miR-126 inhibits growth of SGC-7901 cells by synergistically targeting the oncogenes *PI3KR2* and *Crk*, and the tumor suppressor *PLK2*

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Abstract. MicroRNA (miRNA)-126 (miR-126) was reported to be downregulated and to act as a tumor suppressor in cancers of the lung, cervix, bladder and prostate. However, the functions of miR-126 in gastric cancer appear to be diverse and are largely unknown. MiR-126 was reported to act as a tumor suppressor by targeting the *Crk* gene, or as an oncogene by targeting the *SOX2* gene in gastric cancer. We identified that the expression of miR-126 was decreased in gastric cancer cell lines and tissues. *PLK2*, a tumor suppressor gene, was directly regulated by miR-126 in SGC-7901 cells. Overexpression of miR-126 not only suppressed the growth and clone formation of SGC-7901 cells, but also induced apoptosis *in vitro*, whereas inhibition of miR-126 slightly promoted SGC-7901 cell proliferation. The cell cycle was not affected by miR-126. Moreover, miR-126 suppressed tumor growth *in vivo* in a xenograft model. *PLK2*, *PI3KR2* and *Crk* were regulated by miR-126 in SGC-7901 cells. We infer that the functions of miR-126 in gastric cancer depend on synergistic targeting balance between oncogenes and anti-oncogenes. Our study indicates that miR-126 is a tumor suppressor, which in the future may become a therapeutic target for gastric cancer.

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Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs whose mature products are approximately 22 nucleotides long. MiRNAs may regulate diverse biological processes, including development, cell proliferation, differentiation and apoptosis, through negatively regulating target gene expression at the post-transcriptional and/or translational level (1-3). Lim *et al* showed that each vertebrate miRNA has hundreds of different conserved or non-conserved targets (4). It has also been estimated that approximately 30% of genes are regulated by at least one miRNA (5). Thus, a single miRNA may have substantial biological consequences. Recently, miRNAs have also been reported as key factors in cancer, playing both oncogenic and tumor-suppressing roles (2,6). In 2002, miR-126 was identified in a tissue-specific mouse screen (7). miR-126 is encoded by intron 7 of the *egfl7* gene in all vertebrates, and has been shown to mediate angiogenesis and vascular integrity (8,9). However, the functions of miR-126 in cancers appear to be diverse and remain largely unknown. miR-126 was reported to be downregulated and to act as a tumor suppressor in different cancers of the lung (10,11), stomach (12,13), cervix (14), bladder and prostate (15). miR-126 was also significantly downregulated in ectopic endometria (EC) versus eutopic endometria (EU), suggesting that it may play an initial role in the development and progression of endometriosis (Ems) (16). miR-126 might disturb vascular integrity, leading to a disorganized and abnormal tumor vasculature (17).

To date, the target genes of miR-126 that have been confirmed are *PI3KR2* (18), *IRS-1* (19), *Crk* (12), *SOX2* (13), *ADAM9*, *MMP7* (20) and *SCL7A5* (21). Except for *SOX2*, which is a tumor suppressor, other genes are involved in tumor formation and progression. miR-126 plays a tumor suppressor role in human cancer through the direct or indirect repression of several key oncogenic molecules, such as PI3KR2, IRS-1 and Crk. At present, some data exist showing that miR-126 plays a tumor suppressor role in gastric cancer SGC-7901 cells through targeting *Crk* (12). However, Otsubo *et al* demonstrated that miR-126 acts as an oncogene by targeting *SOX2* in gastric cancer cells (13). Thus, it is controversial as to whether miR-126 is a tumor suppressing or oncogenic miRNA in gastric cancer cells.

PLK2 is a member of Polo-like kinase (PLK) family, which is involved in regulation of cell cycle progression or mitotic progression (22,23). It is well known that PLK2 regulates centriole duplication in mammalian cells (24). PLK2 is also activated by a spindle checkpoint in a p53-dependent manner, and thereby may prevent a mitosis catastrophe following spindle damage (25). Thus, PLK2 seems to inhibit oncogenic transformation (26). A previous study showed that *PLK2* is a target gene of miR-126 (21,27), which is also as a tumor suppressor (12). However, the inhibitory effects of a specific miRNA on a target gene appear to be cell type-specific. For example, miR-126 overexpression does not suppress *PLK2* expression in SCLC cells (21). *TOM1* is a target gene of miR-126 in cystic fibrosis (CF) airway epithelium cells, but not in MCF7 cells (28). Similarly, *SPRED1* is a target gene of miR-126 in HUVEC cells, but not in acute myeloid leukemia (AML) cells (8,27).

Materials and methods

Collection and analysis of clinical samples. Forty-four gastric cancer and adjacent normal tissues were obtained from patients undergoing surgery for gastric cancer at The First and Second Affiliated Hospital of Medical College of Xi'an Jiao Tong University. The samples were placed in liquid nitrogen immediately after excision from the patients and subsequently frozen at -80˚C until RNA extraction. Informed consent was obtained from each patient and was approved by the Institute Research Ethics Committee at the Cancer Center, Xi'an Jiaotong University.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. RNA from gastric cancer tissue samples was extracted using TRIzol (Invitrogen, Life Technologies) according to the manufacturer's recommendations. miR-126 expression was assessed using qRT-PCR. Human U6 small nuclear RNA (snRNA) was used as an internal standard. Primer sequences are listed in Table I. In order to verify that *PLK2* was regulated by miR-126, we collected cells and extracted RNA using TRIzol, according to manufacturer recommendations, after transfecting the miR-126, miR-control and anti-miR-126 individually at 24 h. The *β-actin* mRNA level was used as an internal standard. The *PLK2* and *β-actin* qRT-PCR primers are shown in Table I. Reverse transcription was performed using PrimerScript™ RT reagent kit perfect Real-Time and qRT-PCR using SYBR® Premix Ex Taq™ II (Takara). The qRT-PCR program used for amplification was: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec.

