

## Regucalcin Serves as a Suppressor Protein in Hepatocarcinogenesis

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### Abstract

Regucalcin, which was initially discovered in 1978 as a calcium-binding protein, plays a multifunctional role as a suppressor in signal transduction in various types of cell and tissues. The regucalcin gene (*rgn*) is localized on the X chromosome. Regucalcin is shown to suppress various protein kinases and protein phosphatases activities, protein synthesis, and nuclear deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis in liver cells. Overexpression of endogenous regucalcin reveals suppressive effects on proliferation in the modeled rat hepatoma H4-II-E cells independently on apoptosis *in vitro*. Regucalcin mRNA expression is uniquely downregulated in the development of carcinogenesis in the liver of rats *in vivo*. Regucalcin mRNA and protein expressions were also found to depress in human hepatoma HepG2 cells and hepatoma tissues. The depression of regucalcin gene expression was associated with progression of hepatocarcinogenesis. This review will discuss the role of regucalcin as a suppressor protein in hepatocarcinogenesis.

**Keywords:** regucalcin, cell signaling, gene expression, cell proliferation, carcinogenesis, human hepatoma

### Introduction

Regucalcin was initially discovered in 1978 as a calcium-binding protein [1-4]. The name, regucalcin, was proposed for this calcium-binding protein, which suppresses the activity of various enzymes that are activated by Ca<sup>2+</sup> or Ca<sup>2+</sup>/calmodulin [2,3]. The regucalcin gene (*rgn*) is localized on the X chromosome in consisting of seven exons and six introns [5-7]. Regucalcin and its gene are identified in over 15 species consisting of regucalcin family, and the gene species are highly conserved in vertebrate species [8]. The regucalcin gene expression is regulated through various transcription factors (including AP-1, NF1-A1, RGPR-p117,  $\beta$ -catenin, SP1, and others) [8].

Regucalcin has been demonstrated to play a multifunctional role in cell regulation in various types of cells and tissues [9-16]; regucalcin plays a pivotal role in maintaining of intracellular calcium homeostasis and suppression of nuclear protein kinase and protein phosphatase activities, Ca<sup>2+</sup>-activated DNA fragmentation, and DNA and RNA synthesis. Regucalcin suppresses protein synthesis at translational process [9,10]. Regucalcin has been proposed to play a pivotal role as a suppressor protein in various signal transductions to maintain cell homeostasis for various stimuli [9].

There is growing evidence that endogenous regucalcin plays a role as a suppressor protein in cell proliferation, which 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 *in vitro* [10,12]. Regucalcin mRNA and protein expressions have been shown to suppress in human hepatoma HepG2 cells and hepatoma tissues. Depression of regucalcin gene expression is associated with progression of carcinogens. Suppression of regucalcin gene expression may lead to the promotion of cell proliferation and carcinogenesis. This review will discuss the role of regucalcin as a suppressor protein in the development of hepatocarcinogenesis in animal models and human tumor tissues.

### Hepatocellular Carcinoma

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 [17-19]. Globally, there are approximately 750,000 new cases of HCC reported per year. Features of HCC are an aggressive cancer with a dismal outcome largely due to metastasis and postsurgical recurrence. HCC originates on a background of cirrhosis, a chronic and diffuse hepatic disease that results from continuous liver injury and regeneration [19]. 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 [19]. Increased hepatocyte turnover, inflammation, and oxidative DNA damage is implicated in the pathogenesis of the liver disease including obesity, Type 2 diabetes, insulin resistant and nonalcoholic fatty liver disease. 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 prevalent risk factors for HCC are also the cause of liver cirrhosis and include 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 [20-24]. 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 [25-28]. Viral proteins, in particular X protein (HBx), act as transactivators to upregulate several oncogenes (such as c-myc and c-jun) and transcriptional factors (such as nuclear factor- $\kappa$ B) [13-15]. Additionally, HBx activates promoters of genes encoding interleukin-8, tumor necrosis factor, transforming growth factor- $\beta$  and epidermal growth factor receptor [16]. HBx can also stimulate several signal transduction pathways, including the JAK/STAT, RAS/RAF/MAPK, and Wnt/ $\beta$ -catenin pathways [26,27]. 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 through interaction with p53 or upregulation of Wnt-1 at the transcriptional level [28-31].

