Cancer Letters 452 (2019) 103–118

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

# Sex determining region Y-box 12 (SOX12) promotes gastric cancer metastasis by upregulating MMP7 and IGF1

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ARTICLE INFO

*Keywords:*
SOX12
Gastric cancer
Metastasis
MMP7
IGF1

ABSTRACT

Metastasis is the major cause of poor survival and therapeutic failure in gastric cancer (GC). However, the molecular mechanisms underlying GC metastasis remain unclear. Here, we analyzed the expression patterns of sex determining region Y-box 12 (SOX12) in two independent cohorts of paired GC tissues and adjacent non-tumor tissues and conducted *in vitro* and *in vivo* functional studies to determine the role of SOX12 in GC cells. High SOX12 expression in GC tissues was associated with increased frequency of recurrence and poorer patient survival. SOX12 overexpression increased GC cell migration, invasion and metastasis, whereas SOX12 downregulation decreased these behaviors. Reporter assays revealed that matrix metallopeptidase 7 (MMP7) and insulin-like growth factor 1 (IGF1) are transcriptional targets of SOX12 and indicated their requirement for SOX12-mediated GC metastasis. IGF1 induced SOX12 expression via the PI3K/AKT/CREB pathway, forming an IGF1/CREB/SOX12 feedback loop that contributed to GC metastasis. SOX12 expression correlated with MMP7 and IGF1 expression in GC tissues, and patients expressing SOX12 and either MMP7 or IGF1 had higher metastasis and recurrence rates and shorter survival than patients without that expression pattern. In conclusion, SOX12 is a novel prognostic biomarker and regulator of GC metastasis.

## 1. Introduction

Gastric cancer (GC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide [1,2]. Many GC patients have either inoperable disease at diagnosis or recurrent disease after resection with curative intent, which represents the major contributors to the morbidity of GC [2]. In recent decades, the early detection of GC by screening high-risk patients, as well as targeted therapies such as the anti-HER2 antibody trastuzumab and the anti-VEGFR-2 antibody ramucirumab, has considerably improved the success of GC treatment [3–6]. However, the outcomes of patients with metastasis or recurrence remain poor [7]. Therefore, it is essential to elucidate the underlying mechanisms and pathways of GC progression in order to improve GC patient prognosis and survival.

Members of the sex determining region Y-box (SOX) family of transcription factors are well-established regulators of cell fate decisions and are characterized by a homologous sequence called the high mobility group (HMG) box [8]. The human genome encodes 20 SOX genes, which are divided into subgroups A to H according to homology within the HMG domain and the presence of other structural motifs [8]. The SOXC group proteins—SOX4, SOX11 and SOX12—were found to play key roles in cardiac, neuronal and mesenchymal development processes and, interestingly, in many types of cancers [9,10]. Previous studies reported that SOX4, SOX11 and SOX12 are upregulated in

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https://doi.org/10.1016/j.canlet.2019.03.035
Received 29 November 2018; Received in revised form 2 March 2019; Accepted 21 March 2019

several human cancers and contribute to cancer initiation and progression. SOX4 is a master regulator of epithelial-mesenchymal transition and exerts this activity by controlling Ezh2 expression and activating epigenetic reprogramming; this protein is a key oncogenic target in C/EBP alpha-mutated acute myeloid leukemia [11,12]. In addition, evidence suggests that SOX11 promotes tumor-protective microenvironment interactions and blocks terminal B cell differentiation in aggressive mantle cell lymphoma [13,14]. SOX12 was recently identified as a novel potential target in acute myeloid leukemia and as a cancer stem-like cell marker in hepatocellular carcinoma (HCC) [15,16]. These studies indicate that SOXC proteins are master regulators of cancer progression and metastasis. However, the expression and functional role of SOX12 in human GC remains unknown.

Insulin-like growth factor (IGF) signaling is fundamental for growth and survival. IGF1 and IGF2 stimulate downstream signaling events primarily by binding and activating the type 1 insulin-like growth factor receptor (IGF1R) [17], a transmembrane tyrosine kinase that is frequently overexpressed in tumors and mediates proliferation, protection against apoptosis, invasion and metastasis [18,19]. Up to 50% of patients with GC express the activated form of IGF1R, and overexpression of this protein is considered a useful independent predictor of outcomes in patients with stage II/III GC after they undergo curative resection and adjuvant chemotherapy [20]. A functional genetic approach in a recent study identified IGF1R pathways as one of the most important determinants of lapatinib unresponsiveness in HER2-positive GC [21]. These studies highlighted the important role of IGF1R in cancer progression and suggested IGF1R as an attractive antitumor treatment target in cancers. However, the mechanisms underlying IGF signaling in GC remain unclear.

The aim of this study was to determine the expression and possible tumorigenic role of SOX12 in GC metastasis and to explore the underlying mechanisms and signaling pathways involved. Here, we first reported that SOX12 overexpression is associated with GC progression and indicates poor prognosis. SOX12 promotes GC metastasis by transactivating matrix metallopeptidase 7 (MMP7) and IGF1R expression, which in turn induces SOX12 expression through the PI3K/AKT/CREB pathway.

## 2. Materials and methods

### 2.1. Cell culture

GES-1, AGS and BGC823 cells were obtained from American Tissue Type Culture Collection (ATCC). MKN28 cells were purchased from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank), and SGC7901 cells were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. MKN28-M, SGC7901-M, MKN28-NM and SGC7901-NM sublineage cells were derived from MKN28 and SGC7901 cells and maintained by our laboratory [22]. All cell lines were confirmed to be free of mycoplasma contamination. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, ThermoFisher Scientific, Cambridge, MA, USA) supplemented with 10% bovine growth serum, glutamine, nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) and maintained in an incubator at 37 °C containing 5% $CO_2$.

### 2.2. GC patients and follow-up

This study was approved by the ethics committee of the Fourth Military Medical University. All patients provided full consent for participation in the study. Fresh GC and adjacent tissue specimens were obtained from 389 adult patients (cohort I) who underwent surgery at Xijing Hospital of the Fourth Military Medical University (Xi'an, China) between January 2005 and December 2007 and from 360 adult patients (cohort II) who underwent surgical resection at the Tongji Hospital of Tongji Medical College (Wuhan, China) during the same time period. None of the patients received preoperative chemotherapy or radiotherapy. Pathological staging was performed according to the guidelines of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control. Patients with stage II, III, and IV disease were treated after surgery with adjuvant chemotherapy, but none of the patients received postoperative radiotherapy. The histomorphology of all primary tumor specimens and regional lymph nodes was confirmed via hematoxylin–eosin (H&E) staining by the Department of Pathology of Xijing Hospital. In addition, 120 pairs of frozen GC and nontumor tissues were collected after surgical resection and snap frozen in liquid nitrogen.

The patients were followed up for a minimum of 8 years, during which period they were monitored for recurrence and distant metastasis by endoscopy, ultrasonography, computed tomography, magnetic resonance imaging, and position emission tomography, and, when possible, cytological analyses and biopsy. The disease-free survival time was defined as the time between surgery and the first recurrence of GC, distant metastasis, the development of a second nongastric malignancy (excluding basal cell carcinoma of the skin and carcinoma *in situ* of the cervix), or death from any cause without documentation of a cancer-related event. The overall survival time was defined as the time between surgery and death. For all participants, the follow-up information was updated every 3 months by telephone inquiry and questionnaires. Patient deaths were confirmed by the family and verified by a review of public records.

### 2.3. Tissue microarray construction and immunohistochemistry (IHC)

IHC was performed on routinely processed 4-μm-thick paraffin-embedded sections as previously described [22]. Briefly, after the tissue sections were heated at 60 °C for 1 h, they were deparaffinized with xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide in methanol for 12 min, after which the sections were washed three times with phosphate-buffered saline (PBS). The slides were immersed in 0.01 M citrate buffer solution (pH 6.0) and heated in a microwave oven for 30 min for antigen retrieval. The slides were washed once with PBS and then incubated overnight with primary antibodies at 4 °C. After the sections were washed three times with PBS, they were incubated with a peroxidase-conjugated secondary antibody (Santa Cruz) for 30 min at room temperature. Staining was developed with diaminobenzidine (DAB) for 2 min, and the images were analyzed under a light microscope (Olympus, Japan) equipped with a DP70 digital camera. Antibodies against SOX12 (Abcam ab54371 MA, USA), MMP7 (Abcam ab205525) and IGF1 (Abcam ab9572) were used according to the manufacturer's instructions.

All tissues were scored independently by 2 pathologists blinded to the clinicopathological characteristics of the patients. Both staining intensity and the extent of target protein expression across the specimens were recorded as follows. The immunostaining intensity was scored on a scale of 0–2: 0, negative; 1, weak; 2, strong. The percentage of positive cells was evaluated on a scale of 0–4: 0, negative; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%; The final staining score was calculated by multiplying the intensity (0, 1, 2) and percentage scores (0, 1, 2, 3, 4), with possible scores of 0, 1, 2, 3, 4, 6 and 8. The median of 3 was used as the cut-point score for SOX12 expression. Therefore, each sample was considered ‘low’ if the final score was 0, 1, 2 or 3, and ‘high’ if the final score was 4, 6 or 8.

### 2.4. Plasmid construction

Plasmids were constructed as previously described [23]. The MMP7 promoter construct (−2972/+48 MMP7) corresponding to the sequence from bases −2972 to +48 (relative to the transcription start site) in the 5′-flanking region of the human MMP7 gene was generated from human genomic DNA by PCR amplification. The forward and reverse primers were designed to incorporate KpnI and HindIII sites at the 5′ and 3′ ends, respectively. The PCR product was cloned into the KpnI- and HindIII-digested sites of the pGL3-Basic vector (Promega, CA, USA). Three 5′-flanking MMP7 promoter deletion constructs (−1800/+48 MMP7, -632/+48 MMP7 and -186/+48 MMP7) were similarly generated using the −2972/+48 MMP7 construct as the template. The SOX12 binding sites in the MMP7 promoter were mutated using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, CA, USA). Other promoter constructs were similarly cloned, and all constructs were validated by DNA sequencing. The primer sequences used are listed in Supplementary Table S1.

