Prolonged survival of patients with colorectal cancer is associated with a higher regucalcin gene expression: Overexpression of regucalcin suppresses the growth of human colorectal carcinoma cells *in vitro*

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Received February 23, 2018; Accepted June 12, 2018

DOI: 10.3892/ijo.2018.4458

Abstract. Regucalcin plays a crucial role as a regulator of transcriptional signaling activity, and its decreased expression or activity may contribute to the promotion of human carcinogenesis. A higher regucalcin expression in the tumor tissues has been demonstrated to prolong the survival of patients with various types of cancer, including pancreatic cancer, breast cancer, liver cancer and lung adenocarcinoma. The involvement of regucalcin in human colorectal cancer was investigated in the current study. Regucalcin gene expression and the survival data of 62 patients with colorectal cancer were obtained though the Gene Expression Omnibus (GEO) database (GSE12945) for outcome analysis. The data of gene expression revealed that the prolonged survival of patients with colorectal cancer was associated with a higher regucalcin gene expression in tumor tissues. The overexpression of regucalcin suppressed colony formation and proliferation, and induced the death of human colorectal carcinoma RKO cells cultured in a medium containing fetal bovine serum *in vitro*. Mechanistically, the overexpression of regucalcin induced the G1 and G2/M phase cell cycle arrest of the RKO cells through the suppression of multiple signaling pathways, including Ras, Akt, mitogen-activated protein (MAP) kinase and SAPK/JNK. Of note, the overexpression of regucalcin induced an increase in the levels of the tumor suppressors, p53 and Rb, and the cell cycle inhibitor, p21. Moreover, the levels of the transcription factors, c-fos, c-jun, nuclear factor (NF)-κB p65, β-catenin and signal transducer and activator of transcription 3 (Stat3), were suppressed by the overexpression of regucalcin. On the whole, the findings of this study suggest that regucalcin plays a crucial role as a suppressor in human colorectal cancer, and that the suppressed expression of the regucalcin gene may predispose patients to the promotion of colorectal cancer. The overexpression of regucalcin by gene delivery may thus prove to be a novel therapeutic strategy for colorectal cancer.

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Key words: regucalcin, colorectal cancer, RKO cell, colony formation, cell proliferation, cell death

Introduction

Adenocarcinoma is the predominant malignancy affecting the colon and rectum (1). Colorectal cancer is the third most common cancer diagnosed in the developed world (2,3). The average 5-year survival rate of patients with colorectal cancer remains poor at 55% (3), even though the development of new drugs has improved the survival rate of patients. The prognosis of patients with colorectal cancer remains poor, in spite of the development of novel therapeutic strategies (4-7). Human colorectal cancer represents a heterogenous group of diseases, and its molecular classification is increasingly important (4-7). The characterization of novel biomarker targets may lead to the prolonged survival of patients with colorectal cancer. Biomarkers may play a potential role in the screening, diagnosis, prognosis and monitoring of the disease (4-7). Mutations in the KRAS gene in ~40% of tumors have been reported to be induced by genetic and epigenetic alterations (8-11).

The expression of regucalcin, whose gene is localized on the X chromosome (12-14), has been shown to be suppressed in tumor tissues of mammalian and human subjects *in vivo* (15,16), suggesting that the suppressed expression of regucalcin plays an important role in the promotion development of carcinogenesis (15,16). Regucalcin has been demonstrated to play a pivotal role as a repressor of manifold signaling pathways and transcriptional activity in various types of cells (17,18); this protein plays a regulatory role in the inhibition of various signaling pathways implicated in calcium homeostasis, various protein kinases and protein phosphatases, the suppression of cytosolic protein synthesis, nuclear DNA and RNA synthesis, and the regulation

of nuclear gene expression (17-20). Moreover, regucalcin has been found to suppress the proliferation (15) and apoptotic cell death mediated through diverse signaling molecules in the cells of various types of tissues (20). Thus, regucalcin may play a pivotal role in maintaining cell homeostasis (17,18,21).

Importantly, regucalcin gene expression has been shown to be decreased in various tissues of human cancer (15,16,22), suggesting that a diminished regucalcin gene expression may induce the promotion of carcinogenesis (15,16,22). We have previously demonstrated that survival was prolonged in patients with pancreatic cancer (23), breast cancer (24), hepatocellular carcinoma (25) and lung cancer (26) who had a higher regucalcin expression in their tumor tissues as compared with those with a lower regucalcin expression. In support of these findings, the overexpression of regucalcin exerted repressive effects on the growth of human pancreatic cancer MIA PaCa-2 cells (23), MDA-MB-231 breast cancer cells (24), liver cancer HepG2 cells (25) and lung adenocarcinoma A549 cells (26) *in vitro*. Regucalcin may thus play a potential role as a suppressor of the development of carcinogenesis in human subjects, demonstrating its significance as a novel biomarker in the diagnosis of human cancer.

The involvement of regucalcin in human colorectal cancer has not yet been investigated, at least to best of our knowledge. The current study was thus undertaken to determine whether regucalcin is involved in the suppression of human colrorectal cancer. Notably, we found that the prolonged survival of patients with colorectal cancer was associated with a higher regucalcin gene expression in the tumor tissues, as evaluated by the analysis of gene expression using the Gene Expression Omnibus (GEO) database (GSE12945). In addition, the overexpression of regucalcin was shown to inhibit the growth of human colorectal cancer RKO cells *in vitro*. Our findings thus support the view that the diminished regucalcin gene expression predisposes patients with colorectal cancer, suggesting a novel therapeutic strategy involving regucalcin gene therapy in human colorectal cancer.

Materials and methods

Materials and 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; P/S) were purchased from Corning (Mediatech, Inc. Manassas, VA, USA). Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). Lipofectamine reagent was obtained from Promega, Madison, WI, USA). Tumor necrosis factor-α (TNF-α) was from R&D Systems (Minneapolis, MN, USA). Sodium butyrate, roscovitine, sulforaphane, dibucaine, PD98059, lipopolysaccharoide (LPS), Bay K 8644, wortmannin, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), caspase-3 inhibitor, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Gemcitabine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Gemcitabine and caspase-3 inhibitor were diluted in phosphate-buffered saline (PBS) and thye other reagents were dissolved in 100% ethanol for use.

Patient datasets. Regucalcin gene expression and the survival data of 62 patients with colorectal cancer were obtained though the GEO database, GSE12945, for outcome analysis (27). These datasets contained gene expression data derived from the Affymetrix U133A array. Microarray analysis was performed as previously described (23-26). 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).

Human colorectal cancer RKO cells. We used epithelial RKO cells originating from male adult patients with colorectal carcinoma, which were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The RKO cells were suitable as a transfection host. The cells were cultured using DMEM containing 10% FBS and 1% P/S.

