Prolonged survival of renal cancer patients is concomitant with a higher regucalcin gene expression in tumor tissues: Overexpression of regucalcin suppresses the growth of human renal cell carcinoma cells *in vitro*

MASAYOSHI YAMAGUCHI[1], SATORU OSUKA[2], OLIVER HANKINSON[1] and TOMIYASU MURATA[3]

[1]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA 90095-1732; [2]Department of Neurosurgery, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30333, USA; [3]Laboratory of Analytical Neurobiology, Faculty of Pharmacy, Meijo University, Nagoya 468-8503, Japan

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Abstract. Renal cell carcinoma (RCC), which is a type of cancer found in the kidney tubule, is among the 10 most frequently occurring human cancers. Regucalcin plays a potential role as a regulator of transcriptional activity, and its downregulated expression or activity may contribute to the promotion of human cancers. In this study, we investigated the involvement of regucalcin in human RCC. Regucalcin expression was compared in 23 normal and 29 tumor samples of kidney cortex tissues of patients with clear cell RCC obtained through the Gene Expression Omnibus (GEO) database (GSE36895). Regucalcin expression was downregulated in the tumor tissues. The prolonged survival of patients with clear cell RCC was demonstrated to be associated with a higher regucalcin gene expression in the TCGA dataset. The overexpression of regucalcin suppressed the colony formation, proliferation and the death of human clear cell RCC A498 cells *in vitro*. Mechanistically, the overexpression of regucalcin induced the G1 and G2/M phase cell cycle arrest of A498 cells through the suppression of multiple signaling components, including Ras, PI3 kinase, Akt and mitogen-activated protein (MAP) kinase. Importantly, the overexpression of regucalcin led to an elevation in the levels of the tumor suppressors, p53, Rb and the cell cycle inhibitor, p21. The levels of the transcription factors, c-fos, c-jun, nuclear factor-κB p65, β-catenin and signal transducer and activator of transcription 3, were suppressed by regucalcin overexpression. On the whole, the findings of this study suggest that regucalcin plays a suppressive role in the promotion of human RCC. The overexpression of regucalcin by gene delivery systems may thus prove to be a novel therapeutic strategy for RCC.

Correspondence to: Dr Masayoshi Yamaguchi, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles (UCLA), 700 Tiverton Avenue, Los Angeles, CA 90095-1732, USA
E-mail: yamamasa11555@yahoo.co.jp

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Introduction

Renal cell carcinoma (RCC) is a type of cancer found in the lining of the kidney tubules, and it is among the 10 most frequently occurring human cancers (1-3). RCC is the second leading cause of mortality associated with urological malignant neoplasms (1-3). The prognosis of patients with RCC remains poor, with the 5-year survival rate remaining between 5 and 12% (1,4). RCC results in a number of symptoms, including weight loss, fever, hypertension, hypercalcemia, night sweats and malaise (5,6). The most common histological subtype is clear cell RCC, accounting for approximately 80-90% of all RCC cases (3). Approximately 30% of patients with RCC have metastatic lesions (7). Smoking tobacco, hypertension and obesity are considered as risk factors for RCC (8).

Advances in the treatment of RCC have been derived from agents approved by the Food and Drug Administration (8). These agents target several pathways, including mammalian target of rapamycin (mTOR), multiple pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), and their receptors, VEGFR and PDGFR (8,9). Despite the development of therapeutic regimens (8), the prognosis of patients with RCC remains poor, mainly due to delayed diagnosis and a relatively high incidence of metastasis (10,11). Although the vast majority of patients exhibit a marked clinical response, the therapeutic effects of these inhibitors are limited due to the development of drug-resistant phenotypes (10,11). Therefore, more potent and specific therapeutic strategies are urgently required in order to identify novel diagnostic and therapeutic targets for RCC (8,12-14). However, the molecular mechanisms underlying RCC tumorigenesis remain elusive (12-14).

The gene for regucalcin, which was discovered as a calcium-binding protein (15,16), is localized on the X chromosome (17-19). Regucalcin is expressed in various types of cells and tissues (20,21), and has been demonstrated to play multifunctional roles in the regulation of manifold cells (21-24). Regucalcin has been shown to maintain calcium homeostasis, inhibit various signaling pathways involving various protein kinases and protein phosphatases, suppress cytosolic protein synthesis and nuclear DNA and RNA synthesis, and regulate nuclear gene expression in cells (22-25). Moreover, regucalcin has been shown to suppress proliferation (26) and apoptosis (27) mediated through various signaling factors in various types of cells. Thus, regucalcin has been shown to play a pivotal role in maintaining cell homeostasis (22,23,28).

Importantly, the gene expression and protein levels of regucalcin have been shown to be downregulated in various tumor tissues of mammalian and human subjects (29,30). We previously demonstrated that regucalcin gene expression was decreased in the tumor tissues of human cancer patients, including those with pancreatic cancer (31), breast cancer (32), liver cancer (33), lung cancer (34) and colorectal cancer (35). The prolonged survival of these cancer patients was shown to be associated with a higher regucalcin expression as compared with a lower regucalcin expression in their tumor tissues (31-35). In addition, the overexpression of regucalcin was shown to exert suppressive effects on the proliferation of human pancreatic cancer MIA PaCa-2 cells (31), MDA-MB-231 breast cancer cells (32), liver cancer HepG2 cells (33), lung adenocarcinoma A549 cells (34) and colorectal cancer RKO cells (35) *in vitro*. These findings support the view that regucalcin plays a crucial role as a suppressor in human cancer cells, and that its downregulated gene expression leads to the development of carcinogenesis in various tissues of human subjects. Regucalcin may therefore be a novel target molecule in the diagnosis and therapy of human cancer.