### 2.5. Transient transfection and luciferase assay

Cells were plated at a density of 1 × $10^5$ cells/well in a 24-well plate. After 12–24 h, the cells were cotransfected with 0.6 μg of the expression plasmids, 0.18 μg of the reporter plasmids, and 0.02 μg of the pRL-TK plasmids using Lipofectamine 3000 (Invitrogen, MA, USA) according to the manufacturer's instructions. After 5 h of transfection, the cells were washed and allowed to recover in fresh medium supplemented with 1% fetal bovine serum (FBS) for 48 h, after which the cells were serum starved for the assay. Luciferase activity was detected using a dual luciferase assay (Promega) according to the manufacturer's instructions. The transfected cells were lysed, and the lysates were centrifuged at maximum speed for 1 min in an Eppendorf microcentrifuge tube. Relative luciferase activity was determined using a Modulus™ TD20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA), and the transfection efficiencies were normalized to Renilla luciferase activity.

### 2.6. Generation of lentivirus and stable cell lines

Short hairpin RNAs (shRNAs) were purchased from Sigma (MO, USA) and the sequences are listed in Table S2. The lentivirus production and subsequent infection were performed according to the pLKO.1 lentiviral vector protocol recommended by Addgene. Briefly, the lentiviral plasmid and the packaging plasmids pMD2.G and psPAX2 (Addgene plasmid #12259 and #12260, respectively) were transfected into HEK-293T cells with transfection reagent (Lipofectamine® 3000, Thermo Fisher Scientific) and OPTI-MEM (Invitrogen, Waltham, MA, USA). The lentiviruses were harvested on days 4 and 5, after which they were filtered through a 0.45-μm filter and stored at −80 °C until use. Lentiviral infection of the target cells was performed in cell culture medium supplemented with 5 μg/mL polybrene (Sigma H9268). Beginning 72 h after infection, the cells were selected for 2 weeks with 2.5 μg/mL puromycin (OriGene). Selected pools of cells were used for subsequent experiments.

### 2.7. In vivo metastasis and imaging

Six-week-old BALB/C nude mice were cared for and maintained based on our institution's protocols for ethical animal care. The Committee on the Use of Live Animals in Teaching and Research (CULATR) at the Fourth Military Medical University approved all animal experiments. The indicated luciferase-labeled cells were prepared in Hanks' balanced salt solution (HBSS) (Gibco) at densities of 2 × $10^6$ and 5 × $10^6$ cells/50 μl/injection (10 mice per group), and mice were injected with 5 × $10^6$ cells (see above) via tail vein (10 mice per group). The mice were injected intraperitoneally with D-luciferin (150 mg/kg) at weekly intervals and imaged 10 min after injection with an IVIS 100 imaging system (Xenogen) with a 2-min acquisition time. The mice were observed daily and sacrificed 9 weeks after cell injection. Liver and lung metastases in the mice were examined histopathologically.

### 2.8. In vitro migration and invasion assay

Transwell inserts with polycarbonate membranes with 8.0 μm pores were placed in the wells of a 24-well cell culture plates (Corning, USA). For the invasion assays, the upper chamber was coated with 60 μL of Matrigel (Corning, 200 mg/mL) and dried overnight. For the migration and invasion assays, 5 × $10^4$ and 1 × $10^5$ cells, respectively, were plated in the top chamber, and the lower chamber was filled with 600 μL of complete medium. After incubation for 24 h, the cells on the upper surface of the membranes were removed by swabbing, and the cells on the lower surface were fixed with methanol for 20 min and stained with 0.1% crystal violet for 5 min. The average number of cells in five random fields per membrane was counted on three inserts. The relative invasion and migration levels were expressed as the ratio of the number of counted treated cells to the number of counted control cells.

### 2.9. Real-time PCR

Total RNA was extracted using an RNeasy Plus Mini Kit (50) (QIAGEN), and reverse transcription was performed using an Advantage RT-for-PCR Kit (QIAGEN) according to the manufacturer's instructions. For RT-PCR analyses, cDNA was amplified using a SYBR Green PCR Kit (QIAGEN). The cycling parameters were as follows: 45 cycles of 95 °C for 15 s, 55–60 °C for 15 s, and 72 °C for 15 s. A melting curve analysis was then performed, and Ct was measured during the exponential amplification phase using SDS 1.9.1 software (Applied Biosystems). The expression level was normalized to the fold change in the corresponding control cells or normal tissues, which was set as 1. All reactions were performed in duplicate. The primer sequences used are listed in Supplementary Table S1.

### 2.10. Chromatin immunoprecipitation (ChIP) assay

Cells transfected with the appropriate plasmids were crosslinked in 1% formaldehyde at 37 °C for 10 min. After the cells were washed with PBS, they were resuspended in 300 μL of lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS, and 1 mM PMSF). The DNA was sheared into small fragments by sonication. The supernatants were precleared using a herring sperm DNA/protein G-Sepharose slurry (Sigma, MO, USA) followed by incubation with either specific antibodies or isotype control IgG for 2 h in the presence of herring sperm DNA and protein G-Sepharose beads. The immunoprecipitated DNA was retrieved from the beads with a solution of 1% SDS and 1.1 M $NaHCO_3$ at 65 °C for 6 h. The DNA was then purified using a PCR Purification Kit (Qiagen, USA). The primers used are shown in Supplementary Table S1.

### 2.11. Western blotting

Proteins from lysed cells were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Nonspecific binding was blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST; 120 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated with the specific primary antibodies overnight at 4 °C. Antibodies against SOX12 (ab54371, Abcam, MA, USA), MMP7 (ab205525, Abcam), IGF1 (ab9572, Abcam), phospho-AKT (#13038, Cell Signaling Technology (CST, Danvers, MA), AKT (#4691, CST), phospho-ERK (#4377, CST), ERK (#4695, CST) and the control β-actin (sc-47778; Santa Cruz Biotechnology) were used. The membranes were then washed three times with PBS and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands were visualized using Dura SuperSignal Substrate (Pierce, USA).

### 2.12. Reagents

The ERK1/2 inhibitor SCH772984 (#S7101) and PI3K inhibitor LY294002 (#S1105) were purchased from Selleck Chemicals (Houston, TX, USA). Recombinant human IGF-I protein was purchased from Bio-Techne (#P05019, R&D Systems, MN, USA). All the reagents were used according to the manufacturer's instructions.

### 2.13. Preparation of conditioned medium and enzyme-linked immunosorbent assay (ELISA)

We prepared conditioned medium (CM) from GC cells that were seeded in 100 mm plastic dishes with 10 mL DMEM containing 10% FCS and then incubated for 3 days. To obtain CM, cells were washed with PBS and then incubated for an additional 3 days in 3 mL of serum-free DMEM. Next, the media were collected from each dish, centrifuged, and stored as CM at −20 °C until use. All experiments were performed in medium containing 2% FCS. As a control, DMEM was used instead of CM.

MMP7 and IGF1 levels in CM were quantified using an MMP7 ELISA kit (R&D Systems) and IGF1 ELISA kit (R&D Systems), respectively. The number of cells was counted when the CM was collected, and ELISA data were normalized to a count of $1 \times 10^6$ cells in tested all cell lines.

### 2.14. Statistical analysis

All analyses were performed using SPSS software (version 19.0). Quantitative data were compared using Student's t-test, and categorical data were analyzed using Fisher's exact test. The cumulative recurrence and survival rates were determined using the Kaplan–Meier method and the log-rank test. The Cox proportional hazards model was used to determine the independent factors influencing survival and recurrence based on the variables selected from the univariate analyses. A p value of $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. SOX12 is significantly upregulated in human GC tissues and indicates poor prognosis

SOX12 mRNA expression was analyzed in a series of paired GC tissues and adjacent nontumor tissues and in normal gastric epithelial tissues by RT-PCR. SOX12 levels were significantly higher in the GC tissues than in the adjacent nontumor tissues ($n = 120$) (Fig. 1A). Patients with metastatic ($n = 59$) or recurrent ($n = 58$) GC had significantly higher levels of SOX12 than those with nonmetastatic ($n = 61$) or nonrecurrent ($n = 62$) tumors, respectively (Fig. 1B–C). In addition, SOX12 expression was upregulated in the metastatic foci compared to that in the paired primary malignancies ($n = 30$) (Fig. 1D). The trends in SOX12 upregulation in the GC tissues were also validated by Western blotting and IHC (Fig. 1E–F, Supplementary Figure S1).

To determine the prognostic value of SOX12 expression levels in GC tissues, the patients in both cohort I ($n = 389$) and cohort II ($n = 360$) were stratified on the basis of SOX12 expression. We found that higher SOX12 expression was positively correlated with poor tumor differentiation, increased lymph node and distant metastasis, and more advanced AJCC stage and was an independent risk factor for GC progression (Tables 1–3). Kaplan–Meier analysis showed that patients with SOX12-positive tumors had a significantly higher risk of recurrence and poorer survival than patients with SOX12-negative tumors (Fig. 1G).

### 3.2. SOX12 promotes GC cell invasion and metastasis

The mRNA and protein levels of SOX12 were assessed in a battery of GC cell lines, which included normal gastric epithelial cells (GES-1) and cell sublines with weak (MKN28-NM and SGC7901-NM) or strong (SGC7901-M and MKN28-M) metastatic ability (Supplementary Figure S2). The results showed that SOX12 expression was significantly upregulated in GC cells compared with GES-1 cells and was correlated with the cells' metastatic ability (Fig. 1H), suggesting that SOX12 plays an important role in GC metastasis. To test this hypothesis, transgenic GC cell lines with either overexpression or depletion of SOX12 were generated. Western blot analysis confirmed SOX12 overexpression in MKN28-NM and SGC7901-NM cells and knockdown in MKN28-M and SGC7901-M cells (Fig. 2A). The *in vitro* migration and invasion of GC cells were analyzed by Transwell assays. As shown in Fig. 2B, SOX12 overexpression increased the number of migrating and invading cells, but SOX12 knockdown decreased both values (Fig. 2B). In addition, we also investigated the role of SOX12 on GC cell proliferation. Ectopic SOX12 expression promoted GC cell proliferation and colony formation while downregulation of SOX12 resulted in opposing effects (Supplementary Figure S2). These findings suggested that SOX12 not only promotes GC metastasis but also facilitates GC cell proliferation and colony formation. However, because metastasis is more detrimental to GC patients, we mainly focused the role of SOX12 in GC metastasis in this study.

