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Sox5 induces epithelial to mesenchymal transition by transactivation of Twist1

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ABSTRACT

The epithelial to mesenchymal transition (EMT), a highly conserved cellular program, plays an important role in normal embryogenesis and cancer metastasis. Twist1, a master regulator of embryonic morphogenesis, is overexpressed in breast cancer and contributes to metastasis by promoting EMT. In exploring the mechanism underlying the increased Twist1 in breast cancer cells, we found that the transcription factor SRY (sex-determining region Y)-box 5(Sox5) is up-regulation in breast cancer cells and depletion of Sox5 inhibits breast cancer cell proliferation, migration, and invasion. Furthermore, depletion of Sox5 in breast cancer cells caused a dramatic decrease in Twist1 and chromosome immunoprecipitation assay showed that Sox5 can bind directly to the Twist1 promoter, suggesting that Sox5 transactivates Twist1 expression. We further demonstrated that knockdown of Sox5 up-regulated epithelial phenotype cell biomarker (E-cadherin) and down-regulated mesenchymal phenotype cell biomarkers (N-cadherin, Vimentin, and Fibronectin 1), resulting in suppression of EMT. Our study suggests that Sox5 transactivates Twist1 expression and plays an important role in the regulation of breast cancer progression.

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1. Introduction

Breast cancer is one of most common malignant tumors with 1st incidence and 2nd mortality among all malignant tumors in females [1]. Death from breast cancer primarily results from cancer cells invading surrounding tissues and metastasizing to distal organs followed by formation of secondary tumors [2]. The epithelial to mesenchymal transition (EMT), a developmental process in which epithelial cells lose polarity and develop a mesenchymal phenotype, has been implicated in the initiation of metastasis [3].

It is believed that EMTs endow cancer cells with migratory and invasive properties, and induce cancer stem cell (CSC) properties [3,4]. EMT is characterized by loss of intercellular adhesion (E-cadherin and occludins); down-regulation of epithelial makers (cytokeratins); up-regulation of mesenchymal markers [Vimentin and smooth muscle actin (SMA)]; acquisition of fibroblast-like (spindle) morphology with cytoskeleton reorganization; and increase in motility, invasiveness, and metastasic capabilities [5–9]. In addition, the process known as "cadherin switching" (down-regulation of E-cadherin and up-regulation of mesenchymal cadherins such as N-cadherin [10]) and the accumulation of β-catenin have also been associated with EMT [7,9]. The complex genetic changes necessary to accomplish the phenotypic changes associated with EMT are, at least in part, mediated by a number of specific transcription factors, such as Snail, Slug, Twist1, ZEB1 and ZEB2 [11,12].

Twist1, a member of basic helix-loop-helix (bHLH) transcription factors, plays a key role in the regulation of embryogenesis, gastrulation and mesoderm formation during early embryonic development [13]. Twist1 is overexpressed in various human solid tumors including numerous types of carcinomas as well as sarcomas, gliomas, neuroblastomas, and melanomas [14–17]. Further, increased Twist in cancer cells has been shown to promote metastatic ability *in vivo* and cell survival, angiogenesis, and chemoresistance *in vitro* [18]. In addition, exogenous overexpression of Twist1 increases the invasive and metastatic abilities of cancer cells through promoting the down-regulation of E-cadherin and the induction of EMT [19,20]. However, the molecular mechanism for the up-regulation of Twist in cancer cells is less clear.

Sox5 (sex determining region Y-box protein 5) is a transcription factor that plays a key role in the regulation of embryonic development and in the determination of the cell fate [21]. SOX-5 was found to be associated with human glioma, seminoma, and nasopharyngeal [22–24]. Furthermore, Sox5 enhances nasopharyngeal carcinoma progression by regulation of SPARC expression [24].

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To our knowledge, Sox5 expression has not been characterized in human breast cancer. Here, we aimed to further define the roles of Sox5 in regulating the progression of breast cancer cells. We found that Sox5 increased the MDA-MB-231 cells proliferation and invasion through induction of EMT. Furthermore, this biological effect is dependent on the regulation of Twist1 expression by Sox5. Our results have shed important insights into the biological significance of Sox5 in breast cancer.