pcDNA6.2-GW/EmGFP-miR-126 vector construction. The pcDNA6.2-GW/EmGFP-miR vector was purchased from Invitrogen. The linear structure was converted to a double link-shaped structure by cloning multiple cloning sites (MCS) (using *Eco*RI, *Pst*I, *Nhe*I, *Kpn*I and *Hin*dIII) into pcDNA6.2-GW/EmGFP-miR via the vector's joint. The Hsa-miR-126 clone vector was constructed by Jie Rui Biology Ltd. (Shanghai, China). Hsa-miR-126 mature sequences were cloned into pcDNA6.2-GW/EmGFP-miR, using *Eco*RI and *Hin*dIII, and named pcDNA6.2-GW/EmGFP-miR-126 (miR-126). Finally, for miR-control vector, the miR-126 mature sequence was transformed to clone MCS (short miR-control).

Cell culture and transfection. SGC-7901 cells were obtained from The Central Laboratory of Biomedical Research of Xi'an Jiaotong University Medical School. The cells were grown in Dulbecco's modified Eagle medium (Gibco) with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and incubated at 37˚C in 5% CO_2. There were four experimental groups (control, anti-miR-126, miR-control and miR-126). Anti-miR-126 sequence: 5'-GCATTATTACTCACGGTACGA-3' (Sangon Biotech). Anti-126, miR-control and miR-126 were transfected at a final concentration of 100 nmol/l by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, in all experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. SGC-7901 cells were seeded at $1x10^4$ cells/well in 96-well plates and transfected the following day with miR-126, miR-control and anti-miR-126 using Lipofectamine 2000; the control group was transfected with Lipofectamine 2000 only. MTT was added to cells (Sigma, USA) at 24, 48, 72 and 96 h, respectively, and these continued incubating for 3 h at 37˚C in 5% CO_2, then the supernatant was discarded and dimethyl sulfoxide was added to the cells. Sample absorbance was measured at 490 nm by a high-throughput universal microplate assay (BMG Lab Technologies).

Cell cycle analysis. SGC-7901 cells were seeded at $1x10^5$ cells/well in a 6-well plate and transfected with miR-126, miR-control and anti-miR-126 using Lipofectamine 2000. Cells were harvested at 24 and 48 h post-transfection, stained for DNA content using propidium iodide (PI) and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson). Briefly, cells were harvested by trypsinization and washed once with phosphate-buffered saline (PBS) before fixing overnight in 70% EtOH. For DNA staining, cells were pelleted and stained for 30 min with 300 μl PI solution (0.05 mg/ml PI, 20 μg/ml RNAse A in 0.1% bovine serum albumin).

Cell apoptosis analysis. SGC-7901 cells were seeded at $1x10^5$ cells/well in a 6-well plate and transfected with miR-126, miR-control and anti-miR-126 using Lipofectamine 2000. Cells were harvested 24 and 48 h post-transfection and washed twice with ice-cold PBS before being stained with 7-AAD/PI, and later analyzed on a FACSCalibur flow cytometer (Becton-Dickinson).

Table I. Primer sequences.

Gene	Reverse transcription primers	Real-time PCR primers
U6	5'-CGCTTCACGAATTTGCGTGTCAT-3'	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'
miR-126	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGACCGCATTA-3'	GSP: 5'-GGGGTCGTACCGTGAGT-3' R: 5'-CAGTGCGTGTCGTGGAGT-3'
PLK2		F: 5'-GACCCTATGGGACTCCTCTTT-3' R: 5'-GTATGCCTTAGCCTGTTCTGG-3'
β-actin		F: 5'-TGGCACCCAGCACAATGAA-3' R: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

Colony formation assay in vitro. One thousand cells, which were post-transfected with miR-126, miR-control or anti-miR-126 at 24 h, were seeded into 6-well plates and cultured in common media. Approximately 2 weeks later, colonies that appeared were fixed with pre-chilled methanol and stained with 2% Giemsa solution (Sigma, St. Louis, MO, USA). Experiments were conducted in triplicate.

Tumor xenograft model and tumorigenicity assay. SGC-7901 cells ($1x10^6$ cells/mouse) stably transfected with miR-126 or miR-control lentivirus vector were subcutaneously injected into 6-week-old male nude mice. Tumor size was measured every 3 days. For end-point experiments, the bioluminescence images captured *in vivo* were obtained using a photobiology system (Xenogen). The mice were euthanized 22 days after injection, and the tumors were removed and weighed. The tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E).

Western blot analysis. SGC-7901 cells were seeded at $1x10^6$ cells/6-cm culture dish, and transfected with miR-126, miR-control or anti-miR-126, using Lipofectamine 2000 according to the manufacturer instructions (Invitrogen). Cells were harvested by trypsinization, washed once with ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Igepal, 50 mM Tris-HCl pH 8.0, 2 mM ethylenediamine tetraacetic acid) supplemented with 1X complete mini protease inhibitor cocktail tablets. Then, 25 μg of protein/lane was resolved on 10% polyacrylamide gel electrophoresis gels and transferred to a nitrocellulose membrane (Roche Diagnostics GmbH). Primary antibodies used were PI3KR2 (Cell Signal 9402), Crk (Santa Cruz Sc-53923), Bcl-2 (Sigma V9131), Bax (Santa Cruz Sc-126), PLK2 (sc-25421; Santa Cruz Biotechnology) and β-actin (Bioworld, AP0060). The membranes were further probed with horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO). Working solutions of the Pierce ECL Substrate were prepared and added to polyvinyldifluoride membranes for 1 min. The membranes were removed from the substrates and exposed to ChemiDoc-It 510 (UVP; LLC).

