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Original Article

SDF-1/CXCR4 axis promotes prostate cancer cell invasion and bone metastasis through p38, NF-κB and HIF-1α pathways

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Abstract: Stromal cell-derived factor 1 (SDF-1) and its specific receptor, CXC chemokine receptor 4 (CXCR4), are associated with tumor progression and metastasis in prostate cancer (PCa). The present study aimed to explore the role of SDF-1/CXCR4-mediated metastasis of PCa cells to bone marrow, as well as the underlying molecular mechanisms. The data showed the expression of CXCR4 was significantly higher in PCa tissues than in normal prostate tissues at mRNA and protein levels ($P < 0.05$). CXCR4 expression was associated with vascular endothelial growth factor (VEGF) and matrix metalloprotease-9 (MMP-9) expression, and microvessel density (MVD). In vitro, SDF-1 induced cell invasion and metastasis of CXCR-positive PCa cell by increasing the expression of adhesion molecules and activation of the p38MAPK, NF-κB and HIF-1α pathways. In vivo, histopathological examinations showed SDF-1/CXCR4 axis promoted PCa progression and bone metastasis in SCID mice bearing tumor. In conclusion, CXCR4-promoted PCa progression and bone marrow trafficking were regulated by complex signaling pathways. Thus, our study lays a new foundation for targeting the SDF-1/CXCR4 axis, which could have clinical applications in inhibition of PCa progression.

Keywords: SDF-1, CXCR4, prostate cancer, bone metastasis

Introduction

PCa is the most prevalent diagnosed epithelial tumor type and is the second leading cause of cancer-death among men worldwide [28]. Technological advances in diagnosis have led to high detection rates of PCa, treatment strategy, disease monitoring and prediction of treatment success, but the progression to locally advanced, invasive and metastatic castration-resistant prostate cancers (CRPCs) usually cause disease relapse [27]. Approximately 50% of advanced PCa patients experienced bone metastases, which would increase morbidity and poor prognosis in CRPC. Therefore, the elucidation of underlying mechanism in bone metastases is a central question for PCa therapy.

The binding of SDF-1 to CXCR4 triggered the activation of several downstream pathways related to chemotaxis, cell survival and/or proliferation, which also can increase in calcium influx and gene transcription [12]. The expression of CXCR4 on malignant epithelial cells and on cells from several hematopoietic malignancies indicated that the SDF-1/CXCR4 axis may affect the subsistence of tumor and play a critical role in directing the metastasis of $CXCR4^+$ tumor cells to organs that expressed SDF-1 (e.g., lymph nodes and bones) [1, 20]. Several $CXCR4^+$ cancers metastasize to the lymph nodes and bones in a SDF-1-dependent manner in which the bone marrow can provide a protective environment for tumor cells. In vivo suppression of CXCR4 gene inhibits the metastasis and growth of PCa cells [9]. Bone marrow factors produced SDF-1 to mediate homing, survival, and proliferation of tumor cells and integrin-mediated adhesion sequesters tumor cells to this niche [16].

Several signaling pathways may involve in the metastasis of PCa to bone, such as MAPK pathway [3]. SDF-1 can promote cell survival through the PI3K and MAPK cascades without cell cycle progression. The NF-κB signaling cascade is another marker altered in PCa. Analysis of clinical PCa samples has shown that NF-κB expression increases with the progression of the disease. HIF1α expression is up-regulated in a large proportion of PCa tissues and human PCa cell lines [25]. Normoxic expression of HIF1α has an important role in PCa chemo-resistance, radio-resistance and castrate-resistance. A number of mechanisms may lead to HIF1α overexpression in PCa, but the intriguing question of how HIF1α is up-regulated in PCa is still unanswered [24].

Adhesion molecules and several MMP are key proteins involved in tumor cell invasion and metastasis [7, 29]. In addition, the role of VEGF as a key mediator of tumor angiogenesis is well established [17]. SDF-1 can up-regulate expression of adhesion molecules such as VLA-4 [26]. SDF-1 stimulation has been reported to increase expression of several proteases such as MMP-2, MMP-9 and MMP-14 [32]. However, it is still unclear how PCa cells home to the bone microenvironment, and whether the process is related with adhesion molecular or signal pathways. Hence, we speculate that SDF-1/CXCR4 axis may regulate the expression of these key proteins via complex signaling pathways to affect PCa bone metastases. In this study, we explored the potential roles of SDF-1/CXCR4 axis in PCa bone metastasis.

Materials and methods

Patient prostate cancer specimens

A total of 148 PCa specimens and 10 Benign Prostate Hyperplasia (BPH) specimens were obtained in accordance with the ethical policy of the hospital's Institutional Review Board at Xin Qiao Hospital, Third Military Medical University from 1982 to 2007. The diagnosis of PCa was established by puncture prior to surgical excision. The mean age of the PCa patients was 65.83 years old. The samples were subjected to fixation in 4% formaldehyde solution and embedded in paraffin. Sections of 3 μm were cut and mounted on glass slides. HE staining and immunohistochemistry were carried out for staging and scoring. According to HE staining results and WHO grade system combining Gleason scoring system, 148 PCa specimens were separated into three groups: well (41), moderately (51) and poorly (56) differentiated cancers.

Immunohistochemistry

Specimens sections were dewaxed in H_2O. Endogenous peroxidase activity was blocked by incubation of samples with fresh hydrogen peroxide in methanol. The sections were heated in a microwave oven for 30 min to retrieve the antigens. After being washed with PBS three times, 10% normal goat serum was added for 30 min at 37°C. Specimens were then incubated with anti-human primary antibody (anti-AR, 1:50, ZSGB-BIO, China; anti-CXCR4, 1:100, R&D Systems, USA; anti-MMP-9, 1:80, ZSGB-BIO, China; anti-VEGF, 1:80, FuZhou Maixin Biotech. Co. Ltd., China) at 4°C overnight. The sections were incubated with HRP-linked strepavidin antibody followed by three washes. Slides were treated with 3,3'Diaminobenzidine for staining. BPH specimens were used as positive control and PBS was substituted for primary antibody as the negative control.

