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Original Article

Overexpression of CST4 promotes gastric cancer aggressiveness by activating the ELFN2 signaling pathway

Yi Qiang Zhang[1], Jing Jing Zhang[2], Hong Jie Song[1], Da Wei Li[1]

[1]*Department of Gastroenterology, Zhumadian Central Hospital of Henan Province, Zhumadian, China;* [2]*Department of Laboratory Medicine, The Third Hospital of Xinxiang Medical University, Xinxiang, China*

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Abstract: Gastric cancer is one of the most lethal malignancies of gastrointestinal cancer and its prognosis remains dismal because of the paucity of effective therapeutic targets. Here, we show that cystatin 4 (CST4) is markedly upregulated in gastric cancer cell lines and clinical tissues. Ectopic expression of CST4 in gastric cancer cells promoted proliferation, migration, and invasion of gastric cancer cells in vitro. Furthermore, CST4 overexpression significantly promoted the tumorigenicity of gastric cancer cells in vivo, whereas silencing endogenous CST4 caused an opposite outcome. In addition, extracellular leucine rich repeat and fibronectin type III domain containing 2 (ELFN2) was identified as a downstream target of CST4 in gastric cancer cells and was positively correlated with ELFN2 expression in gastric cancer tissues. Finally, we demonstrated that CST4 enhanced gastric cancer aggressiveness by regulating ELFN2 signaling. Together, our results provide new evidence that CST4 overexpression promotes the progression of gastric cancer and might represent a novel therapeutic target for its treatment.

Keywords: CST4, gastric cancer, ELFN2, metastasis

Introduction

Gastric carcinoma is one of the most common digestive tract cancers worldwide. In spite of advances in surgical treatment and multimodal therapy, the prognosis of patients with gastric cancer still remains poor owing to the recurrence, metastasis and chemotherapy resistance of the disease [1]. Cancer metastasis is a complex, multi-step process, in which cancer cells diffuse from the primary neoplasm to distant tissues. The process starts when primary tumor cells invade adjacent tissue, followed by cell intravasation and extravasation into the surrounding tissue initiating micro-metastases and finally proliferating to form metastatic foci [2]. Therefore, better understanding of the underlying mechanisms behind gastric cancer progression, including cell metastasis and identification of potential therapeutic targets to combat the metastasis are urgently needed to improve the outcomes of patients with advanced gastric cancer.

Cysteine proteases are a group of intracellular proteins with protein degradation activity which are associated with a wide variety of biological process, including inflammation, immune response and facilitating the progression of malignant tumors [3]. Accumulating evidence has demonstrated that cathepsins are overexpressed in tumors and localize to the invasive tumor margin to participate in invasion and metastasis of solid tumors. Previous clinical studies revealed that the upregulation of cysteine proteases influence the response of tumor cells to ionizing radiation and cytostatic agents [4]. In addition, substantive experimental evidence indicates that few members of cysteine proteases, cathepsin B, D, and L in particular, trigger activation of multiple signaling pathways, which play a crucial role in tumor growth and survival [5]. The cystatin 1 (CST1) gene encodes a secretory protein termed CST SN belonging to the type 2 cystatin (CST) superfamily, which includes CST1, CST2, CST3, CST4, and CST5 [6]. Previous studies have reported

that cystatins are involved in tumor invasion and metastasis [7]. Overexpression of CST1 contributes to cell proliferation in gastric cancer and has been identified as a novel molecular marker for colorectal carcinoma [8, 9]. However, CST5 has been reported to function as a tumor suppressor contributing to the antitumor action of 1 alpha, 25(OH)2D3 in colon cancer [9]. These reports suggest that cystatins contribute to the process of carcinogenesis and tumor progression.

In the present study, we determined the expression of cystatin 4 (CST4) in gastric cancer tissues compared with the matched normal tissues. We further elucidated the role of CST4 in promoting gastric cancer cell growth and metastasis *in vitro* and *in vivo* through lentivirus-mediated CST4 upregulation and downregulation experiments. Finally, we found that CST4 induced gastric cancer cell metastasis through its extracellular leucine rich repeat and the fibronectin type III domain containing 2 (ELFN2) signaling pathway. Taken together, these findings suggest that CST4/ELFN2 signaling axis plays a pivotal role in gastric cancer cell progression and serves as a potential therapeutic target.

Materials and methods

Sample specimens and cells culture

Frozen gastric cancer tissue samples (n = 46) and normal tissues (n = 32) collected from 2001 and 2016 were obtained from the Zhumadian Central Hospital of Henan Province. Total RNA was extracted from gastric cancer tissues and normal tissues using TRIzol (Life Technologies, Carlsbad, CA, USA) and was used to conduct qPCR analysis. Human gastric cancer cell lines (MK-45, BGC-823, SGC-7901, MGC-803) and the normal human gastric mucosal cell line GES-1 were obtained from Cobioer Bioscience Co., LTD (Nanjing, Jiangsu, China). Gastric cancer lines were cultured in RPMI-1640 medium or Dulbecco Modified Eagle Medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS).

Cell proliferation assay

Approximately 2×10^5 MKN-45 or SGC-7901 cells were cultured in each well of 96-well plates and incubated in the complete medium for 24, 48, 72, or 96 h. The absorbance (OD) was measured at 490 nm using a Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA) [10].

Colony formation assay

Approximately 1×10^3 MKN-45 or SGC-7901 cells were seeded in each well of 6-well plates and cultured for 14 days. Cell colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cell colonies containing ≥ 50 cells were counted [11].

Soft agar assay

The bottoms of 6-well plates were coated with 2 ml bottom agar mixture (culture medium with 10% FBS, 0.1% agar). After the bottom layer solidified, 2 ml top agar-medium mixture (culture medium with 10% FBS, 1% agar) containing approximately 1×10^3 cells was added and the plates were incubated for two weeks. Plates were stained with 0.1% crystal violet and the number of colonies was counted [12].

Wound healing analysis

MKN-45 and SGC-7901 cells were grown in 6-well plates to until reaching 100% confluence. A cell scratch-wound was generated with a 100 μl tip. After 48 h, the wound width was photographed and the percentage of wound closure was determined based on the wound width at 0 h.

Transwell invasion assays

Cell invasion assays were performed using a modified 24-well Boyden chamber with a membrane which was pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). After MKN-45 and SGC-7901 cells were transfected with shCST4 or vector containing CST4, 200 μl of cells (1×10^5/ml) in FBS-free medium were seeded into the upper chamber, while medium containing 20% FBS was placed in the lower chamber. After 24 h, cells on the bottom surface were fixed and counted in five randomly selected fields [13].

Stable transfection and knock-down

To overexpress CST4 or ELFN2, we transfected MKN-45 and SGC-7901 cells with pcDNA3.1-CST4, pcDNA3.1-ELFN2, or control vector

pcDNA3.1 plasmids. To inhibit CST4 or ELFN2 expression, we transfected MKN-45 and SGC-7901 cells with CST4 shRNA lentiviral transduction particles (sequence CCGGCCACCCACTGTAGTGCTCCCACTCGAGTGGGAGCACTACAGTGGGTGGTTTTT; Sigma-Aldrich, St. Louis, MO, USA) or with ELFN2 shRNA lentiviral transduction particles (sequence, CCGGCAGACACTACTACTCAGGGTACTCGAGTACCCTGAGTAGTAGTGTCTGTTTTTTG; Sigma-Aldrich). Transfection was using lipofectamine 2000 (Invitrogen).

