Journal of Cellular Biochemistry 96:543–554 (2005)

Suppressive Effect of Regucalcin on Cell Differentiation and Mineralization in Osteoblastic MC3T3-E1 Cells

Masayoshi Yamaguchi,* Mayumi Kobayashi, and Satoshi Uchiyama

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-Ku, Shizuoka 422-8526, Japan

Abstract The role of regucalcin in the regulation of osteoblastic cell function was investigated. Osteoblastic MC3T3-E1 cells with subconfluent monolayers were cultured in a medium containing regucalcin (10^{-10}–10^{-8} M) without fetal bovine serum (FBS). The proliferation of osteoblastic cells was not significantly altered in the presence of regucalcin. The results of reverse transcription-polymerase chain reaction (RT-PCR) analysis with specific primers showed that the expression of Runx2 (Cbfa1) and insulin-like growth factor-I (IGF-I) mRNAs in osteoblastic cells was significantly suppressed in the presence of regucalcin (10^{-10} or 10^{-9} M). Transforming growth factor-β1 mRNA levels were significantly enhanced in the 24 h-culture with regucalcin (10^{-10} or 10^{-9} M). α1(I) collagen and glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels were not significantly changed by culture with regucalcin (10^{-10} or 10^{-9} M). Alkaline phosphatase activity was significantly decreased in the lysate of cells cultured for 24 or 48 h with regucalcin (10^{-10}–10^{-8} M). Moreover, the expression of regucalcin in osteoblastic cells was demonstrated by RT-PCR and Western blot analysis. When regucalcin (10^{-7} M) was added into the enzyme reaction mixture containing the lysate of osteoblastic cells cultured in the absence of regucalcin, alkaline phosphatase activity was significantly decreased. Also, Ca^{2+}/calmodulin-dependent nitric oxide (NO) synthase activity in the cell lysate was significantly decreased by addition of regucalcin (10^{-10}–10^{-8} M) into the reaction mixture. The presence of anti-regucalcin monoclonal antibody (5 or 10 ng/ml) in the enzyme reaction mixture caused a significant increase in NO synthase activity in the cell lysate in the presence or absence of Ca^{2+}/calmodulin, suggesting a role of endogenous regucalcin. When osteoblastic cells with subconfluency were cultured in the presence of regucalcin (10^{-10} or 10^{-9} M) for 3, 9, or 18 days, the results with Alizarin red staining showed that the mineralization was markedly suppressed by culture with regucalcin for 3, 9, or 18 days. This study demonstrates that regucalcin regulates the function of osteoblastic cells, and that the protein suppresses cell differentiation and mineralization. J. Cell. Biochem. 96: 543–554, 2005.

Key words: reguclacin; mineralization; osteoblasts; Runx 2; IGF-I; TGF-β1; nitric oxide synthase

Regucalcin, which was found 1978, has been demonstrated to play a multifunctional role as a regulatory protein on intracellular signaling mechanism in many cell types [Yamaguchi and Yamamoto, 1978; Yamaguchi, 2000a,b, 2005; reviews] in recent years. The gene of regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex, and regucalcin is also found in other tissues [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1998; Yamaguchi and Nakajima, 1999]. AP 1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1998, 1999; Misawa and Yamaguchi, 2002].

Regucalcin plays a role in maintaining intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinases and tryrosine kinases, protein phosphatases, nitric oxide (NO) synthase, and the

*Correspondence to: Masayoshi Yamaguchi, PhD, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-Ku, Shizuoka 422-8526, Japan.
E-mail: yamaguch@u-shizuoka-ken.ac.jp

Received 15 February 2005; Accepted 8 April 2005

DOI 10.1002/jcb.20524

control of the enhancement of nuclear deoxyribonucleic acid (DNA) and RNA synthesis in proliferative cell [Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003; Yamaguchi, 2005]. Recent study has demonstrated that regucalcin has a suppressive effect on cell proliferation and apoptosis, which is mediated through many signaling factors, in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Tsurusaki and Yamaguchi, 2003; Izumi and Yamaguchi, 2004a,b]. Regucalcin has been proposed to play a role in the maintaining cell homeostasis and function in many cell types [Yamaguchi, 2005; reviews].

We generated regucalcin transgenic (TG) rats to determine a regulatory role of endogenous regucalcin in vivo using a TG rat model [Yamaguchi et al., 2002a]. Bone loss and hyperlipidemia have been shown to induce in regucalcin TG rats [Yamaguchi et al., 2002b, 2004a], supporting the views that regucalcin has an important role on pathophysiologic state.

The mechanism by which bone loss is induced in regucalcin TG rats has not been fully clarified. Regucalcin is expressed in rat bone marrow cells, and bone loss in regucalcin TG rats is partly involved in osteoclastic bone resorption [Uchiyama and Yamaguchi, 2004; Yamaguchi et al., 2004b]. Regucalcin has been demonstrated to stimulate osteocalst-like cell formation in mouse marrow culture in vitro, and the protein stimulates bone resorption in rat femoral tissues in vitro [Yamaguchi and Uchiyama, 2005]. The effect of regucalcin on bone formation has not been yet determined, however.

The present study was undertaken to determine whether regucalcin could regulate the function of osteoblastic cells, which participate in bone formation and mineralization in vitro. We found that regucalcin plays a regulatory role in the regulation of the function of osteoblastic MC3T3-E1 cells in vitro, and the protein suppresses cell differentiation and mineralization.

MATERIALS AND METHODS

Chemicals

α-minimum essential medium (α-MEM) and penicillin-streptomycin (5,000 U/ml penicillin; penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), *p*-nitrophenylphosphate, arginine, β-nicotinamide adenine dinucleotide phosphate reduced from β-NADPH, citrulline, calmodulin (52,000 U/mg protein from bovine brain), and Alizarin red S was purchased from Sigma (St. Louis, MO). Calcium chloride and other chemicals were of regent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). Reagents used were passed through ion-exchange resin to remove metal ions.

Isolation of Regucalcin

Male Wistar rats, weighing 100–120 g, were obtained commercially from Japan SLC (Hamamatsu, Japan). Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4°C. The livers were removed, cut into small pieces, suspended 1:4 (w/v) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500*g* in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000*g* for 60 min. The resulting supernatant was heated at 60°C for 10 min and recentrifuged at 38,000*g* for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously [Yamaguchi and Yamamoto, 1978].

