

# Prolonged survival in hepatocarcinoma patients with increased regucalcin gene expression: HepG2 cell proliferation is suppressed by overexpression of regucalcin *in vitro*

MASAYOSHI YAMAGUCHI<sup>1</sup>, SATORU OSUKA<sup>2</sup>, M. NEALE WEITZMANN<sup>3,4</sup>,  
BASSEL F. EL-RAYES<sup>1</sup>, MAMORU SHOJI<sup>1</sup> and TOMIYASU MURATA<sup>5</sup>

Departments of <sup>1</sup>Hematology and Medical Oncology, and <sup>2</sup>Neurosurgery, Winship Cancer Institute, Emory University School of Medicine, Atlanta; <sup>3</sup>The Atlanta Department of Veterans Affairs Medical Center, Decatur; <sup>4</sup>Division of Endocrinology and Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 1329 WMRB, Atlanta, GA 30322, USA; <sup>5</sup>Laboratory of Analytical Neurobiology, Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan

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**Abstract.** Hepatocellular carcinoma (HCC) is one of the most common malignant cancers worldwide and ranks third in overall global cancer-related mortality rates. Importantly, in this study gene expression data demonstrate that prolonged survival in HCC patients is associated with increased regucalcin gene expression. Regucalcin has been shown to play a pivotal role as a transcription repressor and diminished expression or activity of regucalcin may play a key role in the development of human carcinogenesis. Indeed, overexpression of regucalcin suppressed the proliferation, cell death, and migration of human HCC HepG2 cells *in vitro*. Mechanistically, regucalcin induced G1 and G2/M phase cell cycle arrest of HepG2 cells through suppression of multiple signaling pathways including Ras, Akt, MAP kinase and SAPK/JNK and by increasing the tumor suppressors p53 and Rb. Furthermore, the oncogenes *c-fos* and *c-myc* were suppressed by overexpression of regucalcin, and overexpression of regucalcin caused an increase in p21 and a decrease in NF- $\kappa$ B p65 and  $\beta$ -catenin. These findings suggest that regucalcin may play a potential role as a suppressor of human HCC, and that diminished expression of regucalcin may predispose patients to development of HCC.

Overexpression of regucalcin may constitute a novel therapeutic approach to treating HCC.

## Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is one of the most prevalent malignant diseases worldwide, and the third most common cause of cancer-related death (1,2). Features of HCC are an aggressive cancer with a dismal outcome largely due to metastasis and post-surgical recurrence. HCC originates on a background of cirrhosis, a chronic and diffuse hepatic disease, which results from continuous liver injury and regeneration (3,4). The majority of HCC cases are also related to chronic viral infections including hepatitis B virus or hepatitis C virus, which induces malignant transformation (5-8).

Hepatocarcinogenesis is a multistep process initiated by external stimuli that leads to genetic changes in hepatocytes or stem cells, resulting in proliferation, apoptosis, dysplasia and neoplasia. HCC is a cancer with a poor prognosis. The prognosis of advanced HCC remains poor in spite of the development of novel therapeutic strategies. In recent years, improved knowledge of the oncogenic processes and signaling pathways that regulate tumor cell proliferation, differentiation, angiogenesis, invasion and metastasis has led to the identification of several potential therapeutic targets, which have driven the development of molecularly targeted therapies (9,10). The most effective therapeutic tool for advanced non-resectable HCC, which can slightly improve patient survival, is based on the multikinase inhibitor sorafenib (11,12). New perspectives in cancer treatment have appeared recently with the advent of the microRNAs, a novel class of non-coding small RNAs (13).

Regucalcin, whose gene is localized on the X chromosome (14,15), plays a potential role as a multi-signaling pathway and transcription repressor in various types of cells and tissues

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**Correspondence to:** Dr Masayoshi Yamaguchi, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, C-5054, 1365 C. Clifton Road, NE, Atlanta, GA 30322, USA  
E-mail: yamamasa1155@yahoo.co.jp

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(16,17). Regucalcin plays a pivotal role in maintaining intracellular calcium homeostasis, inhibiting various protein kinases and protein phosphatases, suppressing protein synthesis, DNA and RNA synthesis, and regulating gene expression (16-20). Moreover, regucalcin has been found to suppress cell proliferation and apoptotic cell death mediated through various signaling factors (19,20) and has been proposed to play a pivotal role in maintaining cell homeostasis and functioning as a suppressor protein of intracellular signaling systems (16,17).

Noticeably, regucalcin has been shown to be involved in carcinogenesis (21). Regucalcin protein expression was found to be downregulated in the tumor tissues from mammalian and human subjects *in vivo* (22), suggesting that diminished regucalcin may play a key role in the development of carcinogenesis. Regucalcin gene expression has been demonstrated to be downregulated in rat hepatoma H4-II-E cells *in vitro*, and enhanced proliferation was reversed by overexpression of regucalcin (23). Moreover, we demonstrated that prolonged overall survival with increased regucalcin expression in pancreatic and breast cancer patients, and that overexpression of regucalcin suppresses the proliferation of human pancreatic cancer MIA PaCa-2 cells and breast cancer MDA-MB-231 cells *in vitro* (24,25). Regucalcin may play an important role as a suppressor of carcinogenesis.

This study was undertaken to determine the involvement of regucalcin in human HCC, which has not been previously investigated. Importantly, we demonstrated that increased regucalcin gene expression is associated with prolonged survival in HCC patients as evaluated by the analysis of gene expression using the Gene Expression Omnibus (GEO) database (GSE17891) in HCC derived from human patients. Moreover, overexpression of regucalcin was found to suppress the proliferation of human hepatoma HepG2 cells *in vitro*. Our findings support the view that regucalcin may play a potential role as a suppressor of human HCC, and that diminished expression of regucalcin may predispose patients to development of HCC.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate and antibiotics (penicillin and streptomycin) were purchased from Corning (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- $\alpha$  (TNF- $\alpha$ ) was from R&D Systems (Minneapolis, MN, USA). Sodium butyrate, roscovitine, sulforaphane, dibucaine, PD98059, lypopolysaccharoide (LPS), Bay K 8644, worthomannin, 5, 6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), 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 other reagents were dissolved in 100% ethanol for use.

