Increased regucalcin gene expression extends survival in breast cancer patients: Overexpression of regucalcin suppresses the proliferation and metastatic bone activity in MDA-MB-231 human breast cancer cells *in vitro*

MASAYOSHI YAMAGUCHI[1], SATORU OSUKA[2], M. NEALE WEITZMANN[3,4],
MAMORU SHOJI[1] and TOMIYASU MURATA[5]

Departments of [1]Hematology and Medical Oncology, and [2]Neurosurgery, Winship Cancer Institute,
Emory University School of Medicine, Atlanta, GA 30322; [3]The Atlanta Department of Veterans Affairs
Medical Center, Decatur, GA 30033; [4]Division of Endocrinology and Metabolism and Lipids, Department of Medicine,
Emory University School of Medicine, 1329 WMRB, Atlanta, GA 30322, USA; [5]Laboratory of Analytical Neurobiology,
Faculty of Pharmacy, Meijo University, Yagotoyama 150, Tempaku, Nagoya 468-8503, Japan

Abstract. Human breast cancer is highly metastatic to bone and drives bone turnover. Breast cancer metastases cause osteolytic lesions and skeletal damage that leads to bone fractures. Regucalcin, which plays a pivotal role as an inhibitor of signal transduction and transcription activity, has been suggested to act as a suppressor of human cancer. In the present study, we compared the clinical outcome between 44 breast cancer patients with higher regucalcin expression and 43 patients with lower regucalcin expression. Prolonged relapse-free survival was identified in the patients with increased regucalcin gene expression. We further demonstrated that overexpression of full length, but not alternatively spliced variants of regucalcin, induces G1 and G2/M phase cell cycle arrest, suppressing the proliferation of MDA-MB-231 cells, a commonly used *in vitro* model of human breast cancer that metastasize to bone causing osteolytic lesions. Overexpression of regucalcin was found to suppress multiple signaling pathways including Akt, MAP kinase and SAPK/JNK, and NF-κB p65 and β-catenin along with increased p53, a tumor suppressor, and decreased *K-ras*, *c-fos* and *c-jun*. Moreover, we found that co-culture of regucalcin-overexpressing MDA-MB-231 cells with mouse bone marrow cells prevented enhanced osteoclastogenesis and suppressed mineralization in mouse bone marrow cells *in vitro*. Taken together, the present study suggests that regucalcin may have important anticancer properties in human breast cancer patients. Mechanistically, these effects are likely mediated through suppression of multiple signaling pathways, upregulation of p53 and downregulation of oncogenes leading to anti-proliferative effects and reduced metastases to bone, a phenotype associated with poor clinical outcome.

Introduction

Breast cancer is the most frequent malignancy and most common cause of cancer-related death in women worldwide. Breast cancer is highly metastatic to bone where it drives bone turnover causing bone damage. Breast cancer bone metastasis occurs in 70-80% of patients with advanced breast cancer (1-4), leading to severe pathological bone fractures, pain, hypercalcemia, and spinal cord and nerve-compression syndromes (3,5), which are a common cause of morbidity and mortality. Tumor invasion into bone tissues is associated with osteoclast and osteoblast recruitment, resulting in the liberation of growth factors from the bone matrix, which can feed back to enhance tumor growth resulting in the vicious cycle of bone metastasis (4,5).

Breast cancer promotes the formation of osteoclasts through secretion of osteoporotic cytokines, such as parathyroid hormone-related peptide, prostaglandin E_2, tumor necrosis factor-α (TNF-α), interleukins and leukemia inhibitory factor (4,6,7). Constitutively activated nuclear factor-κB (NF-κB) in breast cancer cells has been shown to play a crucial role in the osteolytic bone metastasis of breast cancer in stimulating osteoclastogenesis. Enhanced NF-κB stimulates production of granulocyte macrophage-colony stimulating factor (GM-CSF) in breast cancer cells that enhance osteoclast development from monocytes (8). Moreover, breast cancer cells express the receptor activator of NF-κB ligand (RANKL) that mediates epithelial proliferation and carcinogenesis (7).

Correspondence to: Dr Masayoshi Yamaguchi, Department of Hematology and Medical Oncology, Emory University School of Medicine, 1365 C Clifton Road, NE, Atlanta, GA 30322, USA
E-mail: yamamasa1155@yahoo.co.jp

Osteoblasts are negatively affected by breast cancer cells as evidenced by an increase in apoptosis and a decrease in proteins required for new bone formation (6). Breast cancer cell bone metastasis-induced bone loss is due to both activated osteoclastic bone resorption and suppressed osteoblastic bone formation. Bisphosphonates or anti-RANKL antibody (denosumab) have been used as the current standard of care for patients with bone metastasis (9).

The regucalcin, whose gene is localized on the X chromosome (10-12), plays a pivotal role as a suppressor of protein of multi-signaling pathways in various types of cells and tissues (13,14). The regucalcin gene expression is regulated by various hormonal factors including calcium-related process, calcium-regulating hormones, insulin, estrogen and other steroid hormones (15). Regucalcin is translocated from the cytoplasm to nucleus in various types of cells and it regulates nuclear functions (16). Regucalcin has been shown to play a role in the maintaining of intracellular calcium homeostasis and inhibiting of various protein kinases, protein phosphatases and protein synthesis in the cytoplasm and nuclear DNA and RNA syntheses (13-16). Nuclear regucalcin has also been shown to regulate the gene expression of various proteins (16). Moreover, regucalcin has been found to suppress cell proliferation and apoptotic cell death that are mediated through multiple signaling pathways (17,18). Regucalcin has been proposed to play a pivotal role in maintaining cell homeostasis and function as a suppressor protein of intracellular signaling systems (13,14).

There is growing evidence that regucalcin is involved in mitigating human carcinogenesis (17,19). Regucalcin has been reported to be downregulated in human tumor tissues *in vivo* (19-21). We have demonstrated that survival in pancreatic cancer patients is prolonged in subjects with increased regucalcin gene expression (22). Furthermore, overexpression of the human regucalcin gene suppresses the proliferation of human pancreatic cancer MIA PaCa-2 cells *in vitro* (22). Taken together the data suggest that regucalcin may play a potential role as a suppressor of human carcinogenesis.

Because regucalcin has not previously been investigated in the context of breast cancer, the present study was undertaken to determine whether human regucalcin exhibits anticancer effects and anti-bone metastatic activity in human breast cancer. We report significantly improved relapse-free survival in 44 breast cancer patients with higher regucalcin expression. Moreover, overexpression of regucalcin was found to exhibit anti-proliferative effects in MDA-MB-231 cells (23). Regucalcin may play a potential role as a suppressor protein in human breast cancer.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate and antibiotics (penicillin and streptomycin) were purchased from Corning Cellgro (Mediatech, Inc. Manassas, VA, USA). α-Minimum essential medium (α-MEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT, USA). Tumor necrosis factor-α (TNF-α) was from R&D Systems (Minneapolis, MN, USA). Sodium butyrate, roscovitine,

sulforaphane, PD98059, staurosporine, Bay K 8644, wortmannin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), caspase-3 inhibitor, Alizarin red, lypopolysaccharide (LPS) and all other reagents were purchased from Sigma-Aldrich unless otherwise specified. Gemcytabine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Antibodies for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gemcytabine and caspase-3 inhibitor were diluted in phosphate-buffered saline (PBS) and other reagents were dissolved in 100% ethanol to use in experiments.

Patient datasets. Gene expression and survival data of 87 breast cancer patients were obtained through the Gene Expression Omnibus (GEO) database (GSE6532) for outcome analysis (23-25). These datasets contained gene expression data derived from the Affymetrix U133 Plus2 platform. For microarray analysis, expression and raw expression data (CEL files) were summarized and normalized using the Robust Multi-array Average algorithm and the Bioconductor package affy (http://www.bioconductor.org/packages/2.0/bioc/html/affy.html).

Breast cancer MDA-MB-231 cells. Human breast cancer MDA-MB-231 cells lack estrogen, progesterone and human epithelial growth factor type 2 (HER2) receptors, and are therefore considered as triple-negative (26). They express high levels of the epithelial growth factor receptor (EGFR) and activation of this receptor and its downstream signaling events enhance migration, proliferation, invasion and progression of the malignant phenotype of these cells (26). MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA).

