

# The lncRNA-MALAT1/miR-126-5p Axis Promotes Growth and Metastasis of Gastric Cancer through Regulation of VEGFA

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## Research Article

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# Abstract

**Background:** It has been reported that reduction of miR-126 can promote the progression of gastric cancer (GC). However, the regulation of miR-126 in GC is still unclear. This study aims to explore the correlation between lncRNA MALAT1 and miR-126 in gastric cancer and disclose the underlying mechanisms.

**Methods:** We analyzed the correlation of MALAT1 levels and clinical features by analysis of bioinformatic data and human samples. Then we down-regulate the expression of MALAT1 in AGS cells and examined the characteristics of cell proliferation, cycle, apoptosis, migration, invasion, and the effect on miR-126 as well as VEGFA and signaling pathway. In addition, we demonstrated the role of MALAT1/miR-126 axis in GC with dual-luciferase reporter gene assay and treatment of miR-126 inhibitor.

**Results:** The expression of MALAT1 was higher in cancer tissues than para-cancer tissues. In addition, high MALAT1 level suggested greater malignancy and poorer prognosis. Down-regulating the expression of MALAT1 in AGS cells inhibited cell proliferation, migration, and invasion by targeting VEGFA, which is consistent with up-regulation of miR-126. According to dual-luciferase reporter gene assay and treatment of miR-126 inhibitor, we demonstrated that MALAT1 down-regulated miR-126 in GC, which leads to the up-regulation of VEGFA and activation of mTOR signaling pathway.

**Conclusions:** MALAT1/miR-126 axis promotes growth and metastasis of gastric cancer through regulation of VEGFA via mTOR signaling pathway.

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## Introduction

Gastric cancer (GC) is the most common malignant tumors in the digestive system, representing one of the dominant causes of cancer-associated deaths worldwide. Despite the advanced patient management and aggressive surgical techniques, there has been unsatisfactory improvement in the 5-year overall survival rate (1). Moreover, the molecular mechanisms underlying the progress of gastric cancer is still poorly understood. Further investigation is therefore required to elucidate the mechanisms underlying the development of GC and to identify novel therapeutic targets.

Long non-coding RNAs (lncRNAs) were served as critical mediators in various biological processes, including immune responses, angiogenesis, cell proliferation, apoptosis, autophagy, cell migration and invasion (2–4). A variety of studies have demonstrated that lncRNAs present critical roles in the development of various types of cancer, such as breast cancer, colorectal cancer, hepatocellular carcinoma (2–7). In addition, a variety of previous studies have also reported the abnormal expression and functions of lncRNAs in cancers (5, 6).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a kind of lncRNA, which is closely associated with lung cancer progression and poor clinical outcomes (8). The upregulation of MALAT1 level has been discovered in lung cancer, pancreatic cancer, bladder cancer (9–11). Currently, Xia et al. reported that MALAT1 could be served as a diagnostic marker for GC metastasis (12). In addition, it has been reported that lncRNA MALAT1 can up-regulate VEGFA and promote angiogenesis (13). However, the mechanism underlying the promoting function of MALAT1 in GC progression is still unclear.

MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that regulated gene expression by translational repression or mRNA degradation (14). microRNA-126-5p (miR-126-5p), as an endothelial-specific microRNA essential for governing vascular integrity and angiogenesis, participates in a wide range of biological function expression (15). It has been demonstrated that miR-126-5p was down-regulated in GC, and ectopic expression of miR-126-5p could inhibit cell proliferation and metastasis (16). In addition, Chen et al. illustrated that reduced miR-126-5p expression facilitates angiogenesis of GC through its regulation on VEGFA, which may share the same target gene with MALAT1 (15).

In the present study, the effect of MALAT1 on GC and the correlation between MALAT1 and miR-126 were investigated using *in situ* human GC tissue, *in vitro* GC cell lines. We identified the up-regulated expression of MALAT1 in GC tissue compared to the normal gastric tissue as well as an negative correlation between MALAT1 and miR-126-5p by targeting VEGFA and its downstream signaling molecules in AGS cell line. In addition, our present evidences indicated that MALAT1 was served as the competing endogenous RNA (ceRNA) with miR-126-5p.

## Materials And Methods

### Patients and specimens

A total of 7 benign gastric tissue, 29 poor-differentiated and 48 well-differentiated cancerous tissue were collected under surgical resection in the first affiliated hospital of Jinan university during 2015 Jan to 2019 Nov. All of the samples were saved in liquid nitrogen until further analysis. The whole patients didn't received any chemotherapy or radiotherapy before. This research was permitted by Ethics Committee of the first affiliated hospital of Jinan university.

### Bioinformatic analysis

The website of Gene Expression Profiling Interactive Analysis (<http://gepia.cancer-pku.cn>) was applied to inquire and analyze the expression differences of MALA1 between gastric cancer tissues (n = 408) and para-cancer tissue (n = 211). The gastric cancer data set from kaplan-meier Plotter online database (<http://kmplot.com/analysis/>) was used for online data, and the gastric cancer samples were divided into the high-expression group and the low-expression group for survival analysis.

