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Original Article

EGF like domain multiple 7 regulates the migration and invasion of prostate cancer cells

Libin Ma[1*], Kang Jiang[2*], Peiwu Jiang[2], Kean Chen[2], Jia Shao[2], Zhijian Yu[2], Gang Deng[2]

[1]Department of Nephrology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, PR China; [2]Department of Urology, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang, PR China. [*]Equal contributors.

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Abstract: EGF like domain multiple 7 (EGFL7) and vascular endothelial growth factor (VEGF) were overexpressed in prostate cancer tissues. EGFL7 expression was induced by VEGF ex vivo. The aims of the present study were to investigate biological function of EGFL7, as well as the relation between VEGF and EGFL7 in prostate cancer. Here, we found that EGFL7 was significantly elevated in prostate cancer cells compared to prostate epithelial cell. RNA interference experiments demonstrated that knockdown of EGFL7 in two prostate cancer cell lines, DU145 and PC-3, inhibited cell proliferation, migration and invasion. Knockdown of EGFL7 significantly decreased the expression of MMP-2 and MMP-9, which were closely related with migration and invasion. Moreover, VEGF exposure induced the expression of EGFL7 at both mRNA and protein levels. A functional assay revealed that EGFL7 knockdown decreased the promotion effects of VEGF on cell migration, invasion and the expression of MMP-2/9. In summary, EGFL7 was a potential downstream effector for VEGF's function on cancer cell migration and invasion. Our results indicate that EGFL7 is a therapeutic target for prostate cancer.

Keywords: EGFL7, VEGF, migration, invasion, prostate cancer

Introduction

Prostate cancer is the second most frequently diagnosed cancer in males [1] and the sixth leading cause of cancer-related death in males worldwide [2]. The risk factors most commonly associated with prostate cancer are obesity, age and a family history of the disease. Prostate cancer is very rare in men younger than fifty and the average age at the time of diagnosis is 67 [3]. Despite diverse treatment options and extensive researches, hormone resistant [4, 5] or metastatic disease [6] causes prostate cancer to be a major health burden in men. Therefore, better understanding of the molecular basis of prostate cancer and novel therapeutic targets for this disease is urgently required.

Angiogenesis is critical for the development of solid tumors. Angiogenesis is controlled by proangiogenic and antiangiogenic factors [7]. Vascular endothelial growth factor (VEGF) is one of the most important proangiogenic factors. Besides angiogenesis, it is also involved in cellular proliferation, migration and invasion [8]. VEGF was found overexpressed in a variety of human cancers, including prostate cancer [9]. VEGF can mediate angiogenesis in endothelial prostate cancer cells [10]. Through VEGF receptor-2 (VEGFR-2), VEGF regulates the migratory responses of prostate cancer cells [11].

EGF like domain multiple 7 (EGFL7) is a 41-kDa secreted angiogenic factor, which can mediate its angiogenic effects by interfering with the Notch pathway [12, 13]. Prior studies have shown that it plays an important role in the process of vascular tube formation in zebrafish [14]. By using an *ex vivo* Matrigel-embedded mouse eye cup assay, Takeuchi K et al. found that EGFL7 was essential for VEGF-induced tube formation [15]. EGFL7 may protect endothelial cells from hyperoxia-induced apoptosis [16]. Although a previous study has shown that EGFL7 is overexpressed in human epithelial tumor tissues, including prostate cancer [17], the

functions of EGFL7 in prostate cancer have not been defined. It is unclear whether VEGF could regulate EGFL7 expression in prostate cancer cells.

In the present study, we found that EGFL7 knockdown significantly inhibited cell proliferation, migration and invasion of prostate cancer cells. Moreover, EGFL7 expression was induced by VEGF exposure. The effect of VEGF on cell migration and invasion was EGFL7 dependent.

