

# Hormonal regulation of regucalcin mRNA expression in osteoblastic MC3T3-E1 cells

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**Abstract.** The effect of various hormones on regucalcin mRNA expression in osteoblastic MC3T3-E1 cells *in vitro* was investigated. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or various hormones without fetal bovine serum. Regucalcin mRNA expression was significantly increased after culture with parathyroid hormone (synthetic human PTH;  $10^{-7}$  M), insulin-like growth factor-I (IGF-I;  $10^{-8}$  M), or  $17\beta$ -estradiol ( $10^{-10}$  or  $10^{-9}$  M) for 48 h. Culture with 1,25-dihydroxyvitamin D<sub>3</sub> ( $10^{-7}$  M) for 48 h caused a significant decrease in regucalcin mRNA expression. Regucalcin mRNA expression was significantly decreased after culture with tumor necrosis factor- $\alpha$  (1 or 10 ng/ml of medium) for 24 or 48 h. The effect of PTH or IGF-I in increasing regucalcin mRNA expression was not seen in the presence of staurosporine ( $10^{-8}$  M), an inhibitor of protein kinase C, or PD98059 ( $10^{-7}$  M), an inhibitor of mitosis-activated protein kinase (MAP kinase), respectively, suggesting that regucalcin mRNA expression is enhanced through intracellular signaling factors. This study demonstrated that regucalcin mRNA expression in osteoblastic MC3T3-E1 cells is regulated by various hormones.

## Introduction

Regucalcin was discovered in 1978 as a Ca<sup>2+</sup>-binding protein that does not contain the EF-hand motif of the Ca<sup>2+</sup>-binding domain (1-5). The name regucalcin was proposed for this Ca<sup>2+</sup>-binding protein, which regulates the Ca<sup>2+</sup> effect on

various enzyme activities (4,6). In recent years, it has been demonstrated that regucalcin plays a multifunctional role as a regulatory protein in intracellular signaling processes in many cell types (reviewed in 6-9).

The regucalcin gene is highly conserved in vertebrate species (5,10). The rat and human regucalcin genes are localized on chromosome X (11,12). Regucalcin mRNA and its protein are strongly present in liver and kidney cortex (13,14), and only slightly present in other tissues. AP1, NF1-A1, and RGPR-p117 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity (15-18): RGPR-p117 was found to be a novel protein. The expression of regucalcin mRNA is mediated through a signaling mechanism (16,19,20).

Regucalcin has been shown to play a role in the maintenance of intracellular Ca<sup>2+</sup> homeostasis, the inhibitory regulation of various Ca<sup>2+</sup>-dependent protein kinases, tyrosine kinases and protein phosphatases, and nitric oxide synthase (6-9). Regucalcin has suppressive effects on nuclear DNA and RNA syntheses in proliferative cells (9,21,22). Regucalcin is translocated into the nuclei (23), and it regulates the expression of various genes (24).

Overexpression of regucalcin modulated and suppressed cell proliferation in cloned rat hepatoma H4-II-E cells (25) and in cloned normal rat kidney proximal tubular epithelial NRK52E cells (26), and it has been shown to have suppressive effects on cell death and apoptosis induced with stimulation of various factors in H4-II-E cells (27) and NRK52E cells (28). It has been proposed that regucalcin plays a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation (reviewed in 12).

Regucalcin transgenic (TG) rats were generated to determine the regulatory role of endogenous regucalcin *in vivo* (29). Bone loss and hyperlipidemia have been demonstrated in regucalcin TG rats, indicating that regucalcin plays a role in the regulation of bone and lipid metabolisms (30,31).

Regucalcin has been shown to have stimulatory effects on osteoclastogenesis and bone resorption (32) and suppressive effects on osteoblastic differentiation and mineralization *in vitro* (33). Regucalcin may regulate bone mass.

This study was undertaken to determine whether the expression of regucalcin mRNA in osteoblastic cells is hormonally regulated *in vitro*. We found that regucalcin mRNA expression was enhanced after culture with parathyroid

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hormone, insulin-like growth factor-I, or 17 $\beta$ -estradiol, and that it was suppressed with 1,25-dihydroxyvitamin D<sub>3</sub> or tumor necrosis factor- $\alpha$ .

