TRPS1 Suppresses Breast Cancer Epithelial-mesenchymal Transition Program as a Negative Regulator of SUZ12

Abstract
Breast cancer (BC) is among the most common malignant diseases and metastasis is the handcuff of treatment. Cancer metastasis is a multistep process associated with the epithelial-mesenchymal transition (EMT) program. Several studies have demonstrated that transcriptional repressor GATA binding 1 (TRPS1) played important roles in development and progression of primary BC. In this study we sought to identify the mechanisms responsible for this function of TRPS1 in the continuum of the metastatic cascade. Here we described that TRPS1 was significantly associated with BC metastasis using public assessable datasets. Clinically, loss of TRPS1 expression in BC was related to higher histological grade. In vitro functional study and bioinformatics analysis revealed that TRPS1 inhibited cell migration and EMT in BC. Importantly, we identified SUZ12 as a novel target of TRPS1 related to EMT program. ChIP assay demonstrated TRPS1 directly inhibited SUZ12 transcription by binding to the SUZ12 promoter. Loss of TRPS1 resulted in increased SUZ12 binding and H3K27 tri-methylation at the CDH1 promoter and repression of E-cadherin. In all, our data indicated that TRPS1 maintained the expression of E-cadherin by inhibiting SUZ12, which might provide novel insight into how loss of TRPS1 contributed to BC progression.

Introduction
Breast cancer (BC) is one of the most common malignancies in women and metastasis is still the bottleneck of the treatment [1]. Epithelial-mesenchymal transition (EMT) is a crucial event during metastasis to enable the cancer cells to migration. E-cadherin is a prototypical member of type I classical cadherins of adherence junctions. Down-regulation of E-cadherin is one of the key features of EMT, which can be used as a prognostic indicator in several cancers [2]. Its down-regulation is caused by many different mechanisms, ranging from mutations found in lobular BC and gross deletions to repression of gene transcription, as well as signal transduction stimulation of E-cadherin adhesion complex formation [3]. Several studies have shown that Loss of E-cadherin expression in human malignancies is commonly caused by promoter methylation, as occurring in invasive ductal BC, or transcriptional repression due to SNAIL, SLUG, SIP1 and ZEB1 [4,5]. However, the exact molecular mechanisms underlying decreased or lack of E-cadherin expression remains elusive in BC.

TRPS1 gene, located in human chromosome 8q23-24, is characterized by craniofacial and skeletal malformations caused by its mutation [6,7]. TRPS1 gene encodes a novel nuclear transcription factor, belonging to GATA family, with an combination of different types of zinc finger motifs, including one GATA-type DNA-binding domain and two IKAROS-like zinc fingers [6,8]. Function of TRPS1 was predominantly elucidated in EMT of kidney development and renal fibrosis [7,9]. Studies also indicated that TRPS1 promoted the differentiation and apoptosis of ATDC5 chondrogenic cells through MAPK pathway [10]. As a transcription factor, TRPS1 directly repressed Runx1 and Sox9 expression to control proliferation of the normal cartilage formation [11] as well as follicle epithelium [12].
To date, a growing body of evidence suggested the involvement of TRPS1 in a wide variety of functions among human malignancies. We previously reported that TRPS1 promoted angiogenesis in BC though VEGFA activation [13]. A series of studies have identified TRPS1 regulated EMT in several different mechanisms [14–16]. Stinson et al. reported that TRPS1 regulated E-cadherin expression by transcriptional repression of ZEB2, which was DNA-binding transcriptional repressors of E-cadherin gene. Loss of TRPS1 resulted in increased ZEB2 level, leading to repression of E-cadherin and EMT in BC [14]. In addition, a most recent study suggested that TRPS1 transcriptionally activated FOXA1, a negative regulator of EMT [15]. In consistent with these investigations, we have also demonstrated that TRPS1 was related to EMT in BC tissues by immunohistochemical analysis [17].

