SOX4 induces epithelial-mesenchymal transition and contributes to breast cancer progression

Jianchao Zhang¹, Qian Liang², Yang Lei¹, Min Yao³, Lili Li², Xiaoge Gao¹, Jingxin Feng², Yu Zhang¹, Hongwen Gao³, Dong-Xu Liu⁴, Jun Lu¹*, and Baiqu Huang²*

¹The Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China
²The Key Laboratory of Molecular Epigenetics of Ministry of Education (MOE), Northeast Normal University, Changchun 130024, China
³Department of Pathology, The Second Hospital of Jilin University, Changchun 130041, China
⁴The Liggins Institute, the University of Auckland, Auckland 1023, New Zealand

Jianchao Zhang and Qian Liang contributed equally to this work.

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Correspondence Authors:

Baiqu Huang, The Key Laboratory of Molecular Epigenetics of Ministry of Education (MOE), Northeast Normal University, Changchun 130024, China. Phone: +86-431-85099798; Fax: +86-431-85099768; E-mail: huangbq705@nenu.edu.cn

Jun Lu, The Institute of Genetics and Cytology, Northeast Normal University, 5268 Renmin Street, Changchun 130024, China. Phone: +86-431-85098729; Fax: +86-431-85099768; E-mail: luj809@nenu.edu.cn
Abstract

Epithelial-mesenchymal transition (EMT) is a developmental program, which is associated with breast cancer progression and metastasis. Here we report that ectopic overexpression of SOX4 in immortalized human mammary epithelial cells is sufficient for acquisition of mesenchymal traits, enhanced cell migration and invasion, along with epithelial stem cell properties that defined by the presence of a CD44\textsubscript{high}/CD24\textsubscript{low} cell subpopulation. SOX4 positively regulated expression of known EMT inducers, also activating the TGF-\beta pathway to contribute to EMT. SOX4 itself was induced by TGF-\beta in mammary epithelial cells and was required for TGF-\beta-induced EMT. Murine xenograft experiments showed that SOX4 cooperated with oncogenic Ras to promote tumorigenesis in vivo. Finally, in clinical specimens of human breast cancer, we found that SOX4 was abnormally overexpressed and correlated with the triple-negative breast cancer subtype (ER\textsuperscript{-}/PR\textsuperscript{-}/HER2\textsuperscript{-}). Our findings define an important function for SOX4 in the progression of breast cancer by orchestrating EMT, and they implicate this gene product as a marker of poor prognosis in this disease.

Introduction

Breast cancer is the most common cancer in women worldwide (1). Tumor metastasis is the leading cause of mortality associated with cancer
including breast cancer (2, 3). Epithelial-mesenchymal transition (EMT), an early embryonic development program in which cells convert from the epithelial to the mesenchymal state, plays a pivotal role during malignant tumor progression and metastasis. During an EMT, epithelial cells shed their characteristics such as loss of cell-cell contacts, apical-basal polarity and downregulation of epithelial markers, followed by acquisition of mesenchymal features including enhancement of motility and invasiveness, reorganization of cytoskeleton, elevated resistance to apoptosis and increased expression of ECM components like Fibronectin and MMPs (4, 5).

Several developmentally important transcription factors such as Snail, Twist, FOXC1, FOXC2 and LBX1 were reported to act as molecular triggers of the EMT in breast cancer and other cancers (4, 6). In addition to these transcription factors, an EMT program can also be initiated by several signal pathways, including TGF-β, Wnt, Notch and Hedgehog (7); among them, the TGF-β signaling plays a significant role in contributing to the initiation of EMT during embryonic development and cancer pathogenesis (8). Recent studies have demonstrated that EMT endows cancer cell with stem cell-like properties and relates to the highly aggressive basal like breast cancer subtype (9, 10).

SOX4, a member of the C subgroup of SOX (SRY-related HMG box) transcription factor family that are structurally characterized by a highly
conserved HMG box domain that directly binds the minor groove of DNA helix, has been implicated in various development processes, such as embryonic cardiac, central nervous system, lymphocyte development and differentiation, through its transcriptional activity (11-16). Besides functioning as a transcriptional factor in regulation of development processes, SOX4 has been implicated in cancer progression. Increased SOX4 expression is observed in many human tumors, including medulloblastoma, small cell lung cancer, prostate cancer, and breast cancer (17-21). Both TGF-β and Wnt signal pathways have been implicated in cancer progression and EMT (4, 5). TGF-β has been shown to induce SOX4 expression in glioma-initiating cells and glioblastoma multiforme (22, 23). SOX4 has been shown to activate the canonical Wnt signal pathway in cancer cells (24, 25). Therefore, we speculate that SOX4 might play a role in breast cancer progression and aggression through induction of an oncogenic EMT.