Materials and methods

Cell culture

All cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China) and maintained at 37°C in a 5% CO_2/95% air atmosphere. 22RV1, p69 and LNCaP cells were cultured in RPMI-1640 medium (Hyclone; Logan, UT, USA), HEK293, PC-3 and DU145 were grown in DMEM (Hyclone). All culture medium were supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 units/ml penicillin and 100 μg/ml streptomycin.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) analysis was used to determine the relative expression mRNA levels of EGFL7. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was treated with DNase 1 (Sigma, St. Louis, MO, USA) and converted into cDNA using the First-Strand cDNA synthesis kit (Fermentas; Hanover, MD, USA). Subsequently, quantitative PCR was performed in triplicate using SYBR Green PCR Kit (Thermo Fisher Scientific; Rockford, IL, USA) on an ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA). The data was normalized to GAPDH expression levels. Disclosed primers used were: EGFL7, 5'-CCCAAAGCCACATCTGTAG-3' and 5'-CGGAGGAGAATCAGTCATC-3'; GAPDH, 5'-CACCCACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3'.

Western blot analysis

Total cell lysate was prepared using RIPA buffer in the presence of proteinase inhibitor cocktail (Sigma). Proteins were resolved were separated by 10% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bredford, USA). The membranes were hybridized with anti-EGFL7, anti-MMP-2, anti-MMP-9 (Abcam; Cambridge, MA, USA) or anti-GAPDH (Cell Signaling Technology; Danvers, MA, USA) antibodies overnight at 4°C followed by incubation with secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Millipore). The band intensity was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis

EGFL7 concentration in the cultured media was determined by using ELISA assay (Cloud-Clone Corp.; Houston, TX, USA) following the instructions of the manufacturer. Absorbance at 450 nm was read on a using Multiskan MS plate reader (Labsystems, Helsinki, Finland).

Knocking down of EGFL7 by short hairpin RNA (shRNA)

shRNA targeting human EGFL7 mRNA (GGTGCTGCTGATGTGGCT, shEGFL7) or a non-specific scramble shRNA (shNC) was cloned into a lentiviral vector (PLKO.1, Addgene, Cambridge, MA, USA). To produce lentivirus, lentiviral vector and lentiviral packaging vectors were transfected into HEK293T cells by using Lipofectamine 2000 (Invitrogen). At 48-72 h post transfection, viruses were collected to infect DU145 and PC-3 cells.

Assessment of cell proliferation

The cells were seeded in 96-well plates at 2 × 10^3 cells per well and cultured overnight. The cells were further infected with shEGFL7 or shNC. After incubation at 37°C for 0, 24, 48 and 72 h, 10 μl CCK-8 reagents (SAB biotech., College Park, MD, USA) was added to each well and incubated for another 1 h. The optical absorbance at 450 nm was determined using Multiskan MS plate reader. The assay was conducted using three replicate wells per sample and three parallel experiments were performed.

Cell migration and invasion assay

The migratory capacities of human prostate cancer cells were measured using chamber

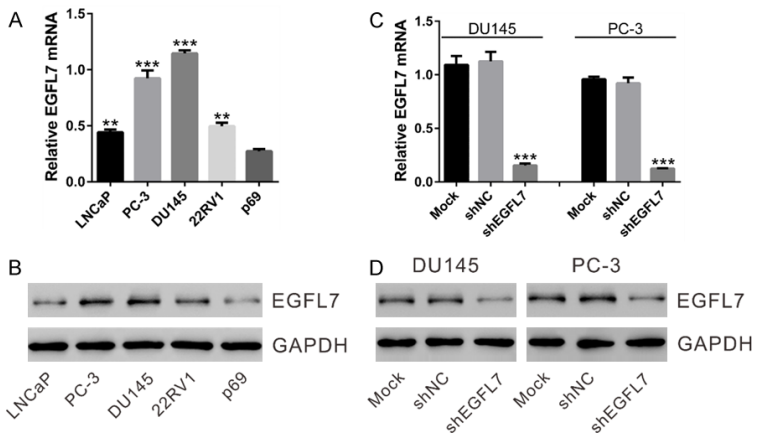

Figure 1. Knockdown of EGFL7 expression in prostate cancer cells. A. mRNA levels of EGFL7 in 5 prostate cancer cell lines were examined by qRT-PCR. ***P*<0.01, ****P*<0.001 versus p69. B. Protein levels of EGFL7 in prostate cancer cell line were assessed by Western blotting. GAPDH was used as a loading control. Representative blots of three independent experiments are shown. C, D. EGFL7 expression was confirmed by qRT-PCR and immunoblotting at 48 h after EGFL7 shRNA (shEGFL7) or control shRNA (shNC) virus infection. EGFL7 expression in DU145 and PC-3 cells was reduced markedly by RNA interference. Cells without any treatment were served as control (Mock). ****P*<0.001 versus Mock and shNC.