Regucalcin transfectants. Stable regucalcin transfectants overexpressing full length or truncated regucalcin proteins in regucalcin in MDA-MB-231 cells were generated as follow. The cDNA encoding human regucalcin with full length (900 bp), deleted exon 4 (684 bp), and deleted exon 4 and 5 (552 bp) were cloned into the pBluescript vector (27,28). The complete regucalcin coding cDNA was cloned into the *Eco*RI site of the pCXN2 expression vector (27). The resultant plasmid was designated as regucalcin/pCXN2 (24). For transient transfection assay, MDA-MB-231 cells were grown on 24-well plates to ~70% confluence. Each of regucalcin (900 bp), deleted exon 4 (684 bp), and deleted exons 4 and 5 (552 bp) and pCXN2 vector alone were transfected into MDA-MB-231 cells using Lipofectamine reagent, according to the manufacturer's instructions (Promega, Madison, WI, USA) (27). After overnight incubation, gemciticin (G418) (600 μg/ml of medium; Sigma-Aldrich) was added to culture wells for selection and cells were cultured for 2 weeks. After that, cells were plated at limiting dilution to isolate stable transfectants. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in medium without G418. The increase in regucalcin in transfectants was 15.5-fold of wild-type cells. In experiments, transfectants were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin for 1-7 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C.

Cell proliferation. MDA-MB-231 wild-type cells ($1x10^5$/ml/well) and MDA-MB-231 cells ($1x10^5$/ml/well) transfected with regucalcin cDNAs of either full length, deleted exon 4 or deleted exons 4 and 5 were cultured using a 24-well plate in DMEM containing 10% FBS and 1% penicillin and streptomycin for 1, 2, 3 or 7 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C (28,29). In separate experiments, MDA-MB-231 wild-type cells ($1x10^5$/ml/well) or full length regucalcin transfectants were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin in the presence of sodium butyrate (10 and 100 μM), roscovitine (10 and 100 nM), sulphoraphan (1 and 10 nM), dibucain (0.1 or 1 μM), Bay K 8644 (1 or 10 μM), PD98059 (1 or 10 μM), wortmannin (0.1 or 1 μM), DRB (0.1 or 1 μM), or gemcitabine (50 or 100 nM) for 3 days. After culture, the cells were detached with trypsin from each culture dishes and counted.

Cell death. MDA-MB-231 wild-type cells ($1x10^5$/ml/well) and MDA-MB-231 cells ($1x10^5$/ml/well) transfected with either full length, deleted exon 4 or deleted exons 4 and 5 regucalcin cDNAs were cultured using a 24-well plate in DMEM containing 10% FBS and 1% penicillin and streptomycin for 5 days. Subconfluent cells were cultured for additional 3 days in the presence or absence of LPS (0.1 or 1 μg/ml), TNF-α (0.1 or 1 ng/ml) (30). In separate experiments, wild-type MDA-MB-231 cells ($1x10^5$/ml/well) or transfectants were cultured for 5 days to confluent, and then for an additional 24 h in the presence or absence of LPS (1 ng/ml) or Bay K 8644 (10 μM) with or without caspase-3 inhibitor (10 μM) (29). After culture, cells were detached with trypsin from each culture dish.

Cell counting. After trypsinization of each culture dish using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37˚C, the detached cells from the dish were collected by centrifugation (28-30). Cells were resuspended on PBS solution and stained with eosin. Cell numbers were quantified by counting under a microscope using a hemocytometer plate. For each dish, we took the average of two counts. Cell number is shown as number of cells per well.

Western blotting. MDA-MB-231 cells, which were transfected with control vector or regucalcin cDNAs with full length, deleted exon 4 and deleted exons 4 and 5 were plated in 35-mm dishes at a density of $1x10^6$ cells/well in 2 ml of medium, and they were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin for 3 days. Cells were washed twice with ice cold PBS and removed from the dish with a cell scraper. Recovered cells were disrupted by sonication in 1.0 ml of ice cold PBS containing protease and phosphatase inhibitors. The homogenate was centrifuged for 5 min at 1,500 x g to obtain cell debris, and then the supernatant including cytoplasm, nucleus and other cell fractions were collected. The concentration of protein was determined using Bradford dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using bovine serum albumin as a standard. Samples 30 μg of the supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using specific antibodies against regucalcin (including 33, 25 and 20 kDa) (21) and other proteins (Santa Cruz Biotechnology). Loading controls consisted of β-actin for cytosolic proteins. A minimum of 3 blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and bands quantitated using ImageJ. Data from independent experiments were normalized as a percentage of control before averaging.

Animals and bone marrow cells. Female mice (CD1-Elite, wild-type, 2 months old), which were purchased from Charles River, were housed in a non-specific pathogen-free facility, and all procedures and protocols were approved through the Institutional Animal Care and Use Committee of Emory University. The femur and tibia were removed immediately after sacrifice (31). Bone marrow cells were isolated under sterile conditions from the femurs and tibias.

Bone cells. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3), was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured as previously described (31).

Mineralization in co-culture of bone marrow, preosteoblastic MC3T3 and breast cancer cells. To determine the effects of breast cancer cells on osteoblastogenesis and mineralization of bone marrow or preosteoblastic MC3T3, we used mineralization medium (MM) containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate in DMEM with 10% FBS and 1% penicillin and streptomycin. Bone marrow cells ($1x10^6$ cells/1 ml/well) or preosteoblastic MC3T3 ($2x10^5$ cells/1 ml/well) were cultured for 3 days at 37˚C in a humidified 5% CO_2 atmosphere, and then the cells were co-cultured with addition of breast cancer MDA-MB-231 cells ($1x10^4$ cells/1 ml/well) of wild-type or transfectant using 12-well plates in α-MEM in the presence or absence of MM containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate for 18 days (31). The medium was changed every 3 days. After culture, cells were washed with PBS and stained with Alizarin red stain. For quantitation, 10% cetylpyridinium chloride solution was added to each well to elute the dye and absorbance was measured at 570 nm on a microtiter plate reader (31).

Osteoclastogenesis in co-culture with bone marrow cell and breast cancer cells. To determine the effects of breast cancer cells on bone marrow osteoclastogenesis, bone marrow cells ($2x10^5$ cells/1 ml/well) were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin using 24-well plates (1.0 ml/well) (31). Bone marrow cells were co-cultured in the presence of wild-type ($1x10^4$ cells/1 ml/well) or transfectant ($1x10^4$ cells/1 ml/well) with full length of regucalcin cDNA for 3 days and then 0.5 ml of the old medium was replaced with fresh medium, and cultures were maintained for an additional 4 days. In other experiments, bone marrow cells ($2x10^5$ cells/1 ml/well) were cultured for 3 days in medium and then fresh medium added. Cells were co-cultured with MDA-MB-231 cells [wild-type ($1x10^4$ cells/1 ml/well) or transfectants ($1x10^4$ cells/1 ml/well)] for additional 4 days (31). After culture for 7 days, the cells adherent to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts (32). Briefly, the cells were washed with phosphate-buffered saline solution and fixed with 10% neutral-

ized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied (32). The fixed cells were incubated for 90 min at room temperature in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a stain for the reaction product, in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells. MNCs scored were the mean ± SDM of six cultures.

Statistical analysis. Survival curves were constructed by Kaplan-Meier analysis and were compared with the log-rank test as performed with IBM SPSS Statistics 18 software (IBM, Chicago, IL, USA; http://www.ibm. com). In the experiments with MDA-MB-231 cells, statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for parametric data as indicated. $P < 0.05$ was considered statistically significant.

Results

Survival in patients with breast cancer. To understand the involvement of regucalcin in human patients with breast cancer, we compared the clinical outcome between 44 patients with higher regucalcin expression and 43 patients with lower regucalcin expression. There was a significant difference of regucalcin expression between the two groups (Fig. 1A). The reduction of regucalcin expression was associated with poor prognosis in patients with breast cancer. Breast cancer patients with the higher regucalcin gene expression were found to have prolonged relapse-free survival (Fig. 1B). These findings support the view that the suppression of regucalcin gene expression partly contributes to the development of carcinogenesis in human breast cancer cells and leads to a worse clinical outcome.