To determine the role of SOX12 in metastasis, nude mice were injected with either MKN28-NM-SOX12 or MKN28-M-shSOX12 cells (along with the corresponding control cells), and lung metastasis was tracked using bioluminescence imaging. Mice injected with cells overexpressing SOX12 showed markedly increased GC metastasis, but cells with downregulated SOX12 exhibited reduced GC metastasis, as indicated by the bioluminescence signals and the incidence of lung metastasis (Fig. 2C–E). Consistent with the increased level of lung metastasis, a significantly shorter survival time was observed in mice injected with MKN28-NM-SOX12 cells than in those injected with MKN28-NM-Control cells. In contrast, the survival of mice injected with MKN28-M-shSOX12 cells was significantly worse than that of mice injected with the corresponding control cells (Fig. 2F). Histopathological examination of the lungs showed a significantly higher number of metastatic nodules in MKN28-NM-SOX12-injected mice than in MKN28-NM-Control-injected mice, while mice injected with MKN28-M-shSOX12 cells presented significantly fewer metastatic nodules than did mice injected with MKN28-M-shControl cells (Fig. 2G–H). At molecular levels, we examine the levels of three well-established metastasis markers—MMP2, MMP9 and fibronectin—in SOX12-overexpressing MKN28-NM and SGC7901-NM cells and in SOX12-knockdown MKN28-M and SGC7901-M cells. The Western blot assays indicated that SOX12 overexpression increase the expression of MMP2, MMP9 and fibronectin, while downregulation of SOX12 reduced the expression of MMP2, MMP9 and fibronectin (Supplementary Figure S4), which supports the role of SOX12 in promoting GC migration, invasion and metastasis. Taken together, these findings indicate that SOX12 increases the invasiveness and metastatic ability of GC cells both *in vitro* and *in vivo*.

### 3.3. MMP7 and IGF1 are direct transcriptional targets of SOX12

To investigate the mechanism by which SOX12 promotes GC progression, we performed a Metastasis RT [2] Profiler PCR Array analysis to examine transcriptome variations mediated by SOX12 overexpression in MKN28-NM cells. Using a fold change of two as the cutoff value, 24 of the 89 metastasis-related genes were upregulated upon SOX12 overexpression in MKN28-NM cells, 20 genes were downregulated, and 45 genes showed no significant changes (Table S3). Among the upregulated genes, *MMP7* and *IGF1* exhibited the strongest induction responses to SOX12 overexpression (Table S3).

To confirm whether SOX12 activates MMP7 and IGF1 expression, the mRNA and protein levels of MMP7 and IGF1 were analyzed in cells with SOX12 overexpression or depletion. SOX12 overexpression significantly increased the MMP7 and IGF1 mRNA levels, whereas the

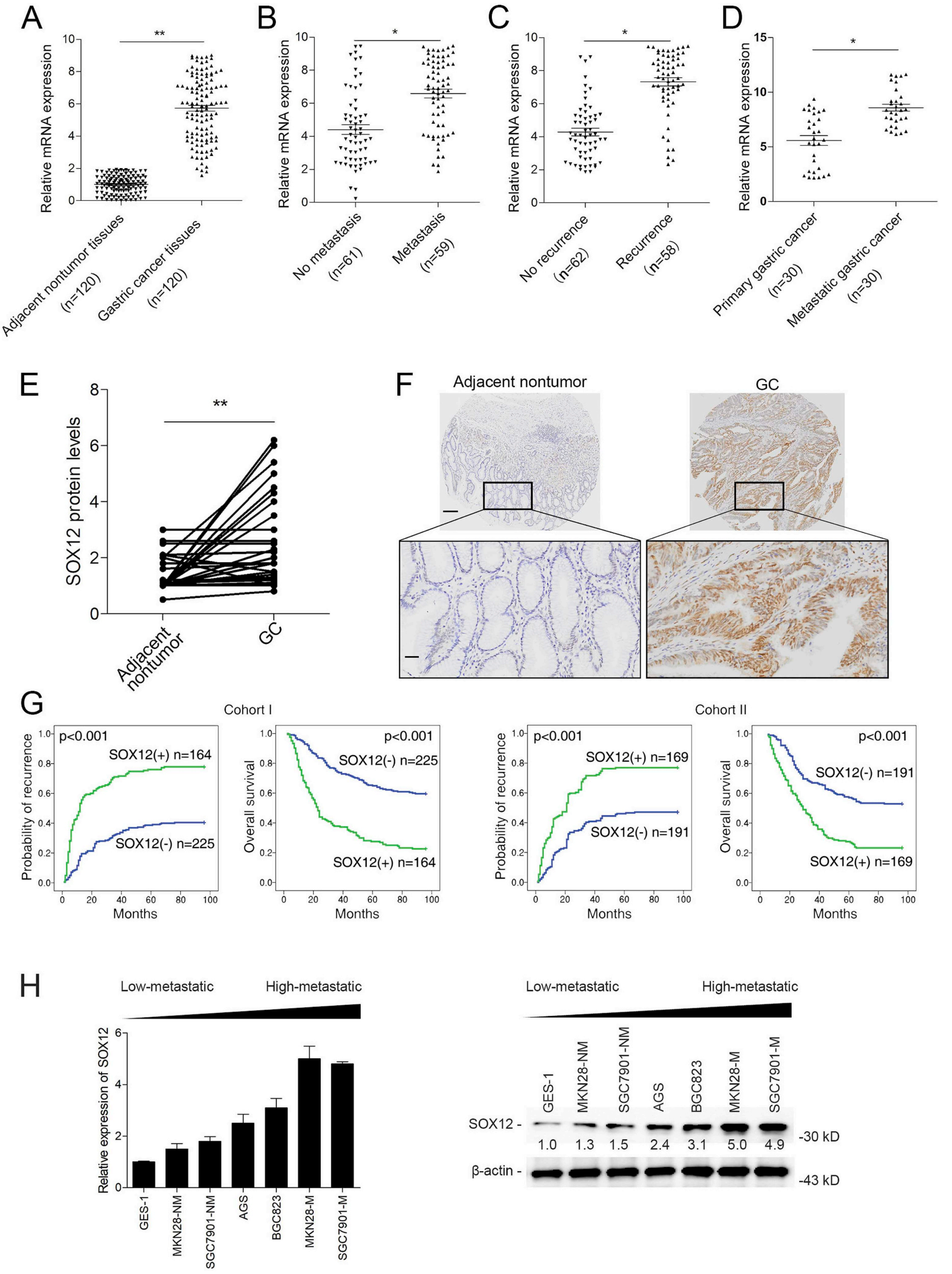


**Fig. 1.** SOX12 is significantly upregulated in human GC tissues and predicts poor prognosis. (A) Real-time PCR analysis of *SOX12* mRNA expression in paired GC tissues and adjacent nontumor tissues (n = 120). (B) Relative expression of *SOX12* mRNA in GC tissue samples from patients with metastasis (n = 59) and without metastasis (n = 61). (C) Relative expression of *SOX12* mRNA in GC tissue samples from patients with recurrence (n = 58) and without recurrence (n = 62). (D) Real-time PCR analysis of *SOX12* mRNA levels in paired primary GC tissues and metastatic GC tissues (n = 30). (E) Western blot analysis of SOX12 expression in 30 cases of paired GC and adjacent nontumor tissues. (F) Representative images showing SOX12 expression in GC and adjacent nontumor tissues. The scale bars represent 200 μm (low magnification) and 50 μm (high magnification). (G) Kaplan–Meier analysis showing the correlation between SOX12 expression and recurrence or overall survival in patients with GC. (H) Real-time PCR and Western blotting analysis of SOX12 expression in different GC cell lines. $^*p < 0.05$.

**Table 1**
Correlation between SOX12 expression and clinicopathological characteristics of gastric cancer in two independent cohorts of human gastric cancer tissues.

| Clinicopathological variables | | Cohort I | | | Cohort II | | |
|---|---|---|---|---|---|---|---|
| | | Tumor SOX12 expression | | *P* value | Tumor SOX12 expression | | *P* value |
| | | Low (n = 225) | High (n = 164) | | Low (n = 191) | High (n = 169) | |
| Age | | 64.79(10.72) | 64.79(11.16) | 0.694 | 61.68(11.66) | 63.11(11.48) | 0.752 |
| Sex | female | 74 | 48 | 0.507 | 59 | 54 | 0.909 |
| | male | 151 | 116 | | 132 | 115 | |
| Tumor size | < 5 cm | 97 | 58 | 0.142 | 86 | 55 | 0.017 |
| | ≥ 5 cm | 128 | 106 | | 105 | 114 | |
| Tumor differentiation | well or moderate | 101 | 59 | 0.095 | 108 | 58 | < 0.001 |
| | poor | 124 | 105 | | 83 | 110 | |
| Tumor invasion | T1 | 18 | 2 | < 0.001 | 16 | 4 | < 0.001 |
| | T2 | 35 | 11 | | 28 | 6 | |
| | T3 | 140 | 91 | | 127 | 123 | |
| | T4 | 32 | 60 | | 20 | 36 | |
| Lymph node metastasis | absent | 81 | 20 | < 0.001 | 63 | 18 | < 0.001 |
| | present | 144 | 144 | | 128 | 151 | |
| Distant metastasis | absent | 213 | 108 | < 0.001 | 186 | 130 | < 0.001 |
| | present | 12 | 56 | | 5 | 39 | |
| AJCC stage | Stage I | 30 | 8 | < 0.001 | 29 | 4 | < 0.001 |
| | Stage II | 93 | 22 | | 77 | 26 | |
| | Stage III | 91 | 78 | | 80 | 99 | |
| | Stage IV | 11 | 56 | | 5 | 40 | |

mRNA levels of both genes were significantly decreased when SOX12 expression was downregulated (Fig. 3A). Similar trends were seen in the MMP7 and IGF1 protein levels (Fig. 3B and Supplementary Figure S3). To determine whether SOX12 mediates the activation of MMP7 and IGF1 transcription, constructs expressing the MMP7 or the IGF1 promoter were cotransfected with pCMV-SOX12, and the relative luciferase activity was determined. The results showed that luciferase activity was significantly increased in cells transfected with either the MMP7 or the IGF1 promoter (Fig. 3C), indicating that SOX12 transcriptionally activated the transcription of both MMP7 and IGF1. We then analyzed the MMP7 and IGF1 promoter sequences and found four potential SOX12 binding sites in both promoters. To identify the specific SOX12 binding sites in their promoters, deletions and selective mutations were introduced into the promoter sequences. The constructs containing serially truncated and mutated MMP7 or IGF1 promoters were then cotransfected with pCMV-SOX12. Although mutations in binding site 3 on the MMP7 promoter did not affect luciferase activity, mutation and complete removal of putative binding site 3 drastically reduced and complete eliminated luciferase activity, indicating that this was the SOX12 binding site responsible for driving MMP7 transcription (Fig. 3D). Using the same approach, we identified binding site 4 on the IGF1 promoter as the putative SOX12 binding site (Fig. 3E). Furthermore, the direct binding of SOX12 to the MMP7 and IGF1 promoters was validated by ChIP (Fig. 3F–G). Taken together, these findings indicate that SOX12 binds directly to specific sites on the MMP7 and IGF1 promoters and transcriptionally activates the expression of these genes.

