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# MicroRNA-106a targets TIMP2 to regulate invasion and metastasis of gastric cancer

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ABSTRACT

**Emerging evidence has shown that microRNA plays an important role in tumor development and progression. Here, we report that miR-106a is frequently up-regulated in gastric cancer tissues and positively correlates with metastasis. Restrained expression of miR-106a in gastric cancer cells significantly reduces their capacity of proliferation, migration and invasion. In tissue sections, the positive signal of miR-106a localized in metastasis-associated regions confirmed this result. Moreover, we show that TIMP2 is a direct downstream target for miR-106a and knockdown of TIMP2 strengthens the beneficial effects of miR-106a. Our study adds miR-106a to the complex mechanisms of tumor metastasis.**


## 1. Introduction

Gastric cancer possesses the fourth highest incidence of human malignant disease and causes the second highest cancer-related death worldwide [1]. Although the research on gastric cancer has made a great advancement, the molecular mechanisms underlying cancer invasion and metastasis are still poorly understood.

MicroRNAs (miRNAs) is a small non-coding RNAs that negatively regulates gene expression at post-transcriptional and translational levels through binding to complementary sequences in the 3′-untranslated regions of their target mRNAs, and is consequently considered to be an important regulator in many human biological processes including human growth and development, cellular differentiation, adhesion, angiogenesis and inflammation [2–4]. Recently, many ectopic expressed miRNAs have been reported to be involved in the initiation and progression of gastric cancer as characters of novel proto-oncogene and tumor-suppressor gene [5]. miR-106a, which is a member of miR-17 family, is mapped to human chromosome Xq26.2 and is derived from the precursor miR-106a∼363. The mature miR-106a is highly expressed in malignant tumors that localized in the digestive tract such as gastric cancer [6,7], esophageal cancer [8] and colorectal cancer [9], etc. Moreover, many studies also indicate that miR-106a has a very wide distribution so that it can be easily detected in tumor tissues, blood [10], fecal [11] and gastric juice [12]. Despite miR-106a harbors a valuable clinical application, the biological function of miR-106a in gastric cancer metastasis remains obscure.

In this study, we demonstrate that miR-106a is frequently up-regulated in human gastric cancer and closely associated with tumor local invasion and distant spread. Furthermore, we identify that miR-106a has a potential to become a novel metastasis-related gene by directly regulating the functional target TIMP2.

## 2. Materials and methods

### 2.1. FFPE samples and cell lines

FFPE samples were collected from General Hospital of Ningxia Medical University with the approval of local ethics committee. Pathological diagnosis was carried out according to the World Health Organization criteria. Exclusion criteria were a previous history of radiotherapy and chemotherapy. Well-, moderately and poorly differentiated human gastric cancer cells MKN-28, SGC-7901 and BGC-823 as well as normal gastric epithelial cell GES-1 were all obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were maintained in

*Abbreviation:* FFPE, Formalin-fixed paraffin-embedded

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RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) cultured at 37 °C in a 5% $CO_2$ incubator.

### 2.2. RNA isolation and real-time PCR

RNA from FFPE samples and cultured cells was extracted by RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) and High Pure miRNA Isolation Kit (Roche) based on the company's specifications, respectively. For detection of miRNA expression, cDNA was obtained from 10 ng RNA using Taqman MicroRNA Assays (Applied Biosystems) and subsequently amplified by TaqMan Universal Master Mix II (Applied Biosystems) with U6 snRNA as an internal reference. For detection of mRNA expression, cDNA was prepared from 300 ng RNA using PrimeScript RT Master Mix (Takara) and then quantified by SYBR *Premix Ex Taq*™ II (Takara) with GAPDH as an internal control. The primers for TIMP2 were: forward 5′-AGCACCACCCAGAAGAAGAG-3′, reverse 5′-GTGACCCAGTC CATCCAGAG-3′. All experiments were performed in triplicates where $2^{-\triangle\triangle Ct}$ was regarded as the analytic formula.

### 2.3. Transfection

Hsa-miR-106a inhibitor, antagomir and negative control were synthesized by RiboBio (China). Small interfering RNA against TIMP2 and negative control were designed by Genepharma (China). The sequences of the si-TIMP2 were: sense 5′-GGCCUGAGAAGGAUAUAGATT-3′, antisense 5′-UCUAUAUCCUUCU-CAGGCCTT-3′. Transfection was carried out using Lipofectamine 2000 (Invitrogen) method. The final concentration was 200 nm for inhibitor or antagomir and 100 nm for siRNA.

### 2.4. MTT assay

Cell proliferation was measured by MTT assay. Cells were seeded in 96-well culture plates at a density of $6 \times 10^3$ cells/well. Transfection was performed the next day at the concentration described above. Cell proliferation was analyzed at 24, 48, 72 and 96 h, and the optical absorbance was read at 490 nm by EnSpire Multimode Plate Reader (PerkinElmer).