2. Materials and methods

2.1. Cell culture

MDA-MB-435, MDA-MB-231, T47D, MCF7, and MCF10A cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-435, T47D, and MCF7 were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA), and MDA-MB-231 was cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen). MCF10A was cultured in DMEM/F12 supplemented with 5% horse serum. The cells were cultured under an atmosphere of 5% CO_2 at 37 °C.

2.2. Plasmids and transfection

To construct the Sox5 expression vector, the entire coding sequence of the Sox5 was amplified by PCR using the Pfu DNA Polymerase (Thermo Scientific, Rockford, IL, USA). The PCR product was cloned into the pcDNA3.1 (Invitrogen). The construct was confirmed by sequencing. shRNAs targeting Sox5 were purchased from RiboBio (Shanghai, China). For transient transfection, cells were kept in antibiotic-free medium for 24 h before transfection and were then transfected with the shRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For generation of stable Sox5-depleted cells, cells were transfected with Sox5 shRNA. Two days after transfection, cells were trypsinized, transferred to 10 cm cell culture dishes and selected by complete medium plus 1 mg/ml of G418 (Sigma–Aldrich, St. Louis, MO, USA) for about 2 weeks.

2.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted with TRIZOL reagent according to the manufacturer's instructions and then was converted to first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen). The RT-qPCR analysis was performed using the Fast SYBR Green Master Mix System (Invitrogen) according to the manufacturer's instructions. The targeted gene relative quantification was given by the CT values, and the CT value of Actin was subtracted to obtain ΔCT. The relative mRNA expression level of targeted genes was determined as $2^{-\Delta CT}$. The experiment was performed in triplicate.

2.4. Immunoblot

Cells were harvested and total protein was extracted. Equal amounts of protein were boiled in sample buffer, separated by electrophoresis on 10% SDS–PAGE gel, and transferred to PVDF membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% skim milk powder in TBS-T (TBS plus 0.5% Tween-20) at room temperature and then incubated overnight at 4 °C with primary antibody. Primary antibodies included: Sox5 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Snail1 (1:1000, Cell Signaling Technology, Beverly, MA, USA), Slug (1:1000, Cell Signaling Technology), Twist1 (1:1000, Cell Signaling Technology), ZEB1 (1:1000, Cell Signaling Technology), Membranes were treated with goat anti-mouse or -rabbit HRP-conjugated secondary antibodies (1:2000, Santa Cruz Biotechnology). The membrane was visualized using an enhanced chemiluminescence (ECL) detection system (Millipore).

2.5. Colony formation and MTT assays

Both MTT and colony formation assays were used to observe and compare cell proliferation ability. For colony formation, 1×10^3 cells were seeded into 6 cm dishes and fresh culture medium was replaced every 3 days. After 15 days of culture, the cells were stained with crystal violet, and the numbers of colonies containing more than 50 cells were counted.

For MTT assay, 2×10^3 cells in 100 μl culture medium were plated into a well of 96-well plates. After culturing cells for an appropriate time, 10 μl of 5 mg/ml MTT was added into each well and continued to culture for 4 h. Then, the cell culture medium was replaced by 100 μl of dimethyl sulfoxide. Thirty minutes after dimethyl sulfoxide addition, the plates were placed on a microplate autoreader (Bio-Rad, Hercules, CA, USA). Optical density was read at 570 nm wavelength and cell growth curves were determined according to the optical density value.

2.6. Migration and invasion assays

Scratch assay was used to observe the migration ability. In briefly, cells were plated in 6-well plates and formed a fluent monolayer. Cell layer was scratched with a 200-μl pipette tip and detached cells were removed. For each sample, at least three scratched fields were photographed immediately and at the time points indicated after the scratch were made.