Transfection of human regucalcin cDNA. The RKO wild-type cells were transfected with pCXN2 vector expressing cDNA encoding human full-length (900 bp) regucalcin (regucalcin cDNA/pCXN2) (28). To assay transient transfection, the RKO cells were grown on 24-well plates to reach subconfluency. Regucalcin cDNA/pCXN2 and empty pCXN2 vector alone were transfected into the RKO cells using the synthetic cationic lipid components, Lipofectamine reagent, according to manufacturer's instructions (Promega) (28). Following overnight incubation, Geneticin (600 μg/ml G418, Sigma-Aldrich) was added to the culture wells, and the cells were cultured to select the transfected cells for 3 weeks. Subsequently, the cells were plated at limiting dilution to isolate transfectants. Survival clones were isolated, transferred to 35-mm dishes, and grown in medium without Geneticin. We obtained clones 1 and 2 of transfectants with the stable expression of regucalcin, and the regucalcin levels in these clones were increased by 7.4- or 10.9-fold as compared with those of the wild-type cells, respectively as shown in Fig. 2. Clone 2 was used in the following experiments.

Colony formation assay. The RKO wild-type cells or transfectants were seeded into 6-well dishes at a density of $1x10^3$/well and cultured in medium containing 10% FBS and 1% P/S under the condition of 5% CO_2 and 37˚C for 7 days, when visible clonies were formed on the plates (29). The obtained colonies were washed with PBS and fixed with methanol (0.5 ml per well) for 20 min at room temperature, and then washed 3 times with PBS. Finally, colonies were stained with 0.5% crystal violet for 30 min at room temperature. Stained cells were washed 4 times with PBS. 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 proliferation assay. The RKO wild-type cells ($1x10^5$/ml per well) or ROK cells ($1x10^5$/ml per well) transfected with regucalcin cDNA were cultured in DMEM containing 10% FBS and 1% P/S using a 24-well plate for 1, 2, 3, 4 or 7 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C, as previously described (23,26,30). In separate experiments, RKO wild-type cells or transfectants were cultured in DMEM containing 10% FBS and 1% P/S in the presence of either sodium butyrate (10 and 100 μM), roscovitine (10 and 100 nM), sulphoraphan (1 and 10 nM),

staurosporin (10 or 100 nM), TNF-α (0.1 or 1 μg/ml), PD98059 (1 or 10 μM), wortmannin (0.1 or 1 μM), DRB (0.1 or 1 μM), or gemcitabine (50 or 100 nM) for 3 days. After culture, the cells were detached from each culture dish by the addition of sterilized 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, and then 0.9 ml of DMEM containing 10% FBS and 1% P/S were added to each well. The cell number in the suspended medium was counted as described below in the section 'Cell counting'.

Cell death assay. The RKO wild-type cells (1x10^5/ml per well) cells or RKO cells (1x10^5/ml per well) transfected with regucalcin cDNA were cultured in DMEM containing 10% FBS and 1% P/S using a 24-wells plate for 3 days until reaching subconfluency, and they were then cultured for an additional 24 h in the presence or absence of either Bay K 8644 (0.1 or 1 μM) or gemcitabine (10 or 100 nM) (31). In separate experiments, RKO wild-type cells (1x10^5/ml per well) or transfectants were cultured for 3 days until reaching subconfluency, and were then cultured for an additional 24 h in the presence or absence of either Bay K 8644 (1 μM) or gemcitabine (10 or 100 nM) with or without caspase-3 inhibitor (10 μM) for 24 h, as previously described (31). After culture, the cells were detached by the addition of sterilized solution (0.1 ml per well) of 0.05% trypsine plus EDTA in Ca^{2+}/Mg^{2+}-free PBS into each well as described above in the section 'Cell proliferation assay', and the cell number was then counted as described below in the section 'Cell counting'.

Cell counting. To detach the cells on each well, the culture dishes were incubated for 2 min at 37˚C after the addition of the solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS, and the cells were then detached through pipetting following the addition of DMEM (0.9 ml) containing 10% FBS and 1% P/S (23,26,30,31). Medium containing the suspended cell (0.1 ml) was mixed by the addition of 0.1 ml of 0.5% trypan blue staining solution. The number of cells with viability were counted under a microscope (Olympus MTV-3, Olympus) using a hemocytometer plate (Sigma-Aldrich) with a cell counter (Line Seiki H-102P; Line Seiki Co., Ltd., Tokyo, Japan). For each dish, we took the average of two counting. The Cell number was shown as the number per well of plate.

Western blot analysis. The RKO wild-type cells, control vector cDNA-transfected cells, or regucalcin cDNA-transfected cells were plated in 100-mm dishes at a density of 1x10^6 cells/well in 10 ml of DMEM containing 10% FBS and 1% P/S, and were cultured for 3 days. Following culture, the cells were washed 3 times with cold PBS and removed from the dish by scraping using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of the 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 10 min. The protein concentration of the supernatant obtained was determined for western blotting using the Bio-Rad Protein Assay Dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as a standard. The supernatant was then stored at -80˚C until use. Samples of 40 μg of supernatant protein per lane were separated by SDS polyacrylamide gel electrophoresis (12%, SDS-PAGE) and transferred onto nylon membranes for immunoblotting using specific antibodies against various proteins, which were obtained from Cell Signaling Technology including Ras (#14429 rabbit), Akt (#9272, rabbit), phospho-Akt (#9271, rabbit), mitogen-activated protein kinase (MAPK; #4695, rabbit), phospho-MAPK (#4370, rabbit), SAPK/JNK (#9252, rabbit), phospho-SAPK/JNK (#9251, rabbit), PI3 p110α (#4255, rabbit), Rb (#9309, mouse), p21 (#2947, rabbit), c-jun (#9165, rabbit), β-catenin (#9581, rabbit), signal transducer and activator of transcription 3 (Stat3; #12640, rabbit), phospho-Stat3 (#9131, rabbit) and β-actin (#3700, mouse) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) including p53 (sc-126, mouse), c-fos (sc-52, rabbit), NF-κB p65 (sc-109, rabbit) β-catenin (sc-39350, mouse) (28). Rabbit anti-regucalcin antibody was obtained from Abcam (Cambridge, MA, USA; diluted 1:1000, ab213459, rabbit), and it has been used previously (22,28,32). The target protein was incubated with one of the primary antibodies (1:1,000) including phosphorylated types of various proteins (as described above) overnight at 4˚C, and followed by horseradish peroxidase-conjugated secondary antibodies (mouse sc-2005 or rabbit sc-2305; Santa Cruz Biotechnology, Inc.; diluted 1:2,000). The immunoreactive blots were visualized with a SuperSignal West Pico Chemiluminescent Substrate detection system (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. β-actin (#3700, mouse; Cell Signaling Technology; diluted 1:2,000) was used as a loading control. Three blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and bands quantified using ImageJ software.

