CXC chemokine receptor 4 (CXCR4) is a cell surface receptor that has been shown to mediate the metastasis of many solid tumors including lung, breast, kidney, and prostate tumors. In this study, we found that overexpression of ets variant gene 4 (PEA3) could elevate CXCR4 mRNA level and CXCR4 promoter activity in human MDA-MB-231 and MCF-7 breast cancer cells. PEA3 promoted CXCR4 expression and breast cancer metastasis. Chromatin immunoprecipitation assay demonstrated that PEA3 could bind to the CXCR4 promoter in the cells transfected with PEA3 expression vector. PEA3 siRNA attenuated CXCR4 promoter activity and the binding of PEA3 to the CXCR4 promoter in MDA-MB-231 and MCF-7 cells. These results indicated that PEA3 could activate CXCR4 promoter transcription and promote breast cancer metastasis.

Keywords PEA3; CXCR4 promoter; transcription regulation

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Introduction

Metastasis continues to be the cause of >90% of human cancer deaths. Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumor [1]. CXC chemokine receptor 4 (CXCR4) is a cell surface receptor that has been shown to mediate the metastasis of many solid tumors including lung, breast, kidney, and prostate tumors [2]. CXCR4 is one of the most important cancer targets discovered in recent years, because it plays a central role in all three stages of cancer progression and in >75% of all cancers. The ligand for CXCR4 is the chemokine stromal cell-derived factor 1 (SDF1). CXCR4/SDF1 also indirectly promoted tumor metastasis by mediating proliferation and migration of tumor cells and enhancing tumor-associated angiogenesis [3].

Some transcription factors can influence CXCR4 transcription. Hypoxia-inducible factor-1 alpha was closely associated with metastasis, which could bind to the promoter of CXCR4 and activate its transcription and promote metastasis [4]. It was reported that the Ets1 (v-ets erythroblastosis virus E26 oncogene homolog 1) cluster of binding sites near the NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) consensus sequence on CXCR4 promoter contributed to CXCR4 transcription, and hepatocyte growth factor induced CXCR4- and CXCL12-mediated tumor invasion through Ets1 and NF-κB [5]. Krüppel-like factor 2 (KLF2), a novel vesnarinone-responsive molecule, can bind to the CXCR4 promoter at positions -300 to -167 relative to the transcription start site. The forced expression of KLF2 led to the downregulation of CXCR4 mRNA and impaired CXCR4 promoter activity [6]. CREB3 was shown to bind to the CRE element in the CXCR4 promoter and to activate the transcription of the CXCR4 gene by causing dissociation of HDAC3 (histone deacetylase 3) and subsequently increasing histone acetylation [7].

The aim of the present study is to explore the activity of PEA3 on CXCR4 transcription and to reveal the role of PEA3 in CXCR4-mediated angiogenesis. Our results showed that PEA3 played an important role in inducing CXCR4 promoter activity by directly binding to the CXCR4 promoter, which is helpful to understand the metastasis mechanism. The inhibition of CXCR4 signaling may provide potential targets for antiangiogenic therapy in several malignancies.
Materials and Methods

Cell lines, culture, plasmids, and transfection
Human breast cancer cell lines MCF-7 and MDA-MB-231 were all obtained from American Type Culture Collection (Rockville, USA). MCF-7 cells were maintained in RPMI 1640 medium 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. MDA-MB-231 cells were routinely maintained in Leibovitz’s L-15 medium with 2 mM of L-glutamine at 37°C in a humidified atmosphere containing 5% CO2. The medium was supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were checked routinely and found to be free of contamination by mycoplasma or fungi.

PEA3 expression vector was kindly provided by Dr Hassell (Department of Biology, McMaster University of Canada). CXCR4 promoter/luciferase construct pGL2-CXCR4 (−2632 to +86) was constructed as previously reported [5].

Briefly, for transient transfection, cells were seeded in the six-well plates at a density of 4 × 10^5 cells/well and cultured overnight. Cells were transfected with 4 μg of PEA3 expression vector or pcDNA3 using Lipofectamine™ 2000 (Gibco BRL, Carlsbad, USA) and then maintained in RPMI 1640 medium or Leibovitz’s L-15 medium containing 10% FBS for 48 h.

