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ORIGINAL ARTICLE

THBS4 predicts poor outcomes and promotes proliferation and metastasis in gastric cancer

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Abstract

Gastric cancer (GC), a common and lethal cancer in the world, has a high risk of metastasis. Our study was to explore the effects of THBS4 on GC progress and metastasis and the underlying mechanisms. The proliferations of MGC-803 and BGC-823 cells were analyzed via cell count, MTT, and soft agar colony formation assay. The migration and invasion of transfected GC cells was investigated via transwell migration and invasion assay. The mRNA abundance of THBS4 and KLF9 was detected by quantitative real-time PCR (qPCR). The analysis of Gene Expression Omnibus (GEO) dataset (GSE26253) suggested that THBS4 was up-regulated in recurrent GC patients and was positively correlated with the increase in pathological stage and poor prognosis in GC. THBS4 stimulated the proliferations of GC cells. Moreover, THBS4 overexpression fostered the migration and invasion of GC cells. Further, the bioinformatics analysis of the cancer genome atlas dataset suggested that there may be a positive correlation between THBS4 and KLF9 expression. QPCR analysis proved that transfected with THBS4 overexpression plasmid enhanced KLF9 expression in GC cells. THBS4 mRNA and protein expression were up-regulated in MGC-803 and BGC-823 cells compared to those in non-tumoral gastric cells. KLF9 overexpression significantly stimulated the proliferation and metastasis of MGC-803 and BGC-823 cells. Besides, KLF9 siRNA inhibited the enhanced viability, migration, and invasion of MGC-803 cells caused by the transfection with THBS4 overexpression plasmid. In conclusion, THBS4 had positive effects on GC proliferation and metastasis via targeting KLF9.

Keywords THBS4 · Gastric cancer · Proliferation · Metastasis · KLF9

Abbreviations

GC	Gastric cancer
GEO	Gene Expression Omnibus
qPCR	Quantitative real-time PCR
TCGA	The cancer genome atlas
siKLF9	KLF9 siRNA

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Introduction

Gastric cancer (GC), also known as stomach cancer, is the fourth leading cause of death from malignancy and accounts for ~ 10% of cancer-associated deaths worldwide [3, 4]. GC cell can invade to other parts of the body, such as the lymph node, liver, and lung. If treated early, GC patients can be cured by surgery. However, the prognoses for people with advanced GC are often poor, with the 5-year survival rate less than 10% [13]. In spite of the great advances in current GC treatment, recurrence and metastasis still often happened. Thus, in depth study of GC development and metastasis is a prerequisite to develop novel diagnostic and therapeutic methods.

The thrombospondins (THBSs), a family of five adhesive glycoproteins, exhibit important roles in cell-to-cell and cell-to-matrix interactions. It is widely accepted that THBS4 forms part of the extracellular matrix and is involved in key cellular processes, such as proliferation, attachment, adhesion, and migration [9]. Moreover, increasing studies suggested that THBS4 was involved in the pathological process of different

types of malignancies. For instance, Greco et al. proved the tumor-suppressing role of THBS4 on the proliferation of colorectal cancer [7]. Dakhova et al. showed that THBS4 was significantly up-regulated during the formation of reactive stroma in prostate cancer [5]. In addition, Förster et al. proved the strong overexpression of THBS4 in gastric adenocarcinomas [6]. However, whether THBS4 contributes to the progress and metastasis of advanced GC remains not known.

Here, we analyzed the normalized Gene Expression Omnibus (GEO) dataset (GSE26253) and found that THBS4 was up-regulated in recurrent GC patients compared to patients without recurrence. Moreover, analysis of GEO dataset (GSE26253) also suggested that increased THBS4 might be significantly associated with the increase in tumor grade and predicted poor prognosis in GC. Hence, we evaluated the potential contribution of THBS4 on GC growth and metastasis and explored the possible mechanisms.

Materials and methods

Cell culture

GC cell lines (MGC-803 and BGC-823) and non-tumoral gastric cell line GES-1 were bought from American Type Culture Collection (ATCC, Manassas, VA). GC cells were kept at 37 °C in RPMI-1640 medium (Life Technologies, Shanghai, China) containing 10% FBS (Gibco, Grand Island, NY) and antibiotic-antimycotic.

