FULL LENGTH ARTICLE

SOX4 contributes to TGF-β-induced epithelial–mesenchymal transition and stem cell characteristics of gastric cancer cells

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Abstract  SOX4 is highly expressed in gastric cancer (GC) and is associated with tumor grade, metastasis and prognosis, however the mechanism is not clear. We report herein that SOX4 was upregulated and overexpression of SOX4 was associated with increased expression of the markers of Epithelial–mesenchymal transition (EMT) and stemness in clinic patient samples. In vitro, overexpression of SOX4 promoted the invasion as showed by Transwell assay and stemness of GC cells as assessed by sphere formation assay, which was suppressed by silencing SOX4 with shRNA. Further studies showed that SOX4 up-regulated the expression of EMT transcription factors Twist1, snail1 and zeb1 and stemness transcription factors SOX2 and OCT4, and promoted the nuclear translocation of β-catenin. Moreover, we revealed that TGF-β treatment significantly up-regulated the expression of SOX4 and silencing SOX4 reversed TGF-β-induced invasion and sphere formation ability of GC cells. Finally, we showed that SOX4 promoted the lung metastasis and tumor formation ability of gastric cancer cells in nude mice. Our results suggest that SOX4 is a target TGF-β signaling and mediates TGF-β-induced EMT and stem cell characteristics of GC cells, revealing a novel role of TGF-β/SOX4 axis in the regulation of malignant behavior of GC.

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Introduction

Gastric cancer (GC) is a common gastrointestinal cancer. Despite the systematic management of GC patients with surgery, chemotherapy, radiotherapy and targeted therapy, most patients finally die of metastasis and recurrence. In order to improve the clinic outcome, it is urgent to elucidate the mechanism of the metastasis and recurrence of gastric cancer.

Epithelial–mesenchymal transition (EMT) is the process of the transformation of epithelial cells to mesenchymal cells, during which the epithelial cells undergo cytoskeleton remodeling and lose cell polarity thereby obtaining the capability of invasion and metastasis. Several transcription factor families including Snail, ZEB and bHLH play an important role in the regulation of EMT by down-regulating the epithelium E-cadherin and up-regulating interstitial molecules N-cadherin and Vimentin.

SOX4 is a sex-determining factor and contains a highly conserved DNA binding high mobility group (HMG) box. SOX4 plays an important role in embryonic development, neurological development, and sex determination. Recent advance suggests an important role for SOX4 in EMT. SOX4 is sex-determining factor and contains a highly conserved DNA binding high mobility group (HMG) box. SOX4 plays an important role in embryonic development, neurological development, and sex determination. Recent advance suggests an important role for SOX4 in EMT. It has been shown that SOX4 up-regulates Snail1/2, Zeb1/2 and Twist1 through the regulation of histone methyltransferase Ezh2 in breast cancer cells, leading to EMT in cancer cells. In colon cancer cells, SOX4 can upregulate the expression of miR-1269a and promote EMT. SOX4-induced tumor cell EMT has also been observed in liver, prostate and esophageal cancers. During the process of EMT, tumor cells often obtain stronger abilities for self-renewal, distant metastasis and drug resistance, accompanied by changes in a series of stem cell markers. Thus, in the multistep processes of tumorigenesis, EMT can promote stem cell characterization and collaborate with CSCs in promoting tumor metastasis, drug resistance and recurrence. Cancer stem cells (CSCs) are a group of cells with self-renewal, multiple differentiation potential and high tumorigenicity. CSCs have been considered the origin of tumor expansion and recurrence. The formation and maintenance of CSCs involves a variety of factors including transcription factors OCT4, SOX2 and Nanog as well as several signal transduction pathways such as Wnt/β-catenin, TGF-β and Notch. These factors often influence the regulation of stemness and are the key regulators of EMT.

Materials and methods

Patients and clinic samples

GC tissues and matched normal mucosa tissues (from cancer tissue ≥ 6 cm) after surgical resection were collected from 84 gastric cancer patients in the First Affiliated Hospital of Chongqing Medical University from January to October 2014. The patients included 49 males and 35 females with age of 30–78 years. The number of patients according TNM staging was as follows: 10 cases of stage I, 26 cases of stage II, 41 cases of stage III and 7 cases of stage IV. All patients did not undergo radiotherapy and chemotherapy before surgery.