The prognosis of advanced HCC remains poor in spite of the development of novel therapeutic strategies [21]. The potential therapeutic target is relatively specific for cancer cells [32]. The target plays an essential role in cancer initiation and progression, and inhibition of expression or activity of the target induces growth suppression and/or apoptosis in cancer cells. The target is "drugable"

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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 [32,33]. The only systemic therapy available for advanced HCC is based on the multikinase inhibitor sorafenib [33], which is the most effective therapeutic tool for advanced nonresectable HCC. The survival of patients with advanced HCC treated with sorafenib depends on the absence of liver dysfunction and on the status of the patient [34]. 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 [35].

### Regucalcin Suppresses Proliferation of Hepatoma Cells in vitro

Regucalcin plays a pivotal role as a suppressor protein in signal transductions in various types of cells and tissues; the protein suppresses signal transduction from the cytoplasm to nucleus in cell regulation, which is mediated through phosphorylation and dephosphorylation of many proteins and nuclear DNA and RNA synthesis [9-12]. Regucalcin was found to suppress the enhancement of hepatoma cell proliferation after serum stimulation. Regucalcin mRNA and protein was expressed in the cloned rat hepatoma H4-II-E cells and human hepatoma HepG2 cells [36-38]. These expressions were lower levels as compared with that in normal rat liver [36-38].

Culture with fetal bovine serum produced an increase in protein kinase activity and a corresponding elevation of cell number in cloned rat hepatoma H4-II-E cells [39]. The increase in protein kinase activity preceded a significant elevation of cell number [39], suggesting that serum factors (including growth factors, cytokines and hormones) stimulate cell proliferation that is partly mediated through cascades of various protein kinases. This enhancement is suggested to be involved in various protein kinases including  $Ca^{2+}$ /calmodulin-dependent protein kinase, protein kinase C, protein tyrosine kinase, extracellular signal-related kinase (ERK), mitogene-activated protein kinase (MAPK) and PI3 kinase [39]. Endogenous regucalcin was also shown to reveal suppressive effects on the enhancement of protein kinase activity in the cytoplasm in H4-II-E cells with cell proliferation by using anti-regucalcin monoclonal antibody [39]. Regucalcin may reveal suppressive effects for overexpression of hepatoma cell proliferation due to inhibiting various protein kinases in the cytoplasm and nucleus.

Regucalcin has been shown to reveal suppressive effects on protein phosphatase activity in the cytoplasm and nucleus of normal rat liver [40,41]. Regucalcin was found to reveal inhibitory effects on  $Ca^{2+}$ /calmodulin-dependent protein tyrosine phosphatase activity in the cells [42,43]. Endogenous regucalcin, which is increased after culture with Bay K 8644, was found to reveal suppressive effects on  $Ca^{2+}$ /calmodulin-activated protein tyrosine phosphatase activity in proliferating cells by using anti-regucalcin monoclonal antibody [42,43]. Processes that are reversibly controlled by protein phosphorylation require not only a protein kinase but also a protein phosphatase [44]. Target proteins are phosphorylated at specific sites by one or more protein kinases and these phosphoproteins are removed by specific protein phosphatase [44]. Regucalcin may play a pivotal role as a suppressor for the enhancement of cell proliferation due to inhibiting the activities of various protein kinases and protein phosphatases that are enhanced in hepatoma cell proliferation.

