Original Research

TRPS1 expression promotes angiogenesis and affects VEGFA expression in breast cancer

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Abstract

Angiogenesis is a hallmark of the malignant process in breast cancer in which vascular endothelial growth factor A (VEGFA) plays an important role. Trichorhinophalangeal syndrome type 1 (TRPS1) is a GATA-type transcription factor and is involved in trichorhinophalangeal syndrome type 1. To investigate the role of TRPS1 in breast cancer angiogenesis, we analyzed the expression of TRPS1 and microvessel density (MVD) marker CD31 by immunohistochemistry in 117 paraffin-embedded breast tissues. TRPS1 expression was positively correlated with CD31. We further investigated whether TRPS1induces human umbilical vein endothelial cell (HUVEC) migration and VEGFA expression of breast cancer cells. The over-expression of TRPS1 induced a significant increase in HUVEC migration accompanied by VEGFA up-regulation in transfected cells. In contrast, knockdown of TRPS1 decreased the induction of HUVEC migration and significantly down-regulated VEGFA expression. Furthermore, endogenous TRPS1 was present in the VEGFA promoter, as determined by chromatin immunoprecipitation assay. Taken together, this study showed that TRPS1 promotes angiogenesis and affects VEGFA expression in breast cancer.

Keywords: Angiogenesis, breast cancer, TRPS1, VEGFA, transcription factor

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Introduction

Angiogenesis is a hallmark of the malignant process, especially in solid tumors.[1] Breast cancer growth and metastasis, which account for the majority of deaths, are both dependent on angiogenesis.[2] Neovasculature provides nutrients and oxygen for the tumor and removes metabolic waste and carbon dioxide. Tumor cells are also distributed to distant tissues by intravasation into circulation, and subsequent colonization and growth form metastatic tumor masses at distant sites.[3,4]

The angiogenic process is well-regulated by a complex growth factor network, including vascular endothelial growth factor (VEGF), fibroblast growth factor,[5] and platelet-derived growth factor.[6] Physiologic angiogenesis only transiently occurs as a response to angiogenic signals from tissues, such as female reproductive organs and injured organs. However, during tumorigenesis, tumor cells outgrow the diffusion limit for oxygen of nearby blood vessels and become hypoxic. This phenomenon results in activation of pathologic angiogenesis, which causes normally quiescent vasculature to sprout new vessels. This process helps sustain the expansion of neoplastic growths.[4,7]

The VEGF family, which stimulates endothelial cell survival, proliferation, and migration, is the most extensively studied angiogenic factor and most potent angiogenesis regulator in human carcinogenesis.[8] This family consists of five glycoproteins, namely vascular endothelial growth factor A (VEGFA), VEGFB, VEGFC, VEGFD (also known as c-fos induced growth factor), and placental growth factor. Notably, MCF-10A cells without VEGF expression cannot stimulate an angiogenic response in NUDE mice.[9] Anti-VEGF therapy using small inhibitors or monoclonal antibodies inhibits pathologic angiogenesis.[10] Unfortunately, this approach is unsatisfactory in multiple tumor types with regard to overall survival[11] and is associated with an increased number of serious side effects, including stroke, wound healing complications, and organ damage or failure.[12] Therefore, there is an urgent need to identify other biomarkers and major factors involved in regulating angiogenesis.

A number of reports suggest that a new GATA family transcriptional regulator called trichorhinophalangeal syndrome type 1 (TRPS1) is highly overexpressed in breast cancer.[13,14] TRPS1 induces the epithelial-to-mesenchymal transition (EMT) by releasing the negative regulation of ZEB2 as a target of miR-221/222 in breast cancer.[15,16] Here we further study the role of TRPS1 in angiogenesis and show that TRPS1 has stimulated effects on angiogenesis in breast cancer.

Materials and methods

Tumor samples

Samples from 117 patients with invasive ductal breast cancer were analyzed at the Department of Pathology, Qilu Hospital, from 2006 to 2009. Patient consent and approval from the Institutional Research Ethics Committee of Shandong Medical University were obtained. The tumor samples were fixed in 4% phosphate buffered formaldehyde directly after the operation and embedded in paraffin.

