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

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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.

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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].

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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 initiating 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 down loaded 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% CO_2.

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 20mL 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).

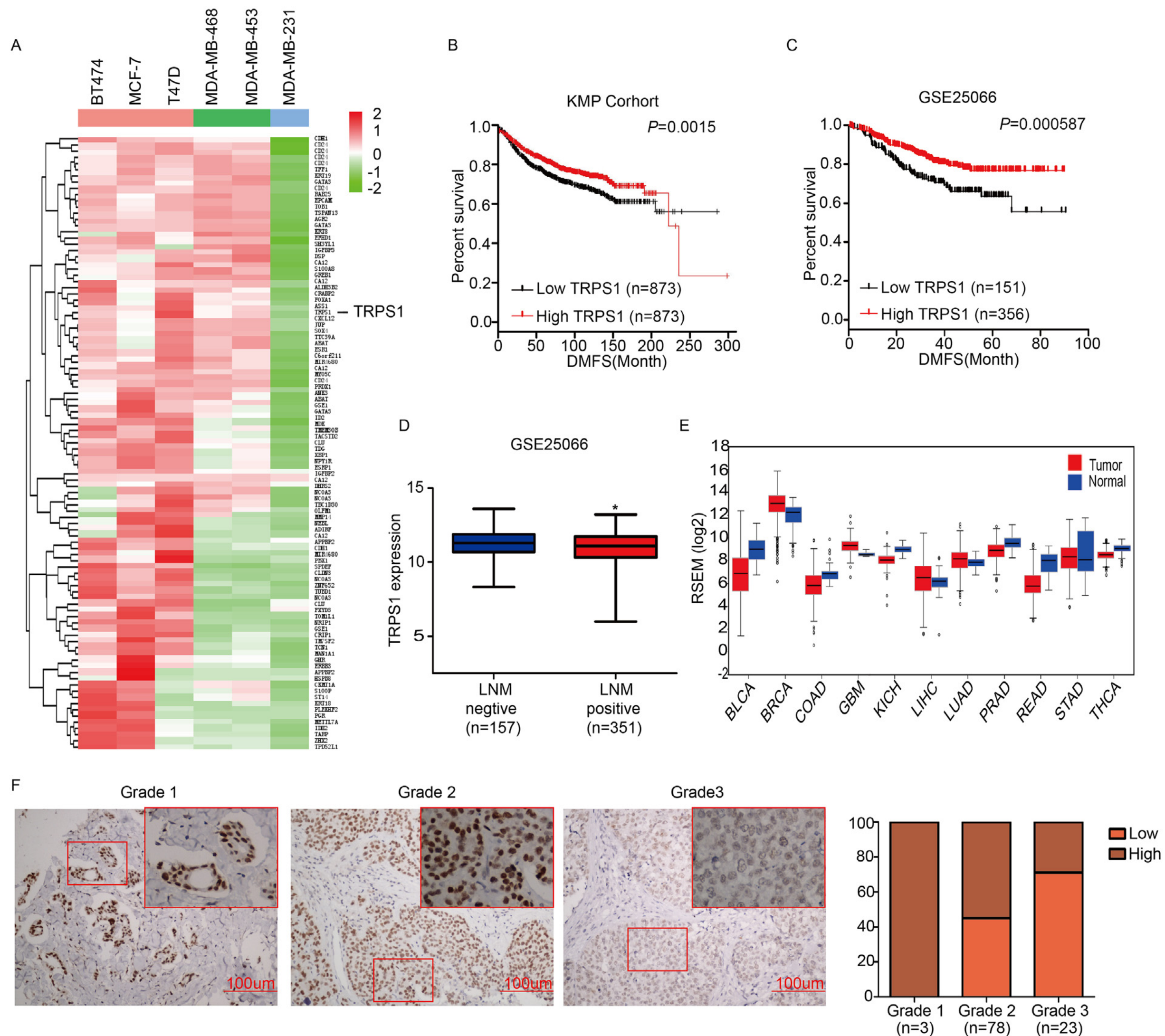

Figure 1. Loss of TRPS1 significantly correlated with metastasis and prognosis in breast cancer. (A) Six common breast cancer (BC) cell lines, selected from MDACC data, were dived into three groups according to the metastatic ability. Heatmap showed the top 100 differential expressed genes in three groups. (B-C) Kaplan–Meier analysis for the disease-free survival rate of patients with BC in TRPS1 low and TRPS1 high groups in the publicly accessible BC KMP cohort and GSE25066. The *P* values for Kaplan-Meier curves were calculated using a log-rank test. (D) The expression of TRPS1 mRNA in BC tissues with LNM and without LNM in GSE25066 ($P = .0103$, based on Student's *t* test). (E) The mRNA levels of TRPS1 in global human cancer tissues (red) and non-tumor tissues (blue) were analyzed using the TCGA database. BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; COAD: Colon adenocarcinoma; GBM: Glioblastoma Multiforme; KICH: Kidney chromophobe; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; STAD: Stomach adenocarcinoma; THCA: yroid carcinoma; (F) Representative immunohistochemistry (IHC) images of TRPS1 expression in Grade 1 to 3 BC tissues. Original magnification, ×200. Scale bar indicates 100 μm. (G) Percentages of different TRPS1 expression levels in Grade 1, Grade 2 and Grade 3 BC tissues.

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 though 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, 1.0×10^7 cells were cross-linked with 1% formaldehyde. The cells were then sonicated, pre-cleaned, mixed with magnetic beads and incubated