Endogenous regucalcin has been shown to reveal suppressive effects on nuclear DNA synthesis enhanced in H4-II-E with cell proliferation [45,46]. The presence of regucalcin in the reaction

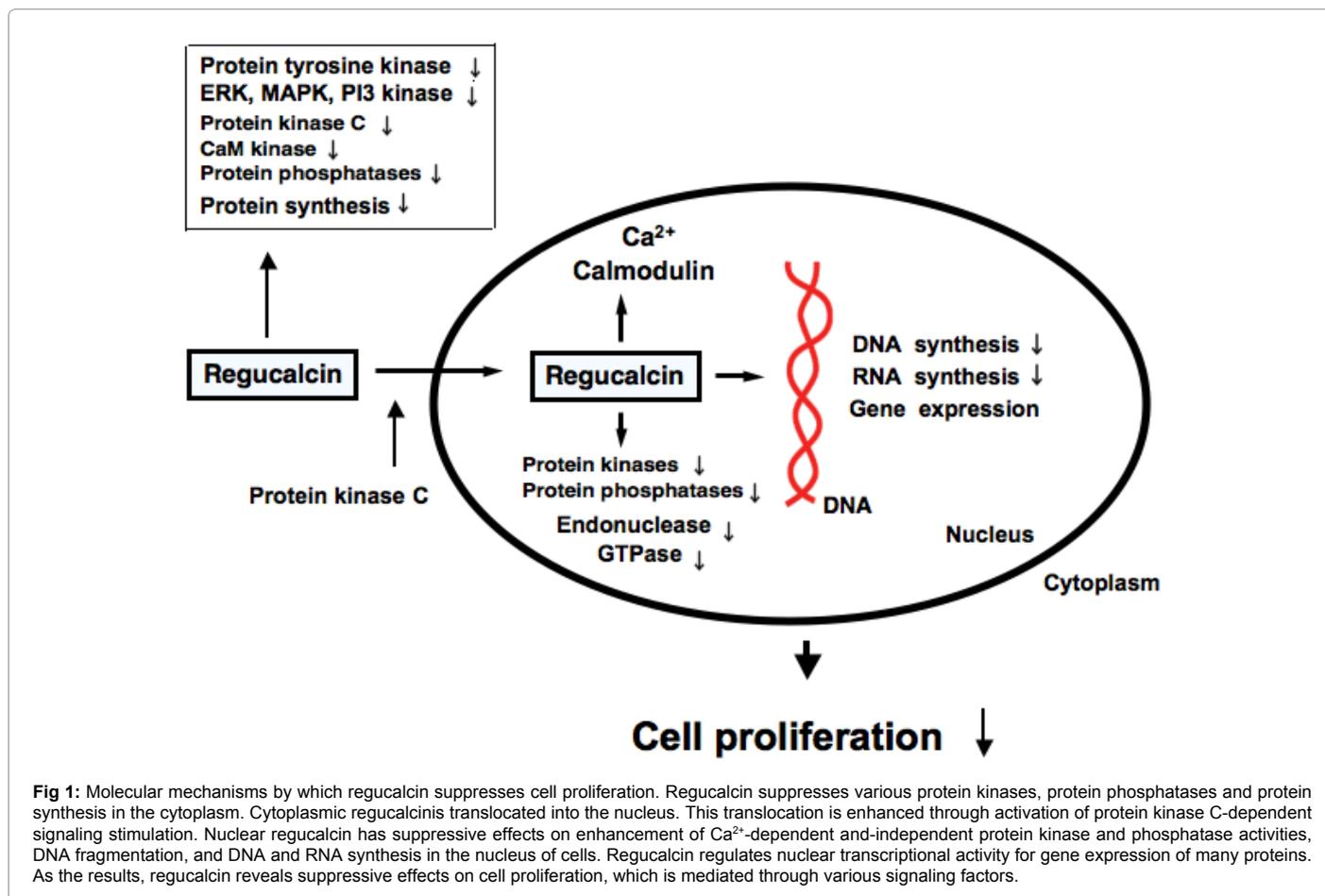
mixture caused a decrease in DNA synthesis activity in the nuclei of H4-II-E cells cultured with serum [45]. Nuclear DNA synthesis activity was increased in the presence of anti-regucalcin monoclonal antibody in the reaction mixture containing the nucleus of H4-II-E cells cultured with serum, and this elevation was depressed after addition of various protein kinase inhibitors in the reaction mixture [45]. These findings support the view that endogenous regucalcin suppresses nuclear DNA synthesis activity enhanced through mechanism by which depresses nuclear protein kinases, and that it has a direct-suppressive effect on DNA synthesis activity in the nuclei of H4-II-E cells with proliferation. Thus, regucalcin may reveal suppressive effects on the enhancement of nuclear DNA synthesis activity in proliferating cells, and it may play a suppressive role for over expression of cell proliferation. This was further supported using H4-II-E cells over expressing regucalcin stably. Regucalcin-over expressing cells were generated, which the regucalcin content of regucalcin/pCXN2-transfected cells showed 19.7-fold as compared with that of the parental wild-type H4-II-E cells and pCXN2 vector-transfected cells (mock type) [46]. Overexpression of endogenous regucalcin was found to reveal suppressive effects on cell proliferation [46]. The presence of anti-regucalcin monoclonal antibody in the reaction mixture caused an increase in DNA synthesis activity in the nuclei obtained from wild-type H4-II-E cells, mock-type cells, and the transfectants with over expression of regucalcin [46]. However, the augmentation of the nuclear DNA synthesis activity was remarkable in the transfectants [46], supporting the view that endogenous regucalcin has a great suppressive effect on the nuclear DNA synthesis activity.