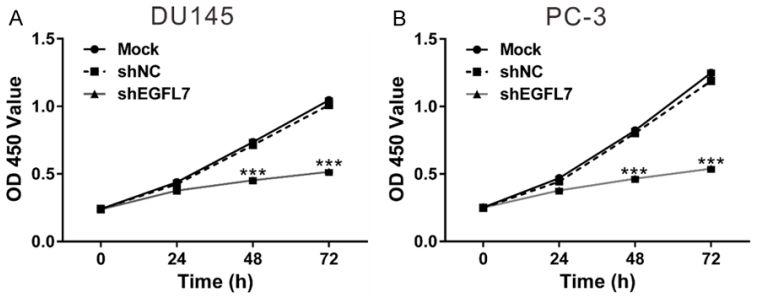

Figure 2. EGFL7 knockdown decreased the proliferation of prostate cancer cells. Proliferation rates of DU145 and PC-3 cells infected with EGFL7 shRNA (shEGFL7) or control shRNA (shNC) were measured by CCK-8 assays. ****P*<0.001 versus Mock and shNC.

with 8 μm pore filters (Corning, New York, NY, USA). Following starvation for 6 h in serum-free DMEM, cells were harvested and seeded in serum-free DMEM to the upper chambers. DMEM supplemented with 10% FBS was added to the lower chamber. After incubating at 37°C for 24 h, the non-migrated cells were completely removed from the upper surfaces of the membranes, and the cells on the lower surface were fixed in 4% paraformaldehyde and stained with crystal violet. The migrated cells were counted in five random field under a microscope. All experiments were performed in triplicate.

The invasive abilities were measured the same as migratory capacities except that the upper chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) prior to cell seeding.

Statistical analysis

All analyses were performed using GraphPad Prism, version 6.0 (GraphPad, San Diego, CA, USA). Statistical analysis was performed with one-way ANOVA followed by Sidak's test. A two-sided *P* value <0.05 was considered statistically significant.

Results

Knockdown of EGFL7 expression in prostate cancer cells

We examined mRNA and protein expression of EGFL7 in a benign immortalized prostate epithelial cell line (p69) and prostate cancer cell lines (PC-3, DU145, 22RV1 and LNCaP), and found that EGFL7 expression was increased in prostate cancer cell lines (**Figure 1A** and **1B**). DU145 and PC-3 cells were selected for knockdown experiments because they expressed high levels of EGFL7. As shown in **Figure 1C** and **1D**, EGFL7 protein and mRNA was efficiently knocked down at 48 h after infection with EGFL7-specific shRNA lentivirus (shEGFL7) as compared to cells infected with control shRNA lentivirus (shNC). EGFL7 expression was comparable in both control cells (Mock) and cells infected with shNC.

EGFL7 knockdown causes decreased proliferation of prostate cancer cells

We next investigated whether EGFL7 knockdown in DU145 and PC-3 cells would affect their growth properties. The results of CCK-8 assays indicated that cell proliferation was sig-