For the transwell invasion assay, cells in 0.2 ml medium without FBS were placed on the top chamber of each insert (8 μm pore size, BD Biosciences, San Jose, CA, USA) with 40 μl of 1 mg/ml Matrigel. The lower chamber was filled with 600 μl of medium with 10% FBS to act as the nutritional attractant. 24 h later, the migrant cells that had attached to the lower, surface were fixed with 20% methanol and stained for 20 min with crystal violet. The membranes were then carved and embedded under cover slips with the cells on the top. Cells in three different fields of view were counted and expressed as the average number of cells per field of view.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was carried out using kit from Upstate Biotechnology according to manufacturer's protocol. The putative binding site of Twist1 was amplified with the following primers: 5′-GAATGGTTT GGGAGGACGA-3′ and 5′-GTGTCATTGGCCTGACGTG-3′. The PCR products were resolved electrophoretically on a 2% agarose gel.

2.8. Luciferase assay

The Twist1 promoter (−500 to +1) was amplified by from genomic DNA of MDA-MB-231 cells and the fragment was cloned into the luciferase reporter plasmids pGL3-basic vector (Promega, Madison, WI, USA). 5×10^4 cells per well in 24-well plates and then the report plasmid were transfected into the cells with or without pcDNA3.1-Sox5. After 24 h, cells were washed with phosphate-buffered saline (PBS), subjected to lysis, and their luciferase activities measured by using a dual luciferase assay kit (Promega). The results were normalized against *Renella* luciferase. All transfections were performed in triplicate.

2.9. Statistical analysis

Results of experiments were depicted as mean ± SD and Student's *t*-test (two-tailed) was used to compare values of test and control samples. All calculations were performed with the SPSS for Windows statistical software package (SPSS, Chicago, IL, USA). The level of significance was set to $P < 0.05$.

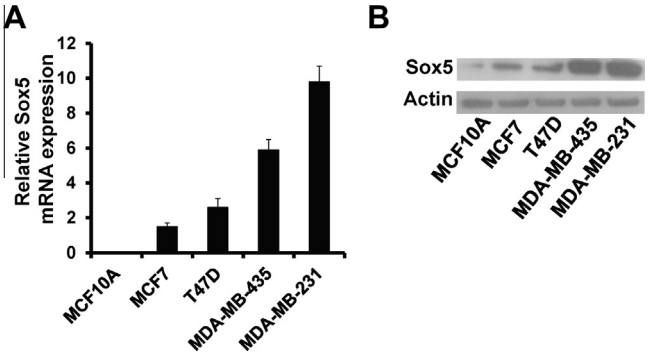

Fig. 1. Sox5 expression in different breast cancer cell lines. (A) Sox5 mRNA levels examined by RT-qPCR. (B) Sox5 protein levels examined by immunoblot.

3. Results

3.1. Sox5 is overexpressed in breast cancer cell lines

To examine the Sox5 expression in breast cancer, we firstly detected the Sox5 mRNA and protein expression in normal breast cell line (MCF10A) and breast cancer cell lines (MCF7, T47D, MDA-MB-435, and MDA-MB-231). According to the results of RT-qPCR, the Sox5 mRNA was significantly up-regulation in the bone metastatic breast cancer cell line, MDA-MB-231 compared to the normal breast cancer cell line, MCF-10A, and the non-bone metastatic breast cancer cell lines, MCF-7, T47D, and MDA-MB-435 (Fig. 1A). The level of Sox5 protein expression was also up-regulation in MDA-MB-231 by immunoblot (Fig. 1B).