Statistical analysis. 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 the Tukey-Kramer multiple comparisons post hoc test for parametric data as indicated. Survival curves were constructed by Kaplan-Meier analysis and were compared with the log-rank test as performed with IBM SPSS software. Other data were analyzed with the paired or unpaired Student's t-test as performed with IBM SPSS Statistics 18 software (IBM, Chicago, IL, USA http://www.ibm.com). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

A higher regucalcin gene expression is associated with the prolonged survival of patients with colorectal cancer. To determine the involvement of regucalcin in patients with colorectal cancer, we analyzed the expression levels of regucalcin in colorectal tumor tissues of human subjects. We compared regucalcin gene expression in the tumor tissues of patients with colorectal cancer. The patients with colorectal cancer were classified into 2 groups: One with a high (31 patients) and another with a low (31 patients) mRNA expression of regucalcin in the colorectal tumor tissues. As the group of patients with a higher regucalcin mRNA expression was compared to the group of patients with a lower regucalcin

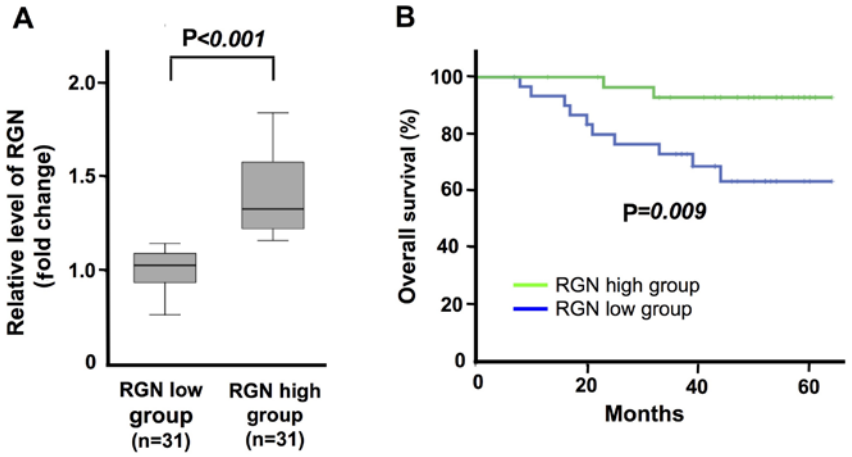

Figure 1. Decreased regucalcin gene expression is assocaited with a poor outcome of patients with colorectal cancer. (A) The group of patients was separated into 2 groups (high and low of regucalcin gene expression). The group with a high regucalcin gene expression exhibited a statistically significant difference as compared with the low expression group. (B) Kaplan-Meier curve indicated that survival curves of the patients with colorectal cancer were significantly prolonged in the high regucalcin gene expression group as compared with those of the low expression group. RGN, regucalcin.

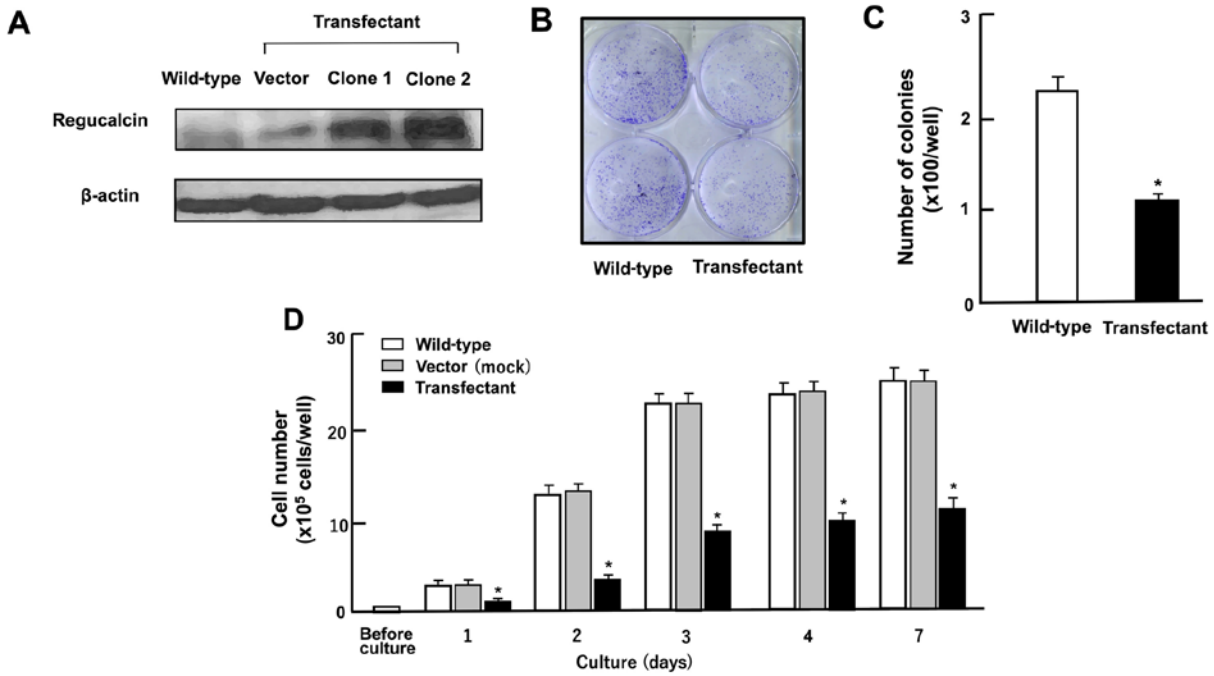

Figure 2. Overexpression of regucalcin suppresses the colony formation and proliferation of human colorectal cancer RKO cells *in vitro*. (A) Regucalcin expression in the cells cultured for 3 days was analyzed by western blot analysis with an anti-regucalcin antibody. The lanes from left to right are as follows: Lane 1, wild-type cells; lane 2, cells transfected with empty vector/pCXN2 (designated as vector); lanes 3 or 4; cells (clone 1 or 2) transfected with the human regucalcin cDNA /pCXN2. (B and C) In colony formation, RKO wild-type cells and transfectants were cultured for 7 days. After culture, colonies were stained cells with 0.5% crystal violet, and stained colonies was counted. (B) Image of colony formation. (C) Colonies containing >50 cells was counted under a microscope. (D) In cell proliferation assay, RKO wild-type cells, vector or clone 2 were cultured in DMEM for 1, 2, 3, 4 or 7 days. After culture, the number of cells attached on the dish was counted. Data are presented as the means ± SD obtained from 8 wells of 2 replicate plates per dataset using different dishes and cell preparation. *P<0.001 vs. wild-type (white bar) or control vector (grey bar), determined by one-way ANOVA with the Tukey-Kramer post hoc test.

mRNA expression, a significant difference was found between the 2 groups (Fig. 1A). The analysis of Kaplan-Meier curve revealed that the survival of the group with a higher regucalcin mRNA expression in the colorectal tumor tissues was predominantly prolonged as compared with that of the group with a lower regucalcin mRNA expression (Fig. 1B). This result supports the view that a decreased regucalcin gene expression leads to the development of carcinogenesis in human colorectal cells, and that this causes a worse clinical outcome. However, further studies using multiple datasets are warranted to confirm the results of this study.