**Patient datasets.** A curated gene expression dataset comprising 35 normal human liver samples and 47 HCC samples were obtained through the Gene Expression Omnibus (GEO) data-

base (GSE45436) for analysis of regucalcin expression (26,27). These datasets contained gene expression data derived from the Affymetrix U133\_plus2 platform. For microarray analysis, expression and raw expression data (CEL files) were summarized and normalized using the Robust Multi-array Average algorithm and the Bioconductor package affy (<http://www.bioconductor.org/packages/2.0/bioc/html/affy.html>). The Spotfire Decision Site for Functional Genomics software package (TIBCO Software, Palo Alto, CA, USA) was used for visualization of microarray data. For protein expression analysis, a dataset of immunohistochemistry was obtained from the Human Protein Atlas (HPA) ([www.proteinatlas.org](http://www.proteinatlas.org)), which is a database of proteins in human normal tissues and cancers (28,29). We also evaluated regucalcin expression in 3 tissues of normal liver, especially at the site of hepatocytes, and 6 tissues of HCC in the liver. A dataset of 2 antibodies (HPA029102 and HPA029103) for regucalcin was used in this analysis. Outcome analysis was based on regucalcin mRNA expression in 162 liver tissue samples from HCC patients (GSE10143) (30). A dataset of regucalcin expression and clinical annotation was obtained from SurvExpress (31).

**Human hepatoma HepG2 cells.** We used HepG2 cells (HB-8065<sup>TM</sup>) cloned from human HCC, which were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). This cell line was isolated from male adolescent (15 years old) with HCC. HepG2 cells were suitable as a transfection host.

**Transfection of regucalcin cDNA.** HepG2 cells were transfected with pCXN2 vector expressing cDNA encoding human full length (900 bp) regucalcin (regucalcin cDNA/pCXN2) (23). For transient transfection assay, HepG2 cells were grown on 24-well plates to ~70% confluence. Regucalcin cDNA/pCXN2 and empty pCXN2 vector alone were transfected into HepG2 cells using the synthetic cationic lipid components, a Lipofectamine reagent, according to the manufacturer's instructions (Promega) (23). After resting overnight, Geneticin (500-700  $\mu$ g/ml G418, Sigma-Aldrich) was added to culture wells for selection, and cells were cultured for 3 weeks. After that, cells were plated at limiting dilution to isolate transfectants. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in the medium without Geneticin. Stable expression of regucalcin was observed in transfectants, and the clone with protein levels of regucalcin increased by 11.2-fold as compared with that of wild-type cells was used in this experiment (Fig. 2A).

**Western blotting.** HepG2 (wild-type) cells transfected with control vector or regucalcin cDNAs were plated in 35-mm dishes at a density of  $1 \times 10^5$  cells/well in 2 ml of medium, and cultured in DMEM containing 10% FBS and 1% P/S for 3 days. Cells were washed twice with ice-cold PBS and removed from the dish by scraping in cell lysis buffer containing protein inhibitors. Lysed cells were centrifuged and the pellet was homogenized by sonication in 0.1 ml of ice-cold cell lyses buffer containing protein inhibitors. The homogenate was centrifuged for 5 min at 1,500 g, and the protein concentration of the supernatant was determined for western blotting using bovine serum albumin as a standard. Samples (30  $\mu$ g)