Immunohistochemistry

We performed immunohistochemistry to investigate altered protein expression in 117 paraffin-embedded breast tissues as described previously.[17] In brief, paraffin-embedded breast cancer sections (4 μm thickness) were deparaffinized in xylene, rehydrated with a grade series of ethanol, subjected to antigen retrieval, and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. The slides were incubated in antibodies to TRPS1 (diluted 1:200; sc-26974, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and CD31 (ZA-0568, Zhongshan Biotechnology Company, Beijing, China) overnight at 4°C. The secondary antibodies were from PV9000/PV9003 IHC reagent kit (Zhongshan Biotechnology Company). Sections were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. For negative controls, the antibodies were replaced with PBS.

Measurement of microvessel density and evaluation of TRPS1 staining

Microvessel density (MVD) was calculated by first identifying the areas of highest vascularization with × 100 magnification in the periphery of CD31 tumor slides with immunohistochemical staining. MVD was determined by counting the number of vessels with × 400 magnification in two independent fields per tumor.[18] Vessels to be counted were identified by CD31. Less than one vessel in average was considered negative. TRPS1 staining was evaluated in the nucleus. In brief, staining was scored semi-quantitatively for intensity (0 = no expression; 1 = weak; 2 = moderate; and 3 = strong) and percentage of positive cells (0 = 0% to 10%; 1 = 20% to 30%; 2 = 30% to 50%; 3 = 50% to 80%; and4 = 80% to 100%). The final score of TRPS1 was the staining score multiplied by the percentage of positive cells. The following cut-off levels were applied: 0–1 for negative, 2–4 for weak positive, and 5–12 for positive.

Cell culture and transfection

Human breast cancer cell lines (MDA-MB-231 and T47D) and human umbilical vein endothelial cell (HUVEC) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained at 37°C in a humidified incubator supplied with 5% CO_2. Lentivirus with TRPS1-specific shRNA was purchased from Shanghai Gene Pharma Co., Ltd. shRNA oligonucleotide sequences targeted at TRPS1 were as follows: forward, 5′-GATCCCC GGCCTTTAAACATCATTAA TTCAAGAGA TTAATGATGTTTAAAGGCC TTTTTGGAAA-3′ and reverse 5′-AGCTTTTCCAAAAA TTAATGATGTTTAAAG GCC TCTCTTGAA GGCCTTTAAACATCATTAA GGG-3′. The human TRPS1 cloning vector was purchased from Open Biosystems (MHS6278-211690440, Thermo Fisher Scientific, Lafayette, 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.[19] Vectors were transfected using a Thermo Scientific Turbofect Transfection Reagent (Thermo Scientific Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. T47D cells transfected with lentivirusare are referred as T47D-nc and T47D-TS, and MDA-MB-231 cells transfected with vectors are referred as 231-vector and 231-TRPS1.

Chemotactic migration assay

Chemotactic migration of cultured HUVEC was assayed using a Transwell chamber with 8 μm pores (24-well insert; Corning Costar, NY, USA). Approximately 2×10^5 breast cancer cells were seeded in 24-well plates and cultured incomplete DMEM and Leibovitz's L-15 for 24 h prior to treatment. The culture supernatants were added in the lower chamber after centrifugation. HUVEC were suspended at a density of 1.25×10^5 cells/mL, and 200 μL was placed on the upper chamber. The chamber was incubated at 37°C for 16 h before the removal of filters, fixation, staining with crystal violet, and counting at ×200 magnifications by light microscopy. We did not consider the margin containing remaining cells that could not be rinsed. Three individual experiments were performed.

Quantitative real-time polymerase chain reaction

Total cellular RNA was isolated using RNAiso Plus (Takara, Dalian, China) following the manufacturer's protocol. cDNA was generated with the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. cDNA was diluted 1:2 and used for 20 μL of quantitative real-time polymerase chain reaction (qPCR) with UltraSYBR Mixture (with ROX) (Beijing CoWin Biotech Co., Ltd., China). Quantification was performed using the ΔΔCt method, and β-actin was used as the reference gene. The following primers were used: β-actin forward primer, 5′-CTCCATCCTGGCCTCG CTGT-3′, reverse primer, 5′-GCTGTCACCTTCACCGTT CC-3′; TRPS1 forward primer, 5′-CAAATCTCAGGCCT GAGTGA-3′, reverse primer, 5′-GTGAAGAGCTGATATC CTGCAG-3′; and VEGFA forward primer, 5′-AGGAG GGCAGAATCATCACG-3′, reverse primer, 5′-CAAGGCC CACAGGGATTTTCT-3′.