Generation of MDA-MB-231 cells overexpressed with regucalcin. The cDNA-encoding human regucalcin with full length (33 kDa), deleted exon 4 (25 kDa), and deleted exons 4 and 5 (20 kDa) was cloned into the expression vector pCXN2. Human breast cancer MDA-MB-231 cells were transiently transfected with the pCXN2 vector or regucalcin/pCXN2 construct by lipofection. To generate the transfectants stably overexpressing regucalcin in MDA-MB-231 cells, pCXN2 vector- or regucalcin/pCXN2-transfected MDA-MB-231 cells were cultured in neomycin-containing medium. Multiple neomycin-resistant clones were selected, and the regucalcin content of these clones was analyzed by immunoblotting with an anti-regucalcin antibody. The regucalcin content in cells transfected with regucalcin cDNA vector of full length (33 kDa) was increased 15.5-fold as compared with that of the parental wild-type MDA-MB-231 cells (Fig. 2A). However, the proteins of 25 and 20 kDa were not expressed in MDA-MB-231 cells transfected with deleted exon 4 (25 kDa) and deleted exons 4 and 5 (20 kDa) (Fig. 2A).

Overexpression of regucalcin suppresses the proliferation of MDA-MB-231 cells. To determine the effects of the over-
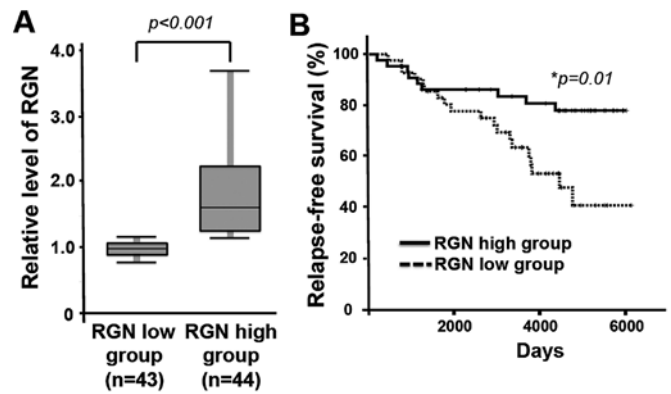

Figure 1. Expanded free survival in breast cancer patients with increased regucalcin gene expression. Reduced regucalcin expression shortens relapse-free survival of patients. (A) The patients group was separated into two groups (RGN high and RGN low) by microarray analysis. RGN high group have much higher RGN expression with statistically difference. (B) Relapse-free survival curves for breast cancer were longed in high expression as compared with low expression of regucalcin. RGN, regucalcin.

expression of endogenous regucalcin on the proliferation of MDA-MB-231 cells *in vitro*, the cancer cells were cultured for 1, 2, 3 and 7 days. Numbers of wild-type cells were increased over time in culture (Fig. 2B-E). This increase was suppressed in MDA-MB-231 cells transfectanted with regucalcin cDNA of full length (34 kDa) for 1 (Fig. 2B), 2 (Fig. 2C), 3 (Fig. 2D) and 7 (Fig. 2E) days. The proliferations of MDA-MB-231 cells transfected with exon 4-deleted regucalcin cDNA or the exons 4 and 5-deleted regucalcin cDNA were not significantly suppressed with culture for 7 days as compared with that of the transfectants with the regucalcin cDNA of full length (Fig. 2B-E). Overexpression of regucalcin with full length was found to specifically exhibit suppressive effects on the proliferation of MDA-MB-231 cells *in vitro*.

Proliferation in MDA-MB-231 cells was determined in the presence of various inhibitors that induce cell cycle arrest *in vitro* (Fig. 3). Wild-type cells were cultured for 3 days in the presence of butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM) (28,33,34). Cell proliferation was suppressed in the presence of these inhibitors (Fig. 3A). Such effects were not revealed in the transfectants (Fig. 3B). Endogenous regucalcin was suggested to induce G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells.

Next, to determine the mechanistic characterization for suppressive effects of regucalcin on cell proliferation, we examined whether suppressive effects of overexpression of regucalcin on the proliferation of MDA-MB-231 cells are modulated by various signaling factors that suppress the proliferation. Proliferation in MDA-MB-231 cells (wild-type) was suppressed in the presence of dibucaine (0.1 or 1 μM), an inhibitor of calcium/calmodulin-dependent protein kinases (28), or Bay K 8644 (0.1 or 1 μM), an agonist of calcium entry into cells (35) (Fig. 4A). Such effects were not seen in transfectants (Fig. 4B). Likewise, the proliferation of MDA-MB-231 cells (wild-type) was suppressed by culture with wortmannin (0.1 or 1 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3K) (36), and PD98059 (1 or 10 μM), an extracellular signal-regulated kinase (ERK) inhibitor (37) (Fig. 4C). Suppressive effects of these inhibitors on cell prolif-
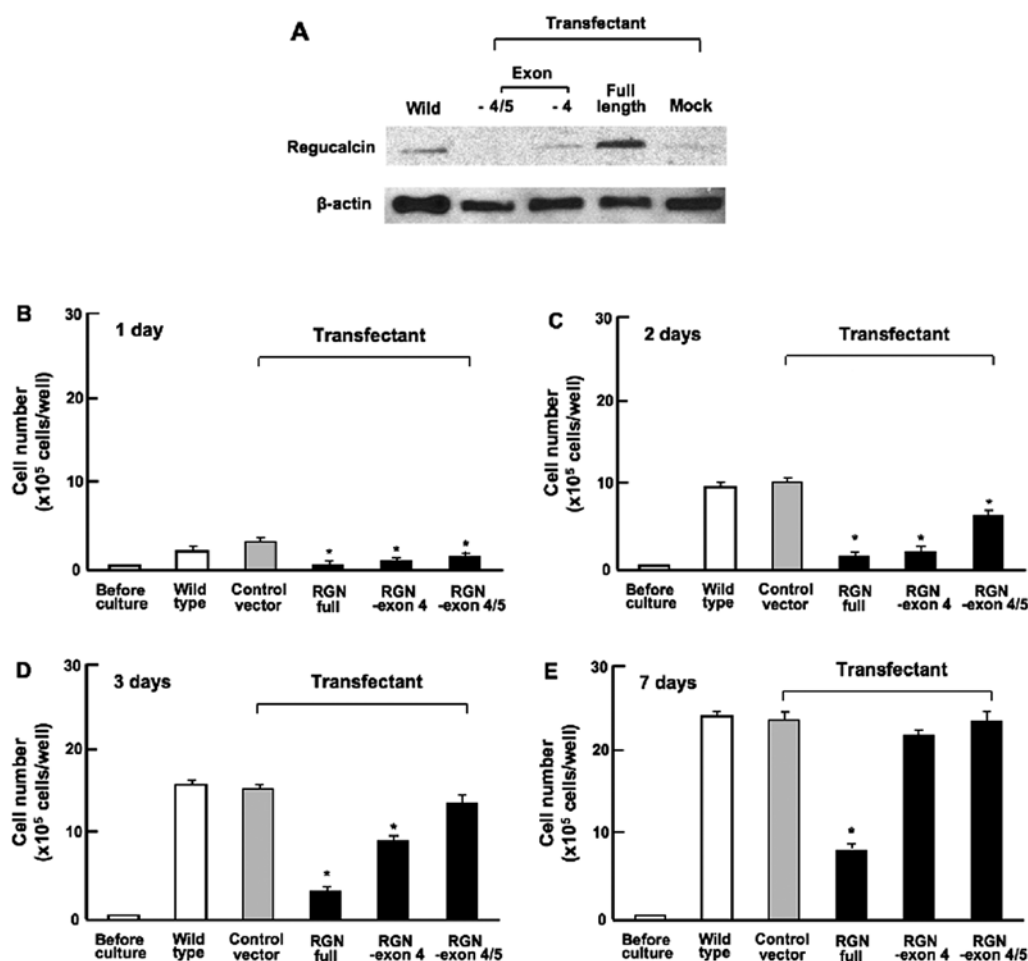
Figure 2. Overexpression of regucalcin suppresses the proliferation in MDA-MB-231 human breast cancer cells transfected with regucalcin cDNA vector including full length, deleted exon 4, and deleted exons 4 and 5 *in vitro*. (A) RGN content of multiple neomycin-resistant cells was analyzed by immunoblotting with an anti-regucalcin antibody. Lane 1, wild-type cells (designated as Wild). Lane 2, cells transfected with RGN (deleted exons 4 and 5)/pCXN2 (designated as -4/5). Lane 3, cells transfected with RGN (deleted exon 4)/pCXN2 (designated as -4). Lane 4, cells transfected with RGN (full length)/pCNX2 (designated as Full length). Lane 5, cells transfected with pCXN2 (designated as Mock). (B-E) Wild-type cells and transfectants were cultured in DMEM for 1 (B), 2 (C), 3 (D) or 7 (E) days. After culture, the number of attached cells on dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. wild-type (white bar) or control vector (grey bar). One way ANOVA, Tukey-Kramer post-test. RGN, regucalcin.
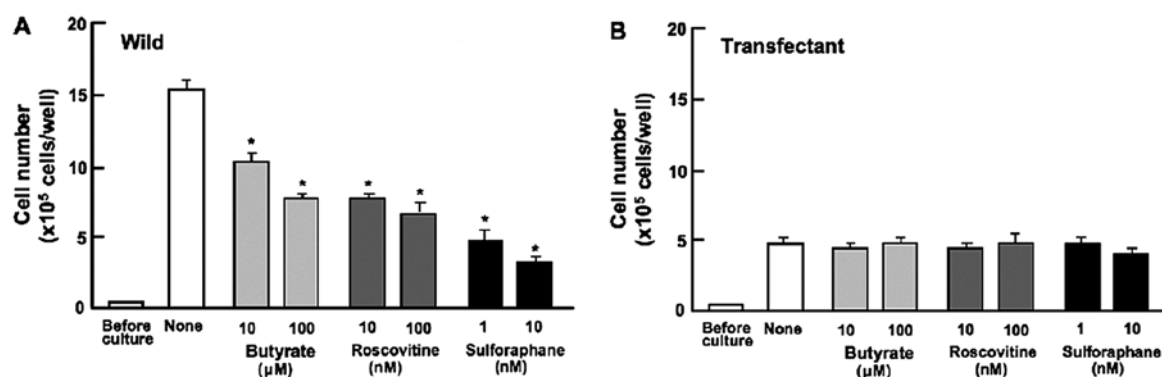