Microvessel detection and counting

A detection procedure for microvessels was performed using mouse anti-human CD34 monoclonal antibody (1:80, ZSGB-BIO, China). A single microvessel was defined as any brown immunostained endothelial cells. The stained sections were screened at × 100 magnification under a light microscope to identify the three regions of section with the highest number of microvessels. Microvessels were counted at × 200 magnification, and the average number of microvessels in these three regions was recorded, defined as microvessel density (MVD).

Matrigel invasion assay

We used 24-well plates inserted with 8.0 μm pore Transwells (Becton Dickinson Bioscience) for cell invasion analysis according to the manufacturer's protocol. Cells (1×10^4) after 72 h transfection were collected and responded with 200 μl serum-free media were placed in the upper well. Bone marrow endothelial cells (BMEC-1) with high expression of SDF-1 were

Table 1. Association between CXCR4 expression and clinicopathology

Criteria	Cases	CXCR4			High expression (++~+++) (%)	P
		+	++	+++		
Pathological grade						
Well	41	29	12	0	29.27	
Moderate	51	7	40	4	86.27	< 0.01
Poor	56	1	22	33	98.21	
Age (yrs.)						
≤ 65	59	9	30	20	84.75	
> 65	89	28	44	17	68.54	< 0.05
Metastasis						
Positive	10	0	3	7	100	< 0.01
Negative	138	37	71	30	73.19	
AR expression						
Positive	76	23	43	10	69.74	< 0.01
Negative	72	14	31	27	80.56	

Groups	Cases	CXCR4				P
		-	+	++	+++	
BPH	10	1	9	0	0	< 0.01
PCa	148	0	38	73	37	

placed in the bottom well the day before. Each group was set three repeats. Cells were co-cultured at 37°C for 24 h. At the termination of the assay, cells on the upper side of the membrane were removed with a cotton swab, while invading cells adhered to the bottom of the membrane and the bottom well were counted with Fluorescence Inversion Microscope System. The cell number of 5 random high power fields in each well was counted.

Generation and transduction of DNA constructs

Peripheral blood monocyte (PBMC) isolated from healthy people was used for total RNA extraction using Tripure one-step method. cDNA was synthesized from total RNA with a AMV reverse transcriptase cDNA first strand synthesis kit (Takara). CXCR4 (GenBank™ NM_001008540) was amplified from the cDNA bank (Primers are in Table S1) and inserted into pEGFP-N1 (Primers are in Table S1). All constructs were verified by direct sequencing.

Generation of stable cell lines

PC-3 cells were transfected with pEGFP-N1 or pEGFP-N1-CXCR4 using Lipofectamine 2000; transfectants were selected in medium containing G418 (800 μg/mL). After 10 to 14 days, single clones exhibiting GFP fluorescence were chosen for analysis. Q-PCR was used to detect the mRNA levels of CXCR4.

Quantitative real-time PCR

Cells were washed three times with PBS and lysed in Tripure solution to extract total RNA according to manufacturer instructions. cDNA was synthesized from total RNA (2 μg per sample) with cDNA first strand synthesis kit (Takara). Quantitative PCR was performed with the SYBR Green I on a Real-Time PCR Detection system. Fold changes were calculated by using the ΔΔCt method with normalization to GAPDH. Primers were designed by using Primer Premier 5; sequences are listed in Table S1.

Immunoblotting

Cells were harvested and lysed in RIPA buffer. Insoluble substances were removed by centrifugation. Bradford assay was used for protein quantitation. Equal amount of Protein was separated using 12% SDS-PAGE and transferred to PVDF (Bio-Rad, #162-0177). After blocking for 1 h at room temperature in 5% nonfat milk in TBST buffer, the membranes were incubated with primary antibodies overnight at 4°C; HRP-conjugated secondary antibodies were applied and immunoreactivity was detected using ECL. Quantitative data were obtained using Chemilmager TM 5500 software and expressed with Relative gray scale (RGS).

PCa metastasis assay in vivo

Male 5-week-old SCID (severe combined immunodeficiency) mice purchased from National Institutes for Food and Drug Control were used for PCa metastasis assays. Approximately 5×10^6 PC-3 or LNcap cells suspended in 1 ml of PBS and equivalent physiological saline were injected subcutaneously into lower right and left abdomen of SCID mice, respectively. About sixty days after the initial inoculation, four mice with tumor nodules from each group were chosen for urethral subcutaneously transplanted

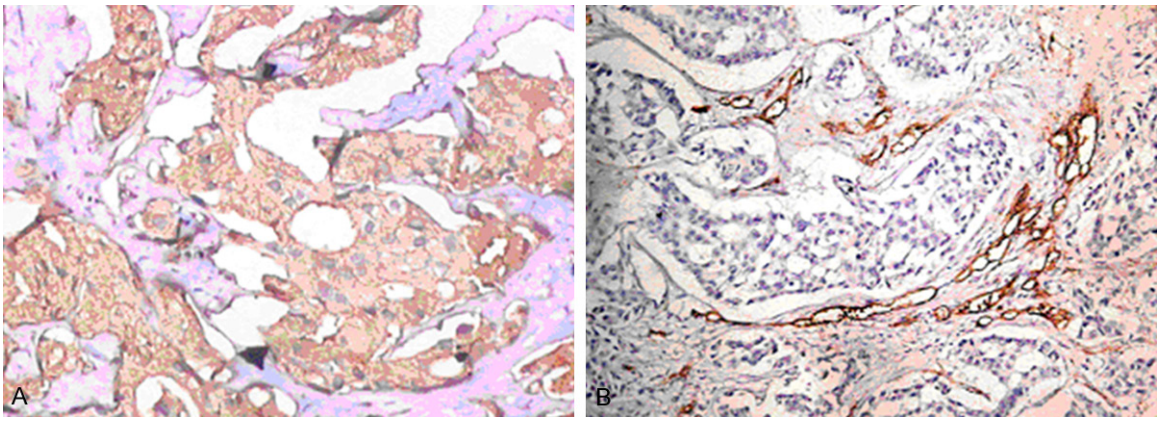

Figure 1. Histological micrographs of PCa tissue specimens. A. Immunohistochemical staining of PCa bone metastasis tissue with antibodies to CXCR4. The cellular membrane and cytoplasm of tumor cells present brown (Magnification × 200). B. Immunohistochemical staining of PCa tissues with antibodies to CD34. Mesenchymal vessels are CD34 positive and distributed in filamentous (Magnification × 200).