### 2.5. Transwell assay

Cell migration and invasion were assessed using transwell assay. For migration, $2 \times 10^4$ cells in serum-free medium were seeded into the top chambers of an insert (8 μm pore size, Merck Millipore), which were soaked into the bottom chambers filled with complete medium. For invasion, the same density of cells was placed into the upper chambers, which were pre-coated with Matrigel (BD Biosciences). After 24 h of incubation, the chambers were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet. The cells passing through the film were counted under microscope.

### 2.6. In situ hybridization

The expression and, more importantly, location of miR-106a was achieved by in situ hybridization (ISH). FFPE specimens were deparaffinized followed by rehydration and digestion with 15 μg/ml proteinase K (Exiqon) at 37 °C for 10 min. The slides were hybridized with 40 nM double-digoxigenin labeled locked nucleic acid (LNA) probe (Exiqon) at 55 °C for 1 h. The anti-Digoxigenin-AP was applied at 1:800 (Roche) for 60 min at room temperature. The colorimetric reaction was performed using BCIP/NBT ready-to-use tablets (Roche). Neutral Red Staining Solution (Sigma–Aldrich) was used for nuclear counter staining. The miR-106a appeared as dark blue particles in the cytoplasm or nucleus after staining.

### 2.7. Luciferase reporter assay

The 3′-UTR of TIMP2 (NM_003255.4) containing the predicted miR-106a binding site was constructed by RiboBio (China). The mutant TIMP2 3′-UTR was created by mutating multiple nucleotides complementary to the miR-106a seed region. HEK293T cells were cultured in 96-well plates with 50–70% confluence 24 h before transfection. A mixture of 100 ng pmiR-RB-Report™ h-TIMP2 wild type (WT) or mutant (Mut) reporter plasmid vector together with 50 nM hsa-miR-106a mimics or negative control (RiboBio) were co-transfected. The luciferase activity was measured 48 h post-transfection using Dual-Glo Luciferase Assay System (Promage) with Renilla (Rluc) luciferase activity as the reporter gene and firefly luciferase (Luc) as the reference gene.

### 2.8. Western blot

Whole-cell proteins were collected with RIPA Buffer (Pierce) and separated by 12% SDS–PAGE before transfer. The PVDF membrane (Merck Millipore) was blocked with 5% non-fat milk and incubated with rabbit anti-TIMP2 monoclonal antibody (1:1000, Abcam). A β-Actin antibody (Santa Cruz Biotechnology) was used as control for normalization. The results were obtained by Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore).

### 2.9. Statistical analysis

Statistical analysis was performed using SPSS statistics 17.0. The results were expressed as mean ± S.E.M. and the level of significance was set at $P < 0.05$. Expression of miR-106a was determined by paired *T*-test, the relationship between miR-106a and clinicopathologic parameters was compared using independent sample T-test and One-Way ANOVA, as well as the results of cellular experiments.

## 3. Results

### 3.1. RNA from FFPE samples has appropriate quantity and quality

To determine whether or not RNA from FFPE samples was suitable for downstream applications, we examined the RNA quantity and quality. The total RNA purity in 82 cases of FFPE samples was 2.022 ± 0.190, and the concentration was 267.062 ± 210.435, indicating that FFPE samples can yield enough total RNA for most applications. The integrity check showed that RNA from FFPE samples was typically recovered as a smear, except in higher quality preparations, which yielded two broad bands representing 18s and 28s rRNA. A majority of the smeared RNA samples ranged from 200 to 300 bases. Moreover, we monitored the effects on PCR efficiency by amplifying the fragments of small sizes (106 bp) from the highly expressed house-keeping gene U6 snRNA. The results showed that this fragment could be amplified without any abnormity and it also had a stable expression in coupled FFPE samples ($P = 0.216$) (data not shown).

### 3.2. miR-106a is up-regulated in gastric cancer

To examine the expression of miR-106a at different levels, we designed a paired sample containing 41 gastric cancer and adjacent non-tumor tissues. As shown in Fig. 1A, the miR-106a expression was significantly up-regulated in gastric cancer tissues when compared with the corresponding non-tumor samples with the fold