Overexpression of regucalcin suppresses the growth of RKO cells. To generate regucalcin-overexpressing cells, RKO cells were transiently transfected with the empty pCXN2 vector or human regucalcin cDNA (coding full-length 33 kDa

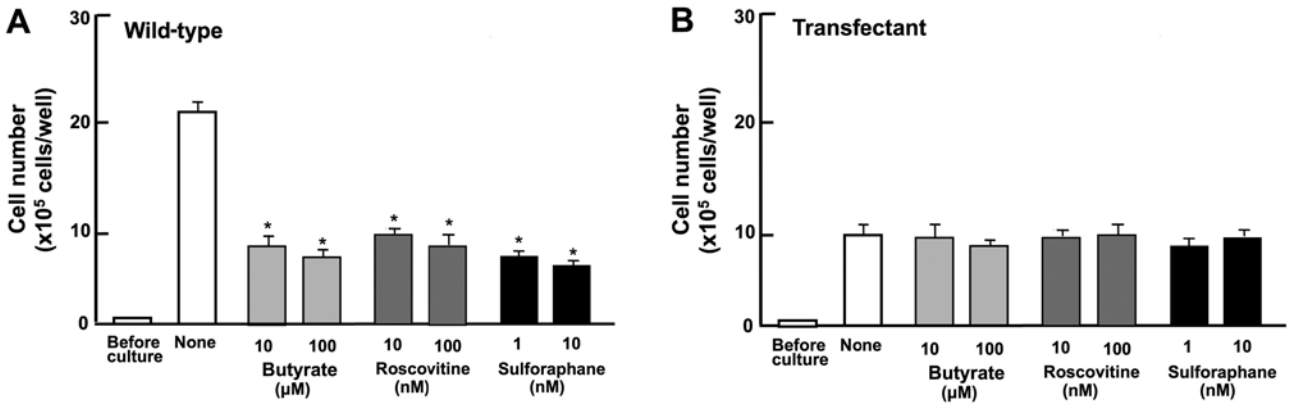

Figure 3. Suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells are implicated in cell cycle arrest. (A) Wild-type cells or (B) transfectants were cultured for 3 days in the absence or presence of butyrate (10 and 100 μM), roscovitine (10 and 100 nM), or sulforaphane (1 and 10 nM). After culture, the number of cells attached on dish was counted. Data are presented as the means ± SD of 2 replicate wells per dataset using different dishes and cell preparation. *P<0.001 vs. control (none; white bar), as determined by one-way ANOVA with the Tukey-Kramer post hoc test.

protein)/pCXN2 construct using Lipofectamine. We isolated clone 1 and 2 of transfectants with a stable expression of regucalcin, and the regucalcin levels were higher in clone 2 as compared with those of clone 1 (Fig. 2A). Thus, clone 2 was used to determine the effects of the overexpression of regucalcin on the growth of RKO cells *in vitro*. Firstly, to determine the effects of the overexpression of regucalcin on colony formation, RKO wild-type cells and transfectants were cultured for 7 days (Fig. 2B and C). The number of colonies formed was found to decrease in the regucalcin-overexpressing transfectants as compared with the wild-type cells (Fig. 2B and C). The proliferation of the wild-type RKO cells was markedly enhanced with increasing days of culture (Fig. 2D). This enhancement of proliferation was clearly suppressed in the transfectants (Fig. 2D). Thus, the overexpression of regucalcin suppressed the colony formation and proliferation of human colorectal cancer RKO cells.

Suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells involve various signaling pathways. To determine the mechanisms through which the overexpression of regucalcin suppresses the proliferation of RKO cells, it was investigated whether the revelation of suppressive effects of the overexpression of regucalcin are attenuated 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) (33), roscovitine (10 and 100 nM) (34) or sulforaphane (1 and 10 nM) (35). The proliferation of the wild-type cells was suppressed in the presence of these inhibitors (Fig. 3A). The effects of these inhibitors were not potentiated in the transfectants (Fig. 3B). Thus, this result suggested that the overexpression of regucalcin induces G1 and G2/M phase cell cycle arrest in the RKO cells, although further experiments using immunofluorescence assay are required to confirm our findings.

Next, we determined the involvement of signaling factors in the suppressive effects induced by the overexpression of regucalcin on cell proliferation. The proliferation of RKO wild-type cells was suppressed by staurosporine (10 or 100 nM), a calcium signaling protein kinase C-related inhibitor (36,37), and PD98059 (1 or 10 μM), an inhibitor of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) (38), and wortmannin (10 or 100 nM), an inhibitor of phosphatidylinositol 3-kinase (PI3K) (39) (Fig. 4A). The blocking of these pathways did not potentiate the suppressive effects of the overexpression of regucalcin on cell proliferation (Fig. 4B).

DRB is an inhibitor of the transcriptional activity with RNA polymerase II inhibition (40). Gemcitabine is a potent antitumor agent that induces nuclear DNA damage (41). In this study, these inhibitors suppressed the proliferation of wild-type cells (Fig. 4C). However, such effects were not potentiated in the transfectants (Fig. 4D). These results suggest that the overexpression of regucalcin suppresses various signaling processes linked to cell proliferation, and that regucalcin-overexpressing cells exhibit a lack of responses to the above-mentioned inhibitors of these pathways.

Suppressive effects of the overexpression of regucalcin on colorectal carcinoma cell proliferation are independent of cell death. The effects of the overexpression of regucalcin on the death of RKO cells were then investigated. The wild-type cells or transfectants were cultured for 3 days until reaching subconfluency, and they were then cultured for 24 h after the addition of various stimulatory factors that induce apoptotic cell death. The number of wild-type cells was decreased by culture with Bay K 8644 (0.1 and 1 μM) or gemcitabine (10 or 100 nM), which are known to induce apoptotic cell death (20,31) (Fig. 5A). The overexpression of regucalcin did not lead to the death of the wild-type cells, and the apoptotic cell death-inducing factors did not cause the cell death of the transfectants (Fig. 5B). This result suggests that the suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells are not a result of cell death.

Moreover, it was investigated whether the effects of overexpressed regucalcin on cell death are mediated through caspase-3. The RKO wild-type cells and transfectants, upon reaching subconfluency, were cultured in the presence of Bay K 8644 (1 μM) or gemcitabine (100 nM) with or without caspase-3 inhibitors (10 μM) for 24 h (Fig. 5C and D). The effects of Bay K 8644 or gemcitabine on cell death were not observed in the presence of the caspase-3 inhibitor (Fig. 5C).