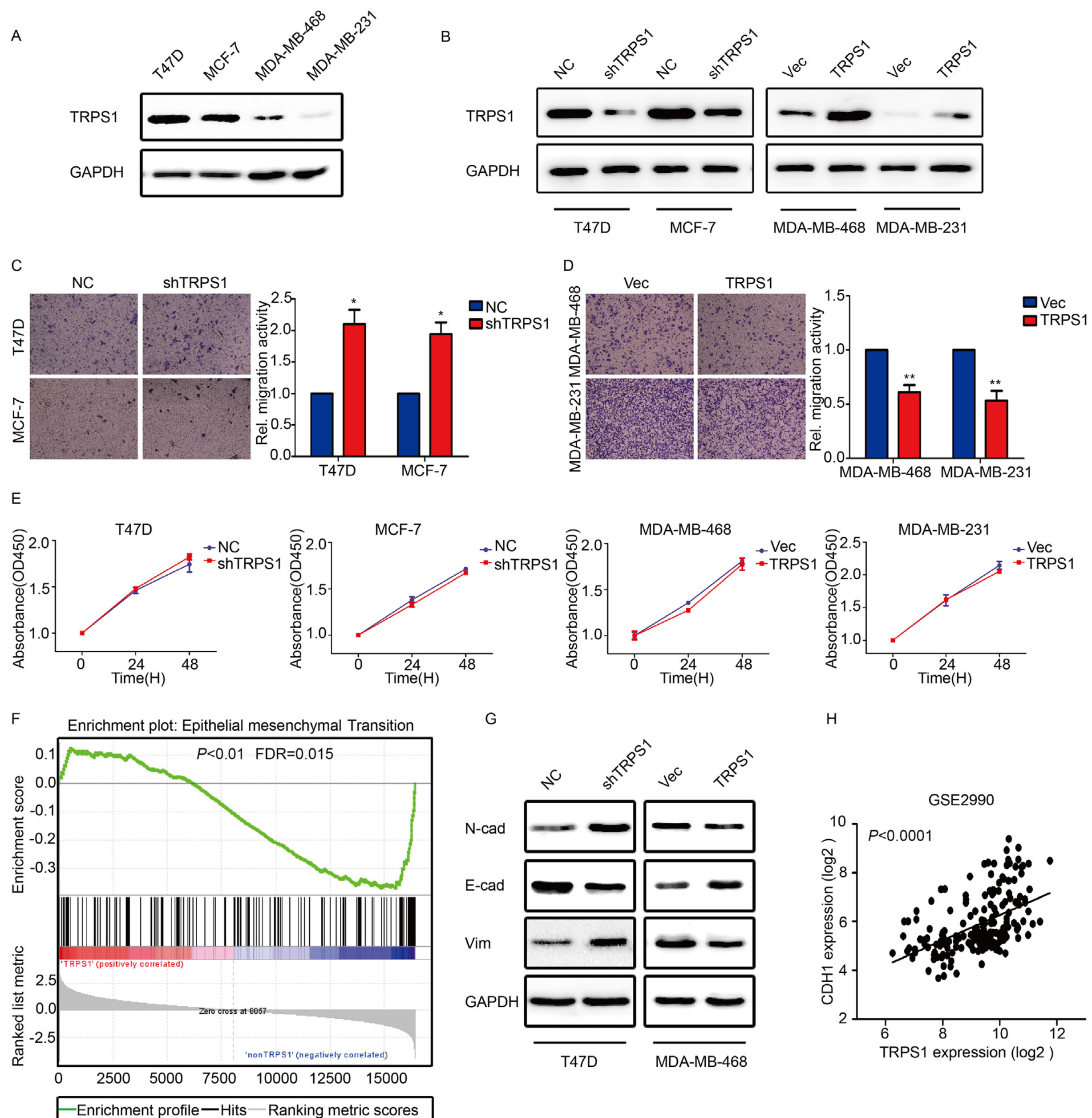

Figure 2. TRPS1 regulates BC cell migration and EMT *in vitro*. (A) The protein levels of TRPS1 in human BC cell lines determined by western blot. (B) Western blot were used to confirm TRPS1 expression in transfected human BC cell lines with different metastatic characteristics. T47D and MCF-7 were transfected with TRPS1-specific shRNA. MDA-MB-468 and MDA-MB-231 were transfected with TRPS1 cloning vector. (C-D) Migration capacity of TRPS1 in transfected BC cell lines T47D, MCF-7, MDA-MB-468 and MDA-MB-231 were examined by transwell migration assay. (E) Cell proliferation was examined by MTS assay. (F) GSEA identified significant association between TRPS1 and EMT. Genes defining epithelial-mesenchymal transition were enriched for shTRPS1. (G) The expression of epithelial marker (E-cadherin) and mesenchymal markers (N-cadherin and Vimentin) in T47D and MDA-MB-468 cells were determined by western blot analysis. GAPDH was used as a loading control. (H) Analysis showing correlation of TRPS1 and CDH1 expression in human breast cancer samples in publicly accessible breast cancer datasets. GSE2990, n = 189,P<0.0001.*P < .05, **P < .01, based on Student's t test.

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 mean±SEM, and the Student's t test