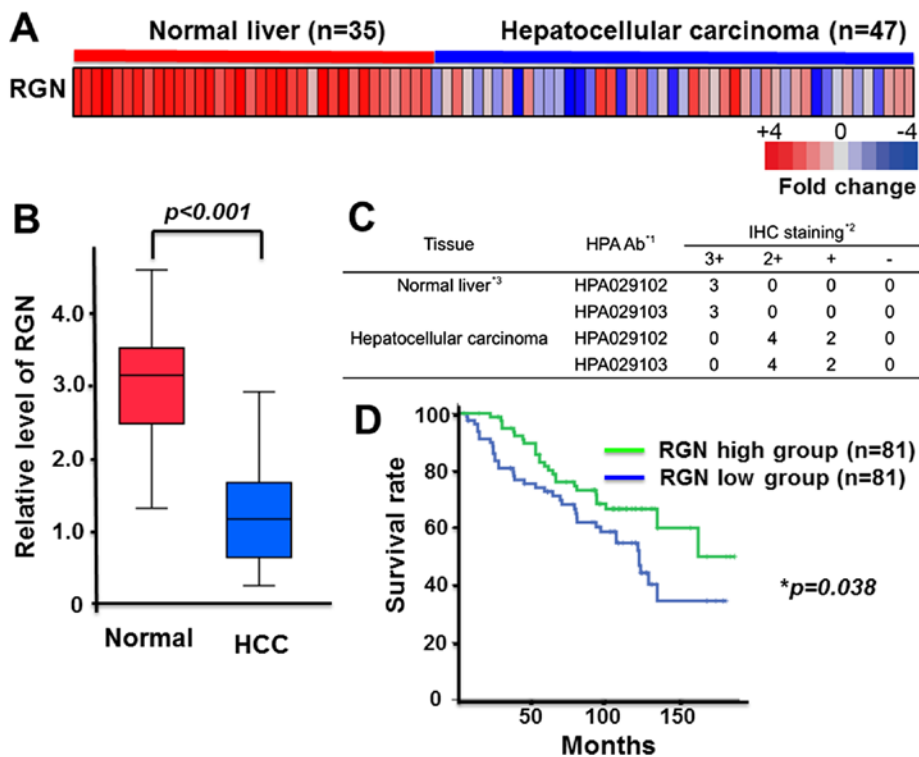


Figure 1. Prolonged survival in hepatocellular carcinoma (HCC) patients with the higher regucalcin gene expression. (A) Microarray expression analysis of regucalcin in 35 normal liver and 47 HCC (26,27). Each colored square on the bottom right represents the relative mean transcript abundance; highest expression is shown in red, average expression in white, and lowest expression is shown in blue. Reduced regucalcin expression weakens survival of HCC patients. (B) Quantification of regucalcin expression in normal liver and HCC similar to that in (A). (C) Degree of immunohistochemical staining for regucalcin into 3 tissues of normal liver and 6 HCC through the immunohistochemistry (IHC) database (The Human Protein Atlas). The results of 2 antibodies are indicated. IHC staining scores: 3+, storong; 2+, moderate; +, weak; -, negative. (D) Survival curves for HCC, and survival was longer in high expression as compared with low expression of regucalcin. HCC, hepatocellular carcinoma; HPA, The Human Protein Atlas; IHC, immunohistochemistry; RGN, regucalcin.

of supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using antibodies against regucalcin (22,23) and other proteins (Cell Signaling Technology, Danvers, MA, USA).  $\beta$ -actin was used as a loading control. A minimum of 3 blots from independent experiments were scanned on an Epson Perfection 1660 Photo scanner, and bands quantified using ImageJ. Data from independent experiments were normalized to a percentage of control before averaging.

**Cell proliferation assays.** Wild-type HepG2 cells ( $1 \times 10^5$ /ml per well) and HepG2 cells ( $1 \times 10^5$ /ml per well) transfected with regucalcin cDNA were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S for 1, 3 or 6 days in a water-saturated atmosphere containing 5% CO<sub>2</sub> and 95% air at 37°C (32,33). In separate experiments, wild-type HepG2 cells or transfectants were cultured in DMEM containing 10% FBS and 1% P/S in the presence of either sodium butyrate (10 and 100  $\mu$ M), roscovitine (10 and 100 nM), sulphoraphane (1 and 10 nM), dibucaine (0.1 or 1  $\mu$ M), Bay K 8644 (0.1 or 1  $\mu$ M), PD98059 (1 or 10  $\mu$ M), worthomannin (0.1 or 1  $\mu$ M), DRB (0.1 or 1  $\mu$ M), or gemcitabine (50 or 100 nM) for 3 days. After culture, cells were detached from each culture dishes and counted.

**Cell death assay.** Wild-type HepG2 cells ( $1 \times 10^5$ /ml per well) and HepG2 cells ( $1 \times 10^5$ /ml per well) transfected with regu-

calcin cDNA were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S for 4 days to confluence, and then were cultured for additional 3 days in the presence or absence of either LPS (0.1 or 1  $\mu$ g/ml), TNF- $\alpha$  (0.1 or 1 ng/ml) or Bay K 8644 (0.1 or 1  $\mu$ M) (34). In separate experiments, wild-type HepG2 cells ( $1 \times 10^5$ /ml per well) or transfectants were cultured for 4 days to confluence, and then were cultured for an additional 24 h in the presence or absence of either LPS (1  $\mu$ g/ml) or TNF- $\alpha$  (1 ng/ml) with or without caspase-3 inhibitor (10  $\mu$ M) for 24 h (34). After culture, cells were detached from each culture dish for counting.

**Cell counting.** Cells were recovered from plates by trypsinization of each of culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS for 2 min at 37°C, detached cells from dish were collected after centrifugation (32-34). Cells were suspended on PBS solution and stained with eosin. Cells were counted under a microscope using a hemocytometer. For each dish, we took the average of two quantifications.

**Migration assay.** We used the *in vitro* scratch assay method for analysis of cell migration *in vitro* (35). HepG2 cells ( $1 \times 10^5$  cells/ml) of wild-type and transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 24 h using 12-well plates to reach confluence, and then a scratch was created in the cell monolayer. Images were captured at the beginning and at 24 or 48 h of culture. Cells in the plate were

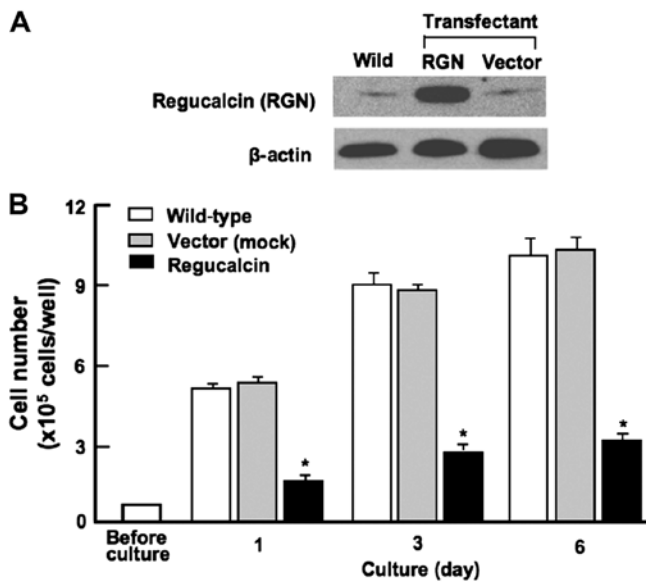


Figure 2. Overexpression of regucalcin suppresses the proliferation in human hepatoma HepG2 cells *in vitro*. Cells were transfected with regucalcin cDNA vector. (A) Regucalcin content in the cells cultured for 3 days was analyzed by immunoblotting with an anti-regucalcin antibody. Lane 1, wild-type cells (designated as wild). Lane 2, the cells transfected with the regucalcin cDNA/pCXN2. Lane 3, cells transfected with empty vector/pCXN2 (designated as vector). (B) These cells were cultured in DMEM for 1, 3 or 7 days. After culture, the number of attached cells on a dish was counted. Data are presented as mean  $\pm$  SD of 2 replicate wells per data set using different dishes and cell preparation. \* $p < 0.001$  versus wild-type (white bar) or control vector (grey bar). One-way ANOVA, Tukey-Kramer post hoc test.

fixed in ice-cold 95% ethanol and stained with crystal violet (1% in PBS). After overnight drying, the migration distance was imaged, and cells were counted under a microscope to determine migration.

