MEDDOCS Open Access Publisher

ISSN: 2640-8198

Annals of Breast Cancer

Open Access | Research Article

Transcription Factor SOX3 Regulates Epithelial-mesenchymal Transition in Human Breast Cancer Cell Line MDA-MB-231

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Received: Jan 06, 2024

Accepted: Jan 31, 2024

Published Online: Feb 07, 2024

Journal: Annals of Breast Cancer

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: SOX3; Breast cancer; EMT; MDA-MB-231.

Abstract

Introduction: Transcription factors proteins play essential roles in regulating cellular events in breast cancer that mediate or contribute to tumorigenesis. Embryonic transcription factors, such as SOX proteins, are key players regulating the balance between Epithelial-Mesenchymal Transition (EMT) and the reverse Mesenchymal-Epithelial Transition (MET), both involved in breast cancer plasticity. In breast cancer, SOX proteins function both as oncogenes and tumor suppressors, depending on each SOX protein involved and in which tissue and cell line its acting.

Objective: Investigate the involvement of SOX3 protein in target gene expression related to the EMT process in a human triple-negative breast cancer cell line.

Methods: Breast cancer cell line MDA-MD-231 transfected with pEF1-SOX3+ and pEF1-Empty vector followed by qPCR for EMT-related gene expression.

Results: MDA-MB-231 transfected with pEF1-SOX3+ presented upregulation of mRNA from ECAD gene, contrasting with downregulation mRNA from NCAD, SNAIL, ZEB1 and ZEB2, compared to non-transfected cells and cells transfected with pEF1-empty vector ($p<0.005$).

Conclusion: SOX3 appears essential for maintaining an epithelial cells phenotype in breast cancer and allowing cells that underwent EMT to transiently re-acquire the epithelial phenotype. This present study revealed that transcription factors regulates EMT-MET in cancer cell phenotype may serve as important targets for breast cancers therapy and markers. Hence, additional studies are needed to understand SOX3 protein involvement the EMT-MET phenomenon.

Cite this article: De Souza Silva FH, Underwood A, Pereira Almeida C, Mendes Lima B, Soares Veloso E, et al. Transcription factor SOX3 regulates epithelial-mesenchymal transition in human breast cancer cell line MDA-MB-231. Ann Breast Cancer. 2024; 7(1): 1026.

Introduction

Epithelial-Mesenchymal Transition (EMT) has become an important factor in tumor development. The quest to understand the mechanisms involved in EMT led to the understanding that different transcription factors are responsible for triggering the stimulation of this event. All these factors promote significant genotypic and phenotypic changes at the cellular level, favoring processes of loss of adhesion, migration, invasion, and resistance to death by apoptosis [1,2]. In breast cancer, EMT is essential in acquiring invasive and metastatic capacity, with cells developing the ability to move freely, surpassing the surrounding extracellular matrix, invading adjacent tissues, and establishing secondary sites [3]. The literature has already described that different transcription factors primarily focus on repressing CDH1 (E-cadherin) expression. As leading examples, the zinc-finger proteins (SNAI1), Zeb1(ZEB1), and Zeb2 (ZEB2) stand out, both acting directly in the repression of CDH1 expression, binding to promoter regions [4,5].

The SOX family contains a highly conserved High-Mobility Group (HMG) domain that regulates their DNA binding capacity and has some members involved in the EMT induction process. Within this family, the Sex-determining region Y-box 3 (SOX3) represents an important member. Studies show that SOX3 favors invasion and proliferation in some tumors, such as osteosarcomas and ovarian cancer, and its expression is related to an oncogene [6-8]. On the other hand, SOX3 overexpression was determined as a promoter of EMT in osteosarcoma tissues, favoring all cellular signs of resistance, such as migration and invasion [9]; and SOX3 expression in human breast cancer cell line triggers the apoptotic pathway [10]. However, the expression of SOX3 in breast cancer and its relationship with EMT is not yet described in the literature. Hence, the present study investigated the effects of SOX3 expression in triple-negative breast cancer MDA-MB-231 cell lines through transient transfection of the pEF-1-human SOX3 expression vector to verify SOX3 involvement in the genes that regulated the EMT process.

Material and Methods

Human SOX3 cloning and amplification

SOX3 sequence was amplified from human DNA (Thermo - MHS6278-202857278) by PCR using the following right primer: 5'-AAAGAATTCTCCGATGTGGGTCAGCGGCA - 3', and left primer: 5'-ATAGGATCCGGAATGCGACCTGTTCGAGAGA-3'at 59.8°C for PCR annealing temperature utilizing Phusion Hot-Start II enzyme (ThermoFisher, USA) for amplification [10]. Protein coding regions of the SOX3 gene were subcloned into the expression vector pEF1/Myc-His (Invitrogen, USA). Restriction enzymes BamHI and EcoRI were used for cloning in the pEF-1 vector. After that, the vector was recircularized with T4 ligase, and the *SOX3* construct was sequence confirmed by BigDye Sanger sequencing on ABI 3130xl genetic analyzer (Applied Biosystems).