Figure 3. Suppressive effects of cell cycle inhibitors on the proliferation in MDA-MB-231 human breast cancer wild-type cells are not exhibited in the transfectants overexpressed with regucalcin of full length. (A) Wild-type cells or (B) transfectants were culture for 3 days in the absence or presence of butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM). After culture, the number of attached cells on the dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. control (none; white bar). One way ANOVA, Tukey-Kramer post-test. Wild, wild-type cells.

eration were not revealed in transfectants (Fig. 4D). DRB is an inhibitor of transcriptional activity with RNA polymerase II inhibition (38). Gemcitabine is a strong antitumor agent that induces nuclear DNA damage (39). Proliferation of
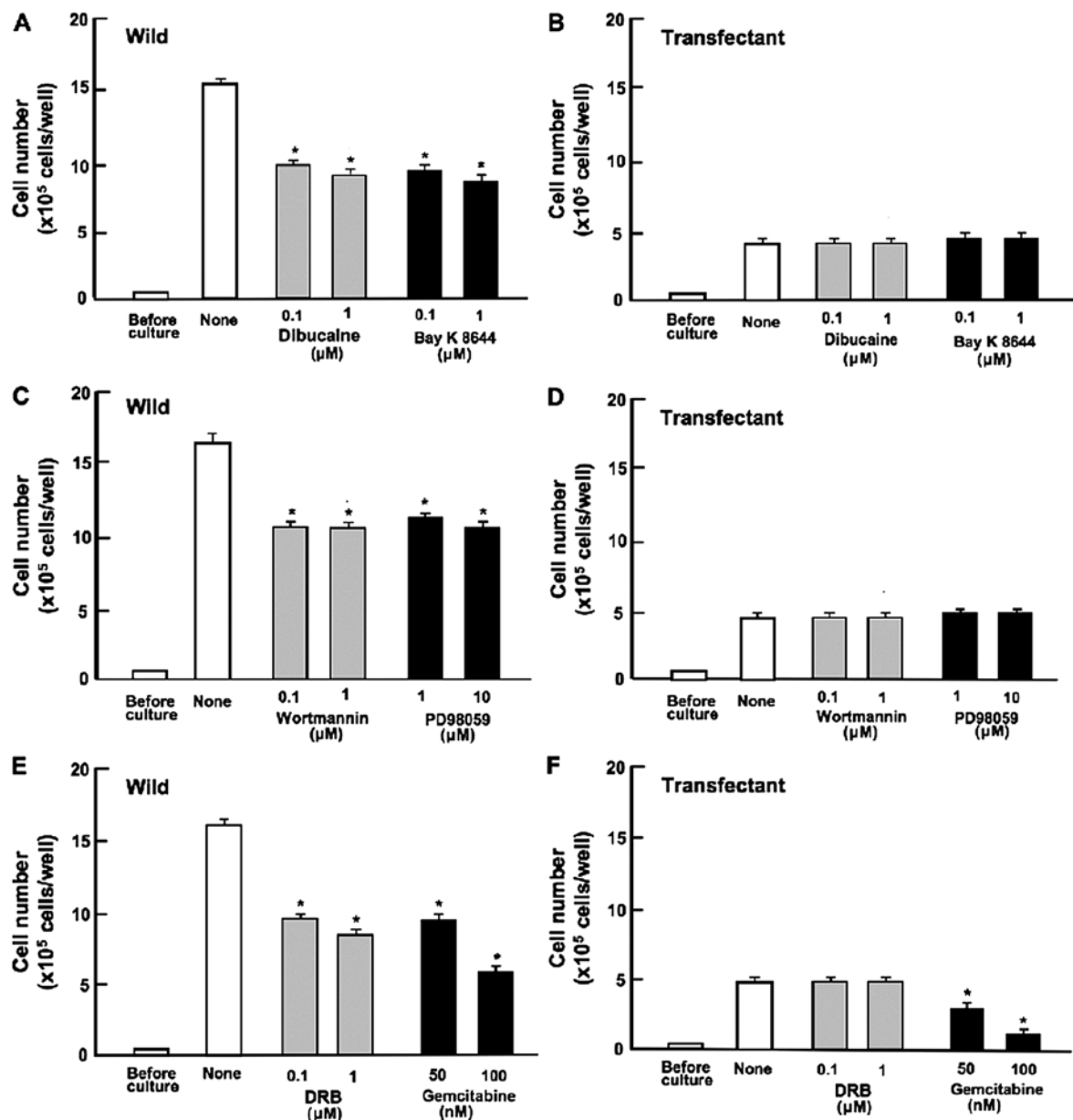
Figure 4. Suppressive effects of various inhibitors of signaling pathways on the proliferation in MDA-MB-231 human breast cancer cells are not exhibited in the transfectants overexpressed with regucalcin full length *in vitro*. Wild-type cells (A, C and E) or transfectants (B, D and F) overexpressed with regucalcin of full length were cultured in the absence or presence of dibucaine (0.1 or 1 μM), Bay K 8644 (0.1 or 1 μM), wortmannin (0.1 or 1 μM), PD98059 (1 or 10 μM), DRB (0.1 or 1 μM), or gemcitabine (50 or 100 nM) for 3 days. After culture, the number of attached cells on the dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *P<0.001 vs. control (none; white bar). One way ANOVA, Tukey-Kramer post-test. Wild, wild-type cells.

MDA-MB-231 cells was suppressed by culture with DRB (0.1 or 1 μM) or gemcitabine (50 or 100 nM) (Fig. 4E). However, the suppressive effects of DRB, but not gemcitabine, were not potentiated in transfectants (Fig. 4F).

Overexpression of regucalcin protects cell death in MDA-MB-231 cells. To determine the effects of the overexpression of regucalcin on cell death in MDA-MB-231 cells, the cells were cultured for 5 days to reach subconfluency. Subconfluent cells were cultured for an additional 24 h. Number of wild-type cells was decreased in the presence of LPS (0.1 or 1 μg/ml) or TNF-α (0.1 or 1 ng/ml), which is known to induce apoptotic cell death (30) (Fig. 5A). Such effects were not exhibited in transfectants overexpressing regucalcin full

length (Fig. 5B). In addition, stimulatory effects of LPS (0.1 or 1 μg/ml) or TNF-α (0.1 or 1 ng/ml) on apoptotic cell death were exhibited in MDA-MB-231 cells transfected with the regucalcin cDNA deleted with the exon 4 or with the exons 4 and 5 (Fig. 5C and D). Thus, overexpression of regucalcin with full length was found to specifically protect cell death induced by LPS or TNF-α in MDA-MB-231 cells.

