

# Knockdown of NCOA2 Inhibits the Growth and Progression of Gastric Cancer by Affecting the Wnt Signaling Pathway–Related Protein Expression

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Zhenlv Lin, MB<sup>1</sup>, Fan Yang, MB<sup>2</sup>, Dong Lu, MB<sup>3</sup>, Wenjie Sun, MB<sup>4</sup>, Guangwei Zhu, MD<sup>4</sup> , and Bin Lan, MD<sup>4</sup>

## Abstract

**Objective:** The aim of the study is to determine the role of nuclear receptor coactivator 2 in cell proliferation and invasion ability of gastric cancer cells and to explore its possible mechanisms. **Methods:** Immunohistochemical staining was used to determine NCOA2 gene expression in gastric cancer. Western blotting was used to detect Wnt signal pathways–related protein expression. Colony formation assays, Cell Counting Kit-8 assays, and transwell assays were used to determine cell proliferation, metastasis, and invasion ability of gastric cancer cells. A flow cytometric apoptosis tests determine gastric cancer cell apoptosis ability after inhibition of the expression of nuclear receptor coactivator 2. Subcutaneous mouse models were used to determine the gastric cancer growth and peritoneal metastasis differences after inhibition the expression of nuclear receptor coactivator 2. **Results:** The expression of nuclear receptor coactivator 2 in gastric cancer cells is high ( $P < .01$ ), including lymph node metastasis, TNM staging, and gender differences in nuclear receptor coactivator 2 expression were statistically significant ( $P < .01$ ). Short interfering nuclear receptor coactivator 2 could inhibit the proliferation and invasion ability of gastric cancer cells. Short interfering nuclear receptor coactivator 2 promotes the apoptosis of gastric cancer cells. Animal experiments showed that short interfering nuclear receptor coactivator 2 could inhibit the growth and invasion of gastric cancer-transplantable tumors. Knockdown of the expression of nuclear receptor coactivator 2 inhibited the Wnt/ $\beta$ -catenin signaling pathway in the gastric cancer cells. **Conclusions:** Knockdown of the expression of nuclear receptor coactivator 2 can inhibit the proliferation and invasion of human gastric cancer *in vitro* and *in vivo*. The underlying mechanism of NOCA2 affects the Wnt signaling pathway.

## Keywords

gastric cancer, nuclear receptor coactivator 2, growth, progression, Wnt signaling pathway

## Abbreviations

BSA, bovine serum albumin; CCK-8, Cell Counting Kit-8; EMT, epithelial–mesenchymal transformation; FBS, fetal bovine serum; NCOA2, nuclear receptor coactivator 2; PBS, phosphate-buffered serum; PI, propidium iodide; TBS, Tris-buffered saline; NC, negative control.

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## Introduction

Gastric cancer is very common worldwide, especially in East Asia.<sup>1</sup> Early gastric cancer often has no obvious symptoms or is associated with some nonspecific symptoms, such as indigestion and loss of appetite. Gastric cancer often reached late stage when symptoms appear. The 5-year survival rate after surgery is only 20% to 40%.<sup>2</sup> Therefore, it is important to discover new biomarkers and broader mechanisms involved in the development and metastasis of gastric cancer.

Nuclear receptor coactivator 2 (NCOA2, aka SRC-2) is a transcriptional regulator of nuclear receptors that plays a key

<sup>1</sup> Department of Emergency Surgery, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

<sup>2</sup> Department of Pediatric Surgery, First Hospital of Quanzhou, Fujian Medical University, Quanzhou, China

<sup>3</sup> Department of Gastrointestinal Medicine, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

<sup>4</sup> Department of Gastroenterological Surgery, The First Affiliated Hospital of Fujian Medical University, Fuzhou, China

## Corresponding Authors:

Bin Lan and Guangwei Zhu, Department of Gastrointestinal Surgery, The First Hospital Affiliated of Fujian Medical University, 20th, Chazhong Road, Fuzhou, Fujian 350005, China.

Emails: whitelanbin@126.com; zgwszy@126.com



role in regulation of energy balance.<sup>3-6</sup> The transcription factor NCOA2 is a member of the p160 family, which does not bind to DNA directly but regulates transcription by interacting with an enhancer or promoter region that regulates target genes.<sup>5-8</sup> Nuclear receptor coactivator 2 also affects different physiological processes, including cell growth, energy metabolism, circadian rhythm, and feeding behavior.<sup>4-8</sup> The expression of the *NCOA2* gene plays a crucial role in the development, progression, and metastasis of many malignant tumors, such as prostate cancer. Amplification or overexpression of NCOA2 occurred in 8% of the patients with prostate cancer and up to 37% in patients with metastatic cancer.<sup>9</sup> More importantly, patients with high expression of NCOA2 are more likely to relapse after androgen-deprivation therapy.<sup>10</sup> Amplification or overexpression of the *NCOA2* gene may play an important role in metastasis. In the present study, we investigated the role of NCOA2 in gastric cancer. Previous study had revealed that NCOA2, also known as SRC-2, is essential for the epithelial-mesenchymal transformation (EMT) in breast cancer cells.<sup>11</sup> Yu *et al*<sup>12</sup> also reported that NCOA2 can inhibit Wnt/ $\beta$ -catenin signaling in colorectal cancer. The Wnt signaling pathway played an important role in the development of numerous human tumors, such as in gastric cancer,<sup>13-16</sup> breast cancer,<sup>17</sup> and melanoma.<sup>18-20</sup> The Wnt pathway may also play a role in leukemia.<sup>21,22</sup> Mutation of the Wnt/ $\beta$ -catenin signaling pathway induces EMT in normal cells and promotes proliferation, invasion, and metastasis of gastric cancer cells.<sup>23-27</sup>

In the present study, we investigate NOCA2 expression in the gastric cancer samples and relationship between expression of NOCA2 and clinicopathological factors of gastric cancer. We investigate that NOCA2 affects the biologic behaviors of gastric cancer cells and the underlying mechanisms.