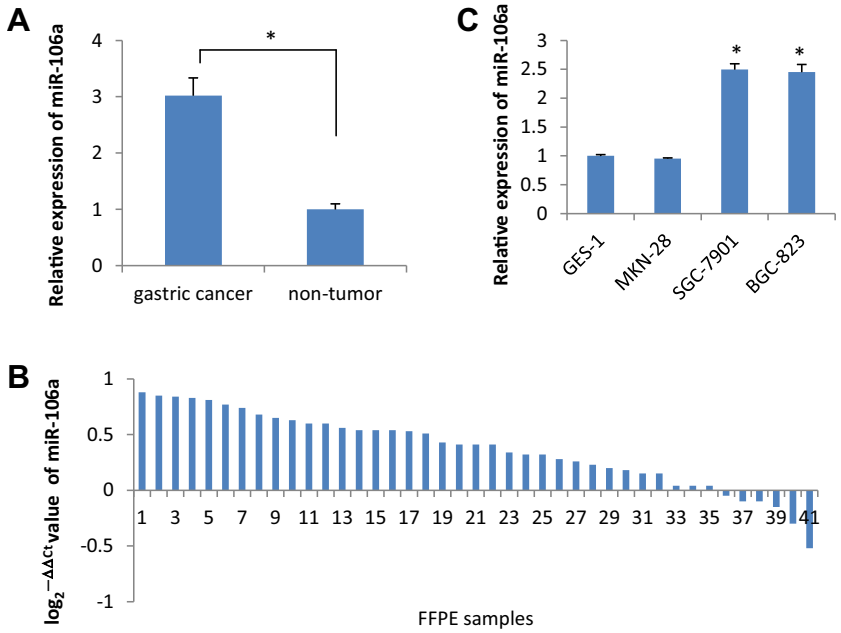


**Fig. 1.** miR-106a is up-regulated in gastric cancer tissues and cells. (A) qRT-PCR for miR-106a in 41 paired human gastric cancer tissues and adjacent non-tumor tissues. *$P = 0.000$. (B) qRT-PCR for the enhanced expression of miR-106a in 85.37% (35/41) of tumor tissues. (C) qRT-PCR for miR-106a in three different gastric cancer cells and GES-1 cell. *$P = 0.000$.

change expressed by $2^{-\triangle\triangle Ct}$ at $3.02 \pm 2.02$, suggesting that the expression level of miR-106a in tumor tissues was higher than that in normal tissues ($P = 0.000$). As shown in Fig. 1B, enhanced miR-106a was observed in 85.37% (35/41) of tumor tissues, indicating that up-regulation of miR-106a was a frequent event in human gastric cancer. In addition, different differentiated tumor cells were also selected to investigate the effects of miR-106a. As shown in Fig. 1C, the expression of miR-106a varied in the three cells ($P = 0.000$). Compared with GES-1 cell, the expression of miR-106a in SGC-7901 and BGC-823 cells were both significantly up-regulated with the fold change of $2.50 \pm 0.18$ and $2.45 \pm 0.23$ ($P = 0.000$), unlike the MKN-28 cell with the fold change of $0.96 \pm 0.20$ ($P = 0.714$).

### 3.3. Relationship between miR-106a and clinical pathology of gastric cancer

It was revealed in Table 1 that the over-expression of miR-106a was closely associated with the clinicopathological parameters of gastric cancer. In our group, the expression of miR-106a did not have significant correlation with patients' age, gender, tumor location, size, degree of differentiation and nerves involvement, but was positively with lymph node metastasis ($P = 0.002$), vascular invasion ($P = 0.017$) and depth of infiltration ($P = 0.009$). These data indicated that the over-expressed miR-106a would create greater opportunities to develop not only local invasion but also distant spread.

### 3.4. Ectopic miR-106a has a diverse effect on cell proliferation

To estimate the potential role of miR-106a in gastric cancer, we constructed the sequence-specific inhibitor for hsa-miR-106a and compared its performance on four different cells. On the basis of results above, we found that in contrast to negative group, the proliferations of SGC-7901 and BGC-823 cells with enhanced expression of miR-106a were attenuated when miR-106a inhibitor was used. However, under the same treatment, MKN-28 cell with silenced expression of miR-106a and normal GES-1 cell were not express significant difference. (Fig. 2).

### 3.5. Ectopic miR-106a triggers cell migration and invasion

In FFPE samples, we found that the markedly expressed miR-106a may lead to the enhancement of gastric cancer metastasis. To gain insight into the biological functions of miR-106a, we conducted transwell assays which indicated that in SGC-7901 and BGC-823 cells infected with miR-106a inhibitor, migration ($16.35 \pm 12.44$ vs. $49.05 \pm 27.63$, $P = 0.000$; $18.80 \pm 17.01$ vs. $94.23 \pm 18.15$, $P = 0.000$) and invasion ($14.25 \pm 9.13$ vs. $35.20 \pm 25.55$, $P = 0.000$; $16.15 \pm 8.42$ vs. $83.00 \pm 42.81$, $P = 0.000$) were both profoundly decreased compared with negative group, but the similar performance was not present in MKN-28, that was, supplanted by no change both in migration ($P = 0.224$) or invasion ($P = 0.374$) (Fig. 3). GES-1 per se did not have metastatic potential, even upon induction by miR-106a. The results from gastric cancer cells approved the conclusion from gastric cancer tissues that miR-106a was highly associated with tumor metastasis.

