Hypoxia increases CX3CR1 expression via HIF-1 and NF-κB in androgen-independent prostate cancer cells

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Abstract. The unique CX3C chemokine CX3CL1 and its cognate receptor CX3CR1 have been implicated in organ-specific metastasis of various types of tumors. Hypoxia, a common phenomenon in solid tumors, is associated with a malignant cancer phenotype. Previous studies have proved that hypoxia facilitates cancer cell metastasis through upregulation of specific chemokine receptors. We hypothesized that hypoxia could upregulate CX3CR1 expression and lead to an increased chemotactic response to CX3CL1 in prostate cancer cells. In the present study, we found that CX3CR1 expression was significantly increased in androgen-independent prostate cancer cells, including DU145, PC-3 and PC-3M, following exposure to hypoxia. This upregulation of CX3CR1 corresponded to a significant increase in migration and invasion of prostate cancer cells under hypoxic conditions, which was attenuated after knocking down CX3CR1 expression. In addition, we examined the possible role of HIF-1 and NF-κB in the process of hypoxia-induced CX3CR1 expression and hypoxia-mediated metastasis. Attenuation of HIF-1 and NF-κB transcriptional activity by siRNAs or pharmacological inhibitors, abrogated hypoxia-induced upregulation of CX3CR1, and also prevented the migration and invasion of DU145 cells under a hypoxic environment. In summary, our study demonstrated that HIF-1 and NF-κB are essential for hypoxia-regulated CX3CR1 expression, which is associated with increased migratory and invasive potential of prostate cancer cells. CX3CR1 signaling is a potential therapeutic target in the adjuvant treatment of prostate cancer.

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Abbreviations: CX3CR1, CX3C chemokine receptor 1; HIF-1α, hypoxia-inducible factor-1α; NF-κB, nuclear factor-κB; Egr-1, early growth response-1; siRNA, small interfering RNA; $CoCl_2$, cobalt chloride; DFX, deferoxamine; PTX, pertussis toxin; YC-1, 3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole; PDTC, pyrrolidine-carbodithioc acid ammonium salt

Key words: hypoxia, prostate cancer, CX3CR1, metastasis, hypoxia-inducible factor-1α, nuclear factor-κB

Introduction

Chemokines were first described as chemoattractant cytokines synthesized at sites of inflammation and are the major regulatory proteins for leukocyte recruitment and trafficking (1,2). Chemokines are subdivided into four families, C, CC, CXC and CX3C, based on the number and spacing of the first two cysteines in a conserved cysteine structural motif (3). CX3CL1 (also known as fractalkine), the sole member of the CX3C class of chemokines, is unique, as it can exist in both soluble and membrane-anchored forms (4-6). The cognate receptor of CX3CL1 is a G-protein coupled receptor, named CX3CR1 (7). Along with strong expression on certain leukocyte populations, such as macrophages, lymphocytes, and natural killer cells, CX3CR1 is abundant on glial cells and astrocytes (8,9). Recently, CX3CR1 expression in tumors was confirmed (10-19). In addition, metastasis of cancer cells to the skeleton mediated by CX3CL1-CX3CR1 binding was also reported in a variety of tumors, including prostate cancer (10,12,15).

Prostate cancer is the second-leading cause of cancer death in western males with an estimated 240,890 new cases and 33,720 deaths in the USA in 2011 (20). Although early stage prostate cancers are not life threatening, development of metastatic prostate cancers is responsible for the majority of cancer-related death. Clinical observations provide evidence that despite current therapies, about one third of prostate cancers

invade surrounding tissue, metastasize to distant organs, and consequently cause death (21,22). Survival of a patient with prostate cancer is directly related to the spread of the tumor (23).

Hypoxia, a common phenomenon in solid tumors, is associated with malignant progression and resistant to radiotherapy and chemotherapy (24-26). Clinical studies also demonstrated that low oxygen tension in neoplasm is an independent prognostic indicator of poor outcome and correlates with an increased risk to develop distant metastasis with prostate cancer, independent of therapeutic treatment (27).

The pathophysiological responses of tumor cells to hypoxia arise from specific alterations in the expression of a large number of genes, which are regulated directly or indirectly by transcription factors. The activity of several transcription factors, including hypoxia-inducible factor-1 (HIF-1), nuclear factor-κB (NF-κB), AP-1, p53 and early growth response-1 (Egr-1) are upregulated in hypoxic cancer cells, which subsequently activate target genes expression to allow the tumor to adapt to decreased oxygen levels (28,29).