Quantitative real-time PCR (qPCR)

Total RNA was isolated using TRIzol reagent (Life Technologies) and the first-strand cDNA was synthesized from total RNA using a PrimeScript RT reagent kit (TakaraBio, Tokyo, Japan). qPCR analysis of the expression of target genes was conducted using 2 μl of cDNA and the FastQuant RT Kit (TIANGEN Biotech, Beijing, China). The comparative cycle threshold (Ct) method was applied to quantify the expression levels using the $2^{(-\Delta\Delta Ct)}$ method. GAPDH was used as an internal control. The primers used for PCR were: CST4 primers 5'-CCTCTGTGTACCCTGCTACTC-3' (sense) and 5'-CTTCGGTGGCCTTGTTGTACT-3' (antisense); ELFN2 primers 5'-TCAAAGCCGTGCTCTACTCCT-3' (sense) and 5'-CGTCAGGTTGCTGAGCTTG-3' (antisense); E-cadherin primers 5'-AAAGGCCCATTTCCTAAAAACCT-3' (sense) and 5'-TGCGTTCTCTATCCAGAGGCT-3' (antisense); N-cadherin primers 5'-TCAGGCGTCTGTAGAGGCTT-3' (sense) and 5'-ATGCACATCCTTCGATAAGACTG-3' (antisense); GAPDH primers 5'-CTCACCGGATGCACCAATGTT-3' (sense) and 5'-CGCGTTGCTCACAATGTTCAT-3' (antisense).

Cell immunofluorescence

Cells were cultured in 24- well plates until reaching 60-70% confluence. Cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. Subsequently, cells were incubated with primary antibodies against CST4 (Santa Cruz Biotechnology, Dallas, TX, USA), E-cadherin (Santa Cruz Biotechnology), N-cadherin (Santa Cruz Biotechnology), CST4 (Santa Cruz Biotechnology), and ELFN2 (Santa Cruz Biotechnology) at 4°C overnight, and subsequently incubated with corresponding Alexa Fluor-conjugated secondary antibodies (Life Technologies). After 1 h, cell nuclei were stained with DAPI (Life Technologies). Microscopic images of cells were obtained using a Leica inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Tumorigenicity and metastasis assay in vivo

Male BALB/c nu/nu mice were purchased from SLAKE Experimental Animal Limited Company (Shanghai, China). All experiments were performed in accordance with the official recommendations of the Zhumadian Central Hospital of Henan province. In the tumor xenograft model, approximately 1×10^6 MKN-45 or SGC-7901 cells were inoculated subcutaneously into the right flank. Tumor volume was assayed every three days, calculated using the formula $V = 0.5 \times L \times W^2$, where L is the mid-axis length, and W is the mid-axis width. Animals were sacrificed 25 days after inoculation. In pulmonary metastasis assays, 100 μl 1×10^6 cells was injected via the tail vein. After four weeks, the lungs with metastatic foci were removed and subjected to hematoxylin and eosin (H & E) staining [14].

Statistical analysis

The data are presented as mean ± SD. Statistical significance was determined using Student's *t*-test (two-tailed) and values of $P < 0.05$ were considered as significant.

Results

Overexpression of CST4 in gastric cancer tissues

By analyzing the published mRNA expression profiles obtained from normal tissues and gastric cancer (GDS1210), we found that CST4 was significantly upregulated in gastric cancer compared with corresponding normal tissue (*P*-value = 4.846e-9) (**Figure 1A**). We queried the Oncomine database (http://oncomine.com) for publicly available gastric cancer microarray data and identified overexpression of CST4 in three independent gastric cancer data sets, which were published in studies by Wang et al. [15] and Cui et al. [16]. We observed significant CST4 upregulation in gastric cancer in two datasets (**Figure 1B**). Analysis of other independent data sets generated using The Cancer Genome Atlas (TCGA) and a study by Deng et al. [17] further supported the idea that CST4 is overexpressed in gastric cancer relative to nor-