Anti-Regucalcin Antibody

Anti-regucalcin antibody was a monoclonal antibody raised against regucalcin prepared using standard methods [Omura and Yamaguchi, 1999]. Mice (BALB/C, Japan SLC) were subcutaneously injected with rat liver regucalcin emulsified with Freund's complete adjuvant, and 19 days later antigen (0.25 mg/animal) was intraperitoneally injected with Freund's incomplete adjuvant. Animals were killed with bleeding at 3 days after the last injection. Spleen cells were prepared from immunized mouse and fused into myeloma cells. Anti-regucalcin monoclonal antibody-producing cells (hybridoma cells) were obtained by screening. The IgG from hybridoma cells was isolated through a protein A-agarose column (Sigma).

Cell Culture

Osteoblastic MC3T3-E1 cells were cultured at 37°C in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). For experiments, about 1.0×10^5 cells per dish were cultured for 3 days to obtain subconfluent monolayers in 35-min plastic containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium without FBS containing either vehicle or regucalcin (10^{-10}–10^{-8} M) and the cells were cultured further for 1–21 days. Cell viability was estimated by staining with trypan blue.

Cell Counting

After trypsinization of the cells in each culture dish using a Ca^{2+}/Mg^{2+}-free PBS containing 0.2% trypsin and 0.02% EDTA for 2 min at 37°C, cells were collected and wash-centrifuged in a Phosphate-buffer saline (PBS) solution at 100*g* for 5 min. The cells were resuspended on 0.5 ml PBS solution, and an aliquot was stained with eosin. The cells were counted under a microscope using a Hemacytometer plate. For each dish, we took the average of two counts.

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M). After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in 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 isoprepanol at −20°C. RNA precipitates were pelted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

RT-PCR was preformed with a Titam™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of mouse regucalcin cDNA were: 5′-AGATGAACAAATCCCAGAT-3′ (sense strand, positions 618–636 of cDNA sequence in reference) [Misawa and Yamaguchi, 2000b] and 5′-ATATTCCTATGCAGGGTGA-3′ (antisense strand, positions 906–924 of cDNA sequence [Misawa and Yamaguchi, 2000]). These oligonucleotid sequences are localized in exon 5 and 7, respectively, of the regucalcin gene. The pair of oligonucleotide primers was designed to amplify a 307 bp sequence from the mRNA of mouse regucalcin. Primers for amplification of mouse Runx2 (Cbfal; type 1) cDNA were: 5′-ATGCGTATTCCTGTAGATCCGAG-3′ (sense strand, positions 1016–1038 of cDNA sequence) and 5′-CATCATTCCCGGCCATGACGGTAAC-3′ (antisense strand, positions 1451–1475) [Seth et al., 2000]. The pair of oligonucleotide primers was designed to amplify a 459 bp sequence from the mRNA of mouse Runx2. Primers for amplification of mouse α1(I) collagen cDNA were: 5′-TTCTCCTGGTAAAGA-TGGTGC-3′ (sense strand, positions 2232-2252 of cDNA sequence) and 5′-GGACCAGCAT-CACCTTTAACA-3′ (antisence strand, positions 2466–2486) [Luppen et al., 2003]. The pair of oligonucleotide primers was designed to amplify a 254 bp sequence from the mRNA of α1(I) collagen. Primers for amplification of mouse IGF-I cDNA were: 5′-GCAAGCTTCAGCCAC-CTTAC-3′ (sense strand, positions 955–974 of cDNA sequence) and 5′-GGGTCGTTTACA-CAGCAGGT-3′ (antisense strand, positions 1466–1485) [Bell et al., 1986]. The pair of oligonucleotide primers was designed to amplify a 511 bp sequence from the mRNA of IGF-I. Primers for amplification of mouse TGF-β1 cDNA were: 5′-CTCTCCACCTGCAAGAC-CAT-3′ (sense strand, positions 633–652 of cDNA sequence) and 5′-CTGCCGTACAACTC-CAGTGA-3′ (antisense strand, positions 1312–1331) [Deryck et al., 1986]. The pair of oligonucleotide primers was designed to amplify a 679 bp sequence from the mRNA of mouse TGF-β1. Glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was used as an internal control to evaluate total RNA input. Primers for amplification of G3PDH cDNA were: 5′-GATTTGGC-CGTATCGGACGC-3′ (sense strand) and 5′-CTCCTTGGAGGCCATGTAGG-3′ (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977 bp sequence from the mRNA of rat G3PDH. RT-PCR was performed using reaction mixture (20 μl) containing 1 or 4 μg of total RNA, supplied RT-PCR buffer,

Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 30 cycles under the following conditions: denaturation for 30 s 94°C, annealing for 30 s at 56°C, and extension for 60 s 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 Fluoro-Imager SI (Amersham Phamacia Biotech).

Western Blot Analysis

The homogenate from osteoblastic MC3T3-E1 cells cultured with 10% FBS was centrifuged for 10 min at 5,500*g* at 4°C, and the supernatant was used for Western blot analysis [Wessendolf et al., 1993]. Aliquots of protein (10 μg) were mixed with 5× Laemmli sample buffer, boiled for 5 min, and SDS–PAGE was performed by the method of Laemmli [1970] using 12% polyacrylamide resolving gel. After SDS–PAGE, the proteins were then transferred onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The membranes were incubated with a polyclonal rabbit anti-regucalcin antibody [Yamaguchi and Isogai, 1993], which was diluted 1:2,000 in 10 mM Tris-HCl, pH 8, containing 150 mM NaCl, 0.1% (w/v) Tween 20 (washing buffer), and 5% (w/v) skim milk for 1 h. The membranes incubated with antibody were washed four times with washing buffer. Then membranes were incubated for 1 h with horseradish peroxidase linked anti-rabbit IgG, which was diluted 1:5,000 with washing buffer containing 5% (w/v) skim milk, and again they were washed. Detection of the protein bands was performed using an enhanced chemiluminescent kit following the manufacture's instruction. The molecular size of the detecting protein was determined by running the standard protein molecules of known sizes in parallel.

Enzyme Assay

To assay alkaline phosphatase in the cells after appropriate treatment periods, the cells were washed three times with PBS, scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. The supernatant, centrifuged at 600*g* for 5 min, was used to measure enzyme activity. The enzyme assay described below was carried out under optimal conditions in the reaction mixture containing 20–40 μg of cell protein/ml of reaction mixture. Alkaline phosphatase activity was determined by the method of Walter and Schutt [1965]. The enzyme activity was expressed as nmol of *p*-nitrophenol liberated per min per mg of protein. Protein concentration in the cell lysate was determined by the method of Lowry et al. [1951].