3.2. Depletion of Sox5 inhibits breast cancer cell proliferation and invasion

To examine the role of Sox5 in breast cancer cells, we ablated Sox5 expression in breast cancer cell line MDA-MB-231, using mammalian vector-based RNA interference. After G418 selection, stable transfectants were obtained. We selected two Sox5-depleted MDA-MB-231 clones as well as their control RNAi clones for further analyses. RT-qPCR and immunoblot analyses indicated that Sox5 expression was markedly decreased in Sox5-depleted cells

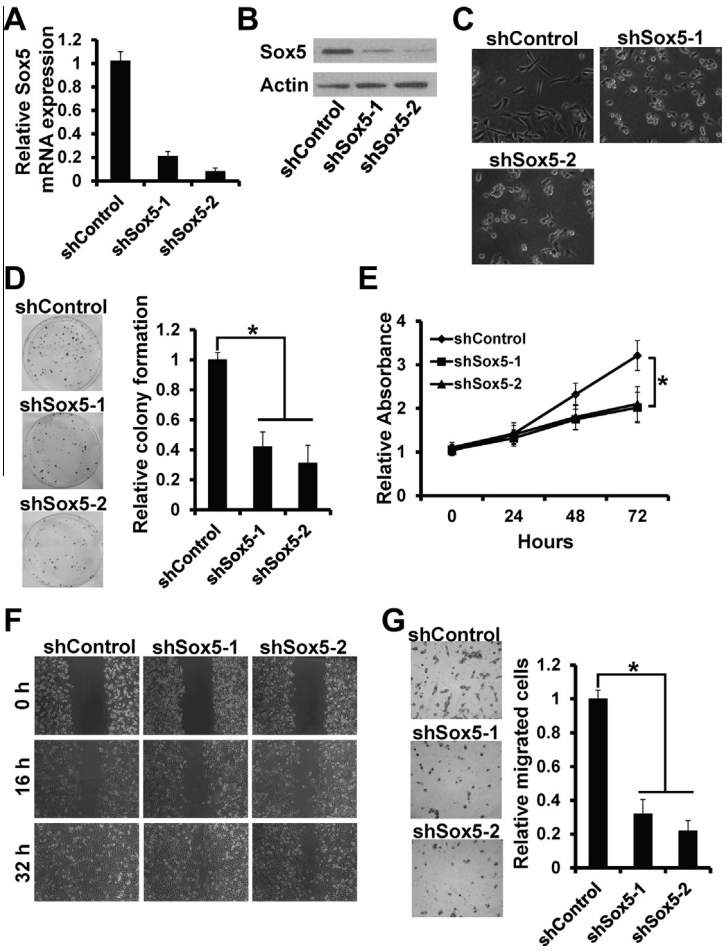

Fig. 2. Suppression of Sox5 inhibits breast cancer cell proliferation, migration, and invasion. (A) RT-qPCR analysis of Sox5 expression in MDA-MB-231 cells stable expressing Sox5 siRNA (shSox5-1 and shSox5-2), as well as shControl cells. (B) Immunoblot analysis of Sox5 expression in shSox5-1 and shSox5-2, as well as shControl. (C) Morphology of shSox5-1 and shSox5-2, as well as shControl cells. (D) Colony formation analysis of shSox5-1 and shSox5-2, as well as shControl cells. (E) MTT analysis of shSox5-1 and shSox5-2, as well as shControl cells. (F) Scratch analysis of shSox5-1 and shSox5-2, as well as shControl cells. (G) Transwell analysis of shSox5-1 and shSox5-2, as well as shControl cells. *$P < 0.01$.

compared with shControl cells (Fig. 2A and B). Compared with shControl cells, depletion of Sox5 made cells underwent a morphological change, which was less spindle-like and fibroblastic, and the pseudopodium was shorter than that of control cells (Fig. 2C). Colony formation assay showed that shSox5-1 and shSox5-2 cells formed smaller and fewer colonies than shControl cells (Fig. 2D). MTT assay also revealed that shSox5-1 and shSox5-2 cells grew much slower than shControl cells (Fig. 2E). Next, we performed scratch assay and transwell assay to examine the role of Sox5 in the migration and invasion of breast cancer cells. The scratch assay indicated that shSox5 cells were defective in migration (Fig. 2F). Furthermore, shSox5 cells displayed much lower migrated cell rates than shControl cells (Fig. 2G). Taken together, these results indicate that depletion of Sox5 inhibits breast cancer cell proliferation, and invasion.