## Cell culture

GES1, MKN45, MGC803, and AGS cells were purchased from the Model Culture Collection (ATCC, Manassas, VA, USA). All of the cells were cultured with RPMI1640 medium. All complete medium consisted of 10% FBS (fetal bovine serum), 1% penicillin (100 U/mL) and streptomycin (0.1 mg/mL). Total cells were cultivated at 37°C with 5% CO<sub>2</sub>. Reagents used in cell culture were bought from Gibco (Grand Island, NY, USA).

## Cell transfection

LncRNA MALAT1 shRNA and shRNA negative control (NC) were bought from GenePharma (Shanghai, China). AGS cells were harvested at confluence of 70–80% and 35nM shRNA were transfected into 10<sup>5</sup> cells in a 2 ml cell suspension. This experiment included untransfected cells as control group. The interval between transfection and the subsequent experiment was 24 h.

## Total RNA and qPCR

Total RNA was isolated from human gastric cancer tissue samples using TRIzol® Reagent Invitrogen (Invitrogen, USA). Then RNA samples were reversely transcribed by PrimeScript RT Master Mix kit (Takara, Dalian, China). The expression levels of mRNA was examined by SYBR Premix Ex Taq™ (Takara Bio, Otsu, Japan). GAPDH was considered as the internal reference gene.  $2^{-\Delta\Delta Cq}$  was used to analyze the relative expression of each genes. Primers were designed as follows: GAPDH: sense, 5'-AGAAGGCTGGGGCTCATTTG -3' and antisense, 5'-AGGGGCCATCCACAGTCTTC -3; MALAT1: sense, 5'-TGGGATGGTCTTAACAGGGA -3' and antisense, 5'-CCTGAAGGTGTTTCGTGCCAA -3; VEGFA: sense, 5'-CGCAGCTACTGCCATCAAT -3' and antisense, 5'-GTGAGGTTTGTATCCGCATAATCT -3; AKT: sense, 5'-CCCAGGAGGTTTTTGGGCTT -3' and antisense, 5'-AAGGTGCGTTCGATGACAGT -3; mTOR: sense, 5'-AACCTCCTCCCCTCCAATGA -3' and antisense, 5'-AATCCCATGAGGCCTTGGTG -3; ERK1: sense, 5'-TGAAAACACACACGTACATGG -3' and antisense, 5'-CCCTGCATTGATCCACCTGT -3; ERK2: sense, 5'-AGTTCTTGACCCCTGGTCCT -3' and antisense, 5'-CCTGGGACATCCCCAGAAAC -3.

## CCK 8 assay

Cell proliferation was analyzed by using CCK 8 assay kit (Beyotime, China) according to the manufacturer instructions. Treated cells were incubated in 96-well plates with or without inhibitor for indicated times and followed by the addition of 10 µL CCK 8 reagent to each well. Samples were further incubated at 37 °C for 1 h. OD values were measured at 450 nm. All of the experiments were performed in triplicate.

## Cell apoptosis and cell cycle assay

Cells were harvested after specific treatment, washed with ice-cold PBS for 3 times, and stained with annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech, Nanjing, China). Cell apoptosis was analyzed in a flow cytometer (BD Biosciences).

After trypsinization, AGS cells were washed with cold PBS. Cells were participated and cold ethanol (75%) was used to dissolve the cells, followed by incubation at 4 °C for 4 h. Cells were washed with cold PBS three times. After washing, cells were stained with BD Pharmingen™ PI/RNase for 30 min at 25 °C, followed by flow cytometer at different cell cycle phases (G1, S, and G2).

## Western blot

After trypsinization, AGS cells were harvested and then lysed in RIPA buffer (RIBO-BIO) with protease inhibitors (Roche, Switzerland). The concentration of protein was examined by using the BCA Protein Assay kit (Genstar, China). Protein samples were separated by 10% SDS-PAGE, and then transferred to PVDF membranes (Millipore, Boston, MA, USA). Next, the membranes were blocked with 5% milk for 1 h at room temperature and following incubation of primary antibodies (anti-VEGFA, anti-AKT, anti-p-AKT, anti-mTOR, anti-p-mTOR, anti-p-ERK, anti-ERK, and anti-GADPH) at 4 °C overnight. The PVDF membranes were incubated for another 1 h at room temperature in secondary antibody after washing three times with TBST. Strips were exposed with StarSignal Plus Chemiluminescent Assay Kit (Genstar, China).

## Transwell assay

Cells were cultured for 72 h, and then in serum-free medium for another 24 h. After detachment with 0.05% trypsin- EDTA the cells were resuspended in a serum-free medium. Upper insert was filled with 100 µl of the cell suspension while reservoir chamber was filled with 600 µl of culture medium. Matrigel invasion assays were carried out in modified Boyden chambers with filter inserts with 8-µm pores in 24-well plates (Corning, NY, USA). The surfaces of the filters were coated with 50mg/L ice-cold Matrigel (Matrigel basement membrane matrix, BD Bioscience, NJ, USA).