## Materials and Methods

### *Immunohistochemistry and Patient Samples Collection*

Immunohistochemistry of tissues chips was carried out as previously described.<sup>26</sup> Briefly, the tissue chips were deparaffinized in xylene and rehydrated through a graded series of ethanol. Then, the chips were incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, followed by bovine serum albumin (BSA) for 10 minutes to block nonspecific binding. Next, the sections were incubated with rabbit anti-human NCOA2 antibody (Abcam) and diluted 200-fold in Tris-buffered saline (TBS; pH 7.6) with 1% BSA, at 4 °C overnight. An LSAB kit was used for staining and visualization by DAB affects counterstaining with hematoxylin.

The human gastric cancer tissue chips study was performed at the Shanghai Jiaotong University. One hundred gastric cancer tissues and normal gastric tissues were located away from the cancer and used as controls. The expression of NCOA2 in tissues is determined by the immunohistochemistry as follows:  $\leq 5\%$ , 5% to 25%, 26% to 50%, 51% to 75%, and  $\geq 75\%$  as positive cells value 0, 1, 2, 3, and 4, respectively, and multiplied with the staining intensity of 0, 1, 2, or 3 to represent no

staining, light yellow, yellow brown, or brown. The score  $< 3$  points indicates NCOA2-positive expression, and  $> 3$  points indicates NCOA2-negative expression. All patients with gastric cancer included in the present study had not been given any preoperative neoadjuvant chemotherapy or other therapy such as radiotherapy.

### *Cell Culture*

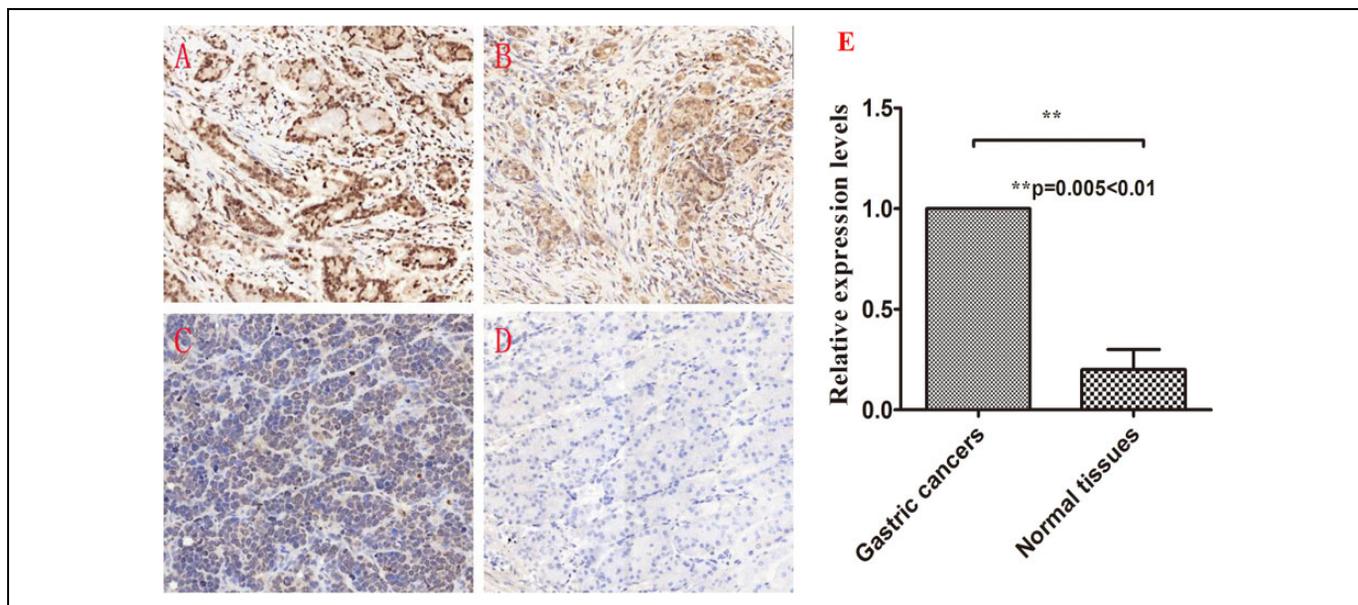
The Hs-746T, SGC-7901, MKN-45, CRL-5822, AGS, CRL-5971, GES-1, MKN-28, and BGC-823 gastric cancer cell lines were purchased from the Chinese Academy of Science (Shanghai, China). The gastric cancer cells were adherent cells; the Hs-746T, SGC-7901, MKN-45, CRL-5822, CRL-5971, MKN-28, and BGC-823 were cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin and placed at 37 °C, 5% CO<sub>2</sub>, in an incubator with saturated humidity.