MTT assay

One thousand transfected GC cells were seeded into 12-well plates. After 48 h, MTT (Sigma, St. Louis, MO) was added for 4 h at 37 °C. Next, the supernatant was replaced with DMSO. The absorbance at 492 nm was read.

Soft agar colony formation assay

Two thousand GC cells were plated into 6-well plates. After 15 days, cells were washed, fixed, and stained by crystal violet. Photos were taken and colony number was counted via computer software (Bio-Rad Quantity One).

Transwell migration and invasion assay

Cells were re-suspended in serum-free culture medium and transferred into the uncoated or Matrigel-coated upper chambers. Medium (10% FBS) was added into lower chambers. After 24 h, non-migrated or non-invaded cells were mechanically removed. Subsequently, the filter was fixed and dyed by 0.2% crystal violet. Images were captured and positively stained cells were counted.

Quantitative real-time PCR (qPCR)

Total RNA was prepared from cells via TRIzol (Qiagen, Valencia, CA) and cDNA was synthesized. Relative abundances of THBS4 and KLF9 mRNA were evaluated by SYBR green qPCR assay (Sigma, St. Louis, MO) on an FTC-3000TM System (Funglyn Biotech, Canada). The primers used in our study were listed below:

THBS4-fwd: 5′-TGCTGCCAGTCCTGACAGA-3′
THBS4-rev: 5′-GTTTAAGCGTCCCATCACAGTA-3′
KLF9-fwd: 5′-GGGAAACACGCCTCCGAAAA-3′
KLF9-rev: 5′-CGTTCACCTGTATGCACTCTGTA-3′

Plasmid construction, siRNA, and cell transfection

To construct the THBS4 or KLF9 overexpression plasmid, THBS4 or KLF9 cDNA was synthesized and cloned into pEGFP-C1. KLF9 siRNA (siKLF9) and matched negative control (NC) were synthesized by GenePharma. Cell transfection was conducted via jetPRIME Kit (Dakewe, Beijing, China), and the medium was changed at 4 h after transfection.

Bioinformatics analysis

The cancer genome atlas (TCGA) database with designed web tool (https://xenabrowser.net/) was employed to examine the relative expression of THBS4 and KLF9, and subsequently explore the associations between THBS4 and KLF9 expression in GC.

Statistical analyses

Data were presented as mean ± SEM from at least three separate tests. Statistical analysis was carried out via SPSS 12.0 software. Difference was considered significant at P values < 0.05. Pearson's r was calculated to evaluate the associations between THBS4 and KLF9 expression.

Results

THBS4 was significantly up-regulated in recurrent GC patients

To find whether THBS4 is associated with the development of human GC, a normalized GEO dataset (GSE26253) was analyzed (Fig. 1a). Figure 1b showed that there was higher level of THBS4 in tumor tissue samples of recurrent GC patients

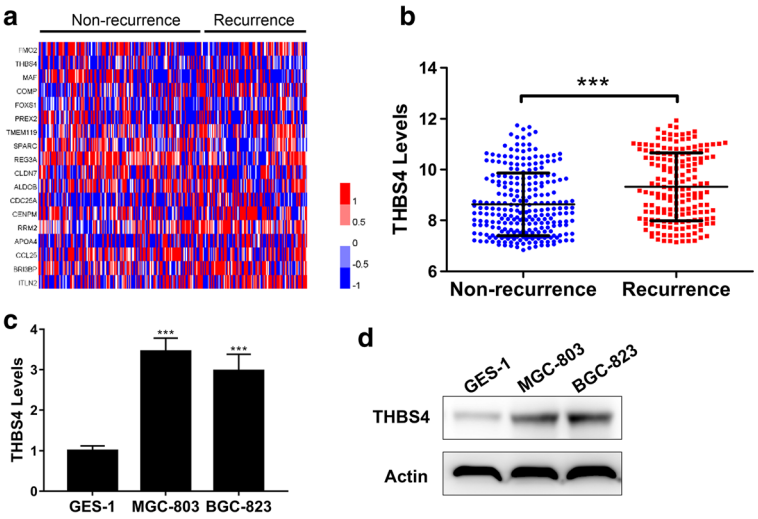

Fig. 1 THBS4 was up-regulated in recurrent gastric carcinoma patients. **a** Heat map represented the expression levels of genes in patients with or without recurrence in GSE26253 gastric carcinoma dataset. **b** The THBS4 level in patients with or without recurrence in GSE26253 GC dataset. **c**, **d** The THBS4 mRNA levels (**c**) and protein levels (**d**) in GC cell lines MGC-803 and BGC-823 compared to non-tumoral gastric cells GES-1. ***$P < 0.001$ (Student's t test)