## Materials and methods

**Chemicals.**  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) and penicillin-streptomycin (5,000 U/ml penicillin; 5,000  $\mu$ g/ml streptomycin) were obtained from Gibco Laboratories. Fetal bovine serum (FBS) was obtained from Bioproducts Inc. Parathyroid hormone [PTH; synthetic human PTH (1-34)], insulin-like growth factor-I (IGF-I), 17 $\beta$ -estradiol, 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), staurosporine, and PD98059 were purchased from Sigma Chemicals (St. Louis, MO). Other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass distilled.

**Cell culture.** Osteoblastic MC3T3-E1 cells were cultured at 37°C in a CO<sub>2</sub> incubator in plastic dishes containing  $\alpha$ -MEM supplemented with 10% FBS. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate-buffered saline (PBS). For experiments, about 1.0 × 10<sup>5</sup> cells per dish were cultured for 72 h to obtain subconfluent monolayers in a 35-mm plastic dish containing 2 ml  $\alpha$ -MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium without FBS containing either vehicle or various hormones, and the cells were cultured further for 24 or 48 h. Cell viability was estimated using staining with trypan blue.

**Cell counts.** After trypsinization of each of the culture dishes using a 0.2% trypsin plus 0.02% EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS for 2 min at 37°C, the cells were collected and centrifuged in a PBS solution at 100 × g for 5 min. The cells were re-suspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a hemacytometer plate. For each dish, we took the average of two counts.

**Preparation of RNA.** Total RNAs were prepared as described previously (34). Osteoblastic MC3T3-E1 cells were homogenized in a buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000 × g for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isopropanol at -20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

**Reverse transcriptional-polymerase chain reaction (RT-PCR) analysis.** RT-PCR was performed with a Titan™ One Tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the supplier to determine the gene expression of regucalcin or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The primers generated were based on the published rat sequences.

Primers for regucalcin cDNA were 5'-AGATGAACAAA TCCCAGAT-3' (sense strand, positions 618-636 of cDNA

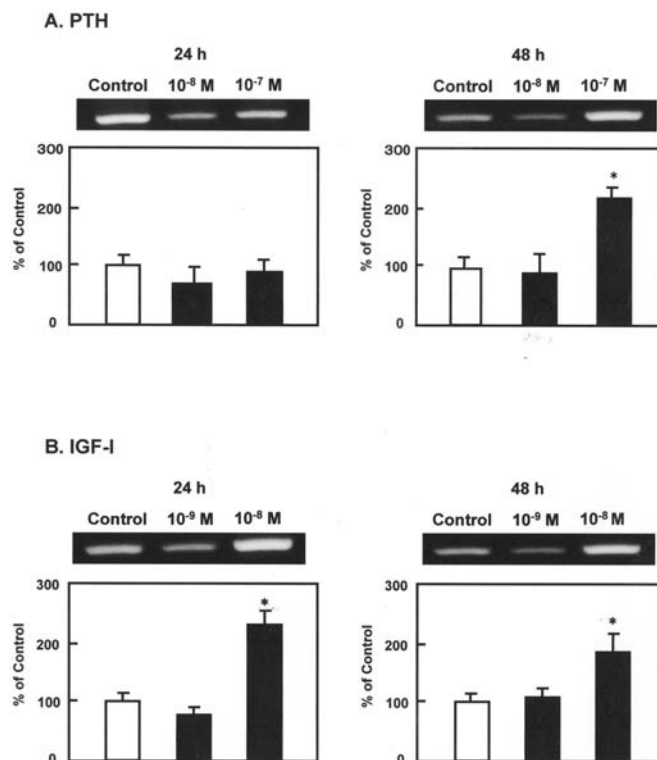


Figure 1. Effect of PTH or IGF-I on regucalcin mRNA expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of PTH (10<sup>-8</sup> or 10<sup>-7</sup> M) or IGF-I (10<sup>-9</sup> or 10<sup>-8</sup> M). After the medium change, cells were cultured for 24 or 48 h. Total RNAs (2  $\mu$ g) extracted from the cells were analyzed using RT-PCR with specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level in the cells cultured for 24 or 48 h in the presence of PTH (A) or IGF-I (B) were indicated as a percentage of control (mean  $\pm$  SEM for four experiments). \*P<0.01 compared with the control value.