Reagents and antibodies

Rabbit anti-human SOX4 (ab80261), SOX2 (ab97959), CD44 (ab157107), EpCAM (ab71916), Nanog (ab80892), Oct4 (ab18976) antibodies were purchased from ABCOM (Cambridge, UK); rabbit anti-human E-cadherin (24E10), N-cadherin (D4R1H) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA); Rabbit anti-human Snail2 (sc-15391), ZEB1 (sc-25388), Twist1 (sc-15393) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Rabbit anti-human GAPDH (10494-1-AP), Vimentin (10366-1-AP), HRP-labeled goat anti-rabbit IgG were purchased from Proteintech (Wuhan, China); Rabbit anti-human CD44-PE (12-0441), and isotype control antibodies (12-4031) were purchased from eBioscience (San Diego, CA, USA). EpCAM-APC (130-098-118) was purchased from Miltenyi Biotec, Beijing, China). Immunohistochemistry kit was purchased from Beyotime (Shanghai, China). Transwell chamber and ultra low adhesion flask were purchased from Corning (NY, USA). PCR-related reagents were purchased from Takara (Dalian, China). Recombinant human TGF-β1, bFGF and EGF were obtained from PeproTech (Rocky Hill, NJ, USA).

Immunohistochemistry (IHC)

Paraffin-fixed tissue sections were deparaffinized with xylol and rehydrated with graded alcohol. After blocking endogenous peroxidase with 3% hydrogen peroxide for 10 min, the slides were subjected to antigen retrieval for 5 min in a pressure cooker using sodium citrate buffer (pH 6.0). Non-specific binding was blocked with normal goat serum. Then the slides were incubated with the primary antibodies at 4 °C overnight. The next day after washing with PBS, the sections were incubated with secondary antibody for 30 min. After washing three times with PBS, the sections were visualized using the ABC substrate buffer for 2 min. Tissue sections were counterstained with hematoxylin, and dehydrated in an ascending series of ethanol (85–100%). After xylol treatment, sections were...
mounted. The staining intensity was scored as follows: Staining intensity: 0 point, no color; 1 point, light yellow; 2 points, brown; 3 points, dark brown. Five fields were randomly viewed in each slide and staining range was scored as follows: 0 point, <5% positive cells; 1 point, 5–25% positive cells; 2 points, 26–50%, positive cells; 3 points, 51–75% positive cells; 4 points, positive cells >75%. The total score is the sum of the intensity and range of staining and the total score >3 points was considered as positive or high expression.

**Cells and lentiviral transduction**

Human GC MKN28, BGC823 cell lines were purchased from Chinese Academy of Sciences Shanghai Cell Bank (Shanghai, China) and cultured in RPMI1640, B27 (Gibco, Grand Island, NY, USA) or DMEM high glucose medium (Hyclone, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Hyclone). Cells were maintained in an incubator with 5% CO₂ at 37 °C. The vector pLV-shRNA SOX4-GFP-puro, pLV-SOX4-GFP-puro and control vector pLV-GFP-puro were purchased from Hanbio Technology (Shanghai, China). The sequences of ShRNA were as follows: sh1: 5′-CGGGTGGCAATCAACGCAACCTCTCCAGATGGGTGCCTTGATCGGCCATTATTATT-3′; sh2: 5′-CGGGAAAGAAGGTGAAGCGCGTCATCTCAAGTGACGCGCTACCTTTTCTTTTCTTTTTT-3′; NC sequence: 5′-TTCTCCGAAAGGTGCACGTAA-3′. Cells were seeded at 2 × 10⁵/ml in 6-well plates (1 ml/well). Culture media were changed after 14 h, and lentiviruses carrying 3 different SOX4 targeting shRNA and NC shRNA were added to the cells at a MOI of 30. Polybrene was added at a final concentration of 5 µg/ml in each well. Culture media were changed after 16 h. Cells were examined under a fluorescence microscope after 72 h to check the transduction efficiency. Transduced cells were transferred to flasks and puromycin was added to the cells at a final concentration of 2 µg/ml. After selecting for 2 weeks, puromycin was removed and total RNA and total proteins were isolated for further experiments.