tumor. Responses to the external disturbance, behavior and diet shift of mice were detected every three days to learn the tumor growth process, and the dead mice were dissected for pathological analysis. One hundred and twenty days after subcutaneous injection, all mice were sacrificed for dissection and taking photos. Tissues were taken and examined macroscopically and microscopically for occurrence of metastasis by histopathology. Fresh transplantation tumor samples were chosen for ultrastructural pathology and images were acquired with JEM-2000EX TEM (transmission electron microscope).

Statistical analysis

All in vitro experiments were performed at least three times. Numerical data are expressed as mean ± standard deviation. Statistical analysis was performed one-way ANOVA or Student's t-test or χ^2-test with significance at $P < 0.05$ and highly significance at $P < 0.01$. Statistical Package for Social Science (SPSS) version 13.0 was used.

Results

Pathological analysis of prostate cancer tissue specimens

To study the association of SDF/CXCR4 axis and cell migration in prostate cancer, we divided the PCa samples into three groups according to the WHO classification and Gleason scoring system. Immunohistochemical analysis was used to investigate the expression patterns of CXCR4. The expression of CXCR4 in adjacent normal tissue of 148 PCa specimens and BPH was minimal or absent, while CXCR4 was high expressed in 148 PCa tissues, especially in Lumen basal cell layers. The high expression of CXCR4 is significantly associated with tumor differentiation, bone metastases ability, patient age and androgen receptor (AR) in PCa patients (**Table 1**). This was consistent with the previous report that androgens induce CXCR4 gene expression in prostate cancer cells and androgen-responsive VCaP cell lines coexpress higher levels of CXCR4 and ERG compared with androgen-unresponsive PC-3 cells [4]. CXCR4 is diffuse distribution or strong positive on the edge of the cancer nests in invasive PC tissues and bone metastases tissues, especially in low differentiated PC tissues (**Figure 1A**).

Previous study indicates that CXCR4 regulates the expression of VEGF and MMP-9 [32]. Immunohistochemical analysis indicated that the expression of VEGF and MMP-9 was increased in samples with low tumor differentiation, high metastases ability ($P < 0.05$), but not related with patient age and AR ($P > 0.05$) (**Table 2**).

We also investigate the relationship between MVD and PCa. Immunohistochemical analysis was used to identify the MVD, and then the numbers were recorded (**Figure 1B**). MVD was significantly associated with CXCR4, VEGF and MMP-9. MVD was significantly increased in

Table 2. Association between VEGF and MMP-9 expression and clinicopathological features from PCa tissues

Criteria	Cases	VEGF				High expression (%)	P	MMP-9				High expression (%)	P
		-	+	++	+++			-	+	++	+++		
Pathological grade													
Well	41	0	36	4	1	12.19	< 0.05	13	25	2	1	7.318	< 0.05
Moderate	51	0	12	31	8	76.47		2	35	12	2	27.45	
Poor	56	0	2	29	25	96.43		1	14	33	8	73.21	
Age (yrs.)													
≤ 65	59	0	16	30	13	72.88	> 0.05	5	30	21	3	40.68	> 0.05
> 65	89	0	34	34	21	61.79		11	44	26	8	38.21	
Metastasis													
Positive	10	0	0	1	9	100	< 0.05	0	0	5	5	100	< 0.05
Negative	138	0	53	63	23	62.32		16	74	42	6	34.78	
AR expression													
Positive	76	0	34	28	14	55.26	> 0.05	11	44	16	5	27.63	> 0.05
Negative	72	0	16	36	20	77.78		5	30	31	6	51.39	
Implanted tumor	30	-6		14	10	80.00	< 0.05	-7		17	6	76.67	< 0.05
Metastatic tumor	168	-21		29	118	87.50		-25		37	106	85.12	

Table 3. Association between MVD and clinicopathology from PCa tissues

Criteria		Cases	MVD (mean ± SD)	F value
Pathological grade	Well	41	34.42 ± 3.91	112.5 (P < 0.01)
	Moderate	51	42.62 ± 3,80	
	Poor	56	49.68 ± 6.36	
Age (yrs.)	≤ 65	59	42.94 ± 7.37	0.009 (P > 0.05)
	> 65	89	43.07 ± 8.21	
Metastasis	Positive	10	48.37 ± 7.15	5.1 (P < 0.05)
	Negative	138	42.63 ± 7.79	
AR expression	Positive	76	40.37 ± 7.19	20.04 (P < 0.01)
	Negative	72	45.82 ± 7.61	
CXCR4	+	37	35.43 ± 5.06	52.52 (P < 0.01)
	++	74	43.45 ± 6.29	
	+++	37	49.75 ± 6.37	
VEGF	+	37	36.06 ± 5.20	49.33 (P < 0.01)
	++	74	46.21 ± 6.41	
	+++	37	47.25 ± 6.73	
MMP-9	-	16	37.73 ± 5.41	23.20 (P < 0.01)
	+	74	40.18 ± 6.48	
	++	47	47.87 ± 6.74	
	+++	11	50.55 ± 7.32	

specimens with low tumor differentiation, high metastases ability, AR-negative and high expression of CXCR4, VEGF and MMP-9 ($P < 0.05$), but not with patient age ($P > 0.05$) (**Table 3**).