Migration or invasion of cells was monitored at 3, 6, and 12 h at 37 °C in 5% CO<sub>2</sub>. Crystal violet was used as the staining solution to distinguish between migrated and non-migrated cells. A cotton swab was used to remove the cells that were left in the upper chamber of the membrane. Those cells that migrated through the insert were examined and counted with bright-field microscope.

## Dual-luciferase reporter gene assay

The relationship between miR-126-5p and MALAT1 was identified using dual-luciferase reporter gene assay. MALAT1 and VEGFA untranslated region was artificially synthesized and inserted into pGL3-control vector (Promega, Madison) between XhoI and BamH sites. Using site-directed mutagenesis, MALAT1 (MALAT1-MUT) and VEGFA mutant (VEGF-MUT) sequence was conducted on the basis of wild-

type (WT) sequence. Recombinant plasmids were co-transfected into HEK 293T cells (Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, Shanghai, China) with miR-126-5p mimic and the negative control (NC) of miR-126-5p, respectively. After transfection for 48 h, the cells were lysed for determination of luciferase activity, which was measured on a Luminometer TD-20/20 (E5311; Promega, Madison, WI, USA) using a dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA).

## Statistical analysis

All assays were conducted in three separate experiments. All data were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed with SPSS 22.0 software (SPSS, Chicago, IL, USA). The *Student's t-test* was used to determine the statistically significant differences between two groups. Kaplan-Meier analysis was used to determine the effects on overall survival of EC patients. Differences were considered statistically significant when P values less than 0.05.

## Results

### MALAT1 leads to poor clinical prognosis

According to the bioinformatic analysis of database, we discovered that the expression of MALAT1 was higher in carcinoma tissue than para-carcinoma tissue (Figure 1A). And the Kaplan-Meier curve suggested the high MALAT1 levels led to shorter overall survival (Figure 1B). Furthermore, we analyzed the MALAT1 levels in gastric cancer tissue with different malignancy and we found that the expression of MALAT1 in carcinoma tissues was positively associated with tumor malignancy. Poor-differentiated carcinoma expressed more MALAT1 than well-differentiated carcinoma, and the gastric cancer expressed more MALAT1 than benign gastric tissue (Figure 1C). Moreover, the Kaplan-Meier curve was consistent with above result that, poor-differentiated carcinoma with higher MALAT1 levels presented poorer prognosis (Figure 1D).

### MALAT1 promotes cell proliferation, migration and invasion.

Because we have discovered that positive association between MALAT1 levels and tumor features, we next try to demonstrate the role of MALAT1 in gastric carcinoma cells. We examined four different gastric cells and discovered that the expression of MALAT1 was highest in AGS cells (Figure 2A). The PCR analysis showed that three Lnc-shRNA exerted good down-regulatory effect, among which Lnc-sh2 was most effective (Figure 2B).

After transfection of Lnc-sh2 in AGS cells, cell viability of AGS cells were significantly decreased at 48 h and 72 h than normal AGS cells and NC transfected AGS cells (Figure 2C). After transfection of Lnc-sh2 in AGS cells, the majority of cells were arrested in G0/G1 phase, which accounts for the results in cell viability assays (Figure 2D). Furthermore, according to cell apoptosis assay, we found that knocking

down the MALAT1 levels in AGS cells could induce cell apoptosis significantly (Figure 2E). In addition, cell migration and cell invasion were also inhibited significantly after knocking down the expression of MALAT1 (Figure 2F and 2G).

## **MALAT1 regulated VEGFA via Akt signal pathway**

Due to the critical role of VEGFA in tumor progression, we examined the expression of VEGFA in four different gastric cancer and we found that AGS cell expressed more VEGFA than the other cells, which is consistent with the level of MALAT1 (Figure 3A). Following PCR and WB analysis showed that knocking down the expression of MALAT1 significantly inhibited the activation of ERK and AKt signal pathway and expression of VEGFA (Figure 3B and 3C).

## **MALAT1 exerts promoting function by regulation of miR-126-5p.**

The result of relative expression of miRNA-126 in AGS cells showed that there was higher level of miRNA-126 in Lnc-sh2 transfected AGS cells, which demonstrated strong negative association between MALAT1 and miRNA-126 (Figure 4A). According to the dual luciferase experimental results, with the increase of miR-126-5p in AGS, the ability of expressing luciferase was suppressed in cell with dual luciferase carrier of MALAT1 target or VEGFA target. On the contrary, with the treatment of miR-126-5p inhibitor, the ability of expressing luciferase was enhanced significantly. However, after mutation of target, the expression capacity of luciferase showed no significant change. Above results demonstrated that MALAT1 was served as a “spongy body” to absorb miR-126-5p, while VEGFA was the target gene of miR-126-5p (Figure 4B and 4C).

After knocking down the expression of MALAT1, cell viability was significantly inhibited, which could be reversed by treatment of miR-126-5p inhibitor (Figure 4D). The result of cell cycle showed that less proportion of cells was arrested in G0/G1 phase after treatment of miR-126-5p inhibitor (Figure 4E). In addition, treatment of miR-126-5p inhibitor significantly inhibited cell apoptosis (Figure 4F). Furthermore, treatment of miR-126-5p inhibitor showed strong promoting function on cell migration and cell invasion, and the inhibitive function of MALAT1 could be totally reversed (Figure 4G and 4H).

