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Exogenous regucalcin suppresses the proliferation of human breast cancer MDA-MB-231 bone metastatic cells *in vitro*

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Abstract. Regucalcin serves a pivotal role as a suppressor protein in signal transduction in various types of cells and tissues. The regucalcin gene, which is localized on the X chromosome, consists of seven exons and six introns. Reductions in the gene expression of regucalcin have been suggested to serve a role in hepatocarcinogenesis in animal models and human patients, indicating a potential role as a suppressor protein in cancer. The aim of the current study was to investigate the effect of exogenous regucalcin on cell proliferation in the cloned human breast cancer MDA-MB-231 bone metastatic cell line *in vitro*. The proliferation of MDA-MB-231 cells was suppressed following the addition of regucalcin (0.1-10 nM) *in vitro*. The suppression of proliferation was not enhanced in the presence of tumor necrosis factor-α, PD98059, staurosporine, Bay K8644, wortmannin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole or gemcitabine. Exogenous regucalcin did not induce cell death in MDA-MB-231 cells *in vitro*. These data suggest that exogenous regucalcin possesses suppressive effects on the proliferation of human breast cancer MDA-MB-231 bone metastatic cells, and that this effect may be mediated through various intracellular signaling pathways *in vitro*. Exogenous regucalcin is suggested to function as a suppressor in cancer cell proliferation.

Introduction

Regucalcin is a calcium-binding protein which has been demonstrated to serve a multifunctional role in the regulation of various types of cells and tissues (1-6). The regucalcin gene (*rgn*)), which is located on the X chromosome, is identified in over 15 species consisting of the regucalcin family, and is highly conserved in vertebrate species throughout evolution (7-11). The expression of the regucalcin gene is regulated by various transcription factors including activator protein 1, nuclear factor I-A1, regucalcin gene promoter region-related protein and β-catenin, which are modulated through intracellular signaling factors associated with the phosphorylation and dephosphorylation of nuclear proteins *in vitro* (11). Regucalcin is expressed in the liver, kidney and various other tissues and is regulated by hormonal factors including calcium-regulating hormones, insulin, estrogen and additional steroid hormones (11,12). Regucalcin is translocated from the cytoplasm to the nucleus in various types of cells (13). Regucalcin has been demonstrated to serve a role in the maintenance of intracellular calcium homeostasis, and it inhibits various protein kinases and phosphatases, in addition to inhibiting protein, DNA and RNA syntheses (3-5,13). Additionally, nuclear regucalcin has been demonstrated to regulate the gene expression of various proteins (13). Furthermore, regucalcin suppresses cell proliferation and apoptotic cell death mediated through various signaling factors in normal kidney NRK52E cells and cloned rat hepatoma H4II-E cells *in vitro* (14,15). Regucalcin has been proposed to serve a physiological role in maintaining cellular homeostasis and function as a regulatory protein of intracellular signaling systems (5,6).

Regucalcin has been demonstrated to possess a pathophysiological role in metabolic disorders (16-18). In addition, regucalcin has been demonstrated to be involved in carcinogenesis (19). The expression of the regucalcin gene and protein have been demonstrated to be reduced in the tumor tissues of animal models and human patients *in vivo* (19,20). Regucalcin gene expression has been demonstrated to be downregulated in carcinogenesis, suggesting a potential role of regucalcin as a suppressor protein in carcinogenesis (19). Overexpression of endogenous regucalcin has been previously demonstrated to suppress the enhancement of cell proliferation in cloned rat hepatoma H4-II-E cells *in vitro* (21).

The aim of the current study was to investigate whether exogenous regucalcin possesses a suppressive effect on the proliferation of human cancer cells *in vitro*.