Western blot analysis

Cells were lysed on ice in RIPA buffer containing 0.1 mM phenylmethylsulfonyl fluoride. Lysates were mixed with reducing sample buffer, boiled, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and blotted

on polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk powder in TBS at room temperature for 2 h and incubated with primary antibodies at 4°C overnight. The blots were washed with TBS containing 0.1% Tween20. The blots were then probed again with species-specific secondary antibodies coupled with horseradish peroxidase. Immunoreactivity was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore). The antibodies were specific for β-actin (diluted 1:1000; Zhongshan Biotechnology Company), VEGFA (diluted 1:1000;Abcam), and TRPS1 (diluted 1:500; Santa Cruz Biotechnology, Inc.).

Enzyme-linked immunosorbent assay

Breast cancer cells were seeded in 24-well plates at a concentration of 4×10^5 cells/mL and a final volume of 500 μL. The culture supernatants were collected after 24 h of incubation at 37°C in 5% CO_2. The supernatants were assessed for VEGFA levels using an enzyme-linked immunosorbent assay (ELISA) kit (NeoBioscience, Beijing, China) according to the manufacturer's instructions. The sample absorbance was read on a Bio-Rad microplate reader (Hercules, Calif., USA) at 450 nm. A standard curve was plotted to calculate the VEGFA concentrations.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP kit (Beyotime, Nantong, China). In brief, 1×10^6 cells were treated with 1% formaldehyde for 10 min for cross-linking, and chromatin samples were immunoprecipitated using an anti-TRPS1 antibody (Santa Cruz Biotechnology, Inc.) or control rabbit IgG antibody (Beyotime) after ultrasonication. DNAs isolated from ChIP assays were analyzed by semi-quantitative PCR. PCR was performed with the following region-specific primers for the VEGFA promoter: –1210 bp to 1200 bp GATA-binding site: 5′-GCTGAGACGAAACCCCCATT-3′ and 5′-GCCAGCACTAAGGAACGTCT-3′. The binding sites were predicted by JASPAR CORE database and TFSEARCH. The specific primers were designed by Primer-BLAST of NCBI.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (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 was used to determine statistical significance. Pearson Chi square test or Fischer's exact test was used to evaluate the correlation in immunohistochemical analysis, if appropriate. $P < 0.05$ was considered statistically significant.

Results

TRPS1 expression positively correlates with MVD

TRPS1 expression was observed in 48 (41%) of 117 breast cancer tissues, whereas 69 cases (59%) did not exhibit TRPS1 expression. To investigate the correlation, we stained for CD31, which is an MVD marker (Table 1); 75% of the 48 TRPS1-positive cases were also MVD positive, while only 56.5% of TRPS1-negative cases were. Therefore, the immunohistochemical expression of TRPS1 correlated with CD31 ($P = 0.001$). These results were further confirmed by Spearman correlation analysis ($R = 0.313$, $P = 0.001$). These data indicate that TRPS1 over-expression contributes to MVD in breast cancer. However, MVD was not up-regulated with increasing TRPS1 expression in CD31-expressing cases.

TRPS1 expression related to breast cancer cells induces HUVEC migration

To determine whether TRPS1 expression affects the ability of breast cancer cells to induce HUVEC migration, we performed a chemotactic migration assay of HUVEC. HUVEC were exposed to either supernatants of negative control cells or transfected cells. In the MDA-MB-231 group, transfection of the TRPS1 expression vector was related to the remarkable increase in HUVEC migration toward the breast cancer cells ($P < 0.0001$; Figure 2a and c. In contrast, T47D-TS showed a decreased ability to induce HUVEC migration compared to T47D-nc ($P < 0.01$; Figure 2b and d).

TRPS1 regulates VEGFA mRNA in breast cancer cells

We investigated the effect of TRPS1 expression on VEGFA mRNA expression by qPCR. The VEGFA mRNA level in 231-TRPS1 was increased over that of 231-vector ($P < 0.05$; Figure 3a). In T47D-TS VEGFA, the mRNA level was clearly decreased compared to T47D-nc ($P < 0.05$; Figure 3b).