In the present study, furthermore, we investigated whether regucalcin plays a role as a suppressor in human RCC. Regucalcin is expressed in rat kidney proximal tubular epithelial cells (20,21) and plays a physiological and pathophysiological role in cell regulation and metabolic disorder in the kidney (24). Of note, the gene expression and protein levels of regucalcin are downregulated in the kidney tumor tissues of human subjects (29,30). Regucalcin may thus be a novel target molecule in the diagnosis and therapy of RCC. The involvement of regucalcin in human RCC has not yet been investigated, to the best of our knowledge. Therefore, the involvement of regucalcin in human RCC was investigated in the current study. Of note, it was demonstrated that the survival of patients with clear cell RCC with a higher regucalcin gene expression in their tumor tissues was prolonged, as evaluated by the analysis of gene expression using the Gene Expression Omnibus (GEO) database (GSE36895). Moreover, the overexpression of regucalcin was found to suppress the growth of clear cell human RCC A498 cells *in vitro*. The current findings furthermore support the view that the downregulation of regucalcin gene expression predisposes patients to various types of cancer. Targeting regucalcin may thus prove to be of clinical significance in the suppression of cancer development. A delivery system with the regucalcin gene may provide a novel therapeutic strategy for human renal cancer.

Materials and methods

Materials and reagents. Dulbecco's modified 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, wortmannin, PD98059, staurosporine, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), lipopolysacchaide (LPS), caspase-3 inhibitor and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Gemcytabine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Gemcitabine and caspase-3 inhibitor were diluted in phosphate-buffered saline (PBS) and the other reagents were dissolved in 100% ethanol prior to use.

Patient datasets. A curated gene expression dataset comprising 23 normal and 29 tumor samples of the kidney cortex tissues of patients with clear cell RCC were obtained through the GEO database (GSE36895) for the analysis of regucalcin expression (36). These datasets contained gene expression data derived from the Affymetrix U133_plus2 platform. For microarray analysis, the 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). The Spotfire Decision Site for Functional Genomics software package (TIBCO Software, Palo Alto, CA, USA) was used for visualization of the microarray data. For protein expression analysis, a dataset of immunohistochemistry was obtained from the Human Protein Atlas (HPA; www.proteinatlas.org), which is a database of proteins in human normal tissues and cancers (37,38). These were non-normally distributed data. We also evaluated regucalcin expression in 3 normal tissues and 12 renal adenocarcinoma tissues from the kidneys of patients by using the dataset of 2 antibodies (HPA029102 and HPA029103) for regucalcin. Moreover, we used the TCGA dataset of 468 patients with clear cell RCC (39) for outcome analysis. The data for regucalcin expression and clinical annotation were obtained by SurvExpress (40).

Human renal cell carcinoma cells. We used A498 cells obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). A498 cells are a renal proximal tubular epithelial cell line originating from a male adult patient with clear cell renal cell carcinoma (RCC), and are non-metastatic cells (41). The A498 cells are suitable as a transfection host. The cells were cultured in a DMEM containing 10% FBS and 1% P/S.

Transfection of regucalcin cDNA. The A498 cells were transfected with the empty pCXN2 vector or pCXN2 vector expressing cDNA encoding human full length (900 bp) regucalcin (regucalcin cDNA/pCXN2). These vectors were prepared as used in our previous study (42). For transient

transfection assays, the A498 cells were grown on 24-well plates to approximately 70-80% confluence. The regucalcin cDNA/pCXN2 or empty pCXN2 vector were transfected into A498 cells using the synthetic cationic lipid, Lipofectamine reagent, according to the manufacture's instructions (Promega, Madison, WI, USA) (42). Following overnight incubation, Geneticin (600 μg/ml G418; Sigma-Aldrich) was added to the wells for selection, and the cells were cultured for 3 weeks. The surviving cells were plated at limiting dilution to isolate transfectants. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in medium without geneticin. We obtained transfectant clones 1 and 2 exhibiting stable expression of regucalcin. The regucalcin levels in these clones were markedly increased as compared with those in the wild-type cells as shown in Fig. 2A and B. The regucalcin levels in clone 1 was higher than that of clone 2. Clone 1 was used in the following experiments.

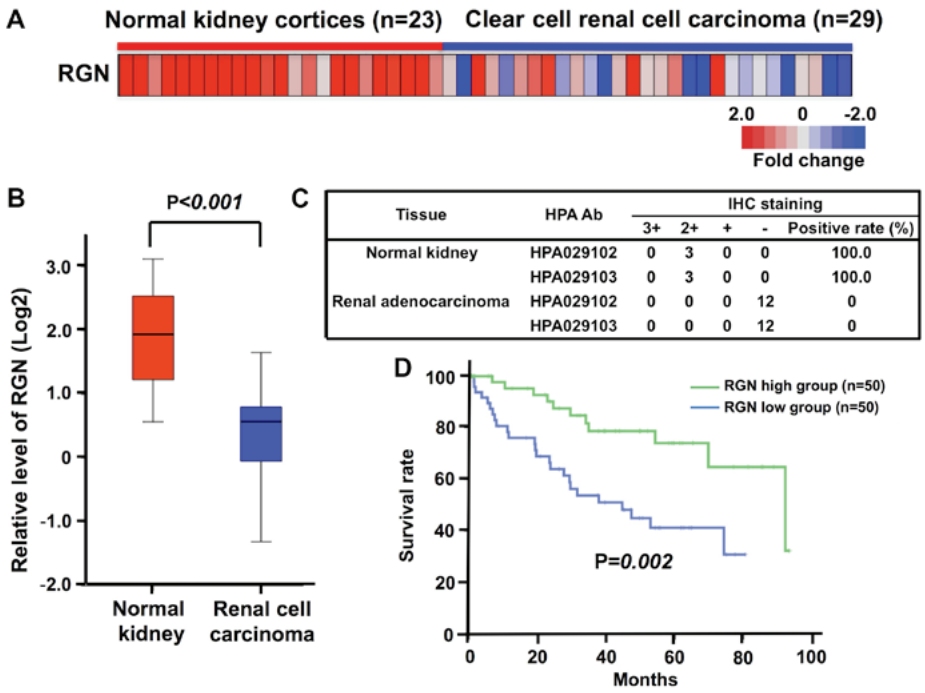
Colony formation assay. The A498 wild-type cells or transfectants were seeded into 6-well dishes at a density of $1x10^3$/well and cultured in medium containing 10% FBS and 1% P/S under conditions of 5% CO_2 and 37˚C for 8 days, when visible clones were formed on the plates (43). The colonies were washed with PBS and fixed with methanol (0.5 ml per well) for 20 min at room temperature, and then washed 3 times with PBS. The colonies were then 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 and growth assays. The A498 wild-type cells ($1x10^5$/ml per well) and A498 cells transfected with the regucalcin cDNA ($1x10^5$/ml per well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S for 1, 2, 3, 4 or 6 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C (42,44). In separate experiments, the A498 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), wortmannin (0.1 or 1 μM), PD98059 (1 or 10 μM), staurosporine (10 or 100 nM), TNF-α (0.1 or 1 ng/ml), DRB (0.1 or 1 μM), or gemcitabine (1 or 10 nM) for 3 days. Following culture, the cells were detached from each culture dishes by adding a sterile solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg-free PBS (Thermo Fisher Scientific, Waltham, MA, USA) with incubation for 2 min at 37˚C. Each well was then supplemented with 0.9 ml of DMEM containing 10% FBS and 1% P/S. The cell number in the cell suspension was counted as described below in the section 'Cell counting'.