sequence) and 5'-TCACCCTGCATAGGAATAT-3' (antisense strand, positions 906-924) (10). The pair of oligonucleotide primers was designed to amplify a 307-bp sequence from the mRNA of mouse regucalcin. Primers for G3PDH cDNA were 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (sense strand) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (antisense strand) from the G3PDH Amplimer Set (Clontech, Palo Alto, CA).

RT-PCR was performed using a reaction mixture (20  $\mu$ l) containing 2 or 4  $\mu$ g of total RNAs, supplied RT-PCR buffer, Titan™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3  $\mu$ M primers. Samples were incubated at 50°C for 30 min, and then amplified for 30 cycles under the following conditions: denaturation for 30 sec at 94°C, annealing for 30 sec at 56°C, and extension for 60 sec at 68°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Image density was quantified with a FluoroImager SI (Amersham Pharmacia Biotech).

**Statistical analysis.** Data are expressed as the mean  $\pm$  SEM. Statistical differences were analyzed using the Student's t-test. P-values <0.05 were considered to indicate statistically

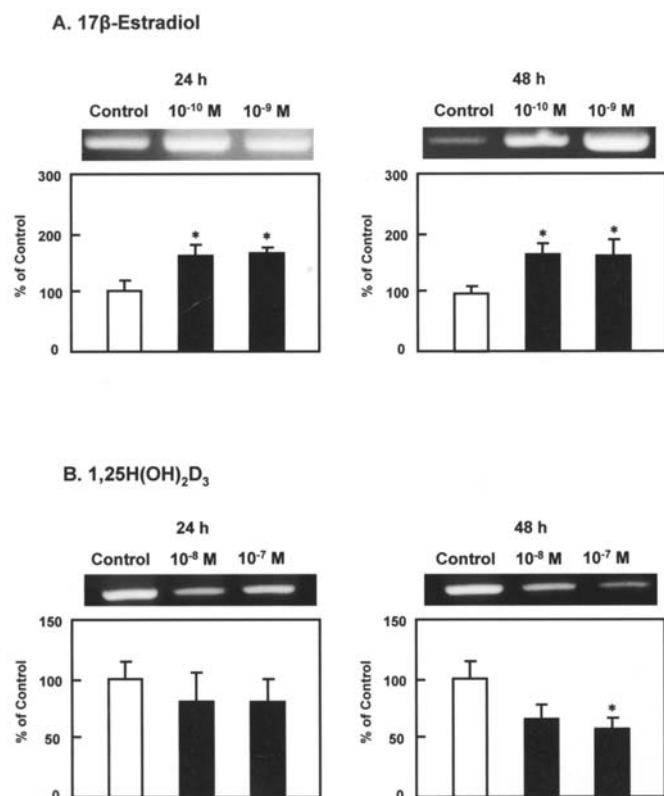


Figure 2. Effect of 17 $\beta$ -estradiol or 1,25(OH)<sub>2</sub>D<sub>3</sub> on regucalcin mRNA expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of 17 $\beta$ -estradiol (10<sup>-10</sup> or 10<sup>-9</sup> M) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-8</sup> or 10<sup>-7</sup> M). After the medium change, the cells were cultured for 24 or 48 h. Total RNAs (2  $\mu$ g) extracted from the cells were analyzed using RT-PCR with specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level in the cells cultured for 24 or 48 h in the presence of 17 $\beta$ -estradiol (A) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (B) were indicated as a percentage of control (mean  $\pm$  SEM for four experiments). \*P<0.01 compared with the control value.