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; P/S) were purchased

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from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Logan, UT, USA). Gemcitabine was obtained from Hospira, Inc. (Lake Forest, IL, USA), and diluted in Dulbecco's modified phosphate-buffered saline (PBS). Tumor necrosis factor-α (TNF-α) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). PD98059, staurosporine, Bay K8644, wortmannin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and all additional reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Regucalcin. Regucalcin was isolated from rat liver cytosol as described previously (1). The livers were perfused with Tris-HCl buffer (pH 7.4), containing 100 mM Tris, 120 mM NaCl and 4 mM KCl, and cooled to 4°C. The livers were subsequently removed, cut into small pieces, suspended 1:4 (w/v) in Tris-HCl buffer (pH 7.4), and homogenized in a Potter-Elvehjem homogenizer (Takashima System Ltd., Tokyo, Japan) with a Teflon pestle (1). The homogenate was spun at 5,500 x g in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000 x g for 60 min at 4°C. The resulting supernatant was purified to electrophoretic homogeneity by gel filtration using Sephadex G-75 and G-50, followed by ion-exchange chromatography on diethylamino-ethyl-cellulose (4). The purity of the isolated regucalcin was analyzed using gel electrophoresis and western blot analysis, which confirmed that it did not contain other proteins.

Breast cancer MDA-MB-231-bone metastatic cells. Breast cancer MDA-MB-231 bone metastatic cells lack estrogen, progesterone and human epidermal growth factor type 2 receptors, and are therefore considered as triple negative (22). However, the cells express epidermal growth factor, transforming growth factor-α and Wnt7B oncogene, and activation of these receptors and its downstream signaling events enhances the migration, proliferation, invasion and progression of the malignant phenotype of these cells. The MDA-MB-231 bone metastatic cells were provided by Dr Toshi Yoneda (The University of Texas, San Antonio, TX, USA) (22).

Cell proliferation in MDA-MB-231 cells. Breast cancer MDA-MB-231 cells ($1x10^5$/ml per well) were cultured in a 24-well plate in DMEM containing 10% FBS and 1% P/S in the presence or absence of regucalcin (0.01, 0.1, 05, 1 or 10 nM) for 1, 2, 3 and 7 days. In separate experiments, MDA-MB-231 cells ($1x10^5$/ml per well) were cultured in DMEM containing 10% FBS and 1% P/S in the presence of TNF-α (1 ng/ml), Bay K8644 (1 μM), PD98059 (1 μM), staurosporine (0.1 μM), wortmannin (1 μM) or DRB (1 μM) for 3 days. Following culture, the number of cells was counted.

Cell death in MDA-MB-231 cells. Breast cancer MDA-MB-231 cells ($1x10^5$/ml per well) were cultured in a 24-well plate in DMEM containing 10% FBS and 1% P/S in the absence of regucalcin for 7 days, until cells were confluent. Subsequently, the cells were cultured in the presence or absence of regucalcin (0.1, 1 or 10 nM) with or without gemcitabine (10, 50, 100, 250, and 1,000 nM) for 7 days. Following culture, the number of cells was counted.

Cell counting. Following trypsinization of each culture dish in 0.2% trypsin with 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37°C, the detached cells were collected by centrifugation at 12 x g for 5 min at 4°C (Eppendorf 5810 R). The cells were resuspended in PBS solution and stained with eosin. Cell numbers were counted under a microscope (Olympus MTV-3; Olympus Corporation, Tokyo, Japan) using a hemocytometer (Brightline; Sigma-Aldrich). For each dish, the cells were counted twice from which the average was calculated. Cell numbers are presented as the number/well of plate.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was determined using GraphPad InStat software, version 3 (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were conducted using a one-way analysis of variance and a Tukey-Kramer multiple comparisons post-test for parametric data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The effect of exogenous regucalcin on the proliferation of breast cancer MDA-MB-231 bone metastatic cells *in vitro* is presented in Fig. 1. MDA-MB-231 cells were cultured in the presence of exogenous regucalcin (0.1-10 nM) for 1-7 days. The number of cells increased with the increasing duration of culture. The addition of exogenous regucalcin reduced the increase in cell number, indicating that cell proliferation was suppressed by the physiological concentrations of serum regucalcin (23).