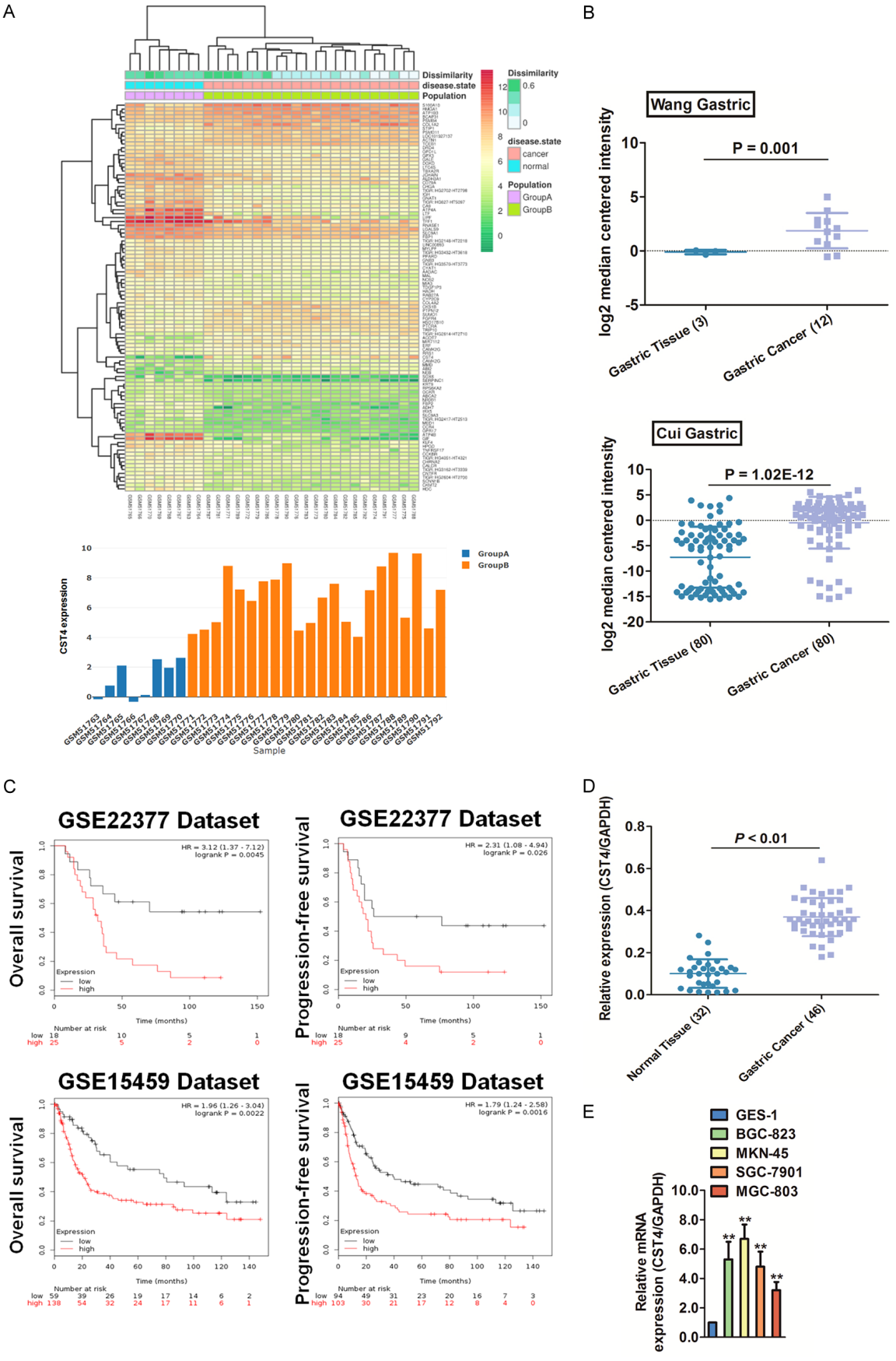

A
Dissimilarity
disease.state
Population
Dissimilarity
disease.state
cancer
normal
Population
GroupA
GroupB
CST4 expression
Sample
B
Wang Gastric
P = 0.001
log2 median centered intensity
Gastric Tissue (3)
Gastric Cancer (12)
Cui Gastric
P = 1.02E-12
log2 median centered intensity
Gastric Tissue (80)
Gastric Cancer (80)
C
GSE22377 Dataset
Overall survival
HR = 3.12 (1.37 - 7.12)
logrank P = 0.0045
Progression-free survival
HR = 2.31 (1.08 - 4.94)
logrank P = 0.026
GSE15459 Dataset
HR = 1.96 (1.26 - 3.04)
logrank P = 0.0022
HR = 1.79 (1.24 - 2.58)
logrank P = 0.0016
Expression
low
high
Time (months)
Number at risk
D
P < 0.01
Relative expression (CST4/GAPDH)
Normal Tissue (32)
Gastric Cancer (46)
E
GES-1
BGC-823
MKN-45
SGC-7901
MGC-803
Relative mRNA expression (CST4/GAPDH)

Figure 1. Expression of CST4 in gastric cancer. A. Expression profiling of mRNAs showing that CST4 was up-regulated in gastric cancer compared to normal tissues (GDS1210). B. Box plots shown increased levels of CST4 in gastric cancer compared with normal tissues in two microarray data sets (Wang gastric and Cui gastric) from Oncomine. C. The Kaplan-Meier curve for gastric cancer patients with high or low CST4 mRNA level was get from Kaplan-Meier Plotter (http://kmplot.com). D. Relative expression of CST4 mRNA in 46 gastric cancer and paired adjacent normal tissues as determined by qPCR. E. qPCR analyzed the expression of CST4 in GES-1 cells and gastric cancer cell lines. GAPDH was used as a loading control.

mal tissues (data not shown). To study the function of CST4 in gastric cancer, Kaplan-Meier curves for gastric cancer patients with high or low CST4 levels were generated from the Kaplan-Meier Plotter tool (http://kmplot.com). As shown in **Figure 1C**, gastric cancer patients with high CST4 expression were associated with poor overall prognosis (OS) and progression-free survival (PFS) as compared to patients with low expression of CST4. We next examined the expression of CST4 in gastric cancer tissues. qPCR analysis demonstrated that the expression of CST4 mRNA was remarkably increased in gastric cancer samples (46 cases) as compared to that in the normal tissue (32 cases) (**Figure 1D**). Finally, we examined CST4 mRNA expression levels in five gastric cancer cell lines and normal human gastric mucosal cell line GES-1 using qPCR (**Figure 1E**).

CST4 enhances growth in gastric cancer cells

To investigate the effect of CST4 in gastric cancer cells, CST4 was knocked down by transfection of shRNA targeting CST4 in MKN-45 and SGC-7901. The efficiency and specificity of CST4 knockdown were shown to be almost complete deletion relative to parental MKN-45 and SGC-7901 cells as assayed by qRT-PCR and cell immunofluorescence analysis (**Figure 2A**). Intriguingly, we observed that the proliferation rates of both MKN-45 and SGC-7901 cells with knockdown of CST4 were significantly decreased compared with the parental cell lines (**Figure 2B**), indicating a positive role of CST4 in regulating gastric cancer cell growth. Consistent with this finding, the colony formation ability of MKN-45 and SGC-7901 cells with disruption of CST4 expression was inhibited dramatically in both regular culture (**Figure 2C**) and soft agar colony formation (**Figure 2D**) conditions. We next examined the effect of CST4 knockdown on gastric cancer cell growth in vivo. Tumor cells with downregulated CST4 expression from both MKN-45 and SGC-7901 cell lines exhibited significantly slower progression, as evidenced by a smaller tumor volume over the treatment period (**Figure 2E**). To further assess the functional role of CST4 in gastric cancer cells, we established lentivirus-mediated upregulation of CST4 in MKN-45 and SGC-7901 cell lines (**Figure 2F**). Cell growth was significantly inhibited in CST4-transfected cells compared with that in control vector transfected cells, as determined by MTT and colony formation assays (**Figure 2G-I**). We next examined the effect of CST4 overexpression on MKN-45 and SGC-7901 cell growth in vivo. As expected, tumor mass from ectopic expression of CST4 in MKN-45 and SGC-7901 cells exhibited significantly faster progression, as evidenced by larger tumor volume over the treatment period (**Figure 2J**). Collectively, these data suggest that CST4 plays an important role in gastric cancer cell growth both in vitro and in vivo.

CST4 accelerates gastric cancer cells metastasis

Given that the invasive property of tumor cells contributes to poor prognosis, we next addressed whether CST4 affects cell migration and invasion. Wound healing assays indicated that knockdown of CST4 in both MKN-45 and SGC-7901 cells resulted in a significant increase in cell migration compared with that in their parental cells (**Figure 3A**). Consistent with this, we observed in a Transwell experiment that more cells invaded into the bottom wells when CST4 was knocked down (**Figure 3B**). These results suggest that CST4 regulates the tumor cell migration and invasion in vitro. To further address whether CST4 regulates the metastasis of tumor cells, we injected the CST4 knockdown cells into nude mice and observed the tumors metastasizing to the lung. The results showed that knockdown of CST4 in the gastric cancer cells significantly suppressed metastasis in lung tissue at four weeks after injection (**Figure 3C**), suggesting that CST4 knockdown inhibited tumor metastasis in vivo. To further determine the effect of altered CST4 expression on metastasis in gastric cancer cells, CST4