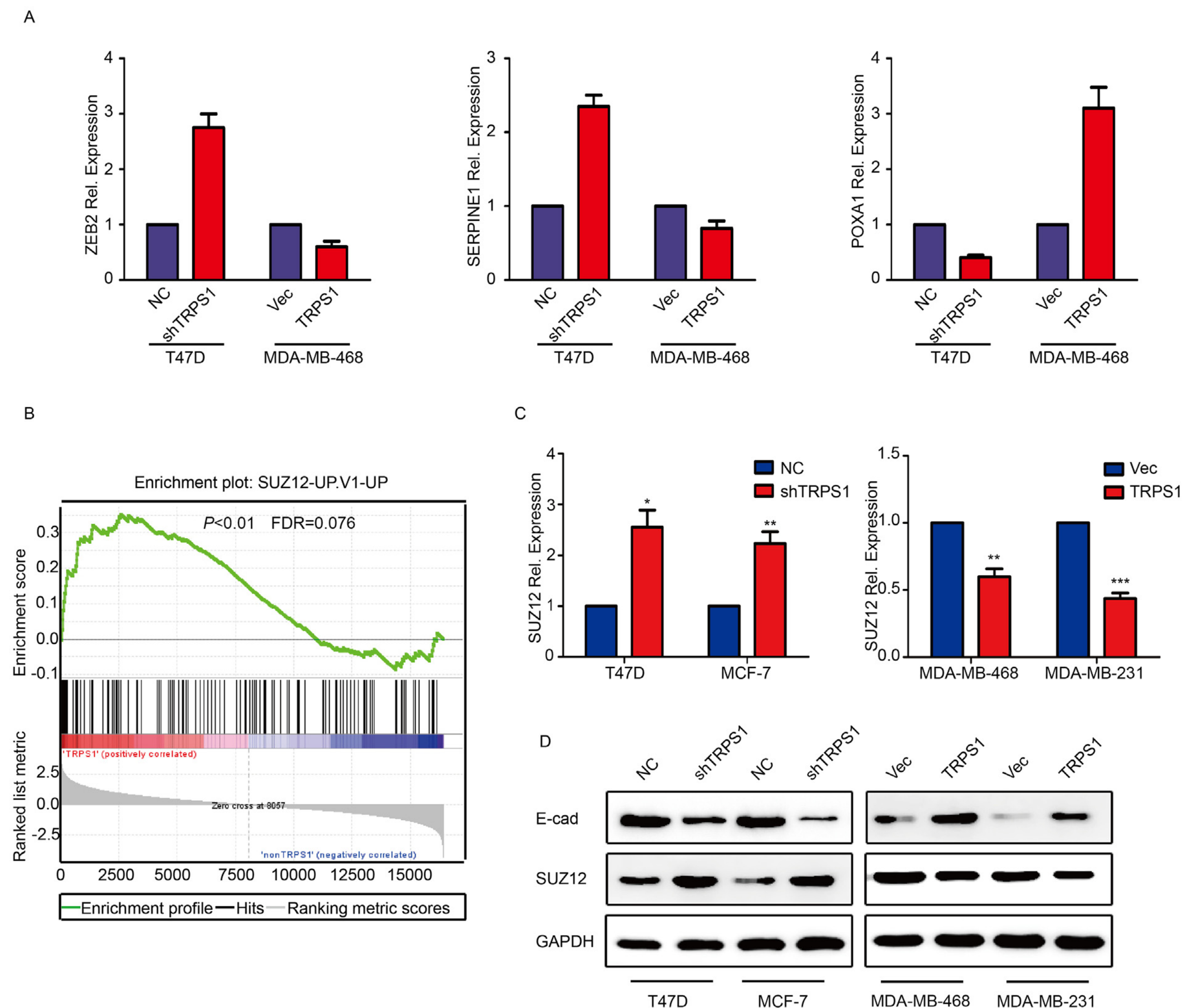

Figure 3. Identification of SUZ12 as a target gene of TRPS1 in BC. (A) mRNA expression of ZEB2, SERPINE1 and FOXA1 were determined in T47D and MDA-MB-468 cells by qPCR. (B) Knockdown of SUZ12 up-regulated gene signatures were enriched for TRPS1. GSEA was carried out to examine the expression of a set of TRPS1-repressed genes in a microarray dataset that profiled T47D cells with control or TRPS1 knockdown. SUZ12-UP.V1-UP: Genes up-regulated upon knockdown of SUZ12 gene. (C) mRNA expression of SUZ12 in transfected breast cancer cell lines were examined by qPCR. (D) Western blot analysis of the expression of E-cadherin and SUZ12 in transfected breast cancer cell lines T47D, MCF-7, MDA-MB-468 and MDA-MB-231.* $P < .05$, ** $P < .01$, based on Student's *t* test.

was used to determine statistical significance. Correlation significance was assessed using chi-square test and Pearson's correlation DE 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 1*A*).

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 1*B*; n = 1746, $P = .0015$ and GSE25066, Figure 1*C*, 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 1*D*, $P = .0103$).

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 1*E*).

We then examined TRPS1 expression by IHC in a cohort of 117 Chinese BC patients. As shown in Figure 1*F*, 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