NO synthase activity in the 5,500*g* supernatant of osteoblastic MC3T3-EI cell homogenate was estimated by the procedure of Lee and Stull [1998] with a minor modification. The enzyme activity was measured for 60 min at 37°C in a reaction mixture (1.0 ml) containing 100 mM HEPES, pH 7.2, 4 mM β-NADPH, 2 mM L-arginine, and the cell protein (30–50 μg/ml) in the presence or absence of calmodulin (2.5 μg/ml). In separate experiments, the reaction mixture contained either vehicle, calcium chloride (10 μM), regucalcin (10^{-10}–10^{-8} M), anti-regucalcin monoclonal antibody (5 or 10 ng/ml), or other reagents. The enzyme reaction was terminated by the addition of 1.0 ml of cold 10% trichloroacetic acid and centrifuged to precipate protein. Produced citrullin in the supernatant was quantified by the method of Boyde and Rahmatullah [1980]. Results were expressed as nmol of citrullin produced per min per mg of cell protein.

Alizarin Red Staining

Osteoblastic MC3T3-E1 cells (2.5×10^5 cells) were cultured for 72 h in an α-MEM containing 10% FBS. Cells with subconfluency were changed to a Dullbecco's modified essential medium (DMEM) containing ascorbic acid (100 μg/ml) and 4 mM β-glycerophosphate in the presence or absence of regucalcin (10^{-10} or 10^{-9} M) with 10% FBS. After medium change, cells were cultured for 3, 9, or 18 days. The medium was changed with every 3 days. At each time point, cells were rinsed with PBS, and fixed on ice with 70% ethanol for 15 min for alizarin red staining of calcium [Kamiya et al., 2002]. The Alizarin red solution (40 mM, pH 4.2) was filtered through Whatman paper and applied to the fixed wells for 30 min at room temperature. Nonspecific staining was removed by several washed in water.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using

Student's *t*-test. *P*-values less than 0.05 were considered to indicate statistically significant differences. Also, we used an ANOVA multiple comparison test to compare the treatment groups.

RESULTS

Effect of Exogenous Regucalcin in Osteoblastic Cells

The effect of regucalcin addition on proliferation of osteoblastic MC3T3-E1 cells reaching subconfluent monolayers was examined. Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS in the presence or absence of regucalcin (10^{-10}–10^{-8} M). The number of osteoblastic cells was not significantly altered in the presence of regucalcin (Fig. 1). Osteoblastic cells were cultured for 72 h in a medium containing 10% FBS without regucalcin addition to obtain subconfluent monolayers, and it was changed to a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) without FBS. After medium change, cells were cultured for 24 or 72 h. The presence of regucalcin did not cause a significant alteration in the number of cells (Fig. 2).

Osteoblastic cells with sobconfluency were cultured for 24 or 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) in the absence of FBS. The change in the expression of Runx 2, α1(I) collagen, IGF-I, or TGF-β1 mRNAs in osteoblastic cells was examined by RT-PCR using specific primers. The housekeeping gene G3PDH was used as an internal control. The signal of bands for Runx2 mRNA expression was significantly reduced in the cells cultured for 24 or 48 h in the presence of regucalcin (10^{-10} or 10^{-9} M) (Fig. 3). The levels of α1(I) collagen or G3PDH mRNAs were not significantly changed in the presence of regucalcin.

The change in IGF-I or TGF-β1 mRNA levels in osteoblastic cells is shown in Figure 4. The levels of IGF-I mRNA were significantly decreased in osteoblastic cells cultured for 24 h in the presence of regucalcuin (10^{-10} or 10^{-9} M). This decrease was also observed by 48h-culture with 10^{-9} M regucalcin. The levels of TGF-β1 mRNA were significantly increased in oseoblastic cells cultured for 24 h with regucalcin (10^{-10} or 10^{-9} M). This increase was not seen with 48 h-culture of regucalcin (10^{-10} or 10^{-9} M).

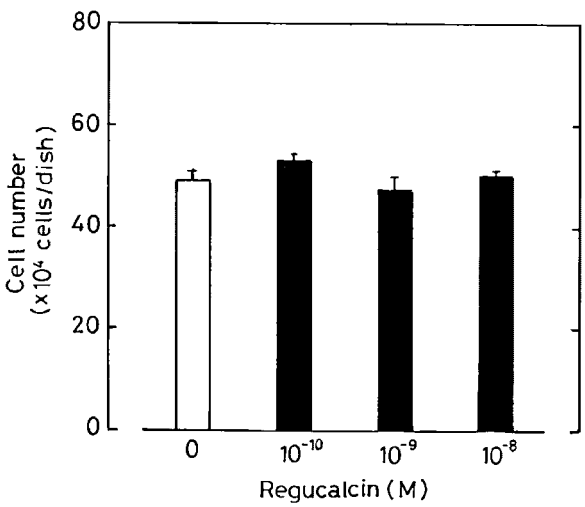

Fig. 1. Effect of regucalcin on the proliferation of osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) in the presence of 10% fetal bovine serum (FBS), and the number of cells was measured. Each value is the mean ± SEM of six experiments. Data were not significant as compared with the control (none).

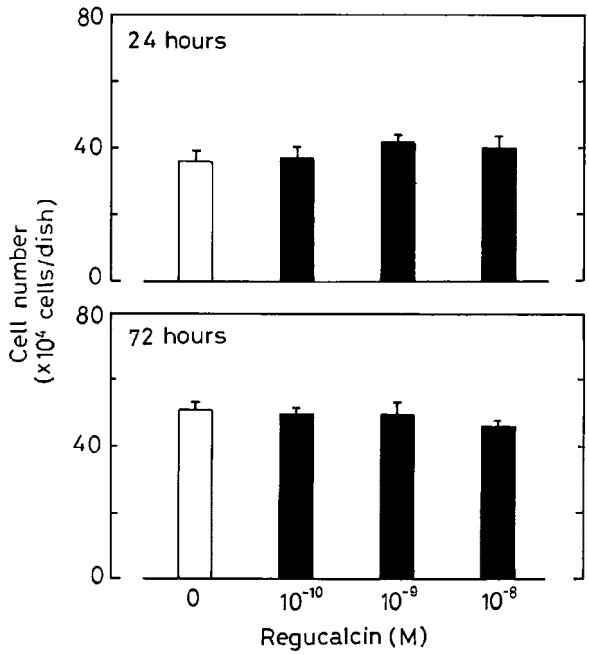

Fig. 2. Effect of regucalcin on the proliferation of osteoblastic MC3T3-E1 cells with subconfluncy. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and it was changed to a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) in the absence of 10% FBS. After medium change, cells were cultured for 24 or 72 h. At each time point, the number of cells was measured. Each value is the mean ± SEM of six experiments. Data were not significant as compared with the control (none) value.