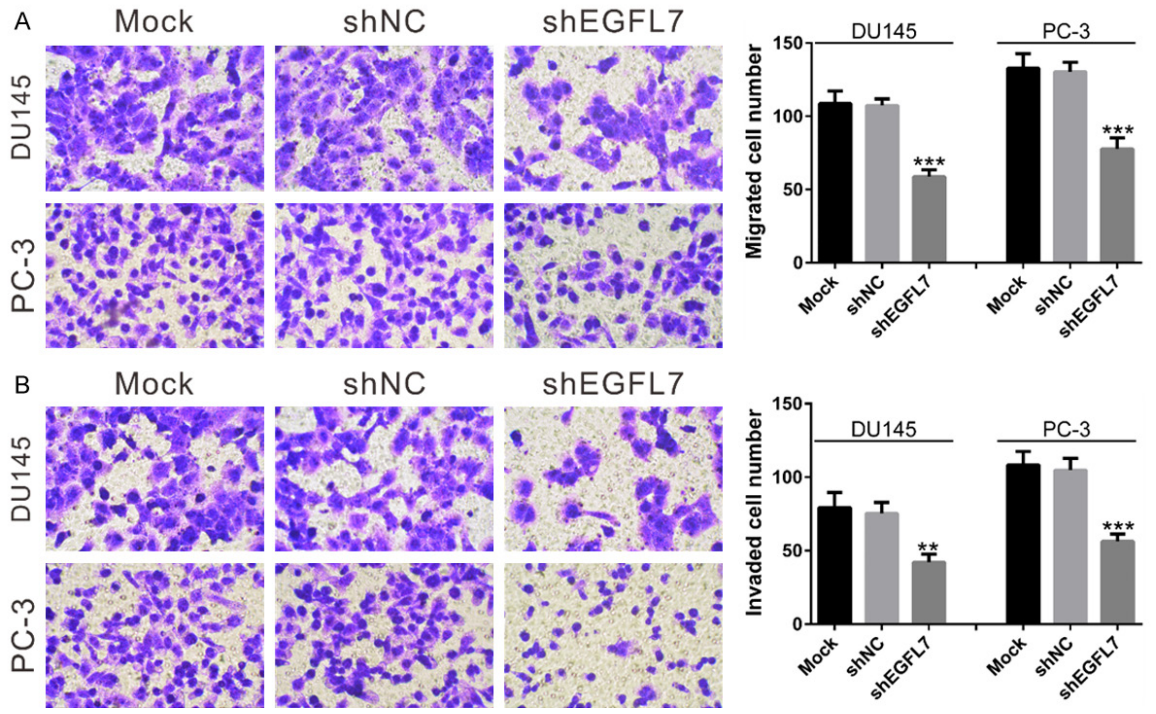

Figure 3. EGFL7 knockdown suppresses cell migration and invasion. Transwell assay was used to examine the migratory (A) and invasive (B) abilities of DU145 and PC-3 cells after infected with EGFL7 shRNA (shEGFL7) or control shRNA (shNC). ***P<0.001 versus Mock and shNC.

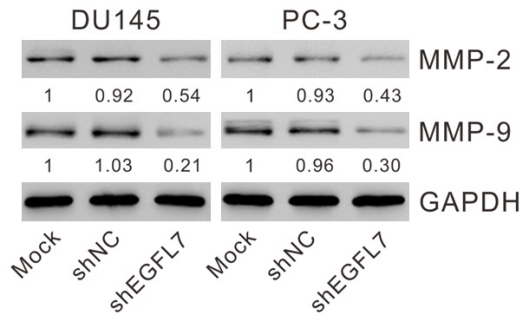

Figure 4. MMP-2/MMP-9 expression is repressed by EGFL7 knockdown. Protein levels of MMP-2 and MMP-9 in prostate cancer cell line were assessed by Western blotting. Representative blots of three independent experiments are shown.

nificantly reduced at 48 and 72 h after shEGFL7 lentiviral infection when compared with control cells (Mock) and cells infected with shNC (**Figure 2**). shNC infection had no effect on the proliferation of DU145 and PC-3 cells.

EGFL7 knockdown suppresses cell migration and invasion

Cell migration and invasion are necessary for cancer metastasis. We next used Transwell assays to test the effects of EGFL7 knockdown on prostate cancer cell migration and invasion. The results showed that the migration (**Figure 3A**) and invasion capacities (**Figure 3B**) of cells infected with shEGFL7 were also decreased compared to those in cells expressing the control shRNA.

MMP-2/MMP-9 expression is repressed by EGFL7 knockdown

Because MMP-2 and MMP-9 are important regulators of cell migration and invasion in the extracellular matrix (ECM), we then assessed the effects of EGFL7 knockdown on their expression. As shown in **Figure 4**, protein levels of MMP-2 and MMP-9 were significantly decreased following shEGFL7 infection in both DU145 and PC-3 cells.