SDF-1/CXCR4 axis promotes the expression of intercellular adhesion molecules

To evaluate the effect of SDF-1/CXCR4 axis on metastases ability, we used CXCR4-GFP-expressing PC-3 cells with PC-3 cells transfected with vector and PC-3 cells as control. The expression of CXCR4 was significantly increased in transfection group (**Figure 2A**, **2B**). Tumor cells can attract or activate tumor-associated stromal cells by releasing cytokines to facilitate their growth, invasion and metastasis. Previous studies suggest VLA-4/VCAM-1, LFA-1/ICAM-1 involve in tumor cells invasion and metastasis [6, 30]. Therefore, we suspected these intercellular adhesion molecules may also mediate the bone metastases of PCa cells. Q-PCR and immunoblotting analysis were used to investigate the expression patterns of VLA-4/VCAM-1 and LFA-1/ICAM-1 in CXCR4-GFP-expressing PC-3 cells with 24 h SDF-1 induction. The mRNA levels of VCAM-1 and LFA-1/ICAM-1 significantly increased except VLA-4 (Figure S1), and the

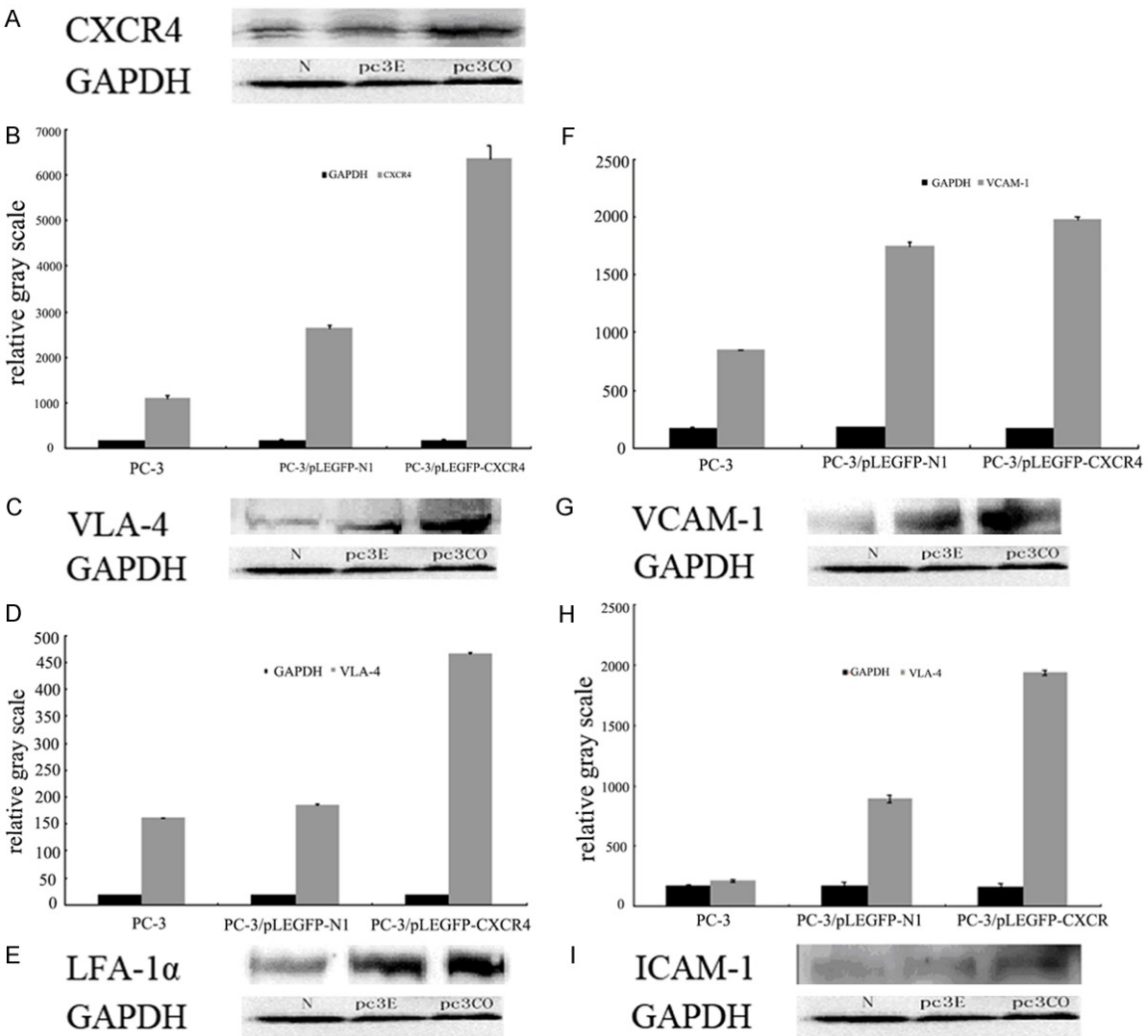

Figure 2. SDF-1/CXCR4 axis promotes upregulation of intercellular adhesion molecules. Expression of CXCR4 in PC-3 cells transfected with none, pEGFP-N1 and pEGFP-N1-CXCR4, and GAPDH served as an internal control (A). Histogram of western data showed CXCR4 under different conditions. Expression of VLA-4 (C), VCAM-1 (E), LFA-1α (G) or ICAM-1 (I) in PC-3 cells, PC-3 cells expressing GFP and PC-3 cells expressing CXCR4-GFP, and GAPDH served as an internal control. Histogram of immunoblots data show VLA-4 (D), VCAM-1 (F), LFA-1α (I) under different conditions (means ± SD, n = 3).

protein levels of VLA-4/VCAM-1 and LFA-1/ICAM-1 were upregulated remarkably (**Figure 2C-I**). These results indicated SDF-1/CXCR4 axis may promote the expression of these adhesion molecules in PC-3 cells, but the regulatory mechanism is still unclear. We need to find the pathways that may affect the expression of these adhesion molecules.