Reverse transcription—polymerase chain reaction
Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, USA) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen) 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 polymerase chain reaction (PCR). The reaction mixture contained 2 μl of cDNA template, 1.5 mM MgCl2, 2.5 U of Tag polymerase, and 0.5 μM of CXCR4 primer (5′-GTCCACGCCACCAAGC-3′; 5′-CTGGTTGTGTCGCG TGGAC-3′), PEA3 primer (5′-GCCAATTATCTCCCCG AA TCGA-3′; 5′-ACCAAGGTTTCCGGACCGTA-3′), GAPDH primer (5′-GCCAAAAGGGTCATCATCCTC-3′; 5′-GTAG AGGCAGGGATGATGTC-3′) designed to knock down PEA3 expression. The siRNA was synthesized by Shanghai GeneChem (Shanghai, China). The cells in the exponential phase of growth were seeded in six-well plates at a density of 5 × 10^5 cells/well. After incubation for 24 h, the cells were transfected with siRNA specific for PEA3 and non-targeting siRNA at a final concentration of 100 nM using Oligofectamine™ reagent and OPTI-MEMI reduced serum medium (Invitrogen) according to the manufacturer’s protocol. Silencing was examined 48 h after transfection.

Enzyme-linked immunosorbent assay detection of CXCR4 level induced by PEA3
Cells seeded in six-well plates were transfected with PEA3 expression vector as described above. After 48 h of growth, 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 products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were carried out according to the manufacturer’s protocol (Active motif, Carlsbad, USA). Briefly, cells in 150-mm tissue culture dishes were fixed with 1% formaldehyde for 10 min at 37°C. The cells were then washed twice with ice-cold phosphate-buffered saline, harvested, and re-suspended in ice-cold Tris-NaCl-Tween lysis buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM Phenylmethanesulfonyl fluoride, and 1% aprotinin]. The lysates were sonicated to shear the DNA to fragments of 200–600 bp, and subject to immunoprecipitation with anti-PEA3 and IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, USA), respectively. Three micrograms of antibodies was used for each immunoprecipitation. The antibody/protein complexes were collected by protein G beads and washed three times with ChIP washing buffer [5% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetra-acetate (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 Milli-Q H2O. The primer corresponding to the CXCR4 promoter region (−401 to −142) (sense: 5′-CAGCAAGTGTCATCCTCC-3′; antisense: 5′-GGAGAGGTGCGCGGA-3′) was used for PCR to detect the presence of the CXCR4 promoter DNA.

Small interfering RNA (siRNA) preparation and transfection
PEA3 siRNA is a target-specific 19 nt siRNA (5′-UGU UAUGCCAGCAAGCUCUA-3′) designed to knock down PEA3 expression. The siRNA was synthesized by Shanghai GeneChem (Shanghai, China). The cells in the exponential phase of growth were seeded in six-well plates at a density of 5 × 10^5 cells/well. After incubation for 24 h, the cells were transfected with siRNA specific for PEA3 and non-targeting siRNA at a final concentration of 100 nM using Oligofectamine™ reagent and OPTI-MEMI reduced serum medium (Invitrogen) according to the manufacturer’s protocol. Silencing was examined 48 h after transfection.
1.5 ml of medium was collected from each well to evaluate CXCR4 level by enzyme-linked immunosorbent assay (ELISA). The supernatants of four wells from each time point were collected and analyzed for CXCR4 expression using anti-CXCR4 antibody (Abcam Inc., Cambridge, USA). The plates were read at 450 nm. CXCR4 concentrations in conditioned media were calculated from a standard curve generated by adding recombinant CXCR4 to the specific unconditioned media.

**Mutation of PEA3 sites in CXCR4 promoter**

CXCR4 promoter/luciferase construct pGL2-CXCR4 was used as a template. Plasmid DNA was methylated with DNA methylase at 37°C for 1 h and amplified in a mutagenesis reaction with two overlapping primers, one of which contained the target mutation. The product was linear, double-stranded DNA containing the mutation. The mutagenesis mixture was transformed into wild-type Escherichia coli. The linear mutated DNA was circularized by the host cell, and the methylated template DNA was digested by McrBC endonuclease in the host cell, leaving only unmethylated, mutated product. For individual mutations, the sequence of PEA3 binding sites (~540 to –535 and –240 to –235) AGGAAA was converted to ACCAAA.

**Luciferase reporter gene assay**

MDA-MB-231 or MCF-7 cells were seeded in six-well plates at a density of 1–2 × 10⁵ cells/well and cultured for 24 h. Cells were then co-transfected with basic reporter plasmid pGL2B (Promega, Madison, USA), wild-type (pGL2-CXCR4), or PEA3 mutant (pGL2-CXCR4mut) CXCR4 reporter construct (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). 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 bicinehinonic acid protein assay (Pierce, Rockford, USA).

