

lncRNA GAS5 inhibits colorectal cancer cell proliferation via the miR-182-5p/FOXO3a axis

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Abstract. Long non-coding RNAs (lncRNAs) are mainly involved in diverse biological processes in human malignancies. The expression profile and underlying mechanism of the lncRNA growth arrest specific transcript 5 (GAS5) in colorectal cancer (CRC) are poorly understood. Here, we investigated the role of GAS5 in human CRC tissues. lncRNA GAS5 was specifically downregulated in CRC tissues and cell lines. Reduced GAS5 levels were significantly associated with advanced clinical stage and lymph node metastasis in CRC. GAS5 overexpression suppressed CRC cell proliferation and promoted cellular apoptosis. A dual-luciferase reporter assay showed that GAS5 could negatively regulate the expression of microRNA (miR)-182-5p. Upregulated miR-182-5p abrogated the effect of GAS5 overexpression on CRC cell proliferation and apoptosis. Furthermore, GAS5 positively regulated the expression of FOXO3a in CRC cells. Taken together, these findings suggest that overexpression of the lncRNA GAS5 inhibits cell proliferation and promotes apoptosis by inhibiting miR-182-5p expression, and thus could be a therapeutic target in CRC.

Introduction

Colorectal cancer (CRC) is the third most common type of tumor worldwide and the fourth leading cause of death. Approximately 1.2 million new cases and 600,000 deaths due to CRC are reported annually (1). An important factor affecting the prognosis of CRC patients is the stage of the disease at the time of diagnosis. The 5-year survival rate has been reported

as 90.1% for patients with *in situ* carcinoma, 69.2% for patients with regional lymph node metastasis, and 11.7% for patients with distant metastases, among all patients diagnosed with CRC in the US between 2001 and 2007 (2). Therefore, identifying specific and effective CRC tumor markers is of great significance for early diagnosis and improved prognosis.

Long non-coding RNA (lncRNA), the focus of this research, does not encode protein but is involved in gene regulation at multiple levels. In recent years, the function of lncRNAs in cancer has gradually been elucidated by researchers on account of new methods that have emerged, such as genome-wide association studies, lncRNA chip-based screening, RIP-RNA sequencing, and gene overexpression and knockdown assays. There is growing evidence that lncRNAs play an important role in the carcinogenesis, invasion and metastasis of cancers of various tissues (3-5). In many different tumors, lncRNAs play a role either as a tumor suppressor or as a carcinogenic agent (6-8). Study of CRC-related lncRNAs has found that they can regulate the formation and development of CRC by stimulating or inhibiting different cellular processes, including tumor cell proliferation, apoptosis, differentiation, invasion and metastasis (9,10).

Growth arrest-specific transcript 5 (GAS5) was initially identified by Schneider and others by screening the tumor suppressor gene library to focus on those genes involved in cell growth arrest. GAS5 is approximately 630 bases long and is located at 1q25 (11). Researchers have proposed that the introns of GAS5 are responsible for its important biological functions. Kino *et al* found that the amount of mature GAS5 was notably higher in serum-starvation and growth-inhibition conditions caused by lack of growth factors. They also found that GAS5 could compete with glucocorticoid response element (GRE) DNA to bind with glucocorticoid receptor (GR) DNA and form a structural domain, causing inhibition of the target genes mediated by glucocorticoid (12). It is becoming increasingly clear that GAS5 acts as a tumor suppressor and is downregulated in certain tumor tissues, including those of breast, kidney and prostate cancers, as well as in non-small cell lung carcinoma (13-16). It has also been reported that GAS5 can induce growth arrest and apoptosis in prostate and breast cancer cells (16). Furthermore, genetic aberration

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of GAS5 has been found in many types of tumor, including melanoma, breast cancer and prostate cancer, but its functional significance remains to be fully elucidated (15). GAS5 exhibits down-regulated expression in colon cancer, and this is likely connected to the poor prognosis of patients (17-19). Meanwhile, GAS5 probably affects the development of colon cancer as a potential cancer-suppressor gene, although its biological functions and mechanism of action in CRC remain ambiguous.

With the discovery of a large number of lncRNAs and the gradual identification of their functions, understanding the mechanisms of lncRNAs and their target molecules has become a focus of research. lncRNAs are able to interact with miRNAs as well as proteins. Sequence analysis has shown that lncRNAs and miRNAs have complementary target sequences. lncRNAs can make miRNAs out of mRNAs by binding the miRNAs. These types of lncRNAs are known as ceRNAs (competing endogenous RNAs), and can reduce the biological effects of multiple miRNAs, resulting in released suppression of the miRNA target genes and therefore increased expression levels (20). Conversely, it has been reported that GAS5 is suppressed by miR-21 through a potential binding site for miR-21 on GAS5; this negative correlation between miR-21 and GAS5 was identified in breast cancer samples (21). Meanwhile, GAS5 in endometrial cancer cells can bind with miR-103, regulating the expression of the downstream target gene PTEN (22). This suggests that the binding of GAS5 with different miRNAs has different functions.

In this study, we reported an interaction between GAS5 and miR-182-5p, which regulated CRC cell growth and apoptosis through the regulation of FOXO3a. Our findings provide a novel understanding of the role of GAS5 in CRC progression and of the underlying mechanism involved.

Materials and methods

Patients and tissue samples. A total of 95 pairs of surgically resected CRC specimens and adjacent normal tissues were collected at the Department of Gastrointestinal Surgery, People's Hospital of Tongling City. All tissue samples were obtained with written informed consent in accordance with the requirements of the Research Ethics Committee at the People's Hospital of Tongling City, and all the experimental protocols were approved by the Research Ethics Committee of the People's Hospital of Tongling City. All of the methods performed in this study were in accordance with the approved guidelines.