than that in the samples of GC patients without recurrence ($P < 0.001$). Besides, THBS4 mRNA (Fig. 1c, $P < 0.001$) and protein expression (Fig. 1d) were up-regulated in GC cells (MGC-803 and BGC-823) compared to those in GES-1 cells.

THBS4 had a positive relationship with poor prognosis in gastric cancer

Kaplan-Meier assay revealed that the prognoses of GC patients bearing low level THBS4 were better than those of patients bearing high level THBS4 (Fig. 2a). In addition, Fig. 2b also showed that there is significant upregulation of THBS4 expression in patients with stage 3 GC compared with those of patients with stage 1 or 2 GC ($P < 0.05$), which suggested that there may be a positive relationship between THBS4 expression and increased malignant potential in GC.

THBS4 promoted the proliferation of MGC-803 and BGC-823 cells

To examine the effects of THBS4 on GC proliferation, MGC-803 or BGC-823 cells were transfected via THBS4 overexpression plasmid. QPCR analysis confirmed there was an increase of THBS4 mRNA in MGC-803 and BGC-823 cells transfected with THBS4 overexpression plasmid compared with those in the control group (Fig. 3a, $P < 0.001$). MTT assay revealed that THBS4 overexpression time-dependently fostered the growth of GC cells (Fig. 3b, c, $P < 0.001$). To further investigate the tumor-contributor effect of THBS4 in GC cells, cell count assay was conducted and the results demonstrated that at day 5, THBS4 overexpression stimulated MGC-803 and BGC-823 cell proliferation by 33.33% (Fig. 3d, $P < 0.01$) and 30.77% (Fig. 3e, $P < 0.05$) compared to control group, respectively. Additionally, colony formation assay indicated that at day 15, GC cells transfected with THBS4 overexpression plasmid displayed higher number of colonies than those transfected with empty vector (Fig. 3f, g, $P < 0.001$ and $P < 0.01$ for MGC-803 and BGC-823 cells, respectively).

THBS4 promoted the migration and invasion of GC cells

We performed transwell experiments on MGC-803 and BGC-823 cells. After 24-h incubation, cells, which did not pass through the pores and were trapped in the upper chamber, were considered to be non-metastatic cells. In contrast, cells in the lower chamber were considered to be metastatic cells. Cells in the upper or lower chamber were collected separately and qPCR analysis indicated that THBS4 was up-regulated in metastasis GC cells compared to that in the non-metastasis GC cells (Fig. 4a, b, $P < 0.001$). To further determine whether

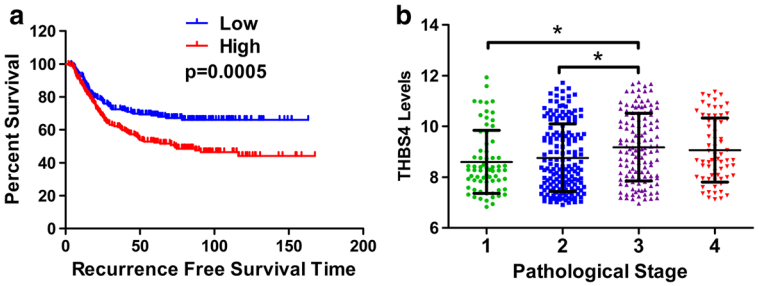

Fig. 2 The positive relationship between THBS4 and poor prognosis in GC. **a** Kaplan-Meier plots of recurrence free survival based on the THBS4 levels in GSE26253 dataset. **b** The THBS4 levels in different pathological stage of gastric cancer patients in GSE26253 dataset. *$P < 0.05$ (ANOVA test)

