SUZ12 promotes gastric cancer cell proliferation and metastasis by regulating KLF2 and E-cadherin

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Abstract SUZ12 is a core component of the polycomb repressive complex 2 (PRC2), which could silence gene transcription by generating trimethylation on lysine 27 residue of histone H3 (H3K27Me3). Meanwhile, SUZ12 has been found to be overexpressed in multiple cancers; however, the clinical significance and molecular mechanisms of SUZ12 controlling gastric cancer cell proliferation and metastasis are unclear. In this study, we found that SUZ12 expression was significantly increased in 64 gastric tumor tissues compared with normal tissues. Additionally, SUZ12 expression was associated with pathological stage, metastasis distance, and shorter overall survival of gastric cancer patients. Knockdown of SUZ12 expression impaired cell proliferation and invasion in vitro, leading to the inhibition of metastasis in vivo. Upregulation of SUZ12 was found to play a key role in gastric cancer cell proliferation and metastasis through the regulation of EMT and KLF2 expression.

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Introduction

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, and gastric cancer remains one of the most common malignancies, especially in East Asia [1]. Recently, although the clinical outcome of gastric cancer has gradually improved, the prognosis of patients with advanced disease is still disappointing, and the 5-year survival rate of patients with gastric cancer still remains relatively low [2]. The carcinogenesis of gastric cancer was complicated involving dysregualtion of a large number of oncogenic and tumor-suppressive genes; however, the molecular mechanisms underlying the development and progression of gastric cancer are still poorly understood [3]. Therefore, it is crucial for us to find novel biomarkers or factors that predict prognosis in patients with gastric cancer and advance development of targeted clinical therapy.

Recently, silencing of lots of genome regions by epigenetic mechanisms was reported to occur frequently in multiple cancers. Epigenetic marks, such as DNA methylation, histone acetylation, and methylation, are altered at these loci [4]. The regulation of histone modification is complex and can result from the interaction of multiple factors including polycomb complexes and long noncoding RNAs [5, 6]. SUZ12 is a core component of polycomb repressive complex 2 (PRC2), which also contains the catalytic subunit EZH2 and EED [7]. PRC2 was reported to take part in the delineation and regulation of gene clusters, such as the HOX clusters through the deposition of H3K27me3 and chromatin compaction mechanisms [8]. SUZ12 has been found to be required for

both histone methyltransferase activity and the silencing function of the EED-EZH2 complex [9].

To date, more and more evidence indicate that SUZ12 plays an important role in carcinogenesis by acting as an oncogene, such as promotes cell proliferation, inhibits cell apoptosis, and stimulates cell metastasis. Moreover, amplified and overexpressed SUZ12 was found in several human cancers, such as ovarian cancer, mantle nonsmall cell lung cancer, and breast cancer [10–14]. As another subunit of PRC2, EZH2 was demonstrated to be involved in pathogenesis of human gastric cancer [15, 16]. However, the SUZ12 expression pattern and biological function in gastric cancer cells are still not well documented.

In this study, we examined the expression of SUZ12 in human gastric cancer tissues and investigated the potential biological roles of SUZ12 in cell proliferation, invasion, and metastasis in vitro and in vivo. Upregulation of SUZ12 was found to play a key role in gastric cancer cell proliferation and metastasis, which may partly through regulating E-cadherin and KLF2 expression. This study advances our understanding of the role of SUZ12 as a regulator of pathogenesis of gastric cancer, suggesting that SUZ12 could be a biomarker for poor prognosis of gastric cancer.

Materials and methods

Tissue collection

Sixty-four gastric cancer samples were obtained from patients who had underwent surgery at Jiangsu province hospital between 2009 and 2010 and were diagnosed with gastric cancer (stages II, III, and IV; seventh edition of the AJCC Cancer Staging Manual) based on histopathological evaluation. No local or systemic treatment was conducted in these patients before the operation. All specimens were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The study was approved by the Research Ethics Committee of Nanjing Medical University, China. Informed consents were obtained from all patients.

Cell lines and culture conditions

Four gastric cancer cell lines (SGC7901, BGC823, MGC803, AGS) and a normal gastric epithelium cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 or DMEM (GIBCO-BRL) medium supplemented with 10 % fetal bovine serum (10 % FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37 °C with 5 % CO2.

RNA extraction and qPCR analyses

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). For qPCR, RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed with Power SYBR Green (Takara, Dalian China). Results were normalized to the expression of GAPDH. The PCR primers are shown in supplementary Table 1. qPCR and data collection were performed on ABI 7500. The relative expression of SUZ12 was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH.

Cell transfection

The siRNAs for SUZ12 [13] or si-NC were transfected into SGC7901 or BGC823 cells. Either SGC7901 or BGC823 cells were grown in 6-well plates until confluent, with transfection facilitated by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48-h posttransfection, cells were harvested for qPCR or Western blot analysis .

Cell proliferation assays

Cell proliferation was monitored using a Cell Proliferation Reagent Kit I (MTT) (Roche Applied Science). The cells transfected with si-SUZ12 (3000 cells/well) were grown in 96-well plates. Cell proliferation was assessed every 24 h following the manufacturer's protocol. For colony formation assays, si-SUZ12-transfected SGC7901 and BGC823 cells ($n=$ 500) were placed in a 6-well plates and maintained in media containing 10 % FBS. The medium was replaced every 4 days; after 14 days, cells were fixed with methanol and stained with 0.1 % crystal violet (Sigma-Aldrich). Visible colonies were then counted. For each treatment group, wells were assessed in triplicate.