### *Lentivirus-Mediated Knockdown of Gastric Cancer Cell Lines*

The negative control (NC) and short hairpin RNA knockdown of NCOA2 lentivirus were provided by Genechem Co Ltd. The 300- $\mu$ L lentivirus particles (titer:  $> 10^8$  TU/mL), which were constructed by Genechem Co and carrying green fluorescence protein, infected MKN-28 and BGC-823 cells that were replanted in 6-well plates with 40% cell confluence, with polybrene at a ratio of 1:1000. After 48 hours, the fluorescence was observed under a microscope. Then, puromycin was added to the culture solution at a ratio of 1:1000, and after continuing to culture for a period of time, all the cells that were successfully transfected with lentivirus particles were selected, the fluorescence was verified under the microscope, and then the cells were stably cultured and passaged to obtain a stable cell line.

### *Western Blot Analysis*

Western blotting was carried as previously described.<sup>28-30</sup> Briefly, an equal amount of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel electrophoresis and transferred onto a 0.45- $\mu$ m polyvinylidene fluoride membranes (PALL). The membranes were blocked in 0.5% BSA at room temperature for 2 hours. The following monoclonal primary antibodies were used in the Western blot analysis: anti-NOCA2 (Abcam), anti-glyceraldehyde 3-phosphate dehydrogenase (CST), anti-Slug (Abcam), anti-N-cad (Abcam), anti-E-cad (Abcam), anti-Vimentin (Abcam), anti- $\beta$ -catenin (Abcam), and anti-TCF-8 (Abcam), overnight at 4 °C. The membranes were washed 3 times with TBST (0.1% Tween-20) for 10 minutes, each at room temperature. The signal was visualized by chemiluminescence kit.



**Figure 1.** Degree of nuclear receptor coactivator 2 (NCOA2) protein expression in human gastric cancer tissues and paired normal gastric tissue determined by immunohistochemical staining. A, Strong positive expression of NCOA2 in gastric cancer. B, Weak NCOA2 expression is in gastric cancer. C, Negative expression of NCOA2 in gastric cancer tissues. D, Negative expression of NCOA2 in nontumor gastric mucosa. E, NCOA2 protein expression is significantly higher in the gastric cancer tissues than in the normal gastric tissues.

### Colony Formation Assay

Human gastric cancer cells MKN-28NC, MKN-28SH, GBC-823NC, and BGC-823SH in logarithmic growth phase were harvested. The cells were seeded onto 6-well plates at a density of  $1 \times 10^3$  cells/well. These cells were cultured in RPMI 1640 complete medium containing 10% FBS in the incubator at 37 °C, 5% CO<sub>2</sub>, for 7 to 10 days. Then, methanol was used to fix cells for 10 minutes and crystal violet was used to stain the cells. Clones were read directly by the naked eye, and the number of clones larger than 10 cells were identified under a microscope (low magnification). Clone formation was calculated as: efficiency = (number of clones/number of cells inoculated)  $\times$  100%. The results represent the mean values from 3 independent experiments.

### Cell Proliferation Assay

MKN-28 and BGC-823 cells cancer cells were planted in 96-well plates at a density of 1500 per well, 3 replicate per group. These cells were cultured in a 10% FBS medium in a 5% CO<sub>2</sub>, 37 °C incubator for 1, 2, 3, 4, and 5 days. The culture medium was then discarded, 10% Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was added in serum-free medium. Results were observed in a microplate reader after culture for 1 hour. Determination of absorbance was at 450 nm. The results represent the mean values from 3 independent experiments.

### Cell Invasion Assay

Transwell chamber (Corning) and Matrigel-coated transwell chamber 8- $\mu$ m and 24-well format were inserted into 24-well

cell culture plates to measure migration and invasion. Cells with density  $1 \times 10^5$  in 0.3 mL of serum-free medium were added in the upper chamber, while 800  $\mu$ L of RPMI 1640 containing 10% FBS was added to the lower chamber. Cells were cultured for 48 hours. Then, the cells with transwell chambers were fixed in methanol for 5 minutes and stained with crystal violet and counted in 3 random fields under microscope. The results represent the mean values from 3 independent experiments.

### Flow Cytometry Apoptosis Experiment

MKN-28NC and BGC-823NC gastric cancer cells and MKN-28SH and BGC-823SH cells of the experimental group were detected for culture with trypsin solution to prepare a single-cell suspension. The cells were counted and seeded in a 24-well cell culture plate at  $1.0 \times 10^5$  cells/well. The cells were cultured in a cell culture incubator at 37 °C (5% CO<sub>2</sub>, saturated humidity). After incubating for 48 hours in an incubator, the suspension cells were collected into a centrifuge tube, and the adherent cells were detected with trypsin without EDTA, collected together with the suspension cells, and centrifuged at 1200 r/min for 5 minutes. The supernatant was discarded and the cells were washed twice with phosphate-buffered serum (PBS). The cells were resuspended in  $1 \times$  binding buffer and transferred to a 5-mL flow tube, centrifuged at 1200 r/min for 5 minute, discarded the supernatant. A 5- $\mu$ L Annexin V-FITC reagent was added to the flow tube and mixed. Then 5- $\mu$ L propidium iodide (PI) reagent was added into the tube, mix by shaking, and incubated for 10 to 15 minutes in the dark at room temperature. One blank control tube (ie, only cells,