### 3.6. Localization of miR-106a confirmed its relationship with metastasis

The findings above illustrated that miR-106a expression was correlated with gastric cancer metastasis such as depth. In order to catch the phenomenon more directly, we employed in situ hybridization to localize gene expression. Since we wanted to mark sure that miR-106a did promote tumor infiltration, it needed a section on which metastasized tumors and tumors that had not yet occurred metastasis were both involved. In view of this, we chose advanced gastric cancer tissues and the results demonstrated that the predominant expression of miR-106a in the endovascular invasion of gastric cancer cells infiltrating into the muscular and serous layers as intravascular emboli. On the contrary, it was less expressed in mucosal surface. (Fig. 4) The result confirmed the relationship between miR-106a and tumor metastasis.

**Table 1**
miR-106a expression and clinical pathology of gastric cancer.

| Characteristics | No. of patients | % | miR-106a (average fold change ± SD) | *P*-value |
|---|---|---|---|---|
| *Age (years)* | | | | |
| ⩾50 | 32 | 78 | 3.009 ± 1.8464 | 0.941 |
| <50 | 9 | 22 | 3.067 ± 2.6935 | |
| *Sex* | | | | |
| Male | 29 | 71 | 2.752 ± 1.6932 | 0.187 |
| Female | 12 | 29 | 3.675 ± 2.6327 | |
| *Tumor site* | | | | |
| Cardia | 9 | 22 | 4.356 ± 2.2528 | 0.078 |
| Body | 6 | 15 | 2.767 ± 2.0801 | |
| Antrum | 26 | 63 | 2.619 ± 1.7989 | |
| *Tumor size (cm)* | | | | |
| $T \leqslant 2$ | 10 | 24 | 2.230 ± 1.5144 | 0.126 |
| $2 < T \leqslant 5$ | 19 | 46 | 3.637 ± 2.2916 | |
| $T > 5$ | 8 | 20 | 2.113 ± 1.7324 | |
| Miss | 4 | 10 | 3.900 ± 1.2138 | |
| *Differentiation* | | | | |
| Well | 2 | 5 | 0.550 ± 0.3536 | 0.206 |
| Moderate | 23 | 56 | 3.209 ± 1.9329 | |
| Poor | 16 | 39 | 3.063 ± 2.1351 | |
| *Lymph node* | | | | |
| Positive | 24 | 59 | 3.800 ± 2.1452 | 0.002* |
| Negative | 17 | 41 | 1.924 ± 1.1956 | |
| *Vessels* | | | | |
| Positive | 11 | 27 | 4.245 ± 2.1815 | 0.017* |
| Negative | 30 | 73 | 2.573 ± 1.7971 | |
| *Nerves* | | | | |
| Positive | 2 | 5 | 3.350 ± 0.2121 | 0.818 |
| Negative | 39 | 95 | 3.005 ± 2.0741 | |
| *Depth* | | | | |
| Mucosa | 4 | 10 | 1.200 ± 0.5292 | 0.009* |
| Submucosa | 4 | 10 | 1.600 ± 0.3559 | |
| Muscular layer | 9 | 22 | 2.244 ± 0.7091 | |
| Serous layer | 24 | 58 | 3.854 ± 2.2368 | |

* Indicated statistical significance ($P < 0.05$).