Cell line culture and transfection. Four human CRC cell lines (HCT-116, HT-29, SW480 and LoVo) and the normal colon epithelial cell line NCM460 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Gibco™ RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories; GE Healthcare Life Sciences, China) at 37°C in a 5% CO₂ incubator. The full-length GAS5 sequence lacking the poly-A tail was synthesized and sub-cloned into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). Small interfering RNAs (siRNAs) against GAS5 (si-GAS5), control siRNA (si-NC), miR-182-5p mimics (miR-182-5p) and

control mimics (miR-NC) were synthesized by GenePharma Co., Ltd. (Shanghai, China). The target sequence for GAS5 was 5'-CU UGCCUGGACCAGCUUAAdTdT-3'. The plasmids, siRNAs and miRNA mimics were transfected into cells separately using Invitrogen™ Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions. At 24, 48 and 72 h after transfection, the transfected cells were harvested and processed for further analysis.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells and tissue specimens using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RNA samples were reverse transcribed into cDNA using a PrimeScript™ II 1st Strand Synthesis kit (Takara Biotechnology, Co., Ltd., Dalian, China). qPCR was performed using SYBR Premix Ex Taq II (Takara Biotechnology) in 96-well optical plates and monitored with a Roche Cobas® z480 system (Roche, Welwyn Garden City, UK). The reaction conditions were as follows: 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 15 sec and 72°C for 30 sec. The relative gene expression of GAS5 was normalized to that of β -actin and calculated using the 2^{- $\Delta\Delta C_q$} method. A TaqMan MicroRNA Assay Kit (Applied Biosystems; Thermo Fisher Scientific) was used for miR-182-5p detection, and the expression level of miR-182-5p was normalized using the 2^{- $\Delta\Delta C_q$} method relative to U6 snRNA expression. The sequences of specific primers used in this study were as follows: GAS5 forward, 5'-CTTCTGGGCTCA AGTGATCCT-3' and reverse, 5'-TTGTGCCATGAGACT CCATCAG-3'; β -actin forward, CTCCATCCTGGCTCGCTG T and reverse, GCTGTACCTTCACCGTTCC; miR-182-5p real-time primer, 5'-GGCTTTGGCAATGGTAGAACTCA-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAA T-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. All assays were performed in triplicate.

Dual-Luciferase reporter assay. The binding sites between GAS5 and miR-182-5p were predicted using DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web). GAS5 fragments containing the predicted wild-type (WT) or mutant (MUT) miR-182-5p binding sites were generated and inserted into the luciferase reporter vector psi-CHECK-2 (Promega, Shanghai, China). HT-29 cells were placed on a 24-well plate and grown to 80% confluence. Cells were then co-transfected with 100 ng miR-182-5p mimics or miR-NC, 50 ng of GAS5-WT or GAS5-MUT plasmid, and 5 ng of pRL-CMV containing *Renilla* luciferase using Lipofectamine 2000. At 48 h after transfection, luciferase activities were detected using a Dual-Luciferase® reporter assay system (Promega). The luciferase activity of each group was normalized to the *Renilla* luciferase activity.

Cell proliferation assay. Cells were seeded into 96-well plates at 2x10³ cells/well and cultured overnight. After transfection for 24, 48 or 72 h, cell viability was analyzed with a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Rockville, MD, USA). CCK-8 reagent was added to each well, and the cells were incubated at 37°C for 1-4 h according to the manu-

