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Original Research

Progression-free survival of prostate cancer patients is prolonged with a higher regucalcin expression in the tumor tissues: Overexpressed regucalcin suppresses the growth and bone activity in human prostate cancer cells

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ABSTRACT

Prostate cancer, which is a bone metastatic cancer, is the second leading cause of cancer-related death in men. There is no effective treatment for metastatic prostate cancer. Regucalcin has been shown to contribute as a suppressor in various types of human cancers. In the present study, furthermore, we investigate an involvement of regucalcin in suppression of prostate cancer. Regucalcin expression was compared in 131 primary tumor tissues and 19 metastatic tumor tissues in prostate cancer patients. Regucalcin expression in the metastatic tumor was found to be reduced as compared with that in primary tumor. The progression-free survival rate was prolonged in patients with a higher regucalcin expression. Translationally, overexpression of regucalcin in bone metastatic human prostate cancer PC-3 and DU-145 cells suppressed colony formation and cell growth *in vitro*. Mechanistically, overexpressed regucalcin enhanced the levels of p*53,* Rb, and p21, and decreased the levels of Ras, PI3 kinase, Akt, and mitogen-activated protein kinase, leading to suppression of cell growth. Furthermore, higher regucalcin expression suppressed the levels of nuclear factor-κB p65, β-catenin, and signal transducer and activator of transcription 3, which regulate a transcription activity. Cell growth was promoted by culturing with the calcium agonist Bay K 8644. This effect was blocked by overexpression of regucalcin. Notably, overexpressed regucalcin suppressed bone metastatic activity of PC-3 and DU-145 cells when cocultured with preosteoblastic or preosteoclastic cells. Regucalcin may suppress the development of human prostate cancer, suggesting that gene delivery systems in which its expression is forced may be a novel therapeutic strategy.

Introduction

Prostate cancer, which is a bone metastatic cancer, is one of the most common malignancy among men in developed countries and is the second leading cause of cancer-related death in men [1–3]. While the 5-year survival rate for localized prostate cancer is close to 100%, metastatic prostate cancer has a 5-year survival rate of only 30%. Bone metastasis represents a common complication of cancer, whose incidence reaches 70–95% in multiple myeloma, up to 65–90% in prostate cancer, and about 65–75% in breast cancer [1,2,4]. Skeletal metastasis is less frequent in other malignancies, ranging from 17% to 64% in lung cancer to approximately 10% in colorectal tumors [4]. Prostate cancer preferentially metastasizes to bone, leading to complications including severe pain, fractures, spinal cord compression and bone marrow suppression [5–7]. Treatment strategies of prostate cancer include surgical removal, chemotherapy, hormonal, castration, immunotherapy, and radioisotope therapies [8–11]. There is no effective treatment for bone metastatic prostate cancer.

Regucalcin was discovered as a novel inhibitory protein of calcium signaling with calcium-binding properties [12,13]. The regucalcin gene localizes on the X chromosome [14], and its protein expresses in various types of cells and tissues [15]. The cytoplasmic regucalcin translocates into the nucleus in cells [16]. Regucalcin plays a multifunctional role in the regulation of cell functions, including maintenance of intracellular calcium homeostasis, inhibitions of protein kinases and protein phosphatases linked to cell signaling process, suppressions of protein production and nuclear DNA and RNA synthesis, regulation of nuclear gene expression, suppression of proliferation and apoptosis [16–22]. Regucalcin may play a pivotal role as a suppressor in the regulation of various signaling processes implicated in promotion of cell growth.

Notably, microarray and proteomics analysis demonstrated that the gene expression and protein levels of regucalcin were downregulated in the tumor tissues of various types of human cancer patients [23–30],

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and that prolonged survival of these cancer patients was associated with a higher regucalcin expression in their tumor tissues [25–30]. Translational studies, furthermore, found that overexpressed regucalcin repressed the growth of various types of human cancer cells, including pancreatic cancer, breast cancer, liver cancer, lung adenocarcinoma, colorectal cancer, renal cell carcinoma, cervical adenocarcinoma, and osteosarcoma *in vitro* [24–30,31,32]. Regucalcin may play a crucial role as a suppressor in human cancer. Downregulated expression of the regucalcin gene may contribute to development of malignancies. Regucalcin may be clinical significance as a novel target molecule in human cancer.

Interestingly, regucalcin has been shown to play a role in the regulation of bone metabolism [33,34], suggesting an involvement in bone metastasis of human cancer cells. Our previous study showed that overexpression of regucalcin suppressed bone metastatic activity of MDA-MB-231 human breast cancer cells [26] and the growth of human osteosarcoma Saos-2 cells [32] *in vitro*. Furthermore, the current study investigates whether or not regucalcin plays a suppressive role in bone metastatic human prostate cancer cells. Notably, microarray analysis with patient's datasets demonstrated that progression-free survival of prostate cancer patients was found to be prolonged in patients with a higher regucalcin gene expression. Moreover, overexpression of regucalcin was found to suppress the growth and bone metastatic activity of human prostate cancer cells, including PC-3 and DU-145 cells *in vitro*. Thus, targeting regucalcin expression may be clinically significant in the suppression of bone metastatic cancer. Moreover, a system for delivery of the regucalcin gene may provide a new therapeutic strategy for human cancer.

Materials and methods

Reagents

Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin; 1% P/S). were obtained from Corning (Mediatech, Inc. Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Lipofectamine reagent was from Promega (Madison, WI, USA). Amphotericin B (fungizone), Geneticin (G418), Bay K 8644, wortmannin, PD98059, staurosporin, dibucaine, caspase-3 inhibitor, lipopolysaccharide (LPS) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tumor necrosis factor-α (TNF-α) was purchased from R&D Systems (Minneapolis, MN, USA). Caspase-3 inhibitor was diluted in phosphate buffered saline (PBS) and other reagents were dissolved in 100% ethanol before use.

Patients' datasets

Microarray analysis of 131 primary tumor and 19 metastatic tumor of prostate cancer patients were performed using the published dataset [35]. The Kaplan–Meier survival analysis were performed using the same dataset. For survival analysis, the patients were separated into 2 groups with higher levels of 70 patients and lower levels of 70 patients of regucalcin expression, respectively. High/Low were defined as median value. The regucalcin expression and clinical data of the published dataset were obtained from SuryExpress [36] and cBioPortal [37].

Human prostate cancer cells

We used PC-3 and DU-145 cells obtained from the American Type Culture Collection (ATCC CRL-1435^{TM}, ATCC; Rockville, MD, USA). PC-3 cells (ATCC CRL-1435^{TM}) are epithelial cell line derived from metastatic bone site originating from a male adult patient (62 years, grade IV, adenocarcinoma). DU-145 cells (ATCC HTB-81) are epithelial cell line derived from metastatic brain site from a male adult prostate patient (69 years, adenocarcinoma). PC-3 and DU-145 cells express androgen receptors [38]. These cells were suitable as a transfection host. The cells were cultured in a DMEM containing 10% FBS, 1% P/S and 1% fungizone.

Transfection of regucalcin cDNA

PC-3 cells and DU-145 cells were transfected with the pCXN2 vector (Addgene, Inc., Cambridge, MA, USA; 600 µg/ml) that expresses cDNA encoding human full length (900 bp) regucalcin (regucalcin cDNA/pCXN2) [39]. For transient transfection assays, PC-3 or DU-145 cells (2×10^5 cells/ml per wells) were grown for 24 h in DMEM containing 10% FBS, 1%P/S and 1% fungizone on 24-well plates to approximately 70–80% confluence. After culture, the medium was changed to DMEM without FBS and antibiotics. The regucalcin cDNA/pCXN2 or empty pCXN2 vector were transfected into PC-3 and DU-145 cells using the synthetic cationic lipid, a Lipofectamine reagent, according to the manufacturer's instructions (Promega, Madison, WI, USA) [39]. This efficiency was in the range of 60–80%. After incubation for overnight, Geneticin (G418, 500 μg/ml, Sigma-Aldrich) was added to culture wells for selection, and the cells were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 weeks. Surviving cells were plated at limiting dilution to isolate transfectants. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in medium with G418 (100 μg/ml). We obtained transfectant clones 1 and 2, which are exhibiting stable expression of regucalcin. The regucalcin levels in these clones were markedly ($p<0.001$) increased as compared with that of wild-type cells, as shown in Fig. 2A and B or Fig. 3A and B. Clone 1 with higher levels of regucalcin was used in the following experiments, respectively.