**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 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. 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 of  $< 0.05$  was considered statistically significant.

## Results

**Prolonged survival in HCC patients with increased regucalcin gene expression.** To understand the involvement of regucalcin in human patients with HCC, we analyzed the expression levels of regucalcin in normal liver tissues and HCC. We analyzed microarray data from the GEO database (GSE45436) (as described in Materials and methods) to evaluate regucalcin expression levels in 35 normal pancreas and 47 HCC patients. Overall regucalcin expression was visually reduced in HCC patients as compared with that of tissues derived from normal liver (Fig. 1A). Quantitative analysis confirmed that expression of regucalcin in HCC patients was remarkably reduced

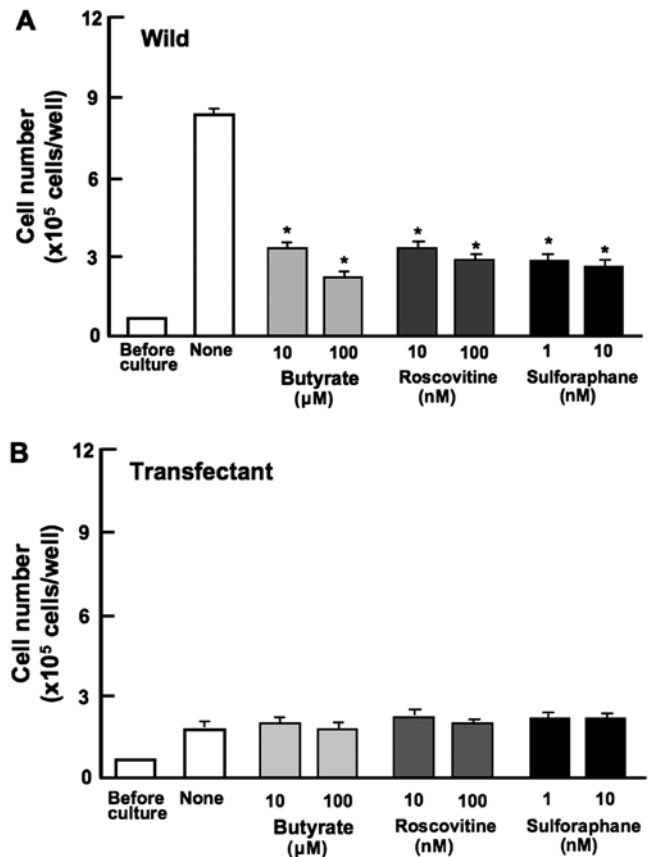


Figure 3. Suppressive effects of regucalcin on the proliferation in HepG2 cells are involved in cell cycle arrest by estimating with cell cycle inhibitors. Wild-type cells (A) or transfectants (B) were cultured for 3 days in the absence or presence of butyrate (10 and 100  $\mu$ M), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM). After culture, the number of attached cells on dish was counted. Data are presented as mean  $\pm$  SD of 2 replicate wells per data set using different dishes and cell preparation. \* $p < 0.001$  versus control (none; white bar). One-way ANOVA, Tukey-Kramer post hoc test.

as compared with that in the tissue of normal liver (Fig. 1B). To confirm the reduction of regucalcin protein levels, we checked the expression of regucalcin in 3 normal liver tissue samples and in 6 HCC samples using the immunohistochemistry database (The Human Protein Atlas). Results from 2 independent regucalcin antibodies revealed that the expression of regucalcin in HCC patients was markedly suppressed as compared to that of normal liver (Fig. 1C). Moreover, we compared the clinical outcome between 81 HCC patients with higher regucalcin expression and 81 HCC patients with lower regucalcin expression. The reduction of regucalcin expression was associated with poor prognosis in HCC patients. Higher regucalcin gene expression was found to prolong survival in HCC patients (Fig. 1D). These findings support the notion that suppression of regucalcin gene expression partly contributes to the development of carcinogenesis in human HCC cells and leads to a worse clinical outcome.

**Generation of regucalcin-overexpressing HepG2 cells.** HepG2 cells were transiently transfected with empty pCXN2 vector or full length (33-kDa protein) regucalcin/pCXN2 construct using lipofection. Regucalcin protein in transfected cells was found to be increased by 11.2-fold as compared with that of