Cell culture and transfection

MDA-MB-231 (ATCC® HTB-26™) were maintained in DMEM (ThermoFisher, USA) with 1% Antibiotic (ThermoFisher, USA) and 10% fetal bovine serum (ThermoFisher, USA). 1 x 10^5 cells were then seeded into six-well plates and incubated in a humidified atmosphere at 37°C and 5% CO_2. Each well was transfected with 500 ng of a pEF-1-SOX3 expression vector or pEF-1 empty vector, using Lipofectamine 3000 (ThermoFisher, USA), according to the manufacturer's protocol. In parallel, a negative control (no transfection) was performed. After 24 h, cells were processed for cell viability protocol and RNA isolation.

Cell viability assay

Cell viability was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA). Cells were seeded in a 96-well plate (8×10^3 cells/well) and cultured for 24h. The cells went through the transfection protocol, and at the end of the incubation period, the cells were incubated with 0,25 mg/ml MTT solution. Four hours later, the absorbance was measured at 570 nm under a microplate spectrophotometer.

RNA extraction, reverse transcription, and real-time PCR

Total RNA was isolated using 1 ml of Trizol® reagent according to the manufacturer's protocol (Invitrogen, USA). The cDNA was synthesized from 1µg of RNA, according to the manufacturer's protocol of the SuperScript III (Invitrogen, USA), using random hexamers primers. Samples were pipetted into 96-well plates with duplicates for each condition in a final reaction volume of 20 µL each, using Sybr® Green Master Mix Kit (Invitrogen, USA). The real-time PCR thermal cycling conditions were as follows: [1] a 50°C/2 min cycle; [2] one cycle at 95°C/10 min; [3] 40 cycles of 95°C/15s, followed by a melting curve from 57°C, for analysis of amplicon specificity. The primers used in qPCR amplification are listed in (Table 1).

Table 1: Primer for PCR and real-time PCR.

Primers	Nucleotide sequence (nt)	Fragment size	GenBank Acession number
ECAD Forward	5'- CCCTTCACAGCAGAACTAAC - 3'	108	NM_001317185.1
Reverse	5'- CACCTCTAAGGCCATCTTTG - 3'		
NCAD Forward	5'- GGACCGAGAATCACCAAATG - 3'	94	NM_ 001308176.1
Reverse	5'- CGTTCCTGTTCCACTCATAG - 3'		
ZEB1 Forward	5'- GGGAGGATGACAGAAAGGAA -3'	100	NM_001323654.1
Reverse	5'- GCATCTGACTCGCATTCATC -3'		
ZEB2 Forward	5'- CCATCTGATCCGCTCTTATC - 3'	125	NM_001171653.1
Reverse	5'- CCTGTGTCCACTACATTGTC - 3'		
GAPDH Forward	5'- TGGGTGTGAACCATGAGAAG - 3'	125	NM_001289746.1
Reverse	5'- GAGTCCTTCCACGATACCAAAG - 3'		
SNAIL Forward	5'- GCAGGACTCTAATCCAGAGTT-TACC -3'	127	NM_005985.4
Reverse	5'- GACAGAGTCCCAGATGAGCATT -3'		
SOX3Hs Forward	5'- GTTGGGACGCCTTGTTTA -3'	126	NM_005634.2
Reverse	5'- TCGCTGCTCCTGACTTAT -3'		

Real-time qPCR data analysis

Data were accessed by comparative CT method ([CT target gene mean]-[CT endogenous control mean]), as the endogenous control was used to normalize target gene expression and generate ΔCT. The ΔΔCT (ΔCT sample - ΔCT of the calibrator) was calculated, and the $2^{-\Delta\Delta CT}$ formula was applied to obtain relative expression levels of each target gene, expressed as fold-change.

Statistical analysis

Statistical data were analyzed using Graph Pad Prism 8.0.2 (Graph Pad Software, USA) and were presented as mean ± standard deviation. The criterion of statistical significance was $P < 0.05$. One-way ANOVA with Tukey's multiple comparisons test was employed to evaluate individual groups' differences.