Regucalcin has been shown to regulate the gene expression of cell cycle-related proteins in proliferating hepatoma cells [47]. Overexpression of endogenous regucalcin has been found to regulate the effect of various factors, which induce cell-cycle arrest, on the proliferation of H4-II-E cells (wild-type) [47]. The expression of p21 mRNA was found to enhance in the transfectants over expressing endogenous regucalcin, although the expressions of *cdc2a* and *chk2* (checkpoint-kinase 2) mRNAs were not changed in the transfectants [47]. P21 is an inhibitor of cyclin-dependent kinases (cdk) [48]. Regucalcin may enhance p21 expression and inhibits G1 progression in H4-II-E cells, although it cannot exclude the possibility, however, that regucalcin directly inhibits cdk activity in the cells. Regucalcin was found to cause G1 and G2/M phase cell cycle arrest in H4-II-E cells [47].

*c-myc*, *c-fos*, *c-jun* and *Ha-ras* are known to be tumor stimulator genes [49]. *p53* and *Rb* are tumor suppressor genes and *c-src* is oncogene [50]. The expression of *c-myc*, *Ha-ras* or *c-src* mRNAs was found to suppress in the transfectant over expressing regucalcin [51]. The expression of *p53* and *Rb* mRNAs was markedly enhanced in the transfectants [51], *p53* has also been known to stimulate the gene expression of p21, an inhibitor of cell cycle-related protein kinases, that induces cell-cycle arrest. The suppression of the expressions of *c-myc*, *Ha-ras* and *c-src* mRNAs and the enhancement of the expressions of *p53* and *Rb* mRNAs in the transfectants over expressing regucalcin may lead to the retardation of proliferation of hepatoma H4-II-E cells. In addition, the gene expression of IGF-I, which is a growth factor in cell proliferation, was found to suppress in H4-II-E cells [47]. Suppression of IGF-I expression may lead to retardation of cell proliferation.

Molecular mechanism by which regucalcin suppresses cell proliferation is summarized (Fig 1).

The revelation of suppressive effects of regucalcin on cell proliferation is involved in the depressive effects on calcium-dependent



signaling factors, various protein kinases and protein phosphatases activities, protein synthesis, nuclear DNA and RNA synthesis, IGF-I expression and the regulatory effects on various tumorigenesis-related gene expressions. Regucalcin, which was translocated into the nucleus, may bind DNA and modulate nuclear transcriptional activity. Over expression of endogenous regucalcin may suppress proliferation of hepatoma cells. Down-regulation of regucalcin gene expression in hepatoma cells may lead to stimulation of cell proliferation with change in various tumorigenesis-related gene expressions.

### Regucalcin Gene Expression is Suppressed in Hepatocarcinogenesis in vivo

Regucalcin gene expression has been shown to down regulate in the development of hepatocellular carcinogenesis in animal models in vivo. Regucalcin gene expression was found to suppress in the hepatoma tissues of rats in vivo [52]. Rat hepatoma was induced after continuous feeding of basal diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) [52]. After 35 weeks feeding, the non-tumorous and tumorous tissues of the livers were removed from the rats [52]. Regucalcin mRNA levels in the tumorous tissues were decreased in comparison with that of the non-tumorous tissues of the chemical-fed rats, while c-myc mRNA was specifically increased in the hepatomas [52].

