SOX5 promotes breast cancer proliferation and invasion by transactivation of EZH2

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Abstract. Sex determining region Y-box protein 5 (SOX5) is a transcriptional factor and serves important roles in various cancer types; however, the pathological role of SOX5 in patients with breast cancer remains unclear. In the present study, the expression and potential role of SOX5 in patients with breast cancer and in breast cancer cells was investigated. The data indicated that SOX5 was highly expressed in breast cancer tissues compared with adjacent healthy tissues, and overexpression of SOX5 was associated with a reduced overall survival rate in patients with breast cancer. Gain and loss of function studies with MTT, colony formation, wound healing and Matrigel invasion assays demonstrated that SOX5 significantly promoted breast cancer cell proliferation and invasion. The chromatin immunoprecipitation (ChIP) assay sequence, quantitative ChIP and luciferase reporter assays were used to identify enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) as a downstream target gene of SOX5. Furthermore, it was determined that ectopic expression of SOX5 increased EZH2 expression at the mRNA and protein level, while the knockdown of SOX5 decreased EZH2 expression. Additionally, the biological effect of SOX5 was investigated, and it was determined to be dependent on the regulation of EZH2 expression. The present results may provide important insights into the biological significance of SOX5 serving as a candidate therapeutic target in breast cancer progression.

Introduction

Breast cancer is one of the most common types among females worldwide in 2014, which cause cancer-associated mortalities globally (1,2). In China, breast cancer remains the most common type of neoplasm in 2014 (3). With the development of medical technologies over the past 20 years, including surgery, radiotherapy and chemotherapy, the diagnosis and treatment of breast cancer have continuously improved (4,5). Metastasis and recurrence remain the major causes of high mortality rates of patients with breast cancer (6); therefore, understanding the mechanisms and investigating novel biomarkers, which are responsible for unfavorable progression, is important. Furthermore, the identification of novel therapeutic targets for breast cancer treatment is essential.

Sex determining region Y-box protein 5 (SOX5) is a member of the SOX family, which was identified based on the conserved homology of the high-mobility group DNA-binding motif (7). It has been reported that SOX5 is involved in the regulation of embryonic development (8), and is associated with various cancer types, including prostate cancer (9), glioblastoma (10), hepatocellular carcinoma (11), osteosarcoma (12) and nasopharyngeal carcinoma (13). In 2014, Pei et al (14) reported that in breast cancer, SOX5 induces epithelial-mesenchymal transition (EMT) by transactivation of Twist1 expression. However, the expression and the precise regulatory mechanism underlying the biological function of SOX5 in breast cancer remain unclear.

Patients and methods

Cell culture and reagents. The normal breast tissue cell line, MCF-10A, and the MCF7, T47D and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MCF-7 and T47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; both Invitrogen; Thermo Fisher Scientific, Inc.). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; both Invitrogen; Thermo Fisher Scientific, Inc.). The cells were incubated in an atmosphere containing 5% CO₂ at 37°C. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection to transfected into MCF-7 or MDA-MB-231 cells. The relative small interfering (si)RNAs targeting SOX5 (si-SOX5-1 and si-SOX5-2) or enhancer of zeste 2 polycomb repressive
complex 2 subunit (EZH2) or negative control and G418 were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), SOX5 and vector plasmid were purchased from GenePharma (Shanghai, China). The 70% confluency of MCF-7 or MDA-MB-231 cells were achieved overnight prior to transfection. In each group, 2 µg oligonucleotide were used for transfection. At 48 h following transfection, the cells were harvested for experimentation.

**Patients.** The present study was approved by the Research Ethics Committee of Weifang People's Hospital (Weifang, China). All patients provided written informed consent. A total of 58 pairs of breast cancer tissues from female patients aged from 40-55 years old and relative adjacent healthy mammary tissues were collected between May 2010 and January 2013. The fresh specimens were frozen immediately at -80°C in liquid nitrogen for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) use. Patients who received tumor-specific therapy prior to diagnosis were excluded. The pathological information was retrieved by the Pathology Department of Weifang People's Hospital. Overall survival times were calculated as the duration between the date of diagnosis and date of cancer-associated mortality in the follow-up period.

