PEA3 activates VEGF transcription in T47D and SKBR3 breast cancer cells

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Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis and a prognostic factor for many tumors, including those of endocrine-responsive tissues such as the breast and uterus. In this study, we found that overexpression of PEA3 could increase VEGF mRNA levels and VEGF promoter activity in human T47D and SKBR3 breast cancer cells. Chromatin immunoprecipitation assay demonstrated that PEA3 could bind to the VEGF promoter in the cells transfected with PEA3 expression vector. PEA3 small interfering RNA attenuated VEGF promoter activity and the binding of PEA3 to the VEGF promoter in T47D and SKBR3 cells. These results indicated that PEA3 could activate VEGF promoter transcription.

Keywords PEA3; VEGF promoter; transcription regulation

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Introduction

Angiogenesis is the formation of new blood vessels from a pre-existing endothelium, which involves proliferation of capillary endothelial cells and their migration toward the angiogenic stimulus [1–3]. Vascular endothelial growth factor (VEGF) plays a pivotal role in the induction of increased microvascular permeability and in angiogenesis [4,5]. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and to the etiology of several additional diseases that are characterized by abnormal angiogenesis. Consequently, inhibition of VEGF signaling abrogates the development of a wide variety of tumors [6,7].

Some transcription factors can influence VEGF transcription. It is reported that the effects of estradiol on VEGF expression in human cancer cells involve estrogen receptor alpha (ER alpha) interactions not with estrogen response elements but with Sp1 and Sp3 on a proximal, GC-rich segment (−66 to −47) of the promoter [8,9]. VEGF expression is also strongly induced in cells by hypoxia, and this occurs via a hypoxia response element on the VEGF promoter, which binds the transcription factor hypoxia-inducible factor 1 [10]. ER alpha induces VEGF transcription activation, and BRCA1 significantly inhibits VEGF gene transcription activation and VEGF protein secretion via direct interaction between BRCA1 and the estrogen receptor [11]. PEA3 is also a transcription factor, and so far there are no reports as to whether PEA3 can activate VEGF transcription.

The purpose of this study is to explore the activity of PEA3 on VEGF transcription and to reveal the role of PEA3 involved in VEGF-mediated angiogenesis. Our results showed that PEA3 played an important role in inducing VEGF promoter activity by directly binding to the VEGF promoter, which is helpful to understand the angiogenic mechanism.

Materials and Methods

Cell lines, culture, plasmids, and transfection
Human breast cancer cell lines, T47D and SKBR3, were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were checked routinely and found to be free of contamination by Mycoplasma or fungi.

VEGF promoter/luciferase construct pGL3-VEGF (−2352 to +955) was kindly provided by Dr Lee...
M. Ellis (Department of Surgical Oncology, UTMD Anderson Cancer Center, Houston, USA). PEA3 expression vector was kindly provided by Dr Hassell (Department of Biology, McMaster University of Canada). Transfections were conducted by the lipofectamine method. Briefly, for transient transfection, cells were seeded in six-well plates at a density of 4 × 10^5 cells/well. The following day, cells were transfected with 4 μg of PEA3 expression vector or pcDNA3 using Lipofectamine 2000 (Gibco BRL, Carlsbad, USA). Following transfection, cells were maintained in RPMI 1640 medium containing 10% FBS and cultured for 48 h.

**Reverse transcription–polymerase chain reaction**

Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.) in a final volume of 20 μl containing 5 μg of total RNA, 200 ng of random hexamers, 1× reverse transcription buffer, 2.5 mM MgCl₂, 1 mM deoxynucleotide triphosphate mixture, 10 mM DTT, RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 50 U of superscript reverse transcriptase, and diethylpyrocarbonate-treated water. After incubation at 42°C for 50 min, the reverse transcription reaction was terminated by heating at 85°C for 5 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1.5 mM MgCl₂, 2.5 U of Taq polymerase, 0.5 μM of VEGF primer (5'-GGATGTCTATCAGCGACGCTAC-3'; 5'-TCA CGGCTCGGCTTGTCACATC-3'), and PEA3 primer (5'-CAGCTCAGCTTCTAGGGTC-3'; 5'-CTCTCTCT GCTTATACCAGC-3'). The GAPDH primer (5'-G CCAAAAAAGGTGTCATCATCTC-3'; 5'-GTAGAGGCGG GATGATGTTT-3') was used as an internal control. Amplification cycles were: 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, followed by 72°C for 10 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChiP) assays were carried out according to the manufacturer’s protocol (Active motif, Carlsbad, CA, USA). Briefly, cells in 150 mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37°C. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS), harvested, and re-suspended in ice-cold TNT lysis buffer [20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 1% aprotinin]. The lysates were sonicated to shear the DNA to fragments of 200 – 600 bp and subjected to immunoprecipitation with the following antibodies, respectively, PEA3 or IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). For each immunoprecipitation, 3 μg of antibodies were used. The antibody/protein complexes were collected by Protein G beads and washed three times with ChIP washing buffer (5% SDS, 1 mM EDTA, 0.5% bovine serum albumin, and 40 mM NaHPO₄, pH 7.2). The immune complexes were eluted with 1% SDS and 1 M NaHCO₃, and the cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and then DNA was purified by mini-column, ethanol precipitation, and re-suspended in 100 ml of H₂O. The primer corresponding to the VEGF promoter region (−339 to −159) (sense: 5'-AGAGGGGAACGCTCAGGC-3'; antisense: 5'-CTCGCGAGCGCTAGTGAA-3') was used for PCR to detect the presence of the VEGF promoter DNA.