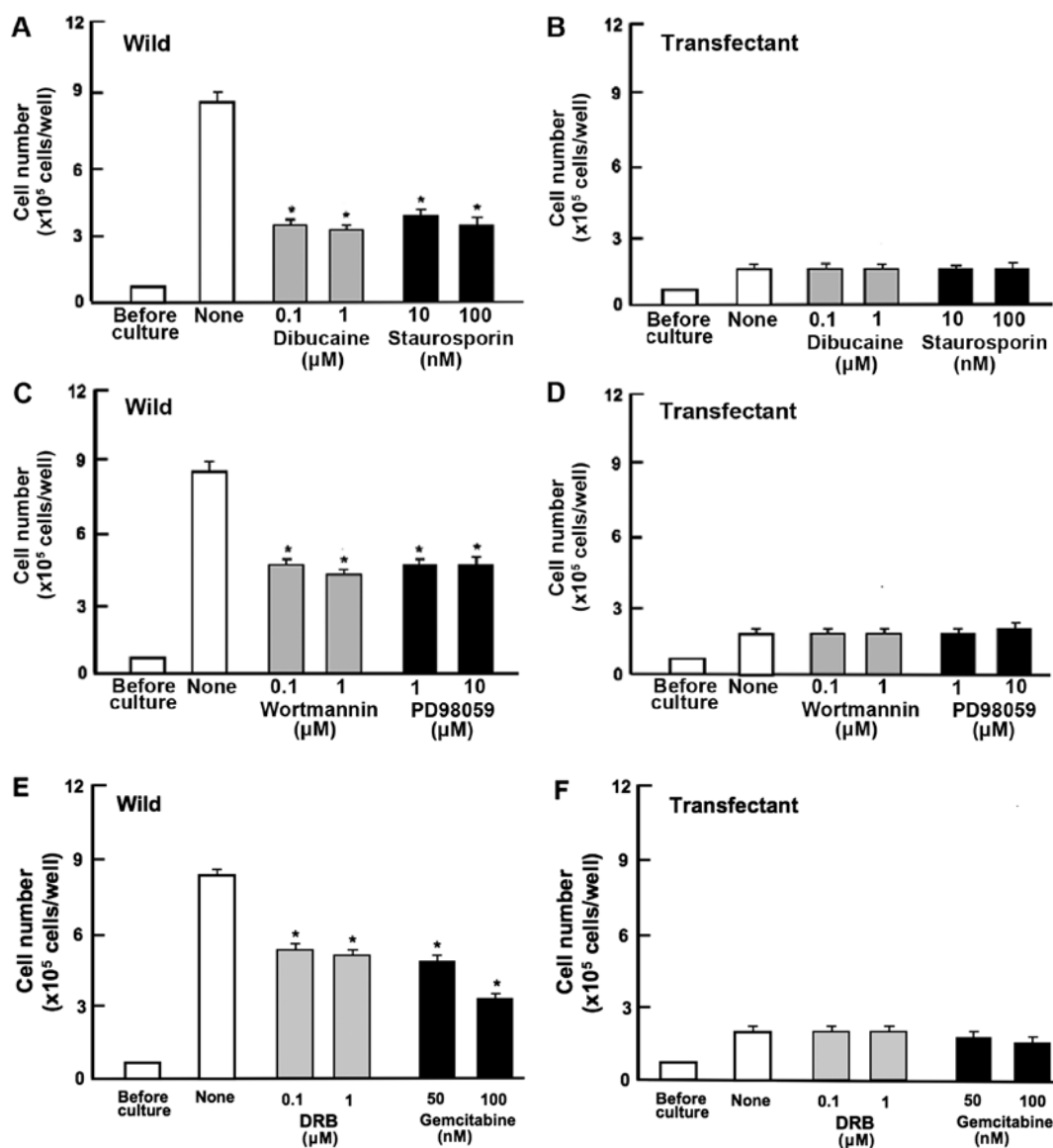


Figure 4. Suppressive effects of regucalcin on the proliferation in HepG2 cells *in vitro* are exhibited through various signaling pathways. Wild-type cells (A, C and E) or transfectants (B, D and F) overexpressed with regucalcin of full length were cultured in the absence or presence of dibucaine (0.1 or 1  $\mu$ M), staurosporine (10 or 100 nM), wortmannin (0.1 or 1  $\mu$ M), PD98059 (1 or 10  $\mu$ M), DRB (0.1 or 1  $\mu$ M) or gemcitabine (50 or 100 nM) for 3 days. After culture, the number of attached cells on a dish was counted. Data are presented as mean  $\pm$  SD of 2 replicate wells per data set using different dishes and cell preparation. \* $p < 0.001$  versus control (none; white bar). One-way ANOVA, Tukey-Kramer post hoc test.

the parental wild-type HepG2 cells (Fig. 2A). To determine the effects of the overexpression of endogenous regucalcin on the proliferation of HepG2 cells *in vitro*, the cancer cells were cultured for 1, 3, and 6 days. Cell proliferation in culture was significantly reduced in regucalcin-overexpressing transfectants at 1, 3, and 6 days (Fig. 2B). Thus, overexpression of regucalcin was found to suppress the proliferation of HepG2 cells *in vitro*.

**Suppressive effects of regucalcin overexpression on HepG2 cell proliferation are related to regulation of various signaling pathways.** Proliferation in HepG2 cells was determined in the presence of various inhibitors that induce cell cycle arrest *in vitro* (Fig. 3). Wild-type cells were cultured for 3 days in the presence of butyrate (10 and 100  $\mu$ M), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM) (32,36,37).

Proliferation of wild-type cells was suppressed in the presence of these inhibitors (Fig. 3A) but not in transfectants (Fig. 3B). This result suggested that endogenous regucalcin induces G1 and G2/M phase cell cycle arrest in HepG2 cells.

Next, to determine the mechanism of regucalcin suppression of cell proliferation, we examined key signaling factors that regulate proliferation. Proliferation in wild-type cells was weakly suppressed by dibucaine (0.1 or 1  $\mu$ M), an inhibitor of calcium/calmodulin-dependent protein kinases (32), and staurosporine (10 or 100 nM), a calcium signaling-related inhibitor (32) (Fig. 4A). Blocking these pathways had no effect on the ability of regucalcin to suppress cell proliferation (Fig. 4B). Likewise, wortmannin (0.1 or 1  $\mu$ M), an inhibitor of phosphatidylinositol 3-kinase (PI3K) (36), and PD98059 (1 or 10  $\mu$ M), an inhibitor of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) (39)