The suppressive effects of regucalcin (1 nM) on cell proliferation in MDA-MB-231 cells were not enhanced in the presence of TNF-α (1 ng/ml), an enhancer of nuclear factor-κB (NF-κB) signaling (24) or Bay K8644 (1 μM), an agonist of Ca^{2+} influx in cells (25), which resulted in significantly reduced cell numbers when applied alone (Fig. 2). In addition, the effects of exogenous regucalcin in reducing cell proliferation were not enhanced in the presence of PD98059 (1 μM), a mitogen-activated protein kinase (MAPK) inhibitor (26) or staurosporine (0.1 μM), an inhibitor of protein kinase C (27), which caused a significant reduction in cell numbers (Fig. 3). Furthermore, the suppressive effects of regucalcin on cell proliferation were not enhanced in the presence of wortmannin (1 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3K) (28) or DRB (1 μM), an inhibitor of transcriptional activity via RNA polymerase II inhibition (29) (Fig. 4).

The suppressive effects of regucalcin on the proliferation of MDA-MB-231 cells were investigated in the presence of gemcitabine, an antitumor agent, which induces nuclear DNA damage (30). The addition of gemcitabine (50-500 nM) to MDA-MB-231 cultures reduced cell proliferation (Fig. 5A). This effect was not altered by the application of regucalcin (1 nM) with gemcitabine (Fig. 5B). The addition of regucalcin (1 nM) significantly reduced cell numbers in the presence of gemcitabine (10 nM).

The effect of regucalcin on cell death in breast cancer MDA-MB-231 cells is presented in Fig. 6. Cells were cultured for 7 days until confluent, and subsequently cultured for 7 days in the presence of regucalcin (0.1 or 1 nM) with or without gemcitabine (100 nM). The addition of regucalcin was not
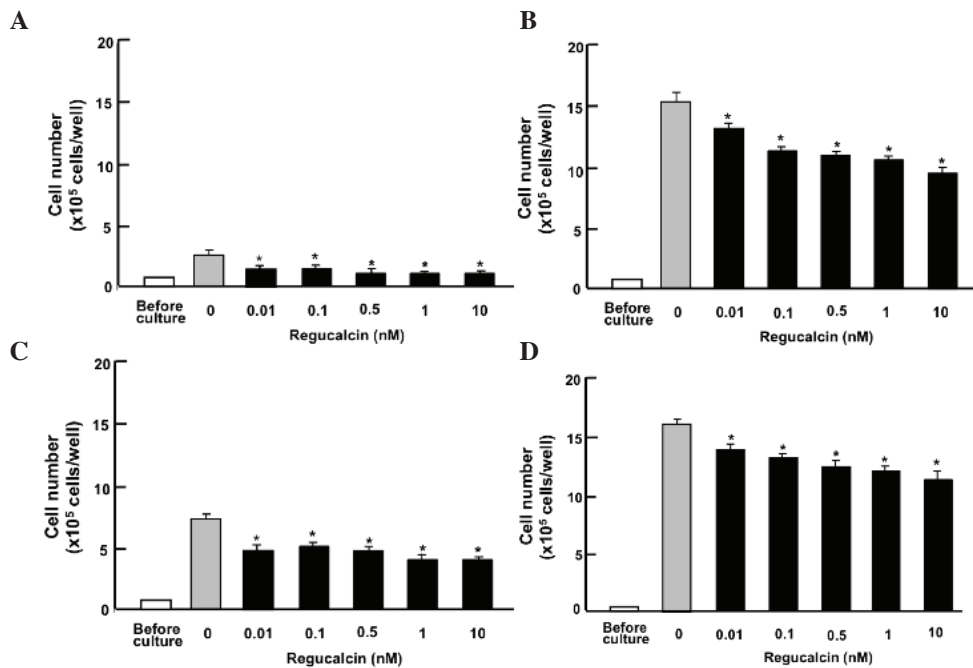
Figure 1. Exogenous regucalcin suppresses the proliferation of MDA-MB-231 cells *in vitro*. Cells were cultured in the presence or absence of regucalcin (0.1-10 nM) for (A) 1, (B) 2, (C) 3 or (D) 7 days. Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way analysis of variance and the Tukey-Kramer post-test were used.
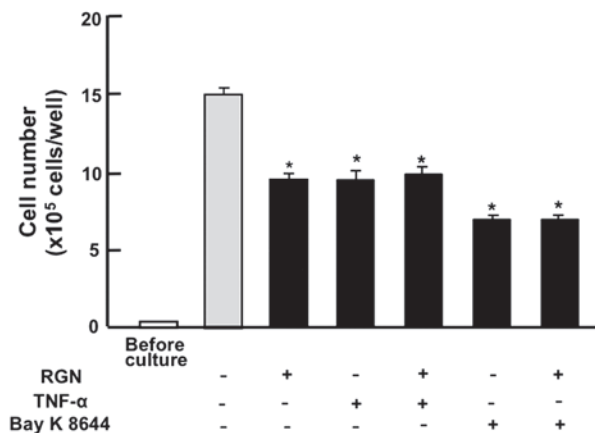