Colony formation assay

PC-3 or DU-145 wild-type cells or transfectants (1×10^3/2 ml per well in 6-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone under conditions of 5% CO_2 and 37 °C for 9 days [40]. Visible clones were formed by culturing for 9 days. After culture, the colonies were washed with PBS (2 ml) and fixed with methanol (95%, 0.5 ml per well) for 20 min at room temperature. After fixation, the plates were washed 3 times with PBS (2 ml). The colonies were then stained with 0.5% crystal violet (1 ml) for 30 min at room temperature. Stained cells were washed 6 times with PBS (2 ml). The plates were air-dried for 2 h at room temperature. The colonies containing >50 cells were counted under a microscope (Olympus MTV-3; Olympus Corporation, Tokyo, Japan).

Cell growth assay

To determine alteration of cell growth, PC-3 or DU-145 wild-type cells (1×10^5/ml per well) and PC-3 cells or DU-145 cells transfected with the regucalcin cDNA (1×10^5/ml per well) were cultured using 24-well plates in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 1, 2, 3, or 5 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37 °C [39,41]. In separate experiments, PC-3 wild-type cells or transfectants were cultured for 3 days in DMEM containing 10% FBS and 1% P/S in the presence or absence of vehicle (PBS) or TNF-α (0.1 or 1 ng/ml). In other experiments, PC-3 wild-type cells or transfectants were cultured for 3 days in DMEM containing 10% FBS and 1% P/S in the presence or absence of either vehicle (1% ethanol as a final concentration) or Bay K 8644 (0.1, 1, 10, 25 or 100 nM). In separate experiments, PC-3 wild-type cells or transfectants were cultured for 3 days in DMEM containing 10% FBS and 1% P/S in the presence or absence of either vehicle (1% ethanol), wortmannin (0.1 or 1 μM), PD98059 (1 or 10 μM), staurosporine (10 or 100 nM), dibucaine (10 or 100 nM) or TNF-α (0.1 or 1 ng/ml) with or without Bay K 8644 (10 nM). After culture, the cells were detached from each well by adding a sterile solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free

PBS (Thermo Fisher Scientific, Waltham, MA, USA) with incubation for 2 min at 37 ˚C. Each well was then added 0.9 ml of DMEM containing 10% FBS and 1% P/S. The number of cells in the cell suspension was counted as described below in the section "Cell counting".

Cell death assay

PC-3 or DU-145 wild-type cells (1×10^5/ml per well in 24-well plates) and PC-3 or DU-145 cells transfected with the regucalcin cDNA (1×10^5/ml per well) were cultured using 24-well plates in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days. On reaching subconfluence, they were cultured for an additional 24 h in the presence or absence of either vehicle (PBS or 1% ethanol as a final concentration), TNF-α (0.1 or 1 ng/ml) or Bay K 8644 (25 or 100 nM) [42]. In other experiments, PC-3 wild-type cells (1×10^5/ml per well) or transfectants were cultured for 3 days, and then the cells reaching on subconfluence, the cells were cultured for an additional 24 h in the presence or absence of either Bay K 8644 (25 or 100 nM) with or without caspase-3 inhibitor (10 μM) for 24 h [42]. After culture, the cells were detached by addition of a sterile solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS per well as described in the section of "Cell growth assay", and the cell number was counted as described below in the section "Cell counting".

Cell counting

After culture, to detach cells on each well, the culture dishes were incubated for 2 min at 37 ˚C after addition of a solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS, and then the cells were detached and mixed through pipetting after addition of DMEM (0.9 ml) containing 10% FBS and 1% P/S [39,41,42]. The number of viable cells was counted under a microscope (Olympus MTV-3) with a Hemocytometer (Sigma-Aldrich) using a cell counter (Line Seiki H-102P, Tokyo, Japan). For each dish, we took the average of two counts. Cell numbers were shown as number per well.

Western blotting

PC-3 or DU-145 cells (1×10^6 cells/10 ml of 100 mm dishes), including wild-type cells, control vector cDNA-transfected cells, or regucalcin cDNA-transfected cells, were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days, and then the cells were washed three times with cold PBS (5 ml) and removed from the dish by scraping with addition of cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing inhibitors of protease and protein phosphatase (Roche Diagnostics, Indianapolis, IN, USA). The lysates were then centrifuged at 17,000 x *g*, at 4 ˚C for 2 min. The concentration of protein in the supernatant was determined using the Bio-Rad Protein Assay Dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as a standard. The supernatant was stored at −80 ˚C until use. Samples of forty micrograms of supernatant protein per lane were separated by SDS polyacrylamide gel electrophoresis (12% SDS-PAGE) and then transferred to nylon membranes. Transferred membranes were immunoblotted using specific antibodies against various proteins obtained from Cell Signaling Technology (Danvers, MA, USA), including retinoblastoma [Rb; catalog number (cat. no.) 9309, mouse], p21 (cat. no. 2947, rabbit), Ras (cat. no.14429 rabbit), PI3 kinase p1100 α (cat. no. 4255, rabbit), Akt (cat. no. 9272, rabbit), mitogen-activated protein kinase (MAPK; cat. no. 4695, rabbit), phosphorylated-MAPK (cat. no. 4370, rabbit), Stat3 (cat. no. 12640, rabbit), and β-actin (cat. no. 3700, mouse), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), including p53 (cat. no. sc-126, mouse), NF- κ B p65 (cat. no. sc-109, rabbit), and β-catenin (cat. no. sc-39350, mouse). Rabbit anti-regucalcin antibody was obtained from Sigma-Aldrich (cat. no. HPA029103, rabbit). Target proteins were incubated with one of the primary antibodies (1:1000) as described above for overnight at 4 ˚C. After incubation, the membranes were additionally incubated for 60 min at room temperature by using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., mouse sc-2005 or rabbit sc-2305; diluted 1:2000), and then the immunoreactive blots were visualized with a Super Signal West Pico Chemiluminescent Substrate detection system (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Three blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and bands quantified using Image J2 software.

Mineralization in coculture with preosteoblastic MC3T3 and prostate cancer cells

To determine the effects of PC-3 or DU-145 wild-type cells and PC-3 or DU-145 cells transfected with the regucalcin cDNA on the mineralization of preosteoblastic MC3T3-E1 (American Type Culture Collection, Manassas, VA, USA), we used mineralization medium (MM) containing ascorbic acid (100 ng/ml) and 4 mM β-glycerophosphate in DMEM with 10% FBS, 1% P/S and 1% fungizone, as described previously [26,42,43]. Preosteoblastic MC3T3-E1 cells (10^5 cells/1 ml per well in 24-well plates) were cultured for 3 days at 37 ˚C in a humidified 5% CO_2 atmosphere, and then the cells were additionally cocultured with addition of PC-3 or DU-147 wild-type cells (10^3 or 10^4 cells/1 ml of medium per well) or regucalcin-overexpressing PC-3 or DU-145 cells (10^3 or 10^4 cells/1 ml of medium per well) in DMEM in the presence or absence of MM containing for 18 days [26,43,44]. The medium was changed every 3 days. After culture, the cells were washed with PBS (2 times), fixed with ethanol (ice-cold 75%), and stained with 40 mM Alizarin red S [26,43,44]. For quantitation of mineralization, 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 [26,43,44].

Osteoclastogenesis in coculture with preosteoclastic cells and prostate cancer cells

To determine the effects of PC-3 or DU-145 wild-type cells and PC-3 or DU-145 cells transfected with the regucalcin cDNA on osteoclastogenesis, we used preosteoclastic RAW264.7 cells (American Type Culture Collection, Manassas, VA), as described previously [26,44]. RAW264.7 cells (10^5 cells/1 ml per well in 24-well plates) were cultured for 3 days in DMEM containing 10% FBS, 1% P/S and 1% fungizone at 37 °C in a humidified 5% CO_2 atmosphere, and then the cells were additionally cocultured by addition of PC-3 or DU-145 wild-type cells (10^3 or 10^4 cells/1 ml of medium per well) or regucalcin-overexpressing PC-3 or DU-145 cells (10^3 or 10^4 cells/1 ml of medium per well) with or without LPS (100 ng/ml of medium) for 3 days. Then, 0.5 ml of old medium was replaced with fresh medium containing LPS (100 ng/ml), and the cultures were additionally maintained for 4 days. After culture, the cells adherent to the plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [45]. Briefly, the cells were washed with PBS solution and fixed with 10% neutralized formalin-phosphate (pH7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied. The fixed cells were incubated for 90 min at room temperature in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma-Aldrich) 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 under a microscope (Nikon TMS, Tokyo, Japan).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc. La Jolla, CA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with