To determine whether the preventive effects of regucalcin on cell death are involved in caspase-3, MDA-MB-231 wild-type cells and transfectants (with full length of regucalcin) were cultured for 5 days to subconfluency, and then the cells were additionally cultured in the presence of LPS (1 μg/ml) or Bay K 8644 (1 μM) with or without caspase-3 inhibitors (10 μM) for 24 h (Fig. 5E and F). Stimulatory effects of LPS or Bay K
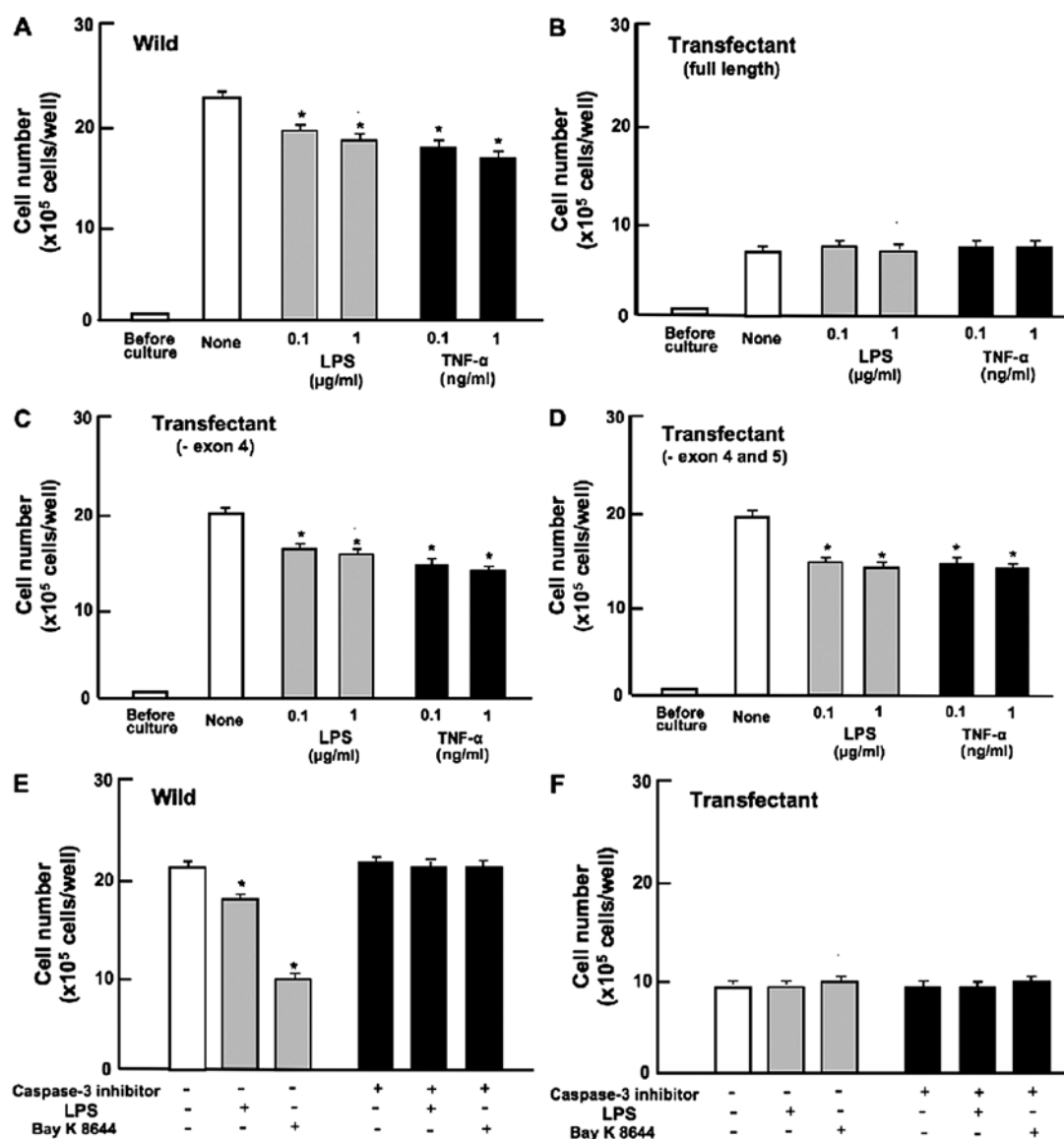
Figure 5. Overexpression of regucalcin prevents cell death induced by stimulation of various factors in MDA-MB-231 human breast cancer cells *in vitro*. Wild-type cells (A) or transfectants overexpressed with regucalcin of full length (B), deleted exon 4 (C) or deleted exons 4 and 5 (D) were cultured for 5 days when reached to confluence, and then the cells were cultured for an additional 24 h in the presence of LPS (0.1 or 1 µg/ml) or TNF-α (0.1 or 1 ng/ml). Likewise, wild-type cells (E) or transfectants (F) overexpressed with regucalcin of full length were cultured for 5 days when reached to confluent, and then the cells were cultured for an additional 24 h in the presence of LPS (1 µM) or Bay K 8644 (1 µM) with or without caspase-3 inhibitor (10 µM). After culture, the number of attached cells on the dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. $^*P<0.001$ vs. control (none; white bar). One way ANOVA, Tukey-Kramer post-test. Wild, wild-type cells.

8644 on cell death were completely prevented in the presence of caspase-3 inhibitor (Fig. 5E). LPS- or Bay K 8644-induced cell death was not seen in transfectants in the presence or absence of caspase-3 inhibitor (Fig. 5F). Thus, overexpression of regucalcin prevents cell death due to decreasing the activity of caspase-3 that activates nuclear DNA fragmentation, which induces apoptosis of cells.

Changes in various protein levels related to cell signaling. It was examined whether overexpression of regucalcin regulates protein levels related to cell signalings in MDA-MB-231 cells *in vitro* using westren blot analysis (Fig. 6). Protein levels of Akt, phospho-Akt, MAPK, phospho-MAPK, SAPK/JNK, and phospho-SAPK/JNK were decreased by overexpression of regucalcin (Fig. 6A). These results suggested that over-

expression of regucalcin suppresses signaling pathways that are related to activation of EGFR in MDA-MB-231 cells. Moreover, overexpression of regucalcin increased protein level of p53, a tumor suppressor protein, and it decreased K-ras, c-fos and c-jun, an oncogene, in MDA-MB-231 cells (Fig. 6B). Notably, overexpression of regucalcin was found to decrease protein levels of β-catenin, a transcription factor related to Wnt signaling, and p65 related to NF-κB signaling (Fig. 6B). In addition, overexpression of regucalcin decreased protein levels of caspase-3 and cleaved caspase-3 (Fig. 6C).