In the present study, we demonstrated that SOX4 was able to activate EMT program in epithelial cells, increase the number of CD44$^{\text{high}}$/CD24$^{\text{low}}$ population and potentiate mammosphere-forming ability. We also demonstrated that in mammary epithelial cells, SOX4 cooperated with the activated oncogenic Ras to endow the cells with the tumorigenicity in vivo. We further demonstrated that human TNBC has increased SOX4 expression. These data implicate a novel role of SOX4 in inducing EMT in
breast cancer progression and its close association with invasive subtypes of human breast cancer.

Materials and Methods

Cell culture

MCF10A, MDA-MB-231, BT549, NMuMG, MDCK and 293T cell lines were obtained from the ATCC (Manassas, VA), where they were characterized by DNA-Fingerprinting and isozyme detection. Cells were immediately expanded and frozen such that they could be revived every 3 to 4 months. MCF10A cells were cultured as previously described (26) in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 μg/ml Hydrocortisone, 100 ng/ml Cholera toxin, 10 μg/ml insulin and pen/strep. MDCK and 293T cells were cultured in DMEM containing 10% FBS (Hyclone). NMuMG cells were maintained in DMEM supplemented with 10% FBS and 10 μg/ml insulin. MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium with 10% FBS at 37°C without CO₂. BT549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 0.023 IU/ml insulin.

Antibodies and reagents

The antibodies and reagents are listed in Supplementary Materials and Methods.

Immunoblotting
Standard procedures for immunoblotting are described in Supplementary Materials and Methods.

**Immunofluorescence**

Experiments were performed as described in Supplementary Materials and Methods.

**Plasmids, viral production and infection of target cells**

A description of procedures are detailed in Supplementary Materials and Methods.

**Human breast tumor specimens and immunohistochemistry**

Human breast tumor specimens were obtained from the Second Hospital of Jilin University, China. Tissue samples were fixed in 10% formaldehyde and embedded in paraffin. Sections were cut and stained using a conventional immunohistochemistry procedure. Briefly, the tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sample in 10 mM citrate buffer (pH 6.0) at 95°C for 15 min. Endogenous peroxidase activity was blocked with peroxidase (DAKO), and sections were blocked with 10% goat serum. Sections were incubated with anti-SOX4 antibody (Abcam, 1:50) for 1 h, followed by incubation with secondary antibodies (DAKO) for 30 min. The immunostaining was developed with 3.3’-diaminobenzidine (DAB). Finally, sections were counterstained with hematoxylin.

**Transwell migration and invasion assays**
Experiments were performed as described in Supplementary Materials and Methods.

**Mammosphere formation assays**

Mammosphere assays were performed as described elsewhere (27) with minor modifications. Single cells were plated at 10,000 cells/ml on 6-well ultra-low attachment plates (Corning) in serum-free DMEM/F12 supplemented with 20 ng/ml bFGF, 20 ng/ml EGF, 4 μg/mL insulin, 4 μg/ml heparin, 1 μg/ml hydrocortisone, 0.4% BSA and B27. Fresh medium was supplemented every three days. The mammospheres were counted at day 14.

**Flow cytometry**

1 × 10^6 cells were resuspended in 100 μl PBS containing 2% FBS (FACS buffer), and then incubated on ice for 10 min. CD44-APC and CD24-PE (BD Biosciences) were added to cell suspension and incubated on ice for 30 min. Cells were washed and resuspended in 500 μl FACS buffer and analyzed using a FACS Calibur Flow Cytometer (BD Biosciences).

**Xenograft mouse experiments**

5 × 10^6 cells in 100 μl PBS were injected subcutaneously into 6-week-old female BALB/c nude mice. Five mice per group were used in each experiment. Tumor volume was measured weekly using a Vernier caliper and calculated according to the formula: \(\pi/6 \times \text{length} \times \text{width}^2\). Eight weeks later, the mice were sacrificed, and tumors were collected and
photographed. All animal experiments were approved by the Animal Care Committee of the Northeast Normal University, China.

**Luciferase reporter assays**

The protocol is described in Supplementary Materials and Methods.

**Cell proliferation assays**

Cell growth rates were assessed by the MTT assay as described in Supplementary Materials and Methods.

**RT-PCR and real-time PCR analysis**

Experiments were performed as described in Supplementary Materials and Methods.

**Statistical analysis**

Data are presented as mean ± SD. The Student’s *t* test (two-tailed) was used to determine statistically the significance of differences between groups. *p*<0.05 was considered statistically significant. SOX4 expression intensities in human breast cancer samples were analyzed by χ² test. Statistical analysis was performed using the SPSS17.0 software.