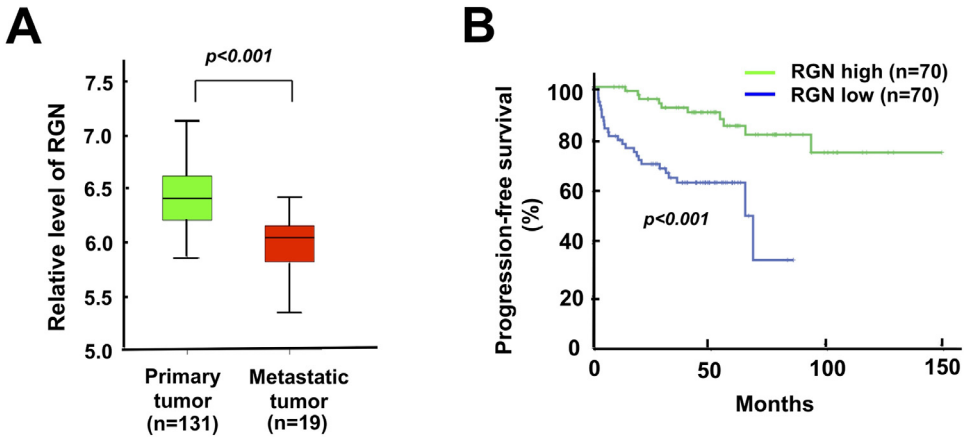

Fig. 1. Progression-free survival is prolonged in prostate cancer patients with a higher regucalcin expression. We analyzed the published dataset of the primary tumors or the metastatic tumors of prostate cancer patients [35]. (A) Microarray analysis showed that the metastatic tumor tissues of prostate cancer patients expressed lower levels of regucalcin as compared with that of primary tumors. (B) Kaplan–Meier curve showed that increased regucalcin mRNA expression was correlated with reduced recurrence in the published database. Abbreviations: RGN; regucalcin.

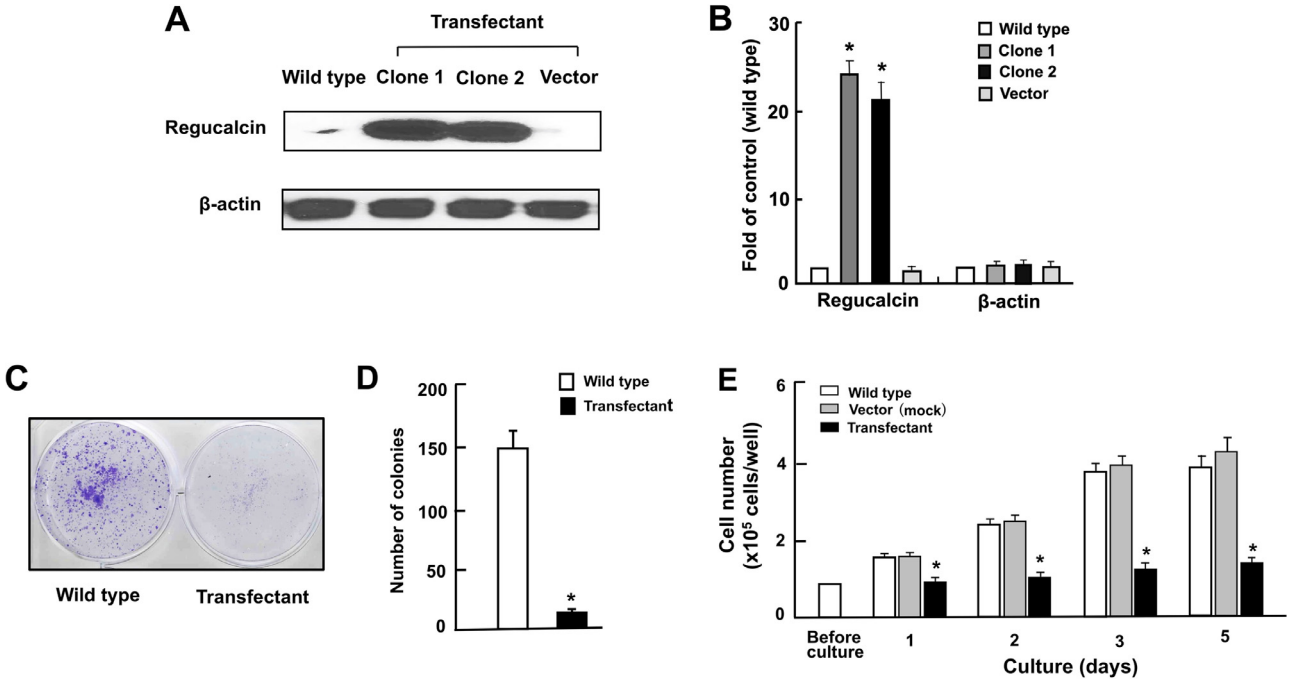

Fig. 2. Overexpression of regucalcin suppresses the growth of human prostate cancer PC-3 cells *in vitro*. Cells (1×10^5 cells/ml per well in 24-well plates) were transfected with full length human regucalcin cDNA vector. PC-3 cells cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days. (A) Regucalcin contents in the cells. Lane 1; wild-type cells. Lane 2 and 3; transfectant (designated as clone 1 and 2). Lane 4; the cells transfected with empty vector/pCXN2 (designated as vector). (B) Regucalcin levels were showed as fold of that in wild-type cells. Data are presented as the mean ± SD. (C) Effects on colony formation. Wild-type cells and transfectants (1×10^3 cells/2 ml per well) were cultured for 9 days. Photo of plates with crystal violet staining is presented. (D) Number of colonies were presented. Data are shown as the mean ± SD of 2 plates (6 wells) using different cell preparations. (E) Effects on cell growth. Cells (1×10^5 cells/ml per well) were cultured for 1–5 days. Number of cells attached on dish was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates using different cell preparations. *$p<0.001$ *versus* wild type (white bar) or control vector (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

Tukey-Kramer multiple comparisons post-test for parametric data as indicated. Kaplan-Meier survival analyses were performed using the log-rank test by using IBM SPSS Statistics 18 software (IBM, Chicago, IL, http://www.ibm. com). A p value of <0.05 was considered statistically significant.

Results

Progression-free survival is prolonged in prostate cancer patients with a higher regucalcin expression

To determine the involvement of regucalcin in tumor malignancy of prostate cancer, we analyzed the published dataset of 131 primary tumors or 19 metastatic tumors [35]. Regucalcin expression was markedly reduced in metastatic tumors as compared with that of primary tumors (Fig. 1A). Furthermore, the Kaplan-Meier survival analysis were performed using the same dataset. The patients were separated into 2 groups with higher levels (70 patients) and lower levels (70 patients) of regucalcin expression, respectively. Progression-free survival was found to prolong in prostate cancer patients with higher regucalcin expression as compared with that of the patients with lower regucalcin gene expression (Fig. 1B). These results suggest that the suppression of regucalcin gene expression contributes to the development of malignancy in human prostate cancer, leading to a worse clinical outcome.

Overexpression of regucalcin suppresses the growth of prostate cancer cells

We generated human regucalcin-overexpressing cells (tansfectants) by using metastatic human prostate cancer PC-3 (Fig. 2). We obtained