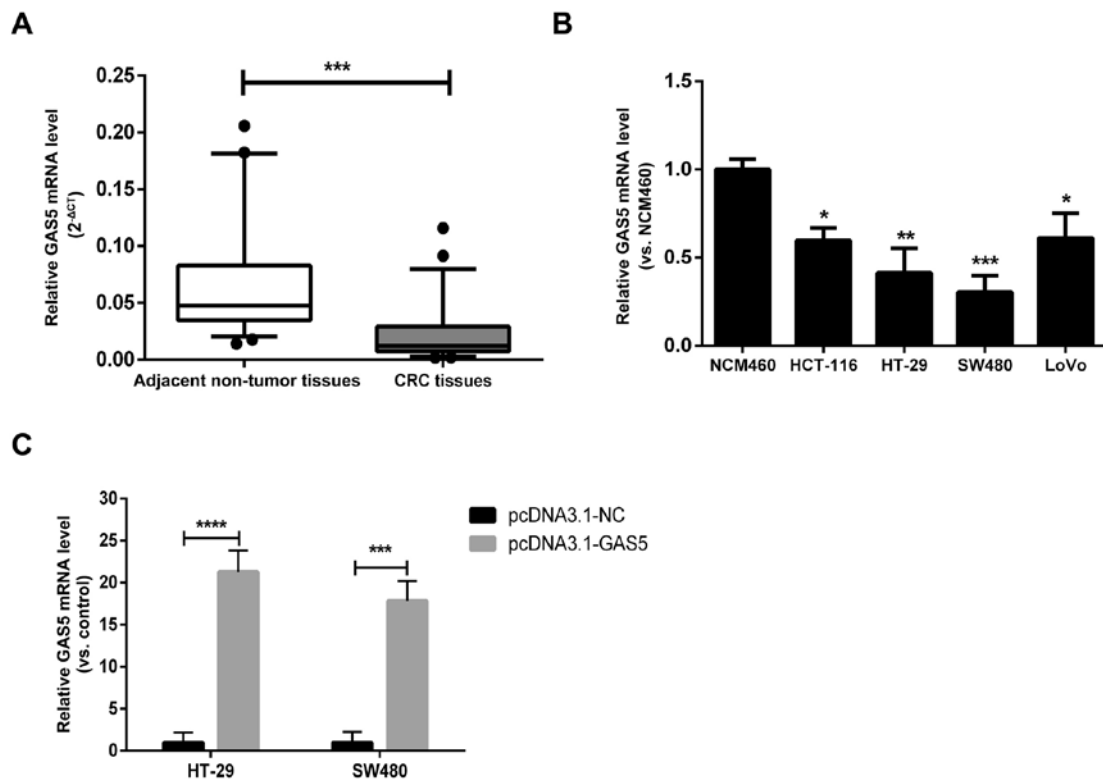


Figure 1. Expression levels of GAS5 in CRC tissues and cell lines. (A) RT-qPCR evaluation of GAS5 expression in 95 paired CRC samples (CRC specimens and corresponding adjacent non-tumor tissues). (B) Expression levels of GAS5 in CRC cell lines (HCT-116, HT-29, SW480 and LoVo) and normal colon epithelial cell line NCM460 were examined by RT-qPCR (compared with NCM460 with Holm-Sidak's multiple comparisons test). (C) The expression levels of GAS5 in HT-29 and SW480 cells transfected with pcDNA3.1-GAS5 or pcDNA3.1-NC were evaluated by RT-qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. GAS5, growth arrest specific transcript 5; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

facturer's protocol. The absorbance value of each group was spectrophotometrically determined at a wavelength of 450 nm. Each group was assayed in a 96-well plate in triplicate.

Western blot analysis. Total protein from transfected cells was extracted in cell lysis buffer (Pierce; Thermo Fisher Scientific, Inc.). Protein concentrations were measured using the Micro BCA Protein Assay kit (Enzyme, Nanjing, China). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Target protein was probed with primary antibodies overnight at 4°C. The primary antibodies used were rabbit anti-FOXO3a (dilution 1:1,000; cat. no. 3938; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti- β -actin (dilution 1:2,000; cat. no. 20536-1-AP; Proteintech, Rosemont, IL, USA). Expression of FOXO3a was quantified using β -actin as the loading control. Then, the membranes were incubated with secondary antibody (dilution 1:2,000; cat. no. 8889; Cell Signaling Technology) for 2 h at room temperature. Protein bands were visualized using an ECL kit (Tanon Science and Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Apoptosis analysis. Cells were transfected in 6-well plates for 48 h, and then washed and resuspended in phosphate-buffered saline (PBS) twice. Flow cytometric analysis was carried out to evaluate apoptosis using an Annexin V-FITC/PI Apoptosis Detection kit (MultiSciences Biotech, Hangzhou, China).

Cells were double-stained with Annexin V-FITC (50 μ g/ml) and propidium iodide (PI) (10 μ g/ml) in the dark for 15 min at room temperature before they were subjected to flow cytometric analysis (FACScan; BD Biosciences, San Diego, CA, USA).

Statistical analysis. Experimental results are presented as the mean \pm standard deviation. The association of GAS5 expression with clinical characteristics was analyzed using a Chi-square test. Comparisons between two groups were conducted using the two-tailed Student's t-test or the Chi-square test (SPSS 18.0; SPSS, Inc., Chicago, IL, USA). The comparison of multiple groups was analyzed by ANOVA with Holm-Sidak's or Dunnett's multiple comparisons test as indicated in the Figure legends (GraphPad Prism 6.0; GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant when $P < 0.05$.

Results

GAS5 is downregulated in CRC tissues and cell lines. The expression levels of the lncRNA GAS5 in 95 paired samples (CRC specimens and corresponding adjacent non-tumor tissues) were examined using real-time RT-qPCR. GAS5 expression was significantly lower in tumor tissues than that noted in the adjacent tissues (Fig. 1A). We next investigated the levels of GAS5 expression in different CRC cell lines (HCT-116, HT-29, SW480 and LoVo) and in the normal colon

Table I. Association between GAS5 expression and clinicopathological characteristics of the colorectal cancer cases.

Characteristics	GAS5 levels		P-value
	Low (n=47)	High (n=48)	
Age, years			0.615
<55	19	17	
≥55	28	31	
Sex			0.576
Male	31	29	
Female	16	19	
Depth of invasion			0.102
T1-T2	13	21	
T3-T4	34	27	
Tumor size (cm)			0.183
≤4	21	28	
>4	26	20	
Lymphatic metastasis			0.027 ^a
N0	14	25	
N1 or above	33	23	
TNM stage			0.027 ^a
I-II	14	25	
III-IV	33	23	

^aP<0.05, significant difference.

epithelial cell line NCM460. GAS5 was expressed at a significantly low level in HCT-116, HT-29, SW480 and LoVo cells in comparison to its level in NCM460 cells; this difference was especially evident in HT-29 and SW480 cells (Fig. 1B). Therefore, HT-29 and SW480 cells were used to establish GAS5-overexpressing cell lines with pcDNA3.1-GAS5 plasmids to investigate the function of GAS5 in the development of CRC. The transfection efficiency was verified by RT-qPCR (Fig. 1C). The median GAS5 expression was used as the cut-off value. When the expression of GAS5 was greater than the median, it was defined as high expression; otherwise it was defined as low expression. As indicated in Table I, low expression of GAS was significantly associated with lymph node metastasis and advanced clinical stage in breast cancer. Therefore, downregulation of GAS5 is associated with the malignant progression of breast cancer.

GAS5 overexpression suppresses CRC cell proliferation and promotes apoptosis. Next, we investigated the association of GAS5 expression with CRC cell proliferation and apoptosis. HT-29 and SW480 cells were transfected with pcDNA3.1-NC or pcDNA3.1-GAS5. Cellular proliferation was evaluated by CCK-8 assay. The results revealed that overexpression of GAS5 significantly attenuated the proliferation rate of both cell lines over time compared with the control group rate (Fig. 2A and B). Flow cytometric analysis was subsequently used to detect the rate of cellular apoptosis. We found that the cellular apoptosis rate was increased significantly in response to GAS5 overex-

pression compared with the rate in the pcDNA3.1-NC control group (Fig. 2C and D). Taken together, these data indicate that GAS5 overexpression suppresses proliferation and promotes apoptosis in CRC cells.