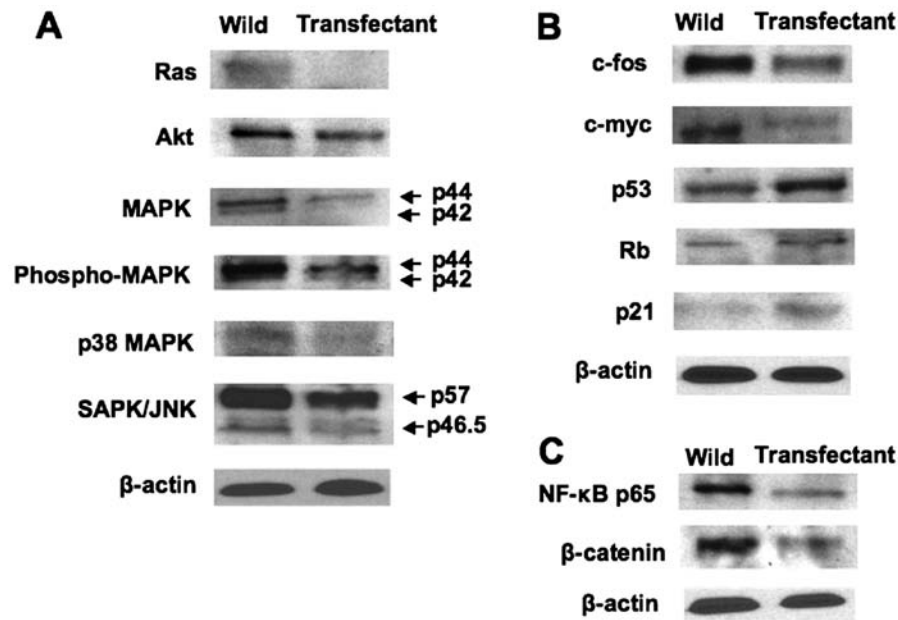


Figure 5. Overexpression of regucalcin regulates various protein levels related to signaling process and transcription activity in HepG2 cells *in vitro*. Wild-type cells or overexpressing transfectants were cultured in DMEM containing 10% FBS and 1% P/S for 3 days. After culture, the cells were removed from the dish with a cell scraper after cell lysis buffer containing protein inhibitors. Samples (30  $\mu$ g) of supernatant protein per lane were separated by SDS-PAGE and transferred to nylon membranes for western blotting using antibodies against various proteins. (A) Cell signaling-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.

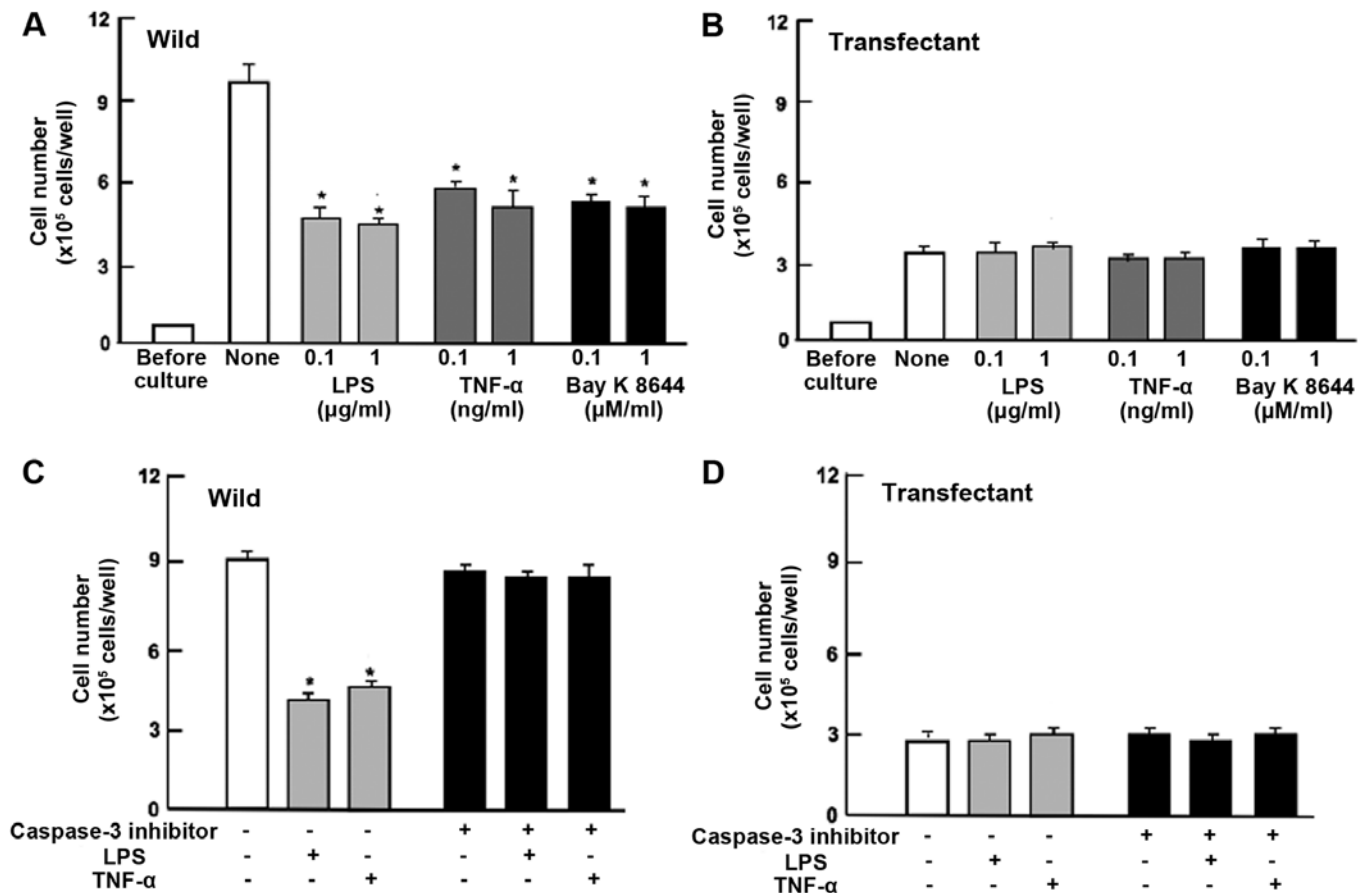


Figure 6. Overexpression of regucalcin prevents cell death induced by stimulation of LPS, TNF- $\alpha$  and Bay K 8644 in HepG2 cells *in vitro*. Wild-type cells or regucalcin-overexpressing transfectants were cultured for 5 days until confluent, and then the cells were cultured for an additional 24 h in the presence of LPS (0.1 or 1  $\mu$ g/ml), TNF- $\alpha$  (0.1 or 1 ng/ml) or Bay K 8644 (0.1 or 1  $\mu$ M) (A or B) or in the presence of LPS (0.1 or 1  $\mu$ g/ml) or TNF- $\alpha$  (0.1 or 1 ng/ml) with or without caspase-3 inhibitors (10  $\mu$ M) (C or D). After culture, the number of attached cells on a dish was counted. Data are presented as mean  $\pm$  SD of 2 replicate wells per data set using different dishes and cell preparations. \* $p$ <0.001 versus control (none; white bar). One-way ANOVA, Tukey-Kramer post hoc test.