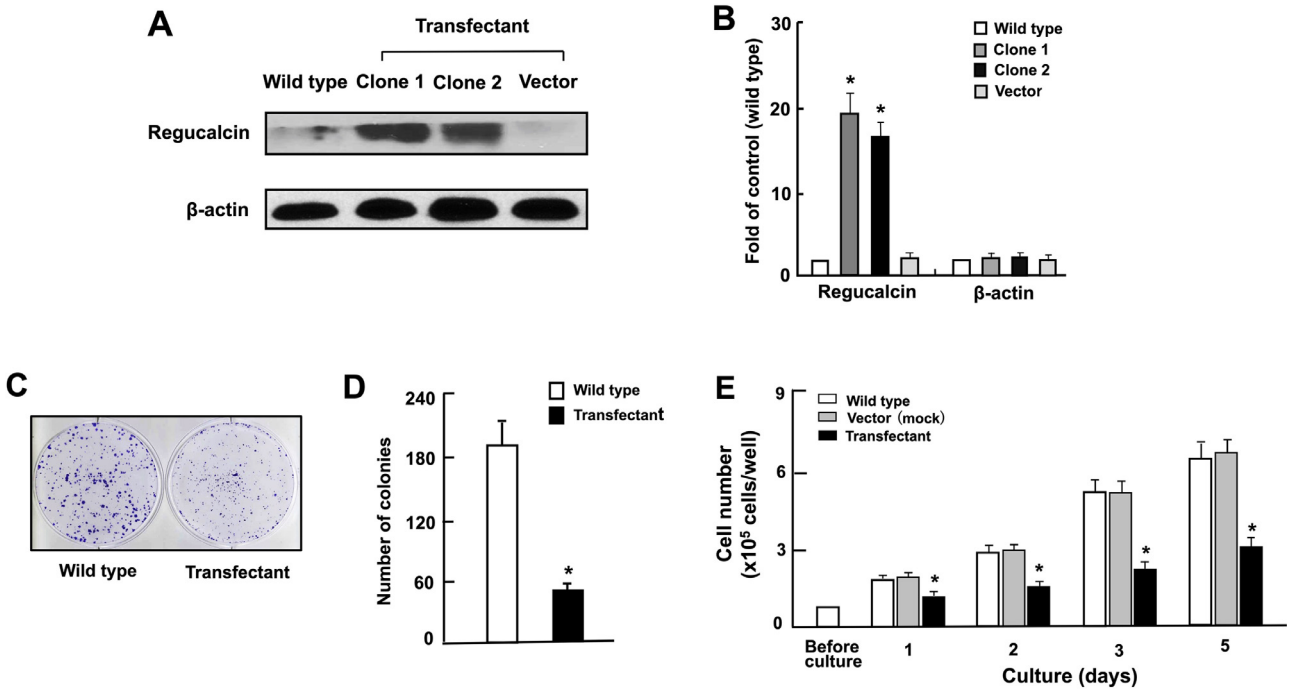

Fig. 3. Overexpression of regucalcin suppresses the growth of human prostate cancer DU-145 cells *in vitro*. Cells were transfected with full length human regucalcin cDNA vector. Wild-type cellas and transfectants (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days. (A) Regucalcin contents in the cells. (A) Content of regucalcin in the cells. Lane 1; wild-type cells. Lane 2 and 3; transfects (designated as clone 1 and 2). Lane 4; the cells transfected with empty vector/pCXN2 (designated as vector). (B) Regucalcin levels were showed as fold of that in wild-type cells. Data are presented as the mean ± SD of blotting using different cell preparations. (C) Effects on colony formation. Cells (1×10^3 cells/2 ml per well) were cultured for 9 days. Photo of plates with crystal violet staining is presented. (D) Number of colonies were presented. Data are shown as the mean ± SD of 6 wells using different cell preparations. (E) Effects on cell growth. Cells (1×10^5 cells/ml per well) were cultured for 1–5 days. Number of cells attached on dish was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates using different cell preparations. $^*p<0.001$ *versus* wild type (white bar) or control vector (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

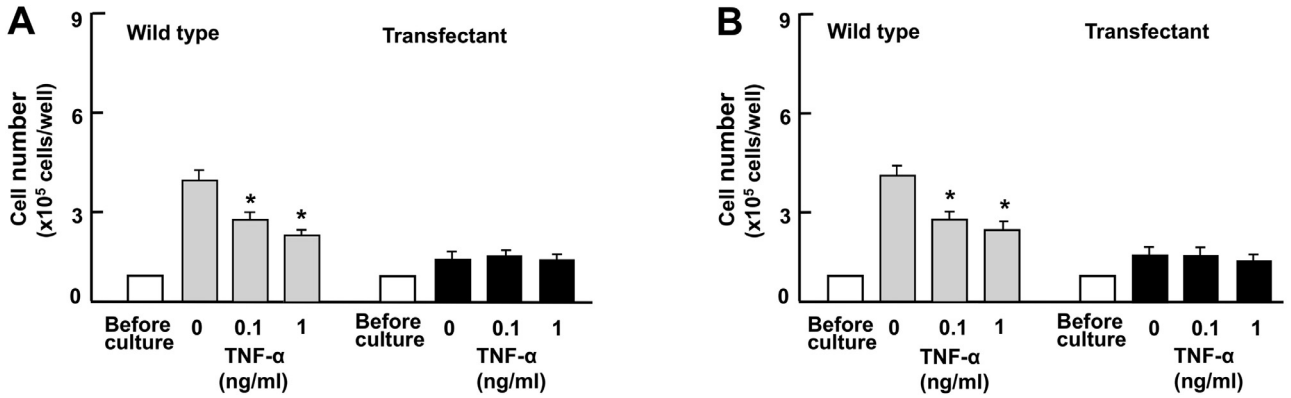

Fig. 4. Suppressive effects of overexpressed regucalcin on the growth are independent of the death of human prostate cancer PC-3 cells *in vitro*. Wild-type cells or transfectants (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone. (A) Effects on cell proliferation. Cells were cultured in the presence or absence of TNF-α (0.1 or 1 ng/ml) for 3 days. (B) Effects on cell death. Cells were cultured without TNF-α for 3 days, and the cells reaching on subconfluence were additionally cultured for 24 h in the presence or absence of TNF-α (0.1 or 1 ng/ml). After culture, the number of attached cells was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates using different cell preparations. $^*P<0.001$ *versus* control (without TNF-α). 1-way ANOVA, Tukey–Kramer post-test.

transfectant clone 1 or 2 with higher stable expression of regucalcin in PC-3 cells. The regucalcin levels in these clones of PC-3 cells were increased about 25- or 22-fold as compared with wild-type cells, respectively (Fig. 2A and B). To determine the effects of overexpressed regucalcin on the growth of PC-3 cells *in vitro*, clone 1 was used in the following experiments. First, we investigated the effects of regucalcin overexpression on colony formation of PC-3 cells. Cells were cultured for 9 days when colony formation clearly appeared. Colony formation of PC-3 cells was repressed by overexpression of regucalcin (Fig. 2C). Furthermore, the effect of overexpressed regucalcin on the growth of PC-3 is shown in Fig. 2E. The growth of PC-3 cells was suppressed by overexpression of regucalcin.

Next, we generated the regucalcin-overexpressing metastatic human prostate DU-145 cells. We obtained two clones, which regucalcin expression were elevated by about 20- or 17-fold as compared with that of wild-type cells, respectively (Fig. 3A and B). We found that overexpression of regucalcin in DU-145 cells suppresses colony formation (Fig. 3C and D) and cell growth (Fig. 3E) by using clone 1.

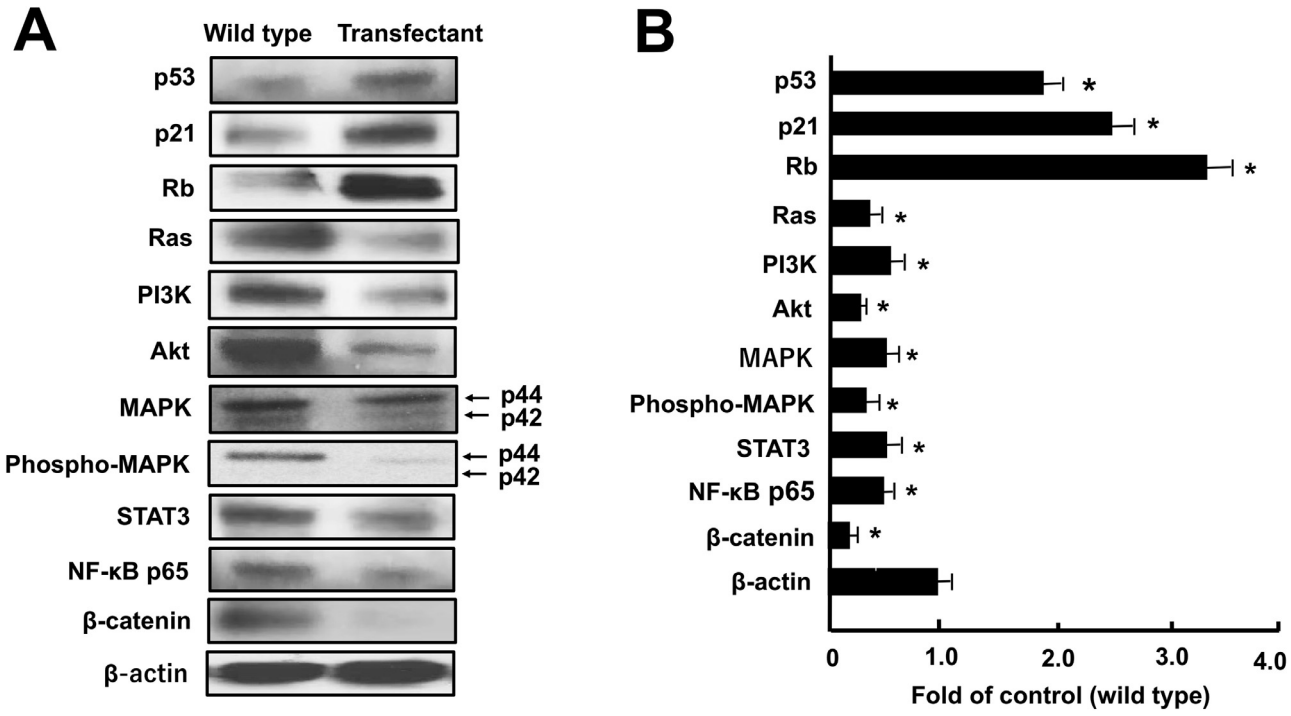

Fig. 5. Overexpression of regucalcin regulates the levels of various proteins linked to cell signaling and transcription processes in human prostate cancer PC-3 cells *in vitro*. Wild-type cells and transfectants (1×10^6 cells/10 ml of medium in 100 mm dishes) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days. After culture, the cells were removed from the dish with a cell scraper in cell lysis buffer containing protease inhibitors. Forty micrograms of supernatant protein per lane were separated by SDS-PAGE (12%) and transferred to nylon membranes for Western blotting using antibodies against various proteins. (A) Representative data are presented. (B) Band was presented as fold of control (wild-type cells). Data are presented as the mean ± SD obtained from 4 dishes by using different cell preparations. $^*P<0.001$ *versus* control (none; gray bar). 1-way ANOVA, Tukey–Kramer post-test.