**Results**

**Exogenous SOX4 expression induced EMT in human mammary epithelial cells**
To investigate the role of SOX4 in EMT, we stably overexpressed SOX4 in the immortalized normal human mammary epithelial cell line MCF10A that lacks endogenous SOX4 expression, by using retroviral infection, as confirmed by immunoblotting (Fig. 1A) and real-time PCR (Supplementary Fig. S1). We observed that MCF10A cells transfected with vector retained their cobblestone-like morphology with tight cell-cell adhesion, whereas cells expressing exogenous SOX4 displayed an elongated fibroblast-like morphology with scattered distribution in culture (Fig. 1B). We then examined both epithelial and mesenchymal markers by immunoblotting (Fig. 1C) and immunofluorescence (Fig. 1D). As can be seen, the SOX4-expressing MCF10A cells exhibited a significant downregulation of β-catenin and complete loss of E-cadherin and Occludin from cell-cell contacts; meanwhile the mesenchymal markers Fibronectin, Vimentin and N-cadherin were dramatically upregulated. Real-time PCR analyses also revealed the repression of E-cadherin mRNA and concomitant induction of N-cadherin, Fibronectin and Vimentin mRNAs in SOX4-expressing MCF10A cells (Fig. 1E). These morphologic and molecular changes suggested an apparent transition of the SOX4-expressing MCF10A cells from an epithelial to mesenchymal status.

To further probe the possible interactions between SOX4 and other EMT-inducing transcription factors, we examined the expression of other known EMT inducers. We showed that the endogenous mRNA levels of
Snail1, FOXC2, GSC, SIX1, Twist1, HOXB7 and ZEB1 were elevated in response to SOX4 overexpression, to a variable extent, while the ZEB2 mRNA level exhibited no detectable change (Fig. 1F). We also ectopically overexpressed other EMT inducers Snail and Twist1 in MCF10A cells. We observed that Snail and Twist1 induced EMT in MCF10A cells (Supplementary Fig. S2A), and upregulated SOX4 mRNA expression in MCF10A cells (Supplementary Fig. S2B). Typically, the EMT phenotype is usually accompanied by the acquisition of cell traits such as greater migration and more invasive ability. As shown in Fig. 1G and 1H, SOX4-expressing MCF10A cells dramatically increased their migratory and invasive behaviors. Similar results were observed in MDCK cells, a prototypic cell model for EMT study (Supplementary Fig. S3A-E). Together, these results demonstrate that SOX4 is a novel inducer of EMT and it promotes cell migration and invasion in MCF10A cells.

Loss of E-cadherin appears to be a critical event in EMT. We next performed a Luciferase reporter assay to investigate whether SOX4 could transcriptionally regulate the E-cadherin expression. The results showed that the relative Luciferase activity of E-cadherin was not changed in 293T cells transiently co-transfected with E-cadherin promoter reporter together with SOX4 expression construct or with empty vector (Supplementary Fig. S4A), indicating that SOX4 may indirectly regulate E-cadherin transcription. In line with these observations, SOX4 increased the
expression of known EMT inducers (including Snail and ZEB1) that act to
directly repress E-cadherin transcription, during SOX4-induced EMT (Fig.
1F). To test the effect of SOX4 on the transcription activity of promoters of
these two EMT inducers, we transiently transfected 293T cells with
Luciferase reporter constructs containing proximal promoter of ZEB1 or
Snail. We found that the relative Luciferase activity of ZEB1 was increased
proportionally to the increasing amount of SOX4 in 293T cells
(Supplementary Fig. S4B). However, the Snail promoter activity was not
changed (Supplementary Fig. S4C). These findings suggest that
E-cadherin transcriptional inactivation during SOX4-induced-EMT may
be a result of induction of ZEB1 expression, a well known upstream
repressor of E-cadherin.

We next performed a loss-of-function assay to further study the role of
SOX4 in invasive mesenchymal breast cancer cells MDA-MB-231 and
BT549. The effect of SOX4 silencing on the invasive ability of these cells
was investigated using transwell invasion assays. The results indicated that
SOX4 knockdown prominently impaired the migration and invasion
ability of these two breast cancer cells, but it did not affect cell
proliferation and apoptosis (Supplementary Fig. S5A-G). Moreover,
suppression of SOX4 expression resulted in Vimentin downregulation in
both cell lines (Supplementary Fig. S5A and S5B). However, no detectable
changes were observed in other EMT markers (data not shown). In
addition, downregulation of SOX4 did not cause significant cellular morphological changes in both MDA-MB-231 and BT549 cells (Supplementary Fig. S5H). Thus, our loss-of-function study suggested that the suppression of SOX4 could partially reverse the EMT phenotype of MDA-MB-231 and BT549 cells.

SOX4-mediated EMT generated stem cell-like cells

Mammary epithelial cells undergoing an EMT program have been linked to stem cell phenotypes such as an increased CD44\textsuperscript{high}/CD24\textsuperscript{low} population and mammospheres formation ability (9). To determine whether SOX4 has the effect to lead to the stem cell phenotypes upon induction of EMT, we performed FACS to identify CD44\textsuperscript{high}/CD24\textsuperscript{low} populations. We observed that the SOX4-expressing MCF10A cells exhibited a significant increase in the CD44\textsuperscript{high}/CD24\textsuperscript{low} stem cell population compared with vector-infected cells (Fig. 2A). Meanwhile, as evidenced in Fig. 2B and 2C, the SOX4-expressing MCF10A cells increased both in size and in number of mammospheres in comparison with the vector-infected cells. We thus concluded that the SOX4-induced EMT generates mesenchymal cells with stem cell-like phenotypes, a feature recently defined for EMT inducers (6, 28).