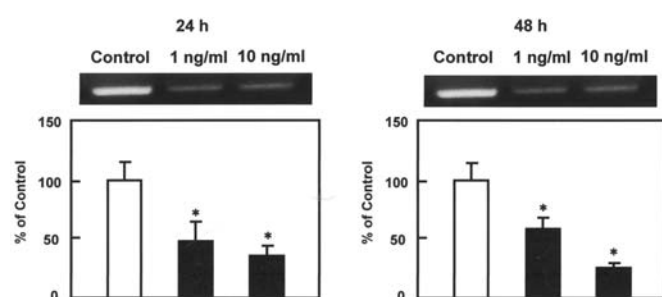


Figure 3. Effect of TNF- $\alpha$  on regucalcin mRNA expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of TNF- $\alpha$  (1 or 10 ng/ml of medium). After the medium change, the cells were cultured for 24 or 48 h. Total RNAs (2  $\mu$ g) extracted from the cells were analyzed using RT-PCR with specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level in the cells cultured for 24 or 48 h in the presence of TNF- $\alpha$  were indicated as a percentage of control (mean  $\pm$  SEM for four experiments). \*P<0.01 compared with the control value.

significant differences. The ANOVA multiple comparison test was also used to compare the treatment groups.

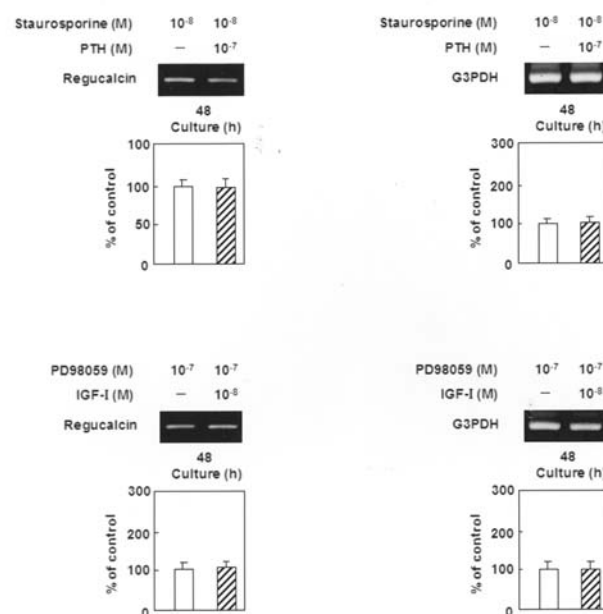


Figure 4. Effect of staurosporine or PD98059 on the PTH- or IGF-I-increased regucalcin mRNA expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of PTH (10<sup>-7</sup> M) or IGF-I (10<sup>-8</sup> M) with or without staurosporine (10<sup>-8</sup> M) or PD98059 (10<sup>-7</sup> M). After the medium change, the cells were cultured for 24 or 48 h. Total RNAs (2  $\mu$ g) extracted from the cells were analyzed using RT-PCR with specific primers. The figure shows one of four experiments with separate samples. The densitometric data for each mRNA level in the cells cultured for 24 or 48 h in the presence of PTH (A) or IGF-I (B) with or without each inhibitor were indicated as a percentage of control (mean  $\pm$  SEM for four experiments).

## Results

Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or various factors without FBS, and the change in regucalcin mRNA expression was examined. Culture with PTH (10<sup>-7</sup> M) for 48 h caused a significant increase in regucalcin mRNA expression as compared with that of the control value (none) (Fig. 1A). Regucalcin mRNA expression was significantly increased after culture with IGF-I (10<sup>-8</sup> M) for 24 or 48 h (Fig. 1B).

The effect of 17 $\beta$ -estradiol or 1,25(OH)<sub>2</sub>D<sub>3</sub> on regucalcin mRNA expression in osteoblastic cells is shown in Fig. 2. Regucalcin mRNA expression was significantly increased after culture with 17 $\beta$ -estradiol (10<sup>-10</sup> or 10<sup>-9</sup> M) for 24 or 48 h (Fig. 2A). Culture with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> M) caused a significant decrease in regucalcin mRNA expression in osteoblastic cells (Fig. 2B).

The effect of TNF- $\alpha$  on regucalcin mRNA expression in osteoblastic cells is shown in Fig. 3. Regucalcin mRNA expression was significantly decreased after culture with TNF- $\alpha$  (1 or 10 ng/ml of medium) for 24 or 48 h.