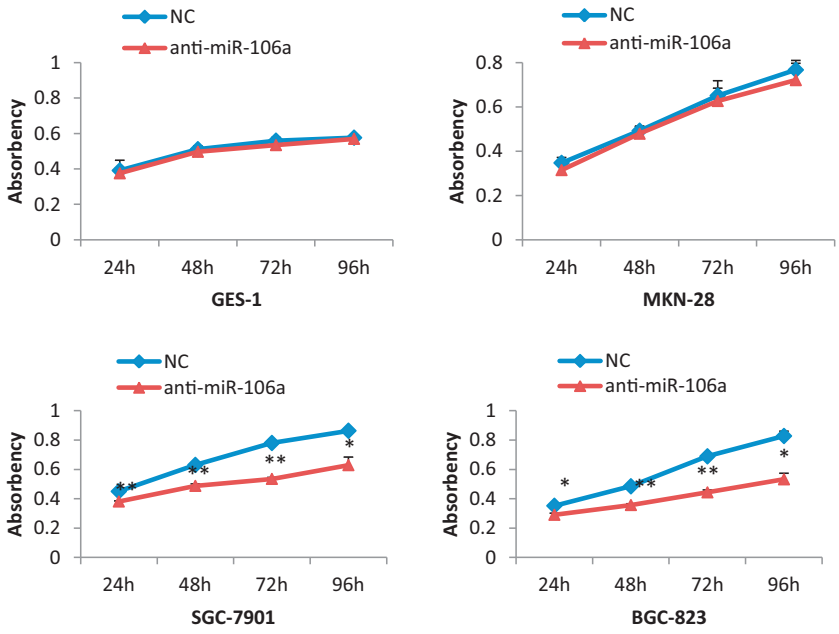


**Fig. 2.** miR-106a regulates human gastric cancer cell proliferation *in vitro*. MTT assays after transduction with miR-106a inhibitor or negative control in four gastric cells. *$P < 0.05$, **$P < 0.001$.

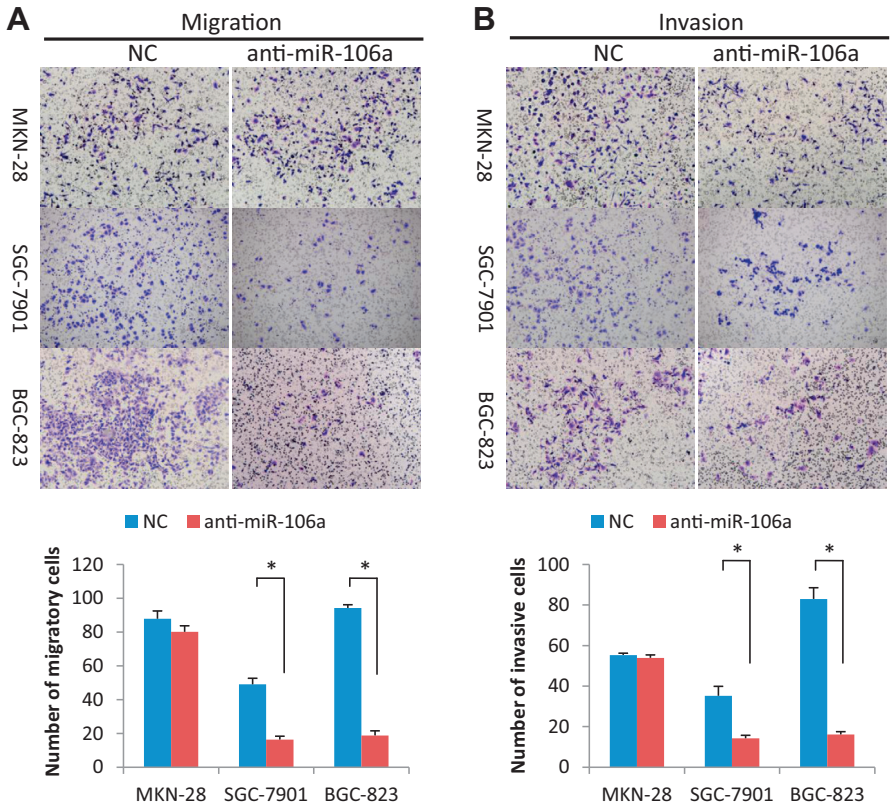


**Fig. 3.** miR-106a promotes human gastric cancer cell migration and invasion *in vitro*. Transwell migration (A) and invasion (B) assays after transduction with miR-106a inhibitor or negative control in three gastric cancer cells. *$P = 0.000$.