Flow cytometry analysis of apoptosis

Cells were harvested at 48-h posttransfection by trypsinization. After staining with FITC-Annexin V and propidium iodide, cells were analyzed by flow cytometry (FACScan; BD Biosciences) using CellQuest software (BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. The ratio of early apoptotic cells was compared to that for controls from each experiment. All samples were assayed in triplicate.

Wound-healing assay

For the wound-healing assay, 3×10^5 cells were seeded in 6-well plates, cultured overnight, and transfected with si-SUZ12 or the si-NC. Once cultures reached 85 % confluency, the cell

layer was scratched with a sterile plastic tip and washed with culture medium, then cultured for 48 h with medium containing 1 % FBS. At different time points, images of the plates were acquired using a microscope.

Cell migration and invasion assays

For the migration assays, at 48-h posttransfection, 5×10^4 cells in serum-free media were placed into the upper chamber of an insert (8-μm pore size; Millipore). For the invasion assays, 1×10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich). Medium containing 10 % FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1 % crystal violet, imaged, and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were independently repeated three times.

Tail vein injections into athymic mice

Athymic male mice (4 weeks old) were purchased from the Animal Center of the Chinese Academy of Science (Shanghai, China) and maintained in laminar flow cabinets under specific pathogen-free conditions. BGC823 cells transfected with sh-SUZ12 or the empty vector were harvested from 6-well plates, washed with phosphate-buffered saline (PBS), and resuspended at 2×10^7 cells/ml. Suspended cells (0.1 ml) were injected into the tail veins of seven mice, which were sacrificed 8 weeks after injection. The lungs were removed and photographed, and visible tumors on the lung surface were counted. This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering [17].

Chromatin immunoprecipitation

BGC823 cells were treated with formaldehyde and incubated for 10 min to generate DNA-protein cross-links. Cell lysates were then sonicated to generate chromatin fragments of 200–300 bp and immunoprecipitated with SUZ12 (Millipore, Cat. #03–179) and H3K27me3-specific antibody (Millipore, Cat. #07–449) or IgG as control. Precipitated chromatin DNA was recovered and analyzed by qPCR.

Western blotting analysis

Cells were lysed using RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with a protease inhibitor cocktail (Roche, CA, USA) and phenylmethylsulfonyl fluoride (Roche). The concentration of proteins was determined using the Bio-Rad protein assay kit. Protein extracts (50 μg) were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes (Sigma), and incubated with specific antibodies. Autoradiograms were quantified by densitometry using Quantity One software (Bio-Rad, CA, USA), with GAPDH (CST, Cat.#2118) used as a control. Antibodies against E-cadherin (1:1000 dilution) was purchased from CST (Cat. #3195). Antibodies against KLF2 were purchased from sigma Technology (Cat.# AV37859).

Statistical analysis

Student's t test (two-tailed), one-way ANOVA, and the Mann–Whitney U test were used to analyze data, along with SPSS 16.0 (IBM, IL, USA). P values of less than 0.05 were considered statistically significant.

Results

SUZ12 expression was upregulated and correlated with poor prognosis of gastric cancer

SUZ12 mRNA expression levels were investigated in 64 paired gastric cancer samples and adjacent histologically normal tissues using quantitative polymerase chain reaction (qPCR) assays. SUZ12 expression was significantly upregulated ($P<0.01$) in cancerous tissues compared with normal tissues (Fig. 1a). In addition, SUZ12 protein expression levels in gastric cancer were also upregulated (Fig. 1b). These data indicate that abnormal SUZ12 expression may be related to gastric cancer pathogenesis.