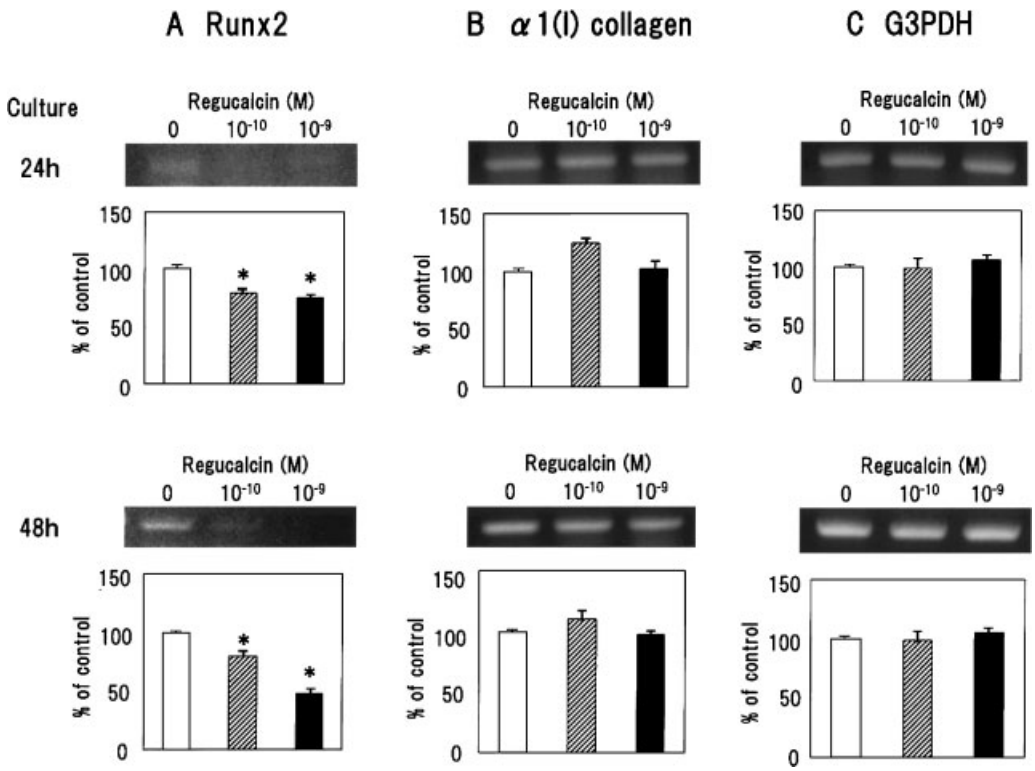

Fig. 3. Effect of regucalcin on the expression of Runx2 or α1(I) collagen mRNAs in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and it was changed to a medium containing either vehicle or regucalcin (10^{-10}–10^{-9} M) in the absence of 10% FBS. After medium change, cells were cultured for 24 or 48 h. Total RNAs [4 μg for Runx2 mRNA and 2 μg for α1(I) collagen mRNA] extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for Runx2, α1(I) collagen, or G3PDH mRNA levels were indicated as % of control (mean ± SEM for five experiments). $^*P < 0.01$, compared with the control level.

The change in alkaline phosphatase activity in osteoblastic MC3T3-E1 cells cultured in the presence of regucalcin was examined. Cells with subconfluency were cultured for 24 or 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) in the absence of FBS. Alkaline phosphatase activity in the lysate of cells cultured for 24 or 48 h was significantly decreased by culture with regucalcin (10^{-10}–10^{-8} M) (Fig. 5).

Expression of Regucalcin and Its Regulatory Effect in Osteoblastic Cells

Osteoblastic MC3T3-E1 cells were cultured for 72 h to obtain subconfluent monolayers in a medium containing 10% FBS. The expression of regucalcin mRNA in osteoblastic cells was examined by RT-PCR using regucalcin-specific primers. The band of RT-PCR products for regucalcin mRNA was observed (Fig. 6). The result of Western blot analysis using cell lysate protein showed that regucalcin was present in osteoblastic cells (Fig. 6). Reguaclcin mRNA and its protein levels were significantly increased by culture with 10% FBS compared with those of 1% FBS.

The effect of regucalcin addition on alkaline phosphatase activity in lysate of osteoblastic cells was examined. The enzyme reaction mixture contained either vehicle or regucalcin (10^{-9}–10^{-7} M). Alkaline phosphstase activity in the cell lysate was significantly decreased by the addition of regucalcin (10^{-7} M) (Fig. 7).

The effect of regucalcin addition on NO synthase activity in the lysate of osteoblastic cells is shown in Figure 8. NO synthase activity was significantly decreased by the addition of regucalcin (10^{-8} M) into the reaction mixture without Ca^{2+}/calmodulin. NO synthase activity was significantly increased by the addition of Ca^{2+}/calmodulin. This increase was significantly inhibited by the addition of regucalcin (10^{-10}–10^{-8} M).

The effect of anti-regucalcin monoclonal antibody addition on NO synthase activity in the lysate of osteoblastic cells is shown in Figure 9.

