Original Article
miR-489 acts as a tumor suppressor in human gastric cancer by targeting PROX1

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Abstract: Dysregulation of microRNAs (miRNAs) are linked to tumorigenesis and tumor progression. In this study, we examined the expression of miR-489 in gastric cancer tissues and cells. Loss- and gain-of-function experiments were done to determine the roles of miR-489 in gastric cancer cell proliferation and invasion. Bioinformatic prediction, luciferase reporter assays, and Western blot analysis were employed to identify the target gene(s) of miR-489. We found that miR-489 was significantly ($P < 0.05$) downregulated in human gastric cancer tissues and cell lines, compared to their non-malignant counterparts. Enforced expression of miR-489 significantly suppressed gastric cancer cell proliferation and invasion, while miR-489 knockdown enhanced the aggressive behaviors of gastric cancer cells. Prospero homeobox 1 (PROX1) was identified to be a direct target of miR-489. A significant negative correlation was seen between miR-489 and PROX1 protein expression in gastric cancer tissues ($r = -0.462$, $P = 0.023$). Silencing of PROX1 phenocopied the suppressive effects of miR-489 in gastric cancer cells. Rescue experiments demonstrated that overexpression of a miR-489-resistant form of PROX1 significantly prevented the reduction in cell proliferation and invasion induced by miR-489 overexpression. In vivo studies confirmed that miR-489 overexpression retarded the growth of xenograft tumors, which was accompanied by reduced PROX1 expression. Overall, these data provide evidence for the suppressive activity of miR-489 in gastric cancer, which is ascribed to targeting of PROX1. The miR-489/PROX1 axis may represent a potential therapeutic target for this disease.

Keywords: Gastric cancer, invasiveness, microRNA, target genes, tumorigenesis

Introduction
Gastric cancer is one of the most common malignancies and a leading cause of cancer-related mortality worldwide [1]. Despite advancements in diagnostic and therapeutic approaches, the 5-year survival rate for patients with gastric cancer, especially at advanced stages of the disease, remains depressed [2, 3]. Understanding the molecular mechanisms for the regulation of gastric cancer development and progression is of significance to develop an effective therapeutic strategy for this disease.

microRNAs (miRNAs) are small endogenous noncoding RNAs that typically repress target gene expression, via mRNA degradation or translational inhibition [4, 5]. miRNAs are implicated in a variety of biological processes including tumorigenesis, invasion, and metastasis [6]. Dysregulation of miRNAs is frequently detected in gastric cancer. For example, reduced expression of miR-203 [7] and miR-198 [8] is associated with aggressive parameters and poor prognosis in gastric cancer. miR-543 expression was found to be positively correlated with tumor size, clinical grade, TNM stage, and lymph node metastasis in gastric cancer [9]. It has been documented that miR-489 is downregulated in breast cancer and restoration of its expression decreases breast cancer cell growth and tumorigenesis [10]. Similarly, overexpression of miR-489 was found to inhibit cell growth in hypopharyngeal squamous cell carcinoma cells [11]. In non-small cell lung cancer, downregulation of miR-489 contributes to the invasiveness of cancer cells [12]. However, loss of miR-489 seems not to be a ubiquitous feature of cancer, as in some types of cancers such as oral cancer, miR-489 is significantly overexpressed [13]. These studies suggest an important role for miR-489 in tumor biology.
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To our best knowledge, there have been few studies on the expression and biological functions of miR-489 in gastric cancer. In this study, we examined the expression levels of miR-489 in gastric cancer tissues and cell lines and investigated its roles in the regulation of aggressive behaviors of gastric cancer cells. The direct target gene(s) involved in the action of miR-489 was identified.

Materials and methods

Tissues and cell lines

Paired human gastric cancer and adjacent non-tumorous gastric tissues were collected from 36 patients with gastric cancer who underwent radical resection in the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) between April 2014 and December 2014. All gastric cancer specimens were pathologically confirmed. Patients who received any anticancer treatments before surgery were excluded from this study. Written information consent was obtained from each patient. This study was approved by the Institutional Ethics Committee of Zhengzhou University.

Human gastric cancer cell lines (MGC-803, SGC-7901, AGS, MKN45, and MKN28) and immortalized human gastric epithelial GES-1 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. Cells were maintain in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from cells and tissues using TRizol reagent (Invitrogen). For miR-489 detection, total RNA (1 μg) was reverse-transcribed to cDNA using a miRNA-specific stem-loop primer (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on the ABI 7900 HT Real-Time PCR System using a TaqMan MicroRNA Assay kit (Applied Biosystems). Cycling reactions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The level of miR-489 relative to U6 (used as an internal control) was calculated using the 2-ΔΔCT method [14].

