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SOX2 promotes tumor metastasis by stimulating epithelial-to-mesenchymal transition via regulation of WNT/β-catenin signal network

Xuefei Li [a], Yingxi Xu [a], Yanan Chen [a], Si Chen [a,b], Xianpei Jia [a], Tongchao Sun [a], Yanhua Liu [a], Xiru Li [c], Rong Xiang [a,d,*], Na Li [a,d,*]

[a] School of Medicine, Nankai University, 94 Weijin Road, Tianjin 300071, China
[b] Division of Biochemical Toxicology, National Center for Toxicological Research, FDA, 3900 NCTR Road, Jefferson, AR 72079, USA
[c] Department of General Surgery, Chinese PLA General Hospital, Beijing 100853, China
[d] Tianjin Key Laboratory of Tumor Microenvironment and Neurovascular Regulation, Tianjin 300071, China

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ABSTRACT

SOX2 was reported to promote metastasis in various tumor tissues; however the underlying mechanisms remain elusive. Here, we disclosed that SOX2 improves metastasis of breast and prostate cancer cells by promoting epithelial-to-mesenchymal transition (EMT) through WNT/β-catenin, but not TGF-β or Snail1 signaling. Dual luciferase assay and chromatin immunoprecipitation revealed activation and binding of SOX2 on promoter region of *β-catenin*. In addition, SOX2 affects the protein expression levels of DKK3, DVL1 and DVL3, which are regulators or downstream molecules of WNT signaling. Taken together, our findings demonstrated β-catenin as one of vital downstream molecules that mediate the EMT induced by SOX2.

1. Introduction

Tumor metastasis is the key factor that compromises the prognosis of tumor patients and accounts for 90% of tumor death [1,2]. Metastasis is a multistep process by which a percentage of primary tumor cells acquire the ability to spread from their initial site to the surrounding normal tissues in the local areas or to secondary tissues/organs [3,4]. It is comprised of multiple steps, which include penetrating the walls of lymphatic and/or blood vessels, infiltration into the circulation system, re-penetration through the vessels, docking and proliferation in the distant organs to form a metastatic tumor. Failure at any one of these steps can block the entire metastatic process. Since tumor metastasis is responsible for the majority of deaths for cancer patients, a better understanding of the molecular mechanism involved in tumor spreading process is important for specific targeting of the tumor metastasis.

Recent studies demonstrated that breast cancer cells with stem/progenitor cell properties exhibit enhanced invasive properties [5,6], supporting the concept that cancer stem cells (CSCs) may play an important role in tumor metastasis. SOX2 is one of the key transcriptional factors that control the unique properties of stem cells, for example self-renewal and pluripotency [7–10]. Recently, additional studies reported the contribution of SOX2 to tumorigenesis and its correlation with clinical progression of various types of tumors, including human breast cancer, rectal cancer and prostate cancer [11–13]. Although these reports revealed the contribution of SOX2 to the metastasis properties of tumor cells, the underlying mechanisms involved in this phenomenon still needed to be explored.

EMT is an important development process in which immotile epithelial cells lose their polarity, tight cell–cell contacts and acquire mesenchymal characters [14,15]. EMT always happens when tumor cells penetrate the lymphatic and/or blood vessels. During this process, tumor cells acquire a migratory behavior and gain more invasive properties to facilitate their departure from the epithelial cell community and to integrate into surrounding tissues, or remote organs. Therefore, EMT plays an important role in tumor migration. The process of EMT can be marked by the decrease in expression levels of epithelial junction proteins, such as E-cadherin, Claudins and Occludin [16–18], as well as increased expression of mesenchymal proteins, such as α-smooth actin, vimentin and fibronectin [19]. It is also embodied in transform of zonula

* Corresponding authors. Tel.: +86 22 23509779; fax: +86 22 23502554 (N. Li), tel.: +86 22 23509505; fax: +86 22 23502554 (R. Xiang).
E-mail addresses: rxiang@nankai.edu.cn (R. Xiang), lina08@nankai.edu.cn (N. Li).

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occludens-1 (ZO-1), which functions as an essential tight junctional protein that directly links tight junction and cytoskeleton [20], from cell–cell contact region to intracellular distribution [21–23]. EMT can be induced by several signal pathways, among them, transforming growth factor (TGF-β) was extensively investigated and demonstrated to regulate EMT through activation of three families of transcriptional factors, including the snail, zinc finger E-box-binding homeobox (ZEB) and basic helix-loop-helix (bHLH) families to control the expression level of epithelial or mesenchymal marker genes [15].

