REVIEW ARTICLE

# Involvement of Regucalcin in Human Carcinogenesis Prevention

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## Abstract

Regucalcin, a calcium-binding protein, was discovered in 1978 and is associated with multifunctional role as a suppressor in signal transduction-related translational activity in various types of cells and tissues. Its gene, *rgn*, is located on the X chromosome in humans. Regucalcin also suppresses nuclear deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis in liver cells. Overexpression of regucalcin suppresses the proliferation of cancer cells by inhibiting G1 and G2/M cell cycle arrest. Regucalcin gene expression is suppressed in cancer tissues of patients, and survival was demonstrated to be prolonged in patients with various types of cancer with higher levels of regulcalcin. Suppressed regucalcin gene expression may play a crucial role in the development of carcinogenesis. Development of regucalcin gene delivery system, as a novel gene therapy, is expected to be beneficial in the clinical aspects in the treatment of cancer patients

## Keywords

Regucalcin, cell signaling, nuclear regulation, cell proliferation, carcinogenesis.



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## Introduction

Regucalcin was discovered in 1978 as a novel calcium-binding protein that suppresses calcium signaling in various types of cells and tissues.[1-6] The regucalcin gene (gene symbol; *rgn*) is located on X chromosome,[7,8] and organization of the regucalcin gene consists of seven exons and six introns,[9] and it comprises regucalcin family of over 15 species of vertebrate and invertebrate.[5,6,10] Regucalcin plays a multifunctional role in cell regulation: maintains intracellular $Ca^{2+}$ homeostasis and suppresses signal transduction, protein synthesis, cell proliferation and apoptosis.[2-4] Regucalcin has been found to play a pivotal role in maintaining cell homeostasis as a suppressor of cell signaling in various types of cells and tissues.[2-4]

Cancer is a pathological condition, where cells display uncontrolled growth, invasion and metastasis. Cell proliferation is mediated through various intracellular signaling transduction-related transcriptional activities that are stimulated by various hormone and cytokines. Enhanced cell proliferation may lead to carcinogenesis. However, mechanism of carcinogenesis is complex and development of novel therapy is required after thorough investigations. Regucalcin has been demonstrated to play a novel suppressing role in cell signaling, and it plays a multifunctional role in regulation of function of various types of cells and tissues.[2-4] Interestingly, overexpression of the regucalcin gene was found to suppress liver cell proliferation and carcinogenesis in animal models.[11-13] Moreover, analysis with multiple gene expression profiles and proteomics has showed that regucalcin gene expression is uniquely suppressed in various human carcinoma tissues.[11-14] Suppressed regucalcin gene expression may lead to the development of carcinogenesis. This mini-review focuses on the potential role of regucalcin as a suppressor in the development of carcinogenesis.

## Role of Regucalcin as a Suppressor in Cell Proliferation

Regucalcin is localized in the cytoplasm and nucleus in cells.[15,16] Nuclear translocation of cytoplasmic regucalcin has been found to passively transport regucalcin to the nucleus through nuclear pore in cells. Immunocytochemical analysis has showed that regucalcin is localized in the nuclei of the cloned normal rat kidney proximal tubular epithelial NRK52E cells.[17] Nuclear localization of regucalcin is enhanced through hormonal $Ca^{2+}$-signaling dependent process, which involves protein kinase C.[17] Regucalcin has been shown to bind protein and DNA and regulate various enzyme activities in the nucleus.[18] Nuclear regucalcin has been found to exhibit suppressive effects on $Ca^{2+}$-activated DNA fragmentation by inhibiting endonuclease activity in isolated rat liver nuclei.[19,20] Regucalcin inhibits the activity of Small GTPase Ran (ras-related nuclear protein) that is required for protein export from the nucleus and protein import into the nucleus.[21] Moreover, regucalcin is found to suppress the activities of tyrosine kinase, protein kinase C and $Ca^{2+}$/calmodulin-dependent protein kinase, which mediate the process of signal transduction from the cytoplasm to nucleus in cells.[22] In addition, nuclear endogenous regucalcin has been shown to play a suppressive role in the regulation of protein tyrosine phosphatases by using anti-regucalcin monoclonal antibody in the reaction mixture.[23] Thus, regucalcin has been shown to play a crucial role in the regulation of the activity of various enzymes in the nucleus.