**RNA extraction and RT-qPCR analysis.** Total RNA was extracted from cells or tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-strand complementary DNA was synthesized using SuperScript II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using the Fast SYBR® Green Master mix system (Roche Applied Science, Penzberg, Germany) on an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR reaction was subsequently performed according to the following conditions: Initial step, 95°C for 5 min; second step, 95°C for 10 sec, 60°C for 30 sec and 72°C for 10 sec for a total of 35 cycles. The primers used were as follows: EZH2 forward, 5'-TTTCCA ACACAAGTCTACCC-3', and reverse, 5'-ATAAACCCCAT TCTTATCCTC-3'; GAPDH forward, 5'-CCGTCCTAGAAAA ACCTGTC-3', and reverse, 5'-GCCAAATTCGTTGCTATA CC-3'. The relative mRNA level was calculated using the 2-ΔΔCq method and normalized to GAPDH (15). The experiment was performed in triplicate.

**Western blot analysis.** MCF-7 or MDA-MB-231 cells were harvested and protein was extracted using radioimmunoprecipitation buffer (50 mM tris-HCl pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl and 0.1% SDS). The Bradford assay reagent (Thermo Fisher Scientific, Inc.) was then used to determine the protein concentration in the lysates. Equal amounts of protein (30 µg) were separated by 10% SDS-PAGE gel, and then transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk in PBS containing 0.5% Tween-20 at room temperature for 1 h and incubated with the primary antibodies overnight at 4°C, and then washed three times with washing buffer Tris-buffered saline Tween-20 (Sigma-Aldrich, Merck KGaA). Horseradish peroxidase-conjugated anti-rabbit (sc-2357; 1:3,000) or anti-mouse (sc-2789; 1:3,000; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies were used as the secondary antibodies at room temperature for 1 h. The signal was visualized using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.). The primary antibodies used were as follows: SOX5 (ab94396; 1:1,000), EZH2 (ab186006; 1:1,000; both Abcam). β-actin (sc-47778; 1:1,000; Santa Cruz Biotechnology, Inc.) was used as the control.

**Chromatin immunoprecipitation assay (ChIP).** The ChIP assay was performed using Chip-IT Express kit (Active Motif; Carlsbad, CA, USA), according to manufacturer's protocols. The PCR products were resolved using a ABI 7500 system. PCR was performed with 5 µl of the immunoprecipitated target DNA, 1 µl primers and 9 µl mixture (1 µl enzyme, 2 µl dNTP and 6 µl SYBR green solution buffer all were included in the ChIPT-IT kit.

**Luciferase report assay.** A luciferase report assay was performed using a dual luciferase assay kit according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). The EZH2 promoter was cloned into the luciferase reporter pGL3-basic vector plasmid, which was part of the kit. A total of 5x10⁵ cells-well were cultured in DMEM at 37°C in 24-well plates for 48 h. The report plasmid was transfected into the cells with the relative plasmid SOX5 or shRNA targeting SOX5 or a negative control plasmid. After 24 h transfection, the luciferase activities were measured according to the aforementioned kit. The result was normalized to Renilla. The transfections were performed in triplicate.

**Cell proliferation assay.** For the MTT assay, 5x10⁵ cells were seeded into 96-well plates with 100 µl culture medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37°C for different periods of time at 0, 24, 48 and 72 h. A total of 10 µl 5 mg.ml MTI reagent (Beyotime Institute of Biotechnology, Shanghai, China) was added into each well and the culture was continued for 4 h. Subsequently, 100 µl dimethyl sulfoxide was used to replace the medium. After 30 min of incubation, the absorbance at 570 nm wavelength was measured on a SpectraMax 190 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and cell growth curves were determined. Experiments were performed in triplicate independently.

**Colony formation assay.** For the colony formation assay, the cells were seeded into 6-well plates with 1x10³ cells-well. Fresh culture medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) was replaced every 3 days and cultured at 37°C in an atmosphere containing 5% CO₂ for 2 weeks, to form colonies. Subsequently, the cells were fixed with 70% methanol at room temperature for 30 mins and stained with 5% crystal violet at room temperature for 10 mins. The colonies containing >50 cells were counted under a Leica DMI 3000B inverted.
microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA) at magnification, x40.

**Scratch assay.** Wound healing was used to observe the migration ability of breast cancer cells. A total of 5x10^4 cells were plated in 6-well plates and cultured until 95% confluency. A plastic 20 µl pipette tip was used to scratch a vertical wound. Detached cells were removed and phase contrast images of the scratched fields were captured at 0 and 24 h. In each group, at least three scratched fields were recorded using an upright light microscope at magnification, x20 (Leica DM4B; Leica Microsystems, Shanghai, China).

