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ORIGINAL ARTICLE

GGN Promotes Tumorigenesis by Regulating Proliferation and Apoptosis in Colorectal Cancer

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Abstract
Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. *GGN* is a germ cell-specific gene, but its function in CRC has been rarely reported to date. The aim of this study was to investigate the potential role of *GGN* in CRC tumorigenesis. Therefore, in this study, we examined the expression of *GGN* in CRC cell lines and tissues and its effects on cellular proliferation and apoptosis. We then explored the underlying mechanism. Our results showed that *GGN* was significantly overexpressed in both CRC cell lines and tissues. Silencing *GGN* robustly inhibited proliferation of CRC cells, and it also promoted apoptosis of CRC cells. Moreover, knockdown of *GGN* inhibited the expression of p-Akt in CRC cells. Taken together, these results showed that knockdown of *GGN* inhibits proliferation and promotes apoptosis of CRC cells through the PI3K/Akt signaling pathway. Our findings revealed for the first time a potential oncogenic role for *GGN* in CRC progress. This finding may provide a unique perspective on how a germ cell-specific gene might serve as a biomarker, or even as a therapeutic target, for CRC.

Keywords Colorectal cancer · Gametogenetin (*GGN*) · Proliferation · Apoptosis

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and women in the United States [1]. Due to changes in lifestyle and dietary behaviors, the incidence and mortality of CRC have been increasing in China, making it the fifth most commonly diagnosed cancer and the fifth leading cause of cancer-related death in men and women combined [2]. Although there have been significant advances in the treatment of CRC, the prognosis of CRC patients remains poor [3–5]. Despite large-scale collections of data on cancer biology, the molecular mechanisms of tumorigenesis and development of CRC are yet to be properly understood [6, 7].

Human gametogenetin (*GGN*) is expressed in the testes and ovaries and is a conserved gene in human and mouse [8, 9]. Recently, *GGN* was found to play a tumor-promoting role in bladder cancer through regulation of NFκB/caspase-3-mediated apoptosis signaling [10]. Furthermore, growing evidence accumulated over the last two decades shows that gametogenetin-binding protein 2 (GGNBP2) is involved in cancer tumorigenesis and therapeutics [11, 12]. However, the function of *GGN* in CRC remains unknown.

Therefore, in this study, we examined the expression of *GGN* in CRC cell lines and tissues to investigate its function in CRC. Furthermore, its effects on cellular proliferation and apoptosis and the underlying mechanism were explored.

Materials and Methods

Tumor Tissue Preparation

Twenty-nine CRC tissues and twelve paracancerous tissues were retrieved from archival material (National Cancer Center/National Clinical Research Center for Cancer/Cancer

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Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). The study protocol was approved by the Institutional Ethics Committee, and all patients gave informed consent for obtaining surgical specimens according to the Declaration of Helsinki.

Cell Lines and Cell Culture

The human CRC cell lines (HCT116, RKO, SW480 and SW620) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific, USA) in a humidified atmosphere incubator with 5% CO_2 at 37 °C.

RNA Preparation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from colorectal cancer tissues and cells using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA was extracted, and its concentration was determined. The PCR conditions consisted of 5 min at 95 °C for one cycle and 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 7 min at 72 °C for 35 cycles. Specific transcripts of *GGN* were amplified by quantitative PCR using the following primers: *GGN*, 5'-CTTCCAGGTGCTTA ACTC-3 (forward) and 5'-CGACTACACTCAGTTGGA-3′ (reverse). GAPDH was chosen as the internal control. GAPDH, 5'-TGACTTCAACAGCGACACCCA-3′ (forward) and 5'-CACCCTGTTGCTGTAGCCAAA-3′ (reverse). The relative expression level of *GGN* was calculated using the ΔΔCT method and normalized against that of GAPDH.

Western Blot

Total protein was extracted and was separated by sodium dodecyl sulfate-polycrylamide gel electrophoresis. Proteins were transferred onto PVDF membranes, and the membranes were then blocked and incubated with rabbit anti-human *GGN* (1:1000) antibody (1:1000) at 4 °C overnight. After 3 washes with TBS-T solution for 10 min, the PVDF membranes were incubated with secondary antibody at room temperature for 2 h. After further washing, the PVDF membranes were visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific, USA).

