



A mechanism of regucalcin knock-down in the promotion of proliferation and movement of human cervical cancer HeLa cells

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Background: Regucalcin (RGN) has been reported to have inhibitory effects on the proliferation and migration of many cancer cells. In this research, RGN expression has been silenced to study its effects on human cervical cancer HeLa cells.

Methods: Lentivirus mediated siRNA was infected into HeLa cells by lentivirus vector. The RGN expression was measured by quantitative real-time PCR (RT-qPCR) and Western blotting (WB). Cell count kit-8, colony formation assay and transwell assay were used to detect cell proliferation, migration and invasion. The expression of Wnt/ β -catenin pathway and epithelial-mesenchymal transition (EMT) related proteins, including β -catenin, GSK-3 β , p-GSK-3 β , matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-7 (MMP-7), matrix metalloproteinase-9 (MMP-9), E-cadherin, N-cadherin and vimentin, were detected by WB.

Results: Compared with HeLa-NC cell line, the expressions of RGN and E-cadherin in HeLa-siRGN cell lines are significantly decreased, while the expressions of β -catenin, MMP-3, MMP-7, MMP-9, p-GSK-3 β , N-cadherin and vimentin increased. The RGN down-regulation has been observed to promote cell proliferation, enhanced cell migration and invasion.

Conclusions: The down-regulation of RGN may enhance the activity of metastasis related signaling pathway Wnt/ β -catenin and EMT in the progression of cervical cancer.

Keywords: Regucalcin (RGN); HeLa; proliferation; migration; invasion

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Introduction

The regucalcin (RGN) has been originally extracted from rat liver homogenate and named by Japanese scientists (1). It plays a key role in maintaining intracellular calcium homeostasis and liver metabolism. It is reported that RGN

affects the activity of several enzymes such as pyruvate kinase, succinate kinase, glycogen phosphorylase and adenosine triphosphatase by the calcium ion (2-5). Recent studies showed that RGN can protect against radiation-induced testicular damage, regulate intracellular Ca²⁺ homeostasis in kidney proximal tubule epithelial cells

and has a cryoprotective effect for buffalo spermatozoa in extender (6-8). Furthermore, more and more reports have demonstrated that the expression of RGN is decreased in cancer cells, such as liver cancer, breast cancer, prostate cancer, lung cancer, pancreatic cancer, colorectal cancer and renal cell carcinoma, compared to normal cells (9-18). Up-regulating RGN expression in the cancer cells above may inhibit proliferation, migration and other biological characteristics. In our previous study, siRNA has been transfected into HepG2 cells and cell proliferation and migration have shown to be promoted (9). In addition to liver cancer, cervical cancer is also one of the most common cancers in the world, especially in developing countries (19-21). The relationship between RGN and cervical cancer has not been reported. In this study, therefore, we have established two cell lines HeLa-siRGN and HeLa-NC by lentiviral siRNA and lentiviral NC. Cell proliferation, migration, invasion abilities and the expression of Wnt/ β -catenin pathway and epithelial-mesenchymal transition (EMT) related proteins have been detected. The aim of this study is to provide theoretical basis for molecular targeted therapy of cervical cancer by targeting RGN.

Methods

Cell lines and lentivirus transfection

HeLa cells were supplied by the Chinese Academy of Sciences. HeLa cells were thawed in 37 °C water bath and cultured in Dulbecco's modified eagle's medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA). The cells were seeded in a 6-well plate (5×10^5 /well) and used for lentivirus infection. The multiplicity of infection (MOI) is 20. SiRNA for RGN interference was designed using Ambion company's online design tool and then linked to lentivirus and packaged by GenePharma company, Suzhou, China. After 24 hours transfection, the virus diluent was removed and cells were incubated in puromycin solution (2 μ g/mL) for 3 days to kill the cells failed in infection.