Another important signaling pathway is Wingless (WNT)/β-catenin [24]. Upon activation, the WNT signals will be transduced to cytoplasmic proteins such as Dishevelled (Dvl) et al. One important consequence was the inhibited activity of glycogen synthase kinase-3β (GSK-3β), which phosphorylates and induces the degradation of β-catenin in the absence of WNT signals. Therefore, activation of WNT signals will stabilize and finally lead to accumulation of β-catenin [25]. It was reported that the cytoplasmic domain of E-cadherin interacts with the catenin complex (α-catenin, β-catenin and γ-catenin/plakoglobin) to form a cadherin–catenin complex [26], which binds to the cytoskeleton and contributes critically to the formation of strong cell–cell adhesion. Upon activation, β-catenin dissociates with E-cadherin, dissembling the adherens and enters the nucleus to turn on the expression of target genes, most of which show stemness-promotion functions [27].

Here we investigated the function of SOX2 in tumor metastasis and found the expression of SOX2 to be closely correlated with the TNM (tumor, lymph node and metastasis) stage and histological grade, as well as dominant overexpression in lymph nodes with tumor metastasis. Overexpression of SOX2 in MCF-7 and DU145 cells stimulated EMT and also enhanced their EMT process under stimulation of TGF-β1. Exploration of the underlying mechanism demonstrated that SOX2 may regulate the EMT through WNT/β-catenin signal pathway. Our research revealed that targeting of SOX2 may be a promising strategy for prevention of tumor metastasis.

2. Materials and methods

2.1. Immunohistochemistry (IHC)

Immunostaining was performed on three pieces of paraffin human breast tissue array (BR1002, BR1005, BR2086, Alenabio Company, Shanxi, China) and the lung and tumor tissues of NOD/SCID mice. Expressions of SOX2, β-catenin and WNT1 in these tissues were detected separately with monoclonal antibody against SOX2 (ab75485, Abcam Inc., Cambridge, UK), polyclonal anti-β-catenin (ab47426, Abcam Inc., Cambridge, UK) and polyclonal anti-WNT1 antibody (ab85060, Abcam Inc., Cambridge, UK) at a 1:100 dilution. The following steps were performed by using an immunohistochemistry kit (Mouse or Rabbit ImmunoCruz™ Staining System, Santa Cruz Biotechnology) according to the manuscript instruction. The expression level of SOX2 in tissue microarray was presented as the percentage of SOX2-positive cells in each breast or lymph node tissue.

2.2. Establishment of cell lines

Breast cancer MCF7 and MDA-MB-231(MDA231) and prostate cancer (DU145) cell lines were obtained from American Type Culture Collection (ATCC) and cultured according to the instructions provided. Establishment of DU145 cells with SOX2 overexpression (DU145-SOX2) was described previously [13]. Parental MDA231 and MCF7 cells were infected with lentivirus carrying pLv-EF1α-SOX2-IRES-Bsd (pLV-SOX2) or the empty vector, then selected by 10 μg/ml Blasticidin (Bsd) to generate stable cell lines with SOX2 overexpression (MDA231-SOX2, MCF7-SOX2) and their control cells (MDA231-Con, MCF7-Con) separately. MCF7-shRNA-SOX2 (MCF7-sh-SOX2) and its control cell MCF7-shRNA-scamble (MCF7-SS) were established by infecting MCF7 cells with lentivirus carrying pLv-H1-shRNA-SOX2-puro and pLv-H1-shRNA-scramble-puro separately [28]. Stable cell line of MCF7-SOX2 with β-catenin downregulation (MCF7-SOX2/sh-β-catenin) and its control (MCF7-SOX2/SS) were established by infecting MCF7-SOX2 cells with pLv-H1-shRNA-β-catenin-puro and pLv-H1-shRNA-scramble-puro plasmid (Biosettia, SanDiego, CA) separately, followed by clonal selection using 3 μg/ml puromycin. The sequences of shRNA1-SOX2 and shRNA-Scramble were described before [28], other shRNAs were designed and chemically synthesized as: shRNA-β-catenin: AAAAGCTTACTG GCCATCTTTAATTGGATCCAATTAAAGATGGCCAGTAAGC; shRNA2-SOX2: AAAAGCTC ATGAAGAAGGATAAGTTGGATCCAACTTATCCTTCTTCATGAGC.