Figure 2. Suppressive effects of exogenous RGN on the proliferation of MDA-MB-231 cells in the presence of TNF-α or Bay K8644 *in vitro*. Cells were cultured for 3 days in the presence of RGN (1 nM) with or without TNF-α (1 ng/ml) or Bay K8644 (1 µM). Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way analysis of variance and the Tukey-Kramer post-test were used. RGN, regucalcin; TNF-α, tumor necrosis factor-α.
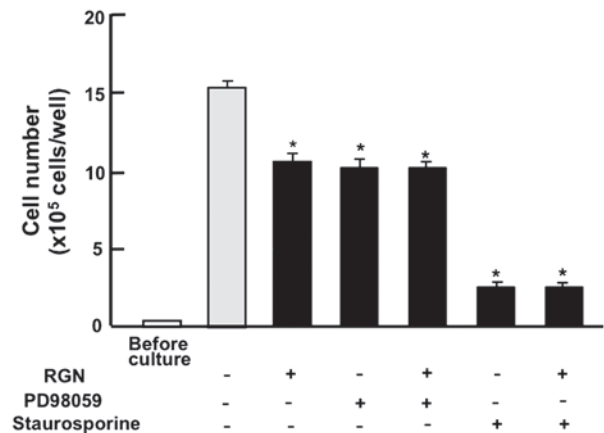

Figure 3. Suppressive effects of exogenous RGN on the proliferation of MDA-MB-231 cells in the presence of PD98059 or staurosporine *in vitro*. Cells were cultured for 3 days in the presence of RGN (1 nM) with or without PD98059 (1 µM) or staurosporine (0.1 µM). Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way analysis of variance and the Tukey-Kramer post-test were used. RGN, regucalcin.

observed to significantly affect the cell number, however culture with gemcitabine reduced cell number. Therefore, it is suggested that regucalcin does not induce cell death.