**Small interfering RNA preparation and transfection**

Cells in the exponential phase of growth were seeded in six-well plates at a concentration of 5 × 10⁵ cells/well. After incubation for 24 h, the cells were transfected with small interfering RNA (siRNA) specific for PEA3 [12] (catalog number: 115237) (Ambion, Austin, TX, USA) and non-targeting siRNA at a final concentration of 100 nM using oligofectamine and OPTI-MEMI-reduced serum medium (Invitrogen Life Technologies, Inc.), according to the manufacturer’s protocol. Silencing was examined 48 h after transfection by reverse transcription–polymerase chain reaction (RT–PCR) and western blotting.

**Western blot analysis**

Cells were washed twice with PBS containing 1 mM phenylmethylsulphonyl fluoride, lysed in mammalian protein extraction buffer (Pierce, Rockford, IL, USA). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 g for 40 min at 4°C. Equal amounts (50 μg of protein) of cell lysates were resolved by SDS–PAGE. The proteins were transferred to nitrocellulose membranes. Membranes were incubated in blocking solution consisting of 5% powered milk in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, and then immunoblotted with anti-PEA3
antibody (Santa Cruz Biotechnology, Inc.) or anti-tubulin antibody (Sigma-Aldrich, St Louis, MO, USA), respectively. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Luciferase reporter gene assay
T47D or SKBR3 cells were seeded in six-well plates at a density of $1 - 2 \times 10^5$ cells/well and cultured for 24 h. Cells were then transfected with the VEGF promoter/luciferase construct pGL3-VEGF (0.5 μg/well) or co-transfected with 0.5 μg of pcDNA3.0, or PEA3 expression vector, together with 20 ng of control Renilla luciferase reporter construct, pRL-TK (Promega, Madison, WI, USA). The total amount of DNA per well was adjusted to 1.5 μg by the addition of sonicated salmon sperm DNA. Luciferase assays were performed as recommended by the manufacturer (Promega) and normalized relative to protein concentration determined by bicinchoninic acid protein assay (Pierce).

Results

Overexpression of PEA3 induced VEGF mRNA level in T47D and SKBR3 cells
To identify the role of PEA3 in regulating VEGF transcription, PEA3 expression vector or pcDNA3 was transfected into T47D and SKBR3 cells and VEGF mRNA was detected. Fig. 1(A) showed that as compared with T47D cells transfected with pcDNA3, the level of VEGF mRNA in the cells transfected with PEA3 expression vector increased as determined by reverse transcription–polymerase chain reaction (RT–PCR).

PEA3 activated VEGF promoter activity in T47D or SKBR3 cells
To identify the role of PEA3 in regulating VEGF promoter transcription, we co-transfected the VEGF promoter/luciferase construct with PEA3 expression vector or pcDNA3 in T47D and SKBR3 cells and detected VEGF promoter activity. Fig. 2(A) and (B) showed that the luciferase activity was enhanced by PEA3 both in T47D cells and in SKBR3 cells, further indicating that PEA3 could activate VEGF promoter activity. In this experiment, exogenous PEA3 could activate VEGF promoter activity, suggesting that PEA3 played a role in regulating VEGF transcription.