### 3.4. *MMP7 and IGF1 are essential for SOX12-promoted GC migration and metastasis*

The effective knockdown and overexpression of MMP7 and IGF1 were validated by Western blotting (Fig. 4A). Downregulation of MMP7 and IGF1 significantly suppressed SOX12-mediated cell migration and invasion (Fig. 4B); however, upregulation of MMP7 and IGF1 reversed the SOX12 knockdown-induced decrease in the migration and invasion abilities (Fig. 4B). In the *in vivo* metastasis assays, GC cells with SOX12 overexpression but either MMP7 or IGF1 knockdown metastasized poorly, with limited metastasis to the lungs and very few metastatic nodules. In contrast, overexpression of MMP7 and IGF1 reversed the decrease in metastasis induced by SOX12 knockdown in MKN28-M cells, as shown by the higher *in vivo* bioluminescence signals, greater incidence of lung metastasis, and higher number of lung metastatic nodules (Fig. 4C–E, G). Compared with mice transplanted with the

**Table 2**
Univerate and multivariate analysis of factors associated with survival and recurrence in Cohort I human GCs.

| Variables | Recurrence | | | | | | Survival | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate analysis | | | Multivariate analysis | | | Univariate analysis | | | Multivariate analysis | | |
| | HR | 95% CI | p value | HR | 95% CI | *P* value | HR | 95% CI | p value | HR | 95% CI | *P* value |
| Age | 1.001 | 0.988–1.014 | 0.885 | | | | 1.001 | 0.989–1.014 | 0.839 | | | |
| Sex (female versus male) | 1.032 | 0.777–1.371 | 0.828 | | | | 1.008 | 0.758–1.341 | 0.957 | | | |
| Tumor size (≤5 versus > 5 cm) | 0.394 | 0.291–0.533 | < 0.001 | 0.631 | 0.460–0.865 | 0.004 | 0.392 | 0.290–0.531 | < 0.001 | 0.640 | 0.466–0.878 | 0.006 |
| Tumor differentiation(well/moderate versus poor) | 0.288 | 0.210–0.394 | < 0.001 | 0.325 | 0.236–0.449 | < 0.001 | 0.278 | 0.203–0.382 | < 0.001 | 0.308 | 0.222–0.426 | < 0.001 |
| Tumor invasion(T1-T3 versus T4) | 0.337 | 0.253–0.448 | < 0.001 | 1.315 | 0.838–2.063 | 0.233 | 0.330 | 0.248–0.439 | < 0.001 | 1.304 | 0.833–2.042 | 0.246 |
| Lymph node metastasis (absent versus present) | 0.176 | 0.111–0.280 | < 0.001 | 0.577 | 0.309–1.075 | 0.083 | 0.175 | 0.111–0.278 | < 0.001 | 0.594 | 0.316–1.117 | 0.106 |
| Distant metastasis (absent versus present) | 0.096 | 0.068–0.134 | < 0.001 | 0.163 | 0.098–0.272 | < 0.001 | 0.090 | 0.064–0.126 | < 0.001 | 0.152 | 0.092–0.251 | < 0.001 |
| AJCC stage(I-II versus III-IV) | 0.147 | 0.102–0.213 | < 0.001 | 0.358 | 0.213–0.599 | < 0.001 | 0.142 | 0.098–0.207 | < 0.001 | 0.340 | 0.200–0.576 | < 0.001 |
| SOX12 expression (low versus high) | 0.322 | 0.245–0.422 | < 0.001 | 0.539 | 0.398–0.731 | < 0.001* | 0.323 | 0.246–0.424 | < 0.001 | 0.561 | 0.414–0.762 | < 0.001* |

**Table 3**
Univerate and multivariate analysis of factors associated with survival and recurrence in Cohort II human GCs.

| Variables | Recurrence | | | | | | Survival | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate analysis | | | Multivariate analysis | | | Univariate analysis | | | Multivariate analysis | | |
| | HR | 95% CI | p value | HR | 95% CI | *P* value | HR | 95% CI | p value | HR | 95% CI | *P* value |
| Age | 0.996 | 0.985–1.008 | 0.520 | | | | 0.996 | 0.985–1.008 | 0.530 | | | |
| Sex (female versus male) | 1.120 | 0.846–1.483 | 0.428 | | | | 1.130 | 0.853–1.497 | 0.393 | | | |
| Tumor size (≤5 versus > 5 cm) | 0.608 | 0.458–0.806 | 0.001 | 0.881 | 0.660–1.175 | 0.388 | 0.608 | 0.458–0.807 | 0.001 | 0.880 | 0.659–1.175 | 0.386 |
| Tumor differentiation(well/moderate versus poor) | 0.471 | 0.357–0.622 | < 0.001 | 0.802 | 0.600–1.071 | 0.135 | 0.467 | 0.354–0.617 | < 0.001 | 0.825 | 0.617–1.104 | 0.195 |
| Tumor invasion(T1-T3 versus T4) | 0.419 | 0.304–0.579 | < 0.001 | 1.062 | 0.632–1.786 | 0.820 | 0.420 | 0.304–0.579 | < 0.001 | 1.126 | 0.663–1.912 | 0.660 |
| Lymph node metastasis (absent versus present) | 0.155 | 0.092–0.263 | < 0.001 | 0.517 | 0.268–1.001 | 0.050 | 0.154 | 0.091–0.261 | < 0.001 | 0.511 | 0.265–0.983 | 0.044 |
| Distant metastasis (absent versus present) | 0.241 | 0.171–0.339 | < 0.001 | 0.454 | 0.258–0.797 | 0.006 | 0.238 | 0.169–0.335 | < 0.001 | 0.427 | 0.240–0.760 | 0.004 |
| AJCC stage(I-II versus III-IV) | 0.151 | 0.104–0.219 | < 0.001 | 0.276 | 0.169–0.451 | < 0.001 | 0.149 | 0.102–0.216 | < 0.001 | 0.271 | 0.166–0.441 | < 0.001 |
| SOX12 expression (low versus high) | 0.425 | 0.324–0.557 | < 0.001 | 0.727 | 0.543–0.973 | 0.032* | 0.427 | 0.326–0.560 | < 0.001 | 0.727 | 0.543–0.974 | 0.033* |

corresponding control cells, mice transplanted with MKN28-NM-SOX12 cells overexpressing MMP7 or IGF1 showed significantly shorter survival times, while mice transplanted with MKN28-M-shSOX12 cells with either MMP7 or IGF1 knockdown showed significantly longer survival times (Fig. 4F). These findings indicate that SOX12 promotes GC migration and metastasis by upregulating MMP7 and IGF1.

### *3.5. IGF1 activates SOX12 expression through the PI3K/AKT/CREB pathway*

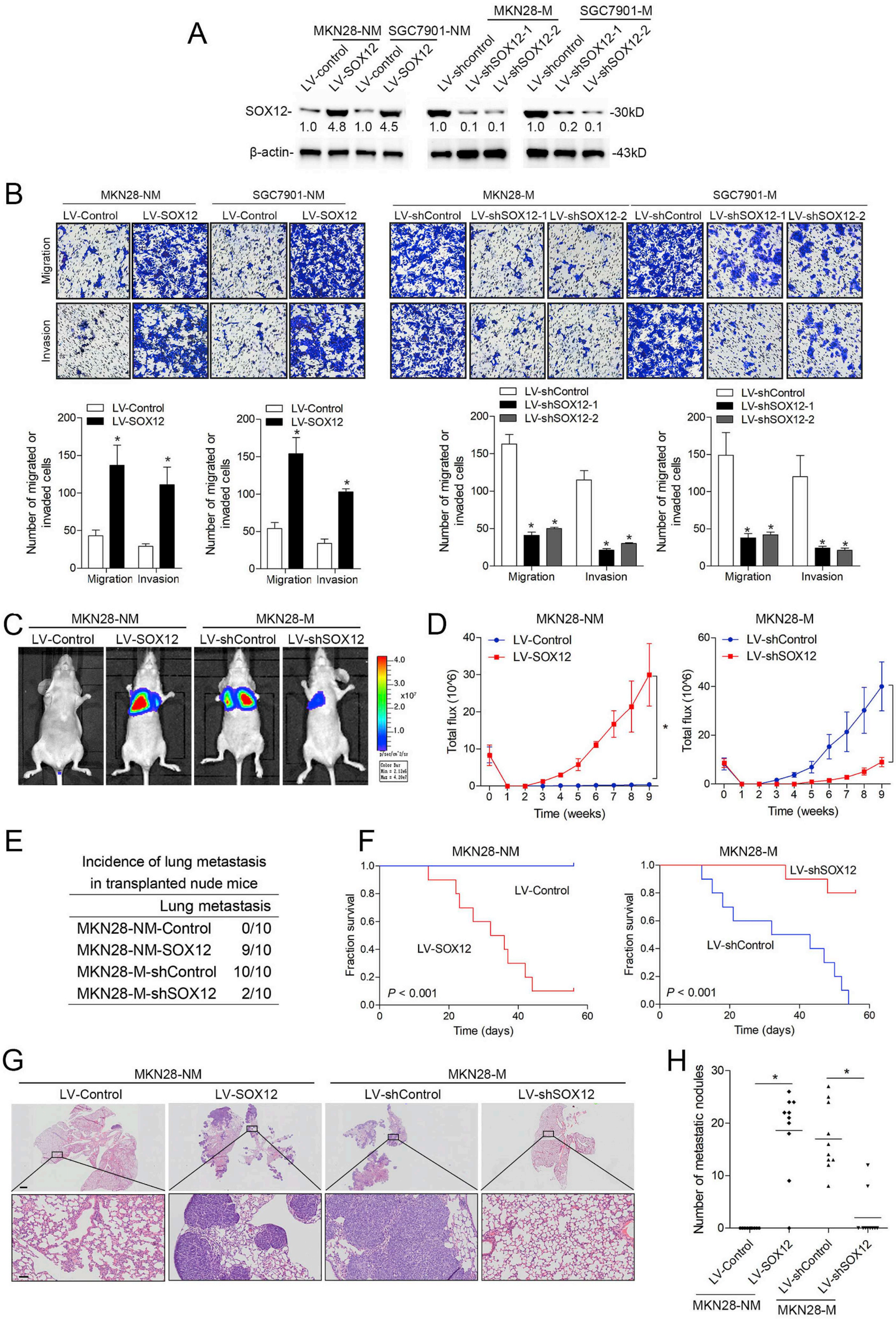
To explore the mechanism regulating SOX12 overexpression in human GC, we analyzed the SOX12 promoter and found several CREB, ELK1, STAT3 and AP-1 binding sites. Previous studies reported that IGF1 signaling activates ELK1 and AP-1 through the ERK and PI3K signaling pathways [24,25]. Based on these studies, we hypothesized that IGF1 regulates SOX12 expression. To test this hypothesis, SOX12 expression was analyzed in GC cells treated with IGF1. As shown in Fig. 5A and B, IGF1 treatment led to SOX12 upregulation at both the mRNA and protein levels. Furthermore, GC cells transfected with a luciferase reporter construct under the control of the SOX12 promoter were treated with IGF1 and showed significantly higher luciferase activity than did GC cells treated with vehicle control (Fig. 5C). This finding indicates that SOX12 is a downstream target of and is transcriptionally activated by IGF1. Because IGF1 is known to signal via the PI3K/AKT and MEK/ERK pathways, MKN28-NM cells were treated with IGF1 and specific inhibitors of either PI3K or ERK1/2. The protein levels of SOX12 and both phosphorylated and total AKT and ERK1/2 were then analyzed by Western blotting, which showed decreased levels of SOX12 following inhibition of the PI3K/AKT pathway (Fig. 5D). To identify the putative transcription factor and binding sites, IGF1-overexpressing GC cells were transiently transfected with a luciferase reporter construct under the control of serially truncated versions of the SOX12 promoter. A drastic reduction in the luciferase signal was observed in cells transfected with the truncated SOX12 promoter, and the (−302∼-45) region, which contains two CREB and two ELK1 binding sites, was identified as the IGF1-responsive site. Site-directed mutagenesis showed that mutation of the ELK1 binding sites had no effect on IGF1-mediated SOX12 promoter activity, but mutation of the CREB binding sites significantly reduced IGF1-mediated SOX12 promoter activity (Fig. 5E), suggesting that the CREB binding sites are required for SOX12 activation. CREB silencing drastically reduced both the expression and promoter activity of SOX12, which were upregulated by IGF1 (Fig. 5F). ChIP assays revealed that CREB binds directly to the SOX12 promoter (Fig. 5G). Taken together, these results suggest that IGF1 upregulates SOX12 expression through the PI3K/AKT/CREB signaling pathway.