## **MALAT1/miR-126-5p axis regulate VEGFA via Akt signal pathway**

After we demonstrated that the MALAT1 could regulate the ability of miR-126-5p and VEGFA was the downstream of MALAT1/miR-126-5p axis, we tried to demonstrated the underlying mechanisms. As is shown in Figure 5, the data of PCR and WB suggested that knocking down the expression of MALAT1 in AGS cell could inhibited the expression of VEGFA and activation of Akt signal pathway, which can be

reversed by treatment of miR-126-5p inhibitor. Above results demonstrated that miR-126-5p was regulated by MALAT1 and MALAT1/miR-126-5p axis regulate VEGFA via Akt signal pathway.

## Discussion

A variety of studies have demonstrated that lncRNAs serve crucial roles in the development of various types of cancer. However, rare studies focused on the function of lncRNAs in GC. Identification of cancer-related lncRNAs and their down-streams is crucial for exploring their roles in tumor progression and for the development of novel therapeutic strategies. The present study investigated the role of lncRNA MALAT1 in GC and its underlying mechanisms.

lncRNA MALAT1 was firstly discovered in non-small cell lung cancer. Previous studies has suggested that MALAT1 promotes GC cell proliferation, migration and invasion, and can be served as a marker of GC in clinical practice (12, 17). In addition, MALAT1 presents a critical role in angiogenesis in GC, which enhanced the growth and metastasis of GC (18). In our present study, we discovered that expression of MALAT1 led to poor prognosis and promoted growth and metastasis of GC by targeting VEGFA, which are consistent with these conclusions.

MicroRNAs (miRNAs) are a type of regulatory noncoding RNAs with a length of 20 to 25 bases, which can target specific mRNA, interact with oncogenes and tumor suppressor factors (19). It has been demonstrated that more than 50% of genes are located in cancer-associated genomic regions, which suggested that miRNA may play a key role in tumor formation and progression (20). Abnormal expression of miR-126-5p is strongly associated with human tumorigenesis. In addition, Chen et al. reported that expression of miR-126-5p associated with higher MVD and VEGFA in GC, and dysfunction of miR-126 leads to tumor growth, which suggested that miR-126-5p shared the same target with MALAT1 (15). However, the regulation of miR-126-5p expression is totally unknown in GC.

Several studies have demonstrated that MALAT1 participated in the progression and development of tumor, served as ceRNA (21-23). To investigate the correlation between MALAT1 and miR-126-5p, we analyzed the expression of miR-126-5p after down-regulation of MALAT1 and we found that miR-126-5p can be down-regulated by MALAT1. In addition, we identified VEGFA as the same target gene shared by MALAT1 and miR-126-5p in GC cells based on the results of the luciferase reporter assay. To further clarify it, we down-regulated the expression of MALAT1 in AGS cells with lentivirus vectors and combined with treatment of miR-126-5p inhibitor. As a result, the expression of miR-126-5p was up-regulated with down-regulation of VEGF, and its downstream signaling molecules (p-AKT, p-mTOR, and p-ERK) as well as cell proliferation reversely correlated with miR-126-5p level. Moreover, the promoting function of MALAT1 can be also reversed by treatment with miR-126-5p.

Previous studies revealed that VEGF could activate ERK and Akt, two well known kinases, signaling pathway in ovarian cancer, hepatocellular carcinoma (24, 25). In our present study, the results of *in vitro* experiment also suggested that Akt and ERK signaling pathway were involved in MALAT1/miR-126-5p/VEGFA axis in GC.

In conclusion, increase of MALAT1 played an important role in promoting tumor growth and metastasis, and MALAT1 could significantly reduce the level of miR-126-5p and enhance the expression of VEGF by Akt signaling pathway, thus counteract the proliferation and invasion of GC cells both *in vitro*. Understanding how MALAT1 is involved in GC will be beneficial to developing potential therapeutic targets in the clinical practice.

## **Declaration**

## **Acknowledgments**

Not applicable.

## **Funding**

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## **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## **Ethics approval and consent to participate**

The First affiliated hospital of Jinan university Ethics Committee approved this study. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study.

## **Authors' contributions**

YZH performed the dominant experiments. QL analyzed and interpreted the patient data regarding the gastric cancer. TYL cultivated and transfected the cell lines. ZLH and HPJ analyzed the experimental data and were major contributor in writing the manuscript. All authors read and approved the final manuscript.