Results

Transfection and expression of pEF1-SOX3+ and its function with cell viability

After the transfection of pEF1-SOX3 in MDA-MB-231 cells, significant morphological alterations were observed in the transfected group, such as a reduction in the number of cells and changes in the membrane (blebs), indicative of cell death (Figure 1A). Regarding cell viability indicated by MTT, in the group transfected with pEF1-SOX3, a significant decrease in cell viability was observed. The group transfected with the pEF-1-Empty vector maintained the cell viability compatible with the control group (Figure 1B). This result indicated that the transfection protocol is not cytotoxic, and the viability reduction is related to the expression of SOX3.

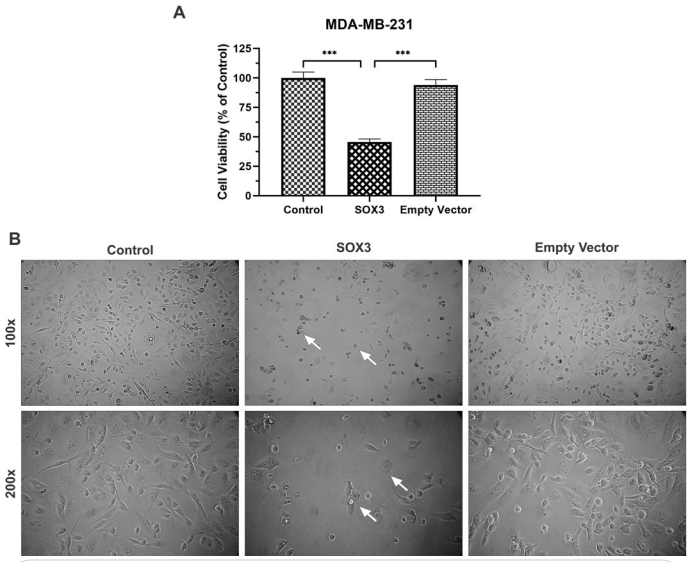

Figure 1

Figure 1. pEF1-SOX3+ on cell morphology and viability: (A) Cytotoxic effect of pEF1-SOX3+ on MDA-MB-231 tumor cells. Cell survival was measured by MTT. Results are presented as cell survival compared with the control group (untreated cells), $p < 0.005$. **(B)** Morphological changes induced by pEF1-SOX3+. Photomicrographs from phase-contrast microscopy of the MDA-MB-231 tumor cells. After 24 hours of transfection protocol, cells showed cell volume reduction, cell rounding, irregularities in the plasma membrane and formation of blebs and vacuoles (white arrows). Images were acquired by the digital camera Nikon Coolpix 4500, coupled to the MO (100x and 200x amplification).

Expression of EMT genes in MDA-MD-231 cells expressing SOX3

MDA-MB-231 cells transfected with pEF1-SOX3 demonstrated ECAD mRNA expression upregulation (27.01-fold change); contrasting with NCAD (0.06-fold change), SNAIL (0.18-fold change), ZEB1 (0.15-fold change) and ZEB2 (0.11-fold change) mRNA downregulation in cells transfected with pEF1-empty vector ($p<0.005$) (Figure 2).