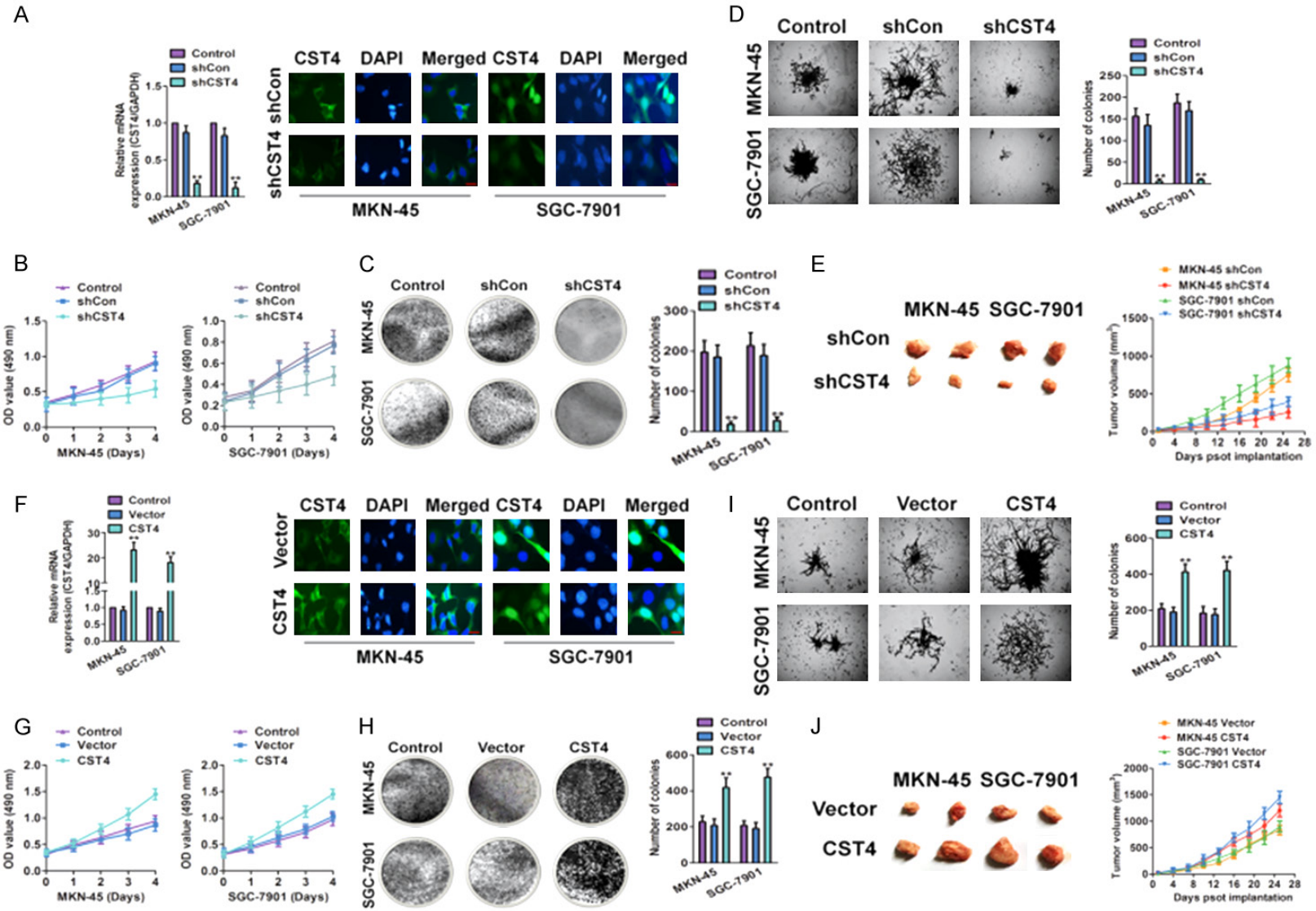


Figure 2. Effect of CST4 on growth gastric cancer cell proliferation and growth. A. qRT-PCR (left panel) and immunofluorescence (right panel) assays were conducted to determine the efficiency of CST4 knocked-down plasmid transfection in MKN-45 and SGC-7901 cells. Scale bar: 50 μm. B. Cell growth rate from parental and CST4 knocked-down MKN-45 and SGC-7901 at indicated time point. C. Colony formation assay of parental and CST4 knocked-down MKN-45 and SGC-7901 cells. D. Soft-agar colony formation assay of parental and CST4 knocked-down MKN-45 and SGC-7901 cells cultured for 14 days and colonies were stained and counted. E. Tumor growth curve for MKN-45 and SGC-7901 xenogaft tumor models. F. qPCR (left panel) and immunofluorescence (right panel) assays were conducted to determine the efficiency of vector containing CST4 transfection in MKN-45 and SGC-7901 cells. Scale bar: 50 μm. G. Cell growth rate from parental and CST4 knocked

down MKN-45 and SGC-7901. H. Colony formation assay of parental and CST4 over-expressing MKN-45 and SGC-7901 cells. I. Soft-agar colony formation assay of parental and CST4 over-expressing MKN-45 and SGC-7901 cells. J. Tumor growth curve for MKN-45 and SGC-7901 xenogaft tumor models.

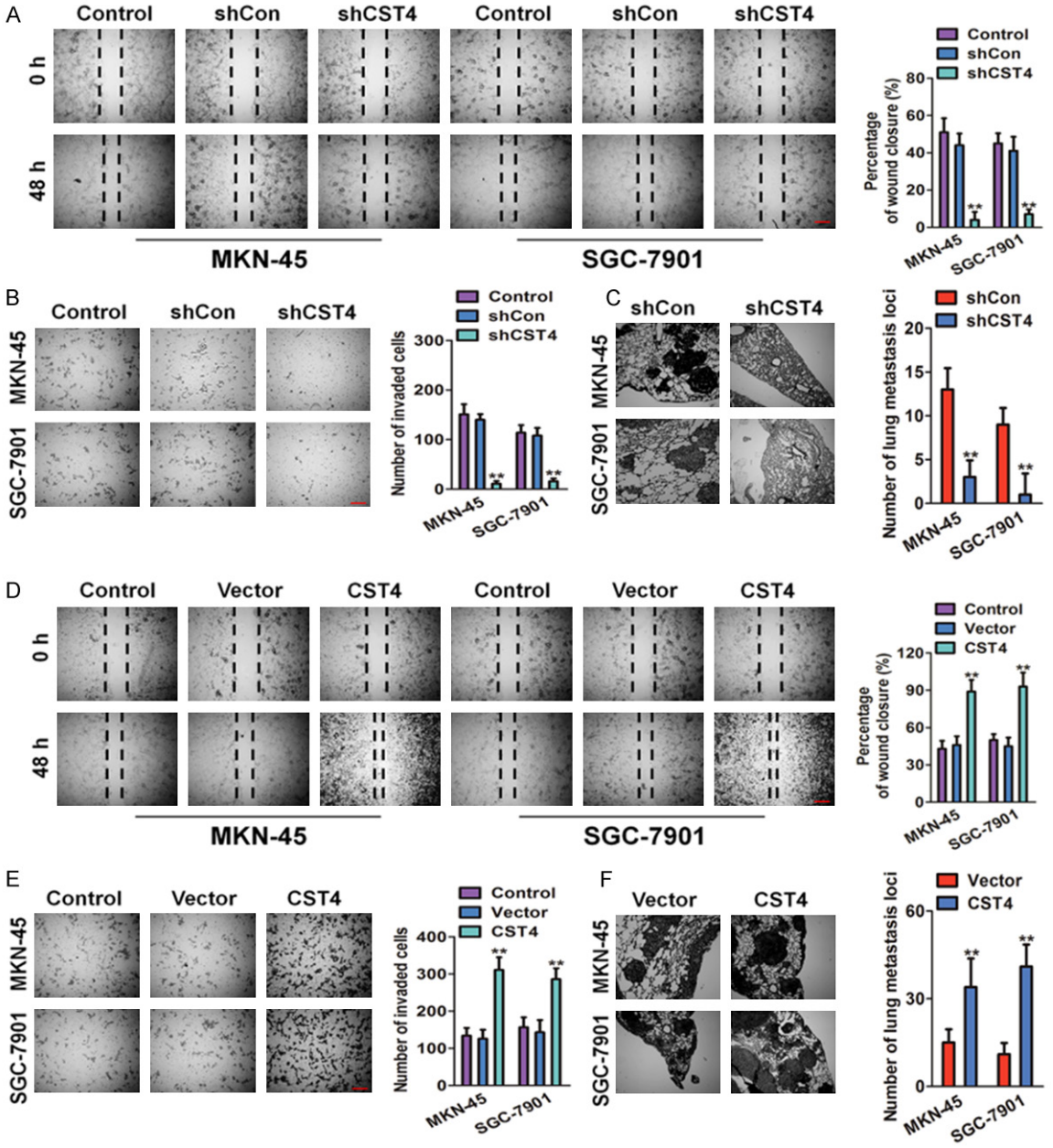


Figure 3. Effect of CST4 on gastric cancer cells metastasis. A. In vitro wound healing assay with MKN-45 and SGC-7901 cells after knocked-down with CST4 expression. Image was acquired at 0 h and 48 h time points after scratching (left panel). Quantification of wound closure was calculated (right panel). Scale bar: 200 μm. B. Representative result of invasive potentials of MKN-45 and SGC-7901 cells in Transwells assay (left panel). Quantification of invasive cells per field was analyzed (right panel). Scale bar: 200 μm. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control. C. CST4 knocked-down inhibited tumor metastasis in gastric cancer cells lung metastasis model in vivo. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to shCon. D. In vitro wound healing assay with MKN-45 and SGC-7901 cells after CST4 over-expression. Image was acquired at 0 h and 48 h time points after scratching (left panel). Quantification of wound closure was calculated (right panel). Scale bar: 200 μm. E. Representative result of invasion of MKN-45 and SGC-7901 cells in Transwells assay (left panel). Quantification of invasive cells per field was analyzed (right panel). Scale bar: 200 μm. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control. F. CST4 over-expressing promoted tumor metastasis in gastric cancer cells lung metastasis model in vivo. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to vector.

