SOX9 promotes epithelial-mesenchymal transition via the Hippo-YAP signaling pathway in gastric carcinoma cells

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Abstract. SRY-box 9 (SOX9) is overexpressed in a number of human tumors, including gastric cancer (GC). However, the function of SOX9 in the development of GC remains unknown. In the present study, SOX9 activated the Hippo-yes-associated protein (YAP) signaling pathway to enhance the epithelial-mesenchymal transition in GC cell lines. The results suggested that SOX9 knockdown inhibited invasion, proliferation and migration of GC cells. Furthermore, SOX9 silencing upregulated the expression of E-cadherin, an epithelial marker, and downregulated the expression of mesenchymal markers, including snail family transcriptional repressor 1, vimentin and N-cadherin. SOX9 overexpression increased the expression of the aforementioned markers. SOX9 significantly affected YAP phosphorylation and total YAP protein levels, suggesting that SOX9 is involved in the Hippo-YAP signaling pathway. The current study revealed that SOX9 may be involved in the pathogenesis of GC, and further elucidation of the pathways involved may support the development of novel therapeutic options for the treatment of GC.

Introduction

Gastric cancer (GC) is the third major contributor of cancer mortality and the fifth most frequently diagnosed cancer in the world (1). GC led to >700,000 mortalities and resulted in 950,000 new cases of GC diagnosed in 2012 (2,3). The majority of patients with GC are diagnosed at a later stage and the five-year survival of GC ranges from 13.1 to 43.8% (4,5). The late detection of the tumor negatively affects the survival rate (6). Therefore, a deeper understanding of the mechanisms underlying the pathogenesis and progression of GC is required.

The SRY-box (SOX) family consists of a DNA binding domain which includes a conserved high mobility group (HMG) (7,8). SOX9, a transcription factor in the HMG-box class, affects the survival, proliferation and differentiation of cells, such as hepatocellular carcinoma and stem cells (9,10). SOX9 has been revealed to have oncogenic properties and its expression was increased in several tumors, including prostatic carcinoma (11), ovarian cancer (12), breast carcinoma (13) and lung carcinoma (14). However, the functions of SOX9 in GC remain unclear.

Epithelial-mesenchymal transition (EMT) provides epithelial cells with the plasticity required to generate a mesenchymal phenotype (15). EMT is involved in multiple physiological and pathological syndromes and processes, including embryo development, fibrosis and cancer progression (16,17). During EMT, morphological transformations are caused by E-cadherin reduction, and increasing of vimentin and N-cadherin expression levels (18,19). Previous studies have investigated the association between SOX9 and EMT in GC (20,21).

The Hippo signaling pathway serves a central role in regulating cell proliferation, cell fate and tissue size (22,23). The pathway has emerged as a tumor suppressive pathway that acts to control the transcriptional activity of two proteins, YAP and WW domain containing transcription regulator 1, also referred to as TAZ (24). YAP and TAZ activity is fundamental for normal organ growth and tissue regeneration; however, it is also involved in cancer pathogenicity (25,26). The YAP/TAZ signaling pathway promotes cancer stem cell characteristics, tumor initiation, progression and metastasis (27,28). In the YAP/TAZ signaling pathway, mammalian sterile 20-like kinase 1/2 kinases phosphorylate and activate a second set of kinases, large tumor suppressor kinase 1/2 (LATS1/2). LATS1/2 subsequently phosphorylate two transcriptional coactivators, TAZ and YAP, leading to their sequestration in the cytoplasm, degradation and functional inhibition. The Hippo signaling pathway effectors, TAZ and YAP, are oncogenes that are commonly dysregulated in cancer (29). Previous studies demonstrated that YAP/TAZ are abnormally overexpressed in tumors, promote
tumorigenesis, and are considered as carcinogenic genes in numerous types of solid cancer (30,31).

In the present study, the roles of SOX9 in the EMT of GC cells were examined to elucidate the underlying mechanisms. The results obtained suggested that SOX9 promoted GC cell invasion, migration and proliferation. In addition, SOX9 may promote the EMT via the Hippo-YAP signaling pathway in GC cells.