Overexpression of regucalcin suppresses the differentiation of bone marrow cells co-cultured with MDA-MB-231 cells. To determine an involvement of regucalcin in the bone metastasis of MDA-MB-231 cells, we examined changes in mineraliza-
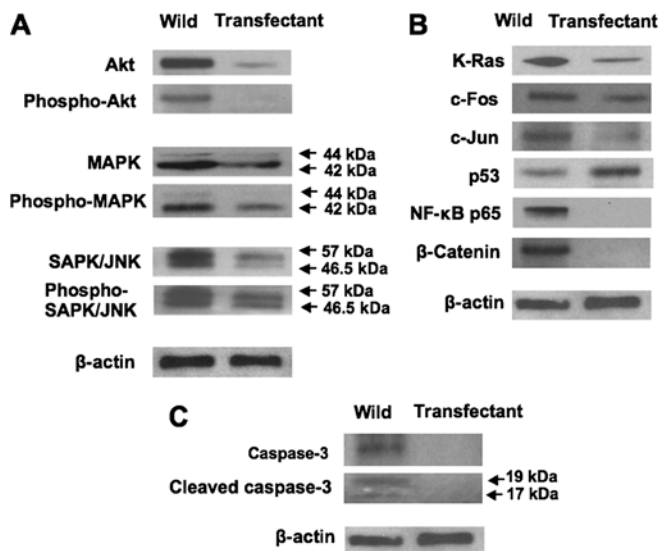
Figure 6. Overexpression of regucalcin regulates various protein levels related to signal transduction in MDA-MB-231 human breast cancer cells *in vitro*. Wild-type cells or transfectants overexpressed with regucalcin of full length were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin for 3 days. After culture, cells were removed from the dish with a cell scraper with cell lysis buffer containing protein inhibitors. Supernatant (30 μg) protein per lane was separated by SDS-PAGE. Data are presented as data set using the cell preparation obtained from different dishes with replicates. (A) Protein levels related to Akt/MAPK signaling. (B) Protein levels related to transcription factors. (C) Protein levels of caspase-3. Wild, wild-type cells.
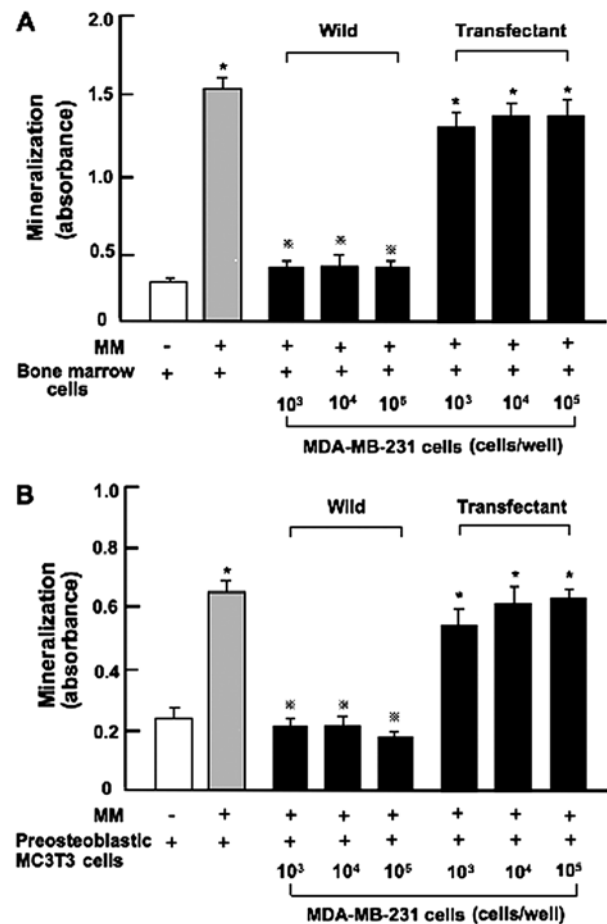

Figure 7. Overexpression of regucalcin prevents osteoblastic mineralization suppressed by coculture with mouse bone marrow cells or preosteoblastic MC3T3 cells in the presence of MDA-MB-231 human breast cancer cells *in vitro*. (A) Bone marrow cells or (B) preosteoblastic MC3-T3 cells were cocultured with or without addition of MDA-MB-231 wild-type cells or transfectant overexpressed with regucalcin full length in the presence of mineralization medium (MM). After culture, cells were stained with Alizarin red to determine mineralization. For quantitation, 10% cetylpyridinium chloride solution was added to each well to elute the dye. After complete elution, the absorbance at 570 nm on a microtiter plate reader for the eluted solution was measured. Data are presented as mean ± SD of 2 replicate wells per data set. *P<0.001 relative to control (white bar). One way ANOVA, Tukey-Kramer post test. Wild, wild-type cells.

tions in bone marrow osteoblasts or of the osteoblastic cell line MC3T3 co-cultured with MDA-MB-231 cells *in vitro* (Fig. 7). Bone marrow cells were cultured in the presence or absence of mineralization medium (MM) (Fig. 7A). After 3 days, bone marrow cells were co-cultured with addition of MDA-MB-231 cells (wild-type) or transfectants for 18 days that revealed mineralization. Mineralization in bone marrow cells was suppressed by co-culture with MDA-MB-231 cells. This suppression was prevented in the presence of transfectants (Fig. 7A). Next, preosteoblastic MC3T3 cells were cultured for 3 days, and then the cells were co-cultured with addition of MDA-MB-231 cells (wild-type) or transfectants in medium containing MM for additional 18 days *in vitro* (Fig. 7B). Co-culture with MDA-MB-231 cells suppressed mineralization in preosteoblastic MC3T3 cells. This suppresstion was not exhibited in the case of transfectants (Fig. 7B).

Moreover, we examined the effects of overexpression of regucalcin on osteoclastogenesis *in vitro* (Fig. 8). Mouse bone marrow cells were co-cultured in the presence or absence of MDA-MB-231 cells (wild-type) or transfectants overexpressed with regucalcin of full length for 7 days (Fig. 8A). Osteoclastogenesis in bone marrow cells was markedly enhanced with MDA-MB-231 cells (wild-type). However, such an effect was not seen in the case of transfectants (Fig. 8A). Next, bone marrow cells were cultured for 3 days, and then MDA-MB-231 cells (wild-type) or transfectants were seeded on bone marrow cells, and those cells were cultured for additional 4 days (Fig. 8B). Overexpression of regucalcin markedly suppressed osteoclastogenesis enhanced by co-culture with MDA-MB-231 cells (Fig. 8B).

Discussion

The present study demonstrates that relapse-free survival was prolonged in the breast cancer patients with increased regucalcin gene expression, and that overexpression of regucalcin with full length (33 kDa) suppresses the proliferation and bone cell effect in culture of MDA-MB-231 human breast cancer cells *in vitro* model. These findings may support the view that regucalcin is involved as a suppressive factor in human breast cancer.

Alternatively spliced variants with the deleted exon 4 (25 kDa) and deleted exons 4 and 5 (20 kDa) of the regucalcin cDNA have been shown to be present in various types of human cells and tissues, although their protein levels were extremely low (21). MDA-MB-231 cells transfected with these cDNA vectors did not exhibit significant suppressive effects on the proliferation and apoptotic cell death. In addition,
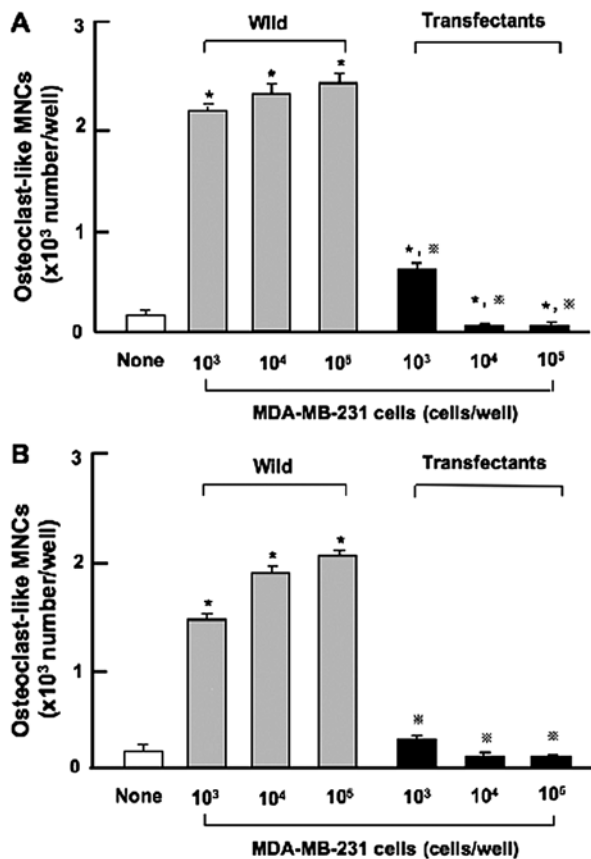
Figure 8. Overexpression of regucalcin suppresses osteoclastogenesis enhanced by co-culture with mouse bone marrow cells and MDA-MB-231 human breast cancer cells *in vitro*. Bone marrow cells were co-cultured for 7 (A) or 4 days (B) after addition of MDA-MB-231 wild-type cells (A) or transfectant overexpressed with regucalcin of full length (B). After culture, TRAP was stained. TRAP+ multinucleated cells (3 or more nuclei) were quantitated and averaged for 8 independent wells for each data point. Data are presented as mean ± SD of 2 replicate wells per data set. *P<0.001 relative to control (white bar). One way ANOVA, Tukey-Kramer post test. Wild, wild-type cells.

the proteins, which corresponded to these variants, were not expressed in MDA-MB-231 cells transfected with regucalcin cDNA of above variants. Thus, overexpression of regucalcin with full length was found to specifically exhibit suppressive effects on the proliferation and death in MDA-MB-231 cells *in vitro*.