**Transwell chamber assay**

Transwell chamber migration assays were performed using Nunc 24-well 8.0 µm pore transwell plates according to the manufacturer’s instructions. Cells were plated at 5 × 10⁴/ml in each well with 400 µl culture medium were added to the upper chamber. The lower chamber was added with 600 µl complete medium. After 36 h incubation, non-invasive cells were removed from the upper surface of the membrane using a cotton-tipped swab. Then the invading cells were fixed in methanol for 10 min and stained with 0.1% Crystal Violet Hydrate (Sigma, St. Louis, MO, USA) for 5 min. The stained cells were counted as cells per field at 10× magnification. For TGF-β1 induction, cells were treated with TGF-β1 2 days before seeding and the upper chamber was added with TGF-β1.

**Sphere formation assay**

Cells were inoculated at a density of 5000 cells/well with 2 ml stem cell culture medium in ultra-low attachment 6-well plates (Corning). 1.5 ml stem cell medium was added every 2 days. The sphere formation of the cells was observed at 10 days. The ingredients of the stem cell culture medium were: DMEM/F12 medium + 20 ng/ml bFGF + 10 ng/ml EGF + 2% B27. In the induction of TGF-β1, TGF-β1 was directly added to the stem cell culture medium.

**Flow cytometry**

CD44 and EpCAM have been widely used in GC stem cell enrichment, we applied CD44+/EpCAM+ as a stem cell marker. At least 1 × 10⁶ cells were resuspended with 100 µl buffer (PBS + 0.5% BSA + 2 mM EDTA) followed by addition 1 µl of CD44-PE antibody and 0.625 µl of EpCAM-APC antibody. After incubation at 4 °C for 10 min, the cells were washed three times and finally resuspended in 300 µl buffer. The percentage of CD44+/EpCAM+ cells was detected by flow cytometry.

**Quantitative real-time PCR**

Total RNA was extracted using TRizol reagent (Invitrogen, USA), and reverse-transcribed was performed using a cDNA Synthesis Kit from Takara. Quantitative real-time PCR was performed in triplicate using SYBR Green PCR Master Mix (Thermo Scientific, USA) and CFX96 Real Time PCR System (Bio-Rad, USA). All the procedures were performed according to the manufacturer’s instructions. The PCR program consisted of an initial denaturation cycle (30 s at 95 °C) followed by 45 cycles of denaturation (5 s at 95 °C) and annealing (30 s at 58 °C) and elongation (60 s at 72 °C). A melting curve analysis was added at the end of the program. The GAPDH was used as endogenous control. The data were analyzed by the 2^(-ΔΔCt}. The sequences of primers are listed in Table 1.

**Immunofluorescence microscopy**

Cells were seeded for 36 h in a confocal small dish. The cells were fixed with 4% parafomaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 5 min, and blocked by 1% BSA for 2 h at room temperature. During each step, cells were washed twice with PBS. The cells were incubated with primary antibody overnight at 4 °C. After washing with PBS, goat anti-rabbit-Cy3 conjugated secondary antibody was added for 1 h at room temperature, followed by DNA staining with DAPI for 15 min. Photographs were taken with a fluorescence microscope (Zeiss, Germany).

**Western blotting**

Total protein was extracted from cells or tissues using RIPA lysis buffer (Pierce). Equal amounts of protein extracts (25 µg) were separated by 1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Membranes were blocked with 5% w/v non-fat dry milk dissolved in Tris buffered saline plus Tween-20 (TBS-T; 0.1% Tween-20; pH 8.3) at room temperature for 1 h, then incubated with primary antibodies at
4 °C overnight. After washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 2 h at room temperature. Immunoblots were visualized using enhanced chemiluminescence (ECL) kit (GE Healthcare, Waukesha, WI, USA) according to manufacturer’s instructions and exposed to X-ray films. GAPDH was used as a loading control. The optical density of bands was analyzed by Fusion software (Vilber Lourmat Deutschland GmbH, Germany).

**Nude mice models**

Nude mice of 4–6 weeks old were purchased from the Animal Experimental Center of Chongqing Medical University. The mice were housed in a temperature-controlled room (24 °C) on a 12-h/12-h light and dark cycle at the animal experimental center. 5 × 10^5 cells in 100 μL PBS were injected subcutaneously into the left flank of the nude mice with 5 in each group. After 6 weeks, the mice were sacrificed by anesthesia and tumor formation rate and tumor weight were calculated. For metastasis analysis, 1 × 10^6 cells in 200 μL PBS were injected into the nude mouse through the tail vein. After 4 weeks, the nude mice were sacrificed to observe the lung metastasis and the lung tissues were stained for hematoxylin-eosin (H&E) for pathology confirmation.