Quantitative real-time PCR

Total RNA was extracted by Total RNA extraction kit (Tiangen, China). PrimeScript™ RT reagent Kit (TAKARA, Japan) was used for DNA removal and reverse transcription. Then, RGN mRNA expression was measured by SYBR Premix Ex Taq™ II (TAKARA, Japan). Primer sequences for RGN are as follows: forward,

5'-GTGGATGCCTTTGACTATGACC-3'; reverse, 5'-CTTCCCCTCAGCATCAATACAC-3'. Primer sequences for internal reference gene GAPDH are as follows: forward, 5'-CGAGATCCTCAACCAATCAA-3'; reverse, 5'-GGTGGTCCAGGGTCGTTACT-3'. All operations were carried out according to the kit instructions. The experimental results were processed by $2^{-\Delta\Delta Ct}$ method.

Western blotting (WB)

Cells were collected with scrapers on ice-bath. The total protein was extracted with cell lysis buffer and protein concentration of each group was determined by bicinchoninic acid method (BCA, Beyotime, China). Forty μ g proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After blocking with skim milk, the membranes were incubated with polyclonal rabbit antibodies at 4 °C for a night and goat anti-rabbit IgG-HRP (1:5,000; E-AB-1003; Elabscience, China) at 37 °C for 1 hour. After washing the membranes with TBST for three times, the membranes were exposed and developed with Super ECL plus hypersensitive luminescence solution (Applygen, China). The relative expression of proteins was analyzed by Image J. The primary antibodies used are as follows: RGN (1:1,000; 17947-1-AP; Proteintech, USA), β -catenin (1:1,000; 51067-2-AP; Proteintech, USA), p-GSK-3 β (1:1,000; ab75814; Abcam, UK), GSK-3 β (1:1,000; 22104-1-AP; Proteintech, USA), MMP-3 (1:1,000; 17873-1-AP; Proteintech, USA), MMP-7 (1:800; 10374-2-AP; Proteintech, USA), MMP-9 (1:800; 10375-2-AP; Proteintech, USA), E-cadherin (1:1,000; 20874-1-AP; Proteintech, USA), N-cadherin (1:1,000; 22018-1-AP; Proteintech, USA), Vimentin (1:1,000; 10366-1-AP; Proteintech, USA), GAPDH (1:1,000; 10494-1-AP; Proteintech, USA).

Cell proliferation assay

Cells were seeded in five 96-well plates at a concentration of 3,000/well. Cell proliferation was determined at 0, 24, 48, 72 and 96 hours, respectively. The culture medium was aspirated and 100 μ L fresh medium with 10 μ L CCK-8 buffer was pipette to each well. The plates were incubated for about 4 hours and the absorbance of formazan produced in the chemical reaction can be detected at 450 nm using microplate reader. The amount of formazan is proportional to the number of living cells.

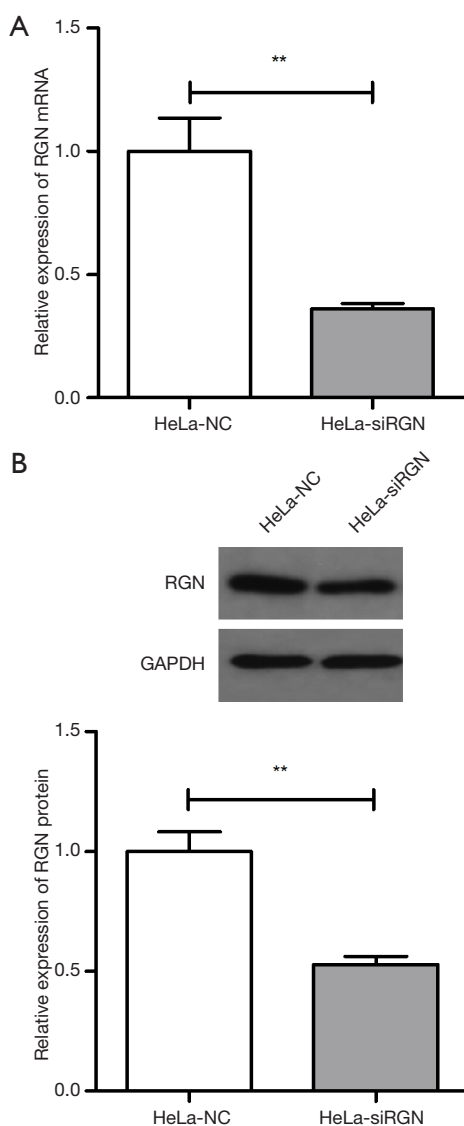


Figure 1 The two cell lines HeLa-siRGN and HeLa-NC have been established successfully. (A) siRNA effectively reduced the expression of RGN mRNA in HeLa cells; (B) siRNA effectively reduced the expression of RGN protein in HeLa cells. **, $P < 0.01$. RGN, regucalcin.

