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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

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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 E2, 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).
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. Gemcycbine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Antibodies for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gemcycbine 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 EcoRI site of pCMXN2 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 at ~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, gemiccitcin (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% CO₂ 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% CO2 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), wortmannin (0.1 or 1 µM), DRB (0.1 or 1 µM), or gencitabine (50 or 100 nM) for 3 days. After culture, the cells were detached with trypsin from each culture dish 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 ng/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 confluence, 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^++/Mg^++-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 1x10^5 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 (1x10^5 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% CO2 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 (1x10^5 cells/1 ml/well) or transfectant (1x10^5 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 (1x10^5 cells/1 ml/well) or transfectants (1x10^5 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 transfected with the pCXN2 vector or regucalcin/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 overexpression 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 transfected 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-
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 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.
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 subconfluence. 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
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 western 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 overexpression 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).

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-
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 suppression 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 apoptotic cell death. In addition, 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 apoptotic cell death.
Bay K 8644, an agonist of Ca\(^{2+}\) 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, winglestype MMTV integration site (Wnt) proteins, hedgehogs, delta/jagged proteins, transcriptional regulators including peroxisome proliferators-activated receptor-gamma (PPARγ) and runt-related 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 Ca\(^{2+}\) entry in cells (35), PD98059, an inhibitor of MEK/ERK signaling pathway (36), 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.

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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-kB 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-kB 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-kB signaling induced by RANKL. Overexpressed regucalcin may suppress the activation of NF-kB 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-kB signaling in MDA-MB-231 cells (47,48). Regucalcin may exhibit potential suppressive effects on signaling process related to β-catenin and NF-kB 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