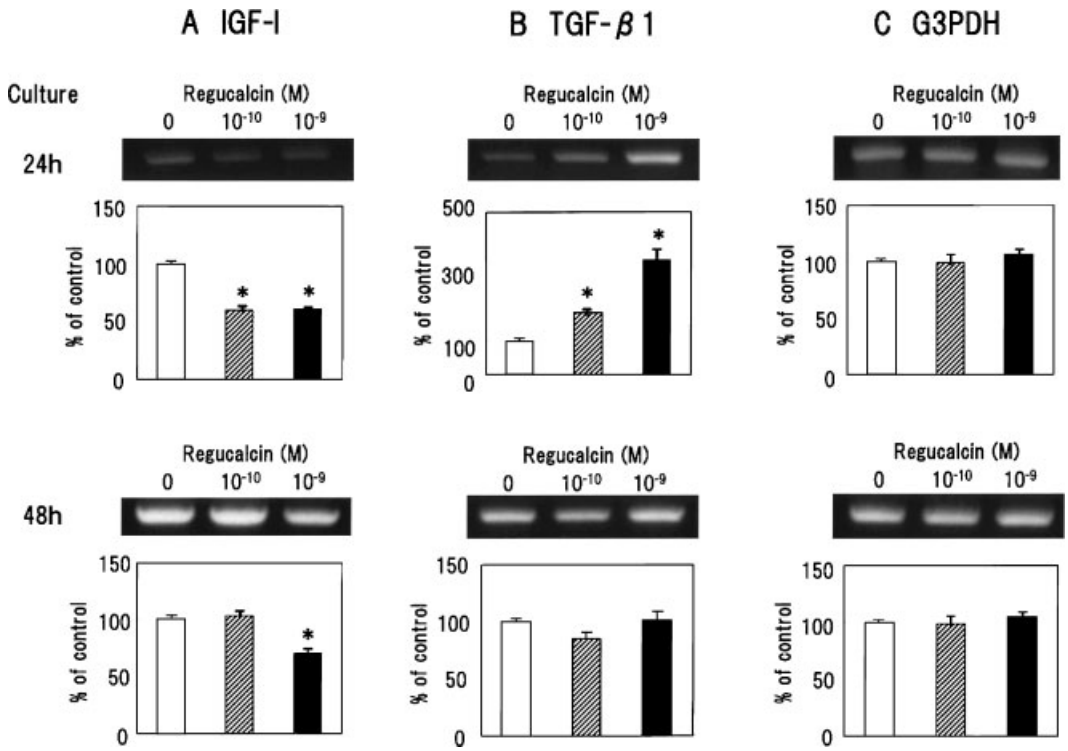

Fig. 4. Effect of regucalcin on the expression of IGF-I or TGF-β1 mRNAs in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and it was changed to a medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) in the absence of 10% FBS. After medium change, cells were cultured for 24 or 48 h. Total RNAs (1 μg for IGF-I and TGF-βI mRNA; 2 μg for G3PDH mRNA) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separates samples. The densitometric data for IGF-I or TGF-β1 mRNA levels were indicated as % of control (without regucalcin) (mean ± SEM for five experiments). $^*P < 0.01$, compared with the control level.

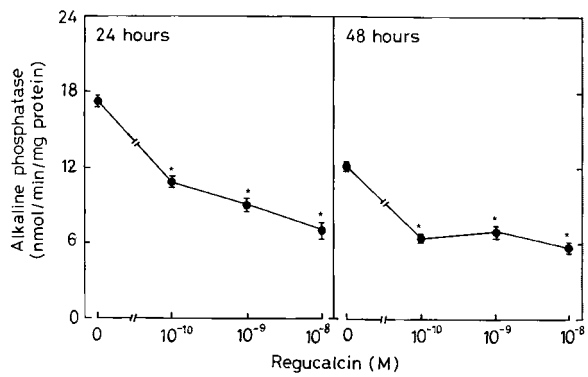

Fig. 5. Effect of regucalcin on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and it was changed to a medium containing either vehicle or regucalcin (10^{-10}–10^{-8} M) in the absence of 10% FBS. After medium change, cells were cultured for 24 or 48 h. At each time point, the enzyme activity was measured. Each value is the mean ± SEM of five experiments with separate samples. $^*P < 0.01$, compared with the control (none) value.

NO synthase activity in the cell lysate was significantly increased in the presence of anti-regucalcin monoclonal antibody (5 or 10 ng/ml of reaction mixture) without Ca^{2+}/calmodulin. The effect of the antibody (10 ng/ml) in increasing NO synthase activity was also observed in the presence of Ca^{2+}/calmodulin.

Mineralization in Osteoblastic Cells Cultured With Regucalcin

Osteoblastic cells with subconfluency were cultured for 3, 9, or 18 days in a medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M), and the effect of regucalcin on the mineralization was examined (Fig. 10). The results with Alizarin red staining for calcium showed that mineralization was significantly suppressed in the presence of regucalcin (10^{-10} or 10^{-9} M). The suppression of mineralization was observed at 3, 9, or 18 days of culture with regucalcin (10^{-10} or 10^{-9} M). The effect was remarkable with 9 or 18 day-culture of regucalcin

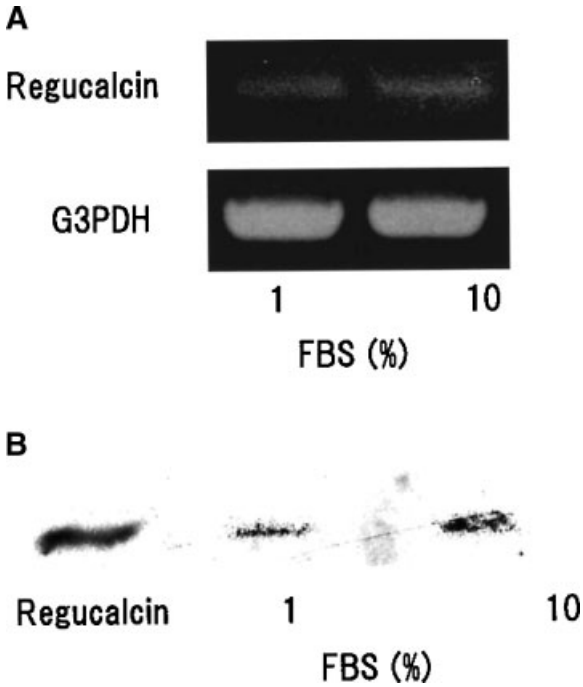

Fig. 6. Expression of regucalcin mRNA and its protein MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing FBS (1 or 10%) to obtain subconfluent monolayers. **A**: Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. **B**: Isolated regucalcin (0.1 μg) or proteins (10 μg) extracted from the cells were analyzed by Western blot analysis. The figure shows one of four experiments with separate samples.

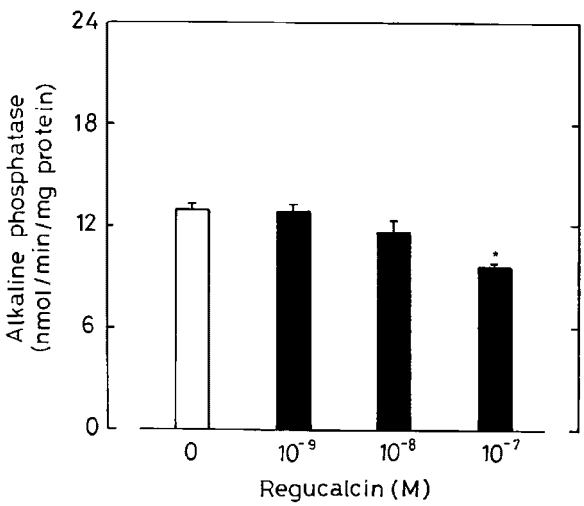

Fig. 7. Effect of regucalcin addition on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers. Cell lysate was used for the enzyme assay. The enzyme reaction mixture contained either vehicle or regucalcin (10^{-9}–10^{-7} M). Each value is the mean ± SEM of five experiments with separate samples. *$P < 0.01$, compared with the control (none) value.