Cell death assay. The A498 wild-type cells ($1x10^5$/ml per well) cells and A498 cells transfected with the regucalcin cDNA ($1x10^5$/ml per well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S for 3 days. Upon reaching subconfluency, they were cultured for an additional 24 h in the presence or absence of either TNF-α (0.1 or 1 ng/ml) or LPS (0.1 or 1 μg/ml) (45). In separate experiments, the A498 wild-type cells ($1x10^5$/ml per well) or transfectants were cultured for 3 days, and upon reaching subconfluency, then cultured for an additional 24 h in the presence or absence of either TNF-α (1 ng/ml) or LPS (1 μg/ml) with or without caspase-3 inhibitor (10 μM) for 24 h (45). Following culture, the cells were detached by the 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 above in the section of 'Cell proliferation assay', and the cell number was counted as described below in the section 'Cell counting'.

Cell counting. To detach cells on each well, the culture dishes were incubated for 2 min at 37˚C following the addition of a solution (0.1 ml per well) of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS, and the cells were detached through pipetting after the addition of DMEM (0.9 ml) containing 10% FBS and 1% P/S (31,42,44,45). Medium containing the suspended cells (0.1 ml) was mixed by the addition of 0.1 ml of 0.5% trypan blue staining solution. The number of viable cells was counted under a microscope (Olympus MTV-3; Olympus Corporation) with a hemocytomete (Sigma-Aldrich) using a cell counter (Line Seiki H-102P, Tokyo, Japan). For each dish, we took the average of 2 counts. Cell numbers are shown as number per well.

Western blot analysis. The A498 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. Following culture for 3 days, the cells were washed 3 times with cold PBS and removed from the dish by scraping using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with the addition of protease and protein phosphatase inhibitors (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 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 stored at -80˚C until used. 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 obtained from Cell Signaling Technology, Inc. including Ras (#14429 rabbit), PI3 p1100 α (#4255, rabbit), Akt (#9272, rabbit), phosphor-Akt (#9271, rabbit), mitogen-activated protein kinase (MAPK; #4695, rabbit), phosphor-MAPK (#4370, rabbit), Rb (#9309, mouse), p21 (#2947, rabbit), c-jun (#9165, 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), nuclear factor (NF)-κB p65 (sc-109, rabbit) and β-catenin (sc-39350, mouse). Rabbit anti-regucalcin antibody was obtained from Abcam (Cambridge, MA, USA; ab213459, rabbit), as has been used previously (22,28,32). Target proteins were incubated with one of the primary antibodies (1:1,000) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., mouse sc-2005 or rabbit sc-2305; diluted



Tissue	HPA Ab	IHC staining				
		3+	2+	+	-	Positive rate (%)
Normal kidney	HPA029102	0	3	0	0	100.0
	HPA029103	0	3	0	0	100.0
Renal adenocarcinoma	HPA029102	0	0	0	12	0
	HPA029103	0	0	0	12	0

Figure 1. Reduced regucalcin expression is involved in the poor outcome of patients with clear cell renal cell carcinoma (RCC). (A) Microarray expression analysis of regucalcin in 23 normal kidney cortices and 29 clear cell RCC (GSE36895). Each colored square on the bottom right represents the relative mean transcript abundance; highest expression shown in red color, average expression showed white, and lowest expression shown in blue. (B) Quantification of regucalcin expression in the kidney tissues of normal subjects and tumor tissues with clear cell RCC presented in (A). Regucalcin expression was suppressed in patients with clear cell RCC. (C) The degree of immunohistochemical staining for regucalcin in 3 tissues of normal kidney and 12 tissues of renal adenocarcinoma in the immunohistochemistry database (The Human Protein Atlas). Regucalcin levels were reduced in clear cell RCC patients. The results of 2 antibodies are indicated. IHC staining scores: 3+, strong; 2+, moderate; +, weak; -, negative. (D) Survival curves for patients with clear cell RCC were prolonged in the subjects with a high expression of regucalcin as compared with those with low expression in TCGA dataset. HPA, The Human Protein Atlas; IHC, immunohistochemistry; RGN, regucalcin.