EGFL7 expression is induced by VEGF treatment

Since VEGF increased EGFL7 expression in Matrigel-embedded mouse eye cup [15], we then tried to explore the effect of VEGF on EGFL7 expression in prostate cancer cells. Similar effects of EGFL7 knockdown had been

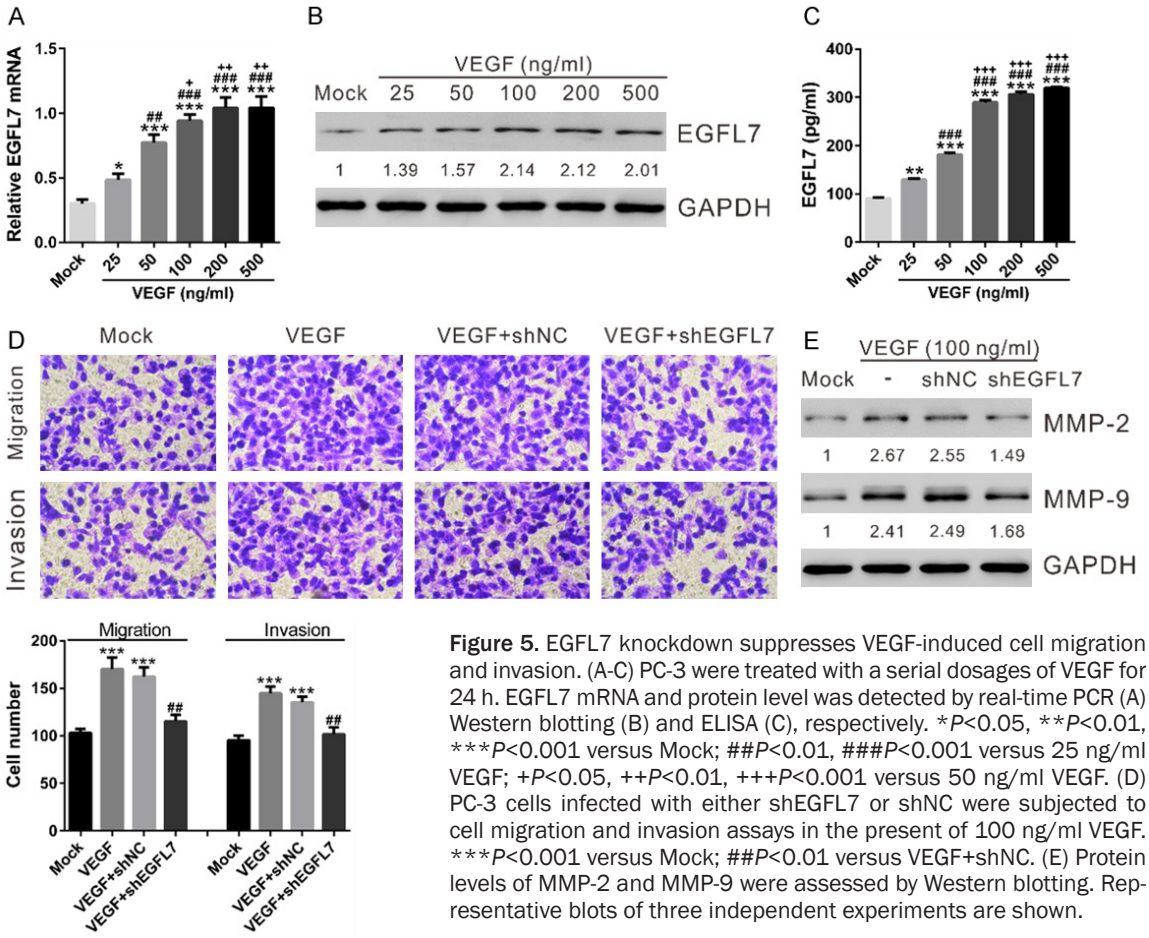

Figure 5. EGFL7 knockdown suppresses VEGF-induced cell migration and invasion. (A-C) PC-3 were treated with a serial dosages of VEGF for 24 h. EGFL7 mRNA and protein level was detected by real-time PCR (A) Western blotting (B) and ELISA (C), respectively. **P*<0.05, ***P*<0.01, ****P*<0.001 versus Mock; ##*P*<0.01, ###*P*<0.001 versus 25 ng/ml VEGF; +*P*<0.05, ++*P*<0.01, +++*P*<0.001 versus 50 ng/ml VEGF. (D) PC-3 cells infected with either shEGFL7 or shNC were subjected to cell migration and invasion assays in the present of 100 ng/ml VEGF. ****P*<0.001 versus Mock; ##*P*<0.01 versus VEGF+shNC. (E) Protein levels of MMP-2 and MMP-9 were assessed by Western blotting. Representative blots of three independent experiments are shown.