Regucalcin has also been demonstrated to exhibit suppressive effects on DNA and RNA synthesis activity in the nuclei of normal rat liver and regenerating rat liver *in vivo*.[24-27] Regucalcin may suppress the enhancement of nuclear DNA and RNA synthesis in proliferating liver cells *in vivo*. Also, regucalcin depressed DNA synthesis activity in the nuclei isolated from rat renal cortex *in vitro*.[28] The presence of anti-regucalcin monoclonal antibody in the reaction mixture containing the liver nucleus causes an increase in nuclear DNA synthesis activity *in vitro*.[24, 25] This increase is completely suppressed in the presence of regucalcin. Thus, endogenous regucalcin is associated with a suppressive effect on DNA synthesis in the nuclei of rat liver and renal cortex.[24,25] The role of regucalcin in inhibiting nuclear RNA synthesis activity in normal rat liver is not observed in the presence of α-amanitin, an inhibitor of RNA polymerase II and III,[26,27] thereby suggesting its suppressive effect, which is partly resulted from the inhibitory action on RNA polymerase II and III. Regucalcin may be also associated with direct inhibitory effects on nuclear DNA and RNA polymerase activity.

Regucalcin was found to suppress nuclear function in proliferating cells using cloned hepatoma H4-II-E cells cultured in the presence of fetal bovine serum (FBS). Culture with FBS induced an increase in cell number and a corresponding elevation of various kinase activities, which are related to $Ca^{2+}$/calmodulin-dependent protein kinase, protein kinase C, protein tyrosine kinase and protein phosphatase activity in H4-II-E cells.[29- 31] These enzymes may contribute to the enhancement of hepatoma cell proliferation after serum stimulation. The presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture using H4-II-E cells cultured with FBS stimulation was found to elevate the activities of protein kinase and protein phosphatase. Such an effect is suppressed by the addition of exogenous regucalcin in the enzyme reaction mixture. Regucalcin plays a crucial role as a suppressor in the enhancement of cell proliferation due to inhibiting the activities of various protein kinases and protein phosphatases in the cytoplasm and nucleus.[29-31] Importantly, nuclear DNA synthesis activity was increased at 6 hours after culture with FBS, which is preceded by an elevation in the number of H4-II-E cells cultured with FBS.[32,33] Nuclear DNA synthesis activity in H4-II-E cells was significantly suppressed by the addition of regucalcin in the reaction mixture, and its activity was enhanced by the addition of regucalcin into the reaction mixture,[32, 33] thereby supporting the view that endogenous regucalcin suppresses DNA synthesis activity by which inhibiting protein kinases in the nuclei of proliferating H4-II-E cells using anti-regucalcin monoclonal antibody.[32]

Moreover, to determine the role of endogenous regucalcin in the regulation of nuclear DNA synthesis, regucalcin/pCXN2-transfected cells, where H4-II-E cells overexpress regucalcin stably, were generated.[33] The increase in cell number and DNA synthesis activity in transfectants was suppressed as compared to those of wild- and mock-type; thereby indicating that overexpression of endogenous regucalcin has suppressive effects on cell proliferation.[33] This finding supported the view that the augmentation of endogenous regucalcin has potent suppressive effects on nuclear DNA synthesis activity in proliferating

hepatoma cells. Regucalcin has been proposed to play a suppressive role for the over-proliferation of liver cells.

Overexpression of regucalcin was demonstrated to induce G1 and G2/M phase cell cycle arrest in transfectants (H4-II-E cells).[34] The mRNA expression of *p21*, an inhibitor of cyclin-dependent kinases (cdk), was increased in transfectants, although cdc2a and chk2 (checkpoint-kinase 2) mRNA levels were not significantly altered.[34] Regucalcin may enhance *p21* expression and inhibits G1 progression in H4-II-E cells. Overexpression of endogenous regucalcin has also been shown to suppress proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells.[35] Endogenous regucalcin was shown to induce G1 and G2/M phase cell cycle arrest in NRK52E cells.[35] Interestingly, expression of *c-jun* and chk2 (checkpoint-kinase 2) mRNAs was suppressed in the transfectants of NRK52E cells,[35] and the expression of *c-myc, c-fos,* cdc2 and *p21* mRNAs was not altered in transfectants.[35] Suppressed c-jun and chk2 mRNA expressions may partly contribute to suppression of cell proliferation induced in regucalcin-overexpressing NRK52E cells. In addition, *c-myc, c-fos, c-ju,* and Ha-ras are known as tumor stimulator genes.[36] *p53* and *Rb* are tumor suppressor genes, and *c-src* is oncogene.[37] Expression of *c-myc*, Ha-*ras* or *c-src* mRNAs was suppressed in regucalcin-overexpressing transfectants.[38] Expression of *p53* and Rb mRNAs was markedly enhanced in transfectants.[38] Suppressed expression of *c-myc*, *Ha-ras* and *c-src* mRNAs and enhanced expression of p53 and Rb mRNAs in transfectants may lead to retardation of proliferation of hepatoma H4-II-E cells. Also, expression of *p53* mRNA was enhanced in regucalcin-overexpressing transfectants of NRK52E cells, while expression of *c-myc, c-fos*, *cdc2* and *p21*mRNAs was not altered in transfectants.[35] Suppressed *c-jun* and chk2 mRNA expressions and enhanced *p53* mRNA expression may lead to retardation of cell proliferation in NRK52E cells overexpressing regucalcin.