Materials and methods

Cell culture. The GC cell lines including BGC823, HGC27, MKN45 and MGC803 (Type Culture Collection of the Chinese Academy of Sciences) were maintained in Dulbecco's Modified Eagle's medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% CO2 humidified incubator at 37˚C. Cell line characterization was performed by short tandem repeat analysis using GeneMapper® software (version 4.0; https://www.atcc.org/) and consolidated using the American Type Culture Collection, German Collection of Microorganisms and Cell Cultures, Japanese Collection of Research Bioreresources Cell Bank and Rikagaku Kenkyusho (Institute of Physical and Chemical Research, Japan) databases (https://www.atcc.org/en/Products/Cells_and_Microorganisms/Cell_Lines.aspx; https://www.dsmz.de/; https://cellbank.nibiohn.go.jp/english/). The cell lines were tested for the presence of Mycoplasma using PCR (32).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using an RNAiso Plus kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's protocol. RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used for reverse transcription, according to the manufacturer's protocols. qPCR was subsequently performed in triplicate using the SYBR® Green PCR master mix (Bio-Rad Laboratories, Inc.) and a CFX-96 Sequence Detection system (Bio-Rad Laboratories, Inc.) prior to transfection with 6 µl of Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). Viral particles were subsequently subcloned into pLenti CMV GFP Zeo (cat. no. 17449; Addgene Inc., Cambridge, MA, USA), replacing GFP. The pLenti CMV GFP Zeo vector was used as a control. For flow cytometry experiments (Aldefluor assay), a control vector with RFP instead of GFP was used. Viral particles were produced as described above. A total of 4x10⁶ cells per well were seeded into six-well plates. Following overnight incubation at 4˚C, the cell culture medium (Invitrogen; Thermo Fisher Scientific, Inc.) was replaced by Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) prior to transfection with 6 µl of Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) and 2 µg of the vector. Following another 48 h of incubation at 4˚C, the cells were harvested for protein expression analysis and migration, invasion and proliferation assays.

Western blot analysis. Cell samples were lysed at 4˚C using radioimmunoprecipitation assay lysis buffer (BioVision; Thermo Fisher Scientific, Inc.) for 10 min and subsequently centrifuged for 15 min at 4ºC and 8,100 x g. Total protein was quantified using a bicinchoninic acid and 40 µg protein/lane was separated via SDS‑PAGE on a 10% gel. The separated protein were subsequently transferred onto a polyvinylidene fluoride membrane and blocked for 1 h at room temperature with 5% non‑fat milk. The membranes were incubated with primary antibodies against SOX9 (cat. no. 82630), N-cadherin (cat. no. 13116), E-cadherin (cat. no. 3195), vimentin (cat. no. 5741), snail family transcriptional repressor 1 (SNAI1; cat. no. 3879), YAP (cat. no. 8418), p-YAP (cat. no. 1619), MOB kinase activator 1 (MOBI; cat. no. 13730), phosphorylated (p)-MOBI (cat. no. 8699), LATS1 (cat. no. 9153), p-LATS1 (cat. no. 9157) and β-tubulin (cat. no. 6181) at a dilution of 1:200, overnight at 4˚C (all

Plasmid construction. The SOX9 short hairpin RNA (shRNA) sequence was obtained from Sigma-Aldrich, Merck KGaA (Darmstadt; Germany) and was subsequently synthesized by Sangon Biotech, Co., Ltd.. The oligo sequence of SOX9 shEGFP included: SOX9 shEGFP forward, 5'-GGT GAA GGT CGG TGT GAA CG-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'. As an internal standard, a fragment of human GAPDH was amplified by PCR using the following primers: forward primer, 5'-GGT GAAGGTGTGTTGAAAGC-3' and reverse primer, 5'-CTC GCTTCTCGTTCAGAAGTCTC-3'.
from Cell Signaling Technology, Inc., Danvers, MA, USA). Following the primary incubation, membranes were incubated with the corresponding secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody #7074, Cell Signaling Technology, Inc.) for 1 h at room temperature. SignalFire™ ECL Reagent (cat. no, 6883; Cell Signaling Technology, Inc.) and ImageJ bundled with 64-bit Java 1.8.0_112 software (National Institutes of Health) were used to visualize and quantify protein expression levels.