Colony formation assay

Five hundred cells of each group were added to the 6-well plates. During the time of culturing, the growth of cells was observed and fresh culture medium was replaced in time. After 14 days, the cells were rinsed twice with PBS, fixed with paraformaldehyde for 15 min and stained with 0.1% crystal violet for 10 min. Crystal violet was washed off with ddH₂O

and let the plates naturally dried. The number of colonies with cell number greater than 50 in each group was then counted.

Cell migration assay

Complete medium was added into the 24-well plates. Cells were collected to make cell suspension using DMEM and seeded in the upper chamber (3×10^4 /well). Then, the plates were incubated at 37 °C for 24 hours to let the cells pass through the pores on the membrane. The cells on the upper layer of the chamber were gently erased, and the cells on the lower layer of the membrane were fixed with 100% methanol and stained with 0.1% crystal violet. Migratory cells were taken pictures, counted in 5 random fields ($\times 100$) and taken average values.

Cell invasion assay

Lay the 1:8 diluted matrigel onto the upper chamber of polycarbonate film and kept for a night at 37 °C. Cells were collected to make cell suspension using DMEM and seeded in the upper chamber (3×10^4 /well). Then, the plates were incubated at 37 °C for 24 hours to let the cells pass through the pores on the membrane. The cells on the upper layer of the chamber were gently erased, and the cells on the lower layer of the membrane were fixed with 100% methanol and stained with 0.1% crystal violet. Invasive cells were taken pictures, counted in 5 random fields ($\times 100$) and taken average values.

Statistical analysis

Spss17.0 statistical software was used to process the data. Measurement data were expressed by mean \pm SD and statistical analyses were performed using *t*-test. $P < 0.05$ was considered to have statistically significant.

Results

RGN expression was down-regulated by siRGN

After lentivirus siRGN infection, the expression level of RGN mRNA and protein of HeLa-siRGN cell line were significantly lower than those of HeLa-NC cell line ($P < 0.01$). The results demonstrated that the two cell lines HeLa-siRGN and HeLa-NC have been established successfully (Figure 1).