As described above, regucalcin suppressed the effects on cell proliferation by regulating many gene expressions that are related to cell proliferation in hepatoma H4-II-E cells and normal kidney NRK52E cells.[39] Regucalcin can bind DNA and modulate nuclear transcriptional activity.[18] Also, regucalcin can bind to the promoter region of various genes, which suppress stimulator gene expression or stimulate suppressor gene expression in cell proliferation.[39] Overexpression of endogenous regucalcin suppresses cell proliferation.[39] Regucalcin may play a pivotal role as a suppressor for over-proliferation of normal and cancer cells by regulating multi-signaling process related to transcription activity. Interestingly, overexpression of regucalcin was shown to protect apoptotic cell death in normal and cancer cells induced by various signaling stimulating-factors,[40] supporting the view that the regucalcin-suppressed proliferation is not critical for apoptotic cell death.

## Suppressive Role of Regucalcin in the Development of Carcinogenesis

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is one of the most prevalent malignant diseases worldwide, and the third most common causes of cancer-related death.[41-43] HCC originates on a background of cirrhosis, a chronic and diffuse hepatic disease, which results from continuous liver injury and regeneration.[43] Cirrhosis is present in approximately 80%-90% of HCC patients and constitutes the largest single risk factor. In cirrhotic liver, changes in fat metabolism associated with the activation of adipocyte-like pathways are thought to be involved in neoplastic transformation.[43] Increased hepatocyte turnover, inflammation and oxidative DNA damage is implicated in the pathogenesis of various liver diseases, obesity, type 2 diabetes, insulin resistant and nonalcoholic fatty liver disease. The prevalent risk factors for HCC that includes viral infections (hepatitis B and C) and alcohol consumption; further risk factors include tobacco smoking, exposure to aflatoxin B1 and vinyl chloride, diabetes, and genetic disorders, such as hemochromatosis and alpha-1 antitrypsin deficiency are also found to be associated with the development of liver cirrhosis.[44-48]

Hepatocarcinogenesis is a multistep process initiated by external stimuli that lead to genetic changes in hepatocytes or stem cells, resulting in proliferation, apoptosis, dysplasia and neoplasia. The majority of HCC cases are also related to chronic viral infections. Hepatitis B virus (HBV) DNA integrates into the host genome, inducing chromosome instability and insertional mutations that may activate various oncogenes, such as cyclin A.[49-52] Viral proteins, in particular X protein (HBx), act as transactivators that upregulate several oncogenes, such

as *c-myc* and *c-jun*, and transcriptional factors, such as nuclear factor-κB.[59-61] Additionally, HBx activates promoters of genes encoding interleukin-8 (IL-8), tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and epidermal growth factor receptor (EGFR).[54] HBx can also stimulate several signal transduction pathways, including the JAK/STAT, RAS/RAF/MAPK, and Wnt/β-catenin pathways.[54,55] The contributions of hepatitis C virus (HCV) to hepatocarcinogenesis are mediated through viral proteins, including core, NS3 and NS5A proteins. HCV core protein can promote apoptosis or cell proliferation by interaction with *p53* or upregulation of *Wnt-1* at the transcriptional level.[56]