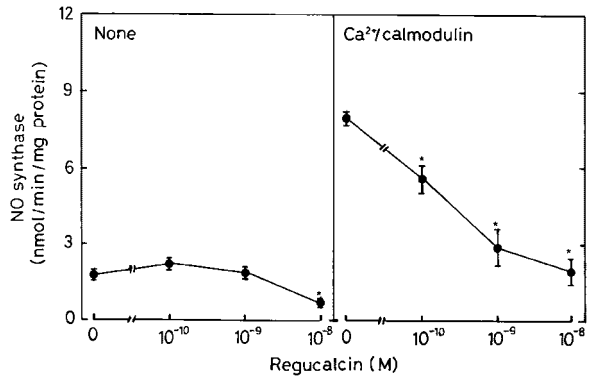

Fig. 8. Effect of regucalcin addition on NO synthase activity in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers. Cell lysate was used for the enzyme assay. The enzyme reaction mixture contained either vehicle or regucalcin (10^{-10}–10^{-8} M) in the presence or absence of calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of five experiments with separate samples. *$P < 0.01$, compared with the control (none) value.

(10^{-10} or 10^{-9} M). Meanwhile, regucalcin did not have a suppressive effect on the number of osteoblastic cells with culture for 18 days (data not shown).

DISCUSSION

Bone loss is induced in regucalcin TG rats in vivo [Yamaguchi et al., 2002b, 2004b]. Osteoclastic bone resorption has been shown to induce in regucalcin TG rats [Uchiyama and Yamaguchi, 2004], and regucalcin has been demonstrated to stimulate directly osteoclast-like cell formation in mouse marrow cultures [Yamaguchi and Uchiyama, 2005]. This study, moreover, was undertaken to determine the effect of regucalcin on the function of osteoblastic cells. We found that culture with regucalcin has a suppressive effect on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells in vitro. This finding may support the view that regucalcin has a suppressive effect on osteoblastic bone formation.

Culture with regucalcin in short term did not have a significant effect on the number of osteoblastic cells reaching subconfluent monolayers, and the additional culture of cells with subconfluency was not significantly changed in the presence of regucalcin. This observation indicates that regucalcin does not have an effect on cell proliferation and apoptosis in osteoblastic cells with culture of short term.

The targeted disruption of Runx2 (Cbfa1) has been shown to result in a complete lack of bone

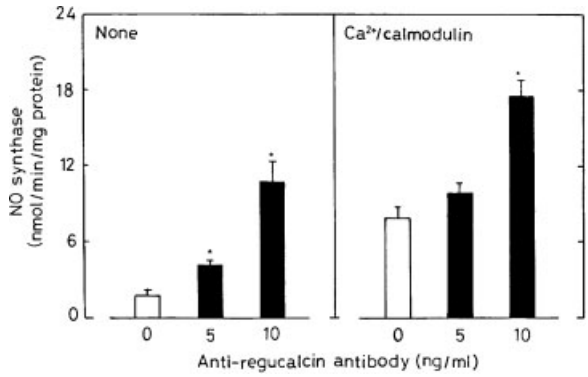

Fig. 9. Effect of anti-regucalcin monoclonal antibody on NO synthase activity in osteoblastic MC3T3-E1 cells. Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers. Cell lysate was used for the enzyme assay. The enzyme reaction mixture contained either vehicle or anti-regucalcin monoclonal antibody (5 or 10 ng/ml) in the presence or absence of calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of five experiments with separate samples. $^*P < 0.01$, compared with the control value without the antibody addition.

formation owing to maturational arrest of osteoblasts [Komori et al., 1997]. The expression of Runx2 mRNA was found to suppress significantly in osteoblastic cells with subconfluency cultured with short term in the presence of regucalcin. The levels of α1(I) collagen and G3PDH mRNAs in osteoblastic cells were not significantly changed in the presence of regucalcin. Regucalcin-induced suppression of Runx2 mRNA expression in osteoblastic cells may lead to a deterioration of cell differentiation and mineralization with culture in short term.

IGF-I plays a role in the stimulation of bone formation in osteoblastic lineage cells [Lian et al., 1999]. The expression of IGF-I mRNA was found to suppress in osteoblastic cells cultured with short term in the presence of regucalcin. This suppression may partly have an effect on the deterioration of osteoblastic bone formation. Menawhile, TGF-β1 mRNA expression in osteoblastic cells was significantly stimulated in the presence of regucalcin. This stimulation was seen with one day-culture. Bone morphogenic protein (BMP) is a potent regulator of osteoblast differentiation, and TGF-β1 can inhibit BMP-2-induced differentiation of osteoblastic cells [de Jong et al., 2002; Lee et al., 2003]. Regucalcin-enhanced TGF-β1 mRNA expression may be partly involved in the suppression of osteoblastic cell differentiation.