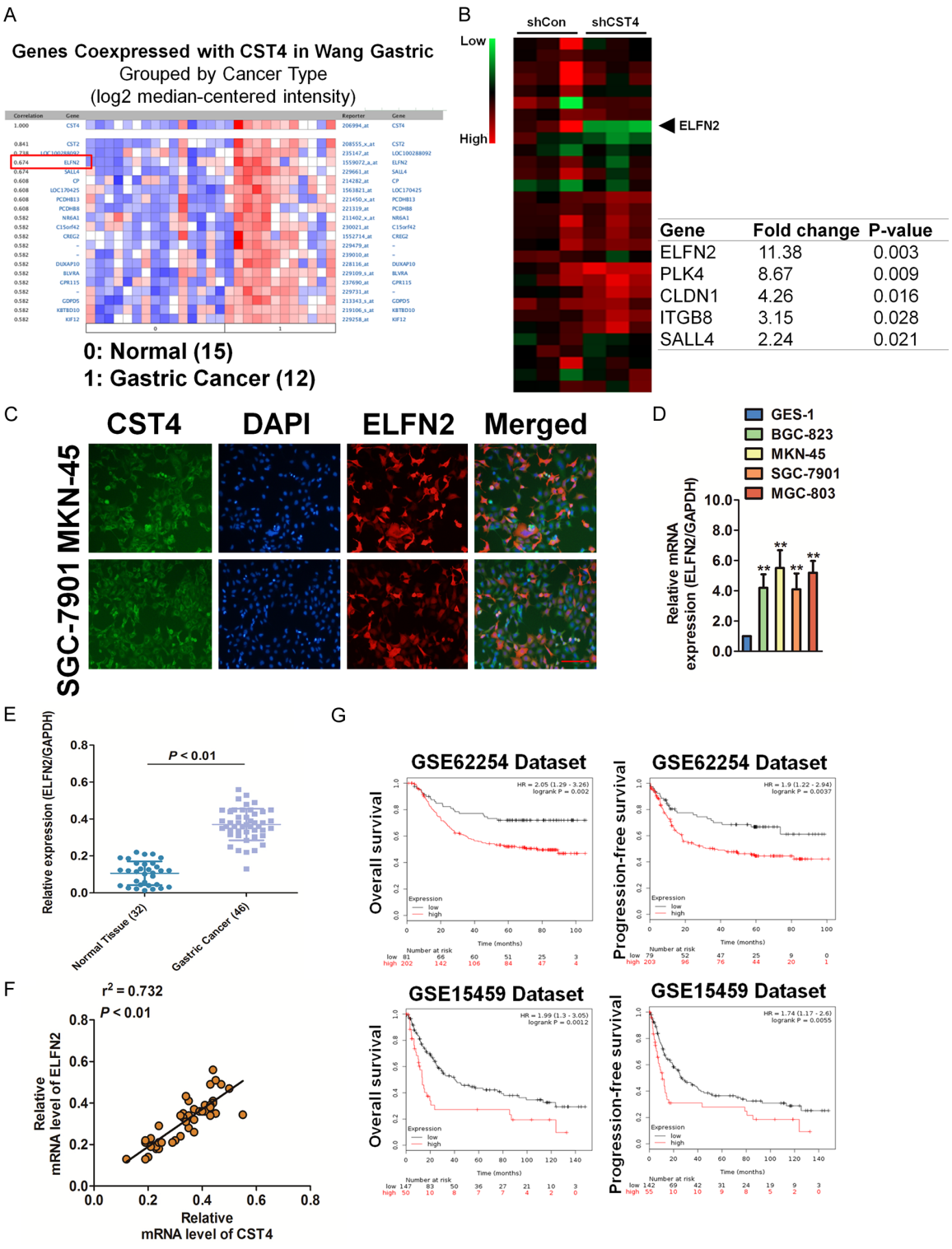


Figure 4. CST4 increases ELFN2 expression in gastric cancer. A. Expression data from Oncomine (www.oncomine.org). We used the following filters: gene 'CST4' Analysis Type: 'Co-expression analysis' Cancer Type: 'Gastric cancer'. The color changed according to a weaker (blue) or higher (red) expression. B. qPCR analysis of genes in parental MKN-45 cells and the CST4 knocked-down MKN-45 cells. C. Double immunofluorescence for CST4 (green) and ELFN2 in MKN-45 and SGC-7901 cells. Scale bar: 200 μm. D. The expression pattern of ELFN2 in gastric cancer cell lines was assessed by qPCR. $^{**}P < 0.01$ compared to GES-1. E. mRNA level of ELFN2 in gastric cancer and non-tumor tissues was analyzed by qPCR. F. The correlation between the CST4 and ELFN2 level was measured in the 46 cases of gastric cancer tissues. G. Kaplan-Meier survival curves of OS and FPS in gastric cancer patients with different levels of ELFN2.

overexpressing MKN-45 and SGC-7901 cells were subjected to wound healing and Transwell invasion assays. We found that elevated expression of CST4 in MKN-45 and SGC-7901 cells significantly increased mobility (**Figure 3D**) and invasion (**Figure 3E**) in vitro. Consistent with these findings, CST4 over-expression in MKN-45 and SGC-7901 cells exhibited more metastatic foci in lung tissues (**Figure 3F**).