Table 1 Correlation between trichorhinophalangeal syndrome type 1 (TRPS1) expression and CD31 in human breast cancer

		TRPS1expression					
CD31	n	≤1	>1	*P* value	Spearman correlation	value (*R*)	*P* value
All cases	117						
Negative	51	39	12	0.001		0.313	0.001
Positive	66	30	36				

The Spearman correlation was used to compare the degrees of correlation. Positive numbers reflected direct correlation, and negative numbers reflected inverse correlation.

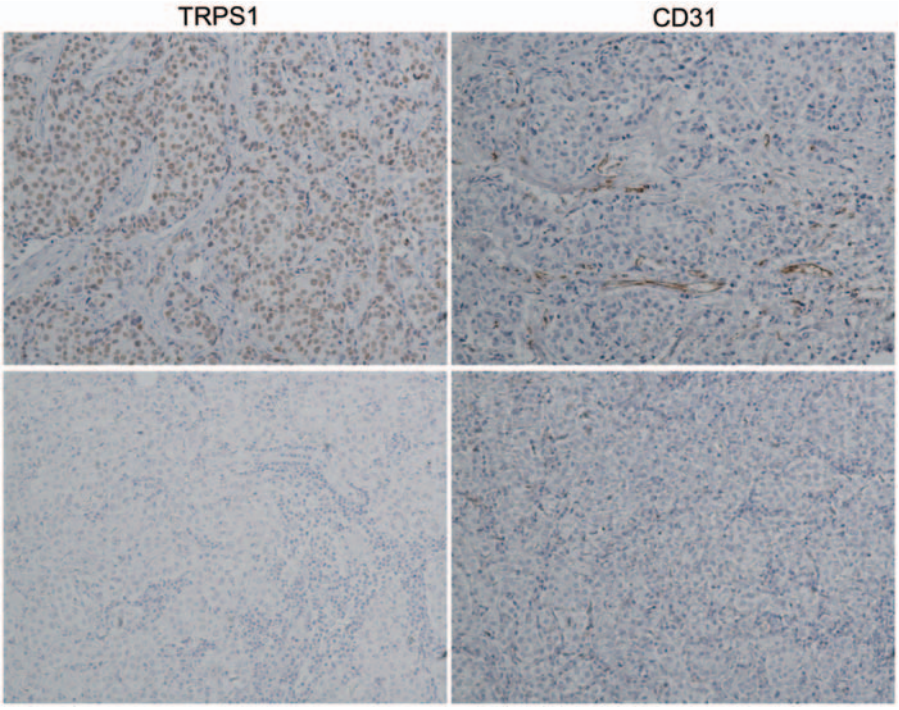

Figure 1 Expression of trichorhinophalangeal syndrome type 1 (TRPS1) and CD31 in human breast cancer. Representative fields of view from breast cancer tissue sections show examples of positive TRPS1 and CD31 (upper row, ×200) and negative TRPS1 and CD31 (lower row, ×200). TRPS1 exhibited nuclear immunoreactivities, and CD31 showed the vessels. The positive group tissues were from a single patient, and the negative group was from another patient. Correlations between both markers from these human cases were analyzed algorithmically. Results are shown in Table 1. (A color version of this figure is available in the online journal.)