Polycomb group (PcG) proteins are evolutionarily conserved regulators of gene silencing, important in development [5], stem cell pluripotency [18,19] and X chromosome inactivation [20,21]. PRC2, including SUZ12 and the catalytic subunit EZH2, functions as a multi subunit complex that tri-methylates histone H3K27 and initiates gene repression [21–23]. SUZ12 was observed that its over-expression coincided with carcinogenesis [24–26]. In cancer cells, CDH1 repression also depends on PRC2 complex, whether recruited by DNA-binding transcriptional repressor, Snail [24] or not [27]. Evidence also showed SUZ12 played key role in gastric cancer progression by directly binding and inducing H3K27 tri-methylation at the CDH1 promoter [28].

In this study, we sought to investigate roles of TRPS1 involving in promoter methylation of E-cadherin in BC. We showed that TRPS1 strongly inhibited expression of the SUZ12, subunit of PRC2, through a direct and evolutionarily conserved interaction with the promoter. Loss of TRPS1 resulted in increased SUZ12 binding and H3K27 tri-methylation at the CDH1 promoter and repression of E-cadherin. In vitro and in vivo data suggested that this pathway is important for tumor progression. Collectively, TRPS1 acted as a tumor suppressor that blocks EMT by inhibiting the SUZ12, and hence prevented the repression of E-cadherin and other critical target genes in BC.

Materials and Methods

Bioinformatics Analysis

The expression data of BC cell lines was obtained from MD Anderson Cancer Center (https://faculty.mdanderson.org/). Expression and clinical data of GSE25066 and GSE2990 datasets was downloaded from Gene Expression Omnibus (GEO) at NCBI.

We further validated the survival analysis using an online tool Kaplan-Meier Plotter (http://kmplot.com/analysis/) [29], with up to 5143 BC patients, containing 13 GEO datasets (Affymetrix microarrays only), European Genome-Phenome Archive (EGA) and The Cancer Genome Atlas (TCGA), with a mean follow-up of 69 months. Using the selected parameters from the website, survival data and curves according to specific genes (mRNA expression) can be auto-generated. For TRPS1 gene expression, Aymetrix probe ID 218502 was chosen based on default suggestion by website.

Tissue Processing and Immunohistochemistry (IHC)

Our cohort consisted of 117 BC patients with invasive ductal carcinoma who underwent surgery from 2008 to 2010 in Qilu Hospital of Shandong University. Lobular carcinoma and other subtypes were excluded in our cohort. A total of two tissue microarrays (TMA) were constructed by incorporating two representative cores of each tumor. Matched adjacent benign breast duct tissues were also included for part of these patients. We performed immunohistochemistry (IHC) to investigate altered protein expression in 117 paraffin-embedded breast tissues as described previously [13]. Briefly, the slides were incubated with antibodies to TRPS1 (1:200, cat no. sc-26974, Santa Cruz), E-cadherin (1:200, cat no. #3195, CST) and SUZ12 (1:100, cat no. 20366-1-AP, Proteintech) overnight at 4°C. For negative controls, the antibodies were replaced with PBS. The diagnosis was confirmed by three pathologists (B.H., P.S., and J.H.). This study was approved by Institutional Review Board of Medical School of Shandong University (Jinan, China). The informed written consent was obtained from each patient.

Cell Culture

Human BC cell lines (T47D, MCF-7, MDA-MB-231 and MDA-MB-468) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were maintained at 37°C in a humidified incubator supplied with 5% CO₂.

Cell Transfection

Lentivirus with TRPS1-specific shRNA was purchased from Shanghai Gene Pharma Co., Ltd. shRNA oligonucleotide sequences targeted at TRPS1 were indicated in Table S1 in the supplemental material. The human TRPS1 cloning vector was purchased from Open Biosystems (MHS6278-211690440, Thermo Fisher Scientific, Lafayette, CO, USA). The target gene was subcloned into a mammalian expression vector, pcDNA3.1 (+) (Life Technologies, NY, USA), and a negative control vector was generated as previously described [13]. Vectors were transfected using a Thermo Scientific Turbofect Transfection Reagent (Thermo Scientific Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. T47D and MCF-7 cells transfected with lentivirus were referred as T47D-NC/ T47D-shTRPS1 and MCF-7-NC/ MCF-7-shTRPS1. MDA-MB-231 and MDA-MB-468 cells transfected with vectors were referred as 231-Vec/ 231-TRPS1 and 468-Vec/ 468-TRPS1.