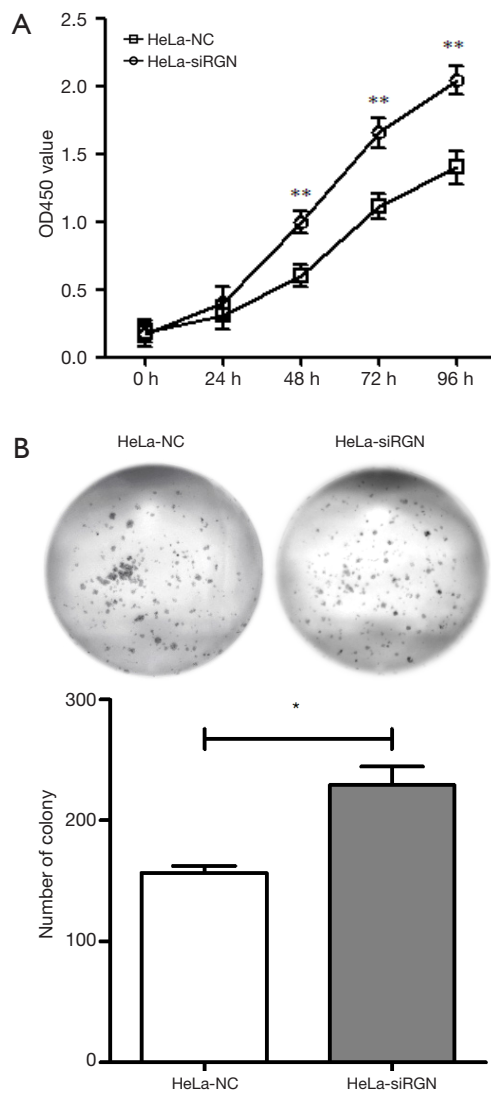


Figure 2 Down-regulation of RGN promoted the proliferation ability of HeLa cells. (A) RGN down-regulation effectively promoted the proliferation rate of HeLa-siRGN cells; (B) RGN down-regulation effectively raised the number of colonies of HeLa-siRGN cells. *, $P < 0.05$; **, $P < 0.01$. RGN, regucalcin.

Down-regulation of RGN promoted proliferation

Figure 2A have shown that the proliferation rate of HeLa-siRGN cells was faster than that of HeLa-NC cells ($P < 0.01$). From the results of clone formation experiment (Figure 2B), it can be seen that after 14 days of culture, the number of colonies (cell number greater than 50) in HeLa-siRGN group was more than what in HeLa-NC group ($P < 0.05$).

Down-regulation of RGN enhanced cell migration and invasion

Cells were cultured in transwell chamber for 24 hours, and the cells passing through the membrane were observed by light microscope. Figure 3A shows that the average migratory cell number in HeLa-siRGN group was 434.33 ± 28.54 , in HeLa-NC group was 231.33 ± 25.93 ($P < 0.001$). Figure 3B shows that the average invasive cell number in HeLa-siRGN group was 405.67 ± 24.13 , in HeLa-NC group was 202.00 ± 27.51 ($P < 0.001$).

Down-regulation of RGN promoted the activation of Wnt/ β -catenin pathway and EMT

We have used WB to detect the effect of RGN down-regulation on Wnt/ β -catenin pathway and EMT. Figure 4 shows that the expression of E-cadherin in HeLa-siRGN decreased ($P < 0.01$) and the expression of β -catenin, p-GSK-3 β , MMP-3, MMP-7, MMP-9, N-cadherin and vimentin increased ($P_{\text{MMP-9}} < 0.05$, $P_{\beta\text{-catenin, p-GSK-3}\beta, \text{MMP-7}} < 0.01$, $P_{\text{MMP-3, N-cadherin, vimentin}} < 0.001$).

Discussion

The role of RGN in tumor has been paid more and more attention by researchers. Its anti-tumor effect may make it a potential target for cancer gene therapy. This study has shown that RGN down-regulation can significantly promote the proliferation, migration and invasion abilities of HeLa cells. Furthermore, RGN down-regulation has decreased the expression of E-cadherin and increased the expression of β -catenin, p-GSK-3 β , MMP-3, MMP-7, MMP-9, N-cadherin and vimentin. From the above results, it seems that, after down-regulation of RGN, the Wnt/ β -catenin pathway of HeLa cells has been activated, and the EMT process has been promoted.

In 1990s, several researchers have found that exogenous double-stranded RNA injected into nematodes is able to induce homologous gene silencing and it has been called RNA interference (22). The siRNA has the abilities to specifically recognize homologous genes and selectively inhibit gene expression when transferred into cells by proper vector. It makes siRNA a good way in studying specific genes (23,24). Nowadays, many developing countries still have a high incidence of cervical cancer due to inadequate