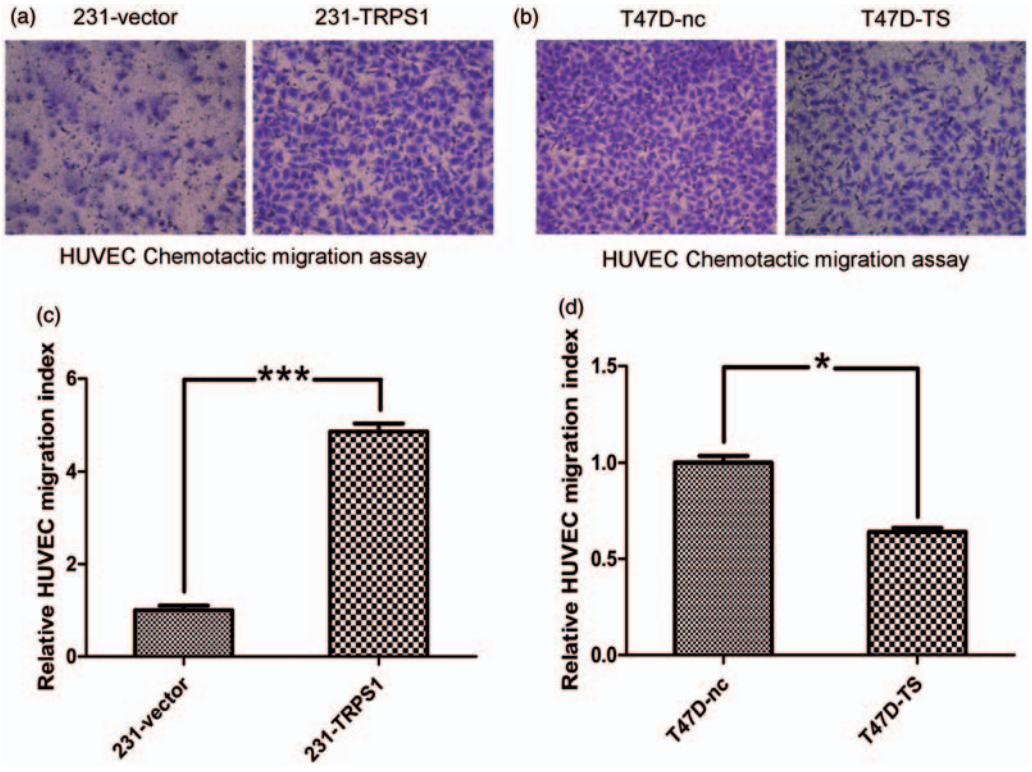

Figure 2 Trichorhinophalangeal syndrome type 1 (TRPS1) expression induces human umbilical vein endothelial cell (HUVEC) migration in breast cancer cells. (a) Over-expression of TRPS1 in MDA-MB-231 cells induced the increase in HUVEC migration. (b) HUVEC migration decreased according to TRPS1 knockdown in T47D cells. (c, d) Histograms indicate significant changes in HUVEC migration. Data are presented as mean ± SEM. P (*) < 0.05, (**) < 0.01. (A color version of this figure is available in the online journal.)