observed in DU145 and PC-3 cells, and one prostate cancer cell line (PC-3) was used in the following experiments. As shown in **Figure 5A-C**, VEGF treatment caused a significant increase in mRNA and protein expression of EGFL7 from 25 to 500 ng/ml as indicated by qRT-PCR, Western blotting and ELISA assays, respectively. Comparable increased ratio was obtained at 100, 200 and 500 ng/ml. Therefore, 100 ng/ml was chosen for the following assays.

EGFL7 knockdown suppresses VEGF-induced cell migration and invasion

To investigate the association between EGFL7 and VEGF in prostate cancer cell migration and invasion, PC-3 cells were infected with shEGFL7 or shNC and then treated with 100 ng/ml VEGF before Transwell assays were carried out. As shown in **Figure 5D**, VEGF treatment significantly promoted cell migration and invasion, while knockdown of EGFL7 significantly suppressed the induction effects of VEGF. The changes of MMP-2 and MMP-9 expression were consistent with the Transwell assay. These data indicate that VEGF may be an upstream regulator for EGFL7 during cell migration and invasion.

Discussion

EGFL7 plays an important role in angiogenesis and tube formation [14, 15]. VEGF can increased EGFL7 expression in Matrigel-embedded mouse eye cup [15]. In this study, we found that EGFL7 expression was enhanced by VEGF and it can regulate the proliferation, migration and invasion of prostate cancer cells.

Recently, EGFL7 has been linked to various cancers. Its expression was up-regulated in several human epithelial tumor tissues, including prostate cancer [17]. Wang et al. reported that EGFL7 played an important role in the regulation of laryngeal carcinoma cells growth

and invasion via regulating the expression of cyclinD1, survivin, MMP-2 and MMP-9 [18]. In the current study, we found that EGFL7 expression was higher in prostate cancer cells than in prostate epithelial cells (p69), which was consistent with the previous report in prostate cancer tissues [17]. EGFL7 knockdown significantly inhibited prostate cancer cell proliferation, migration and invasion as indicated by CCK-8 and Transwell assays, respectively. The expression of MMP-2 and MMP-9, important matrix metalloproteinases involved in tumor invasion and metastasis, was down-regulated by EGFL7 knockdown. Our data and Wang et al.'s findings suggest the oncogenic role of EGFL7 during cancer progression.

Furthermore, EGFL7 was previously identified as a secreted protein and induced by VEGF [15]. VEGF, as a proangiogenic factor, may induce the degradation of the ECM. VEGF was found overexpressed in prostate cancer [9]. It mediated angiogenesis [10] and migration [11, 19] of prostate cancer cells. Presently, EGFL7 expression and secretion was significantly increased by VEGF treatment in prostate cancer cells. Transwell assays demonstrated that VEGF remarkably induced cell migration and invasion, which was in line with previous findings. Knocking down of EGFL7 significantly repressed the promoting-effects of VEGF on cell migration and invasion. Our data suggested a down-stream effector required for VEGF's function on cancer cell migration and invasion. Moreover, the changes of MMP-2 and MMP-9 expression were in accord with the results of Transwell assays. Our data indicate the involvement of EGFL7 in VEGF-induced prostate cell migration and invasion, although the detailed mechanisms require further investigation.

In summary, VEGF exposure significantly enhanced EGFL7 expression. EGFL7 knockdown partially suppressed VEGFL7-induced cell proliferation, migration and invasion of prostate cancer cells. EGFL7 may serve as a therapeutic target for prostate cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Deng Gang, Department of Urology, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou 310006, Zhejiang, PR China. Tel: +86-571-56006932; Fax: +86-571-87914773; E-mail: denggang32@yeah.net

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