**Statistical analysis**

All experiments were repeated three times and the data were analyzed using SPSS 19.0. Measurement data are expressed as mean ± standard deviation (SD). Comparison was made by the t test between two groups and by the one-way ANOVA among multiple groups. The results from immunohistochemistry were analyzed by the χ² test. The correlation among multiple genes was analyzed by linear correlation. P < 0.05 was considered statistically significant.

**Results**

**SOX4 is upregulated and overexpression of SOX4 is associated with EMT and stemness of GC**

Previous studies have shown that overexpression of SOX4 expresses a low level of SOX4, whereas poorly-differentiated GC BGC823 cell line expresses a high level of SOX4 (Supplementary 2). To test whether SOX4 could promote EMT of GC cells or not, we first transduced MKN28 cells with lentiviral vector expressing SOX4 in parallel with an empty control lentiviral vector. When compared to the control group (178.3 vs 468.87) (Fig. 3A), although the control group showed a low level of SOX4, whereas poorly-differentiated GC BGC823 cell line expresses a high level of SOX4 (Supplementary 2). To test whether SOX4 could promote EMT of GC cells or not, we first transduced MKN28 cells with lentiviral vector expressing SOX4 in parallel with an empty control lentiviral vector. When compared to the control group, transduction of MKN28 cells with SOX4 vector significantly increased the mRNA levels of SOX4 by 5.23 fold. Consistently, transduction of SOX4 vector elevated the protein levels of SOX4 in MKN28 cells by 4.94 fold (Fig. 3A). Transwell assay showed that overexpression of SOX4 increased the cell migration capacity by 2.63 times of the control group (178.3 vs 468.87) (Fig. 3A), although the morphological changes were not obvious. We further detected the expression of EMT molecules following SOX4 overexpression in MKN28 cells. It was found that the mRNA expression of EMT molecules following SOX4 overexpression in MKN28 cells. It was found that the mRNA

**Table 1**

The sequences of the PCR primers used in this study.

| Gene   | Primer                  |
|--------|-------------------------|
| GAPDH  | Forward 5′ CTTTGATCTGGAAGGACCTC3′ |
|        | Reverse 5′ GATAGGACGGAGTATGTTCT3′ |
| SOX4   | Forward 5′ GGCTCTAAGCTGGGATGCCG3′ |
|        | Reverse 5′ GCCACACTCGGGGCTTGCAC3′ |
| E-cadherin | Forward 5′ TGCCCTCCTCTTCTACCTCC3′ |
|        | Reverse 5′ TCTAGTGGTAGAACCAGG3′ |
| N-cadherin | Forward 5′ CGTGAAGTGGGGCCAGTGGA3′ |
|        | Reverse 5′ CCTGGGCTTTCTTTACCCC3′ |
| Vimentin | Forward 5′ TCAATGTTAAGATGGCCTT3′ |
|        | Reverse 5′ TCATGTTTGATACCAAGAGG3′ |
| CD44   | Forward 5′ TTACTCTGCTGCTGTCTG3′ |
|        | Reverse 5′ ACAACACCACCCGAGG3′ |
| EpCAM  | Forward 5′ CGGAGCTCAGGAAGATGTG3′ |
|        | Reverse 5′ AGCCATTCTTCTCCTGCT3′ |
| Twist1 | Forward 5′ GGAGTCGGCACTTTACAGG3′ |
|        | Reverse 5′ GCCTGAGGCTGTCACTTGT3′ |
| Snail1 | Forward 5′ TTATCCCTCCACAGCCTCA3′ |
|        | Reverse 5′ GACAGCTCCACCAGTACCT3′ |
| Snail2 | Forward 5′ GGACTTGGACAGAGTCAT3′ |
|        | Reverse 5′ TCTCTCAATCTGCTGAGG3′ |
| ZEB1   | Forward 5′ AAGAACCTCTGGGAGAGTA3′ |
|        | Reverse 5′ TCTTGATCATCTGACTGCATT3′ |
| β-catenin | Forward 5′ TGTCGAGGGAGAGACCC3′ |
|        | Reverse 5′ TGTCACCTGAGGAGCCAA3′ |
of E-cadherin was down-regulated by 48.6%, while the mRNAs of N-cadherin and Vimentin were increased by 3.51 and 3.66 times in SOX4 overexpressing cells compared to that in control cells, were further confirmed by Western blotting and immunofluorescence assays. This indicated that although forced overexpression of SOX4 in MKN28 cells can not further increase their mesenchymal morphology, SOX4 was still able to upregulate the mesenchymal molecules, and downregulate epithelial molecules, thereby promoting the EMT properties in MKN28 cells. Next, we transduced BGC823 cells with lentiviral vector expressing SOX4 shRNA in parallel with a lentiviral vector expressing scrambled control shRNA. When compared to the control shRNA, transduction of BGC823 cells with SOX4 shRNA significantly reduced the mRNA by 73.3% and protein levels of SOX4 by 68.4. The cells transduced with SOX4 shRNA showed highly organized cell—cell adhesion and cobblestone shape with polarity appeared. In addition, Transwell assay showed that silencing of SOX4 suppressed the cell migration capacity of BGC823 cells (241.21 vs 102.39) (Fig. 3B). We further detected the expression of EMT molecules following SOX4 overexpression in MKN28 cells and knockdown in BGC823 cells. qRT-PCR, Western blotting and immunofluorescence assays revealed that silencing of SOX4 increased E-cadherin while reduced Vimentin, although N-cadherin did not change significantly (Fig. 3C).