**Table 1.** Relationship Between Expression of NCOA2 and Clinicopathological Factors of Gastric Cancer.<sup>a</sup>

Variables	Number of cases	NCOA2 immunostaining		P
		Positive, n = 63	Negative, n = 37	
Gender				
Male	67	47	20	.035 <sup>b</sup>
Female	33	16	17	
Age (years)				
≥65	46	30	16	.672
<65	54	33	21	
Tumor differentiation				
Well to moderate	29	10	13	.430
Poor	71	53	24	
Lauren classification				
Intestinal	24	16	8	.670
Diffuse	76	47	29	
Tumor size				
≤5 cm	51	33	18	.541
>5 cm	49	30	19	
T stage				
T1 + T2	36	19	17	.111
T3 + T4	64	46	18	
Lymph node metastasis				
Negative	37	17	20	.007 <sup>b</sup>
Positive	63	46	17	
Distant metastasis				
Negative	77	46	31	.218
Positive	23	17	6	
TNM stage				
I + II	23	10	13	.027 <sup>b</sup>
II + IV	77	53	24	

Abbreviation: NCOA2, nuclear receptor coactivator 2.

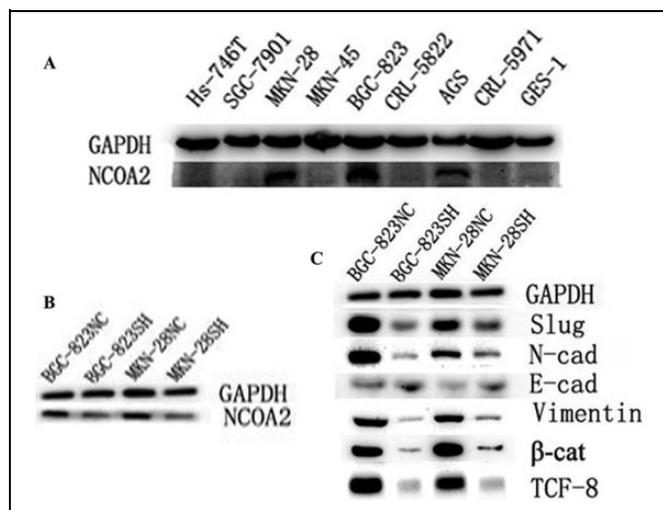
<sup>a</sup>Positive expression of NCOA2 includes all positive cases, such as weak and strong.

<sup>b</sup> $P < .05$ .

without any dye staining) and 2 single-dye adjustment compensation tubes (1 tube is only dyed with Annexin V-FITC dye, and the other tube is stained with PI dye only) were used as controls. Binding buffer (400  $\mu$ L, 1 $\times$ ) was added to each tube. After resuspension, apoptotic cells in each group were detected by flow cytometry (to avoid fluorescence quenching, it should be detected at 1 hour after staining). Analysis of cells by flow cytometry revealed that cells were divided into 4 subgroups: live cells (low fluorescence intensity), early apoptotic cells (higher green fluorescence), advanced cells in apoptosis, and necrotic cells (green and red double fluorescence will appear). The results were analyzed using BD FACSDIVA Software/Cell Quest version 3.0 software. The cell survival rate, early apoptosis rate, middle and late apoptosis rate, and cell mortality were calculated. The experiment was repeated 3 times.

### Animal Experiments

All mice used in the experiment were 4-week-old male nude mice purchased from the Institute of Zoology, Shanghai



**Figure 2.** Western blotting analyzed NCOA2 and Wnt/EMT signal pathway proteins expression. A, NCOA2 expression in 9 gastric cancer cell lines. B, After the knockdown of NCOA2 expression by lentivirus, the difference in protein content between the experimental group and the control group of the 2 gastric cancer cells was tested. C, After knockdown of NCOA2, difference in EMT marker proteins in experimental and control groups of gastric cancer cells. EMT indicates epithelial-mesenchymal transformation; NCOA2, nuclear receptor coactivator 2.