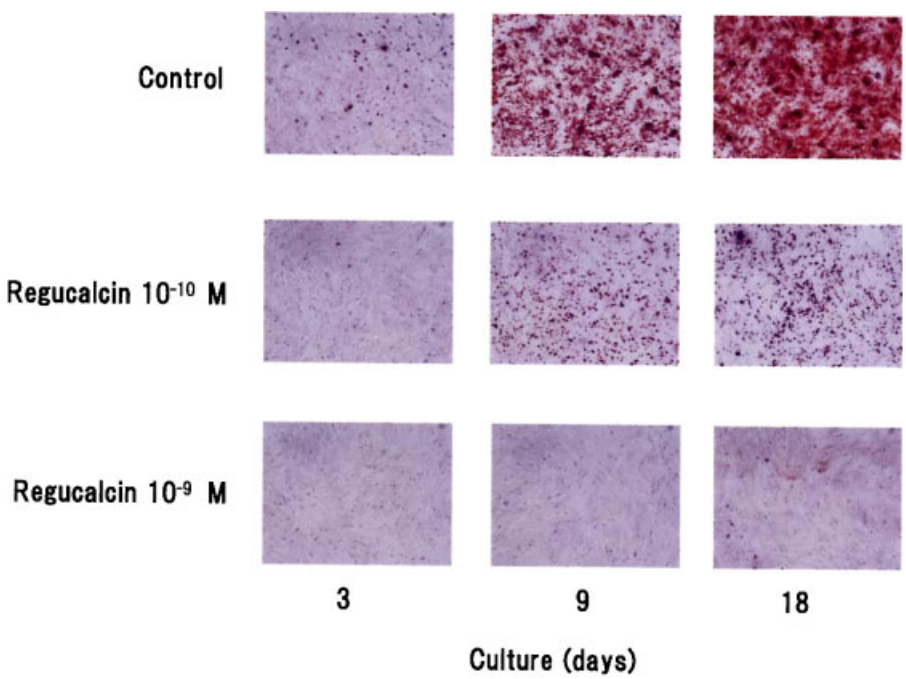

Fig. 10. Effect of regucalcin on mineralization in osteoblastic MC3T3-E1 cells. Osteoblastic (2.5×10^5 cells) were cultured for 72 h in a medium containing 10% FBS. Cells with subconfluency were changed to a medium with 10% FBS in the presence or absence of regucalcin (10^{-10} or 10^{-9} M). After medium change, cells were cultured for 3, 9, or 18 days. Cells were washed with PBS and stained Alizarin red staining. The figure shows one of five experiments with separate culture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Alkaline phosphatase activity was found to decrease markedly in osteoblastic cells cultured with short term in the presence of regucalcin (10^{-10}–10^{-8} M). The enzyme participates in bone mineralization process [Lian et al., 1999]. Regucalcin-induced decrease in alkaline phosphatase activity may influence on osteoblastic mineralization. Meanwhile, the addition of regucalcin (10^{-7} M) in the enzyme reaction mixture caused a significant decrease in alkaline phosphatase activity, although the concentration of 10^{-9} or 10^{-8} M regucalcin did not have a significant effect. Culture with regucalcin may induce the suppressive effect on enzyme activity that is mediated through intracellular factors.

Regucalcin has been shown to express in rat bone marrow cells [Yamaguchi et al., 2004b]. Moreover, the expression of regucalcin in osteoblastic MC3T3-E1 cells was demonstrated by RT-PCR and Western blot analysis. The role of regucalcin in osteoblastic cells was also studied. The lysate was obtained from osteoblastic cells with subconfluency in the absence of regucalcin. Alkaline phosphatase activity in the cell lysate was significantly decreased by addition of regucalcin into the enzyme reaction mixture, suggesting that regucalcin has a direct inhibitory effect on the enzyme activity. Also, NO synthase activity in the cell lysate was significantly decreased by addition of regucalcin into the enzyme reaction mixture. Regucalcin could inhibit activation of NO synthase by Ca^{2+}/calmodulin in the lysate of osteoblastic cells. NO synthase produces NO in many cell types, and NO plays a role in the regulation of cell function [Lowenstein et al., 1994]. Thus, regucalcin has a regulatory effect on the enzyme's activity in osteoblastic cells. Moreover, the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture caused a significant increase in NO synthase activity with and without Ca^{2+}/calmodulin. This finding suggests that endogenous regucalcin has a suppressive effect on NO synthase activity in osteoblastic cells. Regucalcin, which expresses in osteoblastic cells, may play a role in the regulation of cell function.

Prolonged culture with regucalcin was found to induce a remarkable suppression of mineralization in osteoblastic cells. This suppression was observed with culture of regucalcin for 3 days. Short-term culture with regucalcin caused a significant change in some osteoblastic markers. Whether culture with regucalcin in long term induces a change in osteoblastic markers is unknown, at present. It is possible, however, that prolonged culture with regucalcin has an effect on osteoblastic markers. Presumably, regucalcin plays an important role in the regulation of gene expression and mineralization in osteoblastic cells.

Regucalcin has been demonstrated to play a role as a regulator protein of intracellular signaling in many cell types [Yamaguchi, 2005]. Regucalcin may regulate cell signaling in osteoblastic cells. It is speculated that exogenous regucalcin binds to the plasma membranes of osteoblastic cells, and that the protein transmittes a signal into the cells to regulate gene expression and mineralization. Moreover, regucalcin has a suppressive effect on Ca^{2+}/calmodulin-dependent signaling pathway in osteoblastic cells.

Overexpression of regucalcin has been shown to induce bone loss using regucalcin TG rats in vivo [Yamaguchi et al., 2002b, 2004b]; the femur was reduced in mineral density, strength and calcium content in regucalcin-overexpressing rats. This bone loss may be partly related to osteoclastic bone resorption [Uchiyama and Yamaguchi, 2004]. Moreover, we found that regucalcin has a suppressive effect on cell differentiation and mineralization in osteoblastic cells. Regucalcin-overexpressing rats may induce a suppression of osteoblastic bone formation. It is speculated that regucalcin plays a role in the regulation of bone metabolism due to stimulating osteoclastic bone resorption and suppressing osteoblastic bone formation.

In conclusion, it has been demonstrated that regucalcin has a suppressive effect on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells in vitro, and that the protein plays a role in the regulation of osteoblastic cell function.

REFERENCES

Bell GI, Stempien MM, Fong NW, Rall LB. 1986. Sequences of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acids Res 14:7873–7882.

Boyde TRC, Rahmatullah M. 1980. Optimization of conditions for the colorimetric determination of citrulline using diacetylmonoxine. Anal Biochem 107:424–431.

Chomczyshi P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

de Jong DS, van Zoelen EJ, Bauerschmidt S, Olive W, Steegeaga WT. 2002. Microarray analysis of bone morphogenetic protein, transforming growth factor beta,