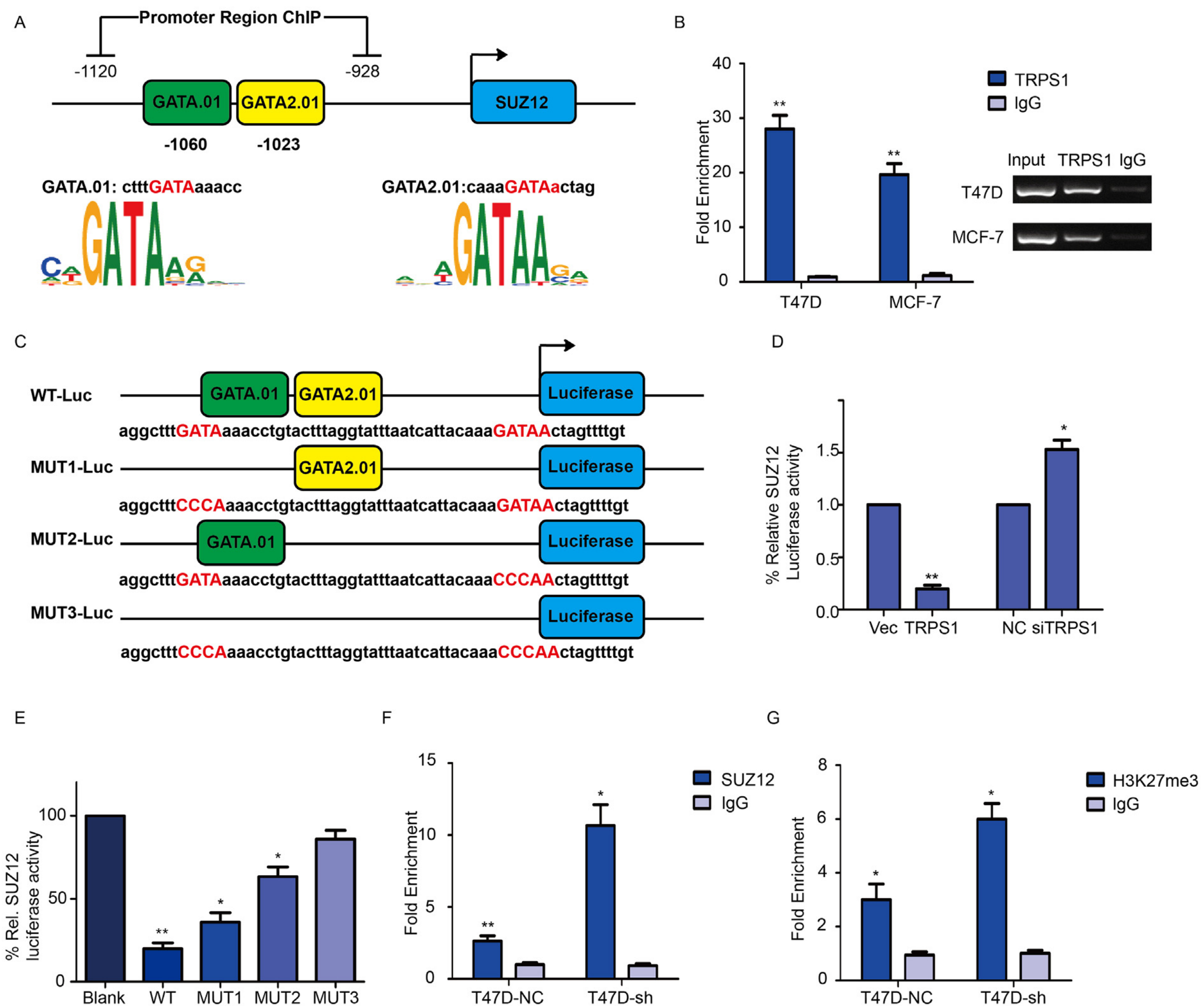

Figure 4. TRPS1 regulates E-cadherin expression through direct SUZ12 binding and H3K27 tri-methylation. (A) Schematic representation of two TRPS1 binding sites in the SUZ12 promoter. (B) ChIP assay with TRPS1 or negative control (IgG) antibodies showed TRPS1 enrichment on SUZ12 promoter in T47D and MCF-7 cells. The y axis represented the fold enrichment of the promoter fragments captured by the two different antibodies. IgG was used as a loading control. (C) Schematic representation of SUZ12 reporter plasmid containing two TRPS1 binding sites. WT-Luc was wild type reporter plasmid; MUT1-Luc was reporter plasmid with GATA.01 binding site mutation; MUT2-Luc was reporter plasmid with GATA2.01 binding site mutation; MUT3-Luc was reporter plasmid with both binding sites mutation. All mutation were performed with GAT changed to CCC using site directed mutagenesis. (D) 293T cells were co-transfected with the SUZ12 reporter plasmid and the indicated concentrations of TRPS1 plasmids, or TRPS1 siRNA, and the luciferase activity was measured. (E) 293T cells were co-transfected with the TRPS1 plasmid and the indicated SUZ12 WT or mutated reporter plasmids, and the luciferase activity was measured. (F) ChIP assay with SUZ12 or negative control (IgG) antibodies showed SUZ12 enrichment on CDH1 promoter in T47D-NC and T47D-shTRPS1 cells. The y axis represented the fold enrichment of the promoter fragments captured by the two different antibodies. IgG was used as a loading control. (G) ChIP assay with H3K27me3 or negative control (IgG) antibodies showing H3K27 tri-methylation enrichment on CDH1 promoter in T47D-NC and T47D-shTRPS1 cells. The y axis represented the fold enrichment of the promoter fragments captured by the two different antibodies. IgG was used as a loading control.*$P < .05$, **$P < .01$, based on Student's *t* test.

proportion of BC cases with histological Grade 1 and Grade 2, as compared with those of Grade 3 (Figure 1*G*). 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 2*A*). 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 2*B*). As shown in Figure 2*C*, knockdown of TRPS1 dramatically increased cell migration (Figure 2*C*,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 2*D*, 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 2*E*).