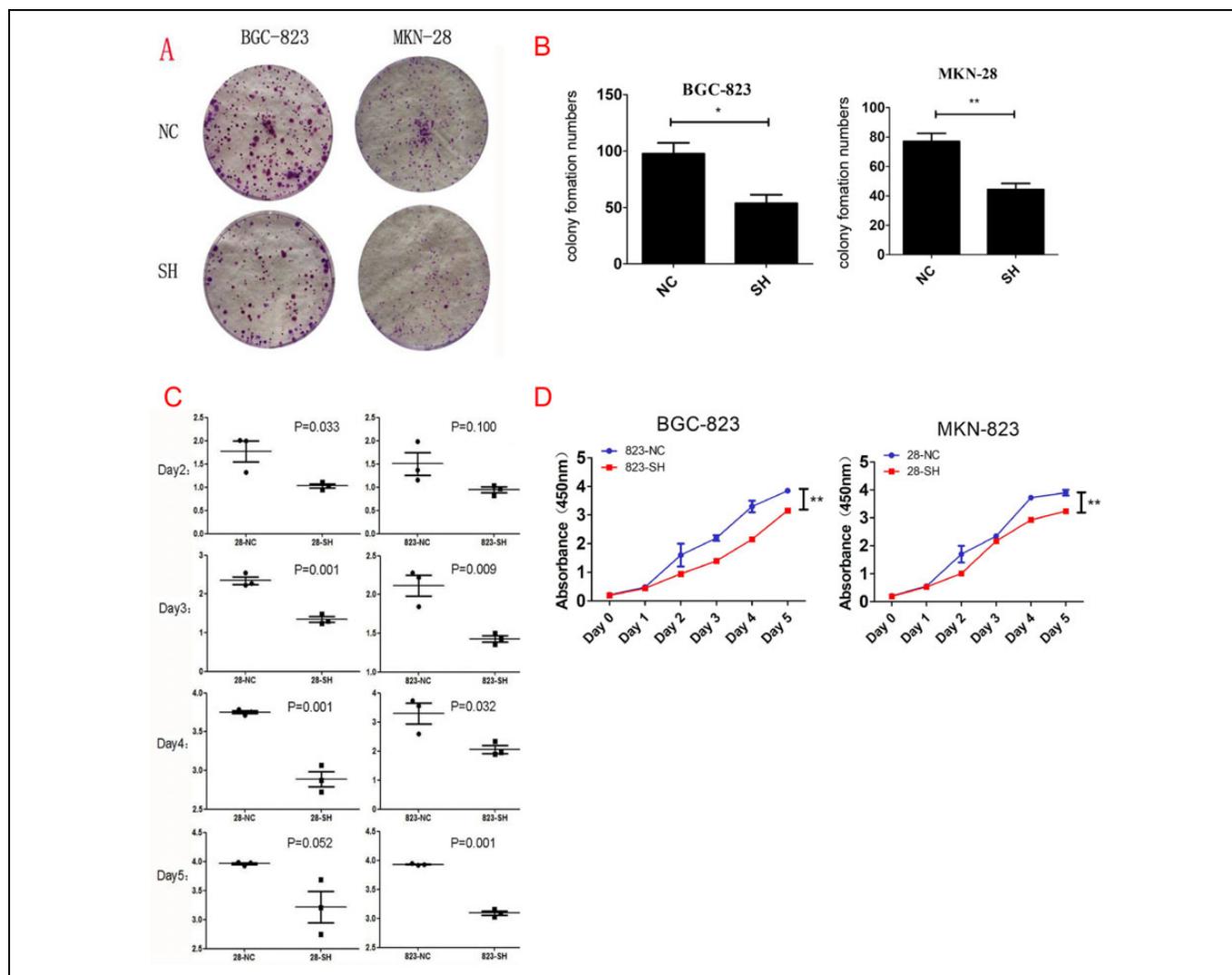
Academy of Sciences, China. All experiments were carried out according to the official recommendations of the Chinese Animal Community. Ten large culture dishes of MKN-28nc and MKN-28sh cells were cultured. Confluence cells were detached with trypsin for 5 minutes and centrifuged for 5 to 10 minutes, and the supernatant was discarded. The cells were washed repeatedly with PBS, centrifuged again, and the pellet was harvested. The PBS 100  $\mu$ L (pH = 7.4) was added, uniformly mixed, and injected into the subcutaneous and intraperitoneal tissues of the nude mice (5 each group; 20 in total). After 30 days, the mice were killed by cervical displacement, and the subcutaneous growth and peritoneal metastasis and spread were observed and photographed.

### Statistical Analysis

Mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ) indicates measurement data and statistical value calculation was done using GraphPad.Prism version 5.0-1 statistical software. Student *t* test was used to compare the mean of multiple samples, and tissue immunohistochemistry results were tested by  $\chi^2$  test before and after cell treatment. All results were found statistically significant at  $P < .05$ .

### Results and Discussion

The expression of NCOA2 in gastric cancer tissues is higher than that in normal tissues. Immunohistochemical staining of gastric cancer and adjacent tissues (Figure 1A-D) demonstrated that the expression of NCOA2 in tumor tissues is significantly