SDF-1/CXCR4 axis affects the expression and activity of p38, NF-κB and HIF-1α

The SDF-1/CXCR4 axis has been reported to regulate tumor cells proliferation and migration through activation of several signaling pathways. In order to find the transcription factors involving PCa cells metastases, we tested several key transcription factors according to previous studies. Q-PCR or immunoblotting analysis was used to investigate the expression status of p38, NF-κB and HIF-1α. The data showed that p38 protein level was significantly increased in CXCR4-GFP-expressing PC-3 cells, while p38 phosphorylation was stimulated and the activity of p38 increased (**Figure 3A** and **3B**). SDF-1/CXCR4 axis promoted increase of NF-κB and HIF-1α mRNA (data not shown). EMSA was used to test the DNA binding ability of NF-κB and HIF-1α and found that CXCR4

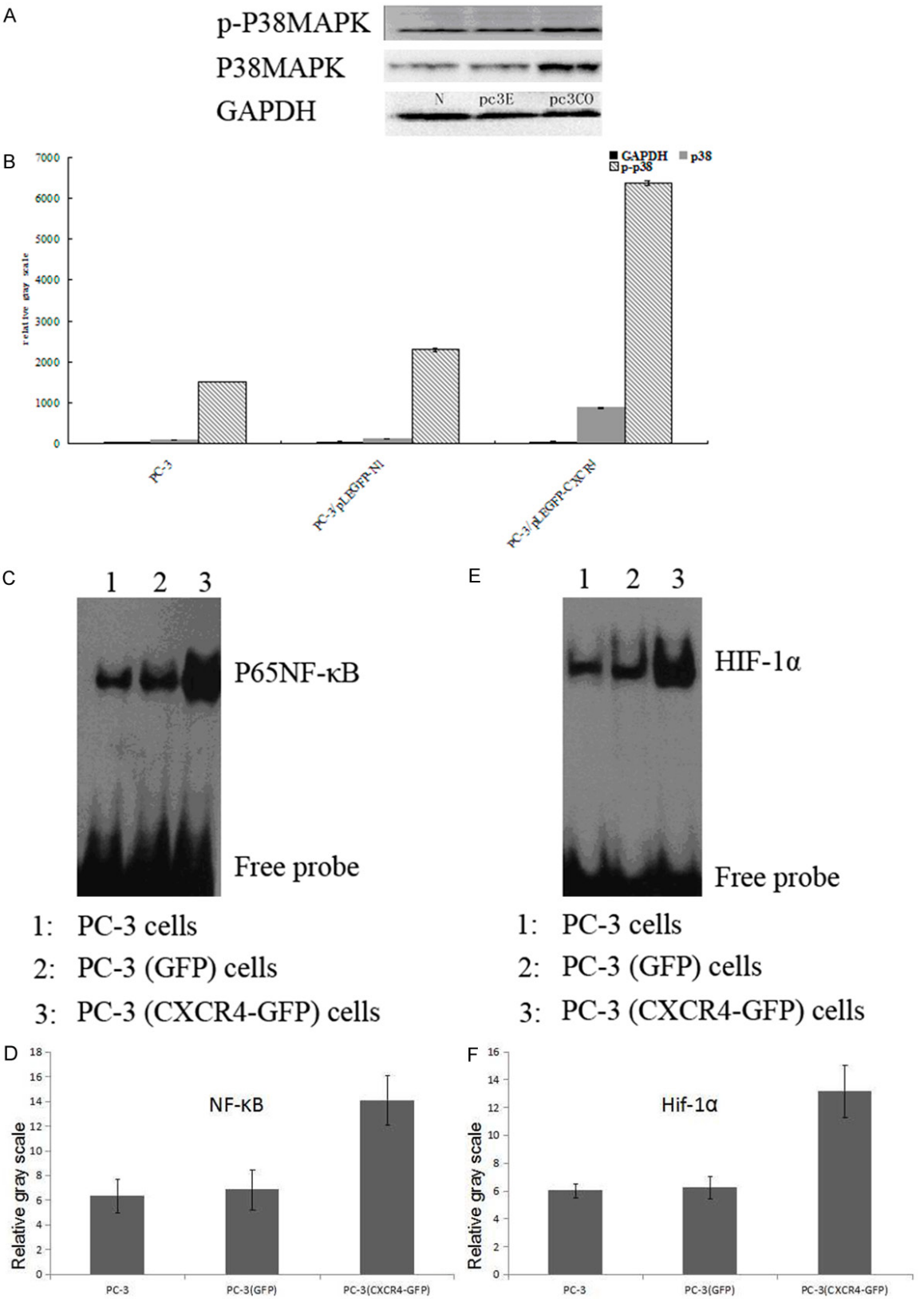

Figure 3. SDF-1/CXCR4 axis promotes expression and activation of p38, NF-κB and HIF-1α. Expression of p38 MAPK and p38 MAPK phosphorylation in PC-3 cells, PC-3 cells expressing GFP and PC-3 cells expressing CXCR4-GFP with GAPDH as an internal control (A). Histogram of immunoblots data show p38 MAPK and phosphorylated p38 MAPK

under different conditions (means ± SD, n = 3). (C~F) EMSA was used to test the DNA binding ability of NF-κB (C) and HIF-1α (E) in PC-3 cells, PC-3 cells expressing GFP and PC-3 cells expressing CXCR4-GFP. Histogram data shows the DNA binding ability of NF-κB (D) and HIF-1α (F).