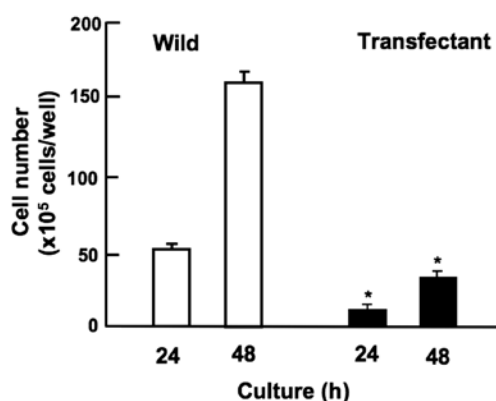


Figure 7. Overexpression of regucalcin suppresses migration of HepG2 cells *in vitro*. HepG2 cells of wild-type and regucalcin-overexpressing transfectants were cultured for 24 or 48 h using 12-well plates until subconfluent, and then a scratch was created in a cell monolayer, capturing the images at 24- or 48-h culture during cell migration. Cells migrated in the space of the scratch was fixed in ethanol and stained with crystal violet. Migrated cells were counted under a microscope. Data are presented as mean  $\pm$  SD of 2 replicate wells per data set using different dishes and cell preparations. \* $p < 0.001$  versus control (none; white bar). One-way ANOVA, Tukey-Kramer post hoc test.

(Fig. 4C) significantly inhibited cell proliferation in wild-type cells. Suppressive effects of these inhibitors on cell proliferation were not exhibited in transfectants (Fig. 4D). DRB is an inhibitor of transcriptional activity with RNA polymerase II inhibition (40). Gemcitabine is a strong antitumor agent that induces nuclear DNA damage (41). Proliferation of HepG2 cells was suppressed by culture with DRB (0.1 or 1  $\mu$ M) or gemcitabine (50 or 100 nM) (Fig. 4E). However, these effects were not seen in transfectants (Fig. 4F).

Overexpression of regucalcin was found to regulate the expression of key proteins related to signaling pathways and transcription activity using western blot analysis. Protein levels of Ras, Akt, MAPK, phospho MAPK, p38-MAPK, and SAPK/JNK were decreased by overexpression of regucalcin (Fig. 5A). These results suggested that overexpression of regucalcin suppresses signaling pathways that are related to activation of ras in HepG2 cells. In addition, overexpression of regucalcin decreased protein levels of *c-fos* and *c-myc* in HepG2 cells (Fig. 5B). Protein levels of p53 and *Rb*, tumor suppressors, and p21, cell cycle regulator, were increased by overexpression of regucalcin (Fig. 5B). Moreover, overexpression of regucalcin suppressed protein levels of transcription factor NF- $\kappa$ B p65 and  $\beta$ -catenin in HepG2 cells (Fig. 5C).

**Overexpression of regucalcin prevents cell death induced by various stimulatory factors.** To determine the effects of the overexpression of endogenous regucalcin on cell death in HepG2 cells, the cells were cultured for 5 days to confluence and then for an additional 24 h. The number of wild-type cells was decreased in the presence of LPS (0.1 or 1  $\mu$ g/ml), TNF- $\alpha$  (0.1 or 1 ng/ml) or Bay K 8644 (0.1 and 1  $\mu$ M), which is known to induce apoptotic cell death (20,34) (Fig. 6A). Such effects were not seen in the regucalcin-overexpressing HepG2 cells that did not exhibit a significant effect on the death (Fig. 6B). Death of HepG2 cells (wild-type) was also induced once the cells were cultured in the presence of TNF- $\alpha$  (1 ng/ml) or

Bay K 8644 (1  $\mu$ M) for 24 and 72 h (Fig. 6A). Such effects were not seen in the transfectants with regucalcin of full length (Fig. 6B).

To determine whether the preventive effects of regucalcin on cell death are mediated via caspase-3, confluent wild-type cells and transfectants were additionally cultured in the presence of LPS (1  $\mu$ g/ml) or TNF- $\alpha$  (1 ng/ml) with or without caspase-3 inhibitors (10  $\mu$ M) for 24 h (Fig. 6C and D). Effects of LPS or TNF- $\alpha$  on cell death were completely prevented in the presence of caspase-3 inhibitor (Fig. 6C). LPS- or TNF- $\alpha$ -induced cell death was not seen in transfectants that were cultured with or without caspase-3 inhibitor (Fig. 6D). These results suggest that endogenous regucalcin prevents cell death due to decreasing the activity of caspase-3 that activates nuclear DNA fragmentation, inducing apoptotic cell death. Thus, suppressive effects of regucalcin overexpression on the proliferation of HepG2 cells were independent of its effect on cell death.

**Overexpression of regucalcin suppresses cell migration.** Overexpression of regucalcin suppressed migration of human pancreatic cancer HepG2 cells *in vitro* (Fig. 7). HepG2 cells of wild-type and transfectants were cultured for 24 h using 12-well plates to reach subconfluence, and then a scratch was created in a cell monolayer. Cells migrating into the space of the scratch were stained and counted. Overexpression of regucalcin prevented migration of cells into the scratch as compared with that of wild-type cells (Fig. 7).