**Invasion assay.** A Matrigel assay was performed to investigate the invasion ability. Transwell chambers (8-µm pore size) were coated with 1 mg/ml Matrigel (both BD Biosciences, San Jose, CA, USA). Cells were seeded into 0.2 ml serum-free medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) at a density of 1x10^5 cells-well and placed on the top chamber of each insert. The lower chamber was filled with 600 µl medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. After 24 h of incubation, cells on the surface were wiped off by mechanical scraping, and the migrant cells attached to the lower surface were fixed with 10% methanol for 30 min at room temperature. Following staining with 5% crystal violet at room temperature for 20 min, the cells were visualized and counted under a Leica DMI 3000B inverted microscope at magnification, x40. A total of three different fields of view in each group were counted.

**Statistical analysis.** SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All data were expressed as the mean ± standard deviation following ≥3 independent experiments. P<0.05 was considered to indicate a statistically significant difference. Kaplan-Meier analysis followed by the log-rank test was used to analyze the association between SOX5 expression and the overall survival rate. Significant differences between two groups were determined with a Student's t-test. One-way analysis of variance followed by Tukey's test was used to analyze the differences between multiple groups to compare values of test and control samples.

**Results**

SOX5 is frequently upregulated in breast cancer tissues and associated with reduced overall survival rate. **In order to identify the role of SOX5 in breast cancer, the expression of SOX5 in 58 pairs of matched breast cancer and adjacent healthy mammary tissues was investigated using RT-qPCR assays. Compared with the adjacent healthy tissues, significantly increased SOX5 mRNA expression levels were determined in breast cancer tissues (Fig. 1A). Furthermore, four pairs**
of breast cancer and relative healthy mammary tissues were selected to detect the protein expression of SOX5. As depicted in Fig. 1B, western blotting demonstrated that SOX5 was notably overexpressed in breast cancer tissues compared with healthy breast tissue. Kaplan-Meier estimator analysis with the log-rank test was used to investigate the prognostic significance of SOX5 in patients with breast cancer. It was determined that an increased expression of SOX5 was significantly associated with a reduced overall survival rate (P=0.00213) (Fig. 1C).

**SOX5 promotes breast cancer cell proliferation in vitro.** In order to examine the role of SOX5 in breast cancer progression, RT-qPCR was performed to investigate the expression of SOX5 in MCF-10A, MCF-7, T47D and MDA-MB-231 cells. Compared with the normal breast cancer cell line MCF-10A, the SOX5 mRNA level was significantly increased in the breast cancer cell lines. Additionally, the triple-negative cell line MDA-MB-231 exhibited the highest SOX5 expression level among all cell lines (Fig. 2A). Therefore, the MDA-MB-231 cell line was selected to perform the loss of function assay, while the MCF-7 cell line was selected for the gain of function assay. As depicted in Fig. 2B, stable transfection of SOX5 lentivirus was obtained following G418 selection and confirmed using RT-qPCR and western blotting analysis.
blotting. An MTT assay was performed, which indicated that cells overexpressing SOX5 proliferated significantly faster, compared with vector control cells (Fig. 2C). The colony formation assay demonstrated that SOX5 formed larger and an increased number of colonies, compared with the vector control group (Fig. 2D).

**SOX5 enhances breast cancer cell invasion in vitro.** To further investigate the role of SOX5 in breast cancer cell invasion, SOX5 expression was silenced in MDA-MB-231 cells using two different siRNAs. Successful depletion of SOX5 expression was confirmed at the mRNA (Fig. 3A) and protein levels (Fig. 3B). As expected, inhibition of SOX5 significantly impeded the MDA-MB-231 cell invasion ability compared with the negative control group (Fig. 3C). Subsequently, a scratch assay was performed to assess the role of SOX5 in the migration of breast cancer cells. As depicted in Fig. 3D, the scratch assay revealed that SOX5 knock down significantly reduced the migratory ability of MDA-MB-231 cells. Therefore, these data indicated that SOX5 exhibited the ability to promote MDA-MB-231 cell invasion and migration in vitro.