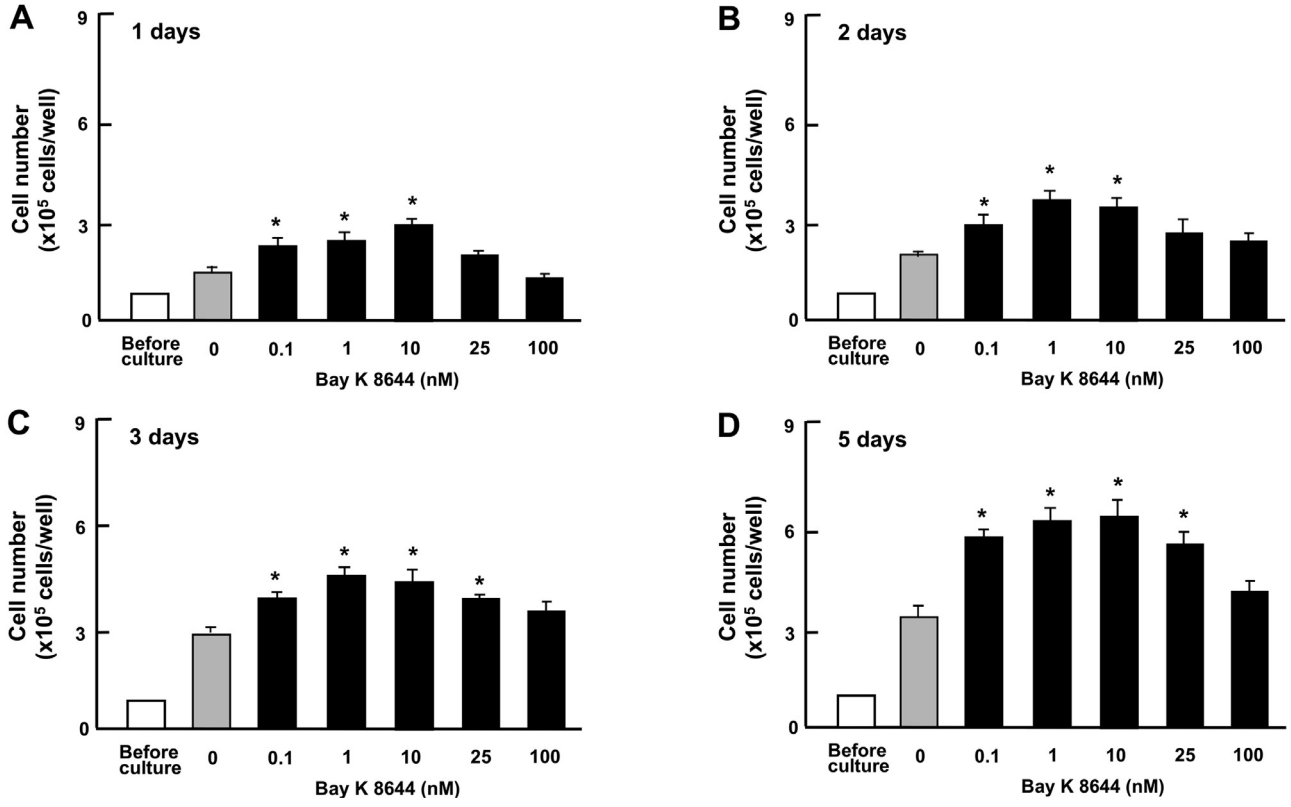

Fig. 6. Effects of Bay K 8644, an agonist of calcium entry in the cells, on the growth of human prostate cancer PC-3 cells *in vitro*. Wild-type cells (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 1 (A), 2 (B), 3 (C), or 5 (D) days in the presence or absence of either vehicle (1% ethanol as a final concentration) or Bay K 8644 (0.1, 1, 10, 25, and100 nM). After culture, the number of attached cells was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates per data set using different cell preparations. $^*P<0.001$ *versus* control (none; gray bar). 1-way ANOVA, Tukey–Kramer post-test.

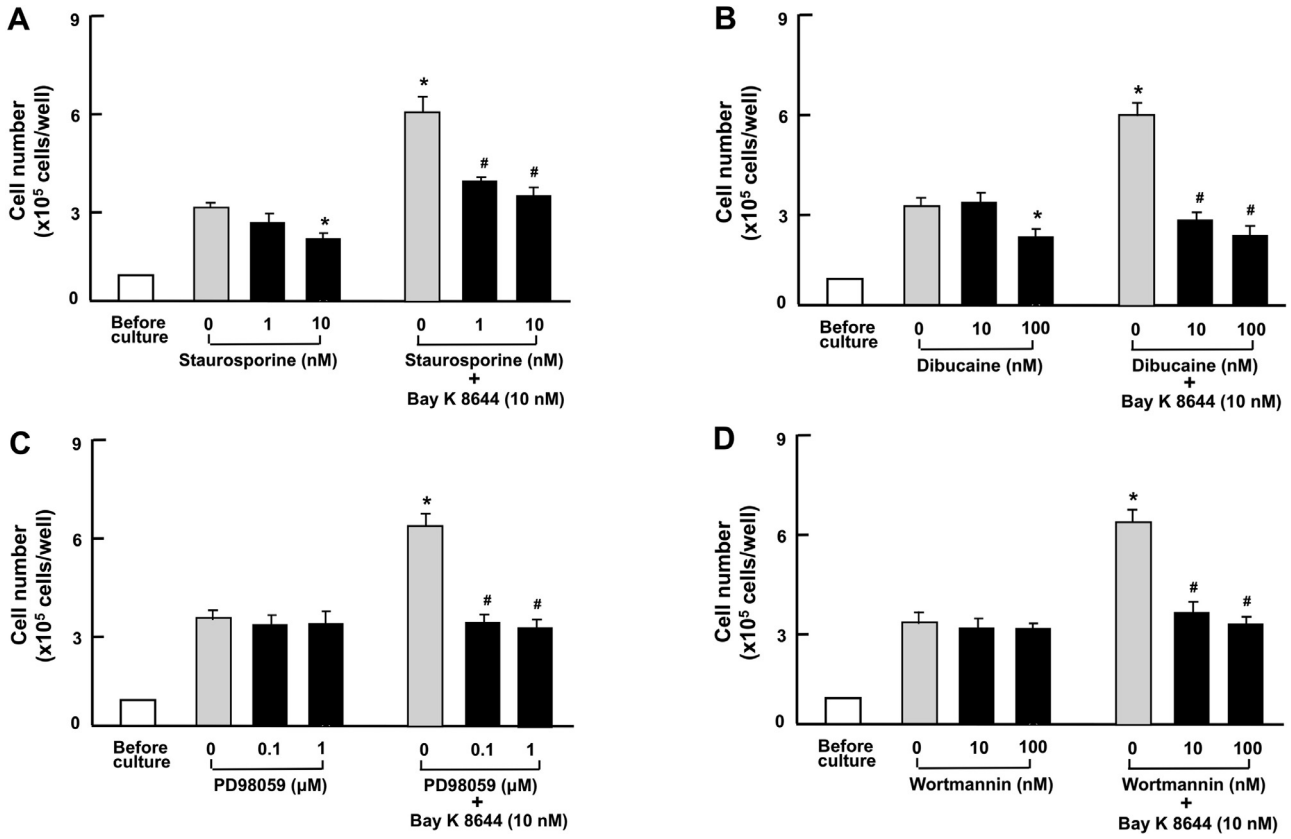

Fig. 7. Stimulatory effects of Bay K 8644 on the growth of human prostate cancer PC-3 cells are blocked by culturing with inhibitors of various signaling pathways *in vitro*. Wild-type cells (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone in the presence or absence of either vehicle (1% ethanol as a final concentration) or Bay K 8644 (10 nM) with or without staurosporine (1 or 10 nM), dibucaine (10 or 100 nM), PD98059 (0.1 or 1 μM) or wortmannin (10 or 100 nM) for 3 days. After culture, the number of attached cells was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates per data set using different cell preparations. $^*P<0.001$ *versus* control (none; gray bar). 1-way ANOVA, Tukey–Kramer post-test.