GAS5 negatively regulates the expression of miR-182-5p in CRC cells. To further investigate the mechanism by which GAS5 regulates CRC cell proliferation and apoptosis, we studied the relationship between miR-182-5p and GAS5. We used a bioinformatics online software program (DIANA tools) to predict the potential miRNAs that interact with GAS5. As expected, we found that miR-182-5p contains a complementary nucleotide sequence for GAS5 (Fig. 3A). We measured the expression level of miR-182-5p in CRC specimens and examined the potential correlation between the RNA expression levels of GAS5 and miR-182-5p. We observed a negative correlation between GAS5 and miR-182-5p levels (Fig. 3B). As we described, GAS5 negatively regulated miR-182-5p, yet, GAS5 regulated miR-182-5p through its function as an miRNA sponger. Thus, we constructed a GAS5 fragment, which contained the miR-182-5p-binding site into luciferase reporter vector, and checked the mimic miR-182-5p effects on luciferase activity. This is a common and standard process as reported previously (23,24). The luciferase activity of the reporter containing GAS5-WT was reduced in cells transfected with miR-182-5, while GAS5-MUT provided resistance to miR-182-5p-induced luciferase reporter repression (Fig. 3C and D). To determine the effect of GAS5 on the expression of miR-182-5p, the CRC cell line HT-29 was transfected with pcDNA3.1-GAS5 and SW480 cells were treated with si-GAS5. Expression levels of miR-182-5p were detected by RT-qPCR in the GAS5-knockdown SW480 cells and GAS5-overexpressing HT-29 cells. The knockdown of GAS5 markedly increased the expression of miR-182-5p in the SW480 cells (Fig. 3E), while GAS5 upregulation significantly decreased miR-182-5p expression in the HT-29 cells (Fig. 3F), compared with expression in the corresponding controls. Taken together, these results imply that GAS5 functions as a sponge, which negatively regulates the expression of miR-182-5p in CRC cells.

GAS5 overexpression suppresses CRC cell proliferation and promotes apoptosis by inhibiting miR-182-5p. Cell proliferation and apoptosis assays were performed on HT-29 cells transfected with miR-182-5p mimics or miR-NC. miR-182-5p overexpression caused a significant increase in cell viability at 48 and 72 h (Fig. 4A), and consequently reduced the proportion of apoptotic cells (Fig. 4B). To further evaluate the effect of miR-182-5p regulation by GAS5 on CRC progression, HT-29 cells were transfected with pcDNA3.1-GAS5, pcDNA3.1-NC, pcDNA3.1-GAS5+miR-182-5p or pcDNA3.1-GAS5+miR-NC. CCK-8 assay results indicated that the reduction in cell proliferation induced by GAS5 overexpression was abrogated by miR-182-5p upregulation (Fig. 4C). Furthermore, flow cytometric analysis indicated that pcDNA3.1-GAS5 transfection led to a significant increase in apoptotic rates; while miR-182-5p overexpression obviously attenuated the GAS5-induced apoptosis in HT-29 cells (Fig. 4D). Taken together, all of these data suggest that GAS5 overexpression inhibits cell proliferation and promotes apoptosis by targeting miR-182-5p in CRC cells.

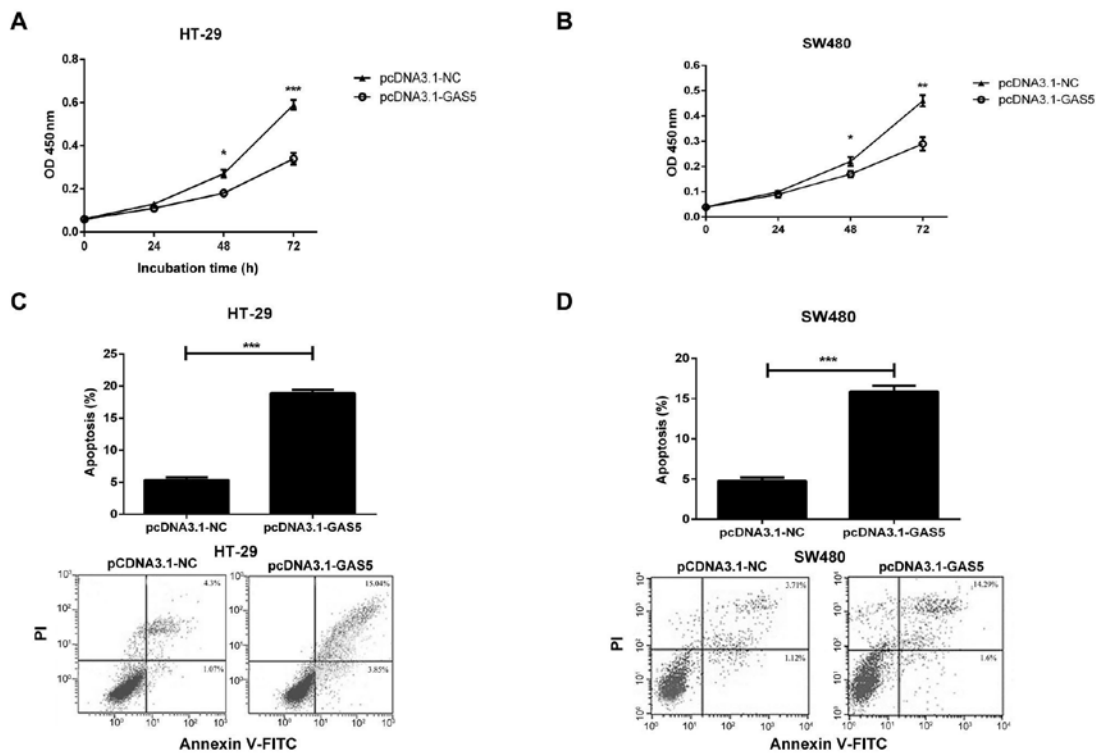


Figure 2. GAS5 overexpression suppresses CRC cell proliferation and promotes apoptosis. Overexpression of GAS5 significantly attenuated the proliferation rate of both the HT-29 (A) and SW480 (B) cell lines over time compared with the control cell group rate. Cell apoptosis was promoted by GAS5 overexpression in the HT-29 (C) and SW480 (D) cells. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. GAS5, growth arrest specific transcript 5; CRC, colorectal cancer.