Lack of oxygen causes the cells of tumor to spread to new locations (30). Chemokines and their receptors have been demonstrated to play major roles in the process of metastasis. Multiple cancers are found to express chemokine receptors, while the corresponding ligands of the receptors are expressed at sites of tumor metastases (10,12,15,31,32). Numerous studies showed that hypoxia can promote cell migration and invasion of several different types of tumor by upregulating the specific chemokine receptors, such as CCR2, CXCR1, CXCR2, CXCR4 and CXCR6 (33-37). However, whether hypoxia mediates the metastatic property of cancer cells through regulating the CX3CR1 expression has not been examined previously. The regulation pattern of CX3CR1 expression in hypoxic cancer cells remains to be elucidated.

The current study was performed to determine whether hypoxia regulates CX3CR1 expression and promotes metastasis of prostate cancer cells by CX3CL1/CX3CR1-axis, as well as the mechanism involved in the process. Our data showed that the expression of CX3CR1 was upregulated by hypoxia, which resulted in an increasing sensitivity to CX3CL1-stimulated migration and invasion in prostate cancer cells.

Materials and methods

Cell culture and hypoxic treatment. Human prostate cancer cell lines DU145 and PC-3 were purchased from American Type Culture Collection (Manassas, VA, USA) and PC-3M was obtained from the Peking University Health Science Center (Beijing, China). Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA) and 1x penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere (5% CO_2/95% air). For experiments involving hypoxia, cells were incubated in a hypoxic chamber (Thermo Scientific) maintained at 1% O_2, 5% CO_2 and 94% N_2 at 37˚C for 24-48 h. $CoCl_2$ (100 μM) (Sigma, St. Louis, MO, USA) or 500 μM DFX (Sigma) was supplemented in the medium to mimic the hypoxic environment under standard culture conditions.

RNA isolation and semi-quantitative real-time PCR. Total-RNA from cells in normoxic or hypoxic conditions was extracted using TRIzol reagent (Invitrogen). RNA was converted to cDNA with Superscript II reverse transcriptase (Invitrogen) by following the manufacturer's instructions. Subsequently PCR was performed using ABI 7500 real-time PCR system (Applied Biosystems). Primers used in this study were: β-actin, sense: 5'-TACCTCATGAAGATCCTCACC-3'; antisense: 5'-TTTCGTGGATGCCACAGGAC-3'; CX3CR1, sense: 5'-CTTCTGGTGGTCATCGTG TT-3'; antisense: 5'-GGTATCTTCTGAACTTCTCCC-3'; CXCR4, sense: AATCTTCCTGCCCACCATCT; antisense: GACGCCAACATAGACCACCT; Bcl-2, sense: AAAGGACCTGATCATTGGGG; antisense: CAACTCTTTTCCTCCCACCA. All the primers were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Annealing temperature was ~58.5-60.5˚C for CX3CR1 and 55˚C for CXCR4, Bcl-2 and β-actin. Amplification cycles were 35 for CX3CR1, 28 for CXCR4 and Bcl-2, and 23 for β-actin. Each sample was tested in triplicate for analysis of relative gene expression and transcript levels of β-actin were monitored as internal control.

Preparation of total cell protein and nuclear fractions. Cells cultured in normoxic or hypoxic conditions, or treated with different chemicals were washed with cold PBS and lysed in ice-cold lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma) for 10 min on ice. The lysate was centrifuged at 12,000 x g at 4˚C for 15 min and the supernatant was collected.

The nuclear protein was prepared using Nuclear and Cytoplasmic Protein Extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, cells were washed in ice-cold PBS and the pellet was resuspended in cytoplasmic protein extraction agent A supplemented with 1 mM PMSF. After incubated on ice for 15 min, the cytoplasmic protein extraction agent B was added and the tubes were incubated on ice for 1 min. Then the samples were centrifuged for 5 min at 14,000 x g at 4˚C. The pellet was resuspended in nuclear protein extraction agent supplemented with PMSF. After vortexing the tubes ~15-20 times for 30 min and centrifuging for 10 min at 14,000 x g, the supernatants containing the nuclear extracts were obtained. The protein concentrations were determined using the BCA Protein Assay Reagent (Beyotime Institute of Biotechnology).