Cell counting kit-8 (CCK-8) assay. Cell viability was measured using a CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China). Cells were seeded at a density of 3,000 cells/well in a 96-well plate and incubated at 5% CO₂ and 37°C. A total of 10 µl of CCK-8 regent was added to each well 1, 2, 3, 4 and 5 days after plating. Following a 2-h incubation at 37°C, the absorbance was measured at a wavelength of 490 nm.

Wound healing assay. Cells were grown in six-well plates to confluence in complete cell culture medium Gibco DMEM containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. At time 0, a wound was created across the diameter of the well using a 10 µl pipette tip. Medium was added to wash the cells and remove dead and
floating cells. The distance between scratch edges was recorded at 0 and 48 h. Images were captured using an inverted microscope (magnification, x200) equipped with a digital camera and the ImageJ bundled with 64-bit Java 1.8.0_112 software (National Institutes of Health) was used.

Migration and invasion assays. Cell migration was measured using a transwell assay A total of 1x10^5 cells were placed into the upper compartment of a transwell insert (Corning Inc., Corning, NY, USA) in serum free media (Gibco DMEM; Thermo Fisher Scientific, Inc.). Medium supplemented with 10% FBS was plated in the lower chambers. Following incubation for 24-36 h in 37°C, the migratory cells were fixed using 4% paraformaldehyde and stained with 1% crystal violet. Stained cells were counted in five randomly selected fields using a light microscope (magnification, x400). The assay was repeated using transwell membranes precoated with Matrigel® to assess cell invasion for 72 h at 37°C.

Statistical analysis. All data are presented as the mean ± standard deviation from at least three independent experiments. All statistical analyses were performed using SPSS software (version 19; IBM Corp., Armonk, NY, USA). Comparisons between groups were analyzed using the Student's t-test (two groups) or a one-way analysis of variance (multiple groups) using the Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results
SOX9 expression level in GC cells. Expression levels of SOX9 in BGC823, HGC27, MKN45 and MGC803 cells were examined using RT-qPCR and western blot analysis. SOX9 expression, at both mRNA and protein levels, was higher in MGC803 and MKN45 cells than that in BGC823 and HGC27 cells (Fig. 1A and B). sh-SOX9 and Flag-SOX9 vectors were constructed to investigate the roles of SOX9 in the development of GC. sh-EGFP and empty vector were used as control groups. The effectiveness of sh-SOX9 vector compared with the sh-EGFP vector was evaluated in MKN45 and MGC803 cells (Fig. 1C and D). Following Flag-SOX9 or empty vector transfection into the BGC823 and HGC27 cell lines, mRNA and protein expression levels of SOX9 were increased in the Flag-SOX9 group compared with the empty vector group (Fig. 1E and F).

SOX9 promotes the proliferation of GC cells. A CCK8 assay was performed to assess the effect of SOX9 on the proliferation of GC cells. The results indicated that the proliferation...
of MKN45 and MGC803 cells was suppressed following knockdown of SOX9 (Fig. 2A and B). The overexpression of SOX9 enhanced the proliferation of BGC823 and HGC27 cells (Fig. 2C and D). The results obtained suggested that SOX9 enhanced the proliferation of GC cells.