## **Competing interests**

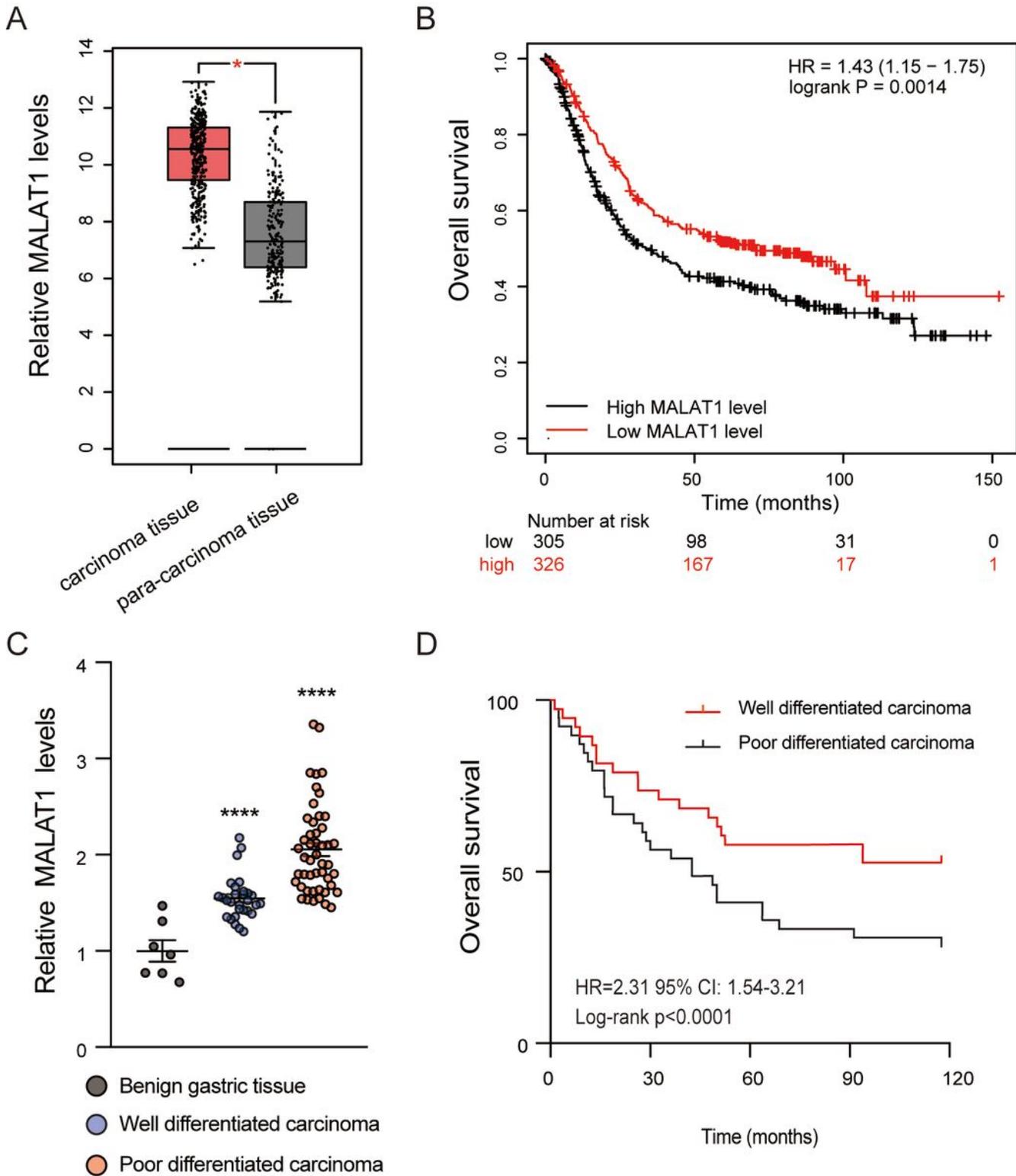
The authors declare that they have no competing interests.