Thus, overexpression of regucalcin was found to suppress colony formation and growth of metastatic human prostate cancer PC-3 and DU-145 cells. These results suggest that overexpressed regucalcin plays a role as a suppressor in metastatic human prostate cancer.

Effects of overexpressed regucalcin on cell death

Next, we investigated whether or not overexpression of regucalcin impacted on the death of PC-3 cells. To determine the effect of TNF-α on cell growth, PC-3 wild-type cells and transfectants were cultured in the presence of TNF-α (0.1 or 1 ng/ml) for 3 days (Fig. 4A). The growth of wild-type cells was decreased by culturing with TNF-α (0.1 or 1 ng/ml) (Fig. 4A). This effect of TNF-α was not exhibited in the transfectants. Furthermore, PC-3 wild-type cells and transfectants were cultured in the absence of TNF-α for 3 days until reaching subconfluence, and then the cells were additionally cultured with TNF-α (0.1 or 1 ng/ml) for 24 h. The number of attached PC-3 wild-type cells were decreased by culturing with TNF-α (0.1 or 1 ng/ml) (Fig. 4B). This effect of TNF-α was not found in the transfectants (Fig. 4B). These results suggest that the suppressive effects of overexpressed regucalcin on cell growth are not mediated via cell death.

Effects of overexpressed regucalcin on the levels of various proteins involved in cell growth

Mechanistically, we investigated whether or not overexpressed regucalcin regulates the expression of key proteins linked to processes of cell signaling and transcription in PC-3 cells (Fig. 5). Overexpressed regucalcin was found to elevate the levels of p53, Rb, and p21, which lead to repression of cell growth (Fig. 5A and B). Overexpression of regucalcin diminished the levels of Ras, PI3 kinase, Akt, MAP kinase and phospho-MAP kinase, which implicate in promotion of cell proliferation (Fig. 5A and B). Furthermore, overexpression of regucalcin decreased the levels of Stat3, NF-κB p65 and β-catenin, which are transcription factors linked to cell proliferation [17] (Fig. 5A and B). Meanwhile, β-actin levels were not altered by overexpression of regucalcin. Thus, the suppressive effects of overexpressed regucalcin on the growth of PC-3 cells were mediated via altered expression of various proteins, which are involved in processes of cell signaling and transcription.

Overexpression of regucalcin suppresses Bay K 8644-promoted cell growth and death

Intracellular calcium signaling is involved in the development of carcinogenesis [46]. We investigated whether or not the suppression of PC-3 cell growth by overexpression of regucalcion was linked to calcium signaling *in vitro*. The L-type calcium channel agonist Bay K 8644 is shown to stimulate calcium entry into the cells [47,48]. This channel is expressed in PC-3 cells [48]. The growth of PC-3 cells was stimulated by culturing with Bay K 8644 (0.1–100 nM) for 1 (Fig. 6A), 2 (Fig. 6B), 3 (Fig. 6C), and 5 (Fig. 6D) days. This is the first report of such an effect on PC-3 cell growth. Interestingly, the stimulatory effects of Bay K 8644 (10 nM) on the growth of PC-3 cells were blocked by culturing with various inhibitors of intracellular signaling pathways, including staurosporine (Fig. 7A), an inhibitor of protein kinase C [49], dibucaine (Fig. 7B), an inhibitor of calcium-dependent protein kinases [50], PD98059 (Fig. 7C), an inhibitor of MAPK [51], and wortmannin (Fig. 7D), an inhibitor of PI3K [52]. This finding may support the view that the stimulatory effects of Bay K 8644 on the growth of PC-3 cells are

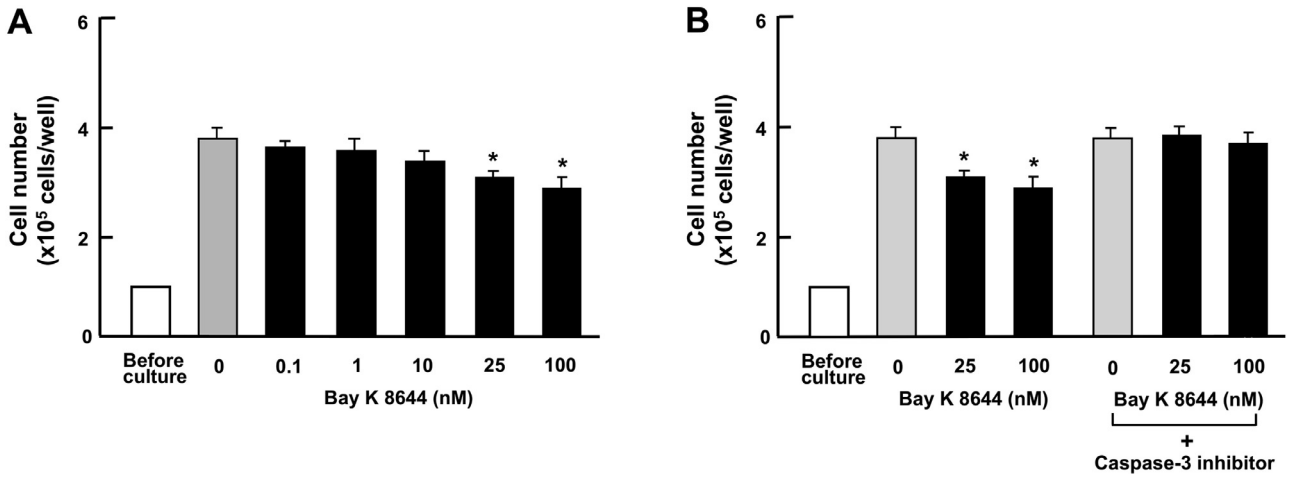

Fig. 8. Effects of Bay K 8644 on the death of human prostate cancer PC-3 cells *in vitro*. Wild-type cells (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone without Bay K-8644 for 3 days, and the cells reaching on subconfluence were additionally cultured for 24 h in the presence of vehicle (1% ethanol as a final concentration) or Bay K 8644 (0.1, 1, 10, 25, and100 nM) without (A) or with (B) caspase-3 inhibitor (10 μM). After culture, the number of attached cells was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates using different cell preparations. *P<0.001 *versus* control (control; gray bar). 1-way ANOVA, Tukey–Kramer post-test.

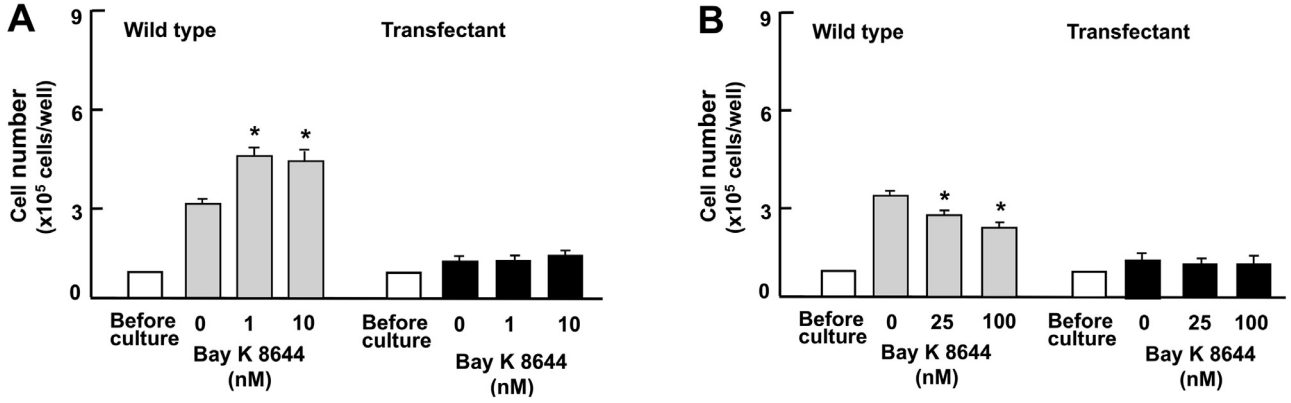

Fig. 9. Overexpression of regucalcin blocks stimulatory effects of Bay K 8644 on the growth and death of human prostate cancer PC-3 cells *in vitro*. (A) Effects on cell growth. Wild-type cells and transfectants (1×10^5 cells/ml per well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone for 3 days in the presence or absence of either vehicle (1% ethanol as a final concentration) or Bay K 8644 (1 or 10 nM). (B) Effects on cell death. Cells were cultured in above medium for 3 days, and the cells reaching on subconfluence were additionally cultured for 24 h in the presence of vehicle (1% ethanol as a final concentration) or Bay K 8644 (25 or 100 nM). After culture, the number of attached cells was counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates using different cell preparations. *P<0.001 *versus* control (without Bay K 8644). 1-way ANOVA, Tukey–Kramer post-test.

mediated via various signaling processes that are activated by calcium signaling.