### *3.6. SOX12 is critical for IGF1-induced GC metastasis*

To test whether SOX12 is involved in IGF1-mediated metastasis, we stably downregulated SOX12 expression in IGF1-overexpressing cells (MKN28-NM-IGF1, Fig. 6A). The *in vitro* migration and invasion of MKN28-NM-IGF1 cells was significantly reduced upon SOX12 knockdown (Fig. 6B), indicating that SOX12 is critical for the IGF1-mediated invasion of GC cells. To confirm the crucial role of SOX12 in IGF1-mediated metastasis, nude mice were injected via caudal vein with MKN28-NM-IGF1 cells with or without SOX12 knockdown, and lung metastasis was tracked (Fig. 6C–D). The overall incidence of lung metastasis was significantly higher in mice injected with MKN28-NM-IGF1-shControl cells than in those injected with MKN28-NM-IGF1-shSOX12 cells (Fig. 6E). Concordantly, the survival of mice transplanted with MKN28-NM-IGF1-shSOX12 cells was significantly improved compared with that of mice injected with MKN28-NM-IGF1-shControl cells (Fig. 6F). Furthermore, metastatic nodule formation in the lungs of mice transplanted with IGF1-overexpressing GC cells was significantly reduced after SOX12 knockdown (Fig. 6G–H). Taken together, these findings indicate that SOX12 is crucial for IGF1-mediated GC invasion and metastasis.

### *3.7. SOX12 expression is positively correlated with MMP7 and IGF1 expression in human GC tissues*

The clinical relevance of the SOX12, MMP7 and IGF1 was determined by analyzing the coexpression of these markers *in situ* in GC tissue samples and correlating their expression levels with the patients' clinicopathological features. Serially sliced tissue sections stained for SOX12, MMP7 and IGF1 indicated colocalization of these markers (Fig. 7A), and a clear positive correlation between SOX12 and MMP7/IGF1 expression in GC tissues was seen in both patient cohorts (Fig. 7B–C). Furthermore, in cohorts I and II, patients with high MMP7 or IGF1 expression in their GC tissues had a significantly higher recurrence risk and shorter survival time than patients with low expression of these markers (Fig. 7D–G, upper panel). Positive expression of MMP7 or IGF1 was also positively correlated with poor tumor differentiation, increased lymph node and distant metastasis, and more advanced AJCC stages (Tables 4–5). Finally, patients expressing high levels of both SOX12 and MMP7/IGF1 had the highest risk of recurrence and lowest survival rates, followed by those positive for either SOX12 or MMP7/IGF1 expression. Patients whose GC tissues were negative for SOX12 and MMP7/IGF1 expression had the lowest recurrence risk and highest survival rates (Fig. 7D–G, lower panel). Taken together, these findings indicate that SOX12 and MMP7/IGF1 coexpression in GC tissues is associated with increased recurrence and predicts poor prognosis and survival of GC patients.



Incidence of lung metastasis in transplanted nude mice

| | Lung metastasis |
|---|---|
| MKN28-NM-Control | 0/10 |
| MKN28-NM-SOX12 | 9/10 |
| MKN28-M-shControl | 10/10 |
| MKN28-M-shSOX12 | 2/10 |

**Fig. 2.** SOX12 promotes GC invasion and metastasis. (A) Western blotting confirming SOX12 overexpression in MKN28-NM and SGC7901-NM cells and SOX12 knockdown in MKN28-M and SGC7901-M cells. (B) Transwell assay showing the migration and invasion of the indicated GC cells. (C–H) Lung metastasis of GC cells injected via the tail vein: (C) Representative bioluminescence images indicating the metastasized cells at 9 weeks after injection. (D) Bioluminescent cells at the indicated time points represented as the total photon flux. (E) The number of mice in each group with lung metastasis. (F) The overall survival of mice in each group. (G) Representative images of H&E-stained lung tissues. The scale bars represent 1 mm (low magnification) and 100 μm (high magnification). (H) The number of lung metastatic nodules in the indicated groups. $^*p < 0.05$.

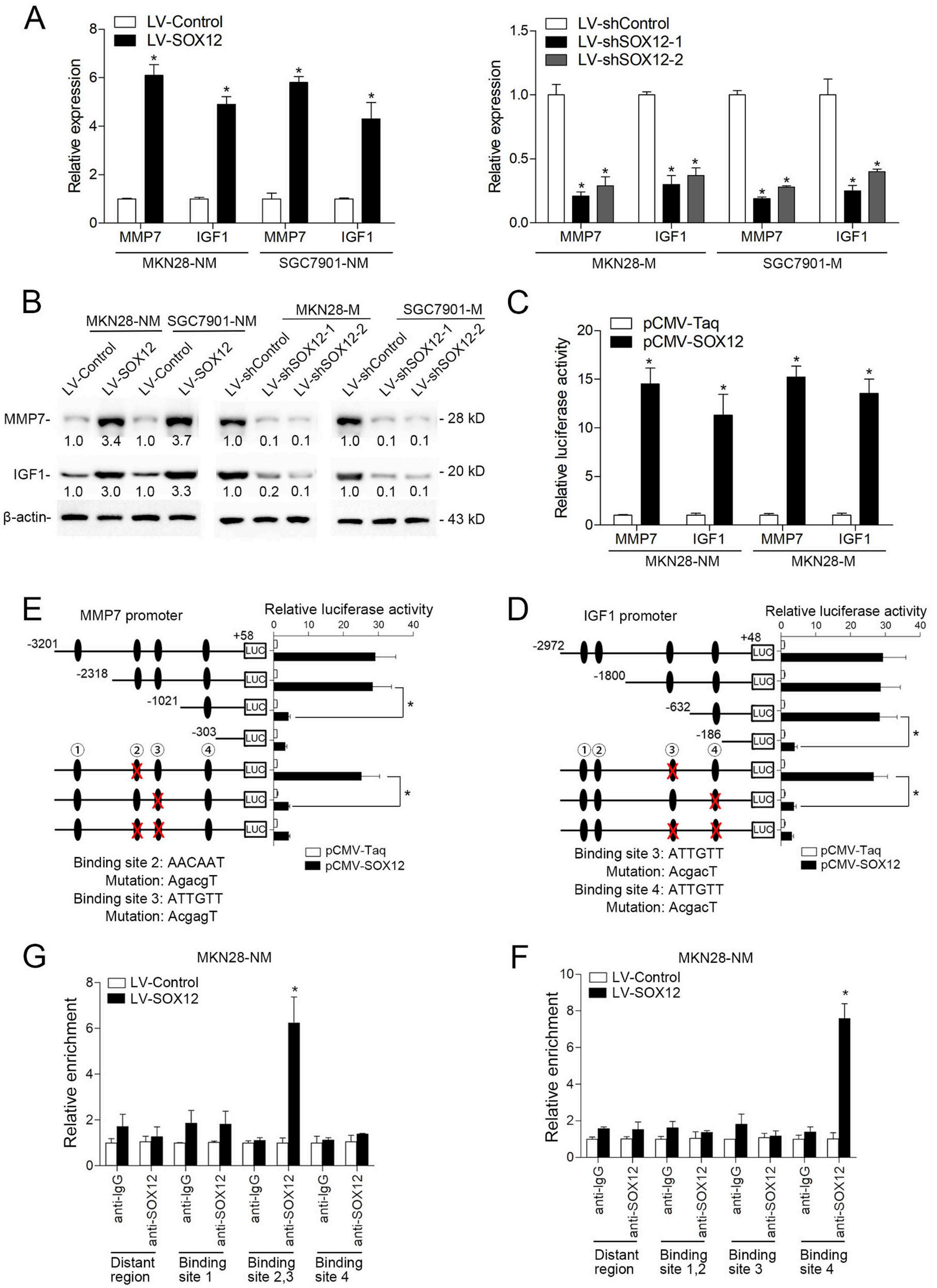


**Fig. 3.** MMP7 and IGF1 are direct transcriptional targets of SOX12. (A) MMP7 and IGF1 mRNA levels in the indicated GC cells. (B) Protein levels of SOX12, MMP7 and IGF1 in the indicated GC cells. (C) Luciferase activity of the reporter gene driven by the MMP7 and IGF1 promoters in the indicated GC cells. (D and E) Luciferase activity of the reporter gene driven by serially truncated/mutated MMP7 and IGF1 promoters indicates the SOX12 binding sites in MKN28-NM cells. (F and G) ChIP assay demonstrating the direct binding of SOX12 to the MMP7 and IGF1 promoters in MKN28-NM cells. The y-axis represents the relative enrichment with the anti-SOX12 antibody compared to that with the IgG control. The data are represented as the means ± SEMs for triplicate experiments. *p < 0.05.