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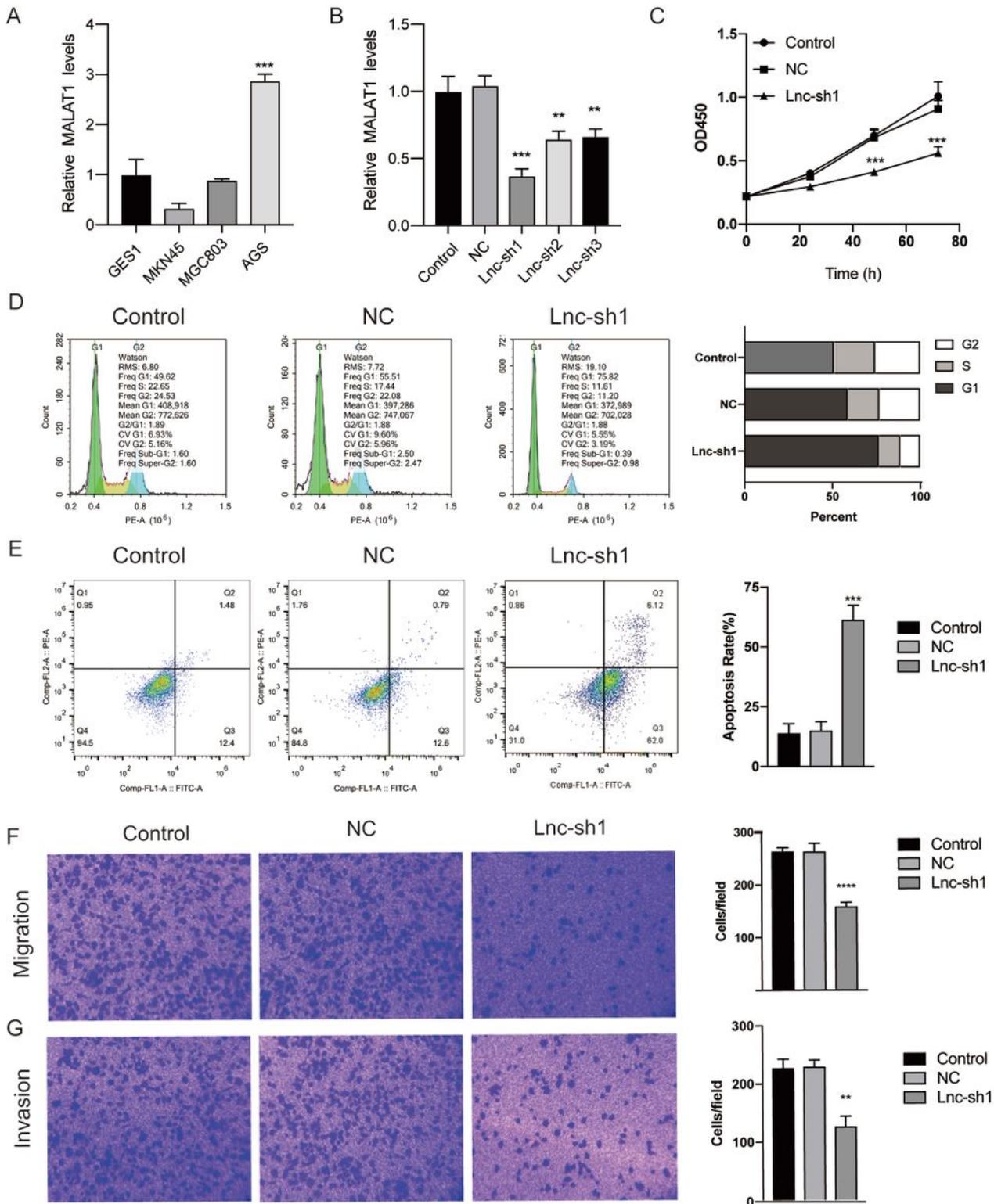
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## Figures



**Figure 1**

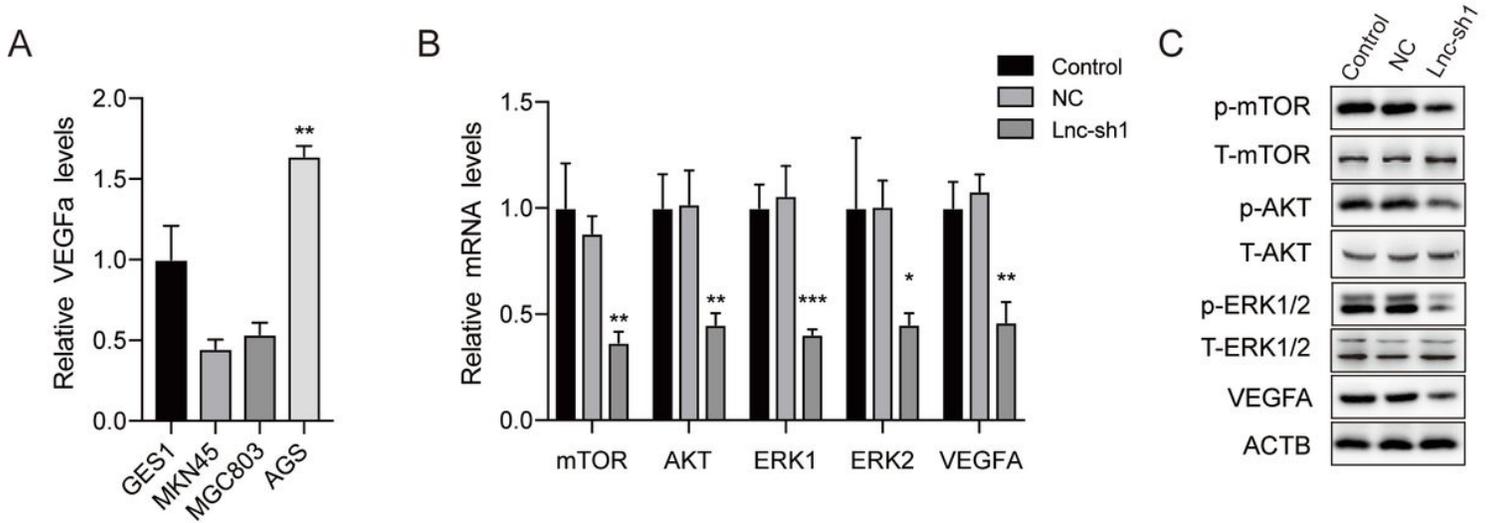
MALAT1 is positively associated with clinical outcomes of patients with gastric cancer. (A) Relative MALAT1 expression in carcinoma tissue and para-carcinoma tissue of gastric cancer. (B) Kaplan-Meier analysis of patients with high or low MALAT1 level gastric cancer. (C) Relative MALAT1 expression in benign gastric tissue, well or poor differentiated carcinoma of gastric cancer. (D) Kaplan-Meier analysis of patients with well or poor differentiated gastric cancer.