The effects of Bay K 8644 on the death of PC-3 cells are shown in Fig. 8. The number of PC-3 cells was reduced by culturing cells in Bay K 8644 with comparatively higher concentrations (25 or 100 nM), indicating that cell death is partly induced by Bay K 8644 (Fig. 8A). Such effects were abolished by the presence of an inhibitor of caspase-3, which induces apoptotic cell death [21,42] (Fig. 8B).

Regucalcin plays an inhibitory role in processes of calcium signaling in various types of cells [15,16]. Furthermore, we investigated whether or not overexpressed regucalcin suppresses the effects of Bay K 8644 on the growth and death of PC-3 cells *in vitro*. Stimulatory effects of Bay K 8644 on the growth (Fig. 9A) and death (Fig. 9B) of PC-3 cells *in vitro* were blocked by overexpression of regucalcin. These results may support the view that regucalcin blocks processes of calcium signaling in PC-3 cells.

Overexpression of regucalcin suppresses bone activity in vitro

We investigated an involvement of regucalcin in bone metastasis of prostate cancer cells *in vitro*. To determine the change in osteoblastic mineralizations, preosteoblastic MC3T3-E1 cells were cocultured with PC-3 or DU-145 wild-type cells or regucalcin-overexpressing PC-3 and DU-145 cells (transfectants) *in vitro*. MC3T3-E1 cells were cultured for 3 days, and then the cells were cocultured with the addition of PC-3 or DU-145 wild-type cells or transfectants in DMEM containing mineralization substrate for 18 days *in vitro* (Fig. 10). Interestingly, mineralization in MC3T3-E1 cells was increased by coculturing with PC-3 (Fig. 10A) or DU-145 (Fig. 10B) wild-type cells. These increases were suppressed in the transfectants (Fig. 10A and B).

We also investigated whether or not overexpression of regucalcin impacts on osteoclastogenesis *in vitro* (Fig. 11). RAW264.7 cells are differentiated to mature osteoclasts by stimulation of LPS [26,44]. RAW264.7 cells were cocultured in the presence of PC-3 or DU-145 wild-type cells or transfectants for 7 days with or without LPS (100 ng/ml). Osteoclastogenesis from RAW264.7 cells was stimulated by coculturing with PC-3 (Fig. 11A) or DU-145 (Fig. 11C) wild-type cells in the absence of LPS. These stimulations were further enhanced in the presence of LPS (Fig. 11B and D). Notably, overexpression of regucalcin suppressed osteoclastogenesis of RAW264.7 cells stimulated by coculturing with PC-3 cells (Fig. 11A and B) or DU-145 cells (Fig. 11C and D) in the presence and absence of LPS. These results suggest that overexpression of regucal-

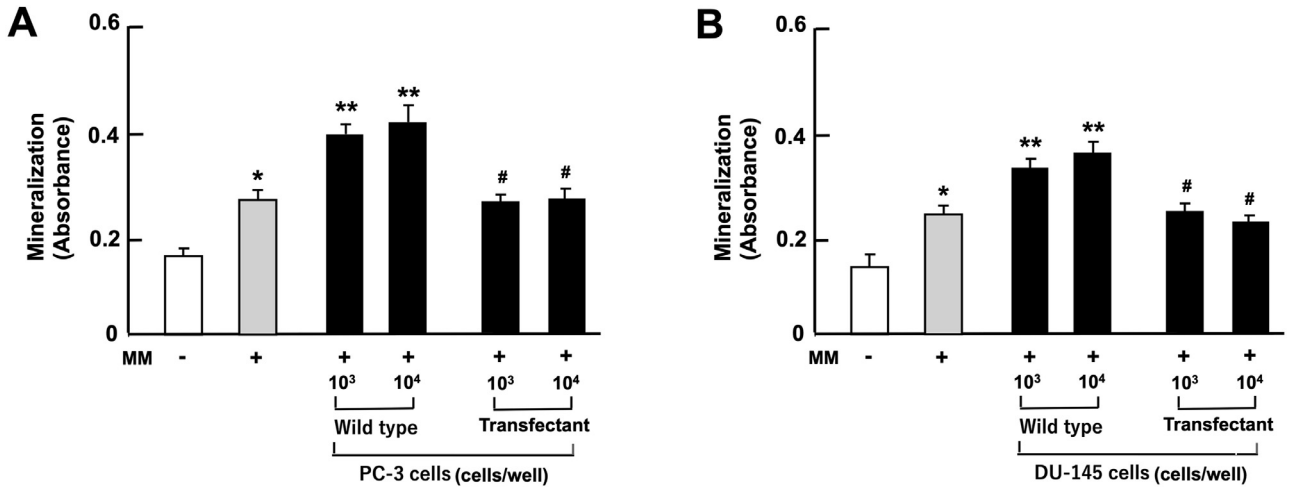

Fig. 10. Alteration of the mineralization in mouse preosteoblastic MC3T3-E1 cells cocultured with human prostate cancer PC-3 or DU-145 cells *in vitro*. MC3T3-E1 cells (10^5 cells/ml/well in 24-well plates) were cocultured for 18 days with or without addition of PC-3 (A) or DU-145 (B) wild-type cells (10^3 or 10^4 cells/ml/well) or transfectants (10^3 or 10^4 cells/ml/well) in DMEM containing 10% FBS, 1% P/S, 1% fungizone, and mineralization medium (MM). After culture, the cells were stained with Alizarin red S 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 the mean ± SD obtained from 6 wells of 2 replicate plates using different cell preparations. $^{*}p<0.001$ *versus* control (white bar). $^{**}p<0.001$ *versus* groups with MM. $^{\#}p<0.001$ *versus* groups of wild type.1-way ANOVA, Tukey–Kramer post-test.

cin suppresses osteoclastogenesis enhanced by human prostate cancer PC-3 and DU-145 cells.

Discussion

In this study, we profiled regucalcin gene expression and related progression-free survival in prostate cancer patients. The data obtained demonstrated that progression-free survival of prostate cancer patients was prolonged with a higher regucalcin gene expression in the tumor tissues. Interestingly, the levels of regucalcin in the metastatic tumor tissues of prostate cancer patients were diminished as compared with that of the primary tumor tissues. This is the first finding concerning an involvement of regucalcin in patients of human prostate cancers. The present study suggests that suppression of the regucalcin gene expression leads to aggressiveness of metastatic human prostate cancer. Translational studies found that overexpressed regucalcin suppressed colony formation, cell growth and bone activity in metastatic human prostate cancer PC-3 and DU-145 cells *in vitro*. Regucalcin may have a significant involvement as a suppressor in metastatic human prostate cancer.

Regucalcin has been shown to regulate nuclear function in cells [23]. Binding of regucalcin to nuclear DNA regulates expression of various genes in the nucleus of cells [23,53–55]. Regucalcin has been shown to be under-expressed in human prostate cancer LNCaP cells *in vitro* [56]. In the present study, overexpression of regucalcin was found to diminish the levels of Ras, PI3K, Akt and MAPK, which are key molecules linked to promotion of the growth in PC-3 cells. Furthermore, overexpressed regucalcin was found to enhance the levels of p53, Rb and p21, which contribute to suppression of cell growth. Interestingly, overexpression of regucalcin decreased the levels of Stat3, NF-κB p65 and β-catenin, which are transcription factors linked to the proliferation of cancer cells. Regucalcin may play a pivotal role as a repressor in the growth of human prostate cancer cells by regulating expression of manifold proteins, which are implicated in the processes of intracellular signaling, transcription, cell cycle, and tumor suppression linked to the development of tumor.

Increased cell calcium may stimulate the proliferation of prostate cancer cells [46]. The L-type Ca^{2+} channel agonist Bay K 8644 is expressed in PC-3 cells [48]. This agonist is shown to elevate Ca^{2+} levels in PC-3 cells [48]. Regucalcin plays an inhibitory role in calcium signaling in various types of cells [15,16,20]. Therefore, we investigated whether or not overexpressed regucalcin suppresses the effect of Bay K 8644 on the growth of PC-3 cells *in vitro*. The growth of PC-3 cells was promoted by culturing with comparatively lower concentrations of Bay K 8644. This effect of Bay K 8644 was blocked by culturing with various inhibitors, which inhibit intracellular signaling processes related to protein kinase C [49], calcium-dependent protein kinases [50], MAPK [51], and PI3K [52]. Stimulatory effects of Bay K 8644 on cell growth may be mediated via activation of diverse signaling pathways in PC-3 cells. Notably, overexpression of regucalcin was found to suppress the stimulatory effects of Bay K 8644 on the growth of PC-3 cells. This result supports the view that overexpressed regucalcin inhibits cell proliferation by inhibiting calcium effects on signaling linked to cell proliferation.