2.3. Western blotting

Cells were lysed as described before [13] in the presence of Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 and 3 (P8340, P5726 and P0044, Sigma-Aldrich, USA). Protein lysates were boiled in the sample-loading buffer and resolved in 4–15% polyacrylamide gels and transferred to nitrocellulose membranes. Antibodies used included: monoclonal anti-α-smooth muscle actin (α-SMA, A2547, Sigma–Aldrich, USA), E-cadherin (610182, BD Transduction Laboratories, USA), β-catenin and Snail (9582 and 3879, Cell Signal Technology Inc., Danvers, MA), anti-β-actin, Smad2, Smad4, polyclonal anti-SOX2, p-Smad2/3 and monoclonal anti-DVL1 (sc-47778, sc-101153, sc-7966, sc-20088, sc-11769 and sc-8025, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti- DVL3 and DKK3 (13444-1-AP, 10365-1-AP, Proteintech, Chicago, IL). The reaction was detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.4. Immunofluorescent and confocal image

Cells were grown on glass coverslip until 60–80% confluent, and then fixed and permeabilized as described before [13]. DU145 and MCF7 cells were each incubated with polyclonal anti-ZO-1(sc-10804, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-E-cadherin (610182, BD Transduction Laboratories, USA) and polyclonal anti-β-catenin (ab47426, Abcam Inc., Cambridge, UK) antibody, which were diluted 1:100 in 5% goat serum overnight at 4 °C. For detection, the cells with incubated with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 546 goat anti-mouse IgG (1:200 diluted in 5% goat serum, Molecular Probes, Inc. Invitrogen, Eugene, OR) at room temperature for 1 hour (h), followed by incubation with DAPI (1:1000 diluted in PBS) for 5 minutes (min) and then subjected to a Lecia laser scanning microscope a 40× objective [13]. The fluorescent intensity of each protein was analyzed by the Image J software.

2.5. Transwell migration assays

Transwell chambers (polycarbonate filters of 8 μm porosity, Milipore) were used in this test. The bottom chamber was filled with culture medium containing 10% FBS. 1×10^5 cells were suspended in serum-free medium and plated in the upper chamber. After incubation for 24 h, the cells were removed from the upper chamber by a cotton swab. Cells penetrated and attached to the bottom of the filter were fixed with 4% formaldehyde in PBS, followed by 20 min staining of 0.5% crystal violet and then subjected to imaging under a 20× objective. For quantification purposes, 50% acetic acid was added to each well to dissolve crystal violet. The stain intensities were measured as absorbance under 560 nm. The number of cells that migrated the filter, dropped and finally attached to the bottom dishes were recorded under a 20× objective. Statistical results of cell numbers per each image field were obtained from three independent experiments averaged from five image fields.

2.6. Wound-healing assay

For each test, 2×10^5 cells were seeded on a 24-well plate. When they grew to full confluence, a 'wound' was made in the middle of a culture plate with a 10 μl pipette tip and the concentration of the serum in culture medium of tumor cells was changed from 10% to low percentage (2% FBS for MDA231 and MCF7, 1% FBS for DU145). The wound-healing process was recorded at 0 h, 24 h or 48 h after the scratch under a 10× objective. The wound healing rate was quantified as the distance of wound recovered versus that of the original wound.

2.7. Enzyme-linked immuno sorbent assay (ELISA)

2×10^5 MCF7 and DU145 cells were seeded separately in 24-well plates and the supernatant was harvested after 24 h of incubation. The ELISA test was performed according to the manufacture's instruction for measurement of human TGF β1 and β2 (DB100B, DB250, R&D Systems, USA).

2.8. Tumor xenografts

All animal experiments were performed strictly under the guidelines on laboratory animals of Nankai University and were approved by the Institute Research Ethics Committee at the Nankai University. Female NOD/SCID mice at 6–8 weeks of age were used for orthotopic implantation of MDA231 cells. They were separated randomly into two groups ($n = 6$ for each group). 3×10^6 MDA231-SOX2 or control cells were transplanted subcutaneously to the 4th mammary fat pads of each mouse. All mice were sacrificed at 10 weeks after xenograft and lung and tumor tissues were all fixed in 4% paraformaldehyde and subjected to IHC and hematoxylin and eosin (HE) staining.