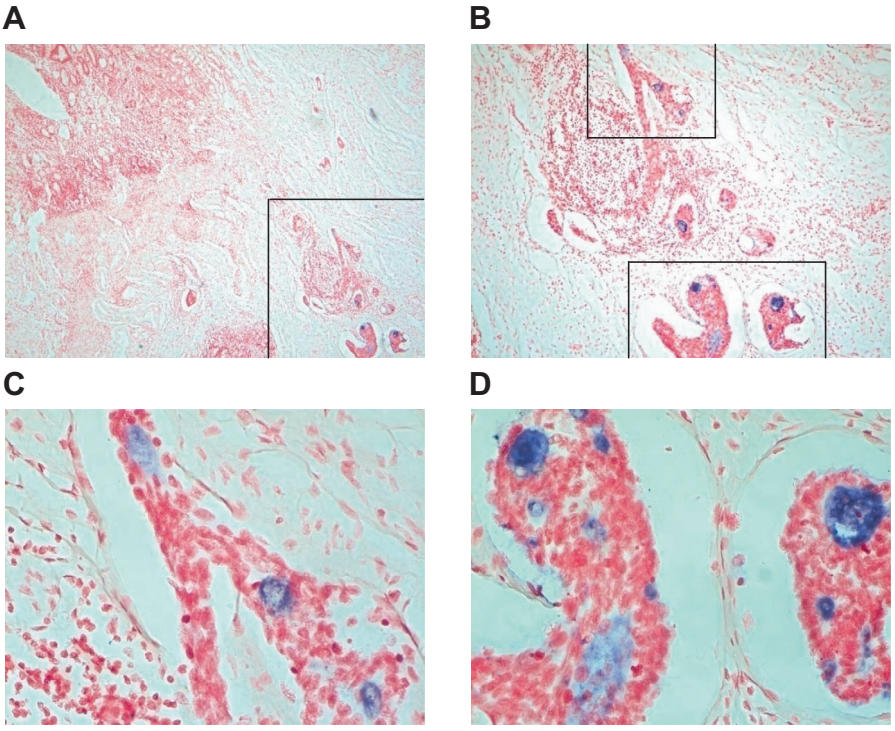


**Fig. 4.** LNA-ISH detection of miR-106a in FFPE tissues from moderately differentiated human gastric adenocarcinoma. (A) Glands infiltrated into the deep gastric wall. (Magnification ×40) (B) Strong miR-106a staining in the infiltrated glands. (Magnification ×100) (C and D) The glands with positive signals concentrated on the vascular cavity. (Magnification ×400) ISH-positive signals stain blue, whereas nuclei stain red.

### 3.7. TIMP2 is a direct target of miR-106a

Three open access programs—TargetScan, miRDB and miRanda—to predict the target of miR-106a. TIMP2, which was one of the metastasis-associated genes, was selected for analysis (Fig. 5A). The 3′-UTR reporter of TIMP2 containing wild and mutant sequences was cloned. When the wild type was transfected with miR-106a mimics and negative, the luciferase activity of WT/miR-106a was significantly decreased compared with WT/NC ($P = 0.000$). When the mutant TIMP2 was transfected with miR-106a mimics and negative, no significant difference can be seen between Mut/miR-106a and Mut/NC ($P = 0.323$) (Fig. 5B). Western blot demonstrated that miR-106a inhibition in SGC-7901 and BGC-823 cells elevated TIMP2 protein level compared with control group (Fig. 5C). To further attest the negative relationship between miR-106a and target gene TIMP2, we measured TIMP2 mRNA and protein levels in gastric cancer cells. Data displayed in Fig. 5D and E showed that the expression of TIMP2 in gastric cancer cells was generally lower. The relative expression in MKN-28, SGC-7901 and BGC-823 was $0.74 \pm 0.01$ ($P = 0.007$), $0.26 \pm 0.08$ ($P = 0.000$), $0.17 \pm 0.02$ ($P = 0.000$), respectively. These evidences deduced that under-expression of TIMP2 may result from up-expression of miR-106a.

### 3.8. Knockdown of TIMP2 promotes cell proliferation, migration and invasion

Since we had observed that miR-106a could meditate gastric cancer SGC-7901 and BGC-823 cells migration and invasion, a specific siRNA should be introduced into these two cells to determine whether down-regulation of TIMP2 had a phenocopy of over-expression of miR-106a. When interference was implemented, the expression level of TIMP2 in SGC-7901 and BGC-823 cells was sharply down ($P = 0.000$) (Fig. 6A). Moreover, silencing of TIMP2 accelerated cell proliferation of high-grade gastric cancer (Fig. 6B). Migration ($58.39 \pm 9.24$ vs. $39.31 \pm 7.31$, $P = 0.000$; $82.13 \pm 7.30$ vs. $66.30 \pm 3.55$, $P = 0.000$) and invasion ($45.00 \pm 11.58$ vs. $32.96 \pm 3.95$, $P = 0.000$; $81.20 \pm 14.20$ vs. $65.36 \pm 3.74$, $P = 0.000$) were also increased in SGC-7901 and BGC-823 cells compared with negative group, respectively (Fig. 6C). These change suggested that inhibition of TIMP2 expression could mimic the effect of miR-106a. Negative regulation of TIMP2 by miR-106a was, at least in part, responsible for miR-106a-induced gastric cancer cell progression.