For measurement of Prospero homeobox 1 (PROX1) mRNA levels, cDNA was synthesized by reverse transcription using an oligo (dT) 14 primer and AMV reverse transcriptase (TaKaRa, Dalian, China). The PCR primers were as follows: PROX1 sense, 5’-CAGATGGAGAAGTACGCAC-3’ and PROX1 antisense, 5’-CTACTCATGAAGCAGCTTG-3’; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5’-GATTGTGACATCAATGAC-3’ and GAPDH antisense, 5’-TTGATTTTGGAGGGATCTCG-3’. The relative PROX1 mRNA expression was determined after normalization to GAPDH.

In situ hybridization (ISH) analysis

ISH analysis was performed with digoxigenin-labeled locked nucleic acid (LNA)-modified probes specific for hsa-miR-489 (Exiqon, Vedbaek, Denmark), as previously described [15]. Briefly, tissue sections were deparaffinized, dehydrated, and treated with 15 μg/ml of Proteinase K (Exiqon) at 37°C for 30 min. The sections were hybridized with 10 nM miR-489 probes at 57°C overnight. After washing, anti-digoxigenin-alkaline phosphatase conjugate (Roche Applied Science, Indianapolis, IN, USA) was applied. Hybridization signals were finally visualized with 4-nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indoly nitro phosphate substrate (Roche Applied Science). The sections were counterstained with nuclear fast red (Roche Applied Science).

Cell transfection

miR-489 mimic, negative control miRNA, anti-miR-489 inhibitor, and control inhibitor were purchased from GenePharma (Shanghai, China). PROX1-targeting siRNA (si-PROX1) and control siRNA (c-siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human miR-489 precursor was amplified by PCR using genomic DNA as a template and cloned into pcDNA3.1(+) vector (Invitrogen). The full length human PROX1 open reading frame lacking 3’-UTR was amplified by PCR and inserted into pcDNA3.1(+) vector. Inserts were confirmed by sequencing.

For transfection studies, cells were plated onto 24- or 12-well plates and allowed to grow to ~70% confluence. Cells were transiently trans-
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infected with miR-489 mimics, anti-miR-489, si-PROX1, or their negative controls (50 nM for each) using Fugene (Promega). In some experiments, cells were co-transfected with 50 nM miR-489 mimic and 0.5 μg pcDNA3.1-PROX1 or vector using Fugene. At 24 h after transfection, cells were collected and subjected to gene expression, cell viability and invasion analysis. To generate a stable miR-489-expressing plasmid, AGS cells were transfected with pcDNA3.1-miR-489 and selected for 2 weeks in the presence of G418 (500 μg/ml; Sigma, St. Louis, MO, USA).

**Cell proliferation assay**

Cells were plated into 96-well plates (4 × 10^3 cells/well) and cultured for 24-72 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli- um bromide (MTT) solution (0.5 mg/ml; Sigma) was added to each well. After incubation for additional 4 h at 37°C, dimethyl sulfoxide was added to dissolve the crystals. Absorbance of each well was measured at 570 nm with a microplate reader.

**Cell invasion assay**

Cell invasion was determined using a transwell chamber pre-coated with Matrigel (Invitrogen). Cells suspended in serum-free medium were added to the upper chamber. The lower chamber was filled with fresh DMEM containing 10% FBS. After incubation for 24 h at 37°C, cells on the upper surface of the membrane were removed. Cells invaded through the Matrigel were fixed and stained with 0.1% crystal violet solution (Sigma). The cells were counted from 5 random microscopic fields for each well.

**Luciferase activity assay**

A PROX1 3’-UTR fragment was obtained by PCR and cloned into pmirGLO vector (Promega, Madison, WI, USA). A mutated form of the PROX1 3’-UTR, where the predicted binding site for miR-489 was disrupted, was generated using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). For luciferase reporter assays, HEK293 cells were transfected with 100 ng pmirGLO constructs, together with 100 ng pcDNA3.1-miR-489 or vector. After 24 h, cells were lysed and tested for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assays (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

**Western blot analysis**

Cells and tissue samples were lysed in radioimmunoprecipitation (RIPA) lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein concentration was determined using a BCA Protein Assay Kit (Beyotime, Nanjing, China). Protein samples were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin and probed at 4°C overnight with rabbit anti-PROX1 polyclonal antibody (ab191019, Abcam, Cambridge, MA, USA; 1:500 dilution) or anti-β-actin polyclonal antibody (#4967, Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution). Membranes were incubated with horseradish peroxidase-conjugated goat-anti rabbit IgG (Santa Cruz Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore). Quantitation of band intensity was performed by densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA). The relative PROX1 protein level was determined after normalization against that of β-actin.