The effect of PTH or IGF-I in increasing regucalcin mRNA expression in osteoblastic cells was examined in the presence of intracellular signaling inhibitors (Fig. 4). Cells were cultured for 48 h in the presence of PTH (10<sup>-7</sup> M) or IGF-I (10<sup>-8</sup> M) with or without staurosporine (10<sup>-8</sup> M), an inhibitor of protein kinase C, or PD98059 (10<sup>-7</sup> M) an inhibitor of mitosis-

activated protein (MAP) kinase. The effect of PTH or IGF-I in increasing regucalcin mRNA expression was not seen in the presence of staurosporine or PD98059, respectively.

## Discussion

Bone loss has been induced in the femoral tissues of regucalcin transgenic rats (30). Regucalcin has been expressed in rat bone marrow cells, and osteoclastic bone resorption has been stimulated in regucalcin transgenic rats with increasing age (35). Osteoblastic cell formation from bone marrow cells has been enhanced in regucalcin transgenic rats (36). Exogenous regucalcin has been shown to have stimulatory effects on osteoclastogenesis in bone marrow cell cultures (32) and suppressive effects on osteoblastic differentiation and mineralization *in vitro* (33). Regucalcin may play an important role in the regulation of bone metabolism.

We examined whether the expression of regucalcin mRNA in osteoblastic cells is hormonally regulated. PTH,  $1,25(\text{OH})_2\text{D}_3$ , or  $\text{TNF-}\alpha$  has stimulated osteoclastic bone resorption (37). PTH, IGF-I, or  $17\beta$ -estradiol has had an anabolic effect on osteoblastic cell function (38,39). The receptors of these hormones and cytokines are expressed in osteoblastic MC3T3-E1 cells. Culture with PTH, IGF-I, or  $17\beta$ -estradiol enhanced the expression of regucalcin mRNA in osteoblastic cells. Meanwhile, regucalcin mRNA expression was suppressed after culture with  $1,25(\text{OH})_2\text{D}_3$  or  $\text{TNF-}\alpha$ .  $\text{TNF-}\alpha$  had a potent suppressive effect on regucalcin mRNA expression, suggesting that the cytokine has a role as the negative regulator in regucalcin mRNA expression.

The effect of PTH or IGF- $\alpha$  in increasing regucalcin mRNA expression in osteoblastic cells was completely prevented after culture with staurosporine, an inhibitor of protein kinase C, or PD98059, an inhibitor of MAP kinase. The expression of regucalcin mRNA in osteoblastic cells may be enhanced through intracellular signaling factors.

$17\beta$ -estradiol had stimulatory effects on regucalcin mRNA expression in osteoblastic cells, suggesting that the gene expression is mediated through the steroid receptors in the cells.

The effect of  $\text{TNF-}\alpha$  is mediated through TNF receptor-associated factor (TRAF6) in osteoblastic cells (37).  $\text{TNF-}\alpha$  was found to suppress regucalcin mRNA expression in osteoblastic cells. The suppressive effect of  $\text{TNF-}\alpha$  on regucalcin mRNA expression in osteoblastic cells may be mediated through the TRAF6 signaling process.

Regucalcin mRNA expression in osteoblastic cells was significantly decreased after a longer culture with  $1,25(\text{OH})_2\text{D}_3$ , suggesting that its expression is suppressed through the steroid receptors.

The endogenous regucalcin in osteoblastic cells has suppressive effects on cell function that is involved in differentiation and mineralization (33). If regucalcin mRNA expression is regulated by various hormones and cytokines which are involved in the regulation of osteoblastic cell function, the endogenous regucalcin may be partly related, in light of hormone and cytokine effects on osteoblastic cells. The present findings support the view that regucalcin may play an important role in the regulation of osteoblastic cell function.

In conclusion, it has been demonstrated that regucalcin mRNA expression is enhanced after culture with PTH, IGF-I, or  $17\beta$ -estradiol in osteoblastic MC3T3-E1 cells, and that it is suppressed with  $1,25(\text{OH})_2\text{D}_3$  or  $\text{TNF-}\alpha$ . The expression of regucalcin mRNA was found to be regulated hormonally in osteoblastic cells.

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