**Identification of EZH2 as a downstream target gene of SOX5.** Subsequently, the potential downstream molecule regulated by SOX5 was identified. A ChIP sequence (ChIP-seq) assay was performed. The ChIP-seq peak distribution is depicted in Fig. 4A, and 17.5% promoters were identified to be targeted by SOX5. To further validate the ChIP-seq results, a qChIP assay and the binding between SOX5 and the EZH2 promoter was demonstrated to be the most significantly enriched among the 10 genes selected (Fig. 4B). To investigate the SOX5-regulated EZH2 promoter activity, a luciferase report assay was performed. The EZH2 promoter reporter or EZH2 binding site mutant promoter reporter was transiently transfected into MCF-7 cells with pcDNA3.1-SOX5 or a vector. As depicted in Fig. 4C, SOX5 significantly activated EZH2 wild type promoter activity, but not the EZH2 mutant reporter activity. No significant changes in EZH2 promoter activity...
were observed in the vector control group. However, in MDA-MB-231 cells transfected with the EZH2 promoter reporter or EZH2 binding site mutant promoter reporter with si-SOX5 or si-control, it was observed that si-SOX5 significantly repressed EZH2 promoter activity, but not the mutant promoter activity (Fig. 4D).

**SOX5 induces breast cancer cell proliferation and invasion by modulating EZH2.** EZH2 is the catalytic subunit of polycomb repressive complex 2, and EZH2 had been demonstrated to serve a role in breast tumor initiation and progression (16-18). Therefore, we hypothesized that the modulation of EZH2 was involved with SOX5, increasing breast cancer cell proliferation and invasion. As depicted in Fig. 5A, the mRNA expression of EZH2 was significantly upregulated in SOX5-transfected MCF-7 cells, compared with vector-transfected cells, as demonstrated by RT-qPCR. This was further confirmed through western blotting. While in MDA-MB-231 cells, the knockdown of SOX5 resulted in significantly decreased EZH2 mRNA expression and markedly reduced EZH2 protein levels (Fig. 5B). These results indicated that SOX5 transactivated EZH2 expression. Notably, the knockdown of EZH2 was able
to overcome the SOX5 promoter effect on the proliferation of MCF-7 cells (Fig. 5C). Additionally, the invasion rate of MDA-MB-231 cells was significantly increased following treatment with EZH2, compared with the SOX5 knockdown group (Fig. 5D). These results indicated that SOX5 may regulate breast cancer cell proliferation and invasion through targeting EZH2 expression.

**Discussion**

Previously, a number of members of the SOX family, including SOX2 and SOX4, have been reported to be involved in tumor progression. SOX2 is a well-established stem cell regulator that is highly expressed in multiple tissue stem cells and sustain the infiltrative behavior in ≥25 different cancer types, including cancers of the ovary, lung, skin, brain, breast, prostate and pancreas (19-21). Increased expression of SOX4 serves as an important role in human tumor development such as through regulating cell growth, invasion, EMT and apoptosis (22-25). However, the research regarding SOX5 remains limited.

To the best of our knowledge, the present study is the first to demonstrate that SOX5 directly regulated EZH2 expression by transactivation, and thus promotes the proliferation and invasion of human breast cancer cells. Using ChIP-seq, qChIP and luciferase reporter assays, EZH2 was identified as a downstream target gene of SOX5. Using RT-qPCR and
western blotting analysis, it was demonstrated that SOX5 regulates the expression of EZH2. The present data added to accumulating evidence regarding SOX family members being involved in breast cancer progression. As reported by Pei et al (14), SOX5 was overexpressed in highly invasive breast cancer cell lines, including MDA-MB-435 and MDA-MB-231 cells, and suppression of SOX5 expression inhibited the proliferation and migration of MDA-MB-231 cells. These data were consistent with the present study. In the present study, SOX5 was demonstrated to be frequently upregulated in breast cancer tissues compared with healthy breast tissue, and associated with a reduced overall survival rate, indicating that SOX5 may serve as a poor prognostic biomarker in breast cancer. Additionally, the function of SOX5 was investigated in different cell lines, including MCF-7, the promotion of breast cancer cells proliferation and invasion indicated that SOX5 may be a potential oncogene. Notably, as reported by Tiwari et al (26), SOX4 directly regulated the expression of EZH2, and thus serves an indispensable role in EMT and cell survival in breast cancer (26). In patients with pancreatic cancer, the SOX4-EZH2 axis was demonstrated to be associated with the clinical outcome (27). Thus, we hypothesized that SOX4 and SOX5 may have a coordinated function on the EZH2 promoter to transactivate its expression. In the future, studies regarding the mechanistic association between SOX5 and EZH2 may be used for development of potential specifically-targeted therapies, and may benefit patients with breast cancer metastasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CS conceived and designed the study. YB provided technical assistance and performed the Transwell assay. YS and ZZ analyzed the data, KW performed the cell culture and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Weifang People's Hospital. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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