Co-expression of CST4 and ELFN2 in gastric cancer

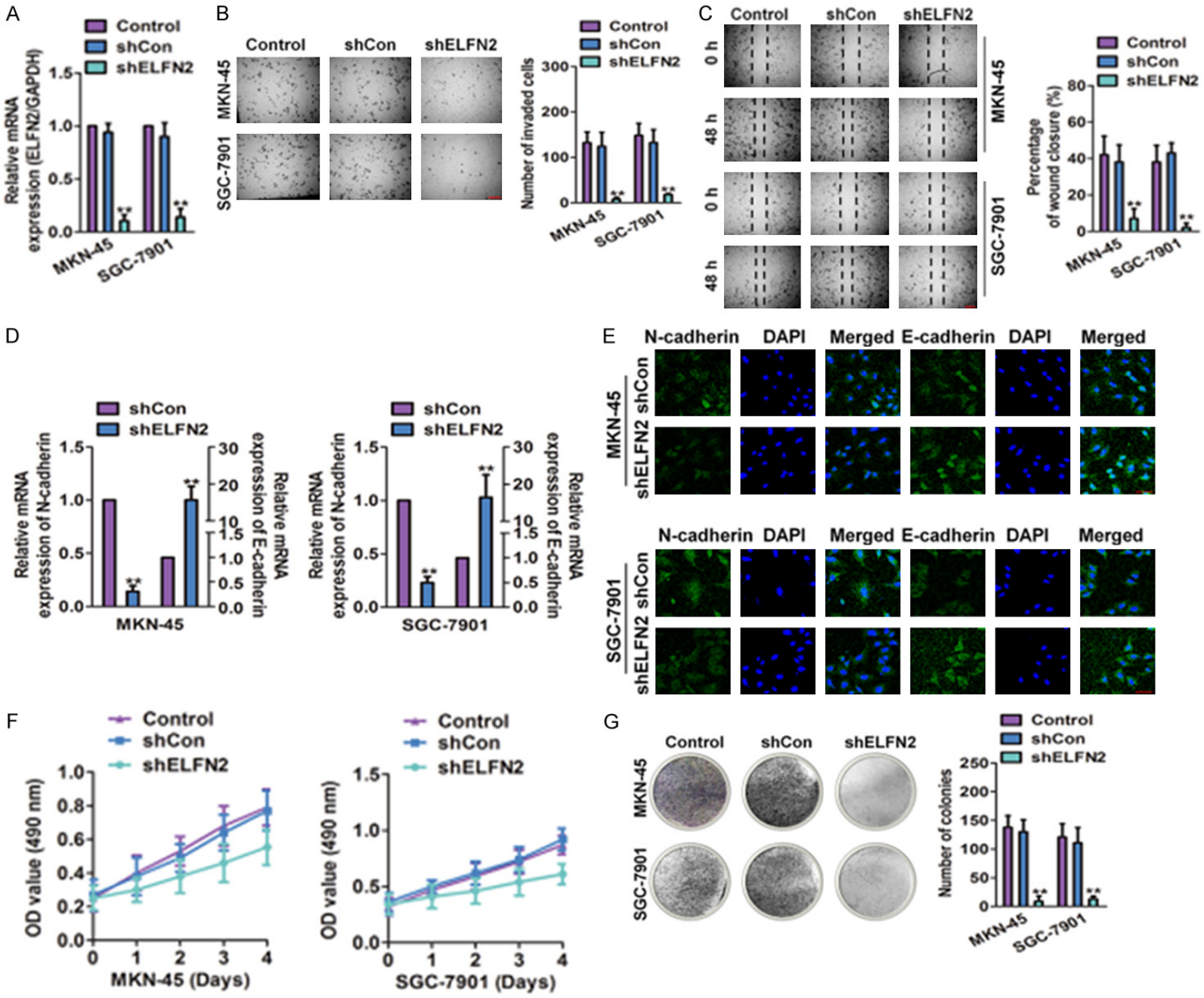
To investigate the mechanisms underlying the effect of CST4 on gastric cancer cell metastasis, we searched for the genes that showed co-expression with CST4 in the Wang gastric cancer dataset from Oncomine [15]. Co-expression analysis showed direct association between 252 genes and CST4 mRNA levels in the Wang dataset (correlation > 0.5) (**Figure 4A**). qPCR assay was conducted to verify the top 30 co-expressed genes which most closely correlated with CST4. Additionally, we found that four genes were significantly decreased in CST4 knockdown MKN-45 cells compared with the parental cells (fold change > 2). Notably, ELFN2 was highly co-expressed with CST4 and its expression was most significantly inhibited after CST4 down-regulation (**Figure 4B**). To verify the co-expression of CST4 and ELFN2 in gastric cancer cells, we immunolabeled MKN-45 and SGC-7901 cells for CST4 and ELFN2. As expected, we found co-expression of CST4 and ELFN2 in both MKN-45 and SGC-7901 cells (**Figure 4C**). In addition, ELFN2 levels were higher in gastric cancer cells than in GES-1 cells (**Figure 4D**). We further determined ELFN2 levels in the 46 clinical gastric cancer tissues and found that ELFN2 was higher in gastric cancer than in normal tissues (**Figure 4E**). Importantly, ELFN2 level was positively correlated with the CST4 level in gastric cancer tissues (**Figure 4F**). Kaplan-Meier analysis (http://kmplot.com/analysis) revealed that patients with high levels of ELFN2 were associated with poor OS and PFS as compared to those with low levels of ELFN2. (**Figure 4G**). These data suggest that ELFN2 is co-expressed with CST4 in gastric cancer.

ELFN2 influenced gastric cell migration, invasion and tumorigenesis

The co-expression of ELFN2 with CST4 in gastric cancer cells suggests that ELFN2 additionally plays a role in its migration and invasion capability. To investigate the function of ELFN2 in gastric cancer cells, we knocked down ELFN2 by transfection of a specific shRNA. The levels of ELFN2 were efficiently depleted in both MKN-45 and SGC-7901 cells (**Figure 5A**). Transwell and wound healing assay were used to examine invasion and migration of gastric cancer cells. In Transwell assays, knockdown of ELFN2 significantly reduced the number of cells which invaded through the membrane compared with control cells (**Figure 5B**). Likewise, wound closure ratio was markedly reduced in ELFN2-shRNA transfected MKN-45 and SGC-7901 cells (**Figure 5C**). Next, we examined the expression of epithelial-mesenchymal transition (EMT) markers following the knockdown of ELFN2. qPCR (**Figure 5D**) and immunofluorescence (**Figure 5E**) analyses showed that the epithelial biomarker E-cadherin was increased in ELFN2-depleted MKN-45 cells, whereas the mesenchymal biomarker N-cadherin was decreased. We then investigated MKN-45 and SGC-7901 cell viability for five consecutive days by MTT assay after cells were transfected with shRNA targeting ELFN2. On day 4, compared with control shRNA transfected cells, the OD value of viable cells infected with shELFN2 was remarkably reduced (**Figure 5F**). In addition, colony formation assay was performed to determine cell proliferation in vitro. Consistent with the results of the MTT assay, the number of cell colonies formed by ELFN2 knockdown cells was decreased compared with that of control cells (**Figure 5G**). These results indicate that knockdown of ELFN2 efficiently inhibited cell proliferation, migration, and invasion of gastric cancer cells.

CST4-enhanced tumor proliferation and aggressive behavior are dependent on ELFN2

We examined whether ELFN2 overexpression could rescue the inhibitory effects of CST4 on gastric cancer cell growth and metastasis. Lentiviral ELFN2 particles and empty vector were introduced to gastric cancer cells which had been transfected with either shCST4 or control shRNA. qPCR confirmed that ELFN2 overexpression markedly and specifically increased ELFN2 expression (**Figure 6A**). Proliferative and colony formation abilities were impaired in shCST4 gastric cancer cells, whereas ELFN2 overexpression rescued the inhibi-


A
Control
shCon
shELFN2
Relative mRNA expression (ELFN2/GAPDH)
MKN-45
SGC-7901
B
Control
shCon
shELFN2
MKN-45
SGC-7901
Number of invaded cells
C
Control
shCon
shELFN2
0 h
48 h
MKN-45
SGC-7901
Percentage of wound closure (%)
D
shCon
shELFN2
Relative mRNA expression of N-cadherin
Relative mRNA expression of E-cadherin
MKN-45
SGC-7901
E
N-cadherin
DAPI
Merged
E-cadherin
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MKN-45
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F
Control
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shELFN2
OD value (490 nm)
MKN-45 (Days)
SGC-7901 (Days)
G
Control
shCon
shELFN2
MKN-45
SGC-7901
Number of colonies