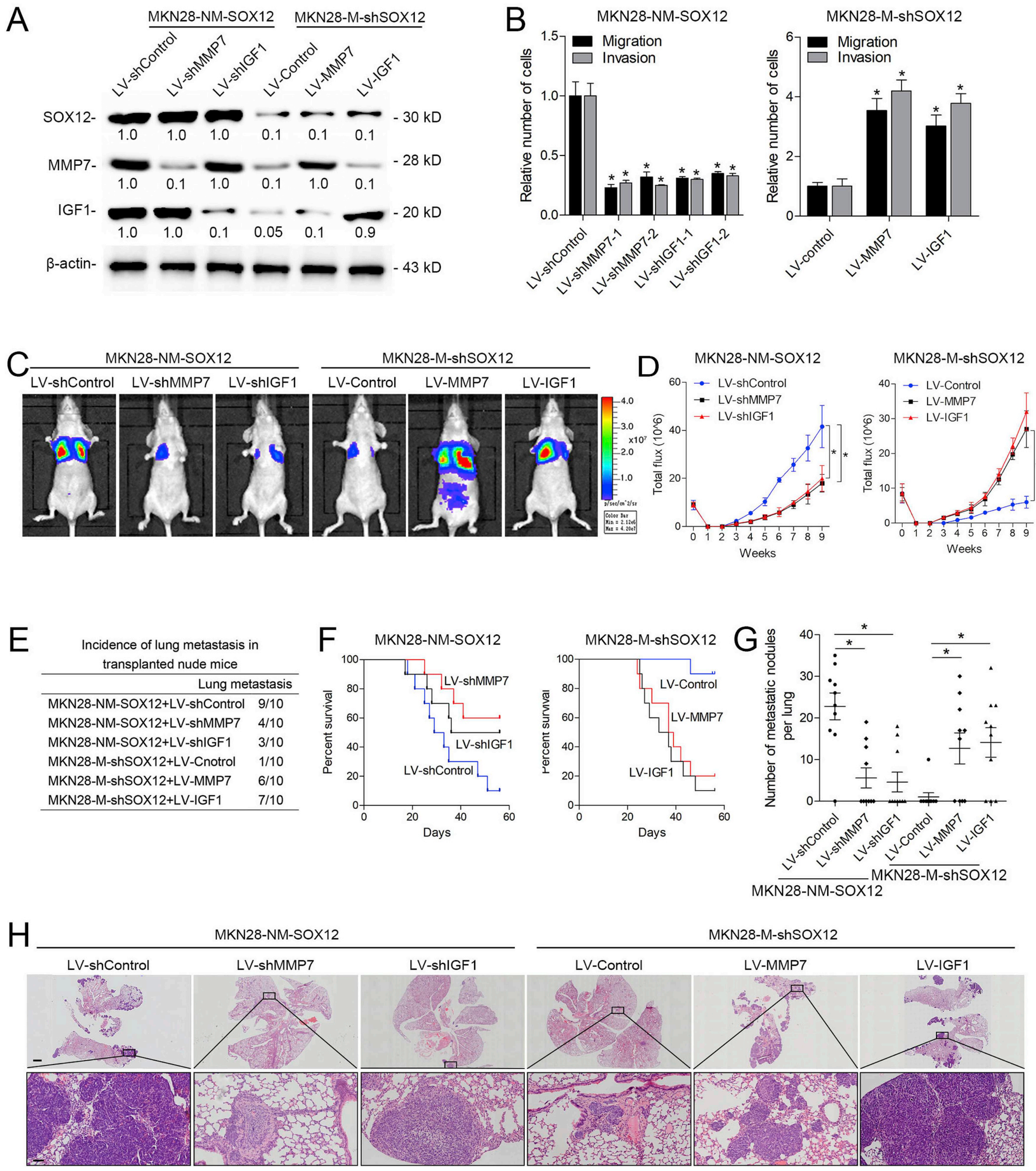


**Fig. 4.** MMP7 and IGF1 are essential for SOX12-promoted GC migration and metastasis. (A) Western blot analysis of MMP7 and IGF1 expression in the indicated cells. (B) Transwell assay with the indicated cells. (C–H) Lung metastasis of GC cells injected via tail vein. (C) Representative bioluminescence images indicating the metastasized cells after 9 weeks. (D) Bioluminescent cells at the indicated time points represented as the total photon flux. (E) The number of mice in each group with lung metastasis. (F) The overall survival of mice in each group. (G) The number of lung metastatic nodules in the indicated groups. (H) Representative images of H&E-stained lung tissues. The scale bars represent 1 mm (low magnification) and 100 μm (high magnification). *$p < 0.05$.

## 4. Discussion

Several developmentally important transcription factors, such as the SOX family of genes, have been reported to act as molecular drivers of human cancers [26,27]. As a member of the SOXC group, SOX12 plays a critical role in embryonic development and cell fate determination during organogenesis and carcinogenesis [28]. In HCC, SOX12 overexpression, which induces epithelial–mesenchymal transition and metastasis by transactivating Twist1 and ZEB1, was significantly correlated with disease progression and poor prognosis [29]. SOX12 was also

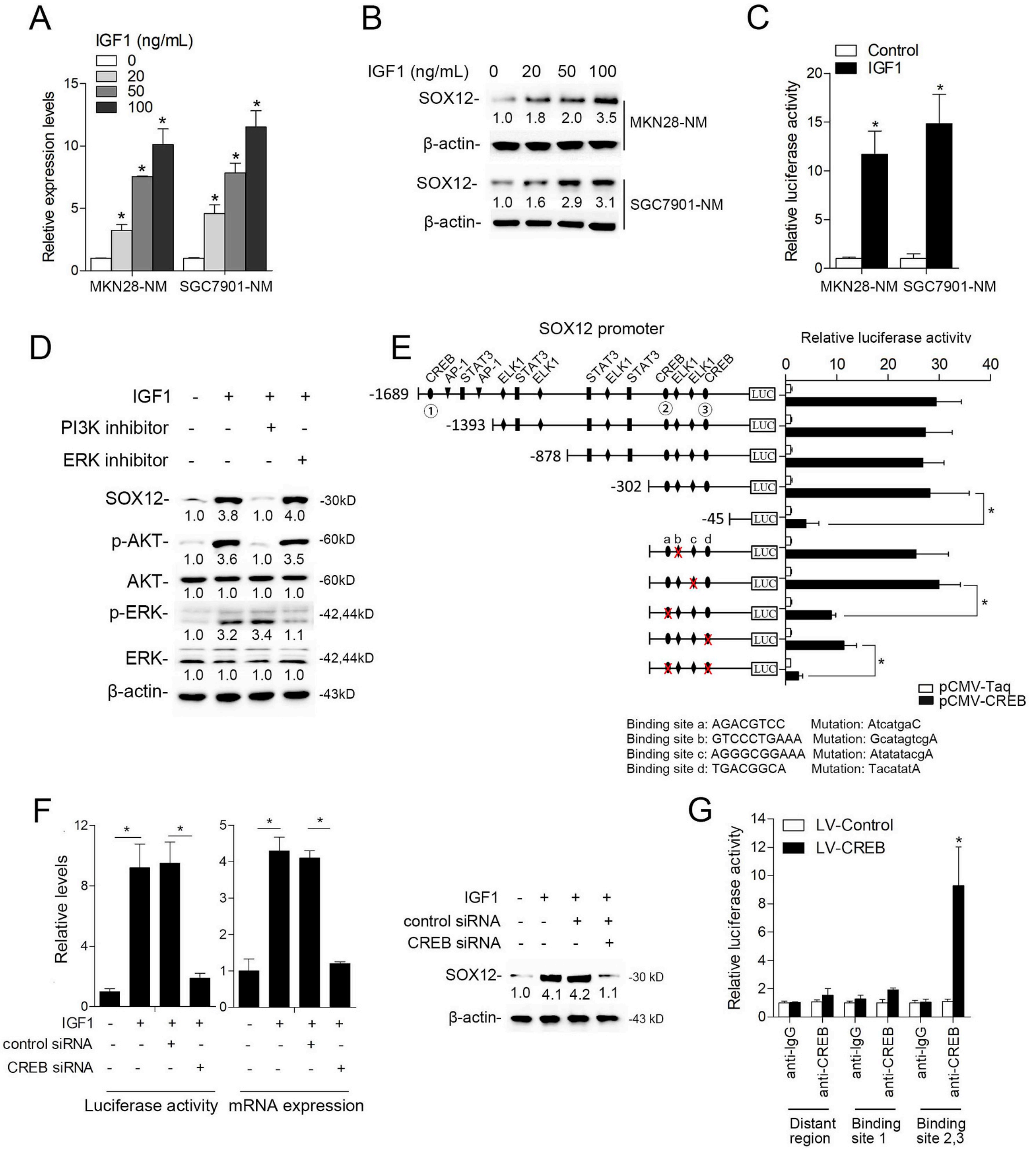
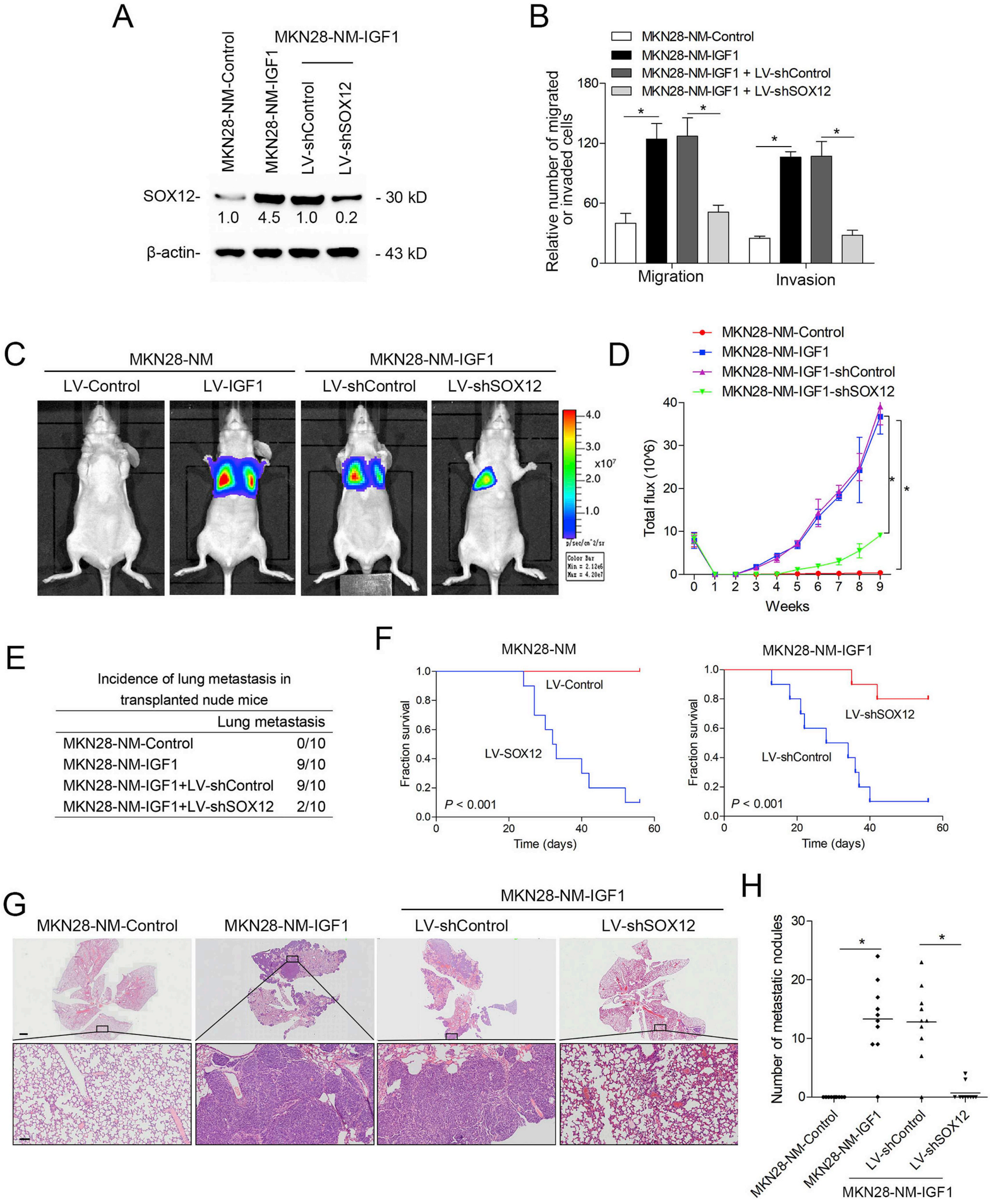