**Tumorigenicity and metastasis assays in athymic mice**

Female athymic BALB/c nu/nu mice, 4–6 weeks old, were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All studies on mice were conducted in accordance with the National Institute of Health (NIH) ‘Guide for the Care and Use of Laboratory Animals’. The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee. Animals were divided into two groups: MDA-MB-231/PEA3 and MDA-MB-231/vector. Each group had 10 mice. Cells (1.5 × 10⁶) were injected into the mammary fat pad. Animals were monitored every 2 days for tumor growth and general health. Animals were sacrificed and autopsied at 6 weeks after cell inoculation. At the end of the experiment, trunk blood was collected and the serum was separated and analyzed by ELISA for CXCR4 expression. The lungs used to evaluate the numbers of metastasis were fixed in Bouin’s solution for 24 h and then stored in 100% ethanol. When the lungs restored their inherent color, the white metastasis deposits could be assessed by macroscopic observation. To confirm the presence of lung metastases, sections were cut at 50 μm intervals and H&E stained. In this study, the number of metastasis nodules on the lung surface was counted. Two independent pathologists calculated the number of metastases.

Microangiography for blood vessels was performed at the Beamline BL13W1, the X-ray imaging, and biomedical application station of the Shanghai Synchrotron Radiation Facility (SSRF) in China. The maximum light size of the beam was 45 mm (horizontal) × 5 mm (vertical) at the object position at 16 keV. All animals were anesthetized by intraperitoneal injection of ketamine (200 mg/kg) (Ketanest; Pfizer, Karlsruhe, Germany). The image contrast agent microfil (Flow Tech Inc., Carver, USA) was injected into the left ventricle. Serial images of tumor blood vessels in nude mice were then recorded using SSRF.

**Results**

**PEA3 induced CXCR4 overexpression in MDA-MB-231 and MCF-7 cells**

To explore the role of PEA3 in regulating CXCR4 transcription, the PEA3 expression vector or pcDNA3 was transfected into MDA-MB-231 and MCF-7 cells for 48 h, and then CXCR4 mRNA and protein were detected. Figure 1(A) showed that as compared with control cells transfected with pcDNA3, the level of CXCR4 mRNA in the cells transfected with PEA3 expression vector was increased in both cell lines as determined by RT–PCR. The level of CXCR4 secretion in the cells transfected with PEA3 expression vector was elevated as determined by ELISA, as compared with control cells transfected with pcDNA3 [Fig. 1(B)]. In this experiment, exogenous PEA3 could induce CXCR4 mRNA and protein expression, indicating PEA3 played a role in regulating CXCR4 transcription.

**PEA3 promoted CXCR4 expression and breast cancer metastasis in vivo**

In the following experiments, we assessed the effect of PEA3 on tumor metastasis by using an orthotopic xenograft tumor model in athymic mice. MDA-MB-231/PEA3 and MDA-MB-231/vector cells were injected into the
mammary fat pad of athymic mice. Six weeks after cells inoculation, we measured the amount of CXCR4 in the mice serum by ELISA. The level of CXCR4 was significantly higher in the serum from mice bearing MDA-MB-231/PEA3 cells compared with the control group [Fig. 2(A)]. Lungs from mice bearing MDA-MB-231/PEA3 and MDA-MB-231/vector cells were examined physically at autopsy and then subject to microscopic examination for morphologic evidence of tumor cells by light microscopy on H&E-stained paraffin sections. The incidence of lung metastasis was increased significantly in mice bearing MDA-MB-231/PEA3 cells compared with the control group [Fig. 2(B)].

Blood vessels of tumors from mice bearing MDA-MB-231/vector and MDA-MB-231/PEA3 cells were also determined. As shown in [Fig. 2(C)], the amount of larger blood vessels of tumors was increased significantly in tumors expressing high levels of PEA3 relative to control groups.

**PEA3 activated CXCR4 promoter activity in MDA-MB-231 and MCF-7 cells**

We also found two PEA3 sites from −1000 to +1 bp on the CXCR4 promoter [Fig. 3(A)]. To identify the role of PEA3 in regulating CXCR4 promoter transcription, we co-transfected the CXCR4 promoter/luciferase construct with PEA3 expression vector or pcDNA3 in MDA-MB-231 cells and MCF-7 cells and detected CXCR4 promoter activity. Figure 3(B) showed that the luciferase activity was enhanced by PEA3 in both MDA-MB-231 cells and in MCF-7 cells, further indicating that PEA3 could activate CXCR4 promoter activity. In this experiment, exogenous PEA3 could activate CXCR4 promoter activity.
activity, suggesting that PEA3 played a role in regulating CXCR4 transcription.