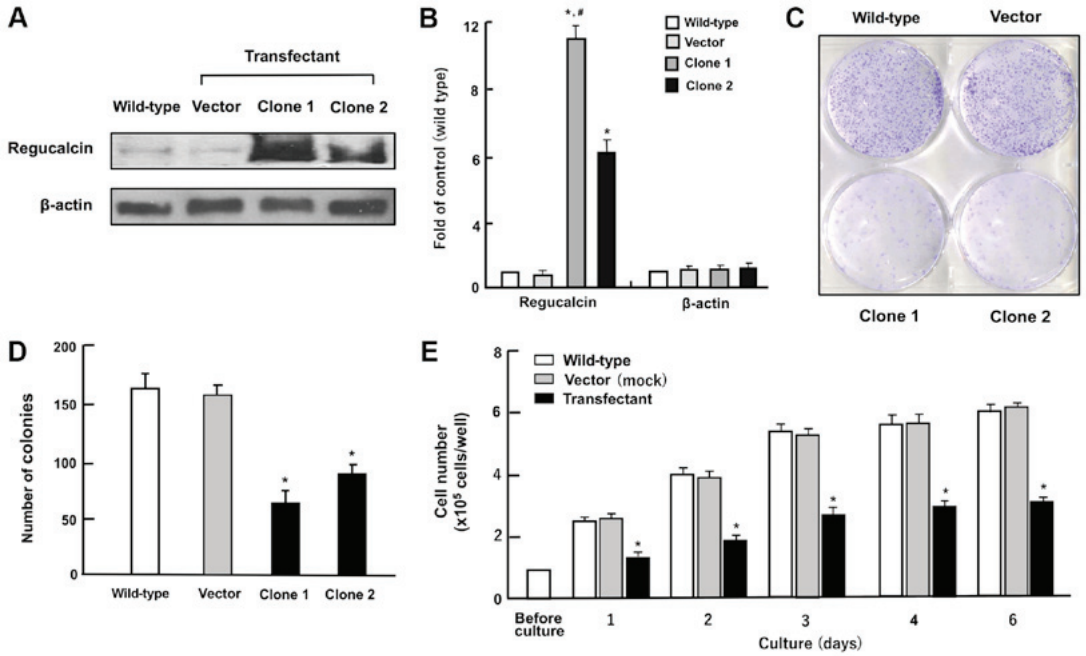


Figure 2. Overexpression of regucalcin suppresses colony formation and proliferation in human clear cell RCC A498 cells *in vitro*. (A and B) Regucalcin content in the cells cultured in DMEM containing 10% FBS and 1% P/S for 3 days as analyzed by western blot analysis with an anti-regucalcin antibody. 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. A representative of 5 films is shown. (A) Representative image. (B) Fold of control; *P<0.001 versus wild-type cells, and $^{\#}$P<0.001 versus clone 2, determined by the Student's t-test. (C and D) Colony formation. The A498 wild-type cells and transfectants (vector, clone 1 or 2) were cultured for 8 days, and the colonies then stained with 0.5% crystal violet and counted. (C) Representative image. (D) Colonies containing >50 cells were counted under a microscope. (E) In the cell proliferation assay, A498 wild-type cells or clone 1 were cultured in DMEM for 1, 2, 3, 4 or 6 days, and the numbers of attached cells were counted. Data are presented as the means ± SD obtained from 8 wells of 2 replicate plates per data set using different dishes and cell preparations. *P<0.001 versus wild-type cells (white bar) or control vector (grey bar), determined by one-way ANOVA with the Tukey-Kramer post hoc test.

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 (diluted 1:2,000; Cell Signaling Biotechnology, Inc.; #3700, mouse) was used as a loading control. Three blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and bands quantified using Image J 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 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. The non-normally distributed data in Fig. 1B were analyzed by the Mann-Whitney test and the rest of the data were analyzed with the Student's t-test to compare the means as performed with IBM SPSS Statistics 18 software (IBM, Chicago, IL, USA; http://www.ibm.com). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Survival of patients with renal cancer with a higher regucalcin gene expression is prolonged. To evaluate the potential involvement of regucalcin in human clear cell RCC, a curated gene expression dataset comprising 23 normal and 29 tumor samples in the kidney cortex tissues of patients with clear cell RCC was obtained through the GEO database (GSE36895) for the analysis of regucalcin expression (36). We compared regucalcin gene expression in the tumor tissues of patients with clear cell RCC using microarray data from the GEO database. Overall, regucalcin expression was found to be visually decreased in the tumor tissues as compared with that in the normal tissues derived from the kidney cortex of patients with clear cell RCC (Fig. 1A). Quantitative analysis confirmed that the expression of regucalcin in the tumor tissues of the kidney cortex of patients with clear cell RCC was markedly decreased as compared with that in the normal tissue of the patient kidneys (Fig. 1B). To confirm the reduction in regucalcin levels, we analyzed the expression of regucalcin in 3 tissues of normal kidneys and 12 tissues of renal adenocarcinoma in the immunohistochemistry database (The Human Protein Atlas). The results from 2 independent regucalcin antibodies revealed that the expression of regucalcin in renal cancer patients was clearly suppressed as compared with that in the normal kidneys (Fig. 1C). Moreover, to determine whether the reduced regucalcin expression is associated with prognosis, we compared the outcome of patients with clear cell RCC with a high or low level of regucalcin mRNA expression in the tumor tissues using Kaplan-Meier curve analysis. To this end, we analyzed the outcome of 468 patients with clear cell RCC using the TCGA dataset, and used data with higher (50 patients)/lower (50 patients) groups defined as top/bottom 10%, respectively (Fig. 1D). A reduced regucalcin expression was found to be associated with a poor prognosis of patients with clear cell RCC (Fig. 1D). These results support the view that the reduced regucalcin gene expression significantly contributes to the development of carcinogenesis in human clear cell RCC cells, leading to a worse clinical outcome.

Overexpression of regucalcin suppresses the growth of A498 cells. To generate regucalcin-overexpressing cells, human clear cell RCC A498 cells were transiently transfected with the empty pCXN2 vector or the vector containing full length (33 kDa protein) human regucalcin using lipofection. We obtained transfectant clone 1 or 2 with stable expression of regucalcin. The regucalcin levels in these clones were increased 11.2- or 6.1-fold as compared with wild-type cells, respectively (Fig. 2A and B). To determine the effects of the overexpression of regucalcin on the growth of A498 cells *in vitro*, clone 1 was used in the following experiments. First, to determine the effects of the overexpression of regucalcin on colony formation, the A498 wild-type cells and transfectants were cultured for 8 days when colony formation clearly appeared (Fig. 2C). The number of colonies was found to be decreased in the regucalcin-overexpressing transfectants (vector, clone 1 or 2) as compared with that of the wild-type cells (Fig. 2D). The mass growth of the wild-type A498 cells was enhanced with the increasing days of culture periods (Fig. 2E). This enhancement was clearly suppressed in the transfectants (Fig. 2E). Thus, the overexpression of regucalcin suppressed the colony formation and proliferation of human renal cancer A498 cells.