**In vivo tumorigenesis**

The experiments involving animals were performed in accordance with the institutional guidelines for animal care and were approved by the Zhengzhou University Committee for the Use and Care of Animals. Male BALB/c nude mice (4 weeks old and weighing about 20 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). miR-489-overexpressing AGS or control cells (5 × 10^6 per mouse, 4 mice per group) were subcutaneously injected into the right flanks of mice. Tumor size was measured every week using a caliper. At 28 days after cell injection, animals were sacrificed and xenograft tumors were excised and weighted. Parts of the tissues were used for Western blot analysis, and the remains were fixed in 4% paraformaldehyde and processed for Ki-67 immunohistochemical staining.

**Immunohistochemical analysis**

Tumor sections (4 μm) were subjected to deparaffinization and rehydration. After inactivation of endogenous peroxidase activity, sections were incubated with rabbit anti-Ki-67 polyclonal antibody (ab15580, Aabcam; 1:100 dilu-
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Figures

**Figure 1.** Downregulation of miR-489 in gastric cancer clinical specimens and cell lines. A. The miR-489 expression in 36 pairs of gastric cancer and corresponding non-cancerous gastric tissues was determined by qRT-PCR analysis. "*P < 0.05, Student's t test. B. Localization of miR-489 in gastric cancer and adjacent non-cancerous tissues detected by ISH. Specific miR-489 hybridization signals were readily detectable in the cytoplasm of gastric mucosal epithelial cells, whereas a weak miR-489 signal was observed in gastric cancer cells. Scale bar, 100 μm. C. Measurement of miR-489 abundance in gastric cancer cells and GES-1 non-malignant gastric epithelial cells. "*P < 0.05 vs. GES-1 cells, ANOVA followed by Tukey test.

Statistical analysis

Data are expressed as mean ± standard deviation and were analyzed with the Student’s t test or one-way analysis of variance (ANOVA), followed by the Tukey test. The relationship between miR-489 and PROX1 protein expression in gastric cancer tissues was determined with Pearson’s correlation analysis. $P < 0.05$ was considered statistically significant.

Results

**Downregulation of miR-489 in gastric cancer clinical specimens and cell lines**

We first examined the expression levels of miR-489 in 36 pairs of gastric cancer and corresponding non-cancerous gastric tissues. qRT-PCR analysis showed that the expression of miR-489 was significantly reduced in tumor samples compared with the non-malignant counterparts ($P < 0.05; \text{Figure 1A}$). ISH analysis showed that miR-489 was readily detected in the cytoplasm of gastric mucosal epithelial cells, whereas a weak miR-489 signal was observed in gastric cancer cells (Figure 1B). We further determined the level of miR-489 in a panel of gastric cancer cell lines. Compared to GES-1 gastric epithelial cells, all the 5 gastric cancer cell lines tested had a significantly lower level of miR-489 ($P < 0.05; \text{Figure 1C}$).

**miR-489 inhibits gastric cancer cell proliferation and invasion**

To explore the biological relevance of miR-489 downregulation in gastric cancer, loss- and gain-of-function experiments were done. Gastric cancer cells were transfected with miR-489 mimic, anti-miR-489, or their corresponding controls, and tested for cell proliferation and invasion. MTT assay showed that the delivery of miR-489 mimic significantly ($P < 0.05$) inhibited the proliferation of AGS cells, compared to control cells (Figure 2A). Moreover, overexpression of miR-489 resulted in a significant ($P < 0.05$) decrease in the invasiveness of gastric cancer cells, as determined by transwell invasion assays (Figure 2B). In contrast, knockdown of endogenous miR-489 significantly ($P < 0.05$) promoted the proliferation (Figure 2C) and invasion (Figure 2D) of SGC-7901 cells. Collectively, these observations demonstrate that miR-489 inhibits the malignant behaviors of gastric cancer cells in vitro.
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PROX1 is a direct target of miR-489