Luciferase assay. The wild-type 3' untranslated regions (UTRs) or mutant 3'UTRs of *PLK2* carrying miR-126-binding sites were cloned downstream of the luciferase reporter in a pmir-GOL vector system (Promega; E1330) through *Sac*I and *Xho*I digestion. The wild-type *PLK2* 3'UTR PCR primer was as follows: sense primer, 5'-GAGCTCGACCCTAT GGGACTCCTCTTT-3' and antisense primer, 5'-CTCGAG GTATGCCTTAGCCTGTTCTGG-3'. The *PLK2* mutant sequence was chemically synthesized by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd., as follows: *PLK2* Mut Top: 5'-cgagtatgttgaagaagatggacatgtggtgatcctaaaacaattccccc-3' and *PLK2* Mut Bottom: 5'-tcgagggggaattgttttaggatcaccacatgtcca tcttcttcaacatactcgagct-3'. The wild-type *PLK2* reporter vector and mutation reporter vector were named pmir-GOL-PLK2 and pmir-GOL-mutPLK2, respectively. SGC-7901 cells were seeded in 96-well plates and transfected with miR-126 or miR-control, and pmir-GOL-PLK2 or pmir-GOL-mutPK2, using Lipofectamine 2000. Cells were harvested 24 h post-transfection and luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega; E2920) according to the manufacturer's recommendations.

Statistical analysis. Quantitative results are presented as the mean ± standard deviation (SD). Statistical analysis was performed by using a t-test, and $P<0.05$ was taken as the level of significance. All results were analyzed using the statistical software SPSS10.0.

Results

Downregulated expression of miR-126 in gastric cancer and gastric cancer cells. To explore miR-126 expression levels in gastric cancer, we collected 44 paired gastric cancer and adjacent normal tissue samples. We used qRT-PCR to determine the miR-126 expression level in gastric cancer. In the 44 paired clinical gastric samples, there were 29 samples with downregulation of miR-126 compared with the matched non-tumor tissue samples (relative expression ratio <1.0), and 18 gastric cancer tissues showed miR-126 expression with significant downregulation (relative expression ratio <0.5) (Fig. 1A). Furthermore, analysis of miR-126 expression in 4 gastric cancer cell lines (GES-1, BGC-823, SGC-7901 and MKN-45) revealed that miR-126 was downregulated in the BGC-823 and SGC-7901 cell lines (Fig. 1B). These data suggest that miR-126 might act as a tumor suppressor in gastric cancer.

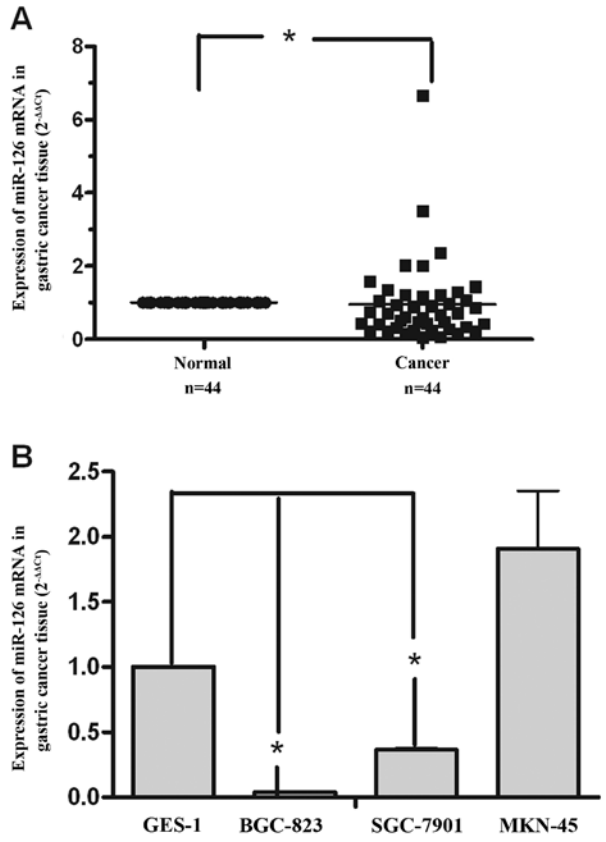

Figure 1. The expression of miR-126 in gastric cancer and gastric cancer cells. (A) The miR-126 relative expression was analyzed by qRT-PCR between gastric cancer tissue and matched normal tissue in 44 patients. miR-126 expression was normalized to U6 expression. (B) The miR-126 expression was analyzed by qRT-PCR between normal gastric cells (GES-1 cells) and gastric cancer cells (BGC-823, SGC-7901 and MKN-45 cells), and miR-126 expression was normalized to U6 expression. Data represent the means of three replicates, where relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (*P<0.05).

miR-126 expression suppresses gastric cancer cell proliferation through inducing apoptosis in vitro. To explore the tumor suppressor role of miR-126 in gastric cancer, SGC-7901 cells were transfected with miR-126 or miR-control. qRT-PCR was performed to examine the expression levels of miR-126 after transfection of the miR-126 or miR-control. As depicted in Fig. 2A, an approximate 10-fold increase in the expression of miR-126 was observed in SGC-7901 cells that were transfected with miR-126 relative to the cells transfected with miR-control. To study the role of miR-126 in SGC-7901 cell proliferation, an MTT assay was used. The results showed that transient overexpression of miR-126 inhibited the proliferation of SGC-7901 cells at 24, 48 and 72 h after transfection (Fig. 2B). However, the cell cycle of SGC-7901 cells was not obviously affected by miR-126 at 24 h (Fig. 2C). In order to further examine the inhibitory role of miR-126 in SGC-7901 cells, a colony formation assay was conducted after similar transient transfection. miR-126-transfected cells displayed fewer colonies compared with the control (Fig. 2D). To further investigate the mechanisms by which miR-126 inhibits the growth of SGC-7901 cells, we tested the apoptosis of SGC-7901 cells induced by miR-126. We found that cells in early apoptosis and late apoptosis (at 24 h) were increased in the miR-126 group. Thus, the inhibitory effect of miR-126 on cell growth may occur through the induction of apoptosis in SGC-7901 cells (Fig. 2E). Altogether, these results indicate that miR-126 was able to inhibit the proliferation of SGC-7901 cells *in vitro*.