According to the median ratio of relative SUZ12 expression in tumor tissues, the 64 gastric cancer patients were classified into two groups: relative high-SUZ12 group ($n=32$, SUZ12 expression ratio≥median ratio) and relative low-SUZ12 group ($n=32$, SUZ12 expression ratio≤median ratio). Clinicopathological factors were compared between the two groups (Table 1). The high-SUZ12 group shown was correlated with advanced pathological stage ($P<0.006$), tumor size ($P=0.023$), and lymph node metastasis ($P=0.045$). SUZ12 expression was not associated with other parameters such as
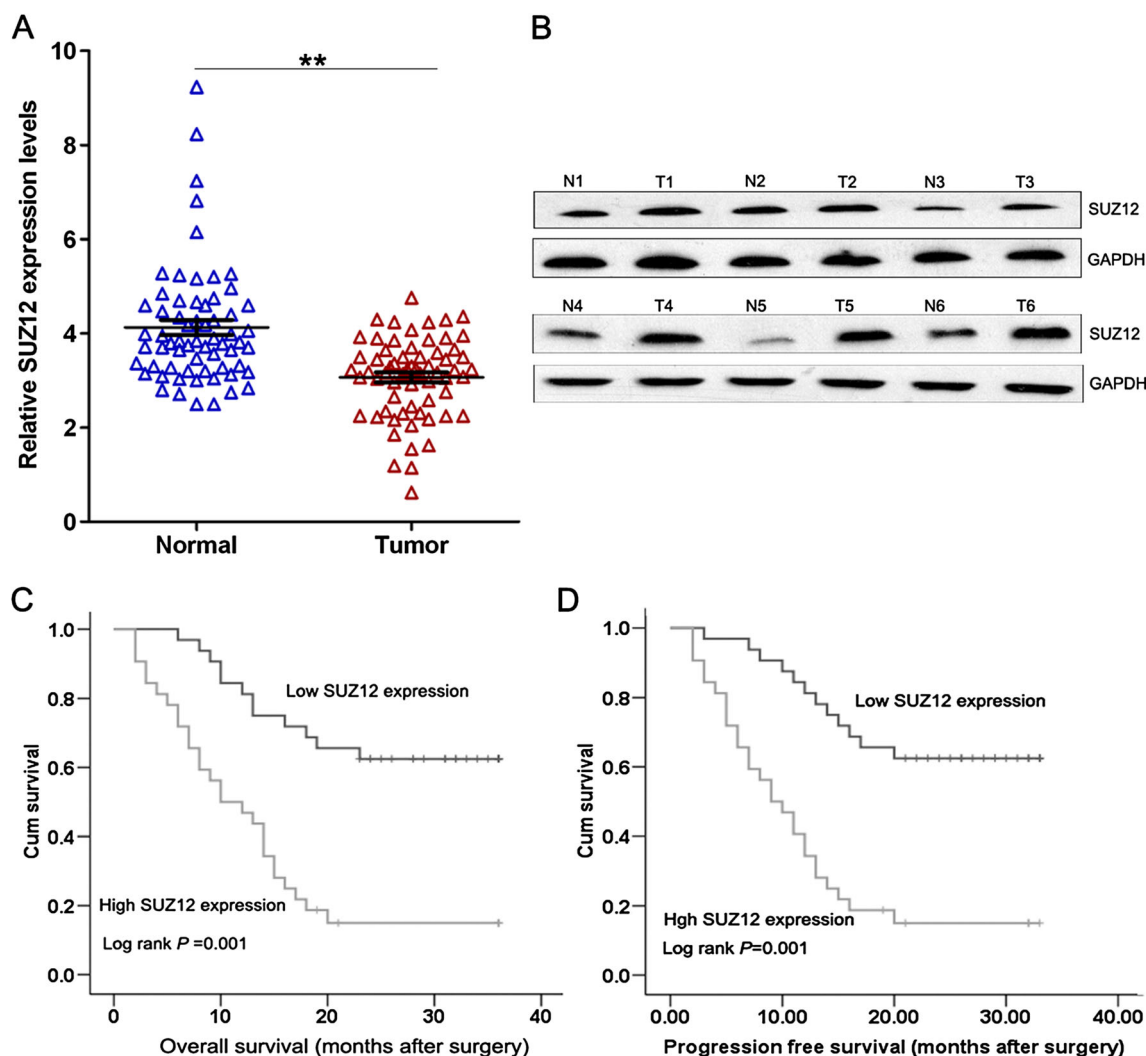
Fig. 1 Relative SUZ12 mRNA expression in gastric cancer tissues and its clinical significance. **a** Relative expression of SUZ12 mRNA in gastric cancer tissue ($n=64$) comparison with corresponding nontumor normal tissues ($n=64$). SUZ12 mRNA expression was examined by qPCR and normalized to GAPDH expression. Data was presented as $^\triangle$CT valus. **b** SUZ12 protein expression levels that are gastric cancer tissues were examined by Western blot. **c, d** Kaplan–Meier overall survival and progression-free survival curves according to SUZ12 expression level. The overall survival and progression-free survival of the high-SUZ12 group were significantly lower than those of low-SUZ12 group ($P<0.001$, log-rank test). ******$P<0.01$

gender ($P=0.446$) and age ($P=0.617$) in gastric cancer (Table 1).

Association between SUZ12 expression and patient survival

Moreover, we investigated whether SUZ12 expression level was correlated with outcome of gastric cancer patients after gastrectomy. Overall survival (OS) and progression-free survival (PFS) curves were plotted according to SUZ12 expression level by the Kaplan–Meier analysis and log-rank test. The results showed that patients with higher SUZ12 expression level had poorer overall survival and progression-free survival ($P=0.001$) (Fig. 1c, d). These results together suggested that overexpression of SUZ12 in gastric cancer was significantly correlated with patients' survival time.

Modulation of SUZ12 expression in gastric cancer cells

Expression levels of SUZ12 in gastric cancer cell lines were determined by Western blot. Compared with that in normal tissues and GES1 cells, relative expression levels of SUZ12 were increased in gastric cancer cells (Fig. 2a, b). In order to manipulate SUZ12 level in gastric cancer cells, three SUZ12 siRNA were transfected into SGC7901 and BGC823 cells to downregulate endogenous SUZ12 expression. QPCR analysis revealed that SUZ12 expression was decreased in si-SUZ12-transfected cells when compared with si-NC control cells (Fig. 2c). Moreover, the results of Western blot assay showed that SUZ12 protein levels were also downregulated in si-SUZ12-transfected cells compared with si-NC control cells (Fig. 2d).