Suppressive effects of regucalcin overexpression on the proliferation of MDA-MB-231 cells were not exhibited in the presence of butyrate, roscovitine or sulphoraphan that induce cell cycle arrest. Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m and cdk5 (33). Sulforaphane induces G2/M phase cell cycle arrest (34). Butyrate induces inhibition of G1 progression (28). Regucalcin was suggested to induce G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells.

To determine the mechanistic characterization of the suppressive effects of regucalcin on cell proliferation, we used various inhibitors that regulate intracellular signaling processes. Suppressive effects of regucalcin overexpression on the proliferation in MDA-MB-231 cells were not potentiated in the presence of TNF-α, an enhancer of NF-κB signaling (40), Bay K 8644, an agonist of Ca²⁺ entry in cells (35), PD98059,

an inhibitor of ERK/mitogen-activated protein (MAP) kinase signaling pathway (36,37), and wortmannin, an inhibitor of PI3/Akt signaling pathway (36), which suppressed the proliferation of wild-type cells. Regucalcin may exhibit suppressive effects on the proliferation by inhibiting various intracellular signaling pathways in MDA-MB-231 cells. We confirmed that the protein levels of Akt, phospho-Akt, MAPK, phospho-MAPK, SAPK/JNK and phospho-SAPK/JNK were decreased by overexpression of regucalcin. Thus, regucalcin may suppress signaling pathways related to EGFR in breast cancer cells. Moreover, overexpression of regucalcin suppressed protein levels of β-catenin and NF-κB p65, which are transcription factors related to cell signaling. These proteins are known to constitutively expressed in breast cancer cells (8). Regucalcin may reveal suppressive effects on transcription activity related to β-catenin and NF-κB signalings.

Moreover, suppressive effects of regucalcin overexpression on cell proliferation were not potentiated in the presence of DRB, an inhibitor of transcriptional activity with RNA polymerase II inhibition (38). Regucalcin has been shown to suppress transcriptional activity in the nucleus of MDA-MB-231 cells (15). Thus molecular mechanism showed similarity to the action of regucalcin in cloned normal rat kidney proximal epithelial cells and cloned rat hepatoma H4-II-E cells *in vitro* (28,29). Suppressive effects of regucalcin on the proliferation were independent on the death in MDA-MB-231 cells, since regucalcin prevents cell death induced by various stimulatory factors.

Gemcitabine is an antitumor agent that induces nuclear DNA damage (39). This agent is known to suppress cell proliferation and stimulate apoptotic cell death in various types of cancer cells (39). Suppressive effects of regucalcin overexpression on the proliferation were furthermore suppressed in the presence of gemcitabine in MDA-MB-231 cells, suggesting that regucalcin partly acts via different pathways in the action mode of gemcitabine.

Overexpression of regucalcin has been shown to prevent apoptotic cell death induced by various stimulatory factors including TNF-α, LPS, thapasigargin, and Bay K 8644 in cloned normal rat kidney proximal epithelial cells and cloned rat hepatoma H4-II-E cells *in vitro* (29,30). Overexpression of regucalcin was found to suppress death induced by various stimulatory factors in MDA-MB-231 cells *in vitro*. This effect was not exhibited in the presence of caspase-3 inhibitor. In addition, overexpression of regucalcin decreased protein levels of caspase-3 and cleaved caspase-3. Regucalcin may prevent cell death through the mechanism by which it decreases the activity of caspase-3 that activates nuclear DNA fragmentation and induces apoptosis. Regucalcin may directly inhibit caspase-3 activity. Regucalcin has also been shown to directly inhibit calcium-activated endonuclease in rat liver nucleus *in vitro* (41).

Bone marrow mesenchymal stem cells are multipotent cells, which among other cell lineages give rise to adipocytes and osteoblasts (42,43). This occurs through cross talk between complex signaling pathways including those derived from bone morphogenic proteins, winglesstype MMTV integration site (Wnt) proteins, hedgehogs, delta/jagged proteins, transcriptional regulators including peroxisome proliferators-activated receptor-gamma (PPARγ) and runt-related

transcription factor 2 (Runx2) and MAPK/ERK signaling pathway (42-45). We determined whether overexpression of regucalcin exhibits suppressive effects on bone metastasis activity of MDA-MB-231 cells using co-culture system with bone marrow cells *in vitro*. This *in vitro* model may be a useful tool in estimation of bone metastasis activity *in vitro* (31). Osteoblastic mineralization in mouse bone marrow cells was markedly suppressed after co-culture with MDA-MB-231 cells *in vitro*. Such an effect was also observed in the case of preosteoblastic MC3T3 cells *in vitro*. Thus, MDA-MB-231 cells were confirmed to directly suppress osteoblastic mineralization *in vitro*. TNF-α, which is produced in breast cancer cells (3,6,7), suppresses osteoblastic mineralization that is mediated through activation of NF-κB signaling (40). MDA-MB-231 cell-induced suppression of osteoblastic mineralization may be partly related to TNF-α, which is produced by breast cancer cells. Moreover, overexpression of regucalcin was found to prevent the suppression of osteoblastic mineralization in bone marrow cells and preosteoblastic MC3T3 cells, which were induced by co-culture with MDA-MB-231 cells. Regucalcin may prevent suppression of osteoblastic mineralization induced by TNF-α in preosteoblastic MC3T3 through suppressing of TNF-α-induced activation NF-κB signaling in preosteoblastic MC3T3 *in vitro*.

Osteoclasts are differentiated from hematopoietic precursors of the monocyte/macrophage lineage by stimulation with a TNF family cytokine, RANKL and macrophage-colony stimulating factor (46). Osteoclastogenesis in mouse bone marrow culture in the absence of bone resorbing-factors was enhanced by co-culture with MDA-MB-231 cells *in vitro*. Breast cancer cells are known to produce RANKL, which plays a pivotal role in formation from preosteoclastic cells to mature osteoclasts (7). Stimulatory effects of MDA-MB-231 cells on osteoclastogenesis in bone marrow culture may be due to RANKL, which may be produced in breast cancer cells. Overexpression of regucalcin was found to suppress osteoclastogenesis in bone marrow cell culture enhanced by co-culture with MDA-MB-231 cells *in vitro*. This suppressive effect may be related by antagonizing activation of NF-κB signaling induced by RANKL. Overexpressed regucalcin may suppress the activation of NF-κB signaling process in MDA-MB-231 cells *in vitro*.

Importantly, overexpression of regucalcin was found to decrease protein levels of β-catenin, a transcription factor related to Wnt signaling, and p65 involved in NF-κB signaling in MDA-MB-231 cells (47,48). Regucalcin may exhibit potential suppressive effects on signaling process related to β-catenin and NF-κB that are transcription factors in MDA-MB-231 cells. Such effects of regucalcin may be related to suppression of metastatic bone activity in MDA-MB-231 breast cancer cells, although further mechanism remains to be elucidated.

Regucalcin gene expression has been shown to be depressed in human breast cancer tissues as compared with that in normal tissues (20). This suggests that downregulated regucalcin gene expression is involved in carcinogenesis of breast cells and that its cell function is disordered. Our findings support the view that suppressed regucalcin gene expression may lead to disturbance of the functions of breast cells and development to carcinogenesis, since regucalcin plays a pivotal role as a suppressor protein in intracellular signaling processes

in various types of cells and tissues (13-17). Overexpression of regucalcin may play a potential role in the prevention and therapy of breast cancer.

In conclusion, the present study demonstrates that the relapse-free survival is prolonged in the breast cancer patients with increased regucalcin gene expression, and that overexpression of regucalcin exhibits anti-proliferation and anti-bone metastatic activity in MDA-MB-231 human breast cancer bone metastatic cells *in vitro*. Overexpression of the regucalcin gene may be a new useful tool in the prevention and therapy in breast cancer bone metastasis *in vivo*.