Western blot analysis. Total protein extraction was subjected to western blot analysis of CX3CR1 and β-actin, and nuclear fraction was subjected to western blot analysis of HIF-1α, p65 and lamin B. Equal amounts of protein (60 μg/lane) was loaded, fractionated by 12% SDS-PAGE and transferred onto nitrocellulose membrane by electro-blotting. After blocked with 5% fat-free milk for 2 h at room temperature, membranes were blotted with rabbit anti CX3CR1 polyclonal antibody (Abcam, Cambridge, MA, USA), rabbit anti β-actin polyclonal antibody (Abmart, Omaha, NE, USA), mouse anti-HIF-1α monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti p65 polyclonal antibody and rabbit anti-lamin B polyclonal antibody (Proteintech group. Inc.) at 4˚C overnight. Bound primary antibody was detected by incubating with appropriate horseradish peroxidase-conjugated secondary antibodies (Proteintech group Inc.) for 2 h. Immunoreactive bands were visualized using the western blot analysis Super ECL plus Detection Reagents

(Applygen Technologies Inc., Beijing, China). The expression levels of β-actin or lamin B were monitored as internal control.

Stable transfection of pcDNA3.1-CX3CR1. The pcDNA3.1-CX3CR1 was constructed by our laboratory. cDNA encoding human CX3CR1 was inserted into the pcDNA3.1-vector. DU145 cells were transfected with 2 μg pcDNA3.0-CX3CR1 (DU145-CX3CR1) or pcDNA3.1 empty vector (DU145-Mock) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. G418 (800 μg/ml) (Gibco-BRL) was applied to select G418 resistant cells.

siRNA transfection. For siRNA treatment, oligonucleotides corresponding to nucleotide sequences of CX3CR1 and HIF-1α were synthesized commercially by Invitrogen and used at the concentration of 100 nM. A scrambled oligonucleotide was included in all experiments as negative control at the same concentration. Sequences are as follows: CX3CR1-siRNA, 5'-UUUGUUUCCACAUUGCGGAGCACGG-3'; HIF-1α-siRNA, 5'-AUAUGAUUGUGUCUCCAGCGGCUGG-3'. Signal Silence NF-κB p65 siRNA kit was purchased from Cell Signaling Technology and trasfection was performed according to the manufacturer's recommendations. The transfected cells were collected at 48 or 72 h for mRNA isolation and protein extraction, respectively.

Flow cytometry analysis. Cells cultured in normoxia or hypoxia, or treated with different chemicals such as siRNAs, DMSO (Sigma), YC-1 (Sigma) and PDTC (Beyotime Institute of Biotechnology) were suspended in ice-cold PBS and washed two times. Then cells were incubated with FITC-conjugated anti-human CX3CR1 antibody (Biolegend, San Diego, CA, USA) or FITC-conjugated rat IgG2b κ isotype control (Biolegend) at 4˚C for 30 min and washed twice with ice-cold PBS. The samples were finally analyzed by flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA) using CellQuest software.

Migration and invasion assay. Cell invasion assay was performed using Matrigel-coated 24-well Transwell inserts containing polycarbonate filters with 8 μm pores (BD Biosciences) according to manufacturer's instructions. Briefly, the inserts were pre-hydrated with 500 μl/well serum-free RPMI-1640 medium for 2 h at 37˚C in a humidified atmosphere (5% CO_2/95% air). Test cells $1x10^5$ in 200 μl serum-free RPMI-1640 medium were seeded onto the upper chambers, whereas 600 μl complete medium containing ~0-400 ng/ml recombinant human CX3CL1 (Proteintech group Inc.) was added to the lower wells. Cells were allowed to invade for 24 h in hypoxic or normoxic environment. In some experiments, cells were preincubated 1 h with 3 μg/ml neutralizing antibody against human CX3CR1 (Abcam), rabbit IgG-matched polyclonal antibody (Abcam), 100 ng/ml PTX (Sigma), 50 μM DMSO, 50 μM YC-1 or 100 μM PDTC before seeding, respectively. In siRNA transfection experiments, cells were transfected with scrambled-siRNA, CX3CR1-siRNA, HIF-1α-siRNA or p65-siRNA for 72 h, then collected and plated onto the upper chamber. The non-invasive cells on the upper surface of the membrane were removed by a cotton swab, and the invaded cells attached to the lower membrane surface were fixed in 4% paraformaldehyde methanol for ~10-15 min, subsequently stained with 0.1% crystal violet for 30 min. The number of invading cells was then counted for a given well under a light microscope (Olympus IX51, Japan) at a magnification of x200. Five random fields were numerically averaged and counted for each assay. The invasion index was calculated by dividing the number of invading cells with the number of cells that invaded in control group and was expressed as percentages of control values, as previously described (38). Cell migration assay was performed through a similar approach without Matrigel coating, and cells in migration chamber were allowed to migrate for 16 h.