Table 1 Correlation between SUZ12 expression and clinicopathological characteristics in patients with gastric cancer

Clinical parameter	SUZ12		Chi-squared test P value
	Low-SUZ12 group, no. of cases	High-SUZ12 group, no. of cases	
Age (years)			0.617
<50	15	18	
>50	17	14	
Gender			0.446
Male	17	21	
Female	15	11	
Location			0.871
Distal	12	13	
Middle	13	12	
Proximal	7	7	
Size			0.023
>5 cm	10	20	
<5 cm	22	12	
Histologic differentiation			0.623
Well	2	2	
Moderately	14	9	
Poorly	13	17	
Undifferentiated	3	4	
Invasion depth			0.012
T1	11	4	
T2	13	7	
T3	4	11	
T4	4	10	
TNM stage			0.006
I	10	3	
II	14	8	
III	6	18	
IV	1	3	
Lymphatic metastasis			0.045
Yes	12	21	
No	20	11	
Regional lymph nodes			0.005
PN0	19	12	
PN1	9	3	
PN2	2	10	
PN3	2	7	
Distant metastasis			0.057
Yes	0	4	
No	32	28	

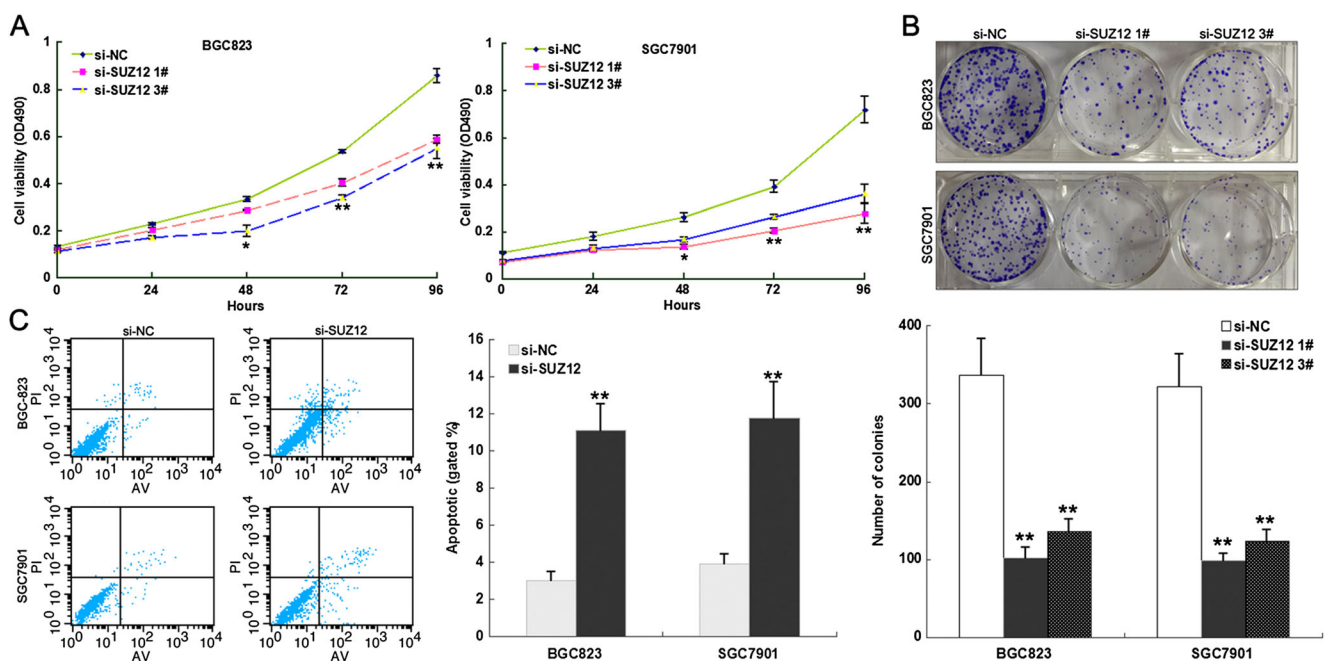### Knockdown of SUZ12 inhibits gastric cancer cell proliferation and induces apoptosis

To assess the biological role of SUZ12 in gastric cancer cells, we investigated the effects of SUZ12 downregulation on the proliferation and apoptosis of SGC7901 and BGC823 cells.