New markers for the liver pre-neoplastic foci in rats treated with diethylnitrosamine and then 2-acetylaminofluorene combined with partial hepatectomy, which induces an increase in proliferating cells, has been investigated [53]. Transaldolase, aflatoxin B1 aldehyde

reductase, and gamma- glutamylcysteine synthetase are found as up-regulated gene, and regucalcin was found as a down-regulated gene, in line with findings for hepatocellular carcinomas [53]. Suppression of regucalcin gene expression was found at the early stage in the development of carcinogenesis [53].

Biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase (CuZnSOD, Sod1) deficient mice have been identified [54]. Liver samples were obtained from 18-month-old 1<sup>-/-</sup> and 1<sup>+/+</sup> mice. Regucalcin showed a divergent alteration in Sod1<sup>-/-</sup> samples [54]. Whereas elevated regucalcin levels were observed in 1<sup>-/-</sup> samples with no obvious neoplastic changes, marked reduction in regucalcin is observed in 1<sup>-/-</sup> samples with fully developed hepatocellular carcinoma [54]. Glutathione S-transferase (GST) mutant 1 (M1) showed a significant increase only in the neoplastic regions obtained from Sod1<sup>-/-</sup> livers [54]. No change in GSTM1 was observed in the surrounding normal tissues [54]. Marked reduction was observed in two intracellular lipid transporters, fatty acid binding protein 1 and major urinary protein 11 and 8 in Sod1<sup>-/-</sup> livers [54]. Thus, regucalcin was identified to be biomarkers associated with the development of hepatocellular carcinoma in CuZn superoxide dismutase deficient mice in vivo [54].

Moreover, the suppression of regucalcin protein expression has also been identified in proteomic analysis that was differentially expressed in the livers of rats fed 5% ethanol for 1 and 3 months [55]. In addition, regucalcin mRNA expression is suppressed by disorder of liver metabolism (including carbon tetrachloride [56], galactosamine [57] and phenobarbital [58] administration, the conditions of diabetes

and ethanol ingestion [59]), which may lead to cirrhosis and HCC. Suppression of regucalcin gene expression may lead to the development of HCC.

Regucalcin gene suppression has been found to be associated with the development of carcinogenesis in animal models *in vivo*. If the chemical feeding-induced suppression of regucalcin expression accelerates the enhancement of various signaling pathways in liver cells, it may generate a possible circumstance for the development of carcinogenesis. In this aspect, the suppression of the regucalcin gene with chemical feeding *in vivo* may play a pathophysiological role in the development of carcinogenesis. Decrease in regucalcin, which plays a multifunctional role as a suppressor in various signaling pathways in proliferating cells, may lead to development in carcinogenesis.

Noticeably, regucalcin was found to be under expressed in human hepatoma HepG2 cells *in vitro* [37,38]. Moreover, the regucalcin gene and its protein levels were found to specifically suppress in human HCC patients using analysis with multiple gene expression profiles and proteomics [60-64]. Suppression of regucalcin gene expression may play a pathophysiologic role in the development of human HCC. Overexpression of regucalcin with the regucalcin gene delivery may be a useful tool in the therapy of human HCC.

## Prospect

Regucalcin plays a multifunctional role as a suppressor protein in cell signaling system in various types of cells and tissues. Regucalcin may be a key molecule in the suppression of the proliferation of hepatoma cells and the development of hepatocarcinogenesis. The enhancement of endogenous regucalcin gene expression may reveal preventive and therapeutic effects in the progression of hepatoma cells. Delivery of the regucalcin gene will be a novel useful tool in the therapy of human hepatocarcinoma. This may provide a new strategy in the development of novel therapeutic tools for human hepatocarcinoma with the regucalcin gene delivery system in clinical aspects.

## Author Disclosures

The author has no conflicts of interest.

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