To determine the molecular mechanism underlying miR-489-mediated suppression of gastric cancer cell proliferation and invasion, in silico studies were conducted to search for its potential target genes using the TargetScan software (http://www.targetscan.org/). The PROX1 3’-UTR was found to contain a putative target sequence for miR-489 (Figure 3A). To test if miR-489 binds directly to the 3’-UTR of PROX1 mRNA, wild-type or mutant PROX1 3’-UTR were cloned into the pmirGLO vector and then co-transfected together with miR-489 mimic or control miRNA into HEK293 cells. As shown in Figure 3B, the relative luciferase activity of the wild-type PROX1 3’-UTR reporter was significantly (P < 0.05) decreased by miR-489 mimic. In contrast, miR-489 overexpression had no significant impact on the expression of the mutant 3’-UTR reporter gene. To further confirm that PROX1 is a direct target of miR-489, we examined the effect of miR-489 on endogenous PROX1 expression in gastric cancer cells. Western blot analysis demonstrated that the delivery of miR-489 significantly (P < 0.05) reduced the protein level of PROX1 in AGS cells (Figure 3C). Additionally, a significant negative correlation was found between miR-489 and PROX1 protein expression in gastric cancer tissues (r = -0.462, P = 0.023; Figure 3D). Altogether, these data indicate PROX1 as a direct target of miR-489 in gastric cancer cells.

The tumor-suppressive activity of miR-489 is mediated by downregulation of PROX1

To determine the roles of PROX1 in gastric cancer cell proliferation and invasion, loss-of-function studies using PROX1-targeting siRNA were performed. qRT-PCR analysis confirmed that transfection with si-PROX1 effectively knocked down PROX1 expression in AGS cells (Figure 4A). Consistent with the findings of transfection with miR-489 mimic, silencing of PROX1 impaired the proliferation and invasion capacities of AGS cells (Figure 4B and 4C). To check whether miR-489 inhibits the malignant behaviors of gastric cancer cells via targeting of PROX1, rescue experiments with a plasmid expressing PROX1 without its 3’-UTR were done. Co-transfection with the PROX1 construct resistant to miR-489 restored the expression of PROX1 in miR-489 mimic-transfected AGS cells (Figure 4D). Most interestingly, PROX1 overexpression significantly (P < 0.05) reversed miR-489-mediated suppres-
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Figure 3. PROX1 is a direct target of miR-489 in gastric cancer. A. Bioinformatic analysis identified a potential miR-489 target site in the 3'-UTR of PROX1. To test if miR-489 can bind to this predicted site, mutation of the target site was achieved as described in Materials and methods. B. HEK293 cells were co-transfected with wild-type or mutated PROX1 3'-UTR reporter constructs and miR-489 mimic or control miRNA (C-miRNA). Firefly luciferase activity was normalized to Renilla luciferase activity. *P < 0.05 vs. C-miRNA, Student’s t test. C. AGS cells were transfected with miR-489 mimic or C-miRNA, and the protein expression of PROX1 was measured. *P < 0.05 vs. C-miRNA, Student’s t test. D. Spearman correlation analysis showed negative correlation between PROX1 protein and miR-489 expression in 36 cases of gastric cancer specimens (r = -0.462, P = 0.023, Pearson’s correlation coefficient test).

Expression of proliferation and invasion of AGS cells (Figure 4E and 4F). These results suggest that PROX1 is a functional target of miR-489 in gastric cancer cells.

miR-489 inhibits tumorigenicity of AGS cells in vivo

Finally, we investigated the role of miR-489 in gastric cancer tumorigenesis in vivo. To this end, AGS cells were stably transfected with miR-489-expressing plasmid or empty vector and subcutaneously injected into nude mice. As shown in Figure 5A, miR-489 overexpression significantly (P < 0.05) inhibited the growth of AGS xenograft tumors, compared with control tumors. At the end of the experiment (4 weeks after cell injection), the average tumor weight in the miR-489 overexpression group was 47.5% lower than that in the control group (P < 0.05; Figure 5B). Immunohistochemical analysis showed that the percentage of Ki-67-positive tumor cells was reduced in miR-489-overexpressing tumors relative to that in control tumors (P < 0.05; Figure 5C). Additionally, Western blot analysis confirmed an inhibition of PROX1 expression in miR-489-overexpressing tumors (Figure 5D). These results collectively validate the growth-suppressive effects of miR-489 against gastric cancer in vivo.