## Discussion

Using HCC microarray analysis of the GEO database (GSE15471) this study demonstrates overall regucalcin expression is reduced in human HCC patients as compared with that of normal human liver tissues. Furthermore, patient survival is prolonged with increased regucalcin gene expression while suppressed regucalcin expression was found to be associated with poor prognosis in HCC patients. These findings support the view that suppressed regucalcin gene expression partly contributes to the development or aggressiveness of carcinogenesis in human HCC and leads to a worse clinical outcome.

Overexpression of regucalcin was demonstrated to exhibit anticancer effects in HepG2 cells from human HCC by suppressing the proliferation of cancer cells *in vitro*. This effect of regucalcin was independent of cell death. In addition, overexpression of regucalcin was found to suppress the migration of HepG2 cells using the scratch assay *in vitro*, supporting anticancer effects of regucalcin *in vitro*. Our findings may support the view that regucalcin plays a potential role as a suppressor protein in human HCC cells.

Suppressive effects of regucalcin overexpression on the proliferation of HepG2 cells were not altered in the presence of butyrate, roscovitine or sulphoraphane that induce cell-cycle arrest. Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m and cdk5 (34). Sulphoraphane induces G2/M phase cell cycle arrest (37). Butyrate induces an inhibition of G1 progression (32). Endogenous regucalcin appears to induce G1 and G2/M phase cell cycle arrest in HepG2 cells. Similar data have been

reported in cloned rat hepatoma H4-II-E cells (32) and normal rat kidney proximal tubular epithelial NRK52E cells (33).

To determine a mechanistic cause of the suppressive effects of regucalcin overexpression on cell proliferation, we used various inhibitors that regulate intracellular signaling pathways. Suppressive effects of regucalcin overexpression on the proliferation in HepG2 cells were not altered in the presence of dibucaine, an inhibitor of calcium/calmodulin-dependent protein kinases (32), wortmannin, an inhibitor of PI3/Akt signaling pathway (38), and PD98059, an inhibitor of ERK/MAP kinase-related to signaling pathway (39). Regucalcin was shown to exhibit suppressive effects on the proliferation by inhibiting various intracellular signaling pathways, which are related to  $\text{Ca}^{2+}$ -dependent kinases, PI3/Akt, and ERK/MAPK in HepG2 cells. Results with western blot analysis confirmed that overexpression of regucalcin led to decrease in protein levels that were involved in the signaling of Ras, Akt and MAPK in HepG2 cells.

In addition, suppressive effects of regucalcin overexpression on cell proliferation were not changed in the presence of DRB, an inhibitor of transcriptional activity with RNA polymerase II inhibition (40). Regucalcin was observed to suppress transcriptional activity in HepG2 cells and to bind to DNA and regulate nuclear gene expression (42). Regucalcin may thus regulate transcriptional activity in HepG2 cells.

Gemcitabine is well known as an antitumor agent that induces nuclear DNA damage, and it is used clinically for the therapy of human pancreatic cancer (41). This agent suppresses cell proliferation and stimulates apoptotic cell death in various types of cancer cells. Suppressive effects of regucalcin overexpression on the proliferation were not potentiated in the presence of gemcitabine in HepG2 cells, suggesting that regucalcin partly acts on pathways related to the action mode of gemcitabine. Regucalcin has been shown to inhibit DNA and RNA synthesis in isolated rat liver nuclei (17-19).

Overexpression of regucalcin was demonstrated to increase the gene expression of p53 and Rb, a suppressor of tumor, and p21, an inhibitor of cell cycle, and suppress the gene expressions of ras, c-jun and c-myc, oncogenes, in cloned rat hepatoma H4II-E cells *in vitro* (42). Overexpression of regucalcin was also found to increase the protein levels of p-53, Rb and p21, and decrease the protein levels of ras, c-fos and c-myc in HepG2 cells *in vitro*. These results may support the view that regucalcin partly mediates a suppressive effect on the proliferation of HepG2 cells by regulating the expression of tumor suppressor proteins and oncogenes. Interestingly, overexpression of regucalcin was found to decrease NF- $\kappa$ B p65 and  $\beta$ -catenin in HepG2 cells. Regucalcin may suppress signaling process and transcription activity related to NF- $\kappa$ B and  $\beta$ -catenin in HepG2 cells. This effect may partly contribute in the revelation of anticancer effects of regucalcin in HCC.

Overexpression of regucalcin was found to prevent cell death induced by various stimulatory factors including LPS, TNF- $\alpha$ , and Bay K 8644 in HepG2 cells *in vitro*, supporting the view that the suppressive effects of regucalcin on the proliferation were not based on cell death. Preventive effect of regucalcin on cell death was not enhanced in the presence of caspase-3 inhibitor. Regucalcin was suggested to prevent cell death through the mechanism by which it increases caspase-3 activity that activates nuclear DNA fragmentation that induces

apoptosis of cells. It is possible that regucalcin directly inhibits caspase-3. In addition, overexpression of regucalcin has been shown to decrease the protein levels of caspase-3 and cleaved caspase-3 in human pancreatic cancer MIA PaCa-2 cells (24) and breast cancer MDA-MB-231 cells (25). Regucalcin has also been shown to directly inhibit calcium-activated endonuclease in isolated rat liver nucleus *in vitro* (43) and to suppress nitric oxide synthetase activity and to regulate the gene expression of various proteins that are involved in apoptosis in cloned rat hepatoma H4-II-E cells *in vitro* (34).

In conclusion, this study demonstrates that prolonged survival is associated with increased regucalcin gene expression in HCC patients, and that the overexpression of regucalcin suppresses the proliferation enhanced through various signaling pathways in human hepatoma HepG2 cells *in vitro*. Our findings may support the view that endogenous regucalcin plays a potential role as a suppressor protein in the development of hepatocarcinogenesis. Overexpression of regucalcin may constitute a novel therapeutic approach to treating HCC.

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