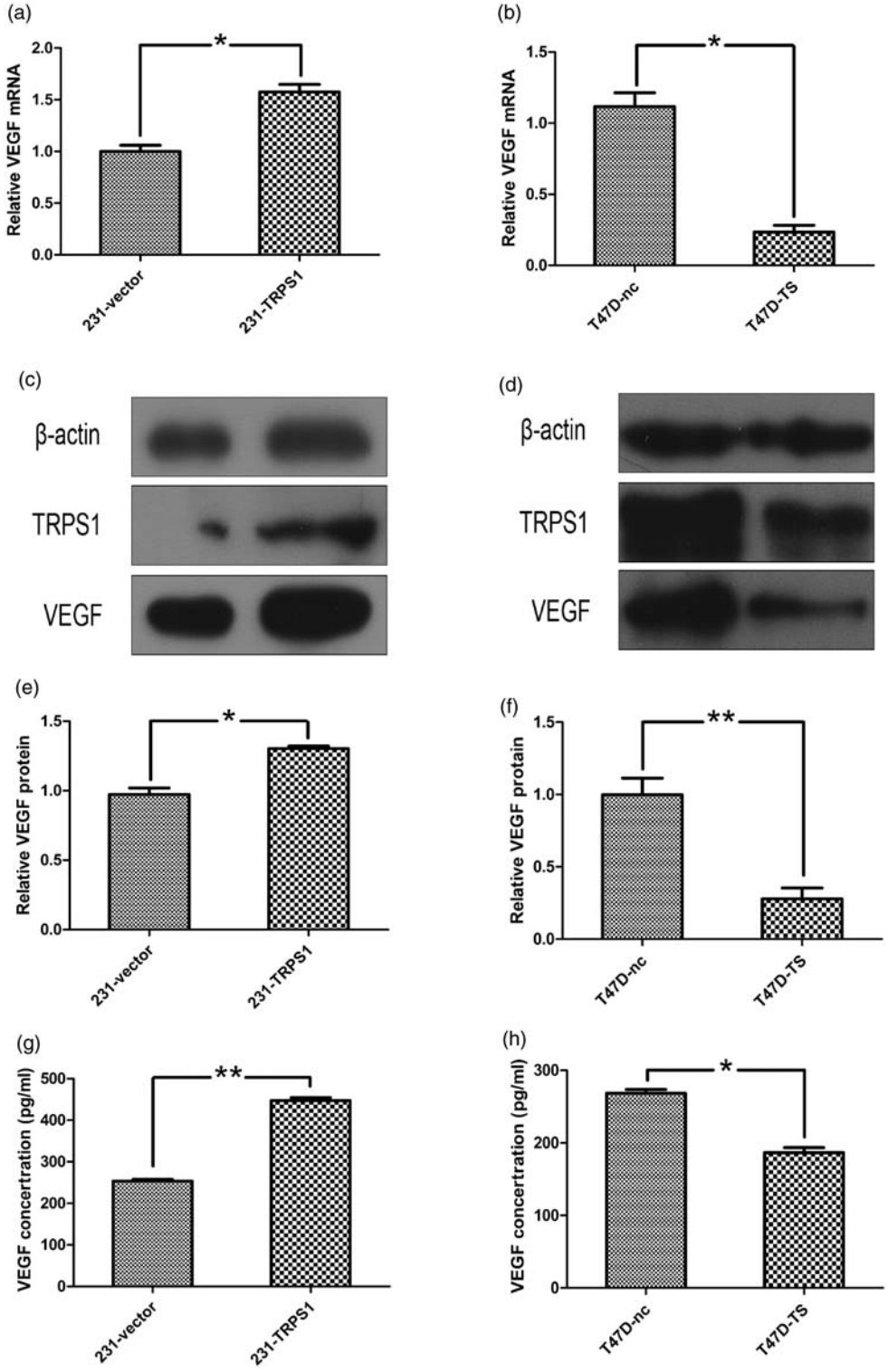

Figure 3 mRNA and protein expression of vascular endothelial growth factor A (VEGFA) correspond to TRPS1. (a) VEGFA mRNA was upregulated following the transfection of TRPS1 expression vector into MDA-MB-231. (b) Compared withT47D-nc, VEGFA mRNA was remarkably decreased. (c, e) The protein level of 231-TRPS1 VEGFA was higher than that of 231-vector. (d, f) VEGFA protein showed a significant decrease that corresponded to TRPS1 knockdown in T47D. (G, H) The VEGFA protein level in cell culture medium analyzed by ELISA was consistent with that of cell lysates both in MDA-MB-231 and T47D

TRPS1 regulates VEGFA protein both in supernatant and cell lysates

Western blot analysis demonstrated that the VEGFA protein level was significantly higher in 231-TRPS1 than that of 231-vector ($P < 0.05$; Figure 3c and e). The VEGFA protein level was lower in T47D-TS than that of the negative control cells ($P < 0.01$; Figure 3d and f). The change in VEGFA protein expression was consistent with that in the VEGFA mRNA level.

To understand the significant differences in HUVEC migration when exposed to different transfected cells, we measured the VEGFA concentrations in the supernatants of breast cancer cells by ELISA. The MDA-MB-231 cells secreted more VEGFA after the transfection of the TRPS1 expression vector ($P < 0.01$; Figure 3g). Knockdown of TRPS1 in T47D significantly inhibited VEGFA secretion compared to the negative control ($P < 0.05$; Figure 3h).

TRPS1 directly regulates VEGFA expression by binding to its promoter region

Given that the transcription factor can influence gene expression directly or indirectly, we investigated whether endogenous TRPS1 is present in the VEGFA promoter. JASPAR CORE database and TFSEARCH indicated that VEGFA promoter −1210 bp to 1200 bp region was possibly bound by TRPS1. Formaldehyde was used to cross-link protein-DNA complexes, and chromatin fragments were subjected to immunoprecipitation with antibodies against endogenous TRPS1. Chromatin fragments immunoprecipitated without antibodies were used as input positive controls, and chromatin fragments immunoprecipitated with non-specific normal rabbit IgG were used as negative controls. As shown in Figure 4, TRPS1 antibody immunoprecipitated the predicted region.

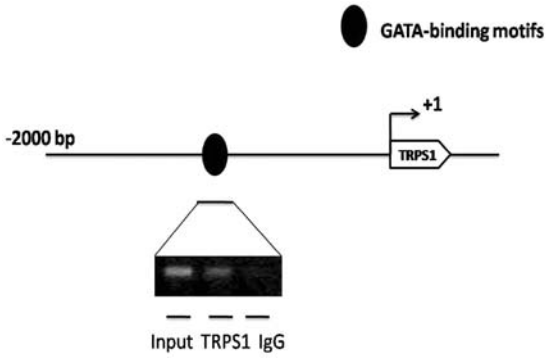

Figure 4 *In vivo* recruitment of trichorhinophalangeal syndrome type 1 (TRPS1) protein at the promoter region of VEGFA. The localization of predicted TRPS1 consensus binding sites in the VEGFA promoter region is indicated with a black oval. Protein–DNA complexes were *in vivo* formaldehyde-cross-linked in T47D cells. Chromatin fragments were subjected to immunoprecipitation with antibodies against endogenous TRPS1. A negative control using non-specific normal rabbit IgG was also included. After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR using the primer pairs that span the reported regions of the VEGFA promoter. Chromatin immunoprecipitated without antibody was used as an input positive control. Experiments were repeated at least three times, and representative images are shown