miR-126 expression suppresses gastric cancer cell proliferation in vivo. We further confirmed the growth inhibitory role of miR-126 *in vivo* in a xenograft model. First, we tested the effects of miR-126 on tumor growth in the *in vivo* xenograft model. miR-126 and miR-control-transfected SGC-7901 cells were injected subcutaneously into either posterior flank of nude mice, and xenograft growth was observed for 22 days. As shown in Fig. 3A and C, the tumors from mice that were injected with miR-126 were significantly smaller than those in control mice on the 13th day after the first injection. On the 22nd day, the tumors were tested using IVIS Spectrum before the mice were euthanized (Fig. 3D), and the tumors were removed and weighed (Fig. 3B). The tumor tissue was fixed in 4% paraformaldehyde, embedded in paraffin and stained with H&E (Fig. 3E). Although, both tissues of miR-126 and control grew tumors, the degree of malignancy in the miR-126 groups was lower than in the miR-control group. These data indicate that miR-126 could inhibit tumorigenicity of SGC-7901 cells in the nude mouse xenograft model.

Inhibition of miR-126 moderately promotes growth of SGC-7901 cells in vitro. The preceding observations demonstrated that miR-126 expression inhibited the growth and increased apoptosis of SGC-7901 cells. We next asked whether downregulated expression miR-126 promotes the growth of SGC-7901 cells. To accomplish this, we transiently inhibited miR-126 in SGC-7901 cells with antisense oligonucleotides. qRT-PCR was performed to examine the expression levels of miR-126 after transfection of anti-miR-126 and control. As shown in Fig. 4A, the expression of miR-126 decreased by ~12% compared with the control. The MTT assay results showed that anti-miR-126 slightly improved the ability of growth at 48 and 72 h (Fig. 4B), but the cell cycle and colony forming ability were unaffected by the inhibitor (Fig. 4C and D). Interestingly, inhibitor-induced apoptosis of SGC-7901 cells was higher than in the control (Fig. 4E), but there was no significant difference, and the percent of apoptosis was very low in both groups. This phenomenon is worth exploring further in the future.

miR-126 regulates its target genes Crk and PI3K2 in SGC-7901 cells. Feng *et al* reported that miR-126 functions as a tumor suppressor in gastric cancer, with *Crk* as a direct target (12). We wanted to know whether any other target genes of miR-126 might also contribute to the function of miR-126 in gastric cancer. Crk and PI3KR2 protein (p85) levels, targets of miR-126, were assayed in miR-126-expressing SGC-7901 cells. As shown in Fig. 5, miR-126 repressed levels of PI3KR2 and Crk protein expression. P13KR2 was upregulated, while the expression of Crk was not affected by the inhibitor of miR-126. These data indicated that miR-126 directly regulates *Crk* and *PI3K2* in SGC-7901 cells. In addition, in overexpressing miR-126 SGC-7901 cells, the Bcl-2 and Bax protein levels were changed; Bax was upregulated and Bcl-2 was downregulated in the miR-126 group. However, protein