3.3. Sox5 regulates genes associated with EMT

Increased motility and invasiveness shown by tumor cells are reminiscent of the events that occur during epithelial mesenchymal transition (EMT) [25]. To investigate the effects of Sox5 on the EMT process of MDA-MB-231 cells, the relative mRNA and protein expression of mesenchymal phenotype cell biomarkers (N-cadherin, Vimentin, and Fibronectin 1), and epithelial phenotype cell biomarker (E-cadherin) in shSox5 cells and corresponding control cells were measured by RT-qPCR and immunoblot. As shown in Fig. 3A and B, The epithelial-specific marker E-cadherin, the loss of which is a key step during EMT [9,26], was significantly decreased. Meanwhile, N-cadherin, Vimentin, and Fibronectin 1 expression levels were increased (Fig. 3A and B). To understand the mechanism by which Sox5 regulates the EMT, we examined if Sox2 modulates the expression of EMT-related transcription factors known to play key roles in regulating EMT in various types of cancers, including Snail1, Slug, Twist1, and ZEB1 [27,28]. We observed that depletion of Sox5 did not result in significant changes in the expression levels of Snail1 and Slug (Fig. 3C and D). However, depletion of Sox5 led to a significant decrease of Twist1 and ZEB1 expression (Fig. 3C and D). These results suggest that Sox5 induces EMT by regulation of Twist1 or/and ZEB1 expression.

3.4. Sox5 transactivates Twist1 expression

We next identified whether Twist1 is the potential downstream molecule regulated by Sox5. To do so, we performed blast analysis and found one conserved putative Sox5-binding site (positions −133 to −138) in the Twist1 promoter (Fig. 4A). Subsequently, the binding between Sox5 and Twist1 promoter was verified by using chromatin immunoprecipitation assay (ChIP) (Fig. 4B). To investigate whether Sox5 regulates Twist1 promoter activity, we transiently transfected Twist1 promoter reporter into MDA-MB-231 cells with pcDNA3.1-Sox5 or control. We observed that Sox5 significantly activate the Twist1 promoter activity (Fig. 4C). Furthermore, the Twist1 expression is up-regulation in Sox5-transfected cells compared with control cells by RT-qPCR and immunoblot (Fig. 4D and E). These results indicate that Sox5 transactivates Twist1 expression.

4. Discussion

Sox5, a member of the Sox family of transcription factors, is involved in the regulation of embryonic development and in the determination of the cell fate [29]. The aberrant expression of Sox transcription factors have been reported to participate in carcinogenesis and progression, such as Sox2 [30] and Sox4 [31]. Sox5 has been reported to correlate indirectly with the invasive tumor growth in testicular seminomas [23]. Furthermore, Sox5 was identified as a tumor antigen of glioma [22]. These findings, together with our observation that Sox5 is overexpressed in high invasive breast cancer cell lines MDA-MB-435 and MDA-MB-231, all suggest that Sox5 may be a potential oncogene. Functional