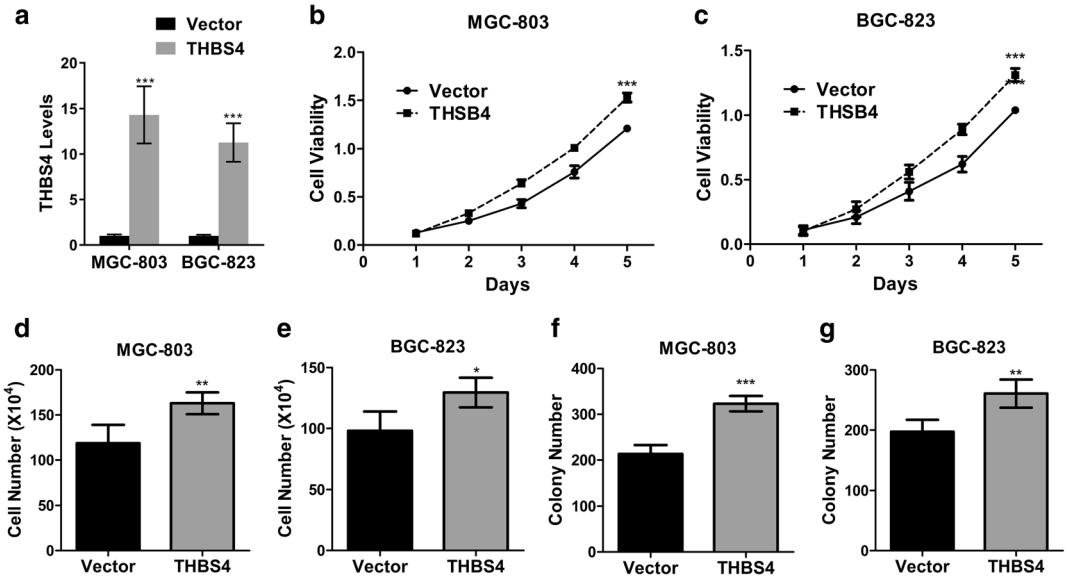

Fig. 3 THBS4 facilitated GC cells proliferation. MGC-803 or BGC-823 cells were transfected with THBS4 overexpression plasmid. **a** THBS4 mRNA abundances in transfected MGC-803 or BGC-823 cells were determined by qPCR. **b**, **c** Cell viability of transfected MGC-803 (**b**) or BGC-823 cells (**c**) was determined via MTT assay. **d**, **e** Cell viability of transfected MGC-803 (**d**) or BGC-823 cells (**e**) was examined via cell count assay. **f**, **g** The viability of transfected MGC-803 (**f**) or BGC-823 cells (**g**) was analyzed via soft agar colony formation assay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (ANOVA test in **b** and **c**, others Student's *t* test)

THBS4 stimulates GC migration and invasion, transwell assay was conducted. Figure 4c, d showed that THBS4 overexpression fostered the migratory and invasion of MGC-803 cells by 81.36% ($P < 0.05$) and 137.5% ($P < 0.01$) by comparison to control groups. In addition, Fig. 4e, f also showed that THBS4 overexpression stimulated the migratory and invasion capacity of BGC-823 cells ($P < 0.01$).

THBS4 up-regulated KLF9 expression in GC cells

The analysis of TCGA database indicated that there may be a positive association between THBS4 and KLF9 expression in GC (Fig. 5a). Moreover, Fig. 5b also showed that THBS4 overexpression plasmid increased KLF9 mRNA level in MGC-803 cells by 233.33% ($P < 0.001$) compared to empty vector.