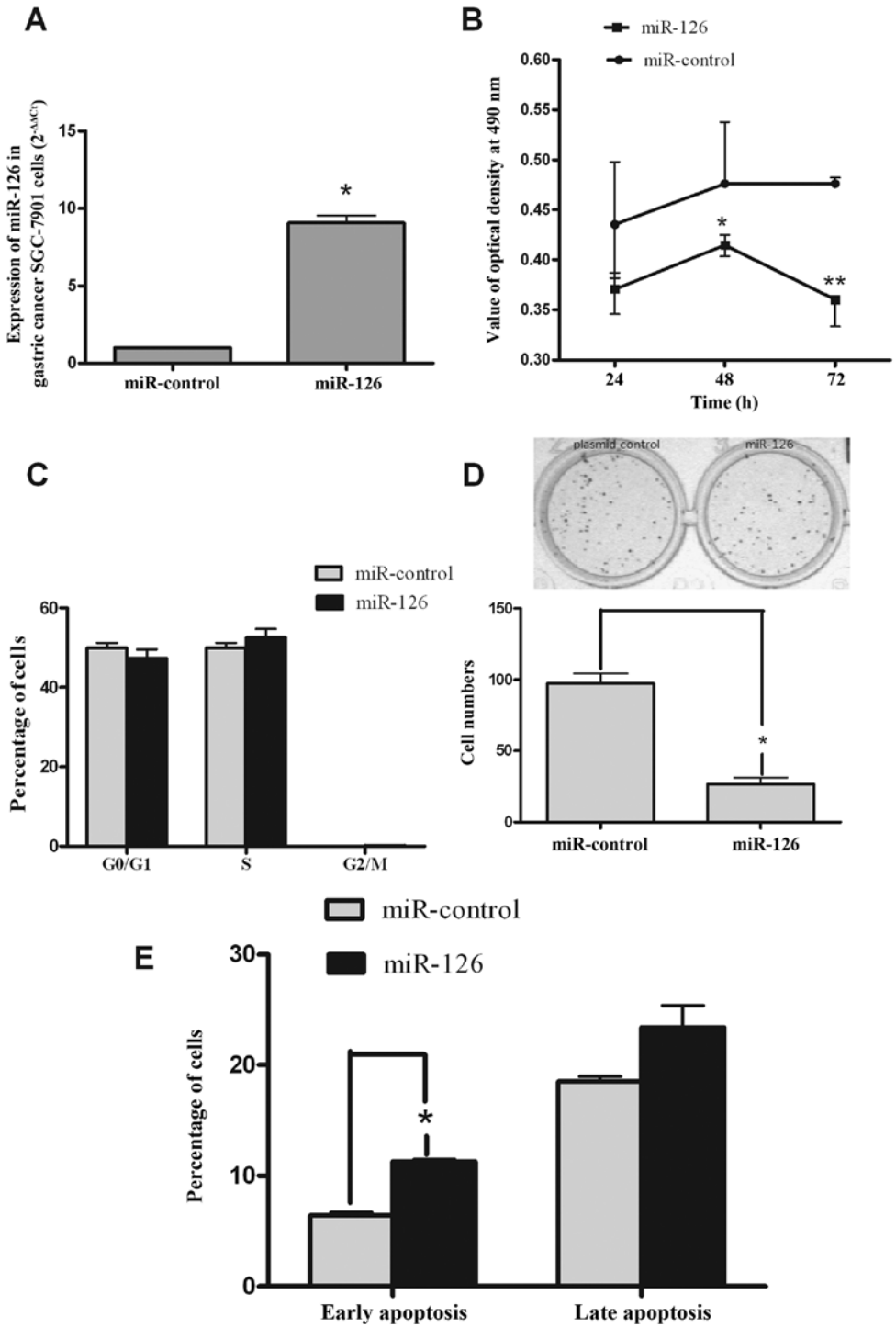

Figure 2. Effect of miR-126 on gastric cancer SGC-7901 cell progression. (A) qRT-PCR analysis of miR-126 in SGC-7901 cells transfected with miR-126 or miR-control, respectively. (B) Proliferation of SGC-7901 cells was determined by an MTT assay, after transfection with miR-126 and miR-control at 24, 48 and 72 h, respectively. (C) Cell cycle was tested by flow cytometry at 24 h after transfection with miR-126 or miR-control, respectively. The cells were stained with propidium iodide. (D) Results of tablet colony formation after transfection with miR-126 or miR-control, respectively. The upper portion of the image shows tablet colony formation after Giemsa staining, while the lower portion shows the histogram of tablet colony formation. (E) Cell apoptosis was analyzed by flow cytometric analysis at 24 h after transfection with miR-126 or miR-control, respectively. The cells were stained with 7-AAD/PI ($^{*}P<0.05$, $^{**}P<0.01$).

levels of Bcl-2 and Bax were unchanged by the inhibitor of miR-126. These data were compatible with the results of the apoptosis analysis.

PLK2 is a target gene of miR-126 in SGC-7901 cells. Through the miRBase (http://www.mirbase.org) and TargetScan (http://www.targetscan.org/) databases, we found matching bases between the 3'UTR of *PLK2* and miR-126 (top of Fig. 6A). In order to determine whether miR-126 regulates *PLK2* in gastric cancer SGC-7901 cells, we performed a luciferase assay to evaluate the relationship between miR-126 and *PLK2*. SGC-7901 cells were co-transfected with miR-126