Suppressive effects of the overexpression of regucalcin on cell growth are independent of cell death. The effect of the overexpression of regucalcin on the death of A498 cells was then investigated. The A498 wild-type cells and transfectants were cultured for 3 days to reach subconfluency. They were then cultured for a further 24 h following the addition of various factor known to induce apoptotic cell death (27,45). The number of wild-type cells was decreased by culture with TNF-α (0.1 or 1 ng/ml) or LPS (0.1 or 1 μg/ml) (Fig. 3A). 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. 3B). This suggests that the suppressive effects of regucalcin overexpression on cell growth do not result from the death of A498 cells.

We then investigated whether the effects of overexpressed regucalcin on cell death are mediated via caspase-3, which activates nuclear DNA fragmentation, inducing apoptotic cell death (16). Regucalcin has been demonstrated to suppress nuclear DNA fragmentation due to the inhibition of caspase-3 activity in isolated rat liver nuclei (16) and to induce apoptotic cell death in cloned rat hepatoma H4-II-E cells induced by TNF-α or thapsigargin (45). The A498 wild-type cells and transfectants, upon reaching subconfluency were cultured in the presence of TNF-α (1 ng/ml) or LPS (1 μg/ml) with or without caspase-3 inhibitor (10 μM) for 24 h (Fig. 3C and D). The effects of LPS or TNF-α on cell death were either not observed or diminished in the presence of caspase-3 inhibitor (Fig. 3C). The stimulatory effects of TNF-α or LPS on cell death were either not observed or diminished in transfectants cultured with or without caspase-3 inhibitor (Fig. 3D). These

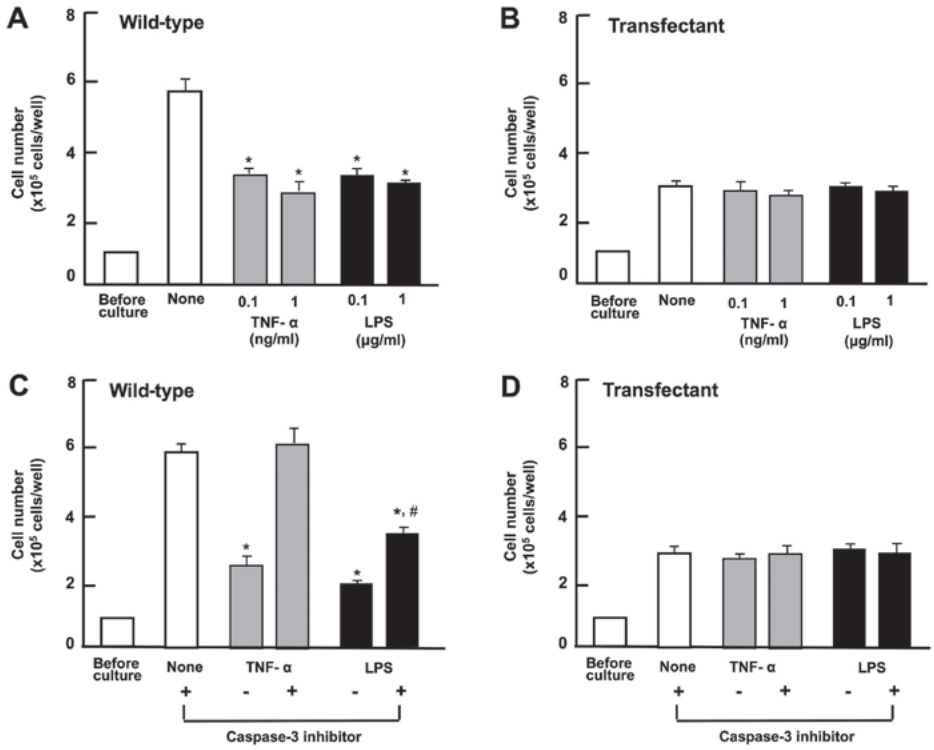


Figure 3. Overexpression of regucalcin prevents stimulation of cell death by TNF-α and LPS in A498 cells *in vitro*. The wild-type cells or transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 3 days, to reach subconfluence, The cells were then cultured for 24 h in the presence of TNF-α (0.1 or 1 ng/ml) or LPS (0.1 or 1 μg/ml) (A or B) with or without caspase-3 inhibitors (10 μM) (C or D), and the number of attached cells counted. Data are presented as the means ± SD obtained from 8 wells of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 versus control (none; white bar), #P<0.001 versus groups without caspase-3 inhibitor (LPS only; black bar), determined by one-way ANOVA with the Tukey-Kramer post hoc test. TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide.

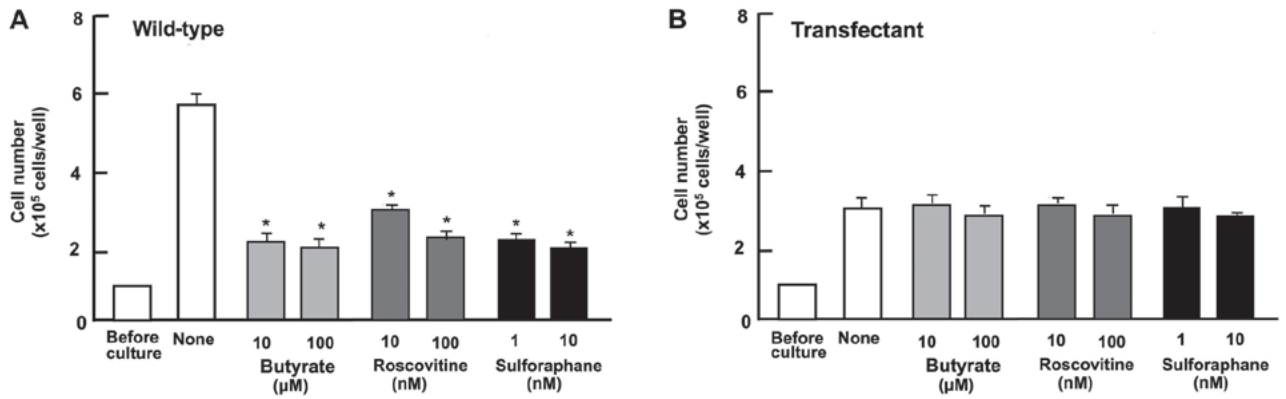


Figure 4. Suppressive effects of regucalcin overexpression on the proliferation in A498 cells act the rough cell cycle arrest as indicated by using various inhibitors of the cell cycle. The (A) wild-type cells or (B) transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 3 days in the absence or presence of butyrate (10 and 100 μM), roscovitine (10 and 100 nM), or sulforaphane (1 and 10 nM), and the number of attached cells on the dish were then counted. Data are presented as the means ± SD obtained from 8 wells of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 versus control (none; white bar), determined by one-way ANOVA with the Tukey-Kramer post hoc test.