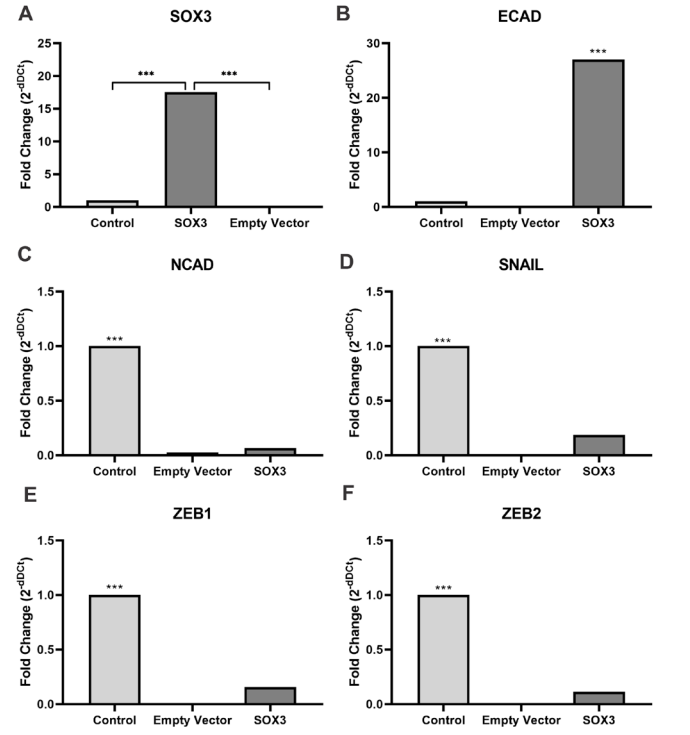

Figure 2

Figure 2. Quantitative PCR -Gene expression: mRNA expression in MDA-MB-231cells: With no transfection (control); transfected with pEF1-empty vector and transfected with pEF1-SOX3+. **(A)** SOX3 **(B)** ECAD, **(C)** NCAD, (**D)** SNAIL, **(E)** ZEB1, and **(F)** ZEB2. Real-time qPCR was expressed as DCt, which is the cycle threshold (Ct) of the target gene minus the reference gene, GAPDH. The relative gene expression was calculated as fold change using the formula 2^{-DCt}, where DCt of the transfected group minus the DCt of the control group. The real-time PCR results demonstrated higher mRNA expression of ECAD (27.01 fold change), and lower mRNA expression of NCAD (0.06 fold change), SNAIL (0.18 fold change), ZEB1 (0.15 fold change), and ZEB2 (0.11 fold change), compared to untransfected control and empty vector-transfected cells, respectively, $p<0.005$.

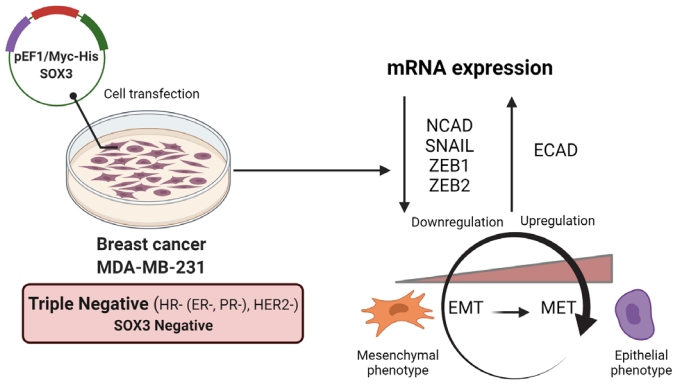

Figure 3

Discussion

Several studies have shown that SOX3 is upregulated in different tumors, affecting tumor progression [6,7,11,12]. Most recently, SOX3 has been demonstrated as an important factor

regulating apoptosis in breast cancer [10]. Epithelial-mesenchymal transition is controlled by several signaling and involves adaptor proteins and gene expression regulation [13]. The results of the present study revealed that inducing SOX3 protein expression in *vitro* is followed by a downregulation in gene expression of mesenchymal phenotype molecule NCAD, as well as the other transcription factors involved in the EMT process, such as SNAIL, ZEB1, and ZEB2. High levels of SOX3 have been previously described as an important factor in inhibiting EMT, acting together with the low expression of SNAIL in the breast cancer cell line, MCF-7 [14]. These results corroborate those found in this work. The tumor cell lineage MDA-MB-231, expressing SOX3 protein, had altered the expression rates of transcription factors related to the induction of EMT and upregulating gene expression of epithelial phenotype molecule ECAD, a key factor in blocking EMT (Figure 3). Even though mRNA expression and levels changes in transcription are associated with translational modifications, the present data indicate that the SOX3 transcriptional factor might have a binding site in promotor regions of genes related to EMT [15]. EMT-MET modulation behavior, induced by the transfection and expression of SOX3 protein in breast cancer cells, may indicate a new key promoter phenotype regulator for future studies in the field. Present data reinforce a possible action of SOX3 as a transcriptional repressor of key genes related to EMT induction, and further studies are essential for understanding its role in this mechanism. SOX3 transfection and expression in the human breast cancer model *in vitro* increased the expression of ECAD and reduced the expression of genes related to EMT induction and phenotype (NCAD, ZEB1, ZEB2, and SNAIL). The EMT-MET modulation behavior, caused by the expression of SOX3 in breast cancer cells, may demonstrate a new path and target for studying new targets for breast cancer. Therefore, cellular changes during the EMT process are very dynamic and microenvironment context-dependent, so it is crucial in future studies to investigate and analyze the EMT using *in vivo* model.

Figure 3: Epithelial-mesenchymal and mesenchymal-epithelial transition phenotype in MDA-MB-231 cells transfected with pEF-1/MycHis/SOX3+. SOX3 protein expression in the triple-negative breast cancer MDA-MB-231 cell line, is followed by a downregulation in NCAD expression, an important mesenchymal phenotype marker, and an upregulation of ECAD expression an important epithelial phenotype marker.

Conflicts of Interest: The authors disclose no potential conflicts of interest.

Funding/Support: Research supported by the Federal University of Minas Gerais (Edital PRPq05/20160); this study was part of a Ph.D. thesis by F.H.S.S. at the Pathology Post-Graduation Program at Universidade Federal de Minas Gerais, a Ph.D. scholarship funded by CAPES.

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