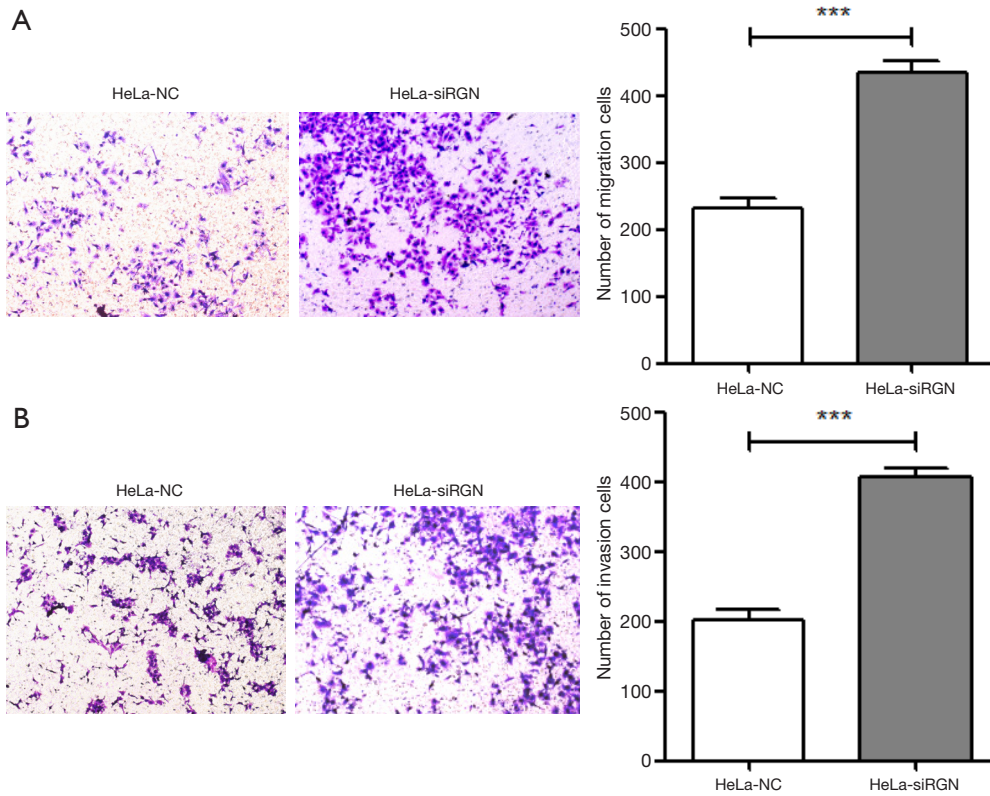


Figure 3 Down-regulation of RGN enhanced cell migration and invasion. The cells on the transwell chamber were stained by 1% crystal violet and taken photos at magnification $\times 100$. (A) RGN down-regulation effectively enhanced the migration ability of HeLa-siRGN cells; (B) RGN down-regulation effectively enhanced the invasion ability of HeLa-siRGN cells. ***, $P < 0.001$. RGN, regucalcin.