findings suggest that the suppressive effects of regucalcin overexpression on cell growth are likely due, at in least part, to the inhibition of caspase-3 activity in A498 cells. Thus, the suppressive effects of regucalcin overexpression on the proliferation of A498 cells are not mediated by cell death.

Suppressive effects of regucalcin overexpression on the proliferation of A498 cells are mediated through various signaling pathways. To determine the mechanisms through which regucalcin overexpression suppresses the proliferation of A498 cells, we investigated whether the suppressive effects of regucalcin overexpression are attenuated in the presence of various inhibitors that induce cell cycle arrest *in vitro* (Fig. 4). Wild-type cells were cultured for 3 days in the presence of butyrate (10 and 100 μM) (46), roscovitine (10 and 100 nM) (47) or sulforaphane (1 and 10 nM) (48). The proliferation of the wild-type cells was suppressed in the presence of these inhibitors (Fig. 4A). The effects of these

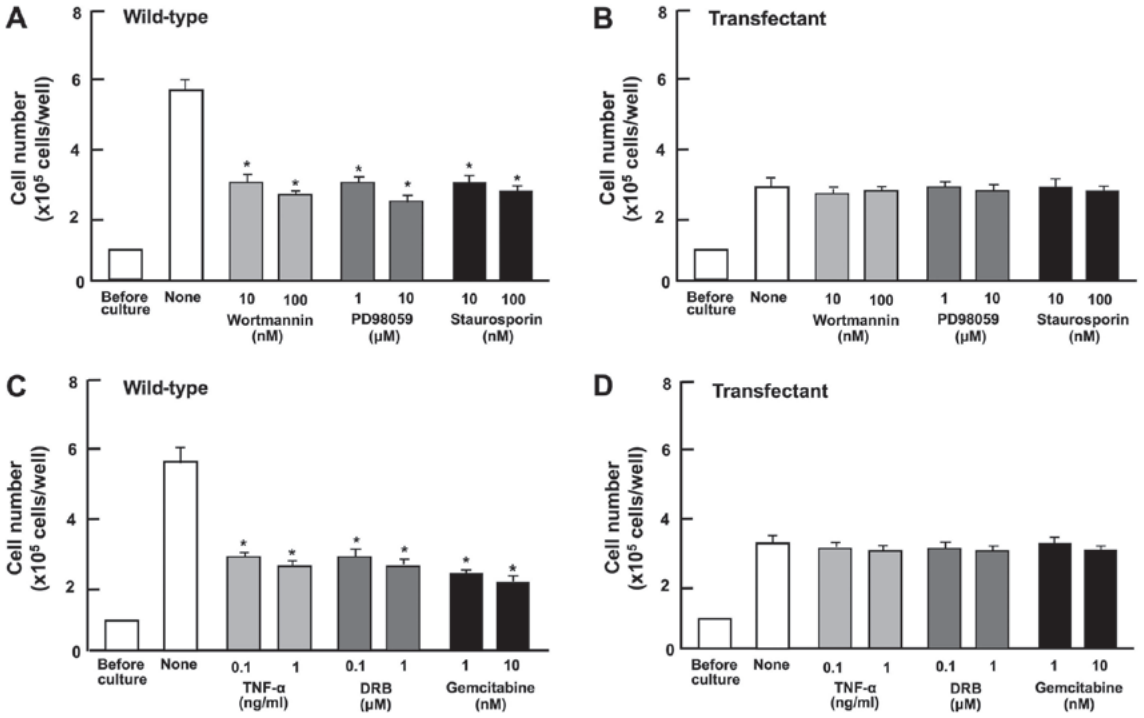


Figure 5. Suppressive effects of regucalcin overexpression on the proliferation of A498 cells *in vitro* is exerted through various signaling pathways. The wild-type cells (A and C) or regucalcin-overexpressing transfectant (B and D) were cultured in DMEM 10% containing FBS and 1% P/S in the absence or presence of wortmannin (10 or 100 nM), PD98059 (1 or 10 μM), staurosporine (10 or 100 nM), TNF-α (0.1 or 1 ng/ml), DRB (0.1 or 1 μM), or gemcitabine (1 or 10 nM) for 3 days, and the number of attached cells were counted. Data are presented as the means ± SD obtained from 8 wells of 2 replicate wells per data set using different dishes and cell preparations. *P<0.001 versus control (none; white bar), determined by one-way ANOVA with the Tukey-Kramer post hoc test. TNF-α, tumor necrosis factor-α; PD98059, MAP kinase inhibitor.

inhibitors were not potentiated in the transfectants (Fig. 4B). These results suggest that the overexpression of regucalcin induces G1 and G2/M phase cell cycle arrest in the A498 cells.

Subsequently, we determined the involvement of signaling factors in the suppressive effect on cell proliferation induced by the overexpression of regucalcin. The proliferation of the A498 wild-type cells was suppressed in the presence of wortmannin (0.1 or 1 μM), an inhibitor of PI3 kinase (50), PD98059 (1 or 10 μM), an inhibitor of extracellular signal-regulated kinase (ERK) and MAP kinase (51), and staurosporine (10 or 100 nM), a calcium signaling-related inhibitor (52) (Fig. 5A). The blocking of these pathways did not potentiate the suppressive effects of regucalcin overexpression of cell proliferation (Fig. 5B).