Immunohistochemistry

CRC tissue and paracancerous tissues were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned and processed for immunohistochemistry staining. The slides were incubated with the primary anti-*GGN* antibody (Bioss, USA) overnight at 4 °C, followed by incubation with second antibody for 30 min. The results were evaluated by 2 pathologists in a blinded manner. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The staining extent was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%), according to the percentage of positively stained cells. The two scores were multiplied to give a final score and divided into the following two groups: low expression (0–6) and high expression (7–12).

GGN Small Interfering RNA Design and Lentivirus Construction

A small interfering RNA (shRNA) specifically targeting *GGN* was designed, and a lentivirus expressing this *GGN* shRNA was constructed and prepared to inhibit *GGN* expression. Briefly, the shRNA (target sequence: CCTCAGGT GCCATCTCTTA) targeting human *GGN* was designed, and a scrambled shRNA sequence (TTCTCCGAACGTGT CACGT) was used as a negative control. Related stem-loop DNA oligonucleotides were synthesized and inserted into the pGCSIL-GFP lentiviral vector (GeneChem, Shanghai, China). Lentivector Expression Systems (GeneChem, Shanghai, China) were used to produce lentivirus expressing *GGN* shRNA or scrambled shRNA.

RNA Interference-Mediated Knockdown of GGN and Cell Transfection

RKO cells were used to examine the knockdown efficiency of *GGN* shRNA. The cells were plated in six-well plates and then infected with lentivirus expressing *GGN* or scrambled shRNA. The cells were cultured in a 5% CO_2 incubator at 37 °C for another 4 days. The cells were then harvested, and the total RNA and protein were extracted to determine the knockdown efficiency by qRT-PCR and Western blot.

Cell Growth Assay

First, RKO cells were infected with lentivirus expressing *GGN* shRNA or scrambled shRNA and then harvested in the logarithmic phase and were seeded at 2000 cells/well into 96-well plates. The cells were then incubated at 37 °C with 5% CO_2 for 5 days. Celigo (Nexcelom Bioscience, MA, USA) was used to count the cells for each day's analysis.

Cell Cycle Analysis by Flow Cytometry

Cell cycle analysis was performed using flow cytometry. After successful transfection, RKO cells infected with lentivirus expressing *GGN* shRNA or scrambled shRNA were collected and fixed overnight with pre-cooled 70% ethanol. The cells

were then washed with ice-cold PBS and stained with propidium iodide (PI) buffer. The fluorescence of DNA-bound PI in the cells was measured using a BD FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA). The cell cycle analysis was carried out using ModFit 2.0 software (Becton Dickinson, San Jose, CA, USA), and triplicate experiments were performed.

Clone Formation Assay

First, RKO cells were infected with lentivirus expressing *GGN* shRNA or scrambled shRNA and then harvested in the logarithmic phase. The cells were resuspended and plated in triplicate into six-well plates at a density of 500 cells per well, and the culture medium was changed at regular time intervals. The inoculated cells were cultured in the incubator for 7 days or until the number of cells in a single clone was larger than 50. Adherent cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS. The colonies were stained with Giemsa solution for 10 min. Then, the cells were washed several times with ddH_2O, dried, and photographed. The clones were counted using a fluorescence microscope (CKX41, Olympus, Tokyo, Japan).

Apoptosis Detection

Apoptosis detection was performed using a Caspase-Glo 3/7 Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. RKO cells were infected with lentivirus expressing *GGN* shRNA or scrambled shRNA and then inoculated in 96-well plates and incubated for 3–5 days. Caspase-Glo 3/7 buffer and Caspase-Glo3/7 freeze-dried powder were placed in a room temperature environment. Then, 10 ml of Caspase-Glo 3/7 buffer was added to a brown flask containing the Caspase-Glo 3/7 substrate. Next, the substrate was completely dissolved to produce a Caspase-Glo reaction. After cell counting, the cell suspension concentration was adjusted to 1×10^4 cells/well at room temperature. The target cells and the negative control cells were added to a new 96-well plate at 100 μl per well, and a cell-free control group was also set up. 100 μl of the Caspase-Glo reaction was added to each well. The incubation was performed for 2 h at room temperature, according to the cell condition, and the fluorescence signal intensity was used to detect apoptosis.

PathScan Analysis

First, RKO cells were infected with lentivirus expressing *GGN* shRNA or scrambled shRNA. After preparing 1× cell lysis buffer to lyse the cells, the PathScan Intracellular Signaling Antibody Array Kit (Cell Signaling, Danvers, MA, USA) was used to detect changes in key signaling molecules from different signaling pathways.