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

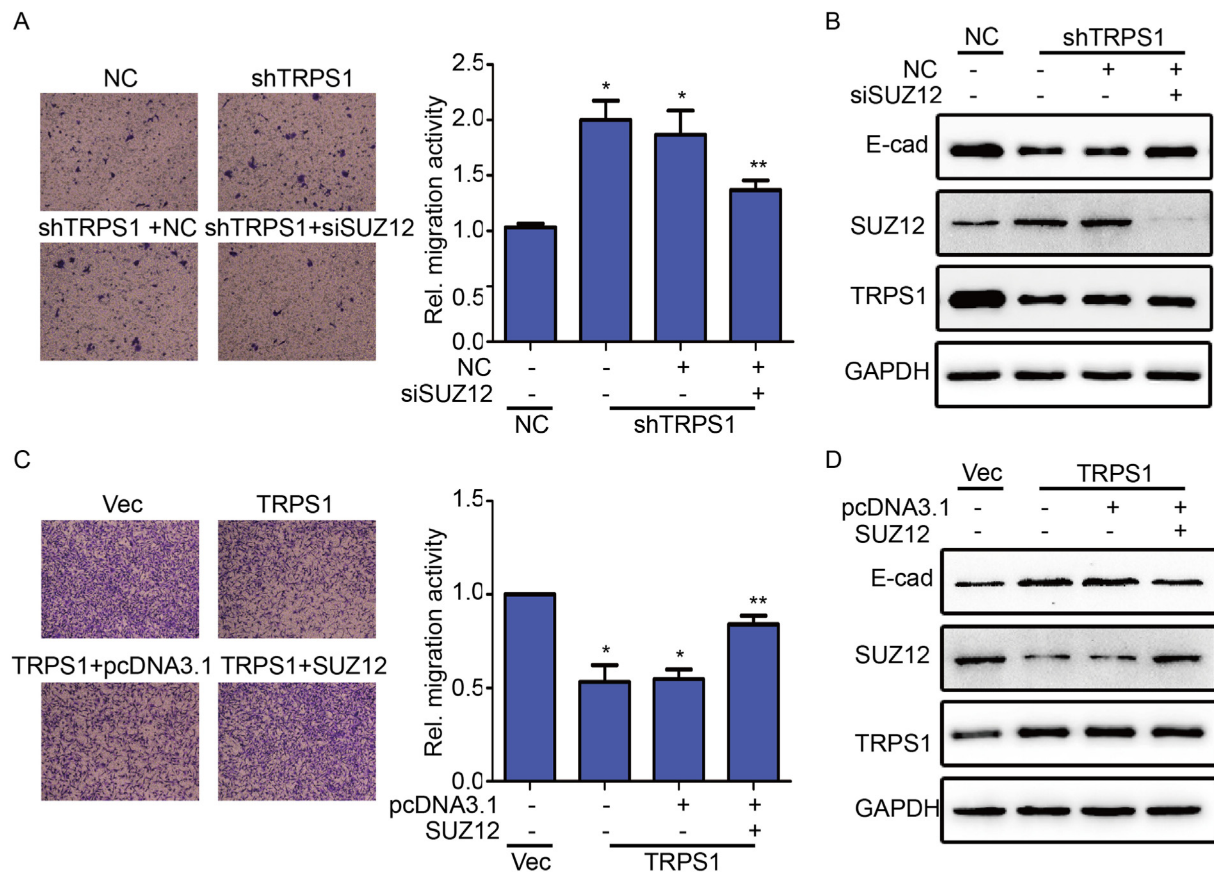

Figure 5. SUZ12 is critical for TRPS1 blocking migration. (A) T47D-NC or T47D-shTRPS1 cells were transiently infected with siSUZ12 or negative control. 48h later migration ability of cells was examined by transwell migration assay. (B) T47D cells were stably/transiently transfected with indicated sh/siRNA respectively. 72 h later, E-cadherin protein levels were determined by western-blot. (C) 231-Vec or 231-TRPS1 cells were transiently infected with plasmid containing either empty control vector (pcDNA3.1) or SUZ12. 48 h later migration ability of cells was examined by transwell migration assay. (D) MDA-MB-231 cells were stably/transiently transfected with indicated plasmids respectively. 72 h later, E-cadherin protein levels were determined by western-blot.**P* < .05, ***P* < .01, based on Student's *t* test.