Figure 1  SOX4 expression was elevated in gastric cancer tissues. A. Representative SOX4 staining in GC and matched non-cancer tissues of 8 typica patients by IHC. The results from IHC were analyzed by the χ2 test. B. The mean score for all GC specimens and matched non-cancer tissues were presented. The results were expressed as mean ± SD and were analyzed by the two-sample t-test. C. The expression of SOX4 mRNA in cancer and adjacent tissues of 30 patients with gastric cancer. Higher ΔCT values indicate lower expression (lower left). Comparison was made by the paired t-test; the ratio of SOX4 expression in cancer to adjacent tissues is expressed as 2−ΔΔCT (lower right). **P < 0.01.
SOX4 was required for the stemness GC cells

SOX4 have been shown to promote EMT and stemness of different cancer cells and EMT enhances the stemness of cancer cells.\textsuperscript{19,20} In order to test whether SOX4 can induce stemness, we examined the percentage of CD44\textsuperscript{+}/EpCAM\textsuperscript{+} cells by flow cytometry and the self-renewal of gastric stem cells using sphere formation assay following SOX4 overexpression or silencing. We found that the percentage of CD44\textsuperscript{+}/EpCAM\textsuperscript{+} cells increased from 0.31\% to 6.45\% (Supplementary 2), and the number of spheres increased by 12.17 times after SOX4 overexpression in MKN28 cells (Fig. 4A). In the BGC823 cells after SOX4 silencing, the number of spheres reduced by 66.7\% (Fig. 4B). Our results suggest that SOX4 contribute to EMT and increase stemness of GC cells.

SOX4 regulates transcription factors to promote EMT and stemness

Multiple factors involve in EMT and stemness. In order to explore the mechanism of SOX4 promoting EMT and stemness, we used qRT-PCR and WB to detect the expression of EMT and stemness related transcription factors after SOX4 overexpression and silencing. After SOX4 overexpression in
MKN28 cells, both the mRNA and proteins of EMT transcription factors Twist1 and ZEB1, and stemness transcription factors Oct4 and SOX2 were up-regulated. In contrast, silencing SOX4 in BGC823 cells resulted in down-regulation of Twist1, snail1, Oct4 and SOX2 (Fig. 5A). This indicated that SOX4 promoted EMT and stemness of GC cells through some key transcription factors.