DRB is an inhibitor of RNA polymerase II-dependent transcriptional activity (53). Gemcitabine is a potent antitumor agent that induces nuclear DNA damage (54). In the current study, these inhibitors inhibited the proliferation of wild-type cells (Fig. 5C). However, these effects did not occur in the transfectants (Fig. 5D). These results suggest that the overexpression of regucalcin suppresses various signaling processes linked to cell proliferation, and that the regucalcin-overexpressing cells exhibit a lack of responses to the above-mentioned inhibitors of these pathways.

Overexpression of regucalcin regulates the expression of various proteins linked to cell signaling and transcriptional activity. We then investigated whether the overexpression of regucalcin affects the expression of key protein involved in signaling pathways and transcriptional activity. The results of western blot analysis revealed that the levels of Ras, PI3 kinase, Akt, phospho-Akt, MAP kinase and phospho-MAP kinase were diminished by the overexpression of regucalcin (Fig. 6A and B). These results suggest that the overexpression of regucalcin suppresses the activation of Ras-linked signaling pathways in A498 cells. By contrast, the overexpression of regucalcin elevated the protein levels of tumor suppressors, p53 and Rb, and p21, an inhibitor of the cell cycle (Fig. 6C and D). In addition, the overexpression of regucalcin diminished the levels of c-fos, c-jun, Stat3, β-catenin and NF-κB p65, which are transcription factors linked to the proliferation of A498 cells (23,25,55) (Fig. 6C and D). We determined the changes in the levels of 14 proteins, which may be major signaling proteins related to the proliferation of cancer cells, although various other proteins are also implicated in the proliferation of cancer cells.

Discussion

In this study, we performed the profiling of gene expression and survival analysis of 52 patients with clear cell RCC using the GEO database (GSE36895) for outcome analysis. The data obtained demonstrated that the prolonged survival of patients with RCC was associated with a higher regucalcin gene expression, and that the diminished regucalcin gene expression was accompanied by the poor prognosis of patients with RCC. This suggests that the diminished regucalcin gene expression may partly contribute to the development or aggressiveness of carcinogenesis in human RCC, and may lead to a worse clinical outcome for patients with RCC. Moreover, to determine a

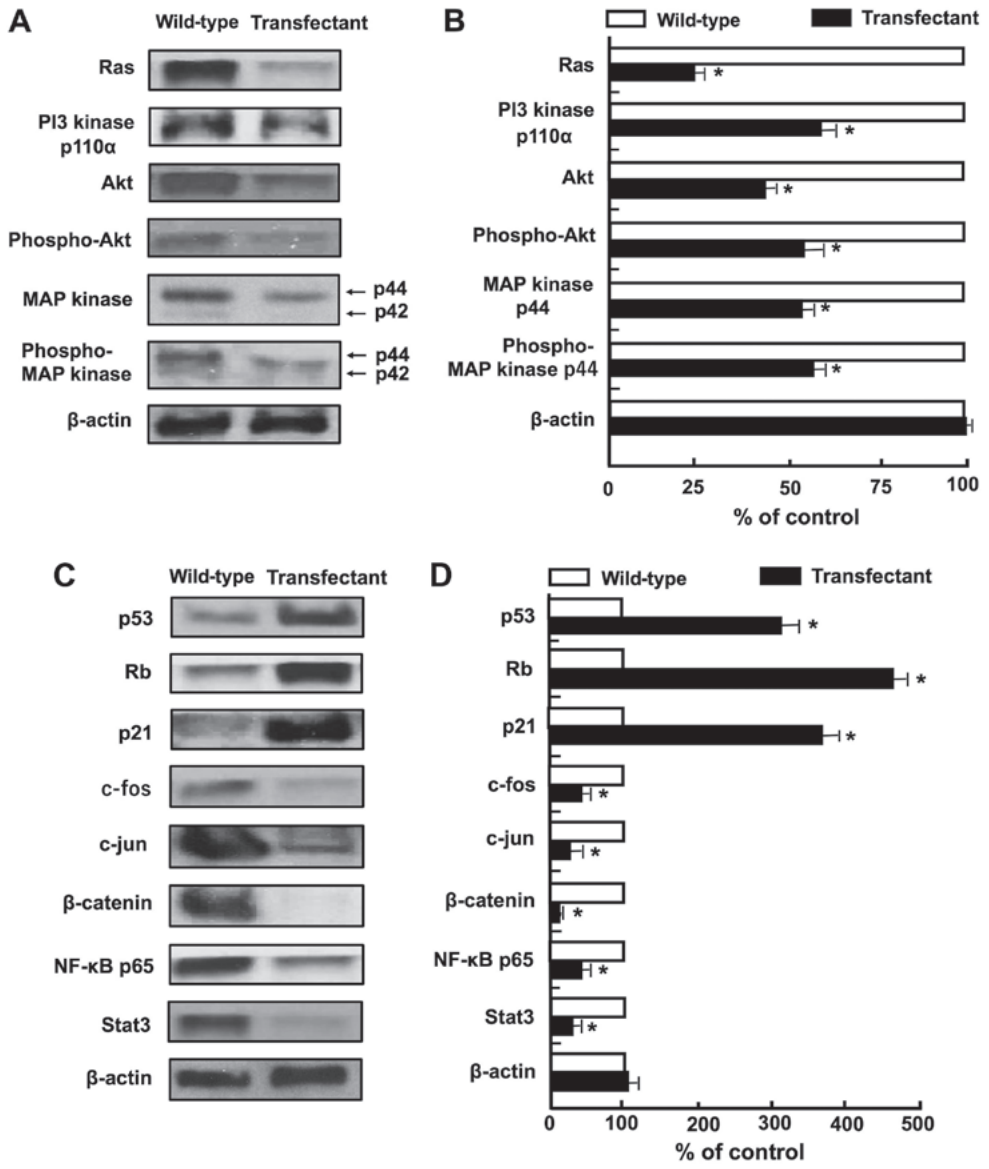


Figure 6. Overexpression of regucalcin regulates various proteins implicated in cell signaling process and transcription activity in A498 cells *in vitro*. The wild-type cells or regucalcin-overexpressing transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 3 days. Following culture, the cells were removed from the dish with a cell scraper in cell lysis buffer containing protease inhibitors. A total of 40 µg of supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blot analysis using antibodies against various proteins. Representative data are presented. (A) Cell signaling-related proteins, and (B) bands are presented as a percentage of the control (wild-type cells). (C) Transcription-related proteins, and (D) bands are presented as the percentage of the control (wild-type cells). *P<0.01versus control (white bar), determined by the Student's t-test.