Figure 5. Knockdown of ELFN2 repress the growth and invasion capacity of gastric cancer cells. A. The effective down-regulation of ELFN2 following shELFN2 transfection was determined by qPCR analyses. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control. B. MKN-45 and SGC-7901 cells transfected with shELFN2 or control shRNA (shCon) were plated in the upper chamber of Transwell. After 24 h, the cells invaded through the membrane were counted in five random microscopic fields. Scale bar: 200 μm. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control. C. The wound closure percentage of shELFN2 and shCon transfected MKN-45 and SGC-7901 cells was quantified 48 h after scratch relative to that at 0 h. Scale bar: 200 μm. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control. D. qPCR analysis of E-cadherin, N-cadherin and vimentin protein expression in gastric cancer cell lines. qPCR results were standardized in relation to GAPDH. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to shCon. E. The expression of E-cadherin, N-cadherin and nuclei in parental MKN-45 cells or knocked-down MKN-45 cells was determined by immunofluorescence staining. Scale bar: 100 μm. F. The number of viable cells was fewer in MKN-45 and SGC-7901 infected with shELFN2 than in cells infected with shCon. G. The number of colonies was fewer in cells infected with shELFN2 than in cells infected with shCon. The data are presented as mean ± SD. $^{**}P < 0.01$ as compared to control.

tion of growth induced by shCST4 (**Figure 6B** and **6C**). Mobility and invasion abilities were impaired in shCST4-transfected MKN-45 and SGC-7901 cells, but were rescued by ectopic expression of ELFN2 (**Figure 6D** and **6E**). In addition, MKN-45 and SGC-7901 cells with co-transfection of ELFN2 shRNA and the vector containing CST4 (**Figure 7A**) were subjected to growth and metastasis assays. MKN-45 and SGC-7901 cell growth (**Figure 7B** and **7C**), migration (**Figure 7D**), and invasion (**Figure 7E**) induced by CST4 overexpression was effectively reversed by ELFN2 down-regulation. Taken together, these findings clearly reveal that CST4 regulates gastric cancer cell proliferation and aggressive behavior and is dependent on ELFN2 expression.

Discussion

Gastric cancer is one of the most invasive forms of cancer and is the leading cause of cancer death worldwide. Advanced gastric cancer is associated with significant mortality because it metastasizes to vital organs [18]. Recent studies suggest that cysteine proteases are frequently overexpressed in a variety of malignancies and contribute to cancer growth and progression [19]. However, the mechanisms by which this occurs in remain unclear. Here, we found that CST4 was significantly overexpressed in gastric cancer tissues compared with normal adjacent tissues. Moreover, overexpression of CST4 was associated with poor overall prognosis (OS) and progression-free survival (PFS). Inspired by these findings, we further explored the role of CST4 in gastric cancer growth and metastasis *in vitro* and *in vivo*. As expected, knockdown of CST4 inhibited gastric cancer cells proliferation *in vitro* and tumorigenesis *in vivo*. Conversely, ectopic expression of CST4 showed the opposite effects. We also investigated the effect of CST4 knockdown on gastric cancer cell metastasis. CST4 knockdown significantly inhibited the migration and invasion abilities of the gastric cancer cell lines MKN-45 and SGC-7901 *in vitro* and pulmonary metastasis *in vivo*, whereas overexpression of CST4 caused opposite effects.

These results suggest a strong association of CST4 expression with gastric cancer metastasis, raising the question on how CST4 contributes to the migration and metastasis of gastric cancer. In this study, we observed a positive correlation between CST4 and ELFN2 in gastric cancer tissues. We found that ELFN2 was overexpressed in gastric cancer tissues and cell lines. Disruption of ELFN2 in gastric cancer cells suppressed cell migration *in vitro* and tumor metastasis in a mouse xenograft model. Importantly, upregulation of ELFN2 in gastric cancer correlates with poor prognosis. To validate the role of ELFN2 in mediating the effects of CST4 in gastric cancer cells metastasis, we treated MKN-45 and SGC-7901 cells with disrupted CST4 expression with a vector driving ELFN2 expression. Overexpression of ELFN2 in CST4 knockdown MKN-45 and SGC-7901 cells effectively rescued cell proliferation, migration, and invasion. CST4 overexpressing MKN-45 and SGC-7901 cells were also transfected with shRNA targeting ELFN2. As expected, disruption of ELFN2 in CST4 overexpressing MKN-45 and SGC-7901 cells effectively abolished the cell proliferation, migration and invasion induced by CST4. These data indicate that ELFN2 serves as a downstream effecter of CST4 in promoting gastric cancer growth and metastasis.

In summary, CST4 expression was upregulated in gastric cancer tissues, which might addition-