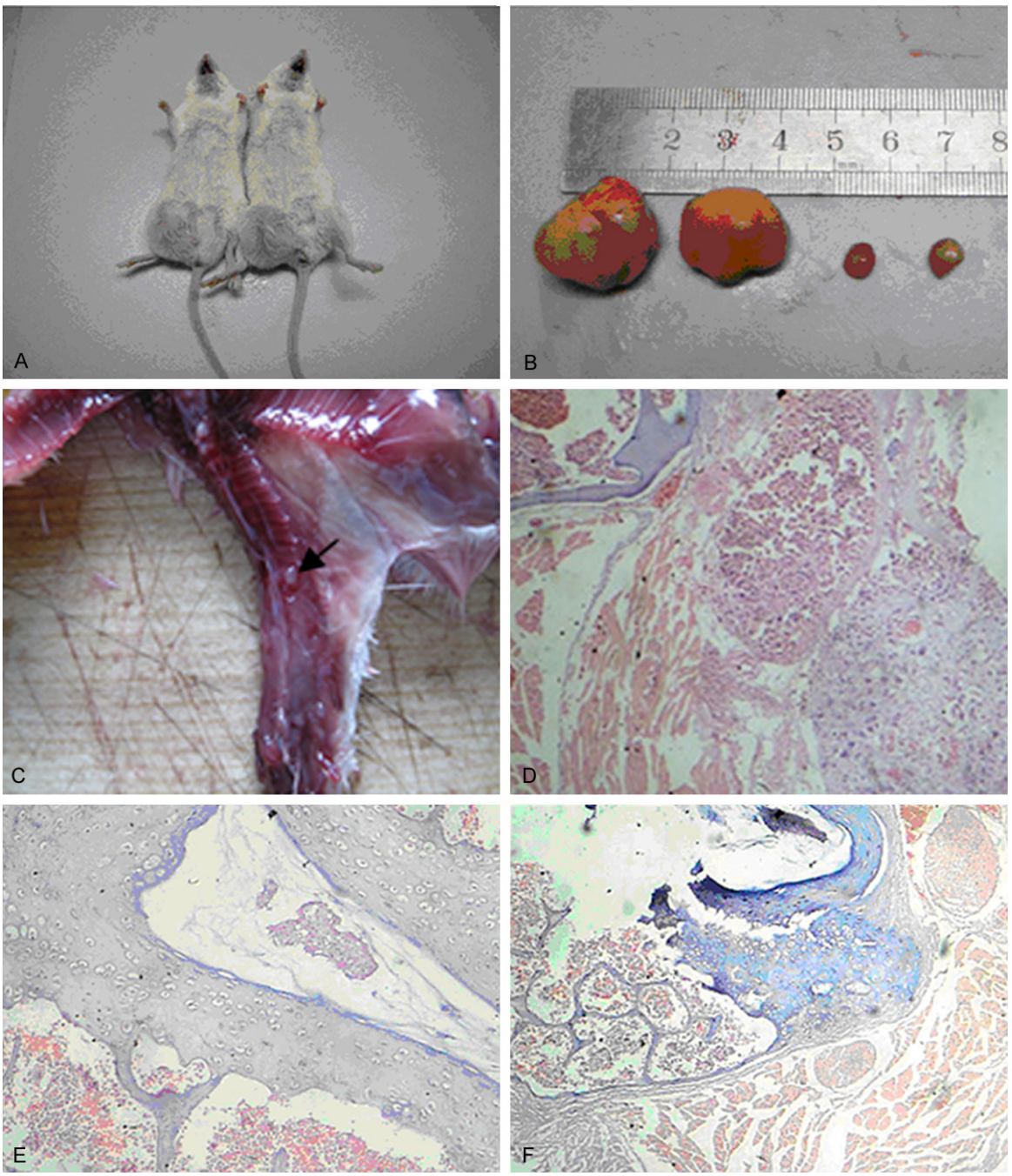

Figure 4. SDF-1/CXCR4 axis accelerates PCa bone metastasis *in vivo.* LNcap cells, PC-3 cells, PC-3 cells expressing GFP and PC-3 cells expressing CXCR4-GFP were used for tumor xenografts in SCID mice. (A) SCID mice with PC-3 cells expressing CXCR4-GFP tumor xenografts (72 d) grew big tumor in lower right abdomen. (B) Comparison of Implantation tumor between SCID mice with PC-3 cells and PC-3 cells expressing CXCR4-GFP, the latter is much bigger. (C) SCID mice with PC-3 cells expressing CXCR4-GFP tumor xenografts (86 d) grew bone metastasis. Metastasis located in periosteal surface; tumor cells arranged in disperse, without envelopes; the edge of tumor infiltrative growth showed abundant capillaries (D). Metastasis located cartilage lacuna inside, presenting sheet distribution (E). Entochondrostosis and chondral-calcification in CXCR4-GFP group (F) (Magnification × 200).

Table 4. Metastasis analysis in SCID mice with tumor xenografts

Groups	Cases	Implanted tumor	Metastatic tumor	Bone metastasis (%)
LNcap	8	7	44	1 (12.5)
PC-3	8	8	37	2 (25)
PC-3 (GFP)	8	7	32	0 (0)
PC-3 (CXCR4-GFP)	8	8	55	3 (37.5)

over-expression simulated the affinity between DNA and NF-κB or HIF-1α (**Figure 3C-F**) under SDF-1 induction. These results suggested these signaling pathways and transcription factors were involved in PC-3 migration and might promote this progress via regulate the expression of adhesion molecules.

SDF-1/CXCR4 axis promotes PCa bone metastasis in vivo

Previously, it has been shown that SDF1-CXCR4 interactions may play a role in the metastasis of prostate cancer to bone, in which expression of adhesion molecules were activated. It also have been demonstrated that human prostate cell lines derived from metastases express functional CXCR4 receptor and that SDF1 enhances their migratory capabilities [2]. In our previous work, it indicated that CXCR4 is essential for PCa metastasis via the up-regulation of VEGF and MMP-9 [32]. These results suggest prostate cancers may be influenced by the CXCR4 during metastasis. To further confirm the role of SDF-1/CXCR4 in PCa bone metastases, we also performed tumor xenografts and metastasis assays in SCID mice.