**SOX9 enhances the migration of GC cells.** A transwell assay was performed to evaluate the migration of GC cells. The results revealed 94±7 migrated MGC803 cells transfected with sh-SOX9 and 322±15 migrated MGC803 cells transfected with sh-EGFP. Furthermore, 92±8 migrated MKN45 cells transfected with sh-SOX9 and 419±11 migrated MKN45 cells transfected with sh-EGFP were observed (Fig. 3A and B). To test the effects of SOX9 knockdown on cell motility, a wound scratch assay was performed. A wound was created on confluent cultures of MGC803 cells expressing either sh-EGFP or sh-SOX9. MGC803 cells expressing sh-SOX9 exhibited reduced motility compared with MGC803 cells expressing sh-EGFP (Fig. 3E). The results suggested that SOX9 knockdown suppressed the migration of MKN45 and MGC803 cells. In addition, the migration ability of BGC823 and HGC27 cells transfected with Flag-SOX9 vector compared with a negative control vector was evaluated. The numbers of migrated cells were 100±7 and 212±10 in Vector and Flag-SOX9 BGC823 cells, and 70±6 and 145±8 in Vector and Flag-SOX9 HGC27 cells, respectively (Fig. 3C and D), indicating that SOX9 overexpression improved the ability of migration in gastric carcinoma cells. To test the effects of SOX9 overexpression on cell motility, a wound scratch assay was performed. BGC823 cells expressing Flag-SOX9 exhibited increased motility compared with BGC823 cells transfected with an empty vector (Fig. 3F). The results suggested that SOX9 overexpression increased the migration ability of GC cells.

**SOX9 enhances the invasion of GC cells.** The effect of SOX9 on the invasion of GC cells was examined using BD Matrigel
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invasion assays. MGC803 and MKN45 cells were transfected with sh-EGFP or sh-SOX9 plasmids, and BGC823 and HGC27 cells with Vector or Flag-SOX9 for 72 h. The numbers of invasive cells were 190±12 and 50±6 in sh-EGFP and sh-SOX9 MGC803 cells, and 183±7 and 48±5 in sh-EGFP and sh-SOX9 MKN45 cells (Fig. 4A and B). The results suggested that the absence of SOX9 suppressed the invasion of MKN45 and MGC803 cells. BGC823 and HGC27 cells were transfected with empty vector or Flag-SOX9 to assess the effects of SOX9 overexpression on invasion. The numbers of invasive cells were 75±5 and 292±7 in vector and Flag-SOX9 BGC823 cells, and 108±6 and 300±8 in vector and Flag-SOX9 HGC27 cells. The data indicated that the upregulation of SOX9 enhanced the invasion of HGC27 and BGC823 cells (Fig. 4C and D). The aforementioned data suggest that SOX9 promotes the invasive ability of gastric cancer cells.

SOX9 enhances EMT via the Hippo-YAP signaling pathway in GC cells. The process of EMT is closely associated with cancer cell invasion and migration (33,34). Therefore, the effect

Figure 4. SOX9 enhances the invasion of gastric cancer cells. Matrigel invasion assays were performed to investigate the effect of SOX9 knockdown on the invasion ability of (A) MGC803 and (B) MKN45 cells. The effect of SOX9 overexpression on the invasion ability of (C) BGC823 and (D) HGC27 cells was also investigated. Magnification, x400. *P<0.001. SOX9, SRY-box 9; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein.
of SOX9 expression on the EMT markers was investigated by western blot analysis. The results suggested that expression of vimentin, SNAI1 and N-cadherin was downregulated following SOX9 knockdown, while E-cadherin was upregulated in MKN45 and MGC803 cells (Fig. 5A). The overexpression of SOX9 upregulated the expression of vimentin, N-cadherin and SNAI1 and downregulated E-cadherin in BGC823 and HGC27 cells (Fig. 5B), suggesting that SOX9 enhanced EMT in GC cells. As the Hippo-YAP signaling pathway is closely associated with EMT in tumors (30,31,33,35,36), the effects of SOX9 on the Hippo-YAP signaling pathway were investigated in the current study. The results obtained suggested that the knockdown of SOX9 downregulated the expression of total YAP, LATS1 and p-MOB1 in MGC803 and MKN45 cells, but upregulated the expression of p-YAP, MOB1 and p-LATS1 in these cells (Fig. 5C). The overexpression of SOX9 resulted in opposite effects in HGC27 and BGC823 cells (Fig. 5D). The results obtained suggested that SOX9 may enhance EMT in GC cells via the Hippo-YAP pathway, however further studies are required to confirm the aforementioned.