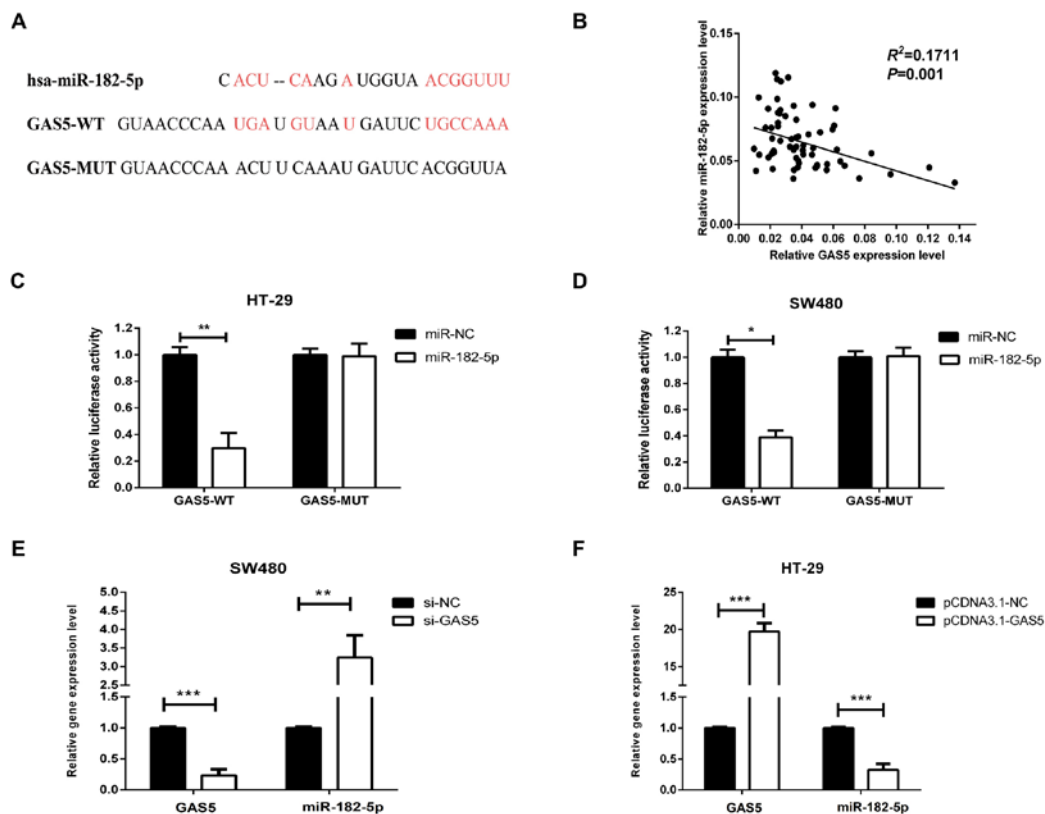


Figure 3. GAS5 negatively regulates the expression of miR-182-5p in CRC cells. (A) Binding sites between GAS5 and miR-182-5p were predicted using DIANA tools. (B) Association analysis was performed to detect the relationship between GAS5 and miR-182-5p in CRC tissues. (C and D) Luciferase activities of reporters containing the GAS5-WT or GAS5-MUT sequence in HT-29 (C) and SW480 (D) cells transfected with miR-182-5p or miR-NC. (E and F) Expression levels of miR-182-5p in the GAS5-knockdown SW480 cells (E) and GAS5-overexpressing HT-29 cells (F) were detected by RT-qPCR. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. GAS5, growth arrest specific transcript 5; miR-182-5p, microRNA-182-5p; CRC, colorectal cancer; WT, wild-type; MUT, mutant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