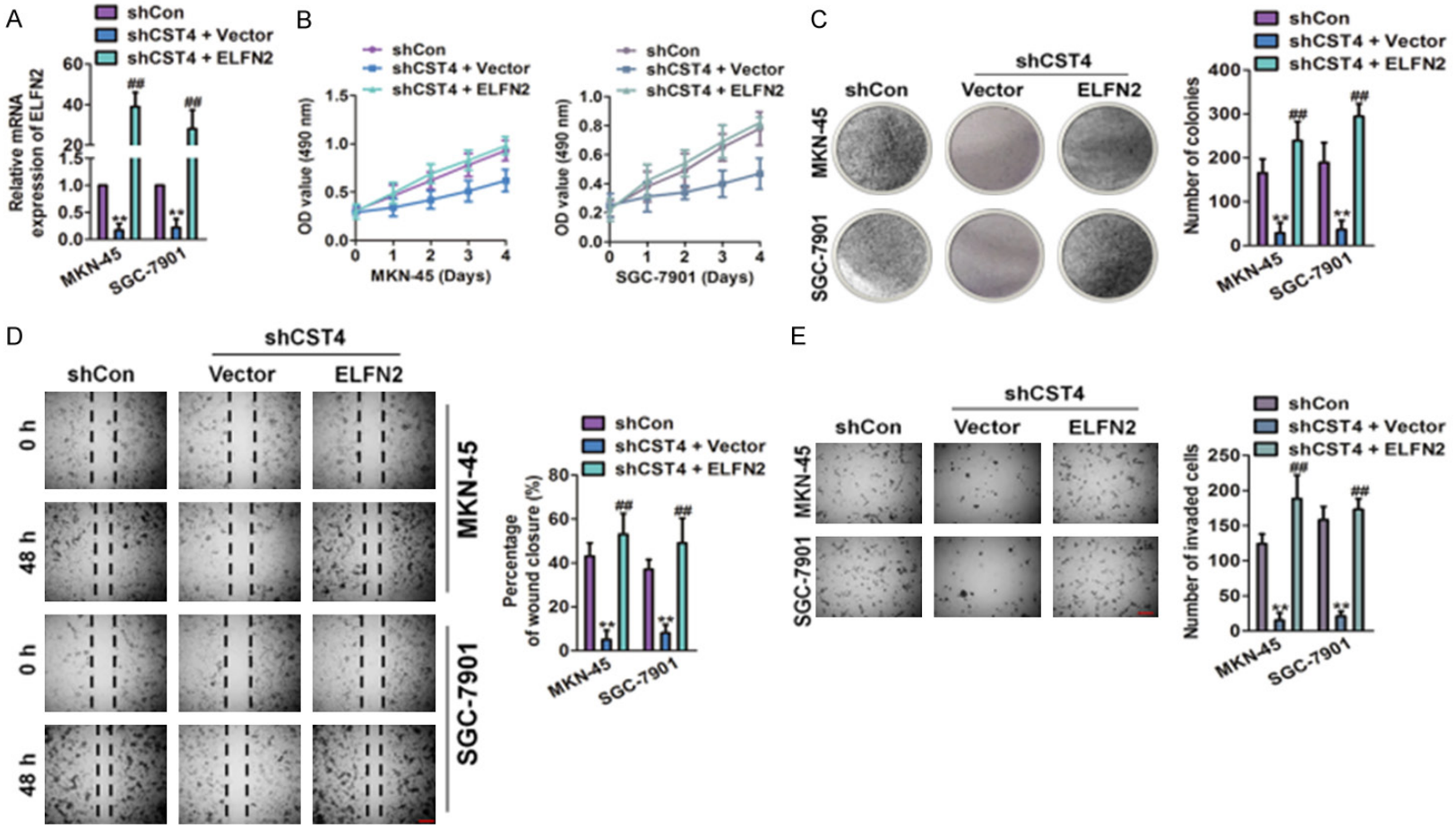

Figure 6. CST4 promotes the growth and metastasis of gastric cancer dependent on ELFN2. A. MKN-45 and SGC-7901 cells were co-transfected with the shCST4 and the ELFN2 plasmid. The levels of ELFN2 in MKN-45 and SGC-7901 cells were analyzed by qPCR. B. After co-transfection of cells with shCST4 and ELFN2 plasmid, cells proliferation was determined by MTT assays. C. ELFN2 overexpression counteracted the inhibition effects of colony formation in CST4 down-expression gastric cancer cells. D. Cells were co-transfected with lentiviral particles containing ELFN2 and shRNA targeting CST4. Cell migration in MKN-45 and SGC-7901 cells were analyzed by wound healing assays. E. Over-expression of ELFN2 may reverse the effect of the CST4 down-regulation on invasion in gastric cancer cells. Scale bar: 200 μm. **P < 0.01 compared to shCon, ##P < 0.01 compared to shCST4 + vector.

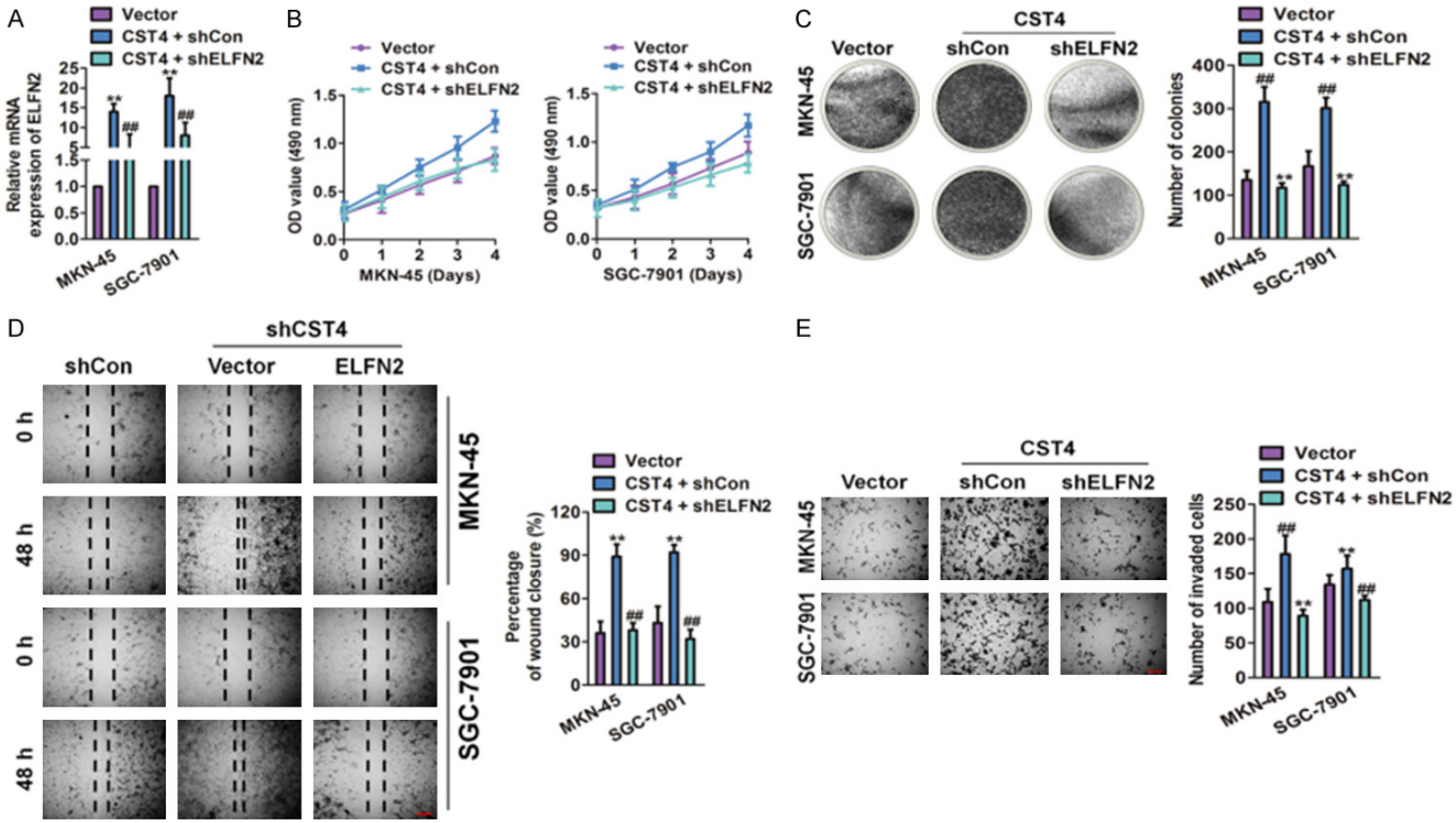

Figure 7. Knock down of ELFN2 inhibits the growth and metastasis of gastric cancer cells accelerated by ELFN2. A. CST4 over-expressing MKN-45 and SGC-7901 cells were transfected with shELFN2. The mRNA levels of ELFN2 were analyzed by qRT-PCR assays. B. ELFN2 down-expression counteracted the positive proliferative effects of CST4 over-expression in MKN-45 and SGC-7901 cells. C. ELFN2 inhibition hampered the colony formation of CST4 over-expression in gastric cancer cells. D. CST4 over-expression MKN-45 and SGC-7901 cells were transfected with shELFN2 and cell migration of gastric cancer cells were analyzed by wound closure assay. E. Cell invasion in indicated MKN-45 and SGC-7901 cells were analyzed by Transwell assays. Scale bar: 200 μm. **P < 0.01 compared to vector, ##P < 0.01 compared to CST4 + shCon group.

ally be significant for the diagnosis of bladder cancer [20]. Moreover, the cell proliferation, migration, and invasion abilities of gastric cancer cells were partially dependent on by CST4 expression. This study also revealed the critical contribution of ELFN2 in CST4-mediated gastric cancer growth and metastasis, which suggests that inhibition of the CST4/ELFN2 signaling axis is a potential therapeutic target for controlling gastric cancer aggression.

Disclosure of conflict of interest

None.

Address correspondence to: Yi Qiang Zhang, Department of Gastroenterology, Zhumadian Central Hospital of Henan Province, Zhumadian, China. E-mail: zhangyiqiangyq@yahoo.com

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