Discussion

Regucalcin has been demonstrated to serve a multifunctional role in the regulation of cell function by suppressing various signaling pathways in various types of cells and tissues (4-6). Previous studies have demonstrated that regucalcin serves a potential role as a suppressor of cell proliferation and carcinogenesis (14,18,19). Regucalcin gene expression was observed to be downregulated in the tumor tissues of human patients (20) and in human cancer cells (18,31). The current study demonstrated that exogenous regucalcin possesses suppressive effects on the proliferation of human breast cancer
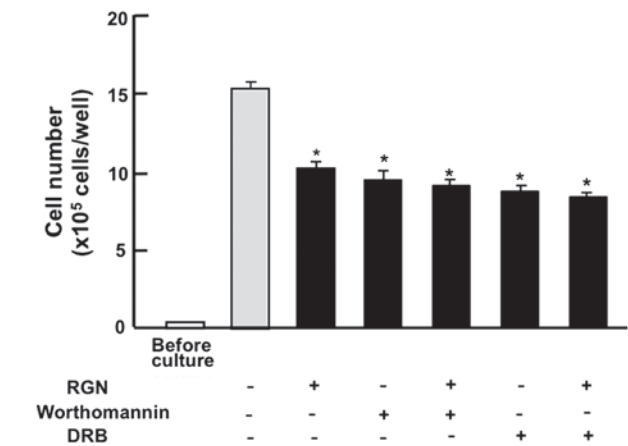
Figure 4. Suppressive effects of exogenous RGN on the proliferation of MDA-MB-231 cells in the presence of wortmannin or DRB *in vitro*. Cells were cultured for 3 days in the presence of RGN (1 nM) with or without wortmannin (1 μM) or DRB (1 μM). Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way analysis of variance and the Tukey-Kramer post-test were used. RGN, regucalcin; DRB, 5,6-dichloro-1-β-D-ribofurano sylbenzimidazole.
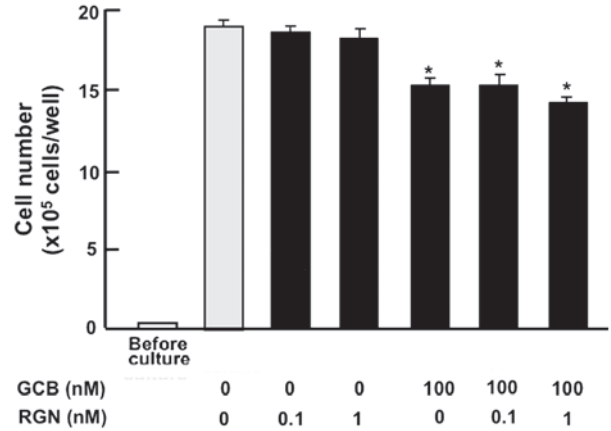

Figure 6. Effect of exogenous RGN on cell death in MDA-MB-231 cells in the presence of GCB *in vitro*. Cells were cultured for 7 days until confluent, and subsequently cultured for 7 days in the presence of RGN (0.1 or 1 nM) with or without GCB (100 nM). Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (grey bar). One way analysis of variance and the Tukey-Kramer post-test were used. RGN, regucalcin; GCB, gemcitabine.
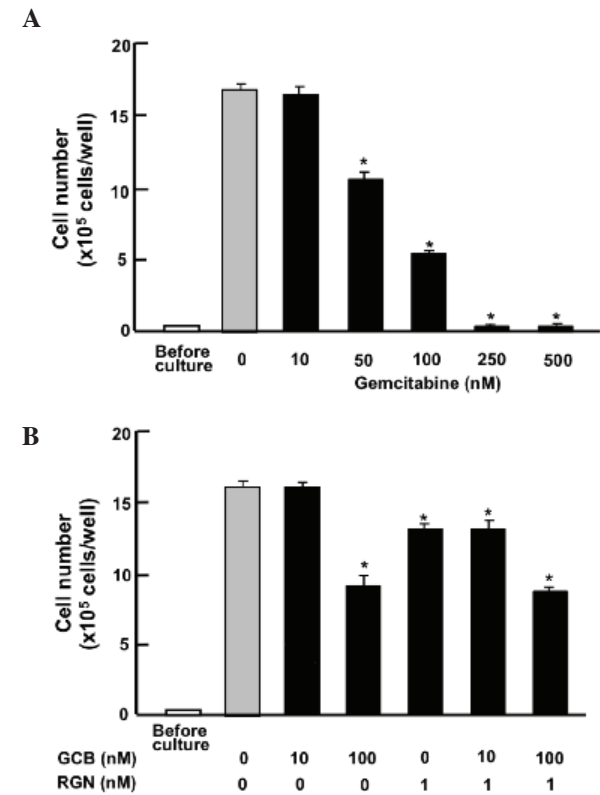

Figure 5. Suppressive effects of exogenous RGN on the proliferation of MDA-MB-231 cells in the presence of GCB *in vitro*. (A) Cells were cultured for 7 days in the presence of GCB (10-500 nM). (B) Cells were cultured for 7 days in the presence of RGN (1 nM) with or without GCB (10 or 100 nM). Following culture, the number of cells were counted. Data are presented as the mean ± standard deviation of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 vs. control (white bar). One way analysis of variance and the Tukey-Kramer post-test were used. RGN, regucalcin; GCB, gemcitabine.