Cells were transiently transfected with siRNA and the corresponding control using Hiperfect transfection reagent (Qiagen) following the manufacturer’s protocol. Transfection efficiency was confirmed by RT-qPCR as well as western-blot assay. The sequences targeting SUZ12 was indicated in Table S1.

RNA Isolation and RT-qPCR Assays

Total cellular RNA was isolated using Trizol reagents (Invitrogen) following the manufacturer’s protocol. cDNA was generated with the ReverTra Ace qPCR RT Master Mix (TOYOBO) according to the manufacturer’s instructions. cDNA was diluted 1:2 and used for 20μL of quantitative real-time polymerase chain reaction (qPCR) with Ultra SYBR Mixture (with ROX) (Roche). Quantification was performed using the ΔCt method [13], and GAPDH was used as the reference gene. Sequences of primers used were indicated in Table S1.

Western Blot Analysis

Western blot analysis was performed as previously described [13]. The antibodies used in this study were: GAPDH (1:1000; cat no. ab9385, Abcam), TRPS1 (1:500; cat no. sc-26974, Santa Cruz), SUZ12 (1:500, cat no. sc-271325, Santa Cruz), E-cadherin (1:1000, cat no. #3195, CST), N-cadherin (1:1000; cat no. 18203, Abcam) and Vimentin (1:1000; cat no.#5741S, CST).
Migration and Proliferation Assays

After transfection with the indicated shRNA or plasmids, migration assay was performed as previously described elsewhere [13]. Transwell assays were used to measure the migration of cells. Cell proliferation was measured by MTS assays (Promega, USA) [30]. Three individual experiments were performed.

Microarrays and Gene Expression Analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). NimbleGen Human Gene Expression Microarrays (KangChen Bio-tech Inc.) was used to investigate the transcriptional profiles of the T47D-NC and T47D-shTRPS1 cells; the array represented more than 45000 genes (http://www.kangchen.com.cn). Microarray data were deposited to NCBI’s GEO Repository and were accessible to readers through GEO series accession number GSE104062. Differentially expressed genes were identified through fold change screening. Enrichment of gene sets was analyzed using the GSEA tool (Broad Institute, Cambridge, MA, USA) as previously described [31].

Chromatin Immunoprecipitation (ChIP) Assay – qPCR Assay

ChIP was performed using the EZ-Magna ChIP assay kit (Millipore, Billerica, MD, USA) follow the manufacturer’s recommendation. Briefly, 1.0×10⁷ cells were cross-linked with 1% formaldehyde. The cells were then sonicated, pre-cleaned, mixed with magnetic beads and incubated...
with 5-10 μg of antibody (TRPS1, cat no.sc-26974, Santa Cruz; SUZ12, cat no. sc-271325, Santa Cruz; H3K27 tri-methylation, cat no. 07-622, Millipore) per reaction overnight. After DNA purification, the enrichment of the DNA template was analyzed by PCR and RT-qPCR. Sequences of primers used were indicated in Table S1.

Luciferase Assay

Constructs of the SUZ12 luciferase reporter or the mutants were co-transfected with TRPS1 expression plasmids, or siRNAs, into 293T cells for 48h. The pRL-TK Renilla luciferase reporter vector was used as an internal control (Promega); Renilla luciferase activity was used to normalize luciferase activity. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL, USA) and Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). All data are presented as means±SEM, and the Student’s t test.
was used to determine statistical significance. Correlation significance was assessed using chi-square test and Pearson’s correlation coefficient test. \( P < .05 \) was considered statistically significant.

**Results**

**Loss of TRPS1 Significantly Correlated with Metastasis and Prognosis in BC**

To investigate the role of TRPS1 in metastasis of BC, a publicly accessible microarray dataset of BC cell line was analyzed. BT474, MCF-7 and T47D were considered as typical epithelial type [32] and low-metastatic cell lines, MDA-MB-468 and MDA-MB-453 were referred as moderately-metastatic cell lines, and MDA-MB-231 referred as high-metastatic cell lines [33]. We showed TRPS1 expression in low-metastatic (MCF-7, T-47D) or moderately metastatic (MDA-MB-468, MDA-MB-453) cell lines were much higher than that of highly metastatic cell lines (MDA-MB-231). Lower expression of TRPS1 accompanied by increased migration ability of BC cells (Figure 1A).