control or shTRPS1 and carried out Gene Set Enrichment Analysis (GSEA). GSEA revealed that EMT was closely correlated with TRPS1 expression. (Figure 2*F*, nominal $P < .01$, FDR = .015). Western blot confirmed TRPS1 negatively regulated to EMT (Figure 2*G*). We also found significant correlation between TRPS1 and CDH1 expression levels in human breast tumors (Figure 2*H*, 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 3*A*). 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 3*B*, nominal $P < .01$, FDR = .076). As shown in Figure 3*C*, 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 3*D*).

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 4*A*). ChIP assay revealed that antibody against TRPS1 efficiently immunoprecipitated this region in both T47D and MCF-7 cells (Figure 4*B*).

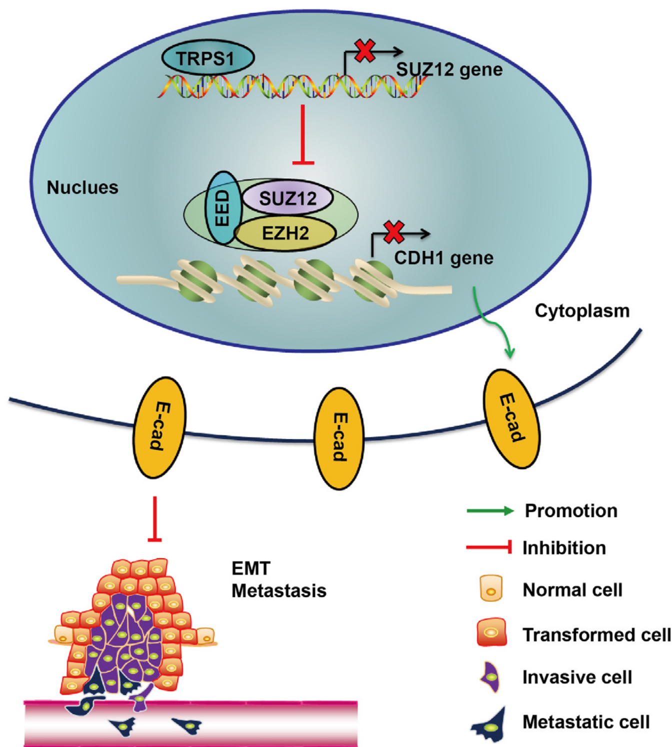

Figure 6. Schematic model of TRPS1 suppress breast cancer progression by SUZ12 repression. TRPS1 contributed to the down-regulation of SUZ12 in BC via transcriptionally repression. SUZ12 further inhibited E-cadherin by H3K27 tri-methylation as subunit of PRC2. TRPS1 maintained the expression of E-cadherin by inhibiting SUZ12. Thereby loss of TRPS1 promoted BC EMT and metastasis.

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 4*C*). As shown in Figure 4*D*, 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 TRPS1over-expression (Figure 4*E*).

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 (Figure 4*F*) and concomitant increase of H3K27 tri-methylation at the CDH1 region (Figure 4*G*).

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 5*A*), and E-cadherin expression (Figure 5*B*) reduced by TRPS1 knockdown. We also ectopicly expressed TRPS1 and SUZ12 in MDA-MB-231cells. In contrast, ectopic expression of SUZ12 partially reversed the attenuation of migration (Figure 5*C*) and E-cadherin expression (Figure 5*D*) induced by TRPS1 over-expression.

Discussion

BC is the primary cause of cancer mortality in woman [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 leaded 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 leaded 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 CDHI 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

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 datasets [34] indicated high TRPS1 predicted better survival. This dual effect on prognosis of TRPS1 due to its function as a positive regulator of cancer cell proliferation [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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