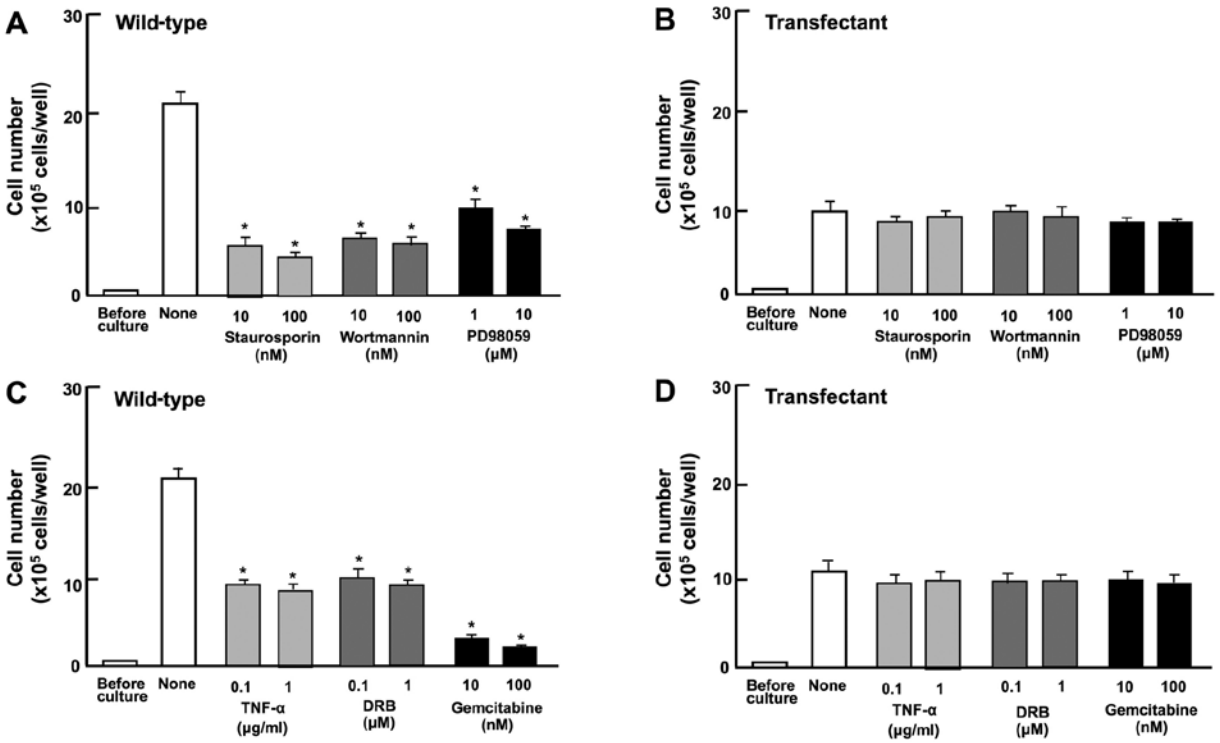

Figure 4. Suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells are exerted through various signaling pathways. (A and C) Wild-type cells or (B and D) regucalcin-overexpressing transfectants were cultured in the absence or presence of staurosporine (10 or 100 nM), PD98059 (1 or 10 μM), wortmannin (10 or 100 nM), TNF-α (0.1 or 1 μg) , DRB (0.1 or 1 μM) or gemcitabine (10 or 100 nM) for 3 days. After culture, the number of cells attached on dish was counted. Data are presented as the means ± SD of 2 replicate wells per dataset using different dishes and cell preparation. *P<0.001 vs. control (none; white bar), as determined by one-way ANOVA with the Tukey-Kramer post hoc test.

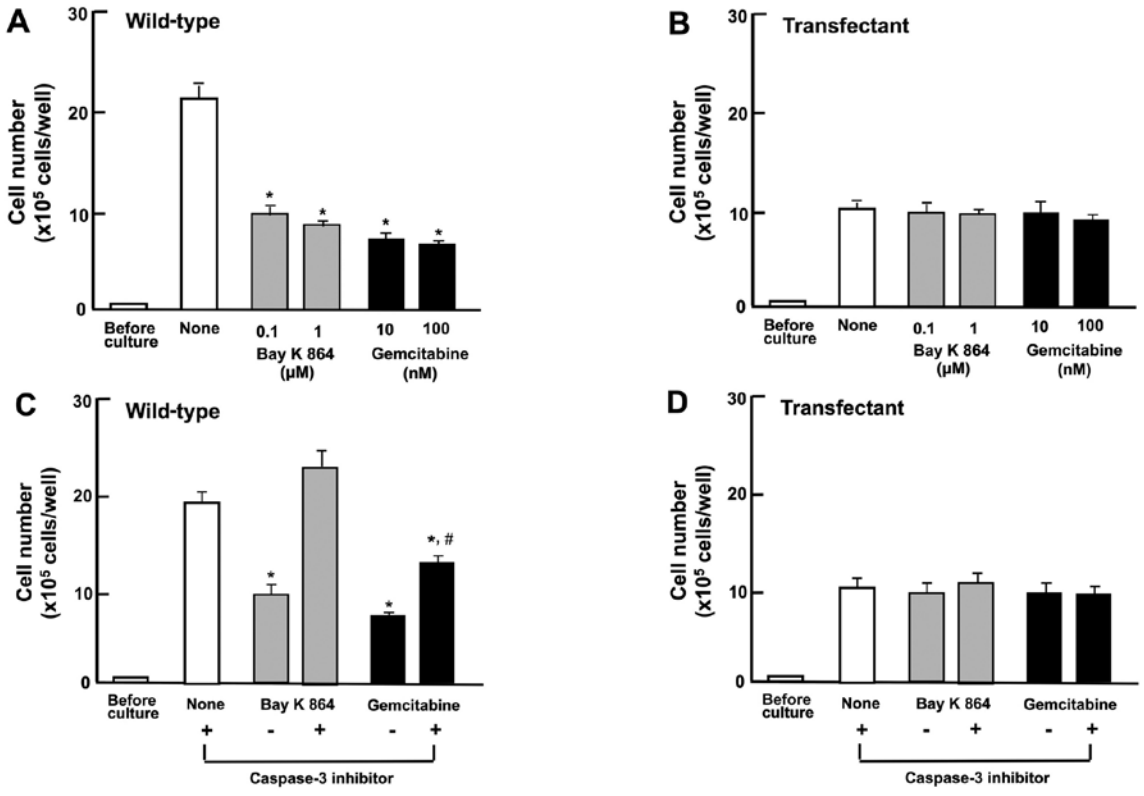

Figure 5. Overexpression of regucalcin prevents the death induced by stimulation with Bay K 8644 or gemcitabine in RKO cells *in vitro*. Wild-type cells or transfectants were cultured for 3 days until reaching subconfluence, and the cells were then cultured for 24 h in the presence of (A and B) Bay K 8644 (0.1 or 1 μM) or gemcitabine (10 or 100 nM) or in the presence of (C and D) Bay K 8644 (1 μM) or gemcitabine (100 nM) with or without caspase-3 inhibitors (10 μM). After culture, the number of cells attached on dish was counted. Data are presented as the means ± SD of 2 replicate wells per dataset using different dishes and cell preparation. *P<0.001 vs. control (none; white bar), as determined by one-way ANOVA with the Tukey-Kramer post hoc test.