Two cells lines (LNcap and PC-3) were chosen, and PC-3 cell line included three groups of mice (PC-3, PC-3/pEGFP-N1 and PC-3/pEGFP-N1-CXCR4; eight mice per group). We found that tumors over-expressing CXCR4 grew significantly earlier and bigger than those in the control group (**Figure 4A**, **4B** and Figure S1A) and more bone metastases (**Table 4**). Compared to LNcap group, tumor was much more widespread in PC-3 groups. Bone metastases mainly happened in lumbosacral vertebrae and located in periosteal surface and cavum medullare (**Figure 4C-E**). In contrast, entochondrostosis and chondral-calcification in PC-3/pEGFP-N1-CXCR4 group were more remarkable (**Figure 4F**). Bone trabecula, branch neovascularization and monocyte in cavum medullare increased too. We also determined the expression of CXCR4, PSA, VEGF and MMP-9. The data showed that CXCR4 was expressed in tumor of all four group mice, and CXCR4 was over-expressed in PC-3/pEGFP-N1-CXCR4 group. The percent of VEGF and MMP-9 over-expression was much higher in metastatic-tumor than implant-tumor ($P < 0.05$) (**Table 2**). Taken together, the SDF1/CXCR4 axis promoted bone metastasis through regulation of VEGF and MMP-4 *in vivo.*

Discussion

The current treatment for recurrent prostate cancer is surgical or medical castration. However, a significant minority of patients will relapse and eventually develop castration-resistant PCa, the majority of which is due to the cancer metastasis [21, 22]. Therefore, there is a considerable gap in our understanding of the mechanisms that underlie prostate cancer progression, metastasis and development of effective therapies. Our results showed that SDF-1 stimulated the adhesion of PCa cells to endothelial cells, consistent with the previous report [35]. This indicated that the SDF-1/CXCR4 axis may be involved in metastasis. In our previous study, the effects of SDF-1 on cell adhesion have been attributed for the most part to changes in the cell adhesion receptors. VCAM-1 and ICAM-1 expression were increased in CXCR4-GFP-expressing PC-3 cells with 24 h SDF-1 induction. The abnormal expression of ICAM-1/VCAM-1 has been reported in multiple cancers [19, 31]. The dysregulation of these adhesion molecules may ultimately increase the risk of PCa onset [30]. Serum ICAM-1 and VCAM-1 are surrogate markers of multiple tumors, which are capable of promoting and maintaining the establishment of tumor vascular system [14, 23]. Thus, it can directly stimulate tumor growth and metastasis. Binding of VCAM-1/VLA-4 and ICAM/LFA-1 promotes tumor anti-angiogenesis and causes tumor cells shedding, migration, invasion and metastatic dissemination [15]. Other investigators analyzing PCa invasion through Matrigel have shown an increase in invasion with SDF-1 stimulation together with increased expression or activation of integrin αvβ3 or α5β1 [10]. The rolling process enables PCa cells to activate integrin signals, triggering additional adhesion

process and resulting in firm adhesions between tumor cells and endothelial cells. This process promotes the migration of tumor cells through the endothelium to the metastatic sites [36]. PCa cells preferentially metastasize to bone through these site-specific interactions.

MAPK and NF-κB are involved in human vascular endothelial cell expression of ICAM-1 and VCAM-1, while stimulation of p38 pathway can activate NF-κB pathway. p38 promotes tumor cells metastasis via upregulation of AP-1 (activator protein) and MMP-9 [5]. p38 may promote PCa metastasis to bone via inflammatory responses [34]. These studies indicate cross-linked activation of p38 MAPK pathway and NF-κB pathway regulates PCa bone metastases through upregulation of VCAM-1/VLA-4 and ICAM/LFA-1 expression. Our pathological analysis of PCa tissue specimens showed that VEGF and MMP-9 expression was increased in samples with low PCa differentiation, high metastasis ability. In vivo, PCa bone metastases results also showed that VEGF and MMP-9 expression was higher in metastatic-tumor than implant-tumor. In tumor angiogenesis, VEGF is released by tumor cells, as well as tumor-infiltrating cells. VEGF is one of numerous genes regulated by HIF1α, which induces proliferation of endothelial cells, tumor angiogenesis, invasion and metastasis [8]. Our results indicate that SDF-1/CXCR4 axis can promote upregulation and activation of HIF1α in PCa. Previous studies have demonstrated that the VEGF expression is markedly up-regulated in the vast majority of human tumors [11]. Depression of VEGF "normalizes" the tumor vessels by reducing the MVD and reversing the pathophysiologic detachment of tumor pericytes from tumor endothelium [18]. In addition, SDF-1 stimulation has been reported to increase expression of several proteases such as MMP-2, MMP-9 and MMP-14, which were related with tumor invasion and metastasis [7]. The increased expression of proteases after SDF-1 stimulation most likely creates a pathway for tumor cell invasion.

How the constitutive expression of VEGF and MMP-9 are regulated in PCa cells is not clear. Recent reports have demonstrated that NF-κB regulated the expression of MMP-9 in several types of cells. Constitutive VEGF expression is suppressed by blocking NF-κB activity in PCa. Importantly, NF-κB is known to be activated in androgen-independent PCa cell lines as well as in PCa tissues [13]. Moreover, blocking of NF-κB activity in PCa cells is associated with suppression of angiogenesis, invasion and metastasis [33]. Our results provided further evidences showing that NF-κB may regulate the expression of VEGF and MMP-9 and HIF1α, which may regulate the expression of VEGF in process of PCa bone metastases. Metastasis is the most horrible stage of tumor and a major cause of death from PC. Therefore, understanding the PCa metastatic process, and identifying the essential molecular events during metastases are important.

Our study, for the first time, plots a complex regulatory network of SDF-1/CXCR4 on VEGF, MMP-9 and adhesion molecules expression via p38, NF-κB and HIF-1α pathways. In vitro and in vivo experiments provide direct evidences of SDF-1/CXCR4 axis promoting PCa progression and bone metastases. Thus, this research may propose gene therapy for suppressing PCa progression, as well as other therapies aimed at blocking SDF-1/CXCR4 axis.