The prognosis of advanced HCC remains poor in spite of the development of novel therapeutic strategies. Traditional therapies are not effective for HCC and are too toxic for patients with cirrhosis. Currently, transarterial chemoembolization and radioembolization remain the main treatments for intermediate-stage HCC. Improved knowledge of the oncogenic processes and signaling pathways, which regulate tumor cell proliferation, differentiation, angiogenesis, invasion and metastasis, has led to the identification of several potential therapeutic targets that have driven the development of molecular-targeted therapies.[57] An ideal cancer target meets the following criteria: the target is relatively specific for cancer cells (not expressed or expressed at very low levels in normal cells but overexpressed in cancer cells).[67] The target is "drugable" as an enzyme (*e.g.,* a kinase) or cell surface molecule (*e.g.,* a membrane-bound receptor) that can be easily screened for small-molecule inhibitors or targeted by a specific antibody.[57,58] The only systemic therapy available for advanced HCC is based on the multikinase inhibitor sorafenib,[58] which is the most effective therapeutic tool for advanced nonresectable HCC. In the past few years, the use of sorafenib in combination with transarterial chemoembolization has improved survival rates in patients with advanced HCC. New perspectives in cancer treatment have appeared with the advent of microRNAs, a novel class of noncoding small RNAs.[59]

Regucalcin, a suppressor protein in various cell signal transductions,[3,4] has been demonstrated to play a pivotal role in the suppression of hepatocarcinogenesis.[35, 60] As introduced in previous section, overexpression of regucalcin was found to play a role as a suppressor protein in cell proliferation that is mediated through various signaling stimulations in the cloned normal rat kidney proximal tubular epithelial NRK52E cells and the cloned rat hepatoma H4-II-E cells, inducing G1 and G2/M phase cell cycle arrest.[11] Regucalcin has also been demonstrated to exhibit direct inhibitory effects on the activities of various $Ca^{2+}$/calmodulin-dependent enzymes, protein kinases and protein phosphatases in the cytoplasm and nuclei, suppressive effects on nuclear DNA and RNA synthesis, depressive effects on the gene expression of *c-myc, Ha-ras* and *c-src*, a tumor-stimulator gene and stimulatory effects on the gene expression of *p53* and *Rb*, a tumor-suppressor gene.[39] Moreover, regucalcin was demonstrated to inhibit protein synthesis by inhibiting aminoacyl-tRNA synthetase and stimulate protein degradation by activating cysteinyl protease.[3,4] Thus, suppressive effects of regucalcin on cell proliferation are mediated by targeting multi-molecules in liver cells.

Importantly, the gene expression of regucalcin was demonstrated to be suppressed in the development of hepatocarcinogenesis. Liver regucalcin gene expression was suppressed at early periods of carcinogenesis in rats treated with diethy lnitrosamine and then 2-acetylaminofluorene combined with partial hepatectomy, which induces an increase in proliferating cells.[12] Suppression of regucalcin protein expression was identified in proteomic analysis that was differentially expressed in the livers of rats fed 5% ethanol for one and three months,[13] Liver regucalcin mRNA expression was suppressed by liver metabolism disorder induced by administration of carbon tetrachloride,[61] galactosamine[62] and phenobarbital[63] in rats. Hepatic regucalcin level was also reduced in diabetes and during ethanol ingestion,[64] which may result in cirrhosis and HCC. Suppressed regucalcin gene expression may lead to the development of HCC. Multiple gene expression profiles and proteomics analysis have showed that the regucalcin gene and its protein levels were found to be significantly suppressed in human HCC. Suppressed regucalcin gene expression may lead to the development of human hepatocarcinogenesis.

## Prospects

We demonstrated that regucalcin mRNA expression is suppressed in various human normal and tumor tissues, including HCC, kidney transitional cell carcinoma, brain malignant

meningioma and lung non-small cell carcinoma in human subjects.[14] Regucalcin plays a key role in suppressing the cell proliferation and carcinogenesis in various types of human cancer cells and tissues. Importantly, survival has been shown to be prolonged in pancreatic cancer patients, with increased regucalcin gene expression,[70] and overexpression of regucalcin was found to suppress the cell proliferation in human pancreatic cancer MIA PaCa-2 cells *in vitro*.[70] Overexpression of the regucalcin gene in cancer cells may exhibit preventive and therapeutic effects on the development of carcinogenesis. Development of the regucalcin gene deliver system will be expected as a novel gene therapy in clinical aspects for cancer treatment.

## Author Disclosure

The author has no conflicts of interest.

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