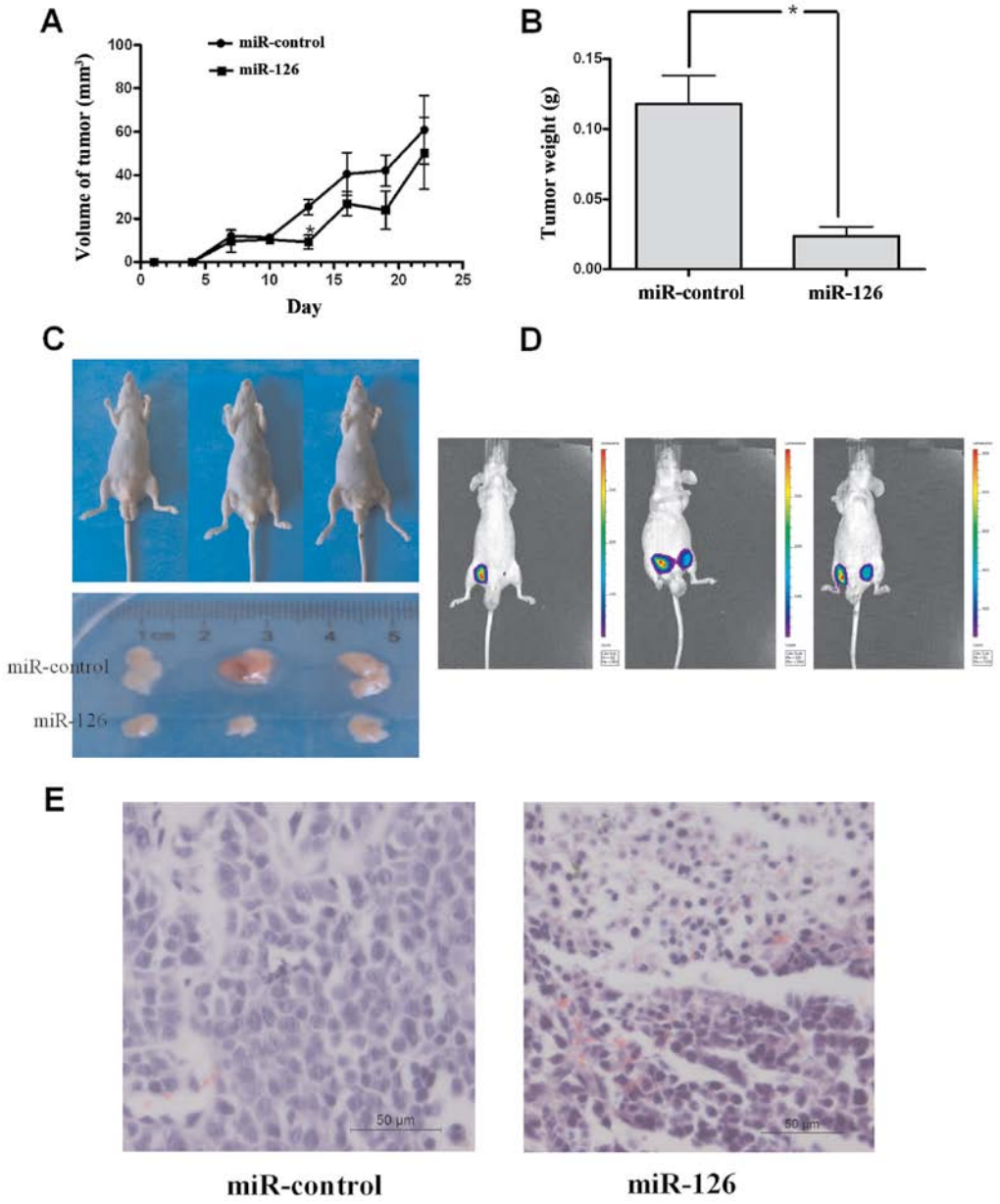

Figure 3. Effect of miR-126 on gastric cancer progression *in vivo*. (A) Nude mice bearing established SGC-7901 tumors. Mean tumor volumes ± SD are shown (n=6 per group). (B) Tumor weight. (C) Photographs of nude mice and tumor tissues. Upper portion of the image shows the tumors derived from miR-126 (right flank) and miR-control (left flank) stably transfected SGC-7901 cells in nude mice. The lower panel shows the tumor tissues retrieved post-mortem (upper line is the miR-control group, lower line is the miR-126 group). (D) The images of tumors derived from miR-126 (right flank) and miR-control (left flank) stably transfected SGC-7901 cells in nude mice, which were evaluated using small animal imaging analysis. (E) H&E staining of tumor tissue 22 days after injection (*P<0.05).

together with pmir-GOL-PLK2 or pmir-GOL-mut-PLK2. miR-126 strongly reduced the expression of *PLK2* (27.73%), but showed almost no effect on the expression of pmir-GOL-mut-PLK2 (81.81%) (bottom of Fig. 6A). As shown in Fig. 6B and C, qRT-PCR revealed that *PLK2* mRNA was decreased in SGC-7901 cells overexpressing miR-126, while it was upregulated in cells treated with anti-miR-126. These results were also confirmed in SGC-7901 cells transfected with miR-126, which showed a lower amount of PLK2 protein when compared to miR-control-treated cells (right of Fig. 6D). The PLK2 protein level did not change significantly between the control and anti-miR-126 groups (Fig. 6D, left). Together, these data indicate that *PLK2* is regulated by miR-126 in SGC-7901 cells.

Discussion

Gastric cancer is the fourth most common human malignant disease and the second most frequent cause of cancer-related death worldwide (29). Improvement of treatment has resulted in good long-term survival for patients with gastric cancer (30). miRNAs, as a new class of small non-coding RNAs, play an important role in cancer development, and have become a new target for cancer therapy. In our previous miRNA microarray study, we found that miR-126 was downregulated in clinical gastric cancer samples. In the present study, we first examined the expression of miR-126 in gastric cancer tissues and cell lines, and confirmed the previous result that miR-126