MTT assay results showed that the growth of SGC7901 and BGC823 cells transfected with si-SUZ12 was impaired compared with that for control cells (Fig. 3a). Colony formation assay results revealed that clonogenic survival was inhibited following knockdown of SUZ12 in SGC7901 and BGC823 cells (Fig. 3b). Flow cytometry analysis of SGC7901 and
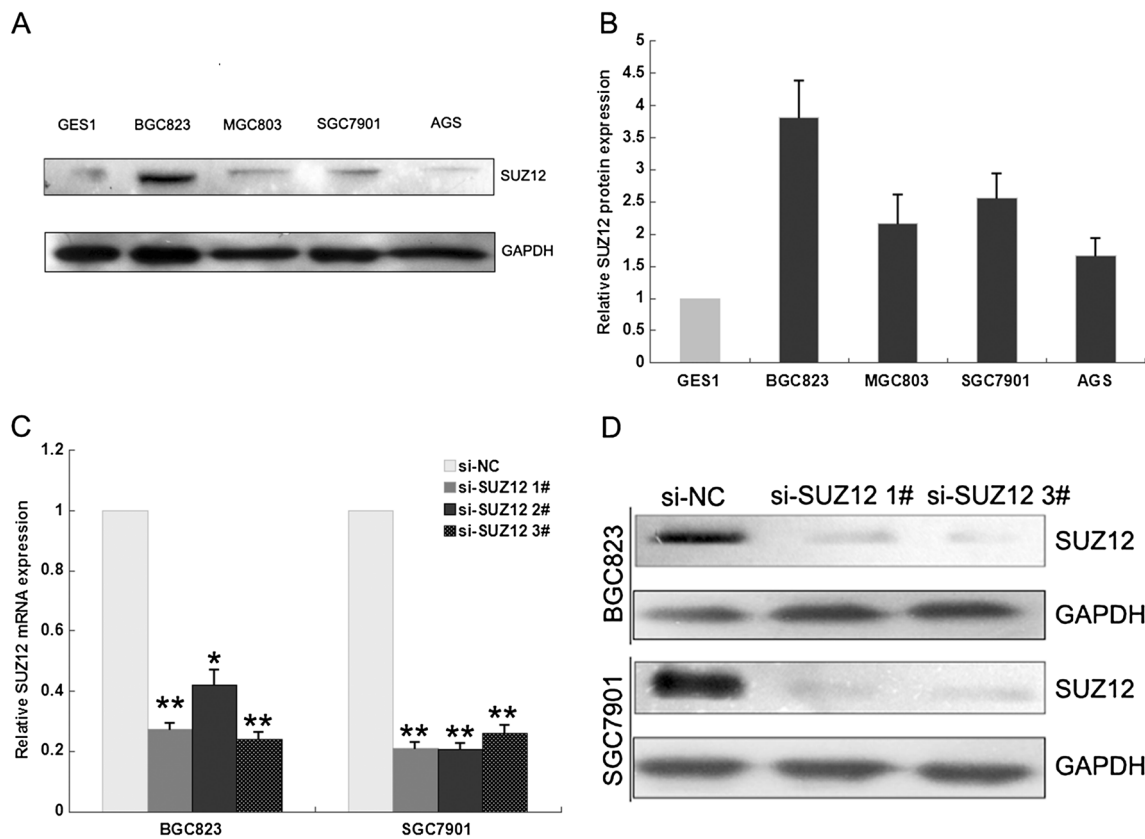
Fig. 2 The level of SUZ12 expression in gastric cancer cells. **a, b** Results from Western blot demonstrating SUZ12 protein levels in gastric cancer in gastric cancer cell lines (SGC7901, AGS, MGC803, and BGC823) and normal human gastric epithelial cell line (GES-1) were detected by Western blot. **c, d** QPCR and Western blot analyses of SUZ12 expression level following treatment BGC823 and SGC7901 cells with si-SUZ12. *P<0.05, **P<0.01

Fig. 3 The effect of knockdown of SUZ12 on gastric cancer cell proliferation and apoptosis in vitro. BGC823 and SGC7901 cells were transfected with si-SUZ12 or si-NC, respectively. **a** MTT assay was performed to determine the proliferation of si-SUZ12-transfected BGC823 and SGC7901 cells. Data represent the mean±sd from three independent experiments. **b** Colony-forming growth assay was performed to determine the colony formation ability of si-SUZ12-transfected BGC823 and SGC7901 cells. The colonies were counted and captured. **c** Flow cytometry analysis of cell apoptosis in BGC823 and SGC7901 cells transfected with si-SUZ12. *P<0.05 and **P<0.01
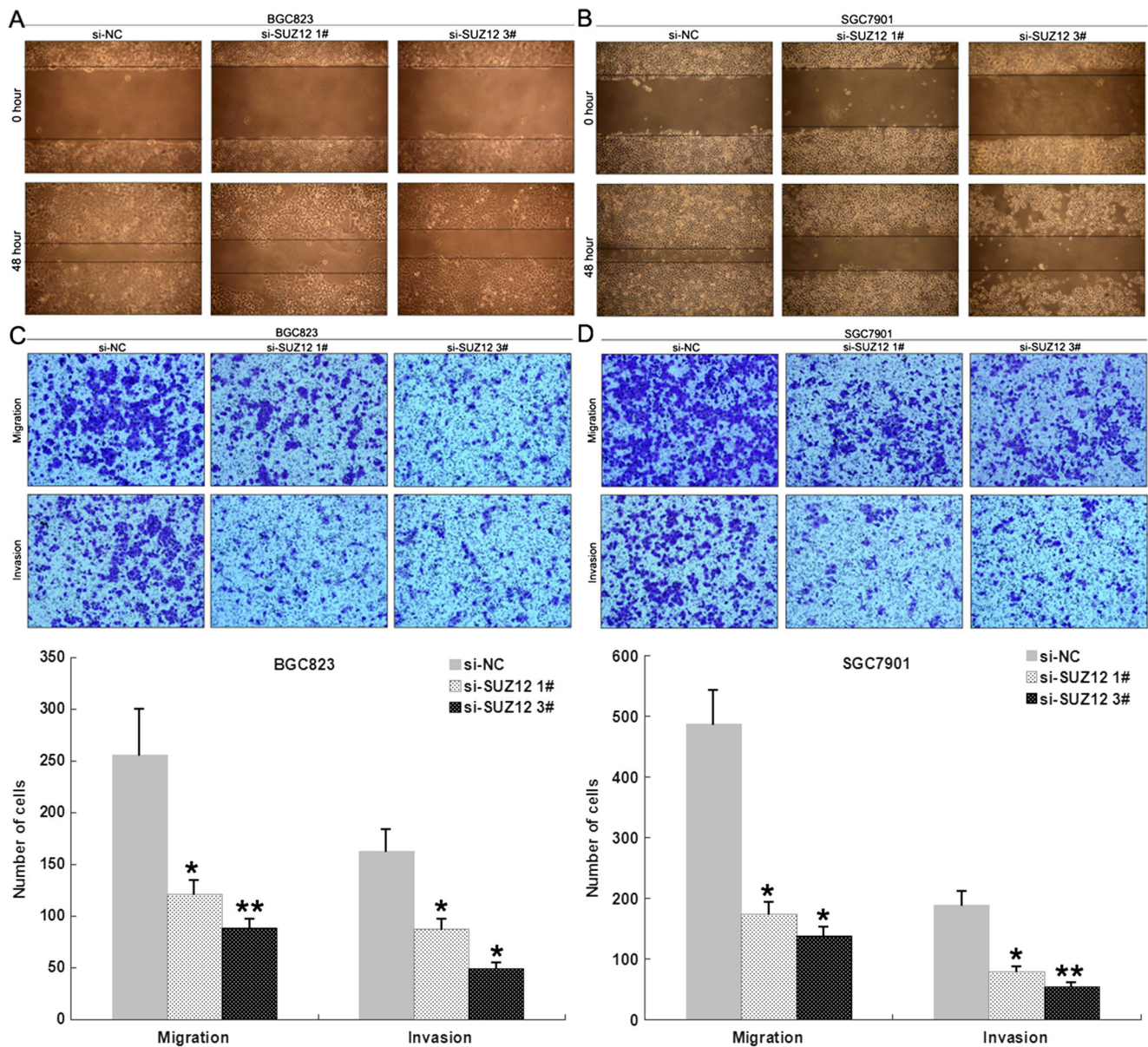
Fig. 4 The effect of inhibition of SUZ12 on gastric cancer cell migration and invasion in vitro. BGC823 and SGC7901 cells were transfected with si-SUZ12 or si-NC, respectively. **a**, **b** Wound-healing assays were used to investigate the migratory ability of BGC823 and SGC7901 cells. **c**, **d** Transwell assays were used to investigate the changes in migratory and invasive abilities of BGC823 and SGC7901 cells. *P<0.05 and **P<0.01