## 4. Discussion

In this study, we focused on miR-106a and used qualitative, quantitative and positioning analysis to investigate its biological functions in gastric cancer. To take full advantage of existing resources, archived FFPE samples were selected. From our experiments, miRNA in FFPE samples was preserved perfectly and can be amplified successfully. The results verify an overview that miRNA species are less affected by autolysis, cross linking and fragmentation during FFPE preparation [13,14]. Above all, we found that the frequently up-regulated miR-106a positively adjusted gastric cancer cell proliferation, migration and invasion. Similarly, miR-17 was found to promote gastric cancer proliferation [15]. As described previously, miR-17 and miR-106a belong to the same family, with the results indirectly corroborating the promotional

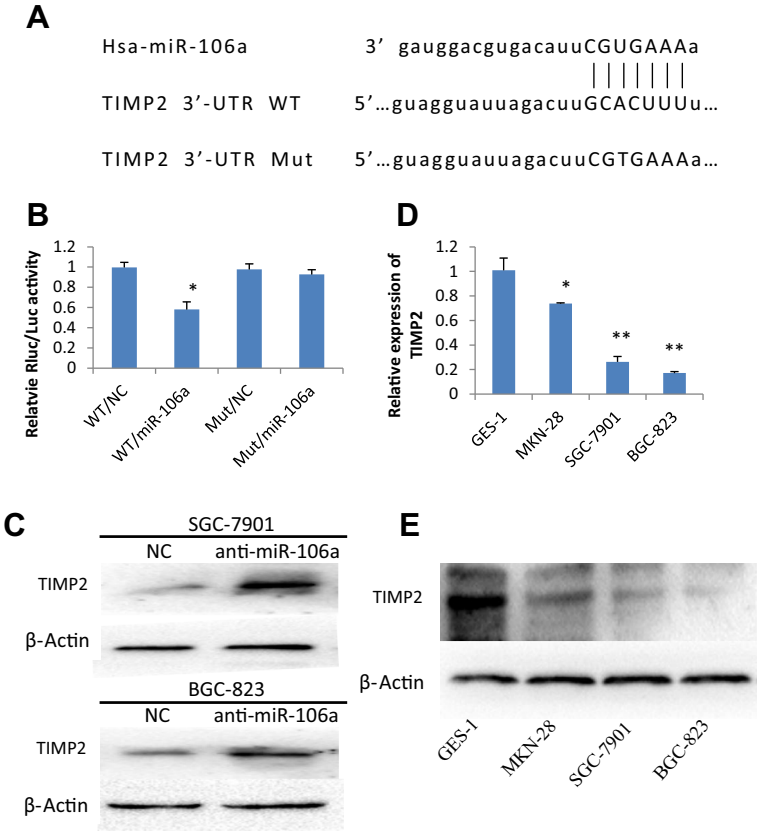


**Fig. 5.** TIMP2 is directly regulated by miR-106a. (A) The wild type and mutant TIMP2 3′-UTR sequences are shown with the miR-106a sequence. (B) Luciferase assays in HEK293T cells with wild-type or mutant TIMP2 3′-UTR vectors and miR-106a mimic or negative control. *$P = 0.000$. (C) Western blot for TIMP2 in SGC-7901 and BGC-823 cells after infection with miR-106a antagomir or negative control. β-Actin is a loading control. (D and E) qRT-PCR and Western blot for TIMP2 in three gastric cancer cells and GES-1 cell. β-Actin is a loading control. *$P = 0.007$, **$P = 0.000$.