SOX4 is a target of TGF-β and silencing SOX4 reverses TGF-β induced EMT and stemness of GC cells

TGF-β is a strong inducer of cancer cell EMT and stemness. To explore the potential relationship between TGF-β and SOX4, we treated MKN28 cells with TGF-β1 for 3 days and detected the expression of SOX4 by qPCR and Western blot. The results showed that SOX4 mRNA and protein levels were significantly up-regulated 3.33 and 4.07 fold compared to untreated control (Fig. 6A), indicating that SOX4 is a potential target gene of TGF-β pathways. To test whether SOX4 plays a role in TGF-β mediated induction of EMT and stemness of cancer cells, BGC823 cells transduced with SOX4 shRNA lentivirus were treated with TGF-β1. After treatment for 5 days, TGF-β1 significantly increased invasion of BGC823-NC cells by 1.98 fold (220.42 vs 435.55), which was associated with the down-regulation of E-cadherin and the up-regulation of N-cadherin and Vimentin; however, all these were attenuated by silencing of SOX4 (Fig. 6B, C). After treatment for 10 days, TGF-β1 significantly increased the percentage of CD44+/EpCAM+ cells and number of spheres, which was reversed by silencing of SOX4 (Supplementary 3, Fig. 6D). These results suggest that SOX4 mediates TGF-β-induced EMT and stemness of gastric cancer cells.

SOX4 promotes EMT and stemness of gastric cancer cells in vivo

To assess the role for SOX4 in the colonization potential of gastric cancer cells in vivo, we used nude mice lung

| Marker expression | SOX4 | Expression | $\chi^2$ | P |
|-------------------|------|------------|---------|---|
| E-cadherin        |      | High       | 4.972   | 0.026 |
|                   |      | Low        | 45      | 14 |
| N-cadherin        |      | High       | 0.144   | 0.705 |
|                   |      | Low        | 34      | 9  |
| Vimentin          |      | High       | 9.575   | 0.002 |
|                   |      | Low        | 46      | 6  |
| EpCAM             |      | High       | 5.327   | 0.021 |
|                   |      | Low        | 40      | 6  |

Figure 3 SOX4 induced EMT of cultured GC cells. A. MKN28 cells were transduced with a lentivirus expressing SOX4 in parallel with an empty control lentivirus for 72 h. The mRNA levels of SOX4 were determined by qRT-PCR and SOX4 protein was assessed by immunoblotting. Cell invasion was assessed by Transwell assay. B. BGC823 cells were transduced with a lentivirus expressing SOX4 shRNA in parallel with a lentivirus expressing control shRNA for 72 h. The mRNA levels of SOX4 were determined by qRT-PCR and SOX4 protein was assessed by immunoblotting. Cell invasion was assessed by Transwell assay. C. MKN28 or BGC823 cells were treated as A and B, the mRNA and protein levels of E-cadherin, N-cadherin and Vimentin were determined by qRT-PCR and qRT-PCR protein was assessed by immunoblotting. Cell invasion of SOX4 expression was assessed by transwell assay. All the experiments were repeated three times. Comparison between two groups was made by the two-sample t-test. *P < 0.05, **P < 0.01.
metastasis model to detect SOX4 on GC cell metastasis ability. MKN28-NC and MKN28-SOX4 cells were injected into the tail vein, and lung tissues were removed after 4 weeks. More lung metastases (19.6 vs 7.8) were found in MKN28-SOX4 compared with MKN28-NC group. In contrast, the lung metastatic nodules of BGC823-sh cells were significantly less than those in BGC823-NC (16.8 vs 36.8) (Fig. 7A). These observations were further confirmed by H&E staining of lung tissues (Fig. 6A).

The enhanced tumorigenesis is hallmark of cancer stem cells. We injected MKN28-NC, MKN28-SOX4, BGC823-NC and BGC823-sh cells subcutaneously in nude mice to observe

Figure 4  SOX4 promoted the stemness of GC cells. A. MKN28 cells were transduced with a lentivirus expressing SOX4 in parallel with an empty control lentivirus for 72 h. The self-renewal of gastric stem cells was assessed using sphere formation assay. B. BGC823 cells were transduced with a lentivirus expressing SOX4 shRNA in parallel with a lentivirus expressing control shRNA for 72. The self-renewal of gastric stem cells was assessed using sphere formation assay. The experiments were repeated three times. The number of spheres between two groups was compared by the two-sample t-test. * *P < 0.01.