**Figure 2**

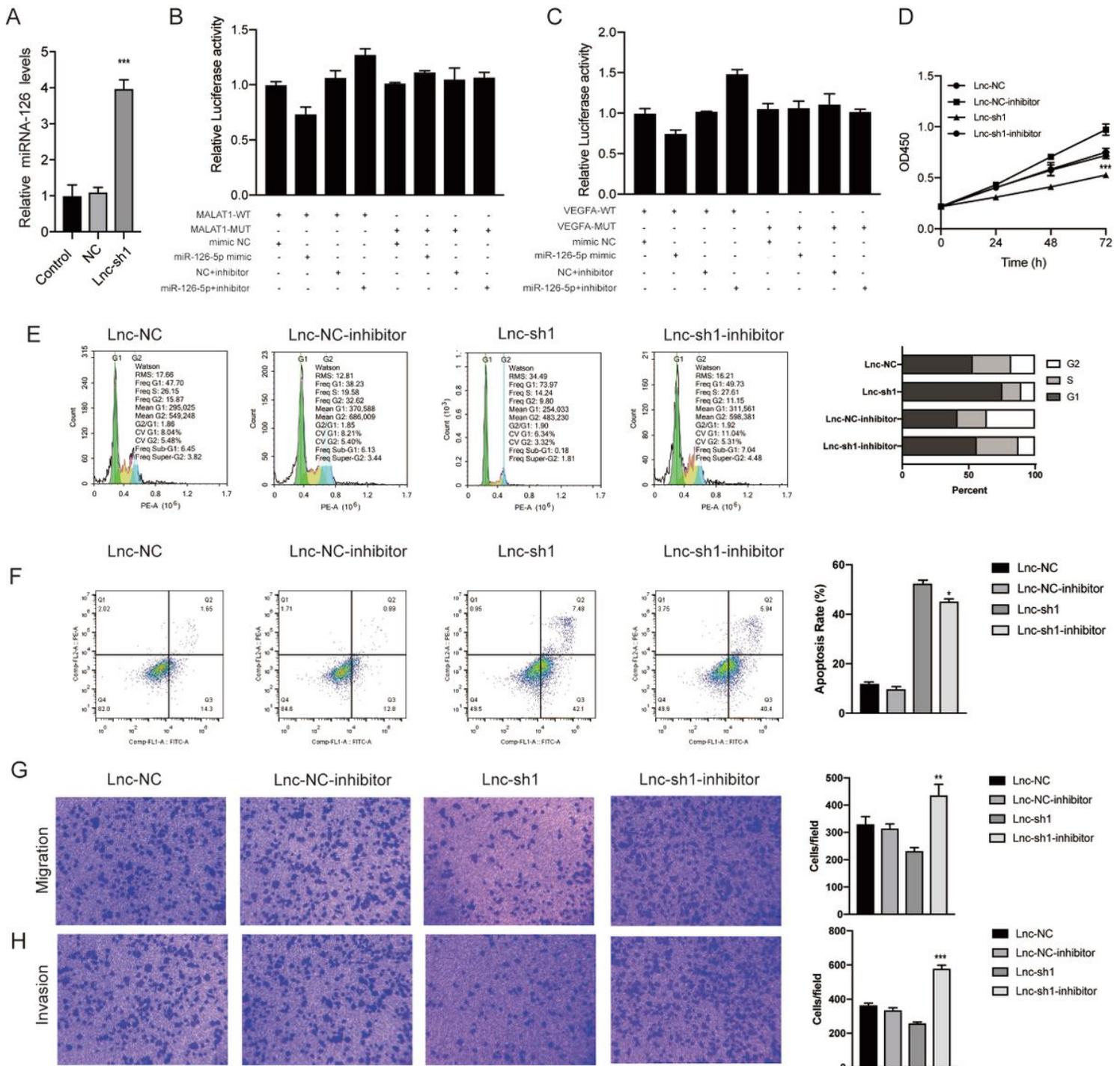
MALAT1 promotes cell proliferation, migration and invasion. (A) Relative MALAT1 expression in GES1, MKN45, MGC803, and AGS cell lines. (B) Relative MALAT1 expression in AGS cell after transfection with NC, Lnc-sh1, Lnc-sh2, and Lnc-sh3. (C) Cell viability of AGC cells after transfection with NC, Lnc-sh1. Representative and quantification of (D) cell cycle and (E) cell apoptosis of AGC cells after transfection

with NC, Lnc-sh1. Representative and quantification of (F) cell migration and (G) cell invasion assays of AGC cells after transfection with NC, Lnc-sh1.