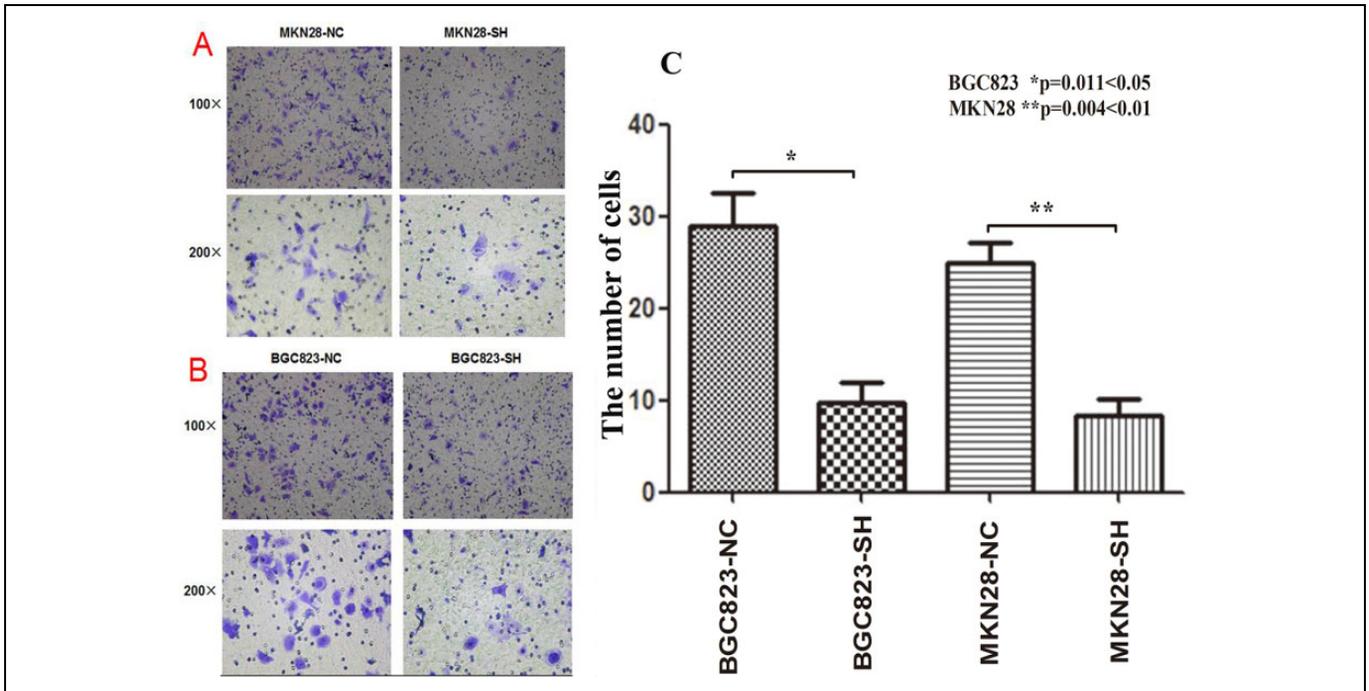


**Figure 3.** Gastric cancer cell proliferation. A, Differences in MKN-28 and BGC-823 colony formation in NC and SH groups. B, The colony formation in SH group is lower than in the NC group in MKN-28 and BGC-823 cells. C, CCK-8 experiments on days 2 to 5 with MKN-28 and BGC-823 cells in both NC and SH groups. D, CCK-8 detected the ability of proliferation of MKN-28 and BGC-823 in both NC and SH groups for 1 to 5 days. CCK-8 indicates Cell Counting Kit-8; NC, negative control; NCOA2, nuclear receptor coactivator 2; SH, short hairpin RNA.

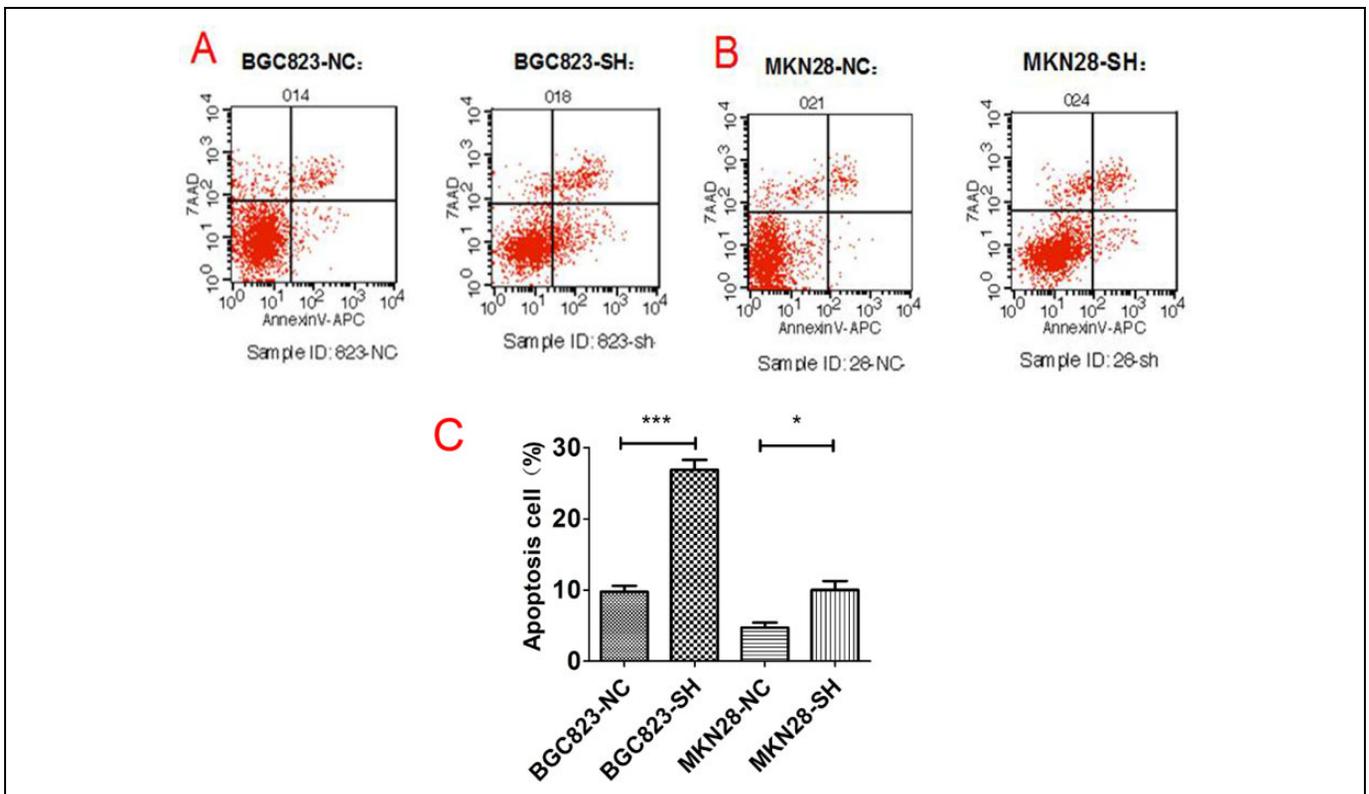
higher than that of nontumor tissues, with patients with gastric cancer having of NCOA2 high expression (Figure 1E;  $P = .005$ ). The patients with gastric cancer have high expression status of NCOA2. We further studied the relationship between the expression of NCOA2 and the pathological features of gastric cancer and found that the expression of NCOA2 is related to lymph node metastasis ( $P = .007$ ), TNM stage ( $P = .027$ ), as well as gender ( $P = .035$ ). The expression of NCOA2 was not related to age, tumor size, and distant metastases (Table 1). The difference in gender is far beyond our expectation because although androgen deprivation is still the most commonly used treatment of prostate cancer, most patients die of castration resistance.<sup>31</sup> Then, whether androgen deprivation therapy can be used for the treatment of gastric cancer and whether there will be a prostate cancer-like outcome in the later stage of the disease are our next questions.