SOX4 cooperated with activated oncogenic Ras to promote tumorigenesis

Although SOX4 was able to induce EMT, MCF10A cells expressing SOX4
were unable to form tumors when injected into nude mice (Supplementary Fig. S6A), suggesting that SOX4 itself lacks the ability to induce neoplastic transformation. It has recently been reported that EMT inducers Twist1, Twist2 and LBX1 cooperate with activated oncogenic Ras to promote even more dramatic characteristics of EMT and enhance tumorigenesis (6, 29). To investigate this possible cooperation, we co-expressed SOX4 with oncogenic H-RasV12 in MCF10A cells. As shown in Fig. 3A and Supplementary Fig. S6B, co-expression of SOX4 and oncogenic Ras led to a more dramatic morphologic change, characterized by prominently elongated spindle-shaped cells. In addition, co-expression of both SOX4 and H-RasV12 triggered a further reduction of β-catenin, relative to either SOX4 or H-RasV12 alone; however, no changes in other epithelial or mesenchymal markers were observed (Fig. 3B). Furthermore, MCF10A cells expressing SOX4+H-RasV12 exhibited higher migration (Fig. 3C) and invasive (Fig. 3D) ability than cells expressing either SOX4 or H-RasV12 alone. Meanwhile, all these cells exhibited roughly the same growth rates with no statistically significant differences (Supplementary Fig. S6C). These data implicated that SOX4 and H-RasV12 worked synergistically in inducing a more prominent EMT phenotype.

To further establish whether SOX4 is able to trigger tumorigenesis in vivo, we tested its effect in a xenograft mouse model. We detected no
primary tumor growth 8 weeks after the nude mice were subcutaneously injected with MCF10A cells stably expressing SOX4 alone. Meanwhile, MCF10A cells expressing oncogenic H-RasV12 alone produced small palpable tumors. Interestingly, co-expression of both SOX4 and H-RasV12 resulted in rapid development of very large tumors (Fig. 3E and 3F). These results clearly indicate that SOX4 and oncogenic H-RasV12 work synergistically to induce tumorigenesis in vivo.

**High expression of SOX4 was correlated with invasive breast cancer and highly aggressive TNBC**

To evaluate the clinical relevance of SOX4 expression in human mammary carcinomas, we performed immunohistochemistry staining of SOX4 in 149 human breast tumor samples representing different subtypes and several normal human breast tissues. 72% (107 out of 149) of breast cancer samples exhibited positive SOX4 immunoreactivity. Among the 107 SOX4 positive tumor samples, 46 (43%) cases showed strong nuclear staining, and 34 (32%) moderate nuclear staining, while non-neoplastic breast tissues only had faint nuclear staining of SOX4 (Fig. 4A-E). Further analysis revealed that high level of SOX4 expression occurred in 56.9% of III grade, 40.9% of PR− and 38.6% of ER− breast cancer samples. In contrast, 14.3% of I/II grade, 16.4% of PR+ and 19.7% of ER+ breast cancer samples showed high expression of SOX4 (Fig. 4F). These data demonstrated that SOX4 overexpression was significantly correlated with...
several poor prognostic parameters such as high tumor grade malignancy ($P<0.001$), negative ER ($P=0.014$) and negative PR ($P=0.001$) (Table 1).

Moreover, we observed that high level of SOX4 expression was associated with the highly aggressive triple-negative breast cancer (TNBC). As shown in Fig. 4F, 45.7% of TNBC samples showed abnormally high SOX4 expression compared with that of 24.3% in non-TNBC samples ($P=0.009$). To further determine the relevance of SOX4 expression with breast cancer progression, we performed an extensive analysis of the expression profile of SOX4 using the Oncomine cancer microarray database. Increased expression of SOX4 mRNA in mammary carcinoma in contrast to normal breast tissues was observed in 15 out of 19 microarray studies (Supplementary Table S1). Further analyses revealed that the expression levels of SOX4 were also significantly correlated to higher tumor grades and stages; and increased SOX4 expression predicted an unfavorable clinical outcome. In particular, among all of the 12 microarray studies in Oncomine data, SOX4 was significantly upregulated in unfavorable ER$^+/PR^-/HER2^-$ triple-negative basal-like subtype (Supplementary Table S2). These observations implicate the potential usefulness of the aberrant high SOX4 expression as a novel prognostic molecular marker for TNBC.