Statistical Analysis

All of the experiments were performed in triplicate. SPSS version 20.0 software (Chicago, IL, USA) was used for all statistical analyses. The data were expressed as the mean ± standard deviation. Student's t test was used for raw data analysis. The χ^2 test was used to test the significance of the differences in *GGN* expression between tumor and paracancerous samples. For all analyses, values of $P < 0.05$ were considered significant.

Results

GGN was distributed mainly in the cytoplasm of CRC cells. More than three-quarters, i.e., 75.86% (22/29) of colorectal tissues were *GGN* positive, while low *GGN* staining was detected in almost all of the paracancerous tissues (Table 1). These results showed that colorectal cancer has higher *GGN* expression than the paracancerous tissues ($P < 0.001$). Next, we performed qRT-PCR to detect *GGN* expression in the CRC cell lines HCT116, SW480, SW620, and RKO. Of these cell lines, RKO cells showed the highest *GGN* expression. Thus, we chose the RKO cell line for further functional experiments.

Three days after infection with shRNA-expressing lentivirus for *GGN* knockdown, both the *GGN* mRNA and protein levels were detected in RKO cells by qRT-PCR and Western blot, respectively. The results showed that *GGN* knockdown significantly inhibited the proliferation of RKO cells (Fig. 1).

As shown in Fig. 2, the *GGN*-siRNA group displayed the following distribution: (G1: 42.27%, S: 20.44%, G2/M: 37.29%), while the control group displayed the following distribution: (G1: 37.25%, S: 30.44%, G2/M: 32.30%). Compared with the control group, knockdown of *GGN* expression increased the number of cells in G1 phase, decreased the number of cells in S phase, and increased the number of cells in G2/M phase. These results suggested that the *GGN* status was significantly correlated with the cell cycle distribution.

Compared with the control cells, clonogenic ability was significantly inhibited in the *GGN* knockdown group. The number of colonies in the *GGN*-siRNA lentivirus-infected cells was statistically less than that of the negative control cells, with 143 ± 6 in the control group vs. 55 ± 3 in the *GGN*-siRNA group ($P < 0.01$, Fig. 3).

Table 1 Expression of *GGN* in colorectal cancer and paracancerous tissues

	Pathological type		*P* value
	Cancer tissue	Paracancerous tissue	
Expression level			0.000
Low expression	7	12	
High expression	22	0	