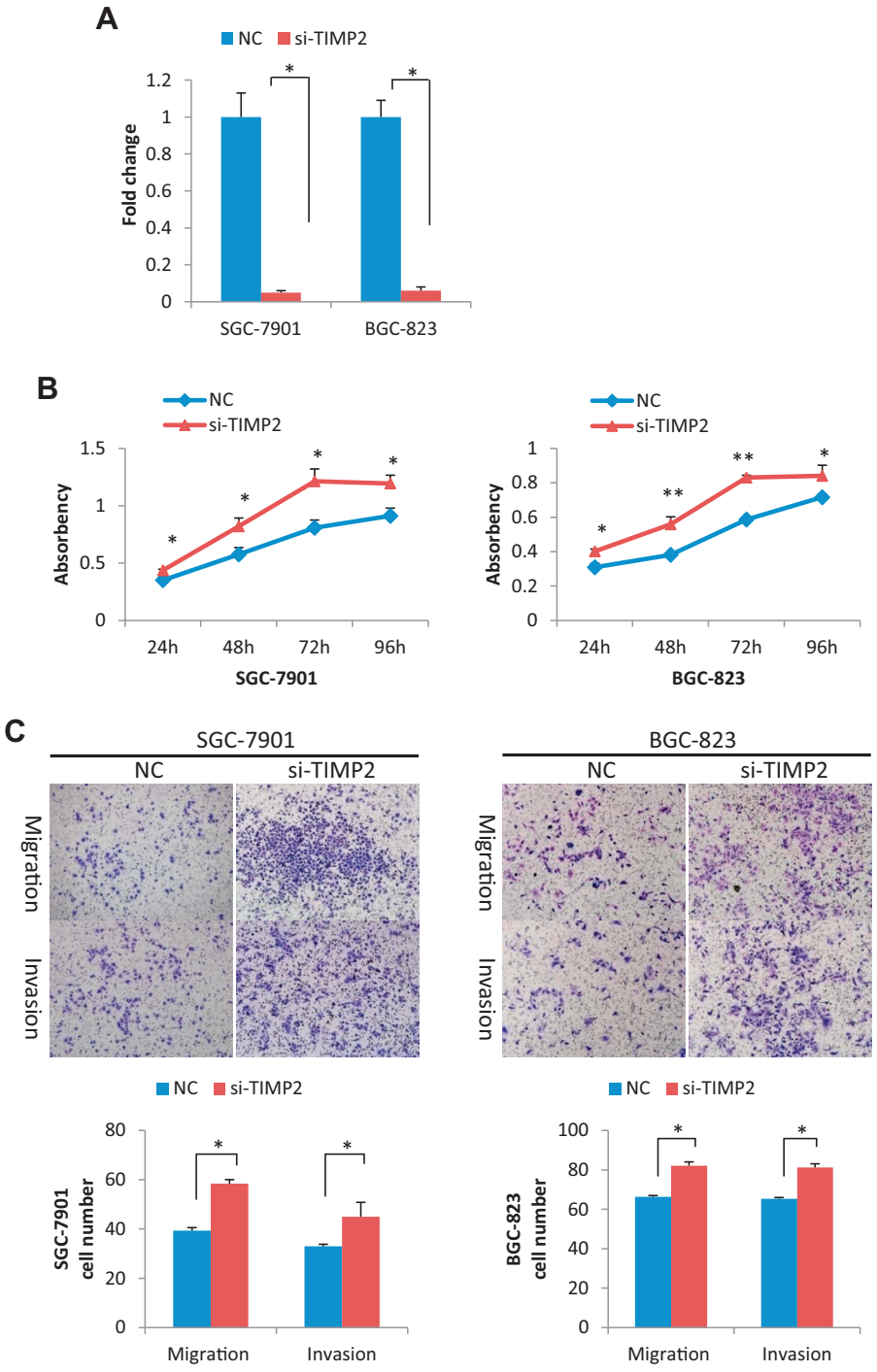


**Fig. 6.** Knockdown of TIMP2 promotes cell proliferation, migration and invasion *in vitro*. (A) qRT-PCR for TIMP2 in SGC-7901 and BGC-823 cells transfected with si-TIMP2 or negative control. $^{*}P = 0.000$. (B) MTT assay in SGC-7901 and BGC-823 cells after siRNA transfection. $^{*}P < 0.05$, $^{*}P < 0.001$. (C) Transwell assay after interference. $^{*}P = 0.000$.

effect of miR-106a. Because the capability of cell migration and invasion is considered to be the most important determinant in the process of cancer metastasis, we perform several methods to visualize miRNA expression and localization in this course. One of the powerful techniques is LNA-ISH, which offers a spatially accurate resolution of miRNA expression unsurpassed by others [16,17]. Using this LNA-modified probe, we found that the positive miR-106a signals appeared exactly in tumor embolus, deep inside the muscular layer. Given the fact that over-expressed miR-106a does promote tumor progression and has a potential to act as a new metastasis-related gene in gastric cancer.

Metastasis is a complicated process that refers to multiple factors and multiple steps [18,19]. Our work elucidates the underlying mechanism of miR-106a in gastric cancer metastasis that is not explored as yet. The exploration of the target genes for clarification of the disorder lead to the identification of TIMP2 which is a member of tissue inhibitor of metallopeptidases (TIMPs) family as a direct and functional downstream mediator for miR-106a. TIMPs family are natural inhibitors of matrix metalloproteinases (MMPs), which can degrade the extracellular matrix (ECM) and basement membrane (BM), the degradation of which is a prerequisite for tumor metastasis [20]. It has been reported that TIMP2 combines with

activated MMP2 through 1 to 1 non-covalent forms and abrogates its collagenases and gelatinases activity, reduces the degradation of ECM, and maintains the integrity of BM [21]. In addition to an inhibitory role against metalloproteinases, TIMP2 has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells with MMP-independent mode [22]. Herein, we show that miR-106a may cooperate with TIMP2 to start gastric cancer invasion-metastasis cascade reaction. The up-regulation of miR-106a may exert oncogenic role with promoting effects on cell proliferation and especially metastasis by targeting TIMP2, and conversely, knockdown of TIMP2 could mimic the miR-106a-induced cancer development benefits. These discoveries prove our speculation that in many gastric cancer types, there is prevalently elevated miRNAs such as miR-106a, which always situated in the upstream direct against anti-metastatic gene like TIMP2 and suppresses its expression at post-transcriptional levels; the regulated target gene subsequently facilitates the tumor cell dissemination. Inverse correlation of miR-106a and TIMP2 ultimately contributes to the enhancement of gastric cancer progression.

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