Discussion

Gene-expression profiling studies revealed that a larger number of miRNAs are differentially expressed in human solid cancers including gastric cancer [16]. The dysregulation of specific miRNAs has been linked to the pathogenesis of tumorigenesis. It was reported that miR-
miR-489 is underexpressed in several types of cancers such as breast cancer [10] and non-small cell lung cancer [12]. Consistently, our data revealed that miR-489 was downregulated in...
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Accumulating evidence indicates that dysregulated miRNAs play an important role in the initiation and progression of cancer, acting as tumor suppressors or oncogenes [17]. Having identification of the downregulation of miR-489 in gastric cancer, we next explored its biological functions in this disease. Notably, ectopic expression of miR-489 was found to cause a significant decline in the proliferation and invasion of gastric cancer cells. In contrast, inhibition of miR-489 enhanced the proliferative and invasive capacities of SGC-790 gastric cancer cells. These results are consistent with observations in several other cancers including breast cancer [10], hypopharyngeal squamous cell carcinoma [11], and non-small cell lung cancer [12]. In vivo studies further confirmed that miR-489 overexpression significantly impaired xenograft tumor formation of AGS gastric cancer cells. These data highlight the tumor-suppressive role for miR-489 in human cancer progression.

miRNAs exert their functions by targeting specific mRNAs [18]. The gene PTPN11, which codes for a src homology 2-containing tyrosine phosphatase, was identified as a target gene of miR-489 [11]. miR-489-mediated downregulation of PTPN11 inhibited the proliferation of hypopharyngeal squamous cell carcinoma cells. It has been reported that the delivery of miR-489 circumvented cisplatin resistance in human ovarian cancer cells by targeting Akt3 [19]. Targeting SUZ12 has been shown to mediate the anti-invasive ability of miR-489 in non-small cell lung cancer [12]. In breast cancer, miR-489 suppresses cell proliferation and invasion by targeting gse1 coiled-coil protein [20]. In this study, we identified PROX1 as a novel direct target of miR-489. We found that miR-489 repressed PROX1 expression via interaction with the 3′-UTR of PROX1. Enforced expression of miR-489 significantly reduced the protein level of PROX1 in AGS gastric cancer cells. Moreover, there was a significant negative correlation between miR-489 and PROX1 protein expression in gastric cancer tissues. PROX1 is known as a critical modulator of malignant progression of cancers [21, 22]. It has been reported that PROX1 expression was significantly associated with lymph node metastasis, cancer stage, and poor survival in gastric cancer [23]. PROX1 overexp

Figure 5. miR-489 inhibits tumorigenicity of AGS cells in vivo. AGS cells stably transfected with miR-489-expressing plasmid or empty vector were subcutaneously injected into nude mice and tumor volumes were measured every week. A. Tumor growth curves from mice inoculated with miR-489-overexpressing and control AGS cells. B. Tumors were resected and weighed at 4 weeks after cell injection. Representative images of the xenograft tumors are shown in top panels. C. Immunohistochemical staining for Ki-67 in the tumors. Representative images are shown in left panels. Bar graphs show quantitative analysis of Ki-67-positive cells. Scale bars, 100 μm. D. Western blot analysis of PROX1 protein levels in all the 4 tumors from each group. Bar graphs show quantitative analysis of the Western blots. The PROX1 protein level was normalized against that of β-actin. *P < 0.05, Student’s t test.

gastric cancer relative to non-cancerous gastric tissues, suggesting its implication in gastric cancer development and progression.
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expression was found to stimulate tumor angiogenesis, lymphangiogenesis, proliferation, and invasion in colorectal cancer [23]. These studies, combined with our findings that miR-489 directly targeted PROX1 expression, suggest that miR-489 likely exerts its anticancer effects through inhibition of PROX1. In support of this hypothesis, our data showed that silencing of PROX1 inhibited the proliferation and invasion of AGS cells and that overexpression of PROX1 reversed the suppressive activity of miR-489 in gastric cancer cell proliferation and invasion. In AGS xenograft tumors, miR-489 overexpression led to a significant decline in the percentage of Ki-67-positive proliferating tumor cells, which was accompanied by reduced expression of PROX1. Collectively, miR-489 inhibits the aggressive phenotypes of gastric cancer cells largely through targeting of RPOX1.

In summary, miR-489 is downregulated in gastric cancer and this miRNA has the ability to suppress the aggressive behaviors of gastric cancer cells in vitro and in vivo by targeting PROX1. These results provide novel insight into the regulation of gastric cancer tumorigenesis and progression by miRNAs. Restoration of miR-489 may have therapeutic potential against gastric cancer.

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Disclosure of conflict of interest

None.

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