Next, the expression of TRPS1 was further analyzed in publicly accessible BC datasets. BC patients with high expression of TRPS1 displayed a lower distance metastasis free survival rate (KMP cohort, Figure 1B; \( n = 1746 \), \( P = .0015 \) and GSE25066, Figure 1C, \( n = 508 \), \( P = .000587 \)). There was an observable decrease of TRPS1 expression in lymph node metastasis (LNM)-positive BC patients compared with LNM-negative patients (GSE25066, Figure 1D, \( P = .0103 \)).

Previous study demonstrated that TRPS1 was up-regulated in BC cell line using Cancer Cell Line Encyclopedia (CCLE) analyses [34]. Here we intend to investigate TRPS1 expression in BC patients. We next analyzed TCGA data with online tool FIREBROWS (http://firebrowse.org/). The results identified TRPS1 was distinctively up-regulated in breast tissues especially BC tissue (Figure 1E).

We then examined TRPS1 expression by IHC in a cohort of 117 Chinese BC patients. As shown in Figure 1F, TRPS1 was predominantly located in the nucleus of BC cells. Notably, loss of TRPS1 expression in BC was related to higher histological grade. Higher expression level of TRPS1 was identified in a much larger
proportion of BC cases with histological Grade 1 and Grade 2, as compared with those of Grade 3 (Figure 1G). The IHC results also confirmed that TRPS1 expression was higher in cancer tissues compared with that of adjacent benign breast tissues (Figure S1). We also found TRPS1 mainly located in glandular epithelium layer rather than myoepithelium layer of benign breast duct.

**Down-Regulation of TRPS1 Promoted Migration and EMT of BC Cells**

TRPS1 was suggested to be a negative regulator of EMT, which often accompanied by increased metastasis [15]. We next asked whether TRPS1 promotes metastasis in BC. The protein expression levels of TRPS1 in BC cells were verified by western blot (Figure 2A). We retrovirally silencing TRPS1 in T47D and MCF-7 cells and established over-expression of TRPS1 in MDA-MB-231 and MDA-MB-468 cells. The levels of TRPS1 in these resultant cell lines were also verified by western blot (Figure 2B). As shown in Figure 2C, knockdown of TRPS1 dramatically increased cell migration (Figure 2C, T47D, \(P = .0424\), and MCF-7, \(P = .0359\)). As expected, the migration capacity of the transfected cells significantly reduced in the TRPS1 cDNA-treated groups compared with the control groups (Figure 2D, MDA-MB-468, \(P = .0073\) and MDA-MB-231, \(P = .00443\), respectively). The proliferation assay showed that alteration of TRPS1 expression did not influence cell proliferation in BC cells (Figure 2E).

In an attempt of characterizing the anti-metastasis effect of TRPS1 in BC, we conducted expression microarray profiling of T47D cells with

![Figure 4](image-url)
control or shTRPS1 and carried out Gene Set Enrichment Analysis (GSEA). GSEA revealed that EMT was closely correlated with TRPS1 expression. (Figure 2F, nominal \( P < .01 \), FDR = .015). Western blot confirmed TRPS1 negatively regulated to EMT (Figure 2G). We also found significant correlation between TRPS1 and CDH1 expression levels in human breast tumors (Figure 2H, GSE2990, \( P < .0001 \)). In consisted with previous studies, our results illustrated that TRPS1 functioned as a negative mediator of EMT in BC cells.