References

1. Boyce BF, Yoneda T and Guise TA: Factors regulating the growth of metastatic cancer in bone. Endocr Relat Cancer 6: 333-347, 1999.
2. Mundy GR: Metastasis to bone: Causes, consequences and therapeutic opportunities. Nat Rev Cancer 2: 584-593, 2002.
3. Roodman GD: Mechanisms of bone metastasis. N Engl J Med 350: 1655-1664, 2004.
4. Akhtari M, Mansuri J, Newman KA, Guise TM and Seth P: Biology of breast cancer bone metastasis. Cancer Biol Ther 7: 3-9, 2008.
5. Coleman RE: Metastatic bone disease: Clinical features, pathophysiology and treatment strategies. Cancer Treat Rev 27: 165-176, 2001.
6. Chen YC, Sosnoski DM and Mastro AM: Breast cancer metastasis to the bone: Mechanisms of bone loss. Breast Cancer Res 12: 215, 2010.
7. Gonzalez-Suarez E, Jacob AP, Jones J, Miller R, Roudier-MeyerMP, Erwert R, Pinkas J, Branstetter D and Dougall WC: RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. Nature 468: 103-107, 2010.
8. Park BK, Zhang H, Zeng Q, Dai J, Keller ET, Giordano T, Gu K, Shah V, Pei L, Zarbo RJ, *et al*: NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat Med 13: 62-69, 2007.
9. Weilbaecher KN, Guise TA and McCauley LK: Cancer to bone: A fatal attraction. Nat Rev Cancer 11: 411-425, 2011.
10. Shimokawa N and Yamaguchi M: Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett 327: 251-255, 1993.
11. Shimokawa N, Matsuda Y and Yamaguchi M: Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151: 157-163, 1995.
12. Thiselton DL, McDowall J, Brandau O, Ramser J, d'Esposito F, Bhattacharya SS, Ross MT, Hardcastle AJ and Meindl A: An integrated, functionally annotated gene map of the DXS8026-ELK1 interval on human Xp11.3-Xp11.23: Potential hotspot for neurogenetic disorders. Genomics 79: 560-572, 2002.
13. Yamaguchi M: Role of regucalcin in maintaining cell homeostasis and function (Review). Int J Mol Med 15: 371-389, 2005.
14. Yamaguchi M: Regucalcin and cell regulation: Role as a suppressor in cell signaling. Mol Cell Biochem 353: 101-137, 2011.
15. Yamaguchi M: The transcriptional regulation of regucalcin gene expression. Mol Cell Biochem 346: 147-171, 2011.
16. Yamaguchi M: Role of regucalcin in cell nuclear regulation: Involvement as a transcription factor. Cell Tissue Res 354: 331-341, 2013.
17. Yamaguchi M: Suppressive role of regucalcin in liver cell proliferation: Involvement in carcinogenesis. Cell Prolif 46: 243-253, 2013.
18. Yamaguchi M: The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis 18: 1145-1153, 2013.
19. Yamaguchi M: Involvement of regucalcin as a suppressor protein in human carcinogenesis: Insight into the gene therapy. J Cancer Res Clin Oncol 141: 1333-1341, 2015.
20. Maia C, Santos C, Schmitt F and Socorro S: Regucalcin is underexpressed in human breast and prostate cancers: Effect of sex steroid hormones. J Cell Biochem 107: 667-676, 2009.
21. Murata T and Yamaguchi M: Alternatively spliced variants of the regucalcin gene in various human normal and tumor tissues. Int J Mol Med 34: 1141-1146, 2014.

22. Yamaguchi M, Osuka S, Weitzmann MN, El-Rayes BF, Shoji M and Murata T: Prolonged survival in pancreatic cancer patients with increased regucalcin gene expression: Overexpression of regucalcin suppresses the proliferation in human pancreatic cancer MIA PaCa-2 cells *in vitro*. Int J Oncol 48: 1955-1964, 2016.

23. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, Ellis P, Harris A, Bergh J, Foekens JA, *et al*: Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol 25: 1239-1246, 2007.

24. Loi S, Haibe-Kains B, Desmedt C, Wirapati P, Lallemand F, Tutt AM, Gillet C, Ellis P, Ryder K, Reid JF, *et al*: Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. BMC Genomics 9: 239, 2008.

25. Loi S, Haibe-Kains B, Majjaj S, Lallemand F, Durbecq V, Larsimont D, Gonzalez-Angulo AM, Pusztai L, Symmans WF, Bardelli A, *et al*: PIK3CA mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. Proc Natl Acad Sci USA 107: 10208-10213, 2010.

26. Yoneda T, Williams PJ, Hiraga T, Niewolna M and Nishimura R: A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res 16: 1486-1495, 2001.

27. Misawa H, Inagaki S and Yamaguchi M: Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84: 143-149, 2001.

28. Yamaguchi M and Daimon Y: Overexpression of regucalcin suppresses cell proliferation in cloned rat hepatoma H4-II-E cells: Involvement of intracellular signaling factors and cell cycle-related genes. J Cell Biochem 95: 1169-1177, 2005.

29. Nakagawa T, Sawada N and Yamaguchi M: Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 16: 637-643, 2005.

30. Izumi T and Yamaguchi M: Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. J Cell Biochem 92: 296-306, 2004.

31. Yamaguchi M, Zhu S, Weitzmann MN, Snyder JP and Shoji M: Curcumin analog UBS109 prevents bone marrow osteoblasto-genesis and osteoclastogenesis disordered by co-culture with breast cancer MDA-MB-231 bone metastatic cells in vitro. Mol Cell Biochem 401: 1-10, 2015.

32. Minkin C: Bone acid phosphatase: Tartrate-resistant acid phos-phatase as a marker osteoclast function. Calcif Tissue Int 34: 285-290, 1982.

33. Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG and Moulinoux JP: Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 243: 527-536, 1997.

34. Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, Kamath R, Brown KD, Zhang L and Baskaran R: Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J Biol Chem 279: 25813-25822, 2004.

35. Cano-Abad MF, Villarroya M, García AG, Gabilan NH and López MG: Calcium entry through L-type calcium channels causes mitochondrial disruption and chromaffin cell death. J Biol Chem 276: 39695-39704, 2001.

36. Serrano-Nascimento C, da Silva Teixeira S, Nicola JP, Nachbar RT, Masini-Repiso AM and Nunes MT: The acute inhibitory effect of iodide excess on sodium/iodide symporter expression and activity involves the PI3K/Akt signaling pathway. Endocrinology 155: 1145-1156, 2014.

37. Chen QW, Edvinsson L and Xu CB: Role of ERK/MAPK in endothelin receptor signaling in human aortic smooth muscle cells. BMC Cell Biol 10: 52, 2009.

38. Palangat M, Grass JA, Langelier MF, Coulombe B and Landick R: The RPB2 flap loop of human RNA polymerase II is dispensable for transcription initiation and elongation. Mol Cell Biol 31: 3312-3325, 2011.

39. Tang SC and Chen YC: Novel therapeutic targets for pancreatic cancer. World J Gastroenterol 20: 10825-10844, 2014.

40. Li Y, Li A, Strait K, Zhang H, Nanes MS and Weitzmann MN: Endogenous TNFalpha lowers maximum peak bone mass and inhibits osteoblastic Smad activation through NF-kappaB. J Bone Miner Res 22: 646-655, 2007.

41. Yamaguchi M and Sakurai T: Inhibitory effect of calcium-binding protein regucalcin on Ca^{2+}-activated DNA fragmentation in rat liver nuclei. FEBS Lett 279: 281-284, 1991.

42. Gharibi B, Abraham AA, Ham J and Evans BA: Adenosine receptor subtype expression and activation influence the differen-tiation of mesenchymal stem cells to osteoblasts and adipocytes. J Bone Miner Res 26: 2112-2124, 2011.

43. Muruganandan S, Roman AA and Sinal CJ: Adipocyte differen-tiation of bone marrow-derived mesenchymal stem cells: Cross talk with the osteoblastogenic program. Cell Mol Life Sci 66: 236-253, 2009.

44. Wu L, Cai X, Dong H, Jing W, Huang Y, Yang X, Wu Y and Lin Y: Serum regulates adipogenesis of mesenchymal stem cells via MEK/ERK-dependent PPARgamma expression and phos-phorylation. J Cell Mol Med 14: 922-932, 2010.

45. Laudes M: Role of WNT signalling in the determination of human mesenchymal stem cells into preadipocytes. J Mol Endocrinol 46: R65-R72, 2011.

46. Zaidi M, Blair HC, Moonga BS, Abe E and Huang CL: Osteoclastogenesis, bone resorption, and osteoclast-based thera-peutics. J Bone Miner Res 18: 599-609, 2003.

47. Xu J, Prosperi JR, Choudhury N, Olopade OI and Goss KH: β-Catenin is required for the tumorigenic behavior of triple-negative breast cancer cells. PLoS One 10: e0117097, 2015.

48. Li L, Zhao F, Lu J, Li T, Yang H, Wu C and Liu Y: Notch-1 signaling promotes the malignant features of human breast cancer through NF-κB activation. PLoS One 9: e95912, 2014.