BGC823 cells showed that downregulation of SUZ12 expression promoted apoptosis in comparison with that in control cells (Fig. 3c).

Downregulation of SUZ12 inhibits migration and invasion of gastric cancer cells

The wound-healing assay results showed that cells transfected with si-SUZ12 resulted in a slower closing of scratch wounds compared with that for control cells (Fig. 4a, b). Furthermore, we evaluated cancer cell invasion through matrigel and migration through transwells. Decreased SUZ12 expression levels impeded the migration of SGC7901 and BGC823 cells compared with controls (Fig. 4c, d). Similarly, invasion of SGC7901 and BGC823 cells was also reduced following downregulation of SUZ12 expression.

Decreased SUZ12 suppresses gastric cancer cell metastasis in vivo

To validate the effects of SUZ12 on the metastasis of gastric cancer cells in vivo, BGC823 cells stably transfected with sh-SUZ12 were injected into nude mice. Metastatic nodules on the surface of the lungs were counted after 8 weeks. Ectopic

Fig. 5 Effects of SUZ12 downregulation on tumor metastasis in vivo. **a** Analysis of an experimental metastasis animal model was performed by injecting SUZ12-knockdown BGC823 cells into nude mice. **b** The lungs from mice in each experimental group, with the numbers of tumor nodules on lung surfaces, were shown. **c** Visualization of the entire lung, and HE-stained lung sections. ****P<0.01**
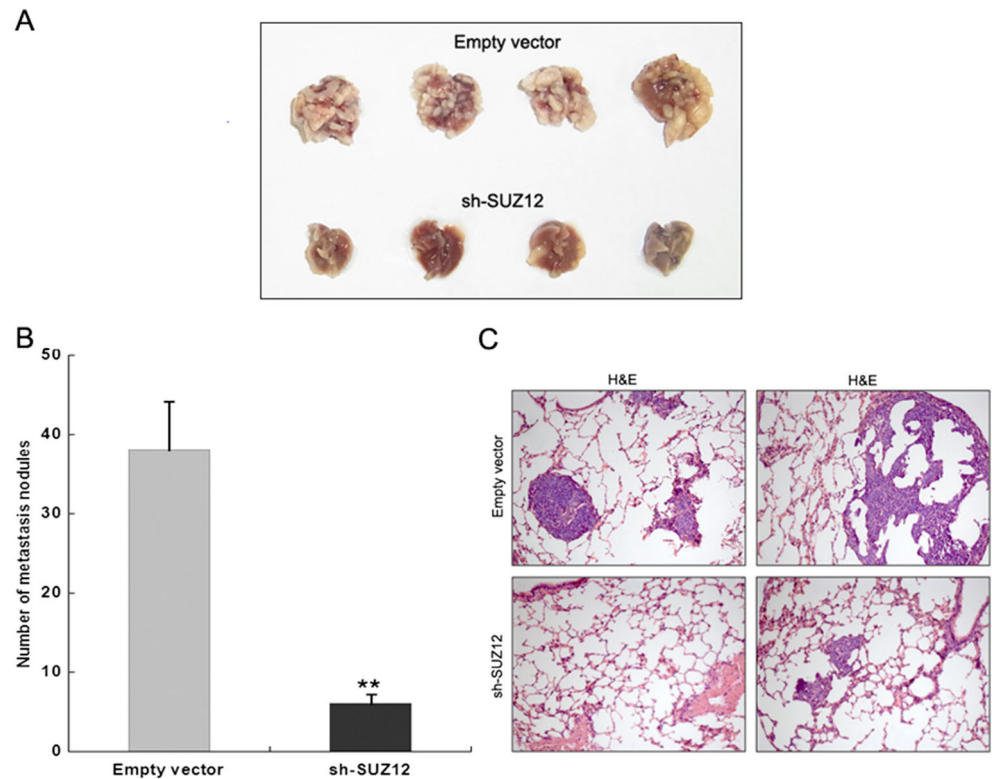
knockdown of SUZ21 resulted in a reduction of the number of metastatic nodules compared with those in the control group (Fig. 5a, b). This difference was further confirmed following examination of the entire lungs and through hematoxylin and eosin (HE) staining of lung sections (Fig. 5c). Our in vivo data complemented the results of functional in vitro studies involving SUZ12.