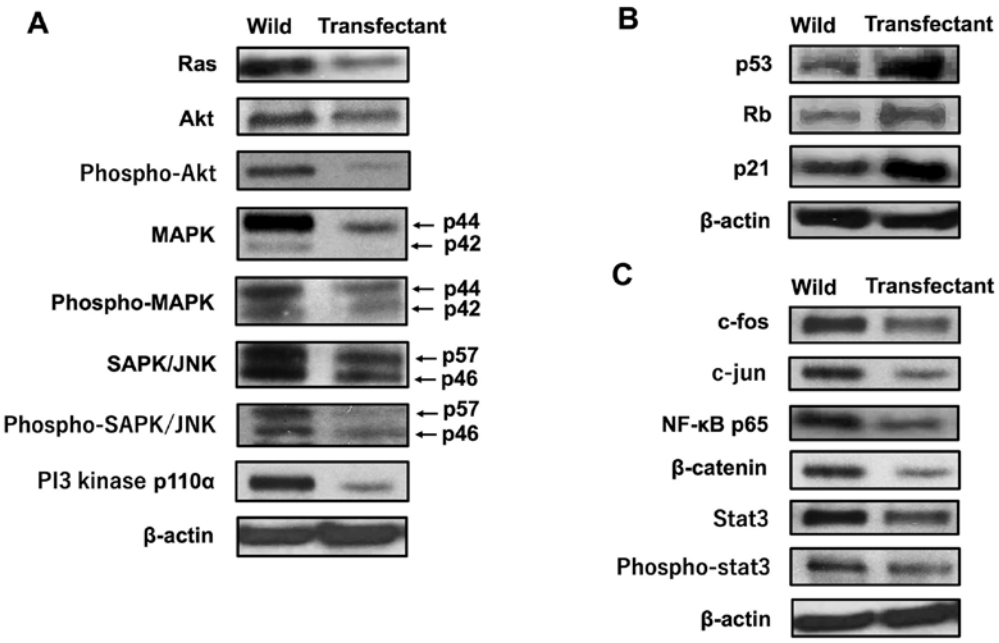

Figure 6. Overexpression of regucalcin regulates various protein levels related to the signaling process and transcriptional activity in RKO cells *in vitro*. Wild-type cells or regucalcin-overexpressing transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 3 days. After culture, the cells were removed from the dish with a cell scraper after cell lysis buffer containing protein inhibitors. Samples of 40 μg of supernatant protein per lane were separated by SDS-PAGE for western blot analysis using antibodies against various proteins. (A) Cell signaling pathway-related proteins. (B) Tumor-related proteins. (C) Transcription-related proteins. Data are presented as data set using the cell preparation obtained from different dishes with replicate.

The stimulatory effects of Bay K 8644 or gemcitabine on cell death were not observed in transfectants cultured with or without caspase-3 inhibitor (Fig. 5D). These findings suggest that regucalcin prevents cell death by decreasing the activity of caspase-3 that activates DNA fragmentation in the nucleus, leading to apoptotic cell death. Thus, the suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells are not a result of cell death.

Overexpression of regucalcin regulates the expression of various proteins related to cell signaling and transcription activity. Mechanistically, it was investigated whether the overexpression of regucalcin regulates the expression of key proteins, which are involved in signaling pathways and transcriptional activity, by western blot analysis. The results revealed that the levels of Ras, Akt, phospho-Akt, MAPK, phospho-MAPK, SAPK/JNK, phospho-SAPK/JNK and PI3 kinase 110α were diminished by the overexpression of regucalcin (Fig. 6A). These results suggest that the overexpression of regucalcin suppresses the activation of Ras-linked signaling pathways in RKO cells. Of note, the overexpression of regucalcin elevated the protein levels of p53 and Rb, tumor suppressors, and that of p21, an inhibitor of the cell cycle (Fig. 6B). In addition, the overexpression of regucalcin diminished the levels of c-fos, c-jun, NF-κB p65, β-catenin, Stat3 and phospho-Stat3 which are transcription factors linked to the proliferation of RKO cells (Fig. 6C). Thus, we determined the changes in the levels of proteins (14 molecules), which may be major signaling proteins related to the proliferation of cancer cells. However, various other proteins are implicated in the proliferation of cancer cells. Thus, further investigations are required to determine involvement of other proteins.

Discussion

In this study, we performed the profiling of gene expression and analysis of the survival of 62 patients with colorectal cancer using the GEO database (GSE12945) for outcome analysis. The prolonged survival of the patients with colorectal cancer was found to be associated with a higher regucalcin gene expression in the tumor tissues. The diminished gene expression of regucalcin was accompanied by a poor prognosis of patients with colorectal cancer. This finding supports the view that the suppression of regucalcin gene expression may contribute to the promotion or aggressiveness of the development of human colorectal cancer. A downregulated regucalcin gene expression may lead to a worse clinical outcome of cancer patients. Moreover, to determine a translational mechanism for this clinical finding, it was investigated whether the overexpression of regucalcin suppresses the proliferation of human colorectal cancer RKO cells *in vitro*. The overexpression of regucalcin was shown to suppress colony formation and the proliferation of RKO cells without inducing direct cell toxicity with necrotic or apoptotic cell death *in vitro*. Thus, this study demonstrated a crucial role of regucalcin as a suppressor in the growth of human colorectal cancer cells. Endogenous regucalcin may play a suppressive role in the development of human colorectal cancer. However, further studies using multiple datasets are warranted to confirm the results of this study.

The mechanistic characterization of the suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells was investigated using various inhibitors that regulate cell signaling pathways. The suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells were not potentiated in the presence of butyrate, roscovitine or sulphoraphan, that induce cell cycle arrest. Butyrate

induces an inhibition of G1 progression (33). Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m and cdk5 (34). Sulforaphane induces G2/M phase cell cycle arrest (35). The overexpression of regucalcin was suggested to cause G1 and G2/M phase cell cycle arrest in RKO cells. Such findings have been shown in various types of cells, including normal rat kidney proximal tubular epithelial NRK52E cells (36), rat hepatoma H4-II-E cells (28) and human cancer cells of various types (23-26) *in vitro*. Importantly, the overexpression of regucalcin has been shown to increase the expression of p21, a cell cycle inhibitor, supporting the view that regucalcin plays a role in cell cycle arrest (23-26).