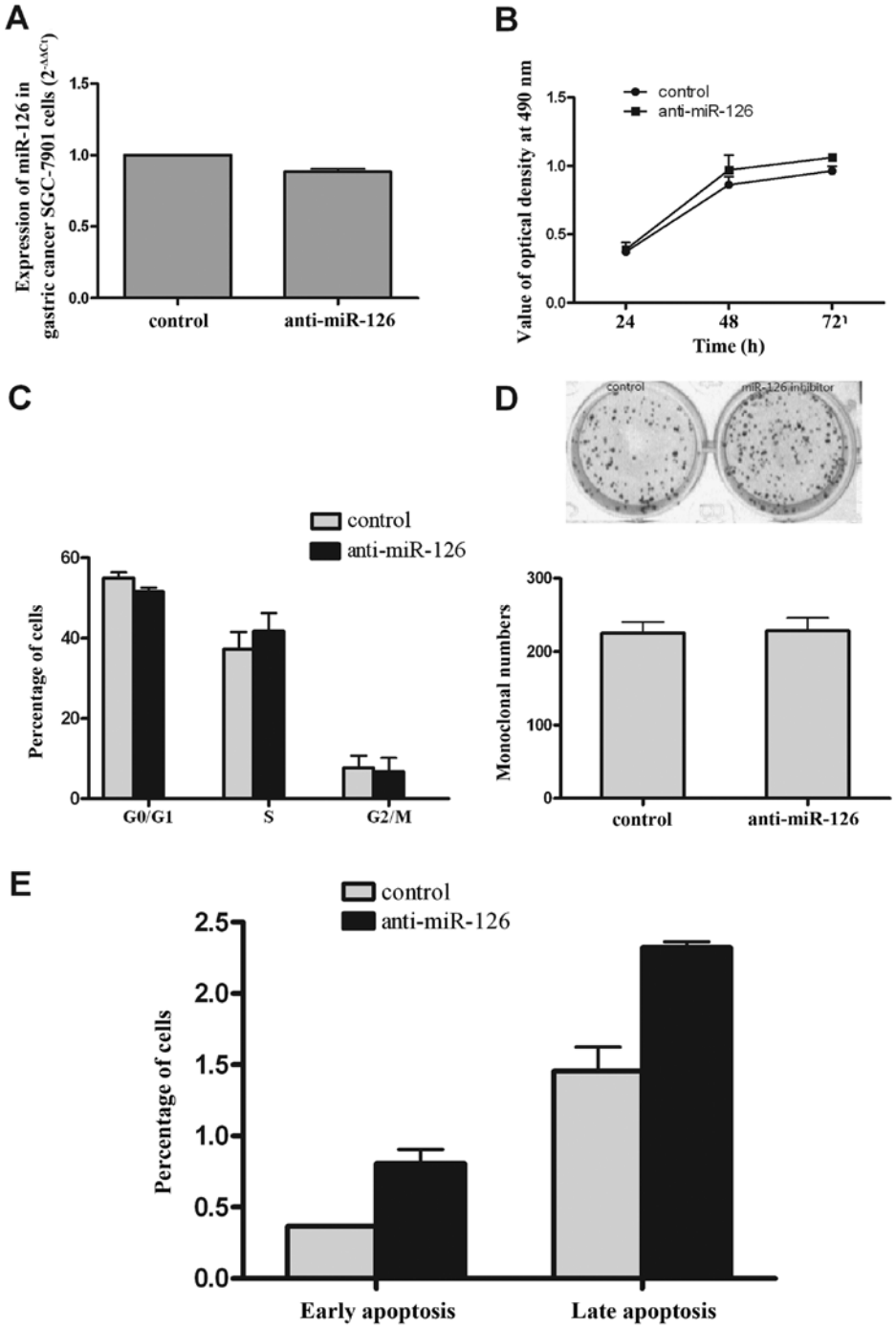

Figure 4. Effect of anti-miR-126 on gastric cancer progression in SGC-7901 cells. (A) qRT-PCR analysis of miR-126 in SGC-7901 cells transfected with anti-miR-126 or control, respectively. (B) Proliferation of SGC-7901 cells determined by an MTT assay after transfection with anti-miR-126 and control at 24, 48 and 72 h, respectively. (C) Cell cycle was tested by flow cytometry at 24 h after transfection with anti-miR-126 or control, respectively. Cells were stained with propidium iodide. (D) Results of tablet colony formation after transfection with anti-miR-126 or control, respectively. Upper portion, the image of tablet colony formation after Giemsa staining. Lower portion, a histogram of tablet colony formation. (E) Cell apoptosis was analyzed by flow cytometric analysis at 24 h after transfection with anti-miR-126 or control, respectively. Cells were stained by 7-AAD/PI ($^{*}P<0.05$, $^{**}P<0.01$).

was downregulated in gastric cancer. Next, we confirmed that miR-126 regulates *PLK2* in SGC-7901 cells. Moreover, we observed a decrease of PLK2 at both the mRNA and protein levels after transfection with miR-126. These data suggest that miR-126 is able to directly regulate *PLK2* in gastric cancer SGC-7901 cells.

It is well known that miRNA mainly performs its regulatory functions through its targets (31). miRNAs also contribute to maintaining the balance among genes that regulate the cell fate, as well as their deregulation, which is a frequent hallmark in a variety of human malignancies. Although the identification and validation of miRNA targets have greatly progressed in the last few years, little is known about the specific cellular and molecular pathways and mechanism involved in these effects (3). Cancer occurrence is tightly linked to a series of genes encoding oncogenic and

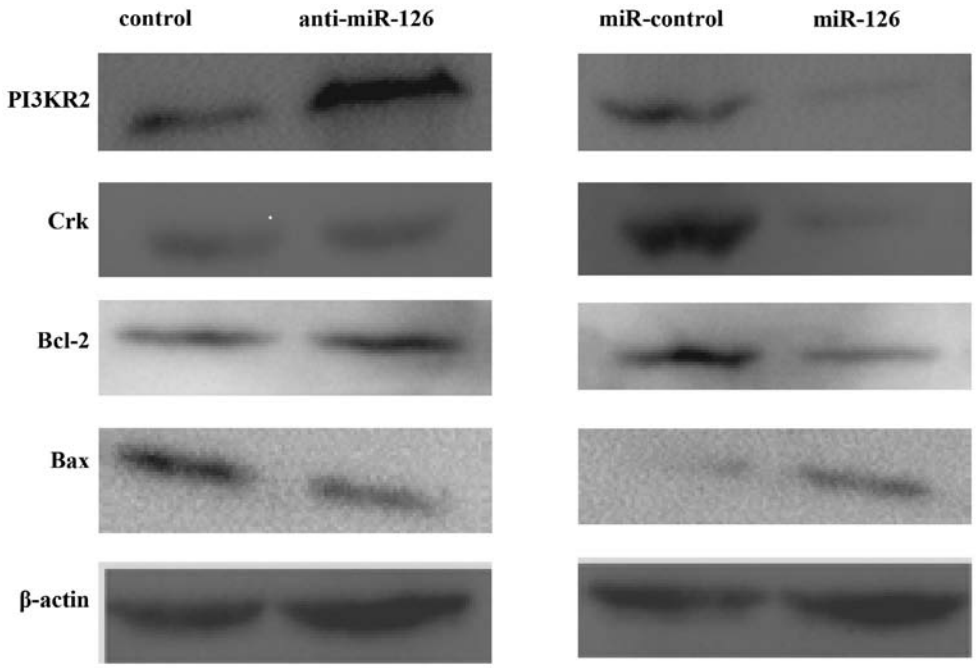

Figure 5. Western blot results of miR-126 targeting Crk and PI3KR2, and apoptosis-related proteins Bcl-2 and Bax in SGC-7901 cells after transfection of miR-126, miR-control, anti-miR-126 and control, respectively. β-actin was used as an internal control.