Furthermore, we found that death of PC-3 cells was caused by TNF-α and higher Bay K 8644. Such effects were abolished by overexpression of regucalcin. Regucalcin suppresses cell death induced by various signaling factors in other types of cells [21,42] and prevents nuclear DNA fragmentation due to inhibiting caspase-3 activity in isolated rat liver nuclei [57]. Overexpressed regucalcin may prevent increased death of PC-3 cells by mechanism which, at least in part, is linked to NF-κB or calcium signaling. In addition, the observed suppressive effects of overexpressed regucalcin on the growth of PC-3 cells were independent of increased cell death.

Prostate cancer preferentially metastasizes to bone, leading to severe pain, fractures, and bone marrow suppression [5–7]. Prostate cancer is known to stimulate osteolytic responses when the cancer cells reside in the bone marrow space. Prostate cancer cells interact with osteoblasts, osteocytes and osteoclasts. Bone metastasis of prostate cancer cells is known to cause osteoblastic lesions composed of hypermineralized bone with multiple layers of poorly organized type I collagen fibrils that have decreased mechanical strength [7,58]. In turn, activated osteoblasts stimulate the growth of prostate cancer cells in the bone microenvironment. Human prostate cancer PC-3 and DU-145 cells have metastatic characterization [1–3]. Furthermore, we investigated whether overexpression of regucalcin regulates bone activity by coculturing with PC-3 or DU-145 cells and bone cells *in vitro*. Interestingly, mineralization of prosteoblastic MC3T3-E1 cells was increased by coculturing with PC-3 or DU-145 wild-type cells. These increases were repressed by overexpression of regucalcin. Thus, overexpression of regu-

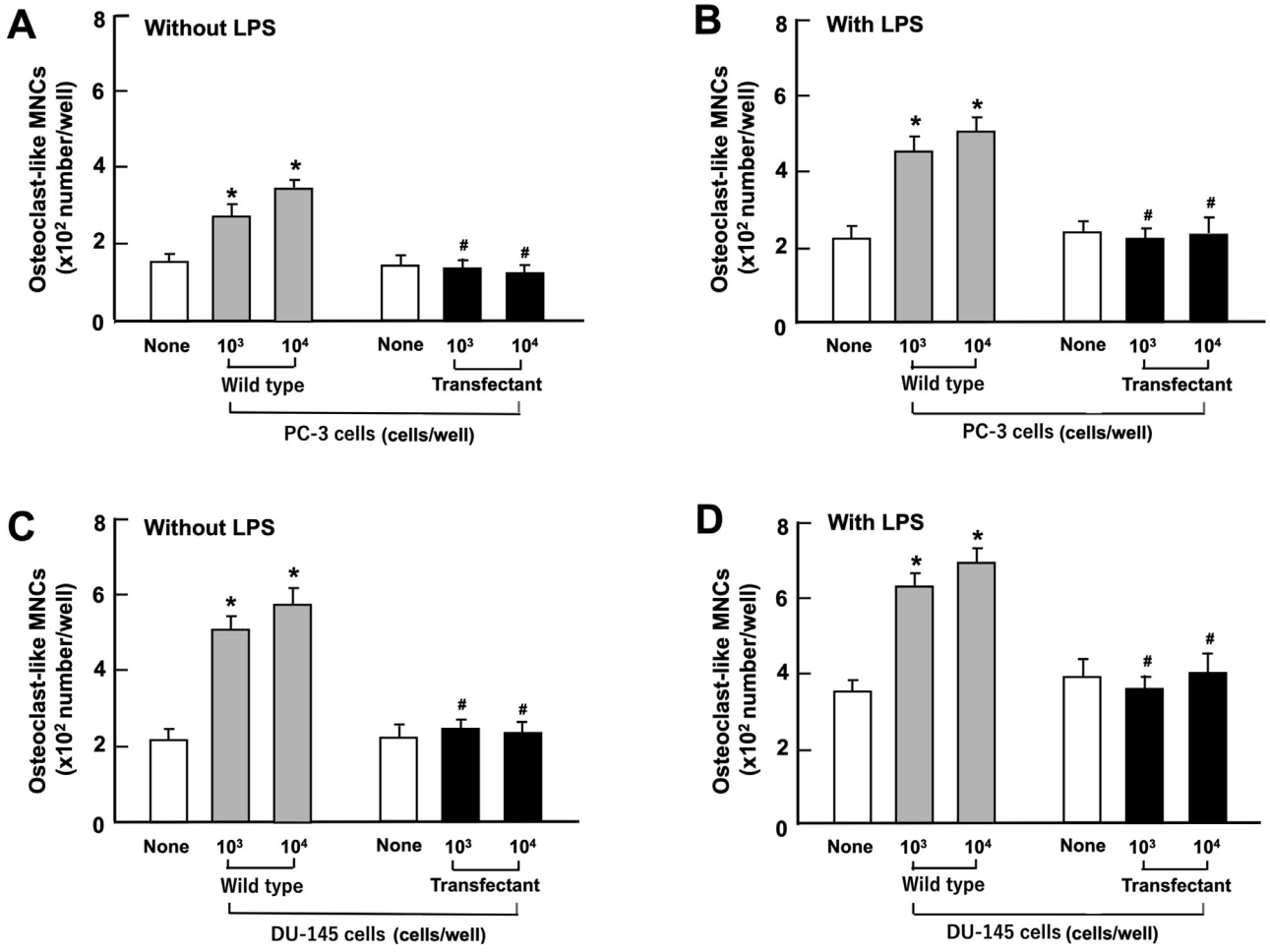

Fig. 11. Overexpression of regucalcin suppresses osteoclastogenesis in coculturing with preosteoclastic RAW264.7 cells and human prostate cancer PC-3 or DU-145 cells *in vitro*. RAW264.7 cells (10^5 cells/ml/well in 24-well plates) were cultured in DMEM containing 10% FBS, 1% P/S and 1% fungizone, and then they were further cultured for 7 days after addition of PC-3 (A and B) or DU-145 (C and D) wild-type cells (10^3 or 10^4 cells/ml/well) or transfectants (10^3 or 10^4 cells/ml/well) with or without LPS (100 ng/ml of medium). After culture, cells were stained for TRACP. TRAP+ multinucleated cell (3 or more nuclei) were counted. Data are presented as the mean ± SD obtained from 8 wells of 2 replicate plates per data set using different cell preparations. $^*p<0.001$ *versus* control (white bar). 1-way ANOVA, Tukey–Kramer post-test.

calcin in PC-3 or DU-145 wild type cells suppressed the ability of the wild type PC-3 or DU-145 cells to enhance osteoblastic mineralization *in vitro*.

Osteoclasts are differentiated from hematopoetic precursors of the monocyte/macrophage lineage by stimulation with the TNF family cytokines, including receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [59,60]. RANKL activates NF- κ B signaling to stimulate formation from preosteoclastic cells to mature osteoclasts [59,60]. Osteoclastogenesis from macrophage RAW264.7 cells is enhanced by stimulation of LPS that activates NF- κ B signaling [44,59,60]. We found that osteoclastogenesis from RAW264.7 cells was enhanced by coculturing with PC3 or DU-145 wild-type cells in the presence and absence of LPS. Notably, this enhancement was repressed by coculturing with regucalcin-overexpressing PC-3 or DU-145 cells in the presence or absence of LPS. Thus, regucalcin overexpressed in PC-3 or DU-145 cells suppressed osteoclastogenesis of RAW264.7 cells. We speculate that PC-3 or DU-145 cells may produce stimulatory factors for osteoclastogenesis, and that this production is suppressed by overexpression of regucalcin in the cells. Thus, overexpression of regucalcin may suppress bone activity of human prostate cancer cells by stimulating osteoblastogenesis and inhibiting osteoclastogenesis.

In conclusion, survival of cancer patients has been shown to prolong with a higher regucalcin expression in the tumor tissues of various types of cancer [25–30]. The present study, furthermore, demonstrates that progression-free survival of prostate cancer patients is prolonged with a higher regucalcin gene expression in the tumor tissues. Translational study found that overexpression of regucalcin suppresses colony formation, cell growth and bone metastatic activity in PC-3 and DU-145 cells of bone metastatic human prostate cancer models *in vitro*. Therefore, regucalcin may play a potential role as a suppressor in the development of bone metastatic human prostate cancer. The regucalcin gene expression may be downregulated in the development of malignancies of various types of human subjects [25–30]. Overexpression of regucalcin may play a role in suppression of tumor growth. Delivery of the regucalcin gene into tumor tissues may be clinically significant as a potential therapeutic tool for human cancer.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors. All experimental protocols used databases or cell culture *in vitro*.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Masayoshi Yamaguchi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Satoru Osuka:** Investigation, Methodology, Resources, Software, Writing - review & editing. **Tomiyasu Murata:** Resources, Writing - review & editing. **Joe W. Ramos:** Funding acquisition, Resources, Writing - review & editing.

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