To determine the potential roles of these PEA3 elements in regulation of CXCR4 gene transcription, PEA3 was co-transfected with either wild-type (pGL2-CXCR4) or PEA3-site-mutated (pGL2-CXCR4mut) CXCR4 reporter construct into MDA-MB-231 cells and MCF-7 cells. As shown in Fig. 3(C), any single mutation of two PEA3 sites decreased the reporter gene activity compared with wild-type CXCR4 promoter construct when co-transfected with PEA3. Mutations of both two PEA3 sites simultaneously caused a further decrease in reporter gene activity. These results suggested that both two PEA3 sites contributed in a concerted mechanism to the PEA3-induced transcription of CXCR4 gene, and PEA3 could activate CXCR4 transcription by binding to PEA3 sites on the CXCR4 promoter.

PEA3 bound to the CXCR4 promoter in PEA3-overexpressed MDA-MB-231 and MCF-7 cells
To investigate whether PEA3 bound to the CXCR4 promoter in the cells transfected with PEA3 expression vector, we performed ChIP experiments. The results showed that PEA3 could bind to the CXCR4 promoter both in MDA-MB-231 cells and in MCF-7 cells transfected with PEA3 expression vector (Fig. 4), indicating that PEA3 activated CXCR4 transcription by binding directly to the CXCR4 promoter.

PEA3 siRNA inhibited CXCR4 expression in MDA-MB-231 and MCF-7 cells
To further identify the role of PEA3 in regulating CXCR4 transcription, expression of PEA3 was knocked down with a gene-specific siRNA, and CXCR4 mRNA level were measured. As shown in Fig. 5(A), PEA3 siRNA inhibited PEA3 and CXCR4 mRNA significantly in MDA-MB-231 and MCF-7 cells after transfection with PEA3 siRNA for 48 h. As indicated in Fig. 5(B), PEA3 siRNA inhibited CXCR4 protein secretion significantly in MCF-7 cells after transfection with PEA3 siRNA for 48 h. These experiments indicated that PEA3 siRNA could knock down PEA3 efficiently and decreased CXCR4 mRNA and protein expression significantly.

PEA3 siRNA repressed CXCR4 promoter activity in MDA-MB-231 and MCF-7 cells
To determine whether the decrease of PEA3 would reduce CXCR4 gene transcription, we knocked down the expression of PEA3 and measured CXCR4 promoter activity. As determined in Fig. 6, PEA3 siRNA attenuated CXCR4 promoter
activity in MDA-MB-231 and MCF-7 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 CXCR4 also decreased.

**PEA3 siRNA attenuated the binding of PEA3 to the CXCR4 promoter**

To determine whether the decrease of PEA3 would influence the binding of PEA3 on the CXCR4 promoter, expression of PEA3 was inhibited by siRNA and the binding status of PEA3 on the CXCR4 promoter was measured. As shown in Fig. 7, PEA3 siRNA attenuated the binding of PEA3 to the CXCR4 promoter in MDA-MB-231 and MCF-7 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 CXCR4 promoter decreased, and PEA3 regulated CXCR4 transcription by binding directly to the CXCR4 promoter.

**Discussion**

The PEA3 transcription factor belongs to the Ets family [8–10]. PEA3 may play a role in human breast cancer. The human PEA3 gene was transcriptionally upregulated in breast tumor cell lines [11] and in 93% of HER2/Neu-positive human breast tumors [12]. The matrix metalloproteinase-7 (MMP-7), MMP-9, and MMP-11, which play pivotal roles in tumor progression, could also be regulated by PEA3 [13,14]. However, transfection of oral carcinoma cells with an antisense sequence of PEA3 resulted in the inhibition of cell invasion and decrease of MMP expression [15]. Previous reports indicated transfection with PEA3 resulted in enhanced motility and invasion in lung cancer cells and human SKBR3 breast cancer cells [15,16]. PEA3 was found to activate VEGF transcription in T47D and SKBR3 breast cancer cells [17]. PEA3 has a close correlation to cancer characteristic, but there are no reports about the interaction between PEA3 and CXCR4 transcription in breast cancer.

In our studies, overexpression of PEA3 could increase CXCR4 mRNA and secretion in MDA-MB-231 and MCF-7 cells. Estrogen receptor is present in MCF-7 cells and absent in MDA-MB-231 cells [18]. CXCR4 transcription was activated by PEA3 in both MDA-MB-231 and
CEXCR4 promoter. PEA3 promotes CXC chemokine receptor 4 expression and breast cancer metastasis and angiogenesis. These investigations are important and offer potential for defining metastasis mechanism regulated by CXCR4 and PEA3. With this information it will be possible to explore potential targets and define appropriate reagents, such as anti-sense or small molecule antagonists for PEA3, to inhibit CXCR4 signaling pathway and prevent cancer metastasis.

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**References**