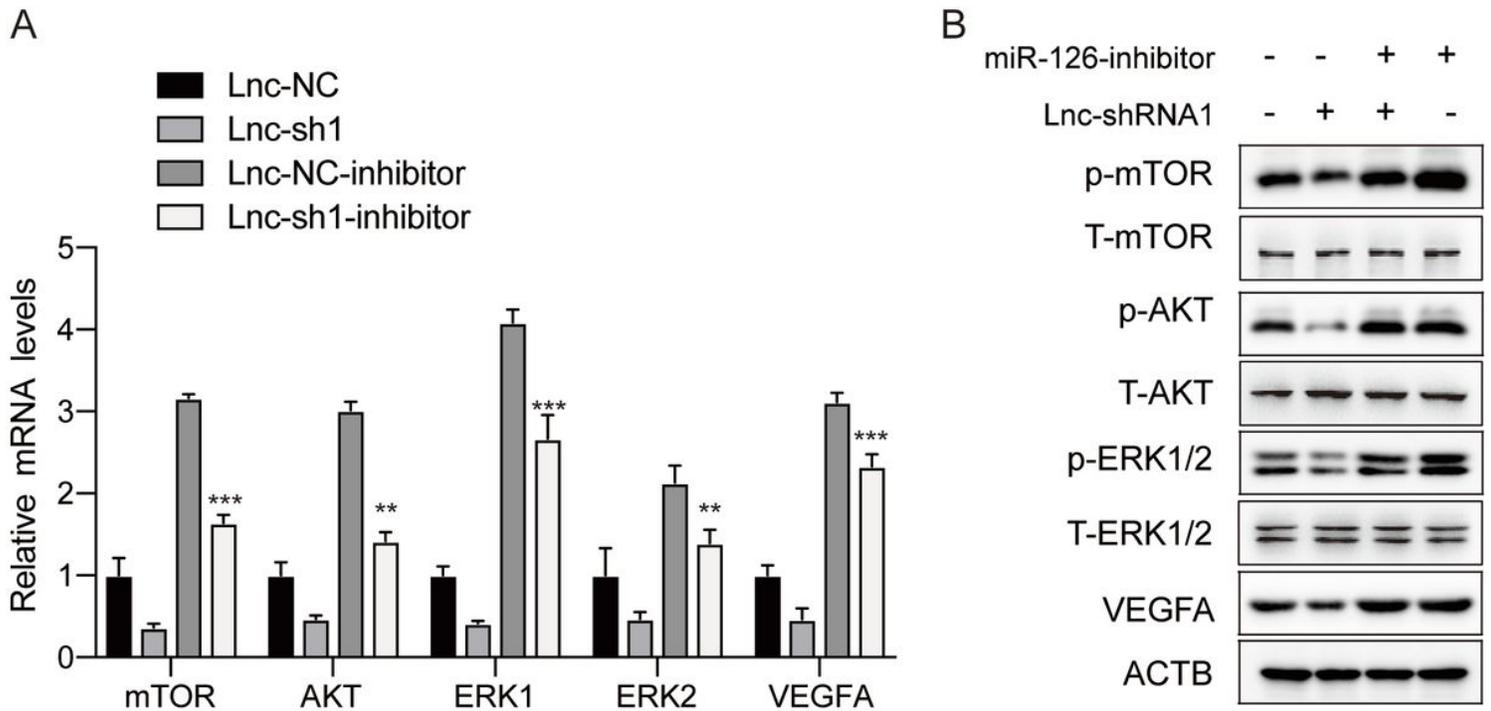
**Figure 3**

MALAT1 targets VEGFA via mTOR signal pathway. (A) Relative VEGFA expression in GES1, MKN45, MGC803, and AGS cell lines. (B) Relative mRNA levels of mTOR, AKT, ERK1, ERK2, and VEGFA in AGS cells after transfection with NC, Lnc-sh1. (C) Western blot analysis of activation of mTOR signal pathway and VEGFA in AGS cells after transfection with NC, Lnc-sh1.



**Figure 4**

MALAT1 exerts promoting function by regulation of miR-126-5p. (A) Relative miRNA-126 levels in AGS cells after transfection of NC, Lnc-sh1. (B-C) Double luciferase experiment. (D) Cell viability of AGS cells after transfection of NC, Lnc-sh1 with or without treatment of miR-126-5p inhibitor. Representation and quantification of (E) cell cycle and (F) cell apoptosis of AGS cells after transfection of NC, Lnc-sh1 with or without treatment of miR-126 inhibitor. Representation and quantification of (G) cell migration and (H) cell invasion of AGS cells after transfection of NC, Lnc-sh1 with or without treatment of miR-126-5p inhibitor.



**Figure 5**

MALAT1/miR-126-5p axis regulate VEGFA and mTOR signal pathway (A) Relative mRNA levels of mTOR, AKT, ERK1, ERK2, and VEGFA in AGS cells after transfection of NC, Lnc-sh1 with or without treatment of miR-126-5p inhibitor. (B) Western blot analysis of activation of mTOR signal pathway and VEGFA in AGS cells after transfection of NC, Lnc-sh1 with or without treatment of miR-126-5p inhibitor.