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Disclosure of conflict of interest

None.

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Table S1. Primers sequences

	Sequence (5'-3')	Length of products (bp)
Primers for CXCR4 cloning from genome DNA		
CXCR4-G-F	ATGTCCATTCCTTTGCCTCTTTTGC	1283
CXCR4-G-R	TGGCCACAGGTCCTGCCTAGAC	
CXCR4 subcloning primers		
CXCR4-F	GAAGATCTATGTCCATTCCTTTGCCTCTTTT	1083
CXCR4-R	GCGTCGACCTGGAGTGAAAACTTGAAGACT	
Q-PCR primers		
CXCR4-Q-F	GCCAACGTCAGTGAGGCAGAT	99
CXCR4-Q-R	GCCAACCATGATGTGCTGAAAC	
GAPDH-Q-F	ACCCATCACCATCTTCCAGGAG	311
GAPDH-Q-R	GCCAACCATGATGTGCTGAAAC	
VLA-4-Q-F	CGGTCTGATTCTGCTGTC	169
VLA-4-Q-R	GAACTTCCTTGCCCTTAT	
VCAM-1-Q-F	GAAATGACCTTCATCCCTAC	116
VCAM-1-Q-R	AGTGTTTGCGTACTCTGC	
LFA-1α-Q-F	TCTGTCCAGGGCATTATCT	186
LFA-1α-Q-R	ATGGTAGTGGCTGAGTTGTC	
ICAM-1-Q-F	CCTGATGGGCAGTCAACAGCTA	200
ICAM-1-Q-R	ACAGCTGGCTCCCGTTTCA	
p38 MAPK-Q-F	CGAAGATGAACTTTGCGAATG	109
p38 MAPK-Q-R	GCTTGGGCCGCTGTAATT	
NF-κB-Q-F	GCCGAGTGAACCGAAAC	141
NF-κB-Q-R	GGTGCTCAGGGATGACG	
HIF-1α-Q-F	TGTGTACCCTAACTAGCCG	157
HIF-1α-Q-R	ACAAATCAGCACCAAGC	

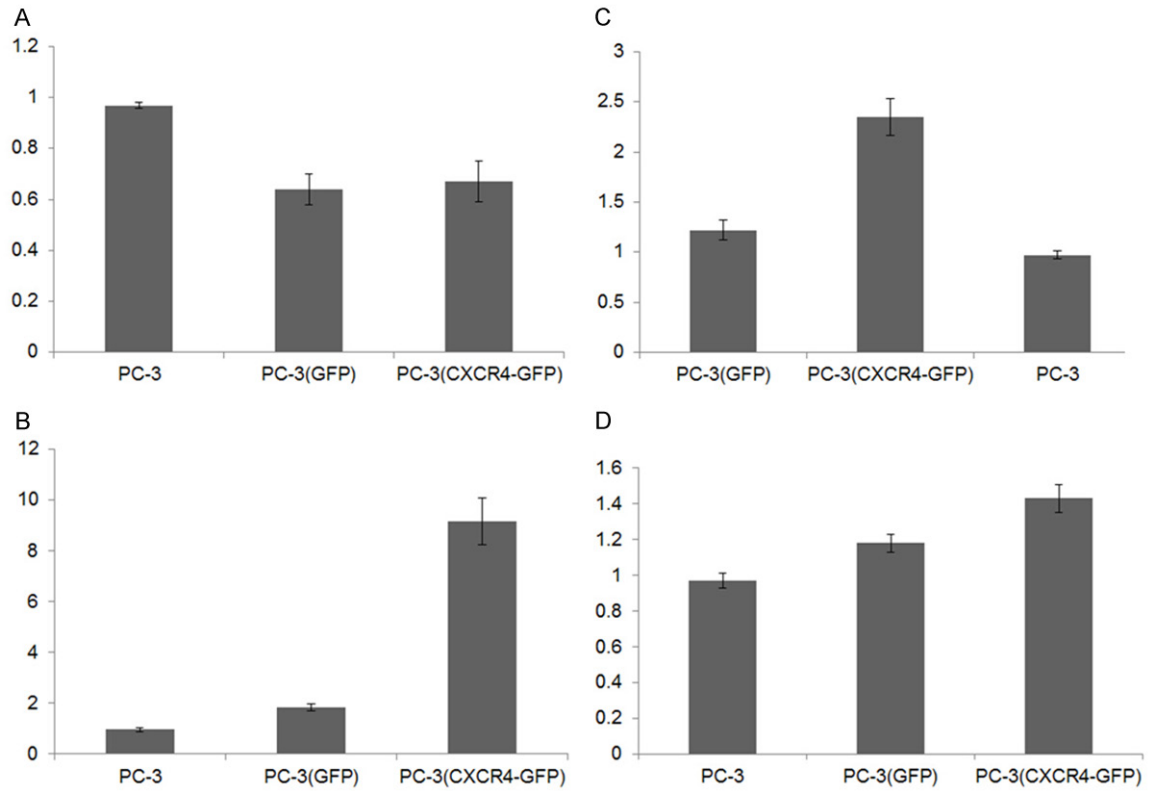

Figure S1. mRNA levels of related genes were measured by Q-PCR. PC-3, PC-3 expressing GFP and PC-3 expressing CXCR4-GFP cells were treated with SDF-1 for 24 h. Transcript levels were measured for VLA-4 (A), VCAM-1 (B), LFA-1α (C) and ICAM-1 (D). (means ± SD, n = 3; $P > 0.05$ between pEGFP-N1 group and pEGFP-N1-CXCR4 group for VLA-4; $P < 0.01$ between pEGFP-N1 group and pEGFP-N1-CXCR4 group for VCAM-1; $P < 0.01$ between pEGFP-N1 group and pEGFP-N1-CXCR4 group for LFA-1α; $P < 0.05$ between pEGFP-N1 group and pEGFP-N1-CXCR4 group for ICAM-1).