### Knockdown of the Expression of NCOA2 Can Inhibit the Wnt/ $\beta$ -Catenin Signaling Pathway in the Gastric Cancer Cells

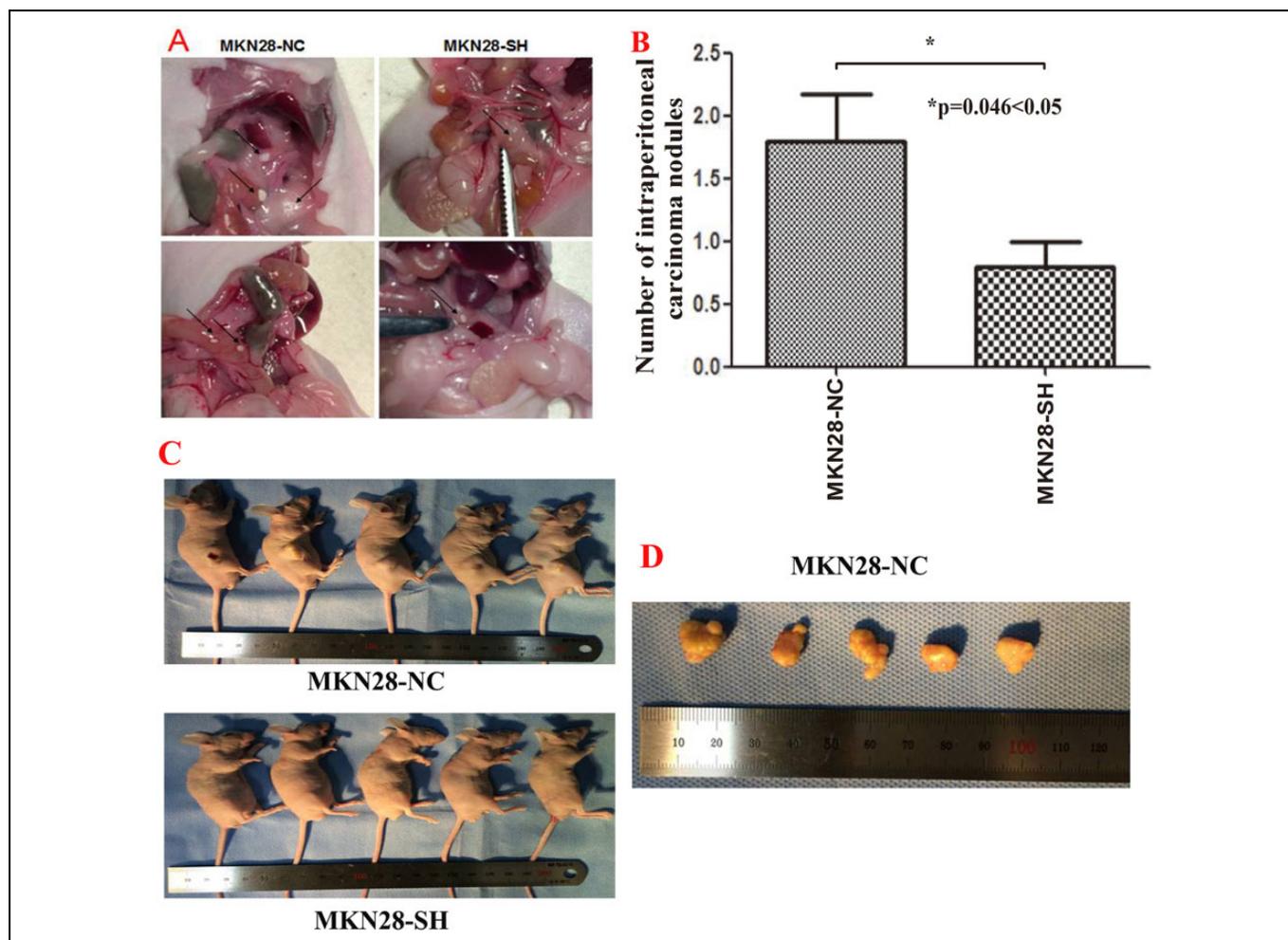
The expression of NCOA2 in human gastric cancer cells MKN-28 and BGC-823 was higher than that of other cell lines (Figure 2A). Therefore, MKN-28 and BGC-823 cell lines were used in next experiments. The NCOA2 small interfering RNA inhibited the expression of NCOA2 in MKN-28sh and BGC-823sh cells compared to MKN-28nc and BGC-823nc cells. The knockdown effect was confirmed by Western blotting (Figure 2B). To explore whether NCOA2 is essential for the EMT in gastric cancer cells, we also detected EMT-related proteins in gastric cancer cells by Western blotting. The levels of  $\beta$ -catenin, N-cad, Vim, and Slug proteins in MKN-28sh and BGC-823sh cells after inhibition of NCOA2 expression were significantly lower than those in the control group. In contrast,



**Figure 4.** Gastric cancer cells metastasis invasion assay. A, Knockdown of the expression of nuclear receptor coactivator 2 (NCOA2) inhibited the invasion ability of MKN-28 cells. B, Knockdown of the expression of NCOA2 inhibited the invasion ability of BGC-823 cells. C, Quantification of the results from (A) and (B), respectively.