Activation of TGF-β was necessary for SOX4-induced cell motility

Several lines of evidence have implicated the involvement of TGF-β in
EMT process both in embryonic development and breast cancer progression (8, 30). We next intended to identify whether TGF-β signaling is activated in SOX4-induced EMT. Our real-time PCR revealed an increased expression of TGF-β1 and TGF-β2 mRNAs in SOX4-expressing MCF10A cells (Fig. 5A). In addition, the level of phosphorylated Smad2 protein, a downstream effector of TGF-β pathway, was significantly increased in SOX4-expressing MCF10A cells (Fig. 5B left). Moreover, SOX4 silencing efficiently decreased the level of phosphorylated Smad2 and the expression of TGF-β1 and TGF-β2 mRNAs in SOX4-expressing MCF10A cells (Fig. 5C); whereas knockdown of SOX4 upregulated E-cadherin expression and partly downregulated N-cadherin expression, presumably due to the residual ectopic SOX4 (Supplementary Fig. S7A). Consistent with these changes, we observed some tight cell clusters upon SOX4 silencing (Supplementary Fig. S7B). Apparently, these experiments pointed to a reinforced TGF-β signaling upon ectopic SOX4 expression in MCF10A cells. To further validate that TGF-β signaling is responsible for the SOX4-induced EMT and the enhanced cell motility, we employed a specific TGF-β receptor kinase inhibitor SB431542 to block the TGF-β signaling in SOX4-expressing MCF10A cells. We found that suppression of TGF-β signaling by the inhibitor reduced Smad2 phosphorylation level and downregulated Vimentin expression (Fig. 5B right), without affecting the morphological and molecular features of SOX4-expressing MCF10A
cells undergoing EMT (data not shown). Moreover, treatment of SOX4-expressing MCF10A cells with SB431542 reduced their migration and invasive ability (Fig. 5D and 5E). These results suggest that the intensified TGF-β signaling induced by SOX4 promotes cell motility, and this partly contributes to EMT.

TGF-β signaling has been shown to be able to induce EMT in many epithelial cells including MCF10A and NMuMG (31, 32). In addition, recent studies have shown that SOX4 is a direct target gene of TGF-β in glioma-initiating cells and is also responsive to TGF-β in glioblastoma multiforme (22, 23). Here we showed that SOX4 mRNA was also induced in MCF10A (Fig. 6A and 6B) and NMuMG (Supplementary Fig. S8) cells, in a dose- and time-dependent manner upon the addition of TGF-β to the cell culture medium. To investigate whether SOX4 is required for TGF-β induced EMT, we knocked down SOX4 expression in MCF10A cells with shRNA and examined their responses to TGF-β1 treatments. MCF10A cells expressing SOX4 shRNA (MCF10A-shSOX4#2) or non-target control shRNA (MCF10A-shCtrl) were treated with TGF-β1. We observed that SOX4 expression was induced 48h after TGF-β1 addition in the MCF10A-shCtrl cells, whereas MCF10A-shSOX4#2 cells exhibited a reduction in the basal expression level of SOX4 as well as in the induction of SOX4 after TGF-β1 stimulation (Fig. 6C). We also found that TGF-β1 treatments induced EMT in MCF10A-shCtrl cells, but not in...
MCF10A-shSOX4#2 cells (Fig. 6D). Consistent with morphological changes, E-cadherin expression was inhibited and N-cadherin expression was increased in MCF10A-shCtrl cells after TGF-β1 treatments. However, under the same conditions E-cadherin and N-cadherin expression were not changed in MCF10A-shSOX4#2 cells (Fig. 6C). These experiments suggest that SOX4 is also involved in and required for TGF-β induced EMT.

Discussion

Increasing evidence suggests that SOX4 overexpression is associated with several human cancers including brain, lung and breast cancers (17). Specifically, it has been reported that SOX4 is expressed in normal breast and breast cancer cells, and progestin can promote SOX4 expression and induce SOX4-mediated transcriptional activity in breast cancer cell lines (21). Moreover, miR-335 suppresses breast cancer metastasis and migration by modulating SOX4 expression (33). An recent analysis of the transcriptional profile of human normal mammary stem cells (hNMSCs), aimed at prediction of biological and molecular features of breast cancers, unravels that SOX4 is upregulated in PKH26-positive cells that possess all the characteristics of hNMSCs (34). These findings implicate the involvement of SOX4 in breast cancer development. Nevertheless, how SOX4 implements its oncogenic effects during breast cancer progression...
remains unclear. We find in this study that SOX4 functions as a trigger for EMT to contribute to breast cancer progression, a previously unreported role of SOX4 in breast cancer. Specifically, our data demonstrate that SOX4 represses the epithelial phenotype, induces the mesenchymal phenotype, and dramatically increases the migration and invasion of MCF10A cells.

