level for controlling the amount of Runx2 proteins in concert with transcriptional regulation [Xiao et al., 2003]. Runx2 is a nuclear protein and inhibition of Runx2 activity could involve sequestration of Runx2 in the cytoplasm as shown for Runx2-Stat-1 interaction [Kim et al., 2003b]. However, in our studies it appears that TGF-β1 did not influence Runx2 mRNA and protein expression levels nor transport of Runx2 from nucleus to cytoplasm (Fig. 3B). These results suggested that effects of TGF-β1 on Runx2 activity most likely occur in the nucleus. Since regulation of gene expression in the nucleus occurs via recruitment or sequestration of transcription factors and/or cofactors on DNA, we speculated that TGF-β1 might influence or alter the DNA binding affinity of Runx2 in osteoblastic cells. However, our ChIP assay indicated that there was no change in binding of Runx2 protein to the Runx binding sites of the rat osteocalcin promoter after TGF-β-treatment (Fig. 3C).

Runx proteins undergo a number of post-translational modifications. Ubiquitin-mediated proteolysis plays a role in the turnover of Runx protein [Tintut et al., 1999] and this is in turn inhibited by interaction of Runx1 protein with PEBP2β/CBFβ [Huang et al., 2001]. Smurf1 (Smad ubiquitin regulatory factor 1) mediates Runx2 degradation in a ubiquitin proteasome-dependent manner [Zhao et al., 2003]. However, since there was no evidence for a decline in Runx2 protein after TGF-β1 treatment, degradation is unlikely to play a role here. Phosphorylation is one of the most versatile post-translational mechanisms that cells use to control the function of proteins. We report here that TGF-β1 stimulated Runx2 phosphorylation, mostly at threonine amino acids in rat differentiating osteoblasts (Fig. 4A). Computer analysis of Runx2 potential phosphorylation sites showed that there may be seven threonine predicted phosphorylation sites (NetPhos 2.0, Denmark). Pretreatment with MAPK inhibitors antagonized the inhibitory effect of TGF-β1 on osteocalcin expression (Figs. 4B and 5A,B).

Even though the physiological role of the MAPK pathway in osteoblasts remains controversial [Lai and Cheng, 2002; Sowa et al., 2002; Schindeler and Little, 2006; Ge et al., 2007], it has been shown that Runx2 is a substrate for the MAPK pathway, and this pathway can be stimulated by a variety of signals including those initiated by ECM, osteogenic growth factors like BMPs, fibroblast growth factor-2 (FGF-2) and mechanical loading [Franceschi and Xiao, 2003; Franceschi et al., 2003]. Pallu et al. [2004] suggested that Runx2 could be phosphorylated by the p38 MAPK pathway. The p38 MAPK inhibitor did not prevent the TGF-β1-inhibitory effect on osteocalcin expression in rat osteoblastic cells (data not shown). There are possibilities that MAPK activates other kinases that directly phosphorylate Runx2 or other accessory factors are required for Runx2 to become a suitable substrate. Even though we observe that TGF-β1 stimulates Runx2 phosphorylation at tyrosine amino acids, tyrosine kinase inhibitor had no effect to relieve the inhibitory effect of TGF-β1 on osteocalcin expression (Fig. 4B). The observed Runx2 phosphorylation at tyrosine amino acids may be a result of interaction between the TGF-β1 and EGF-signaling pathways [Heldin-Feindt et al., 2003].

Phosphorylation of Runx2 protein at multiple sites may serve as an essential way for transducing the molecular signal to recruit other factors and co-factors for the control of its function. The TGF-β1-stimulation of Runx2 phosphorylation observed in osteoblastic cells would allow changes in the nuclear accessory proteins (co-activators and co-repressors) that are involved in RNA polymerase activation on DNA, resulting in inhibition of gene transcription. Recent studies have identified several co-repressor proteins that bind to Runx2 to regulate gene expression [Aronson et al., 1997; Westendorf et al., 2002; Vega et al., 2004; Westendorf, 2006]. Kang et al. [2005] have shown that TGF-β1 represses Runx2 function by recruitment of HDAC-4 and -5 through Smad3 at the osteocalcin promoter. Smad3 is one of the essential components that confers TGF-β-specificity [Shi and Massague, 2003] and it negatively regulates Runx2 activity [Hjelmeland et al., 2005]. The molecular signals responsible for dictating Runx2 to recruit co-repressors via Smad3 are not yet known and our studies suggest that Runx2 phosphorylation may be a primary event for TGF-β1 action that would trigger Smad3 to recruit co-repressors on the osteocalcin promoter, resulting in inhibition of osteoblast differentiation.

Overall our studies indicate that TGF-β1-inhibits expression of osteoblast differentiation genes, most likely by posttranslational modification(s) of Runx2 such as phosphorylation rather than transcriptional, post-transcriptional, and translational mechanisms. In view of the importance of Runx2, TGF-β1-modulation of Runx2 phosphorylation and its role in osteoblastic differentiation would be important in understanding the molecular events governing bone modeling and bone related diseases.

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