It was then determined whether regucalcin regulates cell signaling pathways using various inhibitors. The suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells were not potentiated in the presence of dibucaine, an inhibitor of calcium/calmodulin-dependent protein kinases (30), staurosporine, an inhibitor of protein kinase C (37), wortmannin, an inhibitor of the PI3K/Akt signaling pathway (38) and PD98059, an inhibitor of the ERK/MAP kinase-related signaling pathway (39). The overexpression of regucalcin was shown to exert suppressive effects on cell proliferation due to the inhibition of various signaling pathways, namely Ca^{2+}-dependent kinases, PI3K/Akt, and ERK/MAPK in RKO cells. Thus, regucalcin has potential as a suppressor of diverse signaling pathways in human cancer cells. Furthermore, results of western blot analysis revealed that the overexpression of regucalcin induced a decrease in the levels of various proteins that are involved in signaling pathways linked to Ras, Akt, MAPK, SAPK/JNK and PI3 kinase in RKO cells. The suppressive effects of regucalcin, which are mediated through the regulation of various signaling pathways, have also been observed in various types of human cancer cells, including pancreatic MIA-PaCa2 cells (23), MDA-MB-231 human breast cancer cells (24), human liver cancer HepG2 cells (25) and human lung cancer A549 cells (26) *in vitro*.

The suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells were not altered by culture with DRB, an inhibitor of transcriptional activity with RNA polymerase II inhibition (40). The suppressive effects of the overexpression of regucalcin on the proliferation of RKO cells were not potentiated by culture with gemcitabine, which is used in the therapy of human cancer as an antitumor agent that induces DNA damage in the nuclei (41). This drug inhibits the proliferation and stimulates apoptotic cell death in various types of cancer cells (41). Our results suggest that regucalcin partly regulates pathways implicated in the mode of action of gemcitabine. Regucalcin has been demonstrated to directly suppress DNA and RNA synthesis using isolated rat liver nuclei (17-19).

Regucalcin has been shown to play a role in the regulation of cell nuclear function (19). Importantly, the overexpression of regucalcin has been demonstrated to enhance the gene expression levels of p53 and Rb, tumor suppressors, and those of p21, an inhibitor of the cell cycle, and to suppress the gene expression levels of ras, c-jun and c-myc, oncogenes, due to binding to nuclear DNA in cloned rat hepatoma H4-II-E cells *in vitro* (19,42). Moreover, in this study, the overexpression of regucalcin was found to elevate the protein levels of p53, Rb and p21 and to diminish those of ras, c-fos, c-fos, NF-κB p65, β-catenin and Stat3, which are transcription factors linked to cancer cell proliferation, in RKO cells *in vitro*. These findings may support the view that endogenous regucalcin plays a pivotal role in mediating suppressive effects on the growth of cancer cells due to the regulation of the expression of various proteins linked to transcription factors, tumor suppressors and oncogenes linked to tumor development. Regucalcin binds to DNA (42) and regulates the gene expressions of various proteins in the nucleus of normal and cancer cells (19,42).

The overexpression of regucalcin was shown to prevent the colony formation of RKO cells *in vitro*. Such effects of the overexpression of regucalcin on the colony formation of RKO cells may be a result of the suppression of proliferation induced by the overexpression of regucalcin. Colorectal cancer cells may express a particularly aggressive metastatic phenotype of primary neoplastic cells to regional lymph nodes, liver, adrenal glands, contralateral lung, brain and bone marrow (1,2). The overexpression of regucalcin may lead to the suppression of the colony formation and metastasis of colorectal cancer. In addition, we hypothesize that the overexpression of regucalcin may suppress tumor growth in animal models following the transplantation of cancer cells *in vivo*. This remains to be elucidated.

In conclusion, the current study demonstrates that the prolonged survival of patients with colorectal cancer is associated with a higher regucalcin gene expression in the tumor tissues, and that the overexpression of regucalcin suppresses the proliferation of human colorectal cancer RKO cells *in vitro*. A higher expression of endogenous regucalcin plays a potential role as a suppressor of the development of human colorectal cancer, and the downregulation of regucalcin leads to the progression of carcinogenesis. Previously, we demonstrated that a higher regucalcin expression in tumor tissue prolonged the survival of patients with pancreatic cancer (23), breast cancer (24), hepatocellular carcinoma (25) and lung adenocarcinoma (26), and that the overexpression of regucalcin suppressed the growth of their related human cancer cells *in vitro* (23-26). Thus, regucalcin may play a crucial role as a suppressor of carcinogenesis in various types of human cancer. The downregulation of regucalcin gene expression may predispose patients to the promotion of cancer. The delivery of the regucalcin gene, which is overexpressed in tumor tissues, may provide a novel therapeutic strategy for human cancer.

Acknowledgements

The authors would like to thank Dr Oliver Hankinson (David Geffen School of Medicine, University of California, Los Angeles, USA) for his encouragement.

Funding

The present study was supported in part from the Foundation for Biomedical Research on Regucalcin, Japan.