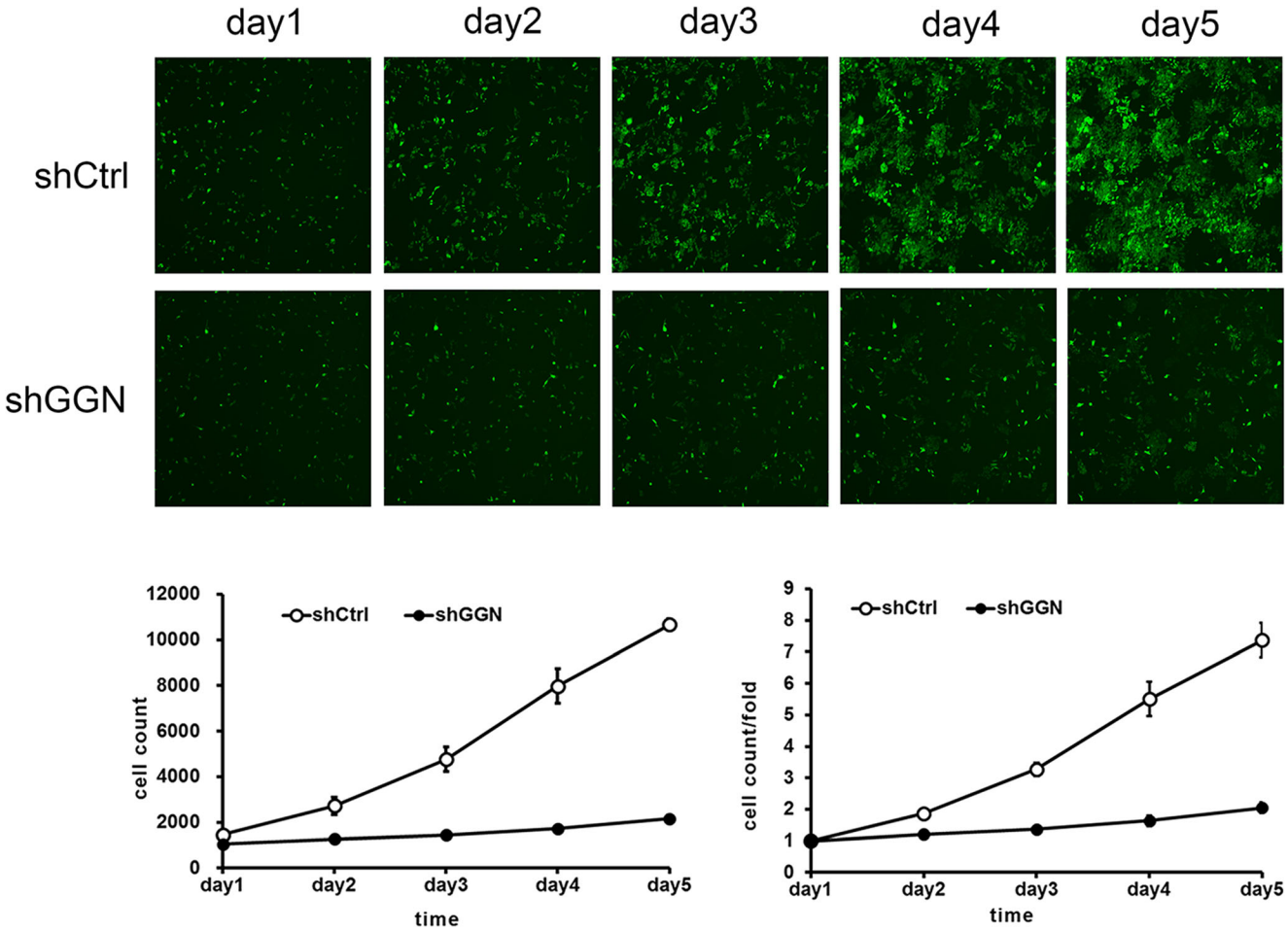

Fig. 1 The rate of RKO cell proliferation was significantly inhibited in the *GGN* shRNA group

Compared with the control cells, *GGN* knockdown significantly increased the caspase-3/7 activity in the RKO cells ($P < 0.01$). After detecting the levels of intracellular signaling pathway-related genes, we found that the expression of p-AMPKa, p-HSP27, p-Bad, p-PRAS40, cleaved-PARP, and cleaved-caspase-3 were elevated in the *GGN* knockdown group, while the expression of p-Akt (Ser473) was reduced (Fig. 4).

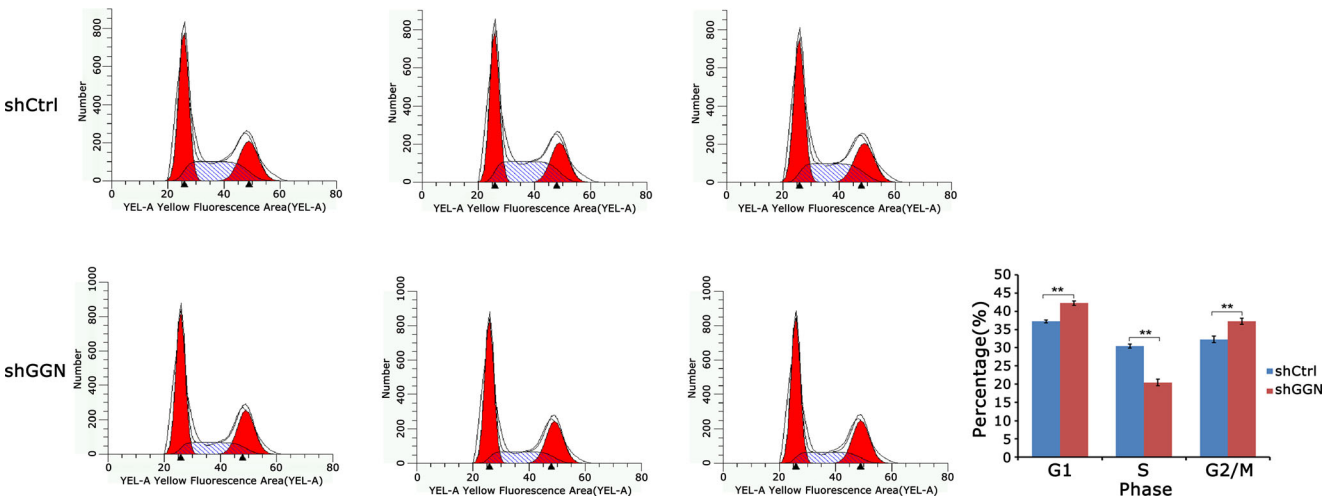

Fig. 2 Knockdown of *GGN* expression induced G2/M phase arrest. Data are shown as the mean ± SD, $P < 0.01$