Besides breast cancer, it has been reported that ectopic overexpression of SOX4 in hepatocellular carcinoma endows an anti-apoptotic effect and contributes to hepatocarcinogenesis (35). Previous study demonstrates that the epigenetic deregulation of microRNA-129-2 leads to the oncogenic overexpression of SOX4 in endometrial cancer. Reactivation of miR-129-2 results in SOX4 downregulation and reduces the proliferation of endometrial cancer cells (36). Accumulating evidence indicates that EMT inducers such as Snail and Twist1 control cell proliferation and survival that are critical in cancer progression (37). These results suggest that SOX4 may act in a similar fashion as Snail and Twist1, exerting dual effects of potent prosurvival function and orchestrating an EMT in tumor progression. Interestingly, SOX4 is also able to promote cell cycle arrest and apoptosis in a p53-dependent manner in HCT116 (38). The distinct physiological functions of SOX4 in different cell lineages and cancer types highlight the importance of cell context and genetic background in various human cancer cell lines and different cancer types that determine the
SOX4 function.

Moreover, we have demonstrated in this work that SOX4 upregulates the mRNA expression of several EMT-inducing transcription factors and SOX4 expression is induced by a large number of known regulators of the EMT program, notably the Snail, Twist, Ras and TGF-β1. Our data are in accordance with a recent study that discovers that overexpression of one of the EMT inducers upregulates a subset of other EMT-inducing transcription factors, implicating the interactions among these EMT inducers (39). E-cadherin plays a pivotal role in epithelial cell-cell adhesion. Functional loss of E-cadherin is considered a hallmark of EMT (4, 5). Our results show that SOX4 may indirectly downregulate the transcription of E-cadherin. It is reported that EMT inducers such as Twist1, Snail, Slug, ZEB1, ZEB2, FOXC1 and FOXC2 are able to transcriptionally repress E-cadherin expression either directly or indirectly (4). Esmeralda Casas and coworkers demonstrate that Twist1 indirectly suppresses E-cadherin transcription to promote EMT through directly binding to the Snail2 gene promoter to activate its transcription (40). We show in this study that SOX4 indirectly downregulates the expression of E-cadherin through activating ZEB1 expression. Interestingly, search of the defined region of ZEB1 promoter with the transcription factor database (TRANSFAC) did not reveal a known SOX4 consensus binding site, suggesting that SOX4 may not be able to bind directly to the ZEB1.
promoter; rather, it may indirectly activate the ZEB1 promoter through as yet unidentified intermediate factor(s).

More importantly, our data have suggested that SOX4 is also involved in and required for TGF-β induced EMT. We demonstrate in our study that the autocrine TGF-β signaling is activated in SOX4-induced EMT; meanwhile TGF-β signaling induces SOX4 expression in breast epithelial cells and knockdown of SOX4 blocks TGF-β1-induced EMT. Thus, we speculate that the SOX4-TGFβ-SOX4 feedback loop presumably functions as a novel signaling pathway in EMT regulation, as well as in breast cancer progression. A recent study by Christina Scheel and coworkers demonstrates that autocrine signaling pathways involving TGF-β and both the canonical and non-canonical Wnt signaling are activated in EMT program induced by diverse stimuli including transcription factors such as Twist, and they propose that these signalings further maintain the EMT program, and disruption of these extracellular autocrine signalings abrogates transcription factor-induced EMT (41). In support of the involvement of additional EMT-promoting pathways, it has been documented that SOX4 activates the Wnt/β-catenin pathway in colon carcinoma and melanoma (24, 25, 42). Whether the canonical Wnt/β-catenin signaling can be activated in SOX4-induced EMT needs to be clarified.