Figure 5  SOX4 positively regulates EMT and stemness transcription factors. A. MKN28 cells were transduced with a lentivirus expressing SOX4 in parallel with an empty control lentivirus. The mRNA and proteins of EMT transcription factors Twist1, Snail1, Snail2 and ZEB1, and stemness transcription factors Nanog, Oct4 and SOX2 were determined by qRT-PCR and Western blotting respectively. B. BGC823 cells were transduced with a lentivirus expressing SOX4 shRNA in parallel with a lentivirus expressing control shRNA. The mRNA and proteins of EMT transcription factors were determined by qRT-PCR and Western blot respectively. The experiments were repeated three times. Comparison of mRNA or protein expression between two groups was analyzed by the two-sample t-test. * P < 0.05, ** P < 0.01.
their tumorigenic ability. After 6 weeks, we found that MKN28-SOX4 had higher tumor formation rate (5/5 vs 3/5), and the tumor weight was significantly higher in MKN28-SOX4 cells than that in MKN28-NC cells. The tumor weight of BGC823-sh group was significantly reduced despite no difference in tumor formation rate between the two groups (5/5 vs 5/5) (Fig. 7B). These results suggest that SOX4 promotes EMT and stemness of gastric cancer cells in vivo.

Discussion

In the present study, we demonstrated that SOX4 was upregulated and overexpression of SOX4 was associated with EMT and acquisition of stemness of gastric cancer cells in clinic patient samples. In vitro, SOX4 promoted the stemness and EMT of gastric cancer cells, which was accompanied with increased expression of EMT and stemness transcription factors. In addition, SOX4 increased the nuclear translocation of β-catenin. Further, we revealed that SOX4 was a target of TGF-β signaling and silencing SOX4 reversed TGF-β induced EMT and stemness of gastric cancer cells. Finally, we showed that SOX4 promoted tumorigenic ability of gastric cancer cells in nude mice.

SOX4 is a member of the sex-determining gene family and located in human Y chromosome 6p22.3, encoding a 47KD SOX4 protein. SOX4 contains HMG cassettes and interacts with other transcription factors to regulate gene expression. In gastric cancer tissues, it was reported that SOX4 nuclear expression was associated with tumor cell infiltration, vascular invasion, lymph node metastasis and distant metastasis. Survival analysis showed that patients with high expression of SOX4 had shorter disease-free survival than those with low expression. In order to explore the mechanism of SOX4 in promoting the malignant behavior of gastric cancer, we first detected the expression of SOX4 in 84 cases of gastric cancer and the matched paracancerous tissues by immunohistochemistry. We found that there was high expression of SOX4 in 65 cases of gastric cancer and that the expression rate of Vimentin and cancer stem cell marker CD44 in SOX4 positive patients was significantly higher than that in SOX4 negative patients, while the expression rate of E-cadherin in SOX4 positive patients was lower than that of SOX4-negative patients. We further analyzed the correlation between SOX4 and EMT and the mRNA levels of stemness markers in 30 cases of gastric cancer tissues. We found that SOX4 was negatively correlated with E-cadherin in gastric cancer tissues but positively correlated with the interstitial molecule Vimentin and stemness molecule CD44. Our results suggest that SOX4 may be involved in gastric cancer cell EMT and stemness regulation.

During EMT, cells undergo cytoskeleton remodeling with polarity disappearance and acquired enhanced migration ability, accompanied by down-regulation of E-cadherin and upregulation of N-cadherin and Vimentin. Cancer stem cells (CSCs) are a group of cells with self-renewal, multiple differentiation potential and high tumorigenicity. CSCs are considered the origin of tumor expansion and recurrence and are identified in various tumors including gastric, colon and breast cancers. Evidence suggests that EMT can induce CSCs characteristics during cancer progression. Both EMT and CSCs are involved in tumor metastasis, drug resistance and recurrence. It has been reported that SOX4 can promote stemness by regulating the EMT of breast cancer cells. Our results showed that the morphology of MKN28 cells was elongated with disappearance of nucleus polarity and obvious spindle shape after overexpression of SOX4, accompanied by down-regulation of E-cadherin, up-regulation of N-cadherin and Vimentin, increased sphere formation ability and percentage of CD44+/EpCAM+ cells. Opposite results were observed in BGC823 cells after SOX4 silencing. Finally, our in vivo nude mice results showed that overexpression of SOX4 increased gastric cancer cell lung metastasis and tumor initiation ability, which was reversed by SOX4 silencing. These findings indicate that SOX4 facilitates the acquisition of stemness through EMT ultimately promoting the progression of gastric cancer.