Identification of SUZ12 as a Novel Target of TRPS1

We next analyzed CDH1 promoter region with three online tools, including JASPAR (http://jaspar.genereg.net/), Genomatrix (http://www.genomatix.de/) and PROMO (http://alggen.lsi.upc.es/), to identify whether TRPS1 could activate E-cadherin expression by directly binding. The result suggested no binding site of TRPS1 in promoter region of CDH1 (data not shown). TRPS1 thus might regulate E-cadherin expression in an indirect manner. Previous studies showed TRPS1 decreased the level of ZEB2, SERPINE1 but increased the level of FOXA1, all of which were transcriptional mediator of EMT [14–16]. Here we also demonstrated that TRPS1 expression was correlated with expression of ZEB2, SERPINE1 and FOXA1 (Figure 3A). To further explore the additional mechanisms of blockage of EMT by TRPS1, we carried out GSEA analysis. Among all the 189 predefined “oncogenic signatures” gene sets, SUZ12, which was a positive regulator of EMT as the core component of PRC2, was identified to be adversely associated with TRPS1 expression (Figure 3B, nominal \( P < .01 \), FDR = .076). As shown in Figure 3C, SUZ12 mRNA level significantly increased in BC cells with TRPS1 depletion but decreased in cells with TRPS1 over-expression. The regulation of SUZ12 by TRPS1 was further verified at protein level in BC cells (Figure 3D).

We next examined whether TRPS1 expression is relevant to SUZ12 and E-cadherin in vivo. Correlations were analyzed algorithmically. As shown in Figure S2 and Table S2, the expression of TRPS1 was positively related to E-cadherin by IHC. We also found a trend that TRPS1 expression was negatively associated with SUZ12, although this did not reach significant difference. Further investigation is needed using larger population cohort.

TRPS1 Regulates E-Cadherin Expression Through Direct SUZ12 Binding and H3K27 Tri-Methylation

We next asked whether TRPS1 could directly bind to the promoter of SUZ12. Using online tools including JASPAR, Genomatrix and PROMO, we analyzed GATA binding site in SUZ12 promoter. Taking intersection of these data, we obtained two candidate GATA binding sites in a region approximately 1.0kb upstream from the transcriptional start site of the SUZ12 gene(Figure 4A). ChIP assay revealed that antibody against TRPS1 efficiently immunoprecipitated this region in both T47D and MCF-7 cells (Figure 4B).
To confirm direct binding of TRPS1 to the promoter of SUZ12 could inhibit SUZ12 transcription, a luciferase reporter containing the SUZ12 promoter was constructed (Figure 4C). As shown in Figure 4D, TRPS1 decreased the promoter activity while TRPS1 knockdown increased the promoter activity. Mutations in the two TRPS1 binding sites blocked the decrease in SUZ12 promoter activity mediated by TRPS1 over-expression (Figure 4E).

It has been shown that promoter methylation of E-cadherin caused by SUZ12 was important to E-cadherin depletion [27,28]. ChiP-qPCR was performed to validate the recruitment of SUZ12 of CDH1 gene. Importantly, shTRPS1 led to a significant increase of recruited SUZ12 by inhibiting SUZ12. Thereby loss of TRPS1 promoted BC EMT and metastasis.

**Loss of TRPS1 Induced Migration by EMT Program was Attenuated by SUZ12 Knockdown in BC**

To further explore whether SUZ12 knockdown was able to attenuate the increase of migration and E-cadherin expression following TRPS1 knockdown, we transfected T47D-shTRPS1 cells with siRNA targeting SUZ12, and then carried out transwell migration assays. Results showed that SUZ12 silencing blocked the augmentation of migration (Figure 5A), and E-cadherin expression (Figure 5B) reduced by TRPS1 knockdown. We also ectopically expressed TRPS1 and SUZ12 in MDA-MB-231 cells. In contrast, ectopic expression of SUZ12 partially reversed the attenuation of migration (Figure 5C) and E-cadherin expression (Figure 5D) induced by TRPS1 over-expression.

**Discussion**

BC is the primary cause of cancer mortality in women [35]. The 5-year survival rate is merely 23% for women with distant metastasis BC [35]. Metastasis is the major cause of morbidity in patients diagnosed with BC. In the current study, we showed that down-regulation of TRPS1 correlated with poor prognosis in patients with BC.