KLF2 and E-cadherin are key downstream mediators of SUZ12

To investigate the potential target genes of SUZ12 in gastric cancer cells, we first analyzed the data of chip-on-chip from GEO and found that there are many SUZ12 and H3K27me3 binding sites in the promoter regions of KLF family members. Meanwhile, the expression levels of cyclin-dependent kinase inhibitors were also detected in gastric cancer cells when transfected with si-SUZ12. The qPCR results showed that the expression levels of KLF2 and E-cadherin were both increased in si-SUZ12-transfected BGC823 and SGC7901 cells, but no significant changes were found in p15 and p16 expression levels (Fig. 6a, b). Moreover, increased KLF2 and E-cadherin protein level were observed. Meanwhile, other EMT regulators including ROCOK1 and ROBO1 levels were detected in si-SUZ12-transfected gastric cancer cells, and the results showed that ROCOK1 and ROBO1 protein levels were deceased in BGC823 cells transfected with si-SUZ12 compared to those with si-NC (Fig. 6c, d).

To further investigate whether SUZ12 silences KLF2 and E-cadherin expression through binding to their promoter regions, we performed ChIP analysis. The results of ChIP assays showed that SUZ12 could directly bind to KLF2 and E-cadherin promoter region (Fig. 6e). However, knockdown of SUZ12 could reduce SUZ12-mediated H3K27me3 modification in KLF2 and E-cadherin promoter (Fig. 6f). These data suggested that SUZ12 promotes gastric cancer cell proliferation and metastasis at least partly via regulation KLF2 and E-cadherin expression.

Discussion

Generally, the development of cancer has been viewed as process that is driven by progressive genetic abnormalities including deletion or mutations in tumor suppressor genes, amplification of oncogenes, and chromosomal abnormalities [18–20]. However, "epigenetic changes" that do not affect the primary DNA sequence have also been found to contribute to alter gene expression in cancer cells [21, 22]. These epigenetic changes involve both DNA methylation or demethylation as well as altered patterns of histone modifications [23]. As an important subunit of PRC2 that regulae target gene expression via the DNA methylation and deposition of H3K27me3, SUZ12 was reported to overexpressed in multiple cancers and silence a lot of tumor suppressor gene transcription. For example,
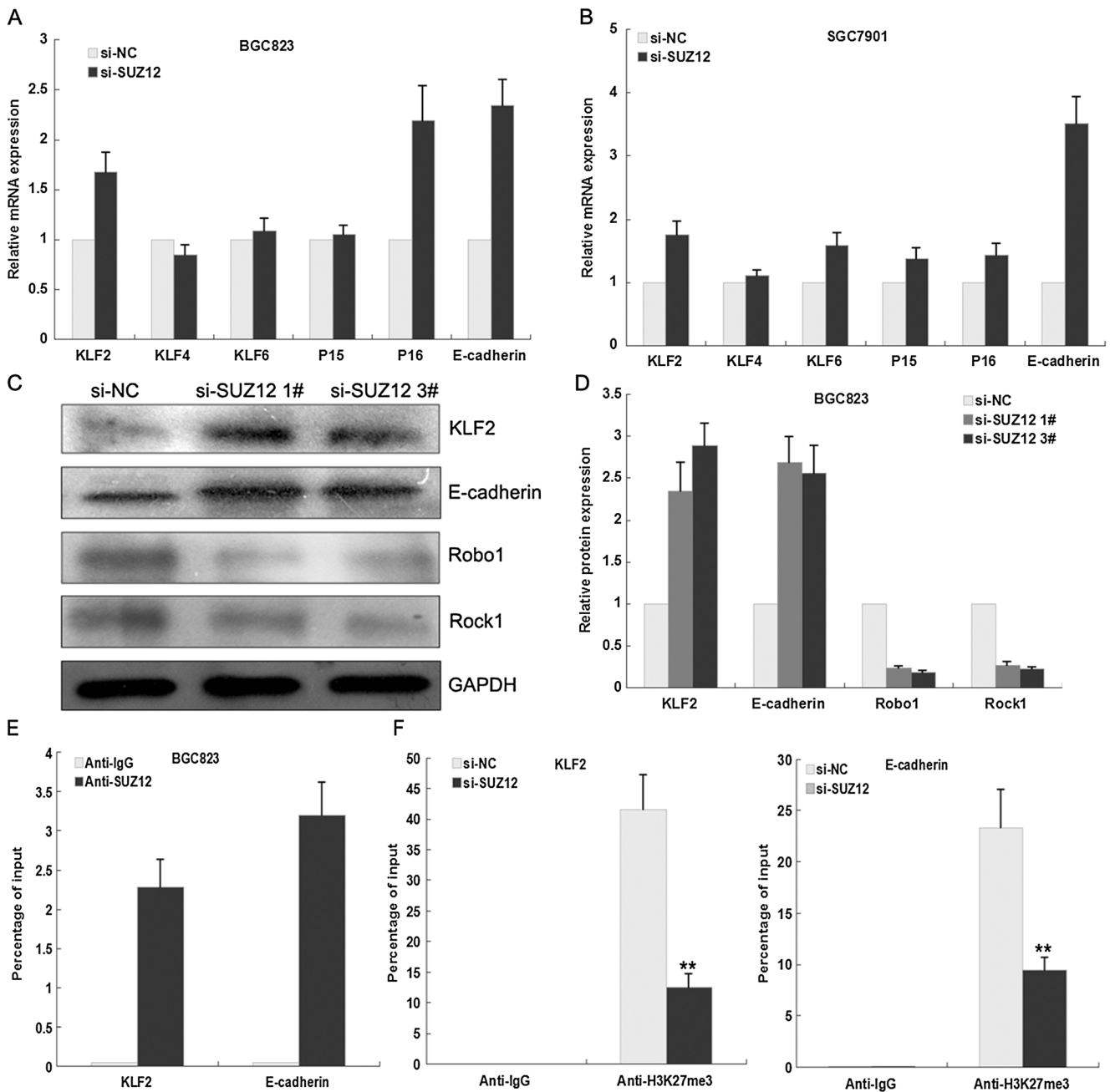
Fig. 6 SUZ12 repressed KLF2 and E-caderin expression by directly binding to promoter regions. **a**, **b** QPCR assays were performed to detect KLF2, KLF4, KLF6, P15, P16, and E-cadherin expression in BGC823 and SGC7901 cells transfected with si-SUZ12. **c**, **d** Western blot analysis of KLF2, Rock1, Robo1, and E-cadherin protein levels in BGC823 cells transfected with si-SUZ12. **e** ChIP–qPCR of SUZ12 occupancy binding in the KLF2 and E-cadherin promoter in BGC823 cell lines. **f** ChIP–qPCR of H3K27-3me binding in the KLF2 and E-cadherin promoter in BGC823 lines treated with SUZ12 siRNA (48 h) or scrambled siRNA. *$P<0.05$, **$P<0.01$