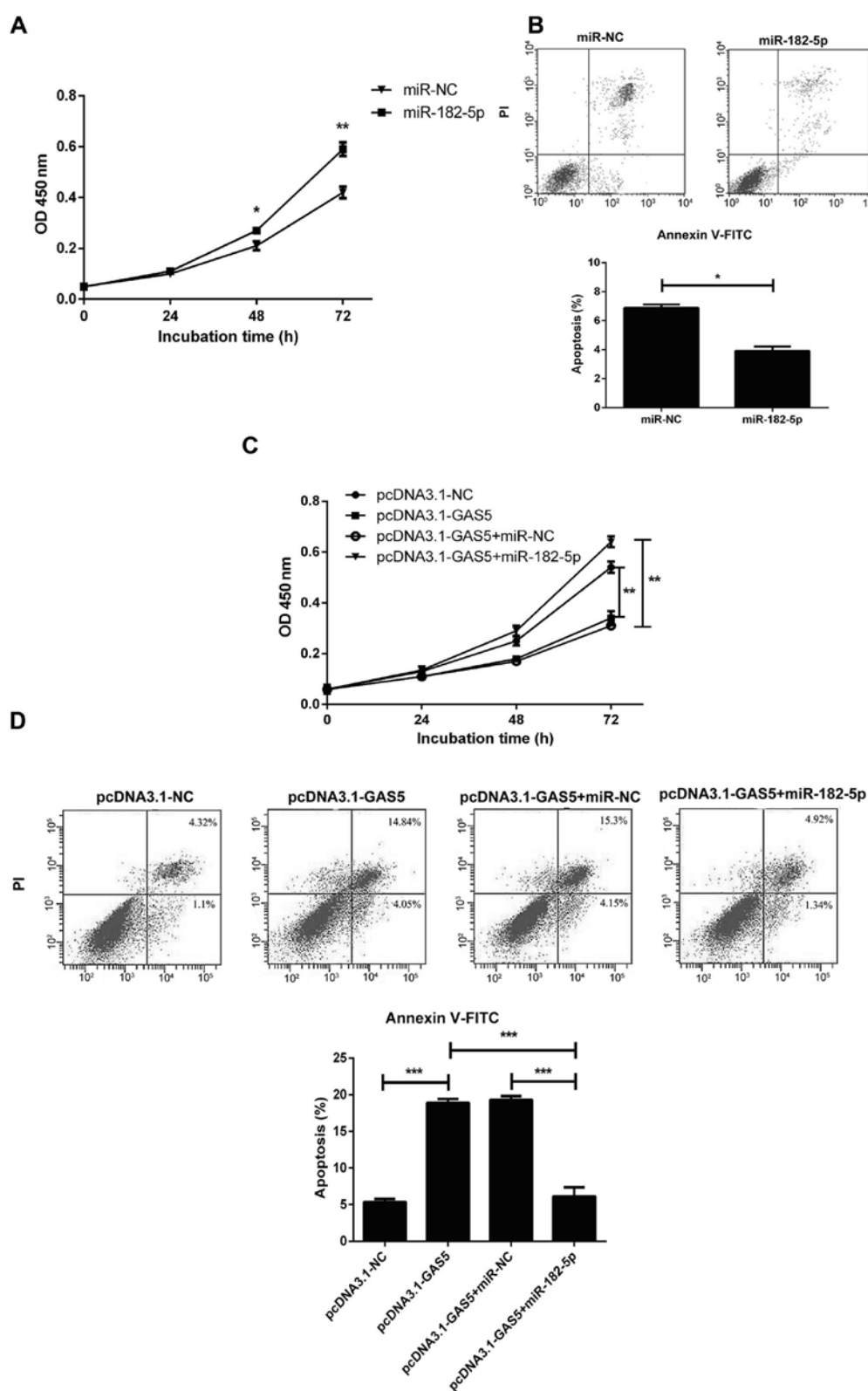


Figure 4. GAS5 overexpression suppresses CRC cell proliferation and promotes apoptosis by inhibiting miR-182-5p. Cell proliferation (A) and apoptosis (B) assays were performed on HT-29 cells transfected with miR-182-5p mimics or miR-NC. HT-29 cells following transfection with pcDNA3.1-GAS5, pcDNA3.1-NC, pcDNA3.1-GAS5+miR-182-5p or pcDNA3.1-GAS5+miR-NC. (C) CCK-8 assay was performed to examine proliferation of the transfected cells. The difference was compared with each other group with Dunnett's multiple comparisons test; significance is shown as indicated. (D) Cell apoptosis was also detected in these cells by flow cytometry. The difference was compared with each other group with Dunnett's multiple comparisons test; significance is shown as indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. GAS5, growth arrest specific transcript 5; CRC, colorectal cancer; miR-182-5p, microRNA-182-5p; miR-NC, microRNA-negative control; CCK-8, Cell Counting Kit-8.

GAS5 inhibits the function of miR-182-5p in CRC cells by regulating FOXO3a. It has been reported that FOXO3a, a

pro-apoptotic transcription factor and direct target of the PI3K-AKT signaling pathway, may be the direct target of