Figure 5. SOX9 may enhance the epithelial-mesenchymal transition via the Hippo-YAP signaling pathway in gastric cancer cells. (A) The expression of E-cadherin, vimentin, N-cadherin and SNAI1 in MGC803 and MKN45 cells following SOX9 knockdown as determined by western blot analysis. (B) The expression of E-cadherin, vimentin, N-cadherin and SNAI1 in BGC823 and HGC27 cells overexpressing SOX9 as determined by western blot analysis. (C) The expression of Hippo-YAP pathway-associated proteins in MGC803 and MKN45 cells following SOX9 knockdown as determined by western blot analysis. (D) The expression of Hippo-YAP signaling-associated proteins in BGC823 and HGC27 cells overexpressing SOX9 as determined by western blot analysis. SOX9, SRY-box 9; SNAI1, snail family transcriptional repressor 1; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein; YAS, yes-associated protein; p, phosphorylated; LATS1/2, large tumor suppressor kinase 1/2; MOB1, MOB kinase activator 1.
Discussion

Previous studies have revealed that SOX9 may function as an oncogene to enhance the growth of cancer cells (37,38). SOX9 expression is increased in different types of cancer, including GC (39-41). The current study demonstrated that SOX9 enhanced the invasion, migration and proliferation of GC cells. Additionally, the present study revealed that SOX9 may enhance the EMT in GC potentially via the Hippo-YAP pathway. 

Previous studies suggested that the prognosis of patients with cancer is significantly influenced by metastasis (42,43). EMT is known to regulate metastasis and acts as a factor in determining the prognosis of cancer patient (44-46). The EMT process includes three major steps: i) Destruction of cell junctions and E-cadherin downregulation; ii) N-cadherin upregulation; and iii) rearrangement of the cytoskeleton for cell invasion (47). During EMT, epithelial cells undergo a number of phenotypic and genotypic changes to obtain a mesenchymal phenotype characterized by increased migration, invasion, resistance to apoptosis and the synthesis of ECM (48,49). Due to the loss of E-cadherin, newly generated mesenchymal cells are associated with poor adhesive properties (50). EMT features the upregulation of fibronectin and N-cadherin as well as vimentin (51). EMT induced by epigenetic and genetic changes in a tumor microenvironment is an important event in the progression and metastasis of different types of cancer (52).

The Hippo signaling pathway, considered as an evolutionarily conserved pathway, is associated with cell polarity, cell proliferation and tumor suppression (53,54). The alterations in this pathway are increasingly recognized to be associated with cancer development (55,56). Nuclear YAP, the downstream effector of the Hippo pathway, is implicated in the processes of EMT, cell proliferation and maintenance of cell polarity (57,58). A previous study confirmed that upregulation of YAP enhanced the EMT and promoted the aggressiveness of colorectal cancer (59). YAP and KRAS proto-oncogene, GTPase have been revealed to work jointly to regulate EMT (60). Another study demonstrated that YAP and tafazzin interact with TEA domain transcription factor 2 to regulate EMT by interacting with specific transcription factors. The results obtained in the current study revealed that SOX9 may regulate EMT and affect the level of YAP phosphorylation and the protein expression of total YAP, suggesting that SOX9 may serve roles in the Hippo-YAP pathway to subsequently promote EMT in GC cells.

In summary, the current study revealed that SOX9 may be implicated in GC cell proliferation, migration and invasion by inducing EMT. The results obtained in the present study suggested that SOX9 may induce EMT by activating the Hippo-YAP signaling pathway. The limitations of the current study included not testing the effect of YAP knockdown/overexpression and using only one shRNA in loss-of-function experiments. Furthermore, another limitation of the current study is that different cell lines were used for knockdown and overexpression experiments. It has been reported that the knockdown of YAP inhibits gastric cancer cell proliferation, migration, invasion and metastasis (62,63). The current study revealed that SOX9 knockdown or overexpression significantly affected YAP phosphorylation and total YAP protein level, indicating that SOX9 may be involved in the Hippo-YAP signaling pathway. SOX9 may potentially be an important target in the development of novel GC treatments.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

AZ and HZ conceived the idea and designed the experiments. HZ and GL analysed data and wrote the paper and HZ, GL, SH and YF collected data and performed the experiments. SH and YF contributed reagents/materials/analysis tools. AZ and HZ prepared the references and managed the data. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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