MDA-MB-231 bone metastatic cells *in vitro*, however was not observed to affect cell death.

Overexpression of endogenous regucalcin has been demonstrated to suppress the proliferation of cloned rat hepatoma H4-II-E cells *in vitro* (14,18,21). Regucalcin has been demonstrated to result in G_1 and G_2/M phase cell cycle arrest in H4-II-E cells (32). In addition, overexpression of endogenous regucalcin has been demonstrated to have suppressive effects on cell proliferation inducing G_1 and G_2/M phase cell cycle arrest in cloned normal rat kidney proximal tubular epithelial NRK52E cells *in vitro* (33). The suppressive effects of endogenous regucalcin on cell proliferation may be mediated through the inhibition of various Ca^{2+} signaling-dependent protein kinases, protein phosphatases and PI3K activities and the suppression of c-Myc, H-Ras, c-Jun and chk2 mRNA expression, or the enhancement of p53 and Rb mRNA expression (14,19,34,35). Furthermore, regucalcin has been demonstrated to suppress cytoplasmic protein synthesis and nuclear DNA and RNA synthesis (13,14).

The current study demonstrated that the suppressive effects of exogenous regucalcin on the proliferation of MDA-MB-231 cells were not modulated in the presence of various inhibitors that regulate intracellular signaling pathways *in vitro*. The suppressive effects of regucalcin on the proliferation of MDA-MB-231 cells were not enhanced in the presence of: TNF-α, an enhancer of NF-κB signaling (24); Bay K8644, an agonist of Ca^{2+} entry in cells (25); PD98059, an MAPK inhibitor (26); staurosporine, an inhibitor of calcium-dependent protein kinase C (27); or wortmannin, an inhibitor of PI3K (28). These data suggest that exogenous regucalcin stimulates various intracellular signaling pathways to suppress cell proliferation in human breast cancer MDA-MB-231 cells. Regucalcin has been demonstrated to bind the plasma membranes of rat liver cells *in vitro* (36). Therefore, it may be possible that exogenous regucalcin binds to the plasma membranes of breast cancer MDA-MB-231 cells,

and potentially regulates intracellular signaling pathways that suppress cell proliferation. In addition, the suppressive effects of regucalcin on cell proliferation were not enhanced in the presence of DRB, an inhibitor of transcriptional activity via RNA polymerase II inhibition (29). The intracellular signals of exogenous regucalcin may be transmitted into the nucleus to suppress transcriptional regulation in human breast cancer MDA-MB-231 cells.

Overexpression of endogenous regucalcin has been demonstrated to have suppressive effects on apoptotic cell death in rat hepatoma H4-II-E cells and normal rat kidney NRK52-E cells, which were increased through various signaling pathways in the cytoplasm and nucleus *in vitro* (15). In the current study, exogenous regucalcin did not induce cell death in human breast cancer MDA-MB-231 cells *in vitro*, indicating that regucalcin does not stimulate cell death. This effect was not enhanced in the presence of gemcitabine, an antitumor agent, which induces nuclear DNA damage (30). This observation may support the theory that the intracellular signaling by exogenous regucalcin may be transmitted to regulate nuclear function in breast cancer cells.

In conclusion, the current study demonstrated that exogenous regucalcin possesses suppressive effects on the proliferation of human breast cancer MDA-MB-231 bone metastatic cells *in vitro*. This suggests that exogenous regucalcin serves a role as a suppressor of the proliferation of human cancer cells.

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