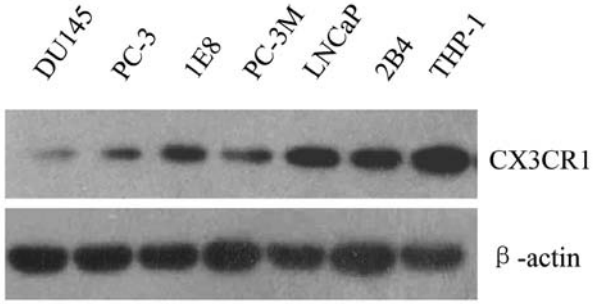

Figure 1. Expression of CX3CR1 in human prostate cancer cell lines. Western blot analysis of CX3CR1 protein expression was assessed in 6 prostate cancer cell lines. Human monocytic THP-1 cells were used as positive control.

Statistical analysis. Statistical analyses were performed using the SAS system, version 9.1.3. Quantification of the bands from western blot analysis were determined by MetaView Image Analyzing System (Version 4.50; Universal Imaging Corp., Downingtown, PA, USA) and each band was normalized by its corresponding control. Each experiment was replicated in triplicate. Results were expressed as mean ± SEM. $p<0.05$ was considered as statistically significant.

Results

Expression of CX3CR1 in prostate cancer cells. CX3CR1 expression was firstly examined in six prostate cancer cell lines, which were derived from metastases localized to different tissues *in vivo*. Human monocytic THP-1 cells, which constitutively express high level of CX3CR1, were used as positive control (Fig. 1). We found that all the cell lines tested were positive for CX3CR1, indicating that CX3CR1 expression is a common character for prostate cancer cells. However, the expression level of the protein was much lower in DU145 cells than that of the other cell lines. Therefore, DU145 cells, together with PC-3 and PC-3M were selected for hypoxia treatment because of their relatively low endogenous CX3CR1 protein expression.

Hypoxia enhances CX3CR1 expression in prostate cancer cells. We exposed DU145, PC-3 and PC-3M cells to 1% O_2 for the indicated times, and performed a dynamic analysis to examine the changes in CX3CR1 expression. As shown in Fig. 2A, the expression of CX3CR1 mRNA was strongly increased with culture time in hypoxia-exposed cells. The expression remained elevated until at least 24 h. Because of the hypoxia-mimicking effect, cobalt chloride ($CoCl_2$) and iron chelator Deferoxamine (DFX) have been used extensively to study the hypoxic signaling pathway. We also incubated pros-