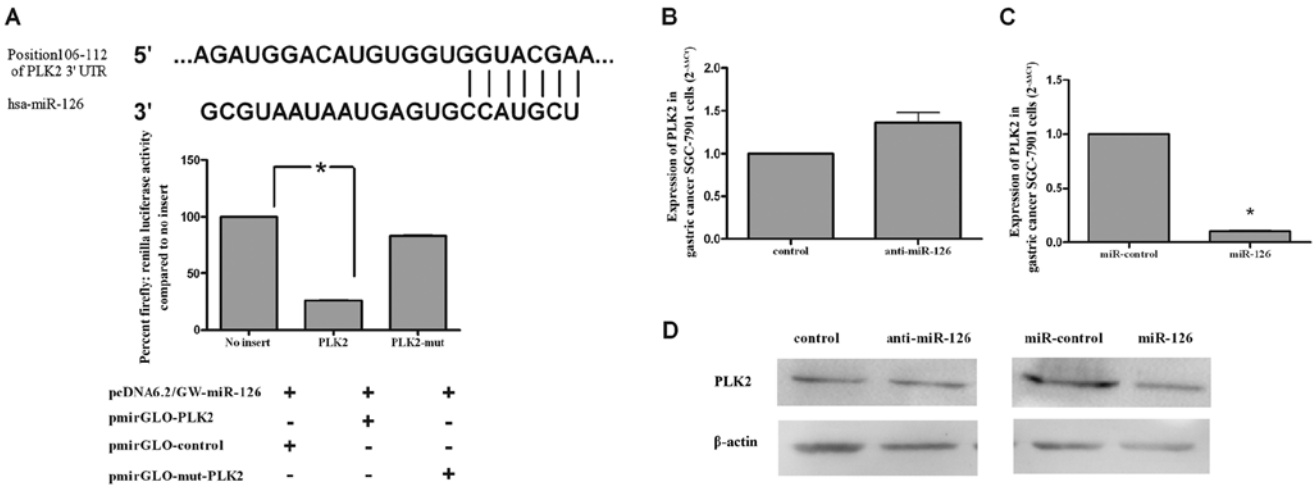

Figure 6. miR-126 targeted *PLK2* in SGC-7901 cells. (A) miR-126 targeted *PLK2* in SGC-7901 cells, as determined using dual luciferase assay. Upper portion shows the miRBase results of predicted miR-126 target sites in the PLK2 3'-UTR. Lower portion shows results of the dual luciferase assay with cotransfection of miR-126 and wild-type PLK2-UTR, PLK2-UTR-mutation and no-PLK2-UTR insert, respectively, in SGC-7901 cells. (B) qRT-PCR analysis of *PLK2* in SGC-7901 cells transfected with control or anti-miR-126. (C) qRT-PCR analysis of *PLK2* in SGC-7901 cells transfected with miR-control or miR-126. (D) Western blot results of PLK2 in SGC-7901 cells after transfection of control, anti-miR-126, miRNA-control and miR-126, respectively. β-actin was used as an internal control ($^*P<0.05$).

tumor-suppressor proteins. In general, oncogenic miRNAs downregulate the tumor-suppressor proteins, while tumor-suppressor miRNAs downregulate oncogenic proteins. It was reported that miR-126 acts as a tumor suppressor in gastric cancer by regulating the oncogene *Crk* (12). However, Otsubo *et al* reported that miR-126 functions as an oncogene in gastric cancer by downregulating the expression of *SOX2*, which is a tumor suppressor (13). Thus, it appeared that the function of miR-126 was mainly determined by one target gene in gastric cancer. Furthermore, previous reports suggested that *PLK2* is a target gene of miR-126, which is also a tumor suppressor. It has been reported that miR-126 inhibits apoptosis of AML cells and enhances the colony-forming ability of mouse bone marrow progenitor cells through targeting *PLK2* (27). Therefore, if miR-126 regulates *PLK2* in SGC-7901 cells, then it must function as an oncogene. However, we showed that the role of miR-126 remains antitumorigenic in SGC-7901 cells. Thus, we inferred that miR-126 must regulate other oncogenes in SGC-7901 cells, and the role of these oncogenes might be more important than that of *PLK2*.

Individual miRNAs each have several hundred different target genes, including oncogenes and anti-oncogenes. The function of a miRNA is not dependent on a single target gene, but rather on the competition or balance among its target genes in specific types of cancer (32). Therefore, there may be a balanced relationship among the oncogenes or tumor suppressor target genes of miR-126 in SGC-7901 cells. *PI3KR2* is a target gene of miR-126 in endothelial cells, and PI3KR2 protein is one of the regulatory subunits of the class IA PI3K enzyme that is activated by tyrosine kinase receptors. PI3KR2 is involved in the PI3K-Akt-mediated survivin signaling pathway (33-35). Crk protein has crucial functions in the signaling pathways regulating cell adhesion, proliferation and migration. Feng *et al* found that miR-126 functions as a tumor suppressor in gastric cancer by targeting *Crk* (12). Thus, we proceeded to measure the protein level expression of PI3KR2 and Crk. Western blot results showed reduced expression of PI3KR2 and Crk in SGC-7901 cells through overexpression of miR-126. We found that miR-126 function was accomplished by regulation of *PI3KR2*, *Crk* and *PLK2* simultaneously. However, the detailed pathway involved in PI3KR2, Crk and PLK2 inhibition by miR-126 in gastric cancer needs to be confirmed in future studies.

Collectively, our study indicates that the ability of miR-126 to inhibit growth of SGC-7901 cells is attributable to the balancing effect through its capacity to inhibit *PLK2*, *PI3KR2* and *Crk* expression. This provides new insight for studies of miRNA function in the future. Of particular note, the function of miRNA is a result of the combined action of many target genes, and not simply a result of a particular gene, as was previously believed.

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