SUZ12 expression is significantly upregulated in human epithelial ovarian cancer (EOC) and increased SUZ12 promotes the proliferation of EOC cells by inhibiting apoptosis via silencing HRK transcription [11]. Moreover, SUZ12 also involved in long noncoding RNA mediated promoting cancer cell invasion and metastasis [24]. Fan Y et al. found that TGF-β-induced upregulation of malat1 promotes bladder cancer cell invasion and metastasis by

associating with SUZ12 [25]. These findings indicated that SUZ12 play an important role in cancer development.

In this study, we found that the SUZ12 expression was significantly upregulated in gastric cancer tissues and cells, and increased SUZ12 expression was correlated with patient tumor size, pathological stage, metastasis distance, and shorter overall survival. Moreover, knockdown of SUZ12 expression inhibited gastric cancer cell proliferation, promoted cell

apoptosis, and impaired cell invasion and metastasis in vitro and in vivo. SUZ12 and other PRC2 members were also reported to be overexpressed in small cell lung cancer (SCLC), and PRC2 might contribute to genesis of SCLC by repressing JUB in SCLC cells [26]. Moreover, SUZ12 is also anomalously expressed in human mantle cell lymphoma (MCL), pulmonary carcinomas and melanoma, and SUZ12 loss increases apoptosis, inhibits cell viability, and targets genes involved in central oncogenic pathways associated with MCL pathogenesis [12]. However, the target genes of SUZ12 in gastric cancer are not well documented, which need to be investigated. Our results showed that knockdown of SUZ12 expression upregulated KLF2 expression. KLF2 repression has an important role in EZH2 oncogenesis via promoting p21 expression [27]. In addition, decreased KLF2 expression is associated with poor prognosis for NSCLC, and KLF2 could inhibit NSCLC cell proliferation and promote apoptosis via regulating CDKN1A/p21 and CDKN2B/p15 protein expression [28]. Meanwhile, SUZ12 could interact with Snail1 and EZH2 increasing Snail1 binding to E-cadherin promoter and the trimethylation of lysine 27 in histone H3, which resulted in repression of E-cadherin transcription [29]. Moreover, increased SUZ12 binding and H3-K27 trimethylation at the E-cadherin promoter and repression of E-cadherin is also observed in metastatic human breast tumors [14].

In this study, we also found that SUZ12 could bind to E-cadherin promoter and repress its transcription. Loss of E-cadherin expression is an important hallmark of EMT and a crucial step in the progression of papillomas to invasive carcinomas [30]. Generally, EMT is proposed and supported by many studies that could be a potent mechanism for promoting the detachment of cancer cells from primary tumors [31]. Furthermore, reduced E-cadherin is associated with local invasion, regional metastasis, and reduced survival in gastric cancer and NSCLC [32, 33]. These findings indicated that increased SUZ12 promote gastric cancer cell proliferation and metastasis partly via repressing KLF2 and E-cadherin transcription.

In summary, the expression of SUZ12 was significantly increased in gastric cancer tissues, suggesting that its upregulation may be a negative prognostic factor for gastric cancer patients, and indicative of poor survival rates and a higher risk for cancer metastasis. We showed that SUZ12 possibly regulates the proliferation and metastatic ability of gastric cancer cells, partially through regulation of KLF2 and E-cadherin. Our findings further the understanding of gastric cancer pathogenesis and development and facilitate the development of diagnostics and therapeutics against cancers.

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