**Fig. 5.** IGF1 activates SOX12 expression through the PI3K/AKT pathway. (A and B) SOX12 mRNA (A) and protein (B) levels in the indicated GC cells treated with different concentrations of IGF1. (C) MKN28-NM and SGC7901-NM cells transfected with a vector containing the reporter gene driven by the SOX12 promoter were treated with IGF1, and the luciferase activity was measured. (D) The protein levels of SOX12 and both phosphorylated and total AKT and ERK1/2 in MKN28-NM cells pretreated with a specific inhibitor for PI3K (LY294002, 20 μM) or ERK1/2 (SCH772984, 100 nM) in the presence or absence of IGF1 (100 ng/mL). (E) The luciferase activity of the reporter gene driven by serially truncated/mutated SOX12 promoters identified the CREB-responsive regions regulating SOX12 promoter activity induced by activated IGF1 in MKN28-NM cells. (F) Knockdown of CREB decreased IGF1-induced promoter activity (left panel), mRNA expression (middle panel), and the protein levels (right panel) of SOX12 in MKN28-NM cells. (G) ChIP assay showing the direct binding site of CREB in the SOX12 promoter in MKN28-NM cells. $*p < 0.05$.

found to be involved in leukemia progression by regulating the expression of beta-catenin and subsequently interfering with the TCF/Wnt pathway, which may be a target in acute myeloid leukemia [15]. In addition, knocking down SOX12 expression inhibits the proliferation, migration, invasion and metastasis of lung cancer and breast cancer cells [30,31]. These studies indicate that SOX12 plays a key role in cancer progression and metastasis. However, the expression and role of SOX12 in human GC has not yet been elucidated. The results of this study showed for the first time that SOX12 expression was significantly upregulated in human GC tissues compared to that in adjacent non-tumorous tissues and that SOX12 overexpression was significantly correlated with disease progression and poor prognosis. Multivariate analysis revealed that SOX12 expression was an independent and significant risk factor for disease recurrence and reduced patient survival after curative resection. Moreover, using a series of *in vitro* and *in vivo* experiments, we found that ectopic expression of SOX12 significantly promoted GC proliferation and metastasis, while SOX12 knockdown produced opposing effects. These observations suggest that SOX12 might be a therapeutic target and prognostic biomarker in GC.

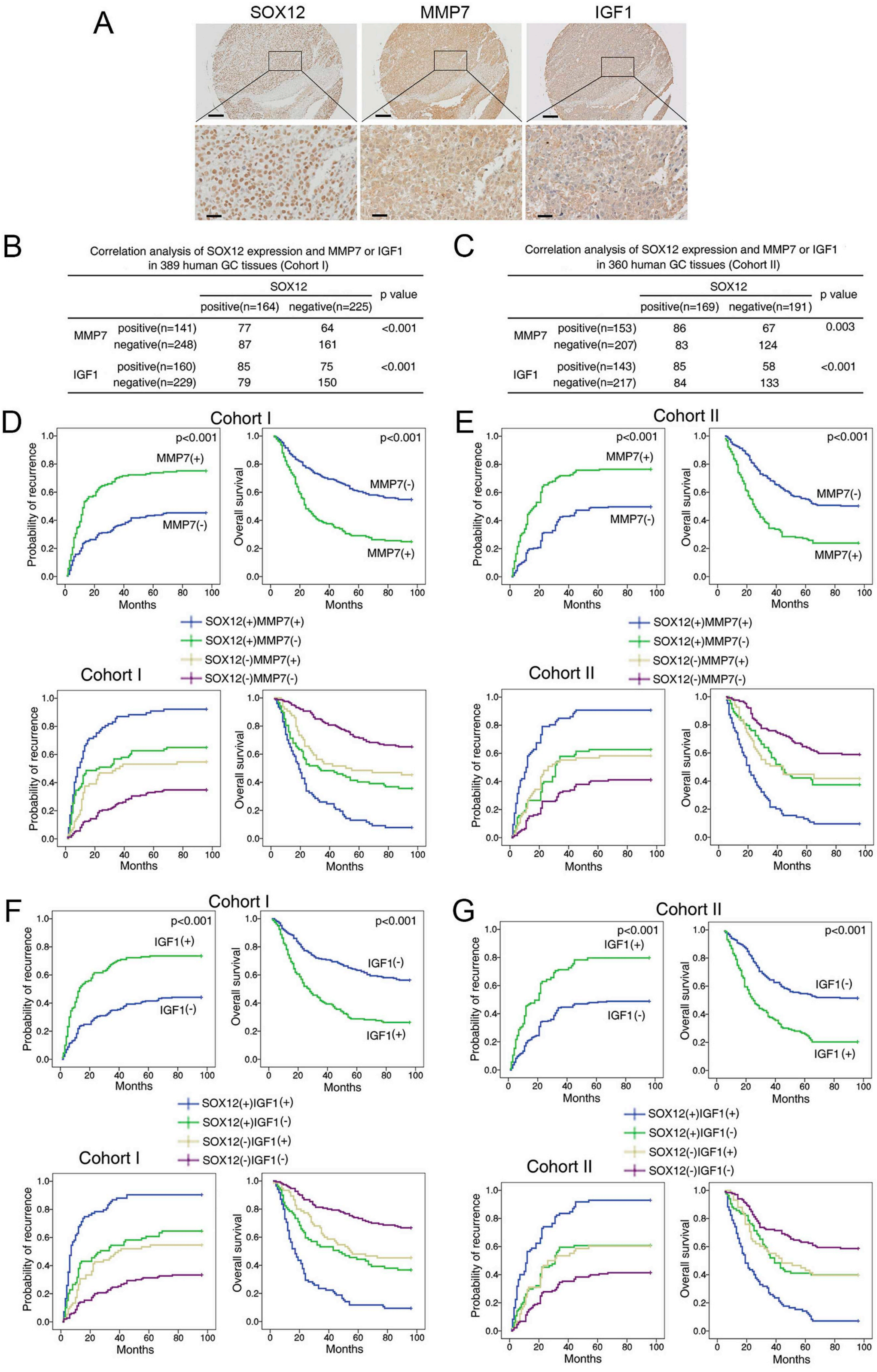
Extensive experimental and biochemical studies on matrix metalloproteinases (MMPs) have implicated MMPs in cancer invasion and metastases [32]. Matrilysin, also known as MMP7, is an important MMP



Incidence of lung metastasis in transplanted nude mice

| | Lung metastasis |
|---|---|
| MKN28-NM-Control | 0/10 |
| MKN28-NM-IGF1 | 9/10 |
| MKN28-NM-IGF1+LV-shControl | 9/10 |
| MKN28-NM-IGF1+LV-shSOX12 | 2/10 |

**Fig. 6.** SOX12 is critical for IGF1-induced GC metastasis. (A) SOX12 protein levels in the indicated GC cells. (B) Transwell assay showing the migration and invasion of the indicated GC cells. (C–H) Lung metastasis of GC cells injected via tail vein: (C) Representative bioluminescence images indicating the metastasized cells after 9 weeks. (D) Bioluminescent cells at the indicated time points represented as the total photon flux. (E) The number of mice in each group with lung metastasis. (F) The overall survival of mice in each group. (G) Representative images of H&E-stained lung tissues. The scale bars represent 1 mm (low magnification) and 100 μm (high magnification). (H) The number of lung metastatic nodules in each group. *$p < 0.05$.