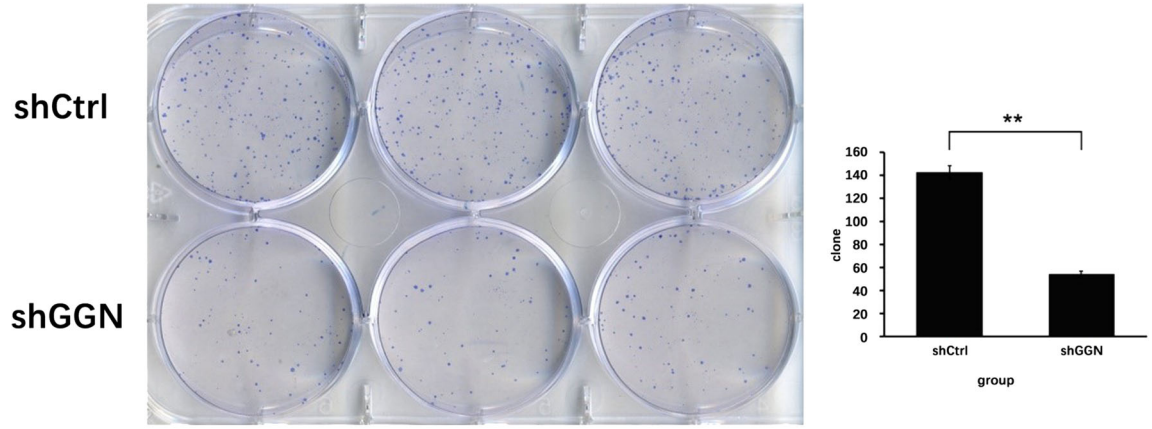

Fig. 3 *GGN* knockdown significantly reduced colony formation in RKO cells, as assessed by a colony formation assay. The data are presented as the mean ± SEM from three independent experiments, each ran in triplicate. $^{**}P < 0.01$

Discussion

The tumorigenesis of CRC is very complex and multi-faceted and is also influenced by many factors [13]. Numerous genetic and epigenetic alterations are directly responsible for a specific event in the adenoma–carcinoma sequence that leads to CRC [14]. *GGN* is known as a critical gene during the development of sperm and embryos [15, 16]. However, the potential role and mechanism of *GGN* in cancer, especially in CRC, has remained elusive and controversial. This study aimed to elucidate the impact of *GGN* on the tumorigenesis of CRC and provides evidence that the downregulation of *GGN* could inhibit cell proliferation and increase apoptosis via the PI3K/Akt signaling pathway. Therefore, it was concluded that *GGN* may be a potential candidate oncogene in CRC.

First, we revealed the existence of high *GGN* expression in CRC patients and CRC cells in vitro. The high expression of *GGN* protein, compared with paired normal tissues, was also shown in bladder cancer and was associated with increased malignant potential behavior of tumors and poor prognosis in patients [10]. To investigate the roles of *GGN* in the mechanism of CRC, we employed RNA interference to knockdown *GGN* expression. These results suggest that targeted blockage of *GGN* activity may be a novel therapeutic strategy in CRC. Furthermore, the in vivo data further support the conclusion that *GGN* can been implicated in the progression of CRC.