TRPS1 gene located in human chromosome 8q23-24, which was highly amplified both in breast and prostate cancer. Several putative target genes, such as MYC, PSCA, EIF3S3 and TRPS1, were proposed for the amplification of 8q23-24 [36]. In consistent with other studies, we showed TRPS1 expression was higher in BC tissues than benign breast ductal epithelium in our cohort and TCGA dataset. However, our IHC results indicated that TRPS1 expression significantly decreased in BC cases with high histological grade. Loss of TRPS1 expression in BC related to higher histological grade and more malignant histological morphology. This expression pattern of TRPS1 also existed in BC cell lines. Mild BC cell lines expressed higher TRPS1, while aggressive cell lines expressed lower TRPS1. These data implied that loss of TRPS1 expression might represent an important stage in BC progression.

Several studies have suggested the association of TRPS1 and EMT in malignancy [14–17]. TRPS1 regulates multiple genes in the EMT program. Studies by Stinson et al. demonstrated that miR221/222 targets the 3′ UTR of the TRPS1 mRNA transcript. This degradation led to increased ZEB2 expression as well as increased migration and invasion of tumor cells [14]. Studies by Huang et al showed that down-regulation of TRPS1 by miR-373 acted as a transcriptional activator to promote EMT and metastasis by repressing FOXA1 transcription, expanding upon its previously reported role as a transcription repressor [15]. Triple-negative BC constitutes 10-20% of all BCs and has a higher rate of distal recurrence and a poorer prognosis than other BC subtypes. Studied showed TRPS1 reduced TNBC cells growth and metastasis by inhibiting SERPINE1 and SERPINE2 mediated EMT [16]. In our study, we focused on E-cadherin, the key marker of epithelial phenotype. Down-regulation of TRPS1 in BC cells led to higher SUZ12 expression, and concomitant H3K27 tri-methylation in promoter region of CDH1 gene. This epigenetic signature silenced E-cadherin expression, leading to aggressive phenotype. We speculated that this epigenetic signature of SUZ12 and PRC2 was not restricted in CDH1 promoter and might be genome-wide. This still need more evidences.

TRPS1 may function as a transcriptional repressor or activator, depending on the cell type, stages of development, or pathological conditions [16]. TRPS1 may behave as a transcriptional repressor, and this process does not occur through a passive mechanism of competing for binding to GATA sites in DNA [37]. It requires C-terminal region that encompasses two Ikaros families. The mechanism of TRPS1 as a transcriptional activator is not explicit. Epigenetic consequences of TRPS1 regulation were observed in cell proliferation. TRPS1 was reported to be able to modulate HDAC activity to control histone3 K9, K18 and histone4 K16 acetylation to promote cell proliferation [38,39].

Preliminary, emerging data suggested that the function of TRPS1 in BC was bilateral. TRPS1 expressed in glandular epithelium layer of breast duct. Carcinogenesis of ductal epithelium accompanied with enhanced expression of TRPS1. No evidence revealed whether TRPS1 was a driver gene in this process. However, TRPS1 expression decreased in BC progression and dedifferentiation. Cells with TRPS1 exhibited lower metastasis ability and better differentiation. As a
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prognosis indicator, TRPS1 was also debatable. Studies in colon cancer confirmed that high TRPS1 expression was significantly associated with higher pathological stage and lymph node metastasis [40]. Wu et al also proved higher TRPS1 associated with worse survival in BC [37]. However, NKI295 cohort [15] and GOBO [40]. Wu et al also proved higher TRPS1 associated with worse prognosis indicator, TRPS1 was also debatable. Studies in colon cancer confirmed that high TRPS1 expression was significantly associated with higher pathological stage and lymph node metastasis [40]. Wu et al also proved higher TRPS1 associated with worse survival in BC [37]. However, NKI295 cohort [15] and GOBO [40]. Wu et al also proved higher TRPS1 associated with worse pathological stage and lymph node metastasis [37] and angiogenesis [13].

Conclusions
In conclusion, TRPS1 acted as a tumor suppressor that adversely correlated with distant metastasis in patients with BC. TRPS1 may maintain the expression of E-cadherin by inhibiting the function of SUZ12 (Fig.6). Our data might provide novel insight into how loss of TRPS1 contributed to BC progression.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.01.009.

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