Correlation analysis of SOX12 expression and MMP7 or IGF1 in 389 human GC tissues (Cohort I)

| | | SOX12 | | p value |
|---|---|---|---|---|
| | | positive(n=164) | negative(n=225) | |
| MMP7 | positive(n=141) | 77 | 64 | <0.001 |
| | negative(n=248) | 87 | 161 | |
| IGF1 | positive(n=160) | 85 | 75 | <0.001 |
| | negative(n=229) | 79 | 150 | |

Correlation analysis of SOX12 expression and MMP7 or IGF1 in 360 human GC tissues (Cohort II)

| | | SOX12 | | p value |
|---|---|---|---|---|
| | | positive(n=169) | negative(n=191) | |
| MMP7 | positive(n=153) | 86 | 67 | 0.003 |
| | negative(n=207) | 83 | 124 | |
| IGF1 | positive(n=143) | 85 | 58 | <0.001 |
| | negative(n=217) | 84 | 133 | |

**Fig. 7.** SOX12 expression is positively correlated with MMP7 and IGF1 expression in human GC tissues. (A) Representative images of SOX12, MMP7, and IGF1 expression in GC tissues. The scale bars represent 200 μm (low magnification) and 50 μm (high magnification). (B and C) The association between SOX12 expression and MMP7 or IGF1 expression in GC tissues from cohorts I and II. (D) Kaplan–Meier analysis showing the correlation of MMP7 expression (upper panels) and SOX12/MMP7 coexpression (lower panels) with recurrence and overall survival in cohort I. (E) Kaplan–Meier analysis showing the correlation of MMP7 expression (upper panels) and SOX12/MMP7 coexpression (lower panels) with recurrence and overall survival in cohort II. (F) Kaplan–Meier analysis showing the correlation of IGF1 expression (upper panels) and SOX12/IGF1 coexpression (lower panels) with recurrence and overall survival in cohort I. (G) Kaplan–Meier analysis showing the correlation of IGF1 expression (upper panels) and SOX12/IGF1 coexpression (lower panels) with recurrence and overall survival in cohort II.

and is strongly correlated with the progression of most cancers, including breast carcinomas, thyroid papillary carcinomas, gastric adenocarcinomas, oral squamous cell carcinomas and gliomas [33]. Notably, MMP7 is often expressed in gastrointestinal cancers and promotes tumor malignancy [34–38]. In colorectal cancer (CRC), MMP7 is correlated with metastasis in patients, and its production and activation are critical for the formation of CRC liver metastases in humans [34]. Evidence indicates that MMP7 expression is upregulated in GC tissues but not in normal gastric mucosa, and patients with MMP7-positive tumors had significantly poorer survival and a higher rate of death from peritoneal recurrence than patients with MMP7-negative tumors [36–38]. These studies imply that MMP7 is an important oncogene in gastric carcinogenesis; however, the mechanism by which MMP7 is deregulated in GC remains unclear. This study showed that MMP7 was

**Table 4**
Correlation between MMP7 expression and clinicopathological characteristics of gastric cancer in two independent cohorts of human gastric cancer tissues.

| Clinicopathological variables | | Cohort I | | | Cohort II | | |
|---|---|---|---|---|---|---|---|
| | | Tumor MMP7 expression | | *P* value | Tumor MMP7 expression | | *P* value |
| | | Low (n = 248) | High (n = 141) | | Low (n = 207) | High (n = 153) | |
| Age | | 64.59(10.62) | 65.15(11.39) | 0.627 | 62.42(11.54) | 62.27(11.68) | 0.905 |
| Sex | female | 78 | 44 | 1 | 69 | 44 | 0.361 |
| | male | 170 | 97 | | 138 | 109 | |
| Tumor size | < 5 cm | 116 | 39 | < 0.001 | 87 | 54 | 0.23 |
| | ≥5 cm | 132 | 102 | | 120 | 99 | |
| Tumor differentiation | well or moderate | 113 | 47 | 0.019 | 115 | 51 | < 0.001 |
| | poor | 135 | 94 | | 91 | 102 | |
| Tumor invasion | T1 | 16 | 4 | < 0.001 | 13 | 7 | 0.009 |
| | T2 | 37 | 9 | | 23 | 11 | |
| | T3 | 157 | 74 | | 150 | 100 | |
| | T4 | 38 | 54 | | 21 | 35 | |
| Lymph node metastasis | absent | 83 | 18 | < 0.001 | 63 | 18 | < 0.001 |
| | present | 165 | 123 | | 144 | 135 | |
| Distant metastasis | absent | 220 | 101 | < 0.001 | 195 | 121 | < 0.001 |
| | present | 28 | 40 | | 12 | 32 | |
| AJCC stage | Stage I | 34 | 4 | < 0.001 | 25 | 8 | < 0.001 |
| | Stage II | 92 | 23 | | 81 | 22 | |
| | Stage III | 95 | 74 | | 89 | 90 | |
| | Stage IV | 27 | 40 | | 12 | 33 | |

a direct and functional target of SOX12, which transactivated MMP7 expression by directly binding to its promoter. MMP7 downregulation significantly suppressed SOX12-enhanced GC proliferation, migration, invasion and lung metastasis, whereas MMP7 overexpression reversed the reduction in GC cell malignant behaviors induced by SOX12 knockdown. In addition, clinical data also confirmed that SOX12 expression was positively correlated with MMP7 expression and that the positive coexpression of these two proteins was correlated with poorer prognosis. These studies suggest that SOX12 promotes GC proliferation and metastasis by transactivating MMP7.

The IGF signaling cascade includes the IGFs, IGF1R, and multiple binding proteins [39]. This growth factor system exerts multiple physiologic effects on cell growth, differentiation, and transformation [40,41]. Dysregulation of IGF system expression and activity is linked to diverse pathologies ranging from growth deficits to cancer development [18,19]. In human cancers, IGF1 overexpression promotes growth and angiogenesis and is associated with aggressive tumor behavior and poor prognosis in several types of cancer [19,42]. However, the expression and function of IGF1 in human GC remains unknown. Here, we found that IGF1 expression was considerably upregulated in GC tissues compared with that in adjacent nontumor tissues. Compared with negative IGF1 expression, elevated IGF1 expression in patients was correlated with poorer tumor differentiation, higher TNM stage, higher recurrence rates and shorter overall survival times. These clinical data strongly indicate that IGF1 plays a role in promoting GC progression. Moreover, we found that IGF1 was transactivated by the direct binding of SOX12 to its promoter. Ectopic IGF1 expression significantly decreased SOX12-mediated GC progression; by contrast, IGF1 downregulation reversed the suppression of GC proliferation and metastasis induced by SOX12 knockdown. IGF1 expression was positively correlated with SOX12 levels, and their positive coexpression was associated with poorer prognosis. Taken together, these results suggest that IGF1 is

**Table 5**
Correlation between IGF1 expression and clinicopathological characteristics of gastric cancer in two independent cohorts of human gastric cancer tissues.

| Clinicopathological variables | | Cohort I | | | Cohort II | | |
|---|---|---|---|---|---|---|---|
| | | Tumor IGF1 expression | | *P* value | Tumor IGF1 expression | | *P* value |
| | | Low (n = 229) | High (n = 160) | | Low (n = 217) | High (n = 143) | |
| Age | | 64.60(10.41) | 65.07(11.58) | 0.676 | 61.88(11.67) | 63.06(11.45) | 0.346 |
| Sex | female | 77 | 45 | 0.268 | 74 | 39 | 0.202 |
| | male | 152 | 115 | | 143 | 104 | |
| Tumor size | < 5 cm | 105 | 50 | 0.004 | 88 | 53 | 0.581 |
| | ≥5 cm | 124 | 110 | | 129 | 90 | |
| Tumor differentiation | well or moderate | 112 | 48 | < 0.001 | 103 | 63 | 0.201 |
| | poor | 117 | 112 | | 114 | 79 | |
| Tumor invasion | T1 | 12 | 8 | < 0.001 | 16 | 4 | 0.007 |
| | T2 | 38 | 8 | | 23 | 11 | |
| | T3 | 144 | 87 | | 151 | 99 | |
| | T4 | 35 | 57 | | 27 | 29 | |
| Lymph node metastasis | absent | 69 | 32 | 0.026 | 64 | 17 | < 0.001 |
| | present | 160 | 128 | | 153 | 126 | |
| Distant metastasis | absent | 211 | 110 | < 0.001 | 200 | 116 | 0.003 |
| | present | 18 | 50 | | 17 | 27 | |
| AJCC stage | Stage I | 28 | 10 | < 0.001 | 22 | 11 | < 0.001 |
| | Stage II | 84 | 31 | | 85 | 18 | |
| | Stage III | 99 | 70 | | 93 | 86 | |
| | Stage IV | 18 | 49 | | 17 | 28 | |

a direct target of SOX12 and is essential for the GC proliferation and metastasis promoted by SOX12.

The aberrantly high expression and oncogenic roles of SOX12 in GC have been clarified; however, the mechanism regulating SOX12 overexpression in human GC remains unknown. In this study, we confirmed the transcriptional upregulation of SOX12 by CREB, which is a master regulator of cancer initiation and progression. CREB was activated by IGF1/PI3K/AKT signaling and subsequently bound to the SOX12 promoter region to transactivate SOX12. Serial deletion, site-directed mutagenesis and chromatin immunoprecipitation (ChIP) approaches identified the specific CREB binding sites within the SOX12 promoter associated with IGF/PI3K/AKT stimulation. Moreover, CREB expression was positively correlated with SOX12 expression, and the positive co-expression of these two proteins in GC patients was associated with poorer prognosis. These studies suggest that CREB-induced SOX12 overexpression plays an important role in promoting GC metastasis and worsening prognosis.

In summary, we report a novel function of SOX12 in gastric carcinogenesis. SOX12 is significantly upregulated in GC and is associated with poor prognosis and reduced survival. SOX12, which is a direct downstream target of IGF1-induced CREB activation, promotes GC proliferation and metastasis by transactivating MMP7 and IGF1. Thus, this study implicates SOX12 as a potential prognostic biomarker and a novel therapeutic target in GC.

## Conflicts of interest

The authors have no conflicts to disclose.

## Author contributions

Feng Du performed the experiments. Weibo Feng and Sheng Chen assisted in immunohistochemical staining and animal experiments. Shengda Wu, Tianyu Cao and Ting Yuan assisted in collecting tissue samples. Dean Tian, Yongzhan Nie, Kaichun Wu and Daiming Fan assisted in conceiving the experiments and analyzing the data. Limin Xia and Feng Du designed the studies and wrote the paper.

## Acknowledgments

This study was supported by combined grants from the National Key Research and Development Program of China (2018YFC1312103[L.X.], SQ2017YFSF090132[K.W.]), National Natural Science Foundation of China (No.81522031[L.X.], No. 81772623[L.X.], No. 81627807[K.W.], No.81430072[D.F.] and No. 81421003[K.W.]), and National Center for Clinical Research of Digestive Diseases (2015BAI13B07[D.F.]).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.035.

## Abbreviations

| | |
|---|---|
| SOX12 | sex determining region Y-box 12 |
| GC | gastric cancer |
| TNM | tumor-node-metastasis |
| ChIP | chromatin immunoprecipitation analysis |
| IHC | immunohistochemistry |
| PI3K | phosphoinositide 3-kinase |
| MMP7 | matrix metallopeptidase 7 |
| IGF1 | insulin-like growth factor 1 |

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