mechanism for this clinical finding, we investigated whether regucalcin overexpression suppresses the proliferation of the RCC A498 human clear cell line *in vitro*. The overexpression of regucalcin was shown to suppress colony formation and the proliferation of A498 cells without inducing necrosis or apoptotic cell death *in vitro*. Thus, this study demonstrated a crucial role of regucalcin in suppressing the growth of human clear cell RCC cells. Endogenous regucalcin may therefore play a suppressive role in the development of human RCC. However, further studies using multi-datasets are warranted to corroborate the results of this study.

The mechanistic characterization of the suppressive effects of regucalcin overexpression on the proliferation of A498 cells was investigated using various inhibitors that regulate cell signaling pathways. This suppressive effect was not potentiated by butyrate, roscovitine or sulphoraphan, which induce cell cycle arrest. Butyrate induces the inhibition of G1 progression (46). Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m and cdk5 (47). Sulforaphane induces G2/M phase cell cycle arrest (48). Our data suggest that regucalcin overexpression causes G1 and G2/M phase cell cycle arrest in A498 cells. Further experiments are required to confirm this finding using other analysis of cell cycle. Similar effects of regucalcin have been shown in various other types of cells, including normal rat kidney proximal tubular epithelial NRK52E cells (49), cloned rat hepatoma H4-II-E cells (44), human pancreatic cancer MIA PaCa-2 cells (31), MDA-MB-231 breast cancer cells (32), liver cancer HepG2 cells (33), lung adenocarcinoma A549 cells (34) and colorectal cancer RKO cells (35) *in vitro*. Notably, 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 (22-26), and we demonstrate herein that thus is also the case in A498 cells.

It was then investigated whether regucalcin regulates cell signaling pathways using various inhibitors. The suppressive effects of regucalcin overexpression on the growth of A498 cells were not potentiated by staurosporine, an inhibitor of protein kinase C (52), wortmannin, an inhibitor of the PI3 kinase (PI3K)/Akt signaling pathway (50), or PD98059, an inhibitor of extracellular signal-regulated kinase (ERK)/MAP kinase (also termed MAPK) (51). The overexpression of regucalcin and its suppressive effects on cell proliferation were associated with the inhibition of various signaling pathways, namely Ca^{2+}-dependent kinases, PI3K/Akt and ERK/MAPK, in A498 cells. Thus, it is suggested that regucalcin is a suppressor of diverse signaling pathways in human RCC cells. Furthermore, the results of western blot analysis revealed that regucalcin overexpression induced a decrease in the levels of various proteins that are involved in signaling pathways linked to Ras, PI3K, Akt and MAPK, in A498 cells (51). The suppressive effects of regucalcin, and its regulation of various signaling pathways, have also been observed in various other types of human cancer cells *in vitro* (31-35).

The suppressive effects of regucalcin overexpression on the proliferation of A498 cells were not altered by culture with DRB, an inhibitor of RNA polymerase II-dependent transcriptional activity (53), and were not potentiated by culture with gemcitabine, which is used in the therapy of human cancer as an antitumor agent that induces nuclear DNA damage (54). This drug inhibits the proliferation and stimulates apoptotic cell death in various types of cancer cells (54). The results of this study suggest that regucalcin partly regulates pathways implicated in the mode of action of DRB and gemcitabine. Regucalcin has been demonstrated to directly suppress DNA and RNA synthesis using isolated rat liver nuclei (25).

Regucalcin has been shown to play a role in the regulation of cell nuclear function (25). Importantly, the overexpression of regucalcin has been demonstrated to enhance the gene expression levels of the tumor suppressor p53 and Rb, and that of p21, an inhibitor of the cell cycle, and to suppress the gene expression of ras, c-fos and c-myc, oncogenes, due to binding to nuclear DNA in cloned rat hepatoma H4-II-E cells *in vitro* (55). Similarly, in this study, regucalcin overexpression was found to elevate the protein levels of the tumor suppressors p53, Rb and p21 (44,56), and diminish those of ras, c-fos, c-jun, Stat3, β-catenin and NF-κB p65, which are transcription factors linked to cancer cell proliferation, in A498 cells. These findings suggest that endogenous regucalcin plays a pivotal role in suppressing the growth of cancer cells due to regulation of the expression of various proteins linked to transcription factors, tumor suppressors and oncogenes involved in tumor development. Regucalcin binds to DNA (55) and regulates the gene expressions of various proteins in the nucleus of normal and cancer cells (25).

In conclusion, the current study demonstrates that the prolonged survival of patients with clear cell RCC is associated with a higher regucalcin gene expression in the tumor tissues, and that the overexpression of regucalcin suppresses colony formation and proliferation in human clear cell RCC A498 cells *in vitro*. Endogenous regucalcin may play a potential role as a suppressor in the development of human renal cancer. Our previous studies have demonstrated that survival is prolonged in patients with pancreatic cancer (31), breast cancer (32), liver cancer (33), lung adenocarcinoma (34), and colorectal cancer (35) exhibiting higher regucalcin gene expression in their tumor tissues. Thus, endogenous regucalcin may play a pivotal role as a suppressor of carcinogenesis in human cancer of various types. The downregulation of the regucalcin gene expression may lead to the development of carcinogenesis in human subjects. Targeting regucalcin may be clinically significant in the diagnosis and potential therapy for human cancer of various types. The delivery of the regucalcin gene, which is overexpressed in tumor tissues, may constitute a novel therapeutic approach to treating human cancer.

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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, OH and TM performed the experiments and discussed the findings. MY wrote the manuscript, and SO, OH 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 work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols used databases or cell culture *in vitro*.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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