Recent studies have demonstrated that EMT can induce non-cancer
stem cells to acquire cancer stem cell (CSC)-like properties in breast cancer cells that exhibit a $CD44^{\text{high}}/CD24^{\text{low}}$ antigenic phenotype (9). In this study, we have shown that ectopic expression of SOX4 in MCF10A cells increases the $CD44^{\text{high}}/CD24^{\text{low}}$ subpopulation and enhances the mammosphere-forming ability, a property of the stem cells. It is reported that normal and neoplastic breast stem-like cells express some EMT inducers such as Twist1, Snail1, Snail2 and ZEB1 (9). A recent study by Chen and colleague unravels that MCF-7 cells cultured in 3D collagen scaffolds acquire the properties of CSCs, and the transcriptions of stem cell markers such as OCT4A and SOX2, and breast cancer stem cell signatures, including SOX4, JAG1 and CD49F, are significantly unregulated (43). Furthermore, SOX4 is also upregulated in PKH26-positive cells that possess all the characteristics of hNMSCs (34). All these data are supportive of a close relationship among SOX4, EMT and CSCs. Previous study reports that the $CD44^{\text{high}}/CD24^{\text{low}}$ subpopulation breast cancer cells enhance tumorigenicity in a xenograft model (44). However, in our xenograft model, MCF10A cells expressing SOX4 alone are unable to form tumors when injected into nude mice, suggesting that SOX4 itself lacks the ability to induce neoplastic transformation, although it promotes the generation of stem cell-like cells. Nevertheless, we have observed a cooperative action between SOX4 and activated oncprotein Ras in MCF10A cells, resulting in the formation of large tumors in nude mice. In
line with our observations, a previous work has demonstrated that SOX4 overexpression alone in NIH3T3 cells do not increases the transforming ability of the cells, but the effect occurs when cells are co-transfected with the weakly oncogenic RHOA-Q63L (45). Thus, our findings provide evidence for the ability of SOX4 to potentiate the oncogenic effect of activated Ras in breast tumorigenesis. Interestingly, we have found that activated Ras itself upregulates the endogenous SOX4 expression, but no synergistic effects between Ras and SOX4 are observed. An earlier study suggests that Ras also induces the expression of other EMT inducer such as Twist1 in MCF10A cells (46). Our result shows that Ras upregulates Snail1 expression in mammary epithelial cells (Supplementary Fig. S6B), but these inducers do not function cooperatively with Ras either. These data seem to support the assumption that only the exogenous EMT inducers and activated Ras are able to exert the cooperative effects to promote even more dramatic characteristics of EMT and cancer progression (6, 9, 29, 41). However, the mechanisms underlying this phenomenon still remain unknown. Conclusively, SOX4 may function as a key tumor progression factor in breast cancer, rather than a tumor-initiating event for breast carcinogenesis.

TNBC is the most aggressive form of breast cancer with high histological grade, aggressive clinical behavior, high incidence of brain and lung metastases and the lack of an effective therapeutic target (47). The
TNBC shares many clinicopathologic and molecular features with basal-like breast cancers (BLBC), a subtype of breast cancer defined by gene-expression profiling, and accounts for approximately 70% of the BLBC cases. Recent studies have linked EMT with the aggressive BLBC (47, 48). Targeting the EMT-like phenotypes would seem to represent the potential strategies for the development of novel anticancer therapeutics. Our results in this study are in favor of a strong correlation between SOX4 and TNBC, and hence point to the prospect of using SOX4 as a novel biomarker for prognosis and diagnosis of TNBC, as well as a potential molecular therapeutic target for this highly aggressive breast cancer.

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References


35. Hur W, Rhim H, Jung CK, Kim JD, Bae SH, Jang JW, et al. SOX4 overexpression regulates the


42. Cai H, Ni A, Li W, Li J. Inhibition of melanoma cell proliferation by targeting Wnt/beta-catenin pathway through Sox4 RNA interference. Journal of Huazhong University of Science and Technology Medical sciences = Hua zhong ke ji da xue xue bao Yi xue Ying De wen ban = Huazhong keji daxue


Table 1. Association between SOX4 expression and tumor subtypes in invasive ductal carcinomas of breast

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total no. of cases</th>
<th>No. with high-level expression for SOX4</th>
<th>Percentage with high SOX4 expression (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor negative</td>
<td>149</td>
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<td>positive</td>
<td>61</td>
<td>12</td>
<td>19.7</td>
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<td>10</td>
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Figure Legends

Figure 1. Ectopic expression of SOX4 in MCF10A cells induced an EMT program. (A) Immunoblotting assessment of the ectopic SOX4 protein expression after retroviral infection in MCF10A cells. (B) Morphologic change of MCF10A cells expressing SOX4 or empty vector. Scale bar =100 μm. (C) Immunoblotting analysis of expression of the epithelial markers E-cadherin, β-catenin and Occludin, and the mesenchymal markers Fibronectin, Vimentin and N-cadherin. (D) Immunofluorescence staining for the epithelial and mesenchymal markers. Scale bar =100μm. (E) The expression of E-cadherin, N-cadherin, Fibronectin and Vimentin mRNAs were assessed by Real-time PCR. (F) mRNA expression levels of known EMT inducers were assessed by Real-time PCR. Error bars represent the mean ± SD of triplicate experiments. (G) Migration (1 d) and (H) invasion (2 d) assays in stable MCF10A cells. The mean was derived from cell counts of five fields, and each experiment was repeated three times (***p<0.001, compared to the control). Representative images of migrated and invaded cells are shown.

Figure 2. SOX4-induced EMT generated stem cell-like cells. (A) FACS analysis of cell-surface markers CD44 and CD24 in MCF-10A
cells expressing SOX4 or empty vector. Percentages of mean CD44^{high}/CD24^{low} subpopulation ± SD based on triplicate experiments are indicated. (B) Phase contrast images of mammospheres formation. Scale bar =100μm. (C) Quantification of mammosphere numbers formed from three independent experiments (Error bar: mean ± SD) (*p<0.05, compared to the control).