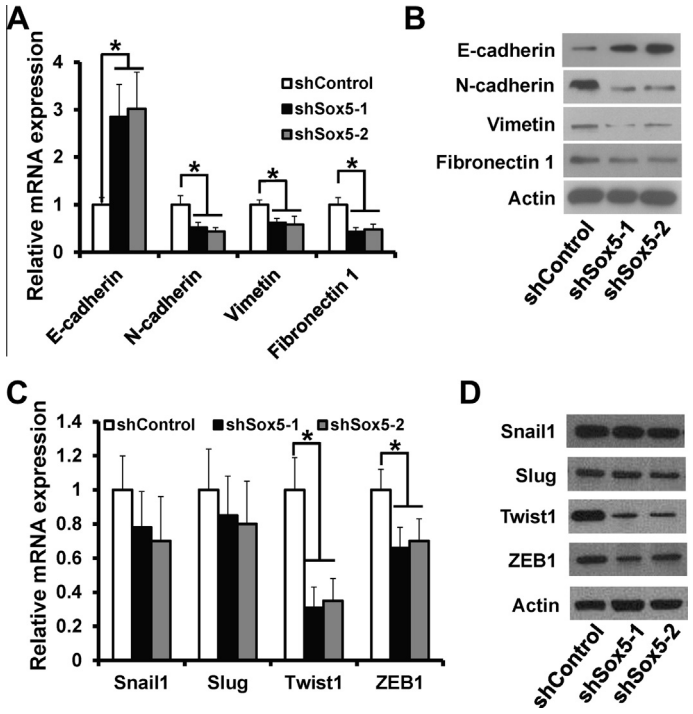

Fig. 3. Depletion of Sox5 inhibits EMT phenotype in breast cancer cells. (A) RT-qPCR analysis of epithelial and mesenchymal markers. (B) Immunoblot analysis of epithelial and mesenchymal markers. (C) RT-qPCR analysis of EMT-related transcription factors. (D) Immunoblot analysis of EMT-related transcription factors. *$P < 0.01$.

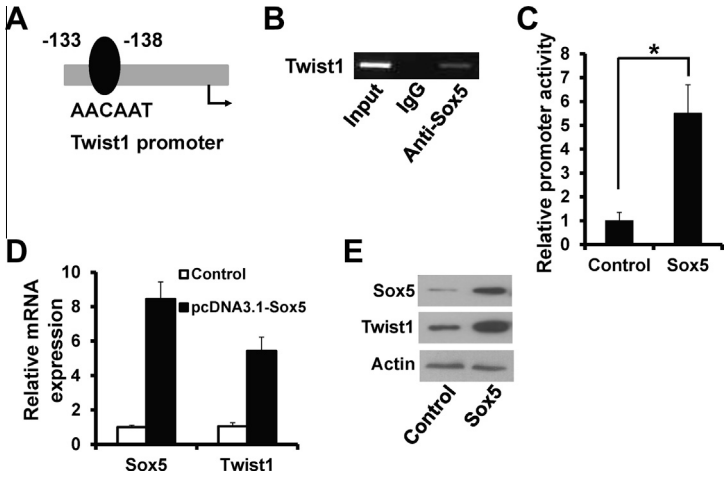

Fig. 4. Sox5 transactivate Twist1 expression. (A) Schematic representation of Sox5 binding site in *Twist1* promoter. (B) ChIP analysis of the human *Twist1* promoter by anti-Sox5 antibody in MDA-MB-231 cells. (C) Luciferase activity of Twist1 promoter in MDA-MB-231 cells. (D and E) RT-qPCR (D) and immunoblot (E) analyses of Twist1 expression in Sox5-transfected and control cells. $^{*}P < 0.01$.

analyses indicated that suppression of Sox5 expression by RNA interference in breast cancer cells inhibits cell proliferation, migration, and invasion *in vitro*.

EMT is accompanied by massive alteration in cell morphology and behavior, and transcription factors play an important role in controlling the cellular functions during EMT, such as cell proliferation, differentiation, adhesion, and migration [32,33]. We report here that Sox5 knockdown inhibit EMT programs of human breast cancer cells, including down-regulation of mesenchymal markers and EMT related transcription factors, up-regulation of epithelial markers, and a decrease in cell migration and invasion *in vitro*. However, its transcriptional targeted genes have not been recognized.

The bHLH transcription factor Twist1, which is essential in the developmental processes, such as gastrulation, has been shown to be oncogenic in various cancers [34–37]. Moreover, Twist1 has been reported to be one of the master regulators of invasiveness and EMT, and dysregulation of Twist1 expression and function has been implicated to be associated with cancer progression [38,39]. In this study, we identify that Twist1 is a novel transcriptional target gene of Sox5 and overexpression of Sox5 transactivates Twist1 expression. Thus, we concluded that Sox5 induces EMT by transactivation of Twist1 expression.

Conflict of interest

The authors declare that they have no competing interests.

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