and activin early response genes during osteoblastic cell differentiation. J Bone Miner Res 17:2119–2129.
Deryck R, Jarrett JA, Chen EY, Goeddel DV. 1986. The murine transforming growth factor-beta precursor. J Biol Chem 261:4377–4379.
Izumi T, Yamaguchi M. 2004a. Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-α or thapsigargin. J Cell Biochem 92:296–306.
Izumi T, Yamaguchi M. 2004b. Overexpression of regucalcin suppresses cell death and apoptosis in cloned rat hepatoma H4-II-E cells induced by lipopolysaccharide, PD 98059, dibucaine, or Bay K 8644. J Cell Biochem 93:598–608.
Izumi T, Tsurusaki Y, Yamaguchi M. 2003. Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 89:800–807.
Kamiya N, Jihho A, Kimata K, Damsky C, Watanabe H. 2002. Establishment of a novel chondrocytic cell line N1511 derived from p53-null mice. J Bone Miner Res 17:1832–1842.
Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfal results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of batcteriophage T4. Nature 22:680–685.
Lee S-J, Stull JT. 1998. Calmodulin-dependent regulation of inducible and neuronal nitric-oxide synthase. J Biol Chem 273:27430–27437.
Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, Sung IM, Wozney JM, Kim HJ, Ryoo HM. 2003. BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem 278:34387–34394.
Lian JB, Stein GS, Roby PG, Boskey AL. 1999. Bone formation: Osteoblast lineage cells, growth factors, matrix proteins, and the mineralization process. In: Favus MJ, editor. Primer on the metabolic bone diseases and diseases and disorders of mineral metabolism, 4th edn. New York: Lippincott Williams & Wilkins Press. pp 14–29.
Lowenstein CJ, Dinerman JL, Synder SH. 1994. Nitric oxide: A physiologic messenger. Ann Intern Med 120: 227–237.
Lowry OH, Rosebrough MJ, Farr AJ, Randall FJ. 1951. Protein measurement with the Folin phenol. J Biol Chem 193:265–273.
Luppen CA, Smith E, Spevak L, Boskey AL, Frenkel B. 2003. Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. J Bone Miner Res 18:1186–1197.
Misawa H, Yamaguchi M. 2000. The gene of Ca^{2+}-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med 6:191–196.
Misawa H, Yamaguchi M. 2002. Identification of transcription factor in the promoter region of rat regucalcin gene: Binding of nuclear factor I-A1 to TTGGC motif. J Cell Biochem 84:795–802.
Murata T, Yamaguchi M. 1998. Ca^{2+} administration stimulates the binding of AP-1 factor to the 5′-flanking region of the rat gene for the Ca^{2+}-binding protein regucalcin. Biochem J 329:157–183.
Murata T, Yamaguchi M. 1999. Promotor characterization of the rat gene for Ca^{2+}-binding protein regucalcin. Transcriptional regulation by signaling factor. J Biol Chem 274:1277–1285.
Omura M, Yamaguchi M. 1999. Enhancement of neutral phosphatase activity in the cytosol and nuclei of regenerating rat liver: Role of endogenous regucalcin. J Cell Biochem 73:332–341.
Seth A, Lee BK, Qi S, Vary CPH. 2000. Coordinate expression of novel genes during osteoblast differentiation. J Bone Miner Res 15:1683–1696.
Shimokawa N, Yamaguchi M. 1992. Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305:151–154.
Shimokawa N, Yamaguchi M. 1993. Molecular cloning and sequencing of the cDNA coding for calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.
Shimokawa N, Matsuda Y, Yamaguchi M. 1995. Genomic cloning and chromosomal assignment of regucalcin gene. Mol Cell Biochem 151:157–163.
Thiselton DL, McDowall J, Brandau O, Ramser J, d′Esposito F, Bhattacharya SS, Ross MT, Hardcastle AK, Meindl A. 2002. An integrated, functionally annotated gene map of the DXS80269-ELK1 interval on human Xp 11.3Xp-Xp 11.23: Potential hospot for neurogenetic disorders. Genomics 79:560–572.
Tsurusaki Y, Yamaguchi M. 2002a. Suppressive role of endogenous regucalcin in the enhamcement of deoxyribonucleic acid synthesis activity n the nucleus of regenerating rat liver. J Cell Biochem 85:516–522.
Tsurusaki Y, Yamaguchi M. 2002b. Role of endogenous regucalcin in nuclear regulation of regenerating rat liver: Suppression of the enhanced ribonucleic acid synthesis activity. J Cell Biochem 87:450–457.
Tsurusaki Y, Yamaguchi M. 2003. Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-Ii-E cells. J Cell Biochem 90: 619–626.
Uchiyama S, Yamaguchi M. 2004. Bone loss in regucalcin transgenic rats: Enhancement of osteoclastic cell formation from bone marrow of rat with increasing age. Int J Mol Med 14:451–455.
Walter K, Schutt C. 1965. Acid and alkaline phosphstase in serum. In: Bergmeyer HV, editor. Methods of enzymnatic analysis, Vols 1-2. New York: Academic Press; pp 856–860.
Wessendolf JHM, Ganfinkel S, Zhan X, Brown S, Maciag T. 1993. Identification of a nuclear localization sequence within the syructure of the human interleukin-l α precursor. J Biol Chem 268:22100–22104.
Yamaguchi M. 2000a. Role of regucalcin in calcium signaling. Life Sci 66:1763–1780.
Yamaguchi M. 2000b. The role of regucalcin in nuclear regulation of regenerating liver. Biochem Biophys Res Commun 276:1–6.
Yamaguchi M. 2005. Role of regucalcin in maintaining cell homeostasis and function. Int J Mol Med 15:371–389.
Yamaguchi M, Isogai M. 1993. Tissue concentration of calcium binding protein regucalcin in rats by enzyme-

linked immunoadsorbent assay. Mol Cell Biochem 122: 65–68.

Yamaguchi M, Nakajima M. 1999. Involvement of intracellular signaling factors in the serum-enhanced Ca^{2+}-binding protein regucalcin mRNA expression in the cloned rat hepatoma cell (H4-II-E). J Cell BIochem 74:81–89.

Yamaguchi M, Uchiyama S. 2005. Regucalcin stimulates osteoclast-like cell formation in mouse marrow cultures. J Cell Biochem (in press).

Yamaguchi M, Yamamoto T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26:1915–1918.

Yamaguchi M, Morooka Y, Misawa H, Tsurusaki Y, Nakajima R. 2002a. Role of endogenous regucalcin in transgenic rats: Suppression of kidney cortex cytosolic protein phosphatase activity and enhancement of heart muscle microsomal Ca^{2+}-ATPase activity. J Cell Biochem 86:520–529.

Yamaguchi M, Misawa H, Uchiyama S, Morooka Y, Tsurusaki Y. 2002b. Role of endogenous regucalcin in bone metabolism: Bone loss is induced in regucalcin transgenic rats. Int J Mol Med 10:377–383.

Yamaguchi M, Igarashi A, Uchiyama S, Sawada N. 2004a. Hyperlipidemia is induced in regucalcin transgenic rats with increasing age. Int J Mol Med 14:647–651.

Yamaguchi M, Sawada N, Uchiyama S, Misawa H, Ma ZJ. 2004b. Expression of regucalcin in rat bone marrow cells: Involvement of osteoclastic bone resorption in regucalcin transgenic rats. Int J Mol Med 13:437–443.