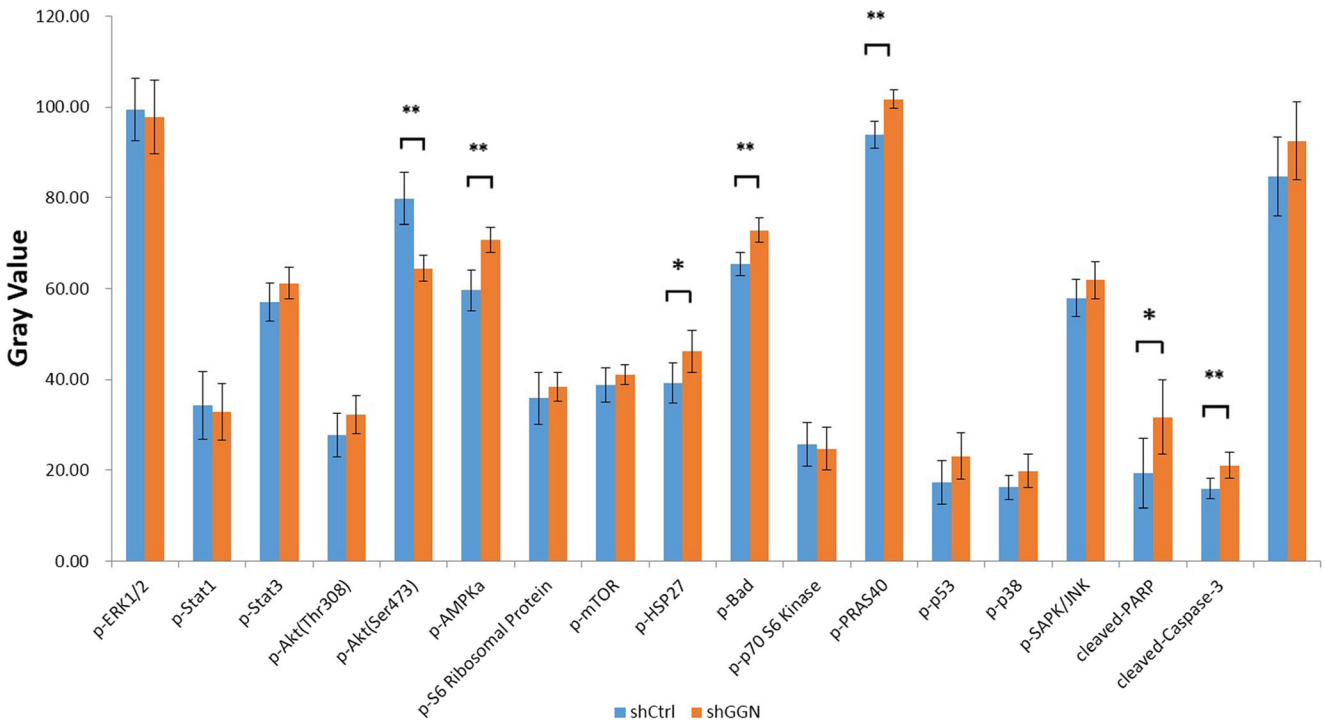

Fig. 4 Changes in key signaling molecules in different signaling pathways after *GGN* knockdown

Apoptosis is a distinctive and important mode of programmed cell death, which involves the genetically determined elimination of cells [17]. Apoptosis is precipitated by the activation of cysteine proteases of the caspase family, including caspase-3, −8 and − 9, and their cleavage into active forms is considered the primary hallmark of apoptosis [18]. Our results revealed that the knockdown of *GGN* significantly increases caspase-3/7 activity in RKO cells. We also observed that *GGN* status was significantly correlated with cell cycle distribution. Similarly, Wang et al. reported that *GGN* induces cell cycle arrest and apoptosis in a bladder cancer cell line [10]. Based on these data, we further proved that *GGN* participates in the regulation of apoptosis signaling.

In addition, we showed that the knockdown of *GGN* could significantly decrease the expression of p-Akt. The PI3K pathway is regulated by a variety of growth factors, and activation of the PI3K/Akt signaling pathway is known to have an important role in the development and progression of CRC [19]. As a downstream effector of PI3K, Akt is closely related to cell survival and anti-apoptotic signaling [20]. In this study, the phosphorylation level of Akt was decreased after *GGN* was knocked down. This observation suggested that *GGN* contributes to the activation of PI3K/Akt signaling in CRC.

In conclusion, our current results showed that the sperm-specific gene *GGN* may be involved in the tumorigenesis of CRC. *GGN* down-regulation with RNA interference can inhibit proliferation, apoptosis, and cell cycle progression of CRC cells by regulating the PI3K/Akt signaling pathway. Uncovering the detailed mechanisms of *GGN* in the regulation of CRC tumorigenesis and progression will require further studies. Taken together, the results of our study provide new insight into the function of *GGN*, which may serve as a potential CRC biomarker or even as a therapeutic target in CRC.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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