Figure 3. SOX4 cooperated with activated oncogenic Ras to promote tumorigenesis. MCF10A cells were infected with empty vector, retroviral vector encoding SOX4, or sequentially infected with retroviral vector encoding H-RasV12 (Ras) and SOX4 as indicated. (A) Phase contrast images of the cells morphology. Scale bar =100μm. (B) Immunoblotting analysis of expression of the epithelial and mesenchymal markers. (C) Migration (1 d) and (D) invasion (2 d) assays. The mean was derived from cell counts of five fields, and each experiment was repeated three times. Representative images of migrated or invaded cells are also shown. (E) Stable MCF10A cells were subcutaneously injected into BALB/c female nude mice (n=5 for each experimental group). (F) Individual tumor volume was measured according to the formula: \( \pi/6 \times \text{length} \times \text{width}^2 \) at week 8 after injection.

Figure 4. SOX4 expression was associated with invasive subtypes of
human breast cancer. Representative images of the immunohistochemical staining of SOX4 in breast cancer samples. (A) Normal human breast tissue. (B) Negative tumor staining. (C) Weak nuclear staining. (D) Moderate nuclear staining. (E) Strong nuclear staining. Scale bar =100µm. (F) Percentages of human breast cancer samples with high level of SOX4 expression in respective tumor subtypes and in different tumor grades. Corresponding p-values analyzed by χ² test are indicated.

Figure 5. Activation of TGF-β was necessary for SOX4-induced cell motility. (A) Expression of TGF-β1 and TGF-β2 mRNAs was determined by real-time PCR in MCF-10A cells expressing empty vector or SOX4. (B, Left) Immunoblots of p-Smad2 and total Smad2/3 protein. (B, Right) SOX4-MCF10A cells were treated with 10 µM SB431542 (SB43) for 24 h. Immunoblots of p-Smad2, total Smad2/3 protein and Vimentin. (C) SOX4-MCF10A cells were stably infected with SOX4 shRNA (shSOX4#2) or non-target vector shRNA (shCtrl). Immunoblotting of SOX4, p-Smad2 and total Smad2/3 protein (left). Real-time PCR analysis of the expression of TGF-β1 and TGF-β2 mRNAs (Right). (D) Migration (1 d) and (E) invasion (2 d) assays of SB431542-treated SOX4-MCF10A cells. The mean was calculated from cell counts of five fields, and each experiment was repeated three times (***(p<0.001, compared to the
DMSO-treated). Representative images of migrated or invaded cells are shown.

**Figure 6. SOX4 was necessary for TGF-β-induced EMT.** (A and B) SOX4 mRNA expression levels in MCF10A cells treated with activated TGF-β1 at indicated time and concentrations. Error bar represents mean ± SD of triplicate assays. (C) Immunoblotting of SOX4, E-cadherin and N-cadherin after 48h treatment of TGF-β1 (10 ng/mL) in MCF10A cells with shCtrl and shSOX4#2. (D) Morphology of MCF10A cells with shCtrl and shSOX4#2 after TGF-β1 treatment as in (C). Scale bar =100μm.
Figure 1
Figure 2

A

Vector

16.87% ± 1.07%

SOX4

75.45% ± 2.38%

B

Vector

SOX4

C

Mammospheres/10,000 cells

Vector

SOX4

*
Figure 3
Figure 4

A. Image of tissue section with immunohistochemistry staining.

B. Image of tissue section with immunohistochemistry staining.

C. Image of tissue section with immunohistochemistry staining.

D. Image of tissue section with immunohistochemistry staining.

E. Image of tissue section with immunohistochemistry staining.

F. Bar charts showing the percentage of high SOX4 expression in tumors for different receptor statuses:

- Estrogen receptor (ER):
  - ER+:
    - Percent of high SOX4 expression: 38.6%
  - ER-:
    - Percent of high SOX4 expression: 19.7%
  - P-value: 0.014

- Progesterone receptor (PR):
  - PR+:
    - Percent of high SOX4 expression: 40.9%
  - PR-:
    - Percent of high SOX4 expression: 16.4%
  - P-value: 0.001

- HER2 receptor:
  - HER2+:
    - Percent of high SOX4 expression: 38.0%
  - HER2-:
    - Percent of high SOX4 expression: 22.9%
  - P-value: 0.046

G. Bar charts showing the percentage of high SOX4 expression in tumors for different tumor grades:

- Tumor Grade:
  - Grade III:
    - Percent of high SOX4 expression: 56.9%
  - Grade I/II:
    - Percent of high SOX4 expression: 14.3%
  - P-value: 0.001

H. Bar charts showing the percentage of high SOX4 expression in tumors for different triple-negative statuses:

- Triple negative:
  - Not Triple:
    - Percent of high SOX4 expression: 45.7%
  - Triple:
    - Percent of high SOX4 expression: 24.3%
  - P-value: 0.009
Figure 6
SOX4 induces epithelial-mesenchymal transition and contributes to breast cancer progression

Jianchao Zhang, Qian Liang, Yang Lei, et al.

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