Discussion

In this study, we showed that TRPS1 is related to breast cancer angiogenesis and VEGFA expression. The TRPS1 gene codes for a 1281-amino acid nuclear transcription factor with an unusual combination of different types of zinc finger motifs, seven classical C2H2-type zinc fingers, including one GATA-type DNA-binding domain, and two IKAROS-like zinc fingers.[20,21] GATA factors serve diverse roles in embryogenesis, cell differentiation, regulation of tissue-specific genes, and carcinogenesis.[22] Over-expression of GATA-3 reduces tumor angiogenesis in mice.[23] It also has been reported that HUVEC treated with conditioned medium from GATA4-over-expressed mesenchymal stem cells exhibited increased formation of capillary-like structures and promoted migration.[24] Previous studies indicated that TRPS1 functions as a potent GATA4-type transcriptional repressor, which is different from all other vertebrate GATA proteins.[25] We demonstrate that over-expression of TRPS1 is associated with higher MVD and more HUVEC migration in breast cancer.

According to our immunohistochemistry, breast cancer with high TRPS1 expression indicates an enhanced expression of the MVD marker, CD31. This result suggests an angiogenesis process during TRPS1-regulated breast cancer development. We transfected full-length TRPS1 into TRPS1-negative MBA-MD-231 cells to further investigate the mechanism and the effect of TRPS1 on angiogenesis. HUVEC treated with 231-TRPS1-conditioned medium promoted migration. In contrast, we knocked down TRPS1 in the strong TRPS1-expressing T47D cell line, and, in correspondence, the ability of T47D to induce HUVEC migration decreased.

VEGFA is the best characterized member of the VEGF family. This glycoprotein is a homodimeric protein of nearly 45 kDa that also acts as a regulator of vascular permeability.[8,26] VEGFA has an important function in the angiogenesis of breast cancer.[9,27] In this study, we confirmed that TRPS1 promoted VEGFA expression and secretion in MDA-MB-231 and T47D cells. ChIP (using an antibody to TRPS1) of the VEGFA promoter from T47D cell lysates indicated a direct endogenous TRPS1 influence on VEGFA expression. Thus, our findings provide new insights on angiogenesis regulation in breast cancer cells.

Breast cancer is the second most common cancer worldwide and the leading cause of cancer deaths in women.[28] Breast cancer growth and metastasis, which account for the majority of deaths, are both dependent on angiogenesis.[3] Targeting angiogenesis is a promising treatment to breast cancer.[29] In this regard, identification of new genes showing specific expression and high prevalence in breast cancer may uncover new molecular regulation of angiogenesis that can be targeted for cancer treatment. RNA expression analysis of histologically diverse breast tumors has been reported to reveal TRPS1 over-expression in breast cancer with little or no expression in normal tissues.[30] In this study, TRPS1 promoted angiogenesis and VEGFA expression. TRPS1 knockdown was associated with the down-regulation of angiogenesis and VEGFA. TRPS1 may therefore be a promising marker and target for angiogenesis in breast cancer.

In conclusion, this study is the first to provide evidence of the direct influence of TRPS1 on VEGFA expression, which contributes to angiogenesis. Thus, further investigation on the functions of TRPS1 in angiogenesis may provide a promising target for breast cancer treatment.

Authors' contributions: JH and GZ conceived of and designed the overall study. JH designed, performed the qPCR, the western blot, the ChIP, analyzed the data, and

wrote the manuscript. PS performed chemotactic migration assay and edited the manuscript. XW, WL, and HZ performed the immunohistochemistry, histopathology characterization of the human tumor samples, and edited the manuscript. MJ did the statistical data analyses. HZ and WL contributed to the manuscript preparation and revision. All authors read and approved the manuscript for publication.

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