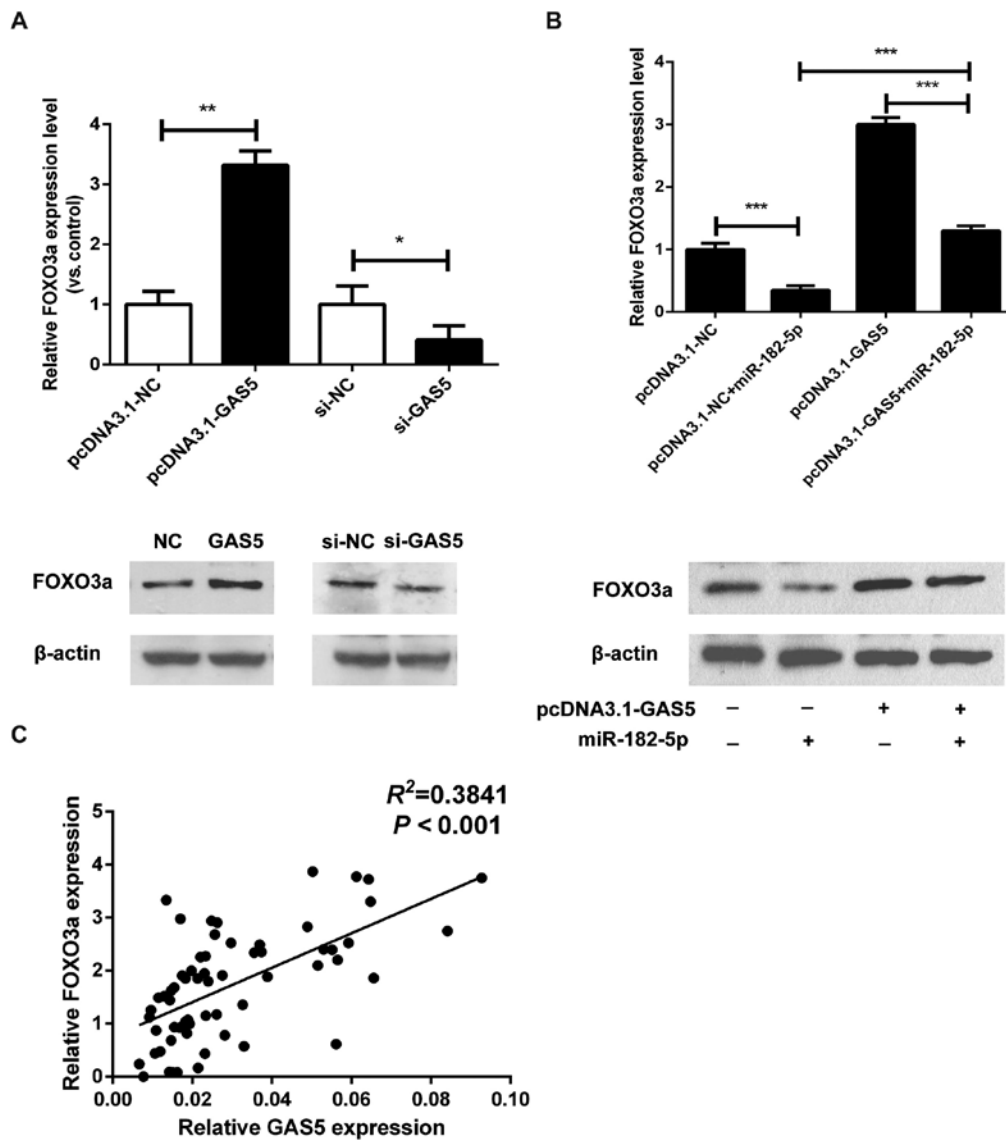


Figure 5. GAS5 inhibits the function of miR-182-5p in CRC cells by regulating FOXO3a. (A) Expression levels of FOXO3a in the GAS5-knockdown SW480 cells and GAS5-overexpressing HT-29 cells were detected by RT-qPCR and western blotting. (B) Expression levels of FOXO3a were detected in HT-29 cells transfected with pcDNA3.1-NC, pcDNA3.1-NC+miR-182-5p, pcDNA3.1-GAS5 or pcDNA3.1-GAS5+miR-182-5p. The difference was compared with each other group with Dunnett's multiple comparisons test, significance was shown as indicated. (C) GAS5 expression is positively correlated with expression of FOXO3a in CRC tissues. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. GAS5, growth arrest specific transcript 5; miR-182-5p, microRNA-182-5p; CRC, colorectal cancer; FOXO3a, forkhead box O3a.

miR-182-5p (25). Therefore, we explored the potential correlation between FOXO3a and GAS5 expression. As shown in Fig. 5A, the mRNA and protein levels of FOXO3a were increased in HT-29 cells transfected with pcDNA3.1-GAS5, and were decreased in GAS5-knockdown SW480 cells. To further investigate the expression correlation between FOXO3a and the GAS5/miR-182-5p axis, we examined the regulation of FOXO3a by miR-182-5p and GAS5. The expression of FOXO3a was significantly downregulated by miR-182-5p overexpression (Fig. 5B). More notably, miR-182-5p mimics reversed the increase in expression of FOXO3a that was induced by GAS5 overexpression (Fig. 5B). Furthermore, in CRC tissues, GAS5 expression was found to be positively association with the expression of FOXO3a (Fig. 5C). These data indicate that GAS5 overexpression inhibits the function of miR-182-5p in CRC cells by regulating FOXO3a.

Discussion

The discovery of ceRNAs was made during the study of miRNA regulation. As it was reported that the lncRNA IPS1 altered the protein level of PHO2 in plants by inhibiting miR-399 activity (26), lncRNA PVT1 was found to regulate HIF-1 α expression through sponge miRNA-199a5p in lung cancer cells (27), lncRNA SNHG1 functions as a ceRNA to antagonize the effect of miR-145a-5p on the downregulation of NUA1 (28). These early studies demonstrated that the process of miRNA targeting may be reciprocal rather than unidirectional. It has been found that multiple lncRNAs can act as ceRNAs and play a role in regulating downstream targets by base pairing with and inactivating miRNAs. For instance, the lncRNA HULC is highly expressed in HCC cells and is implicated in tumor cell proliferation (29); and the

lncRNA Loc285194 can bind to miR-211 by complementary pairing to downregulate the expression of miR-211 and exert an anti-oncogenic function (8). In this study, we found that expression levels of the lncRNA GAS5 were significantly downregulated in CRC tissues compared with that noted in the corresponding adjacent tissues. Further functional and mechanistic studies revealed that GAS5 exerts apoptosis-promoting effects by acting as a ceRNA of miR-18-5p.

To date, many studies have reported the biological roles of GAS5 in multiple cancers and its underlying molecular mechanisms. Li *et al* found that GAS5 was significantly decreased in ovarian cancer, which promoted cell proliferation, migration and invasion partly by regulating cyclin D1, p21 and apoptosis protease activating factor 1 (APAF1) expression, suggesting that lower GAS5 expression may indicate a poor prognosis in ovarian cancer (30). In colorectal cancer (CRC), overexpressed GAS5 was reported to inhibit cell proliferation both *in vitro* and *vivo*, and the expression level of GAS5 was significantly associated with susceptibility to and the progression of CRC (18,19). Other reports have revealed that GAS5 acts as an important regulator of the genesis and development of CRC by influencing inflammatory cytokines via the NF- κ B and Erk1/2 pathways (31). Here, we confirmed that GAS5 expression was lower in both CRC tissues and cell lines, and downregulation of GAS5 was found to be associated with CRC stage and lymphatic metastasis as reported previously (18,19). We studied the effect of GAS5 on cell proliferation and apoptosis using GAS5-overexpressing HT-29 and SW480 cells. We found that the overexpression of GAS5 suppressed proliferation and promoted apoptosis in the CRC cells. Other research has shown that GAS5 plays an important role in the process of cell proliferation (13). To evaluate the underlying mechanism in our study, the expression levels of miR-182-5p were detected by RT-qPCR in GAS5-knockdown SW480 and GAS5-overexpressing HT-29 cells; furthermore, upon analysis of CRC tissues, we found that GAS5 expression was negatively correlated with miR-182-5p expression. Additionally, bioinformatics analysis and a dual luciferase activity assay indicated that GAS5 could directly interact with miR-182-5p and negatively regulate its expression in CRC cells. These findings suggest that the interaction between GAS5 and miR-182-5p may be involved in the regulation of CRC progression. Thus, GAS5 might play a crucial role in inhibiting the proliferation and promoting the apoptosis of CRC cells by regulating miR-182-5p.