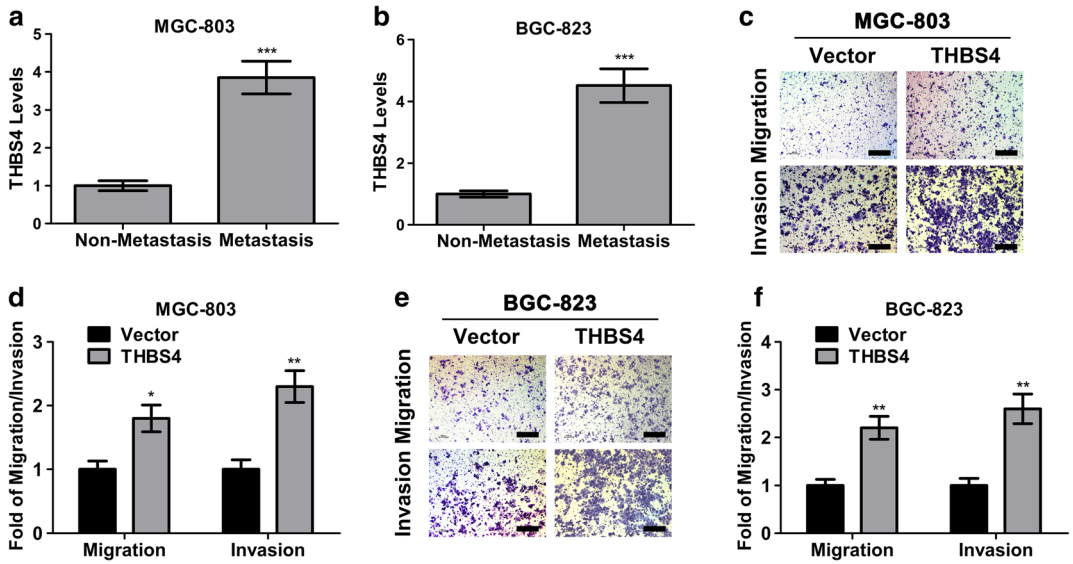

Fig. 4 THBS4 facilitated the migration and invasion of GC cells. **a** High expression of THBS4 in metastasis MGC-803 cells versus non-metastasis MGC-803 cells. **b** High expression of THBS4 in metastasis BGC-823 cells versus non-metastasis BGC-823 cells. **c** Transwell assay of MGC-803 cells transfected with THBS4 overexpression plasmids. Scale bars, 200 μm. **d** The statistical data of **c**. **e**, Transwell assay of BGC-823 cells transfected with THBS4 overexpression plasmids. Scale bars, 200 μm. **f** The statistical data of **e**. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t* test)