Availability of data and materials

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

Authors' contributions

MY conceived and designed the study. MY, SO and TM performed the experiments and discussed the findings. MY wrote the manuscript, and SO and TM reviewed and edited the manuscript. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Porter MG and Stoeger SM: Atypical colorectal neoplasms. Surg Clin North Am 97: 641-656, 2017.
2. American Cancer Society: Cancer facts & figures 2016. https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2016.html.
3. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
4. Brenner H, Kloor M and Pox CP: Colorectal cancer. Lancet 383: 1490-1502, 2014.
5. Alnabulsi A and Murray GI: Integrative analysis of the colorectal cancer proteome: Potential clinical impact. Expert Rev Proteomics 13: 1-11, 2016.
6. Alnabulsi A, Swan R, Cash B, Alnabulsi A and Murray GI: The differential expression of omega-3 and omega-6 fatty acid metabolising enzymes in colorectal cancer and its prognostic significance. Br J Cancer 116: 1612-1620, 2017.
7. Carini F, Mazzola M, Rappa F, Jurjus A, Geagea AG, Al Kattar S, Bou-Assi T, Jurjus R, Damiani P, Leone A, *et al*: Colorectal carcinogenesis: Role of oxidative stress and antioxidants. Anticancer Res 37: 4759-4766, 2017.
8. Colussi D, Brandi G, Bazzoli F and Ricciardiello L: Molecular pathways involved in colorectal cancer: Implications for disease behavior and prevention. Int J Mol Sci 14: 16365-16385, 2013.
9. Kudryavtseva AV, Lipatova AV, Zaretsky AR, Moskalev AA, Fedorova MS, Rasskazova AS, Shibukhova GA, Snezhkina AV, Kaprin AD, Alekseev BY, *et al*: Important molecular genetic markers of colorectal cancer. Oncotarget 7: 53959-53983, 2016.
10. Jones RP, Sutton PA, Evans JP, Clifford R, McAvoy A, Lewis J, Rousseau A, Mountford R, McWhirter D and Malik HZ: Specific mutations in KRAS codon 12 are associated with worse overall survival in patients with advanced and recurrent colorectal cancer. Br J Cancer 116: 923-929, 2017.
11. Downward J: Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3: 11-22, 2003.
12. Shimokawa N and Yamaguchi M: Molecular cloning and sequencing of the cDNA coding for a calcium-binding protein regucalcin from rat liver. FEBS Lett 327: 251-255, 1993.
13. Shimokawa N, Matsuda Y and Yamaguchi M: Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151: 157-163, 1995.
14. Thiselton DL, McDowall J, Brandau O, Ramser J, d'Esposito F, Bhattacharya SS, Ross MT, Hardcastle AJ and Meindl A: An integrated, functionally annotated gene map of the DXS8026-ELK1 interval on human Xp11.3-Xp11.23: Potential hotspot for neurogenetic disorders. Genomics 79: 560-572, 2002.
15. Yamaguchi M: Suppressive role of regucalcin in liver cell proliferation: Involvement in carcinogenesis. Cell Prolif 46: 243-253, 2013.
16. Yamaguchi M: Involvement of regucalcin as a suppressor protein in human carcinogenesis: Insight into the gene therapy. J Cancer Res Clin Oncol 141: 1333-1341, 2015.
17. Yamaguchi M: Role of regucalcin in maintaining cell homeostasis and function (review). Int J Mol Med 15: 371-389, 2005.
18. Yamaguchi M: Regucalcin and cell regulation: Role as a suppressor protein in signal transduction. Mol Cell Biochem 353: 101-137, 2011.
19. Yamaguchi M: Role of regucalcin in cell nuclear regulation: Involvement as a transcription factor. Cell Tissue Res 354: 331-341, 2013.
20. Yamaguchi M: The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis 18: 1145-1153, 2013.
21. Yamaguchi M (ed): The Role of Regucalcin in Cell Homeostasis and Disorder. Nova Science Publishers, Inc., New York, NY, 2017.
22. Murata T and Yamaguchi M: Alternatively spliced variants of the regucalcin gene in various human normal and tumor tissues. Int J Mol Med 34: 1141-1146, 2014.
23. Yamaguchi M, Osuka S, Weitzmann MN, El-Rayes BF, Shoji M and Murata T: Prolonged survival in pancreatic cancer patients with increased regucalcin gene expression: Overexpression of regucalcin suppresses the proliferation in human pancreatic cancer MIA PaCa-2 cells in vitro. Int J Oncol 48: 1955-1964, 2016.
24. Yamaguchi M, Osuka S, Weitzmann MN, Shoji M and Murata T: 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. Int J Oncol 49: 812-822, 2016.
25. Yamaguchi M, Osuka S, Weitzmann MN, El-Rayes BF, Shoji M and Murata T: Prolonged survival in hepatocarcinoma patients with increased regucalcin gene expression: HepG2 cell proliferation is suppressed by overexpression of regucalcin in vitro. Int J Oncol 49: 1686-1694, 2016.
26. Yamaguchi M, Osuka S, Shoji M, Weitzmann MN and Murata T: Survival of lung cancer patients is prolonged with higher regucalcin gene expression: Suppressed proliferation of lung adenocarcinoma A549 cells in vitro. Mol Cell Biochem 430: 37-46, 2017.
27. Staub E, Groene J, Heinze M, Mennerich D, Roepcke S, Klaman I, Hinzmann B, Castanos-Velez E, Pilarsky C, Mann B, *et al*: An expression module of WIPF1-coexpressed genes identifies patients with favorable prognosis in three tumor types. J Mol Med (Berl) 87: 633-644, 2009.
28. Misawa H, Inagaki S and Yamaguchi M: Suppression of cell proliferation and deoxyribonucleic acid synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84: 143-149, 2001.
29. Fang Z, Tang Y, Fang J, Zhou Z, Xing Z, Guo Z, Guo X, Wang W, Jiao W, Xu Z, *et al*: Simvastatin inhibits renal cancer cell growth and metastasis via AKT/mTOR, ERK and JAK2/STAT3 pathway. PLoS One 17: e62823, 2013.
30. Yamaguchi M and Daimon Y: Overexpression of regucalcin suppresses cell proliferation in cloned rat hepatoma H4-II-E cells: Involvement of intracellular signaling factors and cell cycle-related genes. J Cell Biochem 95: 1169-1177, 2005.
31. Izumi T and Yamaguchi M: Overexpression of regucalcin suppresses cell death in cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-alpha or thapsigargin. J Cell Biochem 92: 296-306, 2004.
32. Yamaguchi M and Isogai M: Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay. Mol Cell Biochem 122: 65-68, 1993.
33. Charollais RH, Buquet C and Mester J: Butyrate blocks the accumulation of CDC2 mRNA in late G1 phase but inhibits both the early and late G1 progression in chemically transformed mouse fibroblasts BP-A31. J Cell Physiol 145: 46-52, 1990.
34. Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, Inagaki M, Delcros JG and Moulinoux JP: Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 243: 527-536, 1997.
35. Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, Kamath R, Brown KD, Zhang L and Baskaran R: Sulforaphane-induced G2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J Biol Chem 279: 25813-25822, 2004.
36. Nakagawa T, Sawada N and Yamaguchi M: Overexpression of regucalcin suppresses cell proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells. Int J Mol Med 16: 637-643, 2005.

37. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F: Staurosporine, a potent inhibitor of phospholipid/Ca^{++}dependent protein kinase. Biochem Biophys Res Commun 135: 397-402, 1986.
38. Serrano-Nascimento C, da Silva Teixeira S, Nicola JP, Nachbar RT, Masini-Repiso AM and Nunes MT: The acute inhibitory effect of iodide excess on sodium/iodide symporter expression and activity involves the PI3K/Akt signaling pathway. Endocrinology 155: 1145-1156, 2014.
39. Pelech SL, Charest DL, Mordret GP, Siow YL, Palaty C, Campbell D, Charlton L, Samiei M and Sanghera JS: Networking with mitogen-activated protein kinases. Mol Cell Biochem 127-128: 157-169, 1993.
40. Palangat M, Grass JA, Langelier MF, Coulombe B and Landick R: The RPB2 flap loop of human RNA polymerase II is dispensable for transcription initiation and elongation. Mol Cell Biol 31: 3312-3325, 2011.
41. Tang SC and Chen YC: Novel therapeutic targets for pancreatic cancer. World J Gastroenterol 20: 10825-10844, 2014.
42. Tsurusaki Y and Yamaguchi M: Role of regucalcin in liver nuclear function: Binding of regucalcin to nuclear protein or DNA and modulation of tumor-related gene expression. Int J Mol Med 14: 277-281, 2004.