In recent years, miRNAs, which have been identified to act as both oncogenes and cancer-suppressor genes, have been widely implicated in the occurrence and development of CRC as well as in tumor pathological processes including metastasis (32-34). Furthermore, miRNAs may also serve as prognostic target molecules for patients with CRC. A study by Tazawa *et al* revealed that high expression of miR-34a could inhibit the proliferation of CRC cells by negatively regulating the E2F1 signaling pathway; E2F1 may affect the cell cycle by promoting cellular transition from the G1 phase to the S phase (34). Based on comparison and analysis of miR-195 expression between CRC and normal tissues, Liu *et al* noted downregulation of miR-195 in cancer tissues, and their further experiments demonstrated that miR-195 could function as a tumor-suppressor gene to induce the apoptosis of tumor

cells (33). Therefore, we may conclude that miRNAs play an important role in the occurrence and development of CRC. As a member of the miR-183 family, also composed of miR-183 and miR-96, miR-182-5p exhibits a highly conserved coding sequence across animal species (35,36). Most previous studies suggest that miR-182-5p is highly expressed in various types of human cancer, including prostate, breast, bladder, liver, colon, cervical and ovarian cancers and glioma, and indicate an oncogenic role of miR-182-5p in tumor progression (37-42). In patients with early-diagnosed colon cancer, expression of miR-182-5p and miR-21-3p has been reported to be significantly upregulated (43). Our data are consistent with previous studies and, furthermore, confirm that miR-182-5p overexpression significantly promotes CRC cell proliferation while suppressing apoptosis. In the present study, to further evaluate the effect of miR-182-5p regulation by GAS5 on CRC progression, HT-29 cells were transfected with pcDNA3.1-GAS5, pcDNA3.1-NC, pcDNA.1-GAS5+miR-182-5p or pcDNA3.1-GAS5+miR-NC. CCK-8 and flow cytometric analyses showed that GAS5 overexpression obviously inhibited cell proliferation and induced apoptosis in CRC cells, while miR-182-5p overexpression markedly abolished these effects, suggesting that GAS5 served as a tumor suppressor by inhibiting miR-182-5p expression in CRC cells. With increasing studies on the relationship between cancers and miR-182-5p-mediated regulation in recent years, it has been found that miR-182-5p can promote the proliferation of kidney cancer cells by activating the AKT/FOXO3a signaling pathway (44). miR-182-5p is also capable of regulating FOXO1 and FOXO3 genes and contributing to the metastasis of breast cancer and melanoma (35). Furthermore, by acting on the downstream target gene FOXO1 in liver cancer, miR-183, miR96 and miR-182 activate the Wnt/ β -catenin pathway, thereby accelerating cell migration and invasion (45). Other reports have also detected elevated expression of miR-182 in hepatocellular carcinoma and found that this may promote liver tumor metastasis by regulating the MTSS1 gene (46).

FOXO3a has a pivotal role in both oncogenesis and tumor suppression (47). Loss of FOXO3a has been observed in various cancers, and its cellular localization and phosphorylation status are considered to be prognostic factors for breast, prostate, bladder and ovarian cancers (48-52). It has been reported that FOXO3a, a pro-apoptotic transcription factor and direct target of the PI3K-AKT signaling pathway, might be a direct target of miR-182-5p (25,44). In this study, we analyzed the expression level of FOXO3a and found that the expression level of GAS5 was positively association with FOXO3a in CRC tissues. In addition, we found that FOXO3a expression was increased in GAS5-overexpressing CRC cells and decreased in GAS5-knockdown SW480 cells. Furthermore, miR-182-5p abrogated the upregulation of FOXO3a induced by GAS5. Thus, we suggested that GAS5 overexpression inhibited the function of miR-182-5p in CRC cells by regulating FOXO3a expression. This adds to previous research findings that the ectopic expression of miR-182-5p has a tumor-promoting effect in melanoma, lung cancer and stomach cancer by targeting MITF, BCL2, cyclin D2, RGS17 and CREB1 (53-55).

Overall, this study demonstrated that GAS5 was down-regulated in CRC tissues and cell lines. GAS5 overexpression in CRC cells suppressed cell proliferation and promoted

apoptosis by inhibiting miR-182-5p. We also found that GAS5 inhibits the function of miR-182-5p in CRC cells by regulating FOXO3a. In conclusion, the GAS5/miR-182-5p/FOXO3a axis might play a key role in CRC growth and serve as a target for potential therapeutic applications.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

KWC and WMZ conceived and designed the study. KWC, ZGZ, GW and JW performed the experiments. WMZ and KWC wrote the paper. KWC, ZGZ, GW and JW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All tissue samples were obtained with written informed consent in accordance with the requirements of the Research Ethics Committee at the People's Hospital of Tongling City, and all the experimental protocols were approved by the Research Ethics Committee of the People's Hospital of Tongling City. All of the methods performed in this study were in accordance with the approved guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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