PEA3 bound to the VEGF promoter in PEA3-overexpressed T47D or SKBR3 cells
To investigate if PEA3 bound to the VEGF promoter in the cells transfected with PEA3 expression vector, we performed ChIP experiments. The results showed that PEA3 could bind to the VEGF promoter both in T47D and SKBR3 cells transfected with PEA3 expression vector [Fig. 3(A) and (B)]. In this experiment, PEA3 could bind to the VEGF promoter in PEA3-overexpressed T47D or SKBR3 cells, indicating PEA3 activated VEGF transcription by binding directly to the VEGF promoter.
PEA3 small interfering RNA inhibited PEA3 expression in T47D or SKBR3 cells

To further identify the role of PEA3 in regulating VEGF transcription, we knocked down the expression of PEA3 with a gene-specific siRNA and measured PEA3 mRNA and protein. As shown in Fig. 4(A) and (B), PEA3 siRNA inhibited PEA3 mRNA significantly in T47D and SKBR3 cells after transfection with PEA3 siRNA for 48 h. As indicated in Fig. 4(C) and (D), PEA3 siRNA inhibited PEA3 protein significantly in T47D and SKBR3 cells after transfection with PEA3 siRNA for 48 h. This experiment indicated that PEA3 siRNA could knock down PEA3 expression efficiently.

PEA3 small interfering RNA repressed VEGF promoter activity in T47D and SKBR3 cells

To determine if the decrease in PEA3 would reduce VEGF gene transcription, we knocked down the expression of PEA3 and measured VEGF promoter activity in normal T47D and SKBR3 cells after transfection with PEA3 siRNA for 48 h. This experiment indicated that when endogenous PEA3 was knocked down by siRNA, the promoter activity of endogenous VEGF also decreased.

PEA3 small interfering RNA attenuated the binding of PEA3 to the VEGF promoter

To determine if the decrease in PEA3 would influence the binding of PEA3 on the VEGF promoter, we knocked down the expression of PEA3 and measured the binding status of PEA3 on the VEGF promoter. As determined in Fig. 6(A) and (B), PEA3 siRNA attenuated the binding of PEA3 to the VEGF promoter in normal T47D and SKBR3 cells after transfection with PEA3 siRNA for 48 h. This experiment showed that when endogenous PEA3 was knocked down by siRNA, the binding of PEA3 to the VEGF promoter decreased, also indicating that PEA3 regulated VEGF transcription by binding directly to the VEGF promoter.

Discussion

The PEA3 subfamily includes PEA3, ER81, and ERM [13–15]. PEA3 may play a role in human breast cancer. The human PEA3 gene is transcriptionally upregulated in breast tumor cell lines [16] and in 93% of HER2/Neu-positive human breast tumors [17]. PEA3 expression is also found in the majority of both clinical specimens and cell lines of lung and oral carcinoma [18–20]. PEA3 can regulate the transcription of several such proteases including the matrix metalloproteinases (MMP) collagenase-IV/gelatinase B (MMP-9), matrilysin (MMP-7), and stromelysin-3 (MMP-11), and the serine protease urokinase-type plasminogen activator [21,22]. However, transfection of oral carcinoma cells with an antisense sequence of PEA3 can result in the inhibition
of invasion and MMP expression [23]. Transfection with PEA3 results in enhanced motility and invasion in lung cancer cells and human SKBR3 breast cancer cells [18,23]. Expression of PEA3 dominant-negative form reduces tumor growth in this model [24]. All these reports indicated that PEA3 is related to cancer motility and invasion, but there are no reports about the relationship between PEA3 and VEGF transcription in breast cancer.

In our studies, overexpression of PEA3 could increase the VEGF mRNA level in T47D and SKBR3 cells. In order to analyse the putative effects of PEA3 on VEGF transcription, we performed luciferase assay, and our results demonstrated that PEA3 activated VEGF promoter activity. ChIP assay demonstrated that PEA3 could bind to the VEGF promoter in the PEA3-overexpressed cells. Bioinformatic analysis of the 5'-flanking region of the human VEGF gene showed that there existed a PEA3 binding site (TTTCCCT) in the VEGF promoter region from −298 to −293. It has been reported that PEA3 could elevate PEG-3 promoter activity by binding to the PEA3 binding site (TTTCCCT) in the PEG-3 promoter [25,26].

A functional interaction between PEA3 and VEGF promoter was strengthened by PEA3 siRNA. We further found that PEA3 siRNA attenuated VEGF promoter activity in T47D and SKBR3 cells. At the same time, PEA3 siRNA attenuated the binding of PEA3 to the VEGF promoter. These results further indicated that PEA3 could influence VEGF promoter activity by binding to the VEGF promoter.

We conclude that PEA3 plays an important role in inducing VEGF promoter activity by directly binding to the VEGF promoter. These investigations are important and offer potential for defining the angiogenic mechanism regulated by VEGF and PEA3. With this information, it will be possible to demarcate potential targets and define appropriate reagents, such as antisense or small molecule antagonists, for inhibiting or preventing cancer development and progression.

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