**Figure 5.** FCM assay tested the apoptosis in knockdown of NCOA2 of gastric cancer cells. A, Knockdown of the expression of NCOA2 promotes the cells apoptosis of BGC-823 cells. B, Knockdown of the expression of NCOA2 promotes the cells apoptosis of MKN-28 cells. 188 × 97 mm (300 × 300 DPI). C, The quantity of apoptosis cells in SH group is larger than in NC group in BGC823 and MKN28 cells. FCM indicates flow cytometry; NC, negative control; NCOA2, nuclear receptor coactivator 2; SH, short hairpin RNA.



**Figure 6.** Effect of NCOA2 on the growth and peritoneal metastasis of MKN-28 cells. A, Comparison of the number of cancer nodules of MKN-28 cells in peritoneal metastasis of mice in NC and SH groups of MKN-28 cells. B, Quantification of the results from (A). C, Comparison of MKN-28 groups subcutaneously in NC and SH groups of MKN-28 cells. D, Stated subcutaneous tumor of the MKN-28nc group. The MKN28-SH group had not tumor growth. NC indicates negative control; NCOA2, nuclear receptor coactivator 2; SH, short hairpin RNA.

E-cad expression in the experimental group was higher than the control group (Figure 2C). These results indicate the functional role of NCOA2 in regulating EMT in gastric cancer cells.

### Knockdown of NCOA2 Can Suppress the Proliferation of Gastric Cancer Cells

The cancer MKN-28sh and BGC-823sh cell clusters with knockdown *NCOA2* genes were significantly smaller than those of MKN-28nc and BGC-823nc cells. The proliferation ability was significantly lower than that of the control (Figure 3A). The CCK-8 assay analyzes cell proliferation ability. The results of plate cloning were identical to CCK-8 assay (Figure 3C). After knocking down NCOA2, the absorbance at 450 nm of MKN-28 and BGC-823 cells was significantly decreased, showing that cell proliferation was inhibited ( $P < .05$ ; Figure 3D). These results also indicate the role of NCOA2 in the proliferation of gastric cancer cells.

### Knockdown of NCOA2 Expression Inhibits the Invasion Ability of Gastric Cancer Cells

MKN-28sh and BGC-823sh cells with knockdown NCOA2 expression had a lower number of cells penetrating the transwell cell membrane compared to MKN-28nc and BGC-823nc (Figure 4A and B;  $P < .05$ ). Thus, the invasion ability of gastric cancer cells with stably knockdown of NCOA2 was significantly reduced.

### Knockdown of NCOA2 Expression Promotes Apoptosis of Gastric Cancer Cells

The apoptosis rate of MKN-28nc and BGC-823nc with NCOA2 expression was significantly lower than that of MKN-28sh and BGC-823sh cells after NCOA2 knockdown. Microscopically, knockdown of *NCOA2* gene expression in gastric cancer cells simultaneously increased the number of early apoptosis (LR) and late-stage apoptosis (UR) cells in

gastric cancer cells, thus more NCOA2 knockdown had a pronounced effect on early-stage apoptosis than late-stage apoptosis (Figure 5A and B).

### NCOA2 Knockdown Inhibited Subcutaneous Growth and Peritoneal Spread of Gastric Cancer Cells *In Vivo*

In order to confirm whether NCOA2 knockdown can suppress the proliferation of gastric cancer cells *in vivo*, we injected MKN-28nc and MKN-28sh cells subcutaneously and in the abdomen of nude mice. The cancer nodules in the peritoneal cavity of the mice inoculated with the control group MKN-28nc cells were significantly larger than those in the mice inoculated with the NCOA2 MKN-28sh cells (Figure 6A and B;  $P = .046$  and  $<.05$ ). The subcutaneous cancer nodules of the mice inoculated with the control group MKN-28nc cells were significantly larger than those of the mice inoculated with the NCOA2 MKN-28sh cells (Figure 6C and D).

In summary, NCOA2 plays an important role in the occurrence and development of gastric cancer. Knockdown of NCOA2 expression can not only inhibit the proliferation of gastric cancer cells but also weaken the survival of gastric cancer cells by promoting apoptosis, inhibiting metastasis and invasion via Wnt signaling pathway, affecting the progression of gastric cancer.

#### Authors' Note

Zhenlv Lin and Fang Yang contributed equally to this work. The data sets used or analyzed during the current study are available from the corresponding author on reasonable request. This study was approved by the Ethics Committee of The First Affiliated Hospital of Fujian Medical University, China (protocol number: IEC-FOM-008-2.0, B Lan).

#### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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#### ORCID iD

Guangwei Zhu  <https://orcid.org/0000-0003-3011-9101>

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