Snail, ZEB and Twist family promotes EMT by regulating E-cadherin, N-cadherin and Vimentin. Previous studies have shown that SOX4 does not directly regulate the

Figure 6  SOX4 mediated TGF-β-induced EMT and stemness of GC cells. A. MKN28 cells were cultured in medium containing TGF-β1 (3 ng/ml) for 3 days. QRT-PCR and Western blotting were used to detect the expression of SOX4. The experiments were repeated three times and the data was analyzed by the two-sample t-test. *P < 0.01. B–C. BGC823-NC and BGC823-sh cells were treated with TGF-β1 (3 ng/ml) for 5 days. Cell invasion was assessed by Transwell assay. The expression of E-adhering, N-cadherin and Vimentin were determined by qRT-PCR, immunoblotting and IF. The experiments were repeated three times and the data was analyzed by one-way ANOVA. *P < 0.05, **P < 0.01. D. BGC823-NC and BGC823-sh cells were treated with TGF-β1 (3 ng/ml) for 10 days, the percentage of CD44+/EpCAM- cells was determined by flow cytometry. The stemness self-renewal of gastric stem cells was assessed using sphere formation assay. The experiments were repeated three times and the data was analyzed by one-way ANOVA. *P < 0.05, **P < 0.01.
Figure 6 (continued).
transcription of Snail, ZEB and Twist, possibly by elevating their expression through an intermediate protein or epigenetic modification. We found that SOX4 upregulated Twist1, ZEB1 and snail1 expression in MKN28 and BGC823 cells, but the mechanism remains to be further studied. The transcription factors Nanog, OCT4 and SOX2 regulate the transcription of genes required for maintenance of the self-renewal and pluripotent potential of stem cells. Although there is no evidence that SOX4 directly regulates the expression of these three major transcription factors, our study showed that SOX4 did increase Oct4 and SOX2, thereby maintaining the stemness of gastric cancer cells. The Wnt/β-catenin pathway is closely related to gastrointestinal neoplasm. These results indicate that SOX4 may maintain gastric cancer EMT and stemness through regulating the relevant transcription factors.

Figure 6 (continued).

Figure 7 In vivo assessment of SOX4 in colonization potential and stemness of GC cells. A. 2 × 10⁶ of the indicated gastric cancer cells were injected into nude mice tail vein with 5 mice in each group. After 4 weeks, lung tissues were removed to observe the metastatic nodules and the lung tissues were stained for hematoxylin-eosin (H&E) for pathology confirmation. B. Nude mice were injected subcutaneously with 5 × 10⁵ of the indicated gastric cancer cells with 5 mice in each group. After 6 weeks, tumor tissues were removed and weighed. The experiments were repeated three times and the data was analyzed by the two-sample t-test. *P < 0.05, **P < 0.01.
It has been well documented that TGF-β is a strong inducer of cancer cell EMT and stemness. Studies in breast cancer have shown that SOX4 is an essential factor for TGF-β-induced EMT, and in glioma cells TGF-β increases the sphere formation ability and stem cell ratios through TGF-β-Sox4-Sox2 axis. Consistent with these previous studies, we found that TGF-β1 significantly up-regulated SOX4 in MKN28 cells. We further observed that TGF-β1 significantly promoted the invasion of BGC823-NC cells associated with the down-regulation of E-cadherin and the up-regulation of N-cadherin and Vimentin, which was attenuated after SOX4 silencing. At the same time, TGF-β1 enhanced the self-renewal ability of BGC823-NC cells and increased the ratio of CD44+/EpCAM+ cells, which was reversed by SOX4 silencing. These results suggest that SOX4 is a target of TGF-β and mediates TGF-β-induced EMT and stemness behavior of gastric cancer cells.

In summary, we demonstrate that SOX4 promotes the progression of gastric cancer by controlling the EMT and stemness acquisition of gastric cancer cells through EMT transcription factors (Twist1, ZEB1, snail1) and stemness transcription factors (Oct4, SOX2), and the activation of the Wnt pathway. In addition, SOX4 is a target gene of TGF-β signaling and mediates TGF-β-induced EMT and stemness behavior, confirming the role of TGF-β/SOX4 axis in gastric cancer cell EMT and stemness regulation. This study has revealed the molecular mechanism of SOX4 in the progression of gastric cancer and may provide new targets for gastric cancer therapy.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.j.endis.2017.12.005.

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