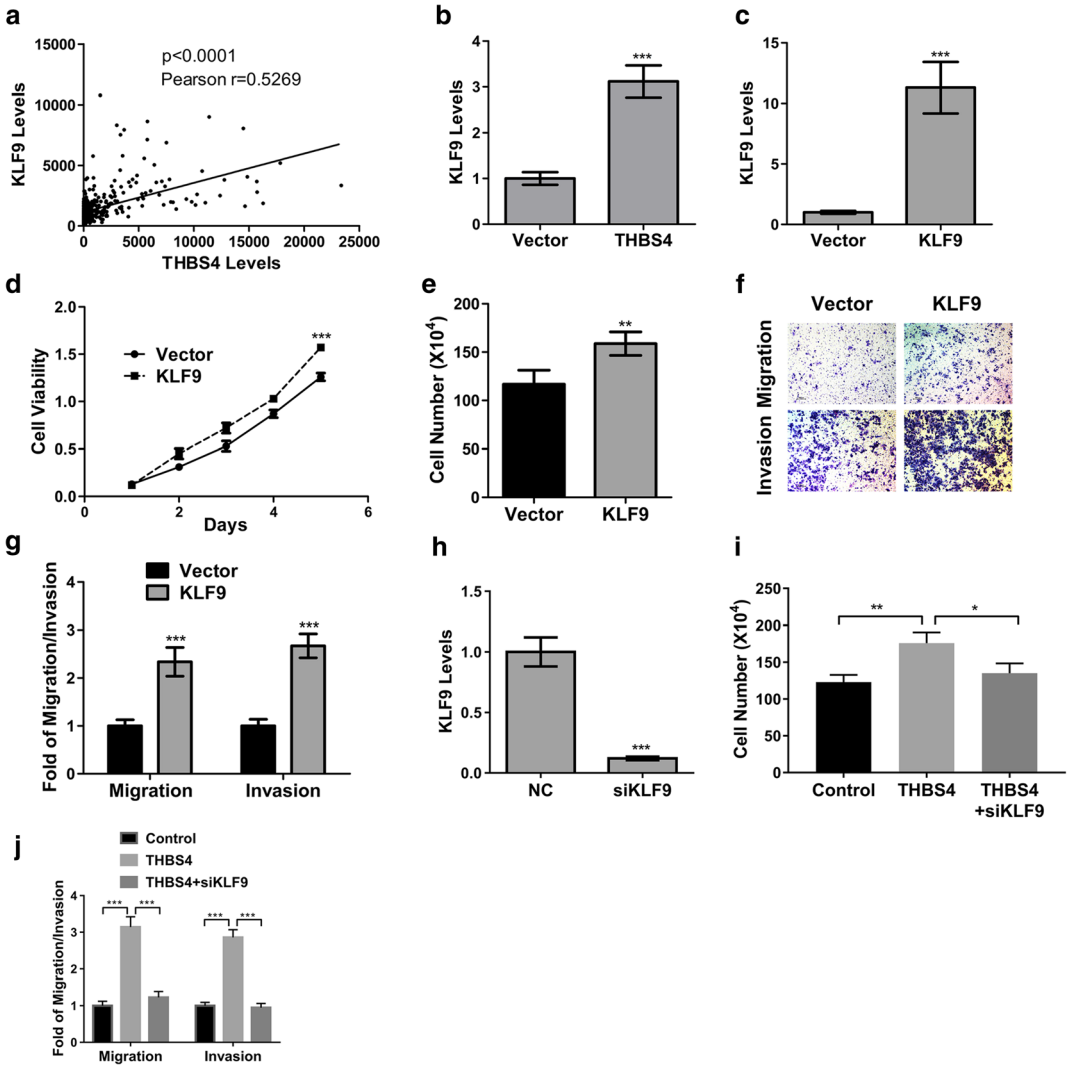

Fig. 5 THBS4 promoted KLF9 expression in GC cells. **a** The correlation of THBS4 and KLF9 expression in TCGA gastric cancer dataset. **b** KLF9 mRNA abundance in MGC-803 cells transfected with THBS4 overexpression plasmid was determined by qPCR. **c** KLF9 mRNA abundance in MGC-803 cells transfected with KLF9 overexpression plasmid was determined via qPCR. **d**, **e** The viability of MGC-803 cells transfected with KLF9 overexpression plasmid was determined via MTT assay (**d**) and cell count assay (**e**), respectively. **f** Transwell assay of MGC-803 cells transfected with KLF9 overexpression plasmids. Scale bars, 200 μm. **g** The statistical data of **f**. **h** KLF9 mRNA abundance in MGC-803 cells transfected with siKLF9 was determined via qPCR. **i** The viability of MGC-803 cells transfected with THBS4 expression plasmid with or without siKLF9 was determined via cell count assay. **j** Transwell assay of MGC-803 cells transfected with THBS4 overexpression plasmid with or without siKLF9. $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA test in **d**, others Student's *t* test)

Next, MGC-803 and BGC-823 cells were transfected via KLF9 overexpression plasmids. Figure 5c confirmed the higher mRNA abundance of KLF9 in MGC-803 cells transfected via KLF9 overexpression plasmid in comparison with that in cells of control groups ($P < 0.001$). Further analysis indicated that transfection with KLF9 overexpression plasmid time-dependently stimulated the proliferation of MGC-803 and BGC-823 cells (Fig. 5d and Fig. S1A, $P < 0.001$). In addition, cell count assay also proved the tumor-promoting effect of KLF9 on the viability of MGC-803 and BGC-823 cells (Fig. 5e and Fig. S1B, $P < 0.01$). On the other hand, KLF9 overexpression enhanced the migration and invasion of MGC-803 and BGC-823 cells (Fig. 5f, g and Fig. S1C, $P < 0.001$).

Figure 5h confirmed the lower level of KLF9 mRNA in MGC-803 cells transfected via siKLF9 than that in cells of NC groups ($P < 0.001$). Next, MGC-803 cells transfected with THBS4 overexpression plasmid with or without siKLF9. Cell count assay indicated that siKLF9 inhibited the enhanced viability of MGC-803 cells caused by the transfection with THBS4 overexpression plasmid (Fig. 5i, $P < 0.05$). Moreover, transwell assay found that the transfection with

THBS4 overexpression plasmid enhanced the migration and invasion of MGC-803 cells, whereas co-transfection with siKLF9 abolished the above phenomena (Fig. 5j, $P < 0.001$).