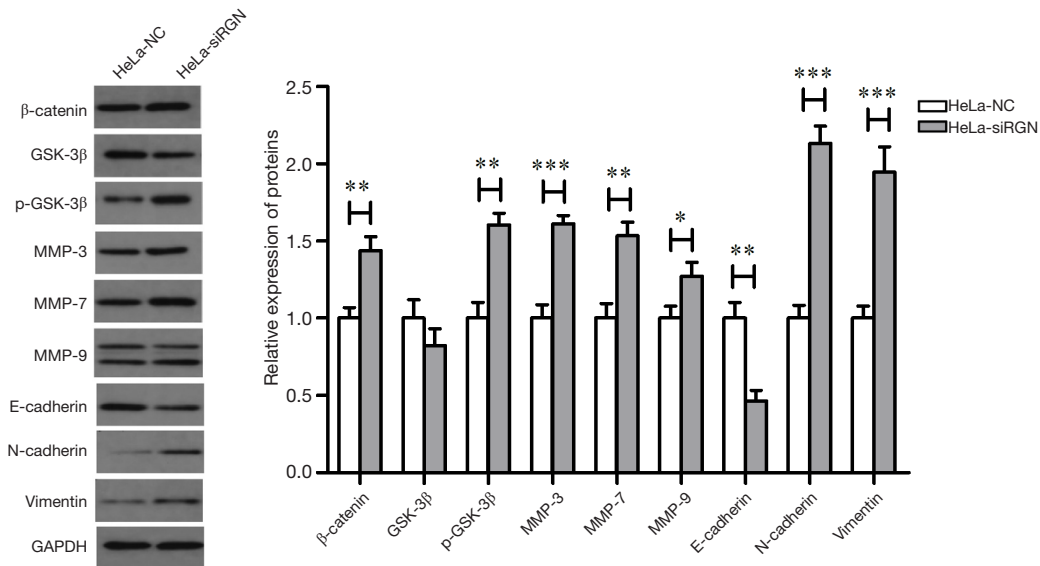


Figure 4 Down-regulation of RGN may promote the activation of Wnt/ β -catenin pathway and EMT. Compared with HeLa-NC cell line, the expression of E-cadherin significantly decreased, while the expression of β -catenin, p-GSK-3 β , MMP-3, MMP-7, MMP-9, N-cadherin and vimentin increased. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. RGN, regucalcin; EMT, epithelial-mesenchymal transition.

screening and prevention. Gene therapy is undoubtedly one of the most promising methods in cervical cancer treatment. A common method to know a gene is to down-regulate it by siRNA. Many researchers have demonstrated that RGN can weaken the growth and migration of cancer cells. It is reported that overexpressing RGN suppresses the proliferation of rat hepatoma H4-II-E cells, which was approached by suppressing various protein kinase activities such as Ca^{2+} /calmodulin-dependent protein kinase, protein kinase C, and protein tyrosine kinase (10-11,25). It can also suppress the growth of liver cancer cells HepG2 when culturing it with medium supplemented with regucalcin (12). Ricardo Marques established transgenic rats overexpressing RGN (Tg-RGN) and found Tg-RGN rats had lower incidence of carcinogen-induced mammary gland tumors, lower proliferation of tumor cells, and less invasive forms, compared to wild type rats (13). Furthermore, Masayoshi Yamaguchi has found overexpression of RGN can suppress cell proliferation, death and migration when transfected RGN cDNA into non-small-cell lung cancer (NSCLC) A549 cells, human colorectal carcinoma RKO cells and human clear cell RCC A498 cells (15-17). Our study is to investigate if the biological characteristics can reverse when endogenous RGN expression is down-regulated, compared to RGN transfection cancer cells. As expected, our study shows that the proliferation, migration and invasion abilities of HeLa-siRGN cells have increased significantly. It has been demonstrated that the proliferation suppression effect of RGN is due to the inhibition of various signaling pathways such as Akt, MAPK, SAPK/JNK and Ras (15,16,18,26). In our research, RGN under-expression enhanced cell migration significantly. So, we have studied the effect of RGN on the signaling pathways proteins associated with metastasis. E-cadherin, vimentin and N-cadherin are markers of EMT (27-29). Our studies have shown that the expression of E-cadherin in HeLa-siRGN cells decrease and the expression of vimentin and N-cadherin increase, indicating that the EMT process has been activated. Furthermore, by detecting the key genes of the Wnt/ β -catenin pathway, it has been found that RGN down-regulation led to the increase of p-GSK-3 β expression. This means that GSK-3 β activity has reduced, and less β -catenin has been degraded by ubiquitination, which leads to excessive β -catenin entering the nucleus, forming more β -catenin/LEF/TCF complex, promoting the transcription and expression of downstream genes such as MMP3\MMP7\MMP9, and promoting the invasion and migration of HeLa cells (30-35). It seems that deficiency

of RGN can promote cervical cancer. Reports have shown that RGN mRNA and protein expression was diminished in breast and prostate cancer tissues, and RGN expression was negatively correlated with the histological grade of breast infiltrating ductal carcinoma (13,14). The studies above seem to suggest that the loss of RGN can make various cancers to get worse. While, higher RGN expression has been seen in lung cancer and pancreatic ductal adenocarcinomas patients with prolonged survival (15,18). In addition, RGN overexpression decreases the oncogenes c-fos, c-myc, Ha-ras and elevation of the tumor suppressors p53 and Rb (15,36). It is no doubt that RGN is a protective factor in cancer progression (37).

In conclusion, our study shows the decreased expression of RGN promotes the progression of cervical cancer and its effect is probably achieved by activating Wnt/ β -catenin pathway and EMT. Our research provides the theoretical basis of RGN for anti-cancer molecular therapy.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.02.01>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional Review Board review was waived.

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