Discussion

THBS4, an extracellular calcium-binding protein, exhibited inhibitory activities against colorectal cancer [7]. In contrast, increasing studies have demonstrated that THBS4 has tumor-promoting effects on many other type of tumors, such as hepatocellular carcinoma [17], prostate cancer [9], and breast cancer [1, 11]. However, the effect of THBS4 on GC is less well known. Therefore, we attempted to investigate the biological activities of THBS4 in GC development and metastasis to provide more clues to develop new powerful targets for GC treatment.

Bioinformatics analysis of GEO dataset (GSE26253) showed that THBS4 expression was up-regulated in recurrent GC patients compared to GC patients without recurrence, which indicated the carcinogenic potential of dys-regulated THBS4 expression. Patients bearing high level THBS4 had statistically poorer prognosis than those bearing low-level THBS4. Moreover, THBS4 expression in patients with stage 3 GC was significantly up-regulated compared to that of patients with stage 1 or 2 GC. Thus, we speculated that THBS4 upregulation might be linked to GC development and metastasis. In current study, the results of MTT, cell count, and colony formation assays showed that THBS4 overexpression stimulated tumor cell proliferation of GC cells. Moreover, transwell assay revealed that stable overexpression of THBS4 fostered the migration and invasion of GC cells. On the other hand, previous study found that THBS4 was primarily expressed and secreted by cancer-associated fibroblasts in diffuse-type gastric adenocarcinomas [6]. Here, we found that MGC-803 and BGC-823 cells also expressed THBS4 mRNA. Moreover, metastasis GC cells had higher abundance of THBS4 mRNA than non-metastasis GC cells.

Further bioinformatics analysis suggested that there was a significant positive relationship between THBS4 and KLF9 expression in GC. Moreover, qPCR analysis proved that THBS4 overexpression significantly up-regulated KLF9 expression in GC cells, which suggested that THBS4 may exhibit positive effects on GC growth and metastasis via modulating KLF9 in vitro.

KLF9, also known as BTEB Protein 1, is a member of Krüppel-like factor (KLF) family of transcription factors [16, 18]. KLF family has important activities in a wide range of biological events, such as cell proliferation, adhesion, and apoptosis [12, 20]. Like other members of the KLF family, KLF9 has conserved C2H2-type zinc finger DNA-binding domain [2, 14]. Moreover, KLF9 is down-regulated and has tumor-suppressing effects on the carcinogenesis and progression of various human malignancies, such as lung carcinoma [19], pancreatic ductal adenocarcinoma [10], glioma [8, 21], and prostate cancer [15]. On the other hand, KLF9 expression was enhanced in tumor samples of ovarian cancer patients by comparison to neighboring normal tissues, and KLF9 knockdown inhibited ovarian cancer cell growth [22]. However, the biological activities of dys-regulated KLF9 on GC progression are less well known.

Therefore, we overexpressed KLF9 and found that KLF9-treated MGC-803 cells had a significant accumulation of KLF9 mRNA. Moreover, KLF9 overexpression resulted in stimulated cell proliferation and improved migration and invasion in GC cells. Besides, co-transfection with siKLF9 inhibited the enhanced viability, migration, and invasion of GC cells caused by the transfection with THBS4 overexpression plasmid. Hence, the above results suggested that THBS4 may contribute to GC development and metastasis through modulating KLF9 related signaling pathways in vitro.

In summary, our study confirmed THBS4 upregulation was positively linked with increased malignant potential and poor clinical outcome in GC. Moreover, our study also suggested that THBS4 acted as an oncogenic driver during GC progress and metastasis through regulating KLF9 expression. Therefore, all these findings not only suggested that THBS4 might have clinical potential as a novel prognostic biomarker, through which patients with risk of poor prognosis could be confirmed, but also suggested that both THBS4 and KLF9 could be employed as new powerful therapy targets for future treatment of GC.

Conclusion

Our findings suggested that THBS4 contributed to the proliferation and metastasis of GC cells via mediating KLF9 expression.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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