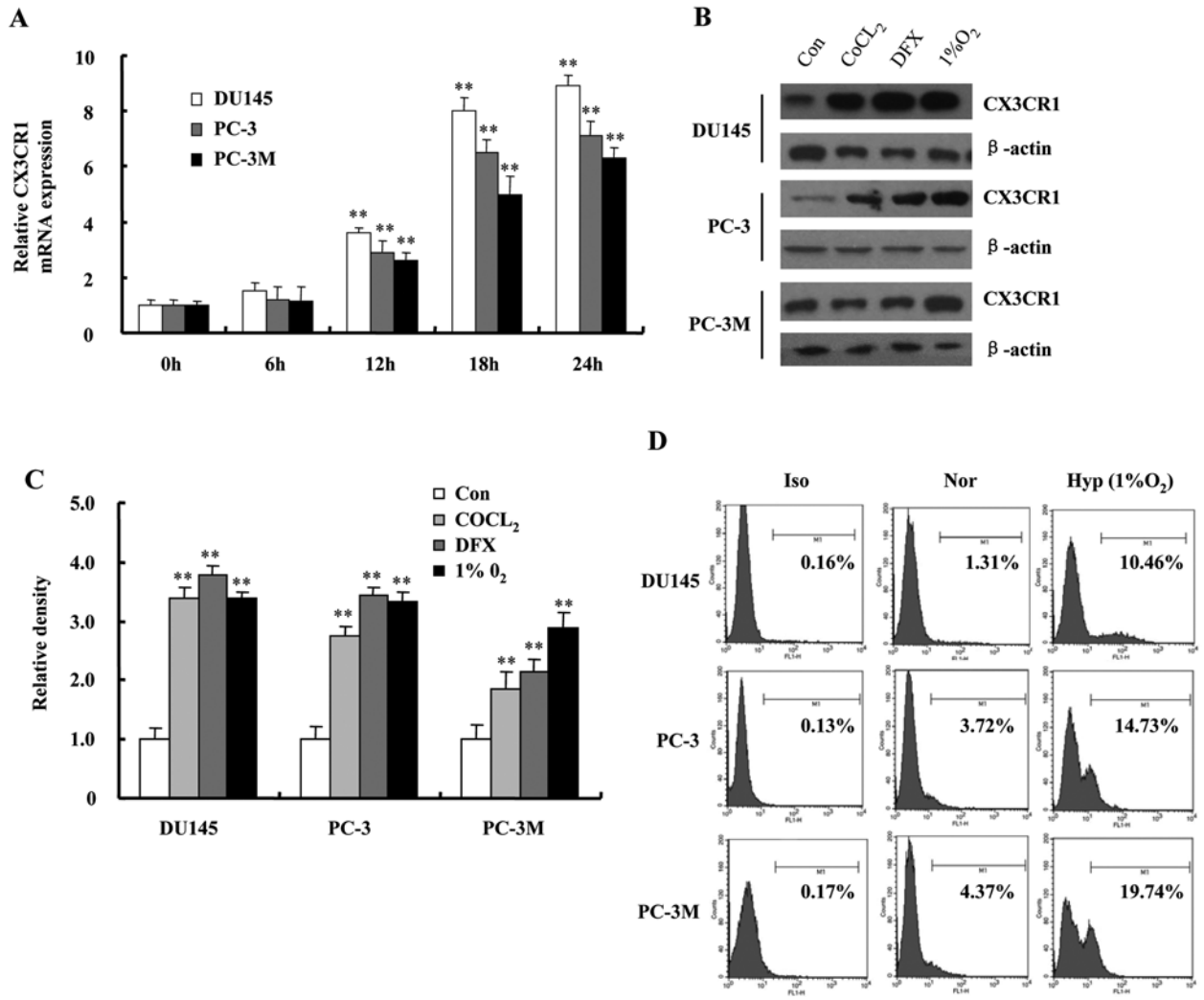

Figure 2. Hypoxia increases CX3CR1 expression in prostate cancer cells. (A) Cells were exposed to 1% O_2 for different times as indicated. CX3CR1 mRNA expression was determined by real-time PCR. $^{**}p<0.01$ vs 0 h. (B) DU145, PC-3 and PC-3M cells were treated with 1% O_2, 100 μM $CoCl_2$ or 500 μM DFX for 24 h, respectively. CX3CR1 protein expression levels were determined by western blot analysis. (C) Quantitative analysis of band density normalized to β-actin of the same sample was expressed graphically. $^{**}p<0.01$ vs control. (D) Surface expression of CX3CR1 was determined by flow cytometry using a FITC-conjugated anti-human CX3CR1 antibody.

tate cancer cells with either 100 μM $CoCl_2$ or 500 μM DFX under normoxic conditions for 24 h. The results showed, that both $CoCl_2$-treatment and DFX-treatment increased CX3CR1 protein expression in DU145, PC-3 and PC-3M cells similarly to 1% O_2 (Fig. 2B and C). Increased surface expression levels of CX3CR1 in the three cell types after exposure to 1% O_2 were also confirmed by flow cytometry (Fig. 2D).

Hypoxia activates HIF-1 and NF-κB and induces their target gene expression in DU145 cells. HIF-1 is one of the major regulators of the cellular response to hypoxia. Western blot analysis revealed a rapid increase in HIF-1α protein accumulation in nuclear extracts from hypoxia-exposed DU145 cells. HIF-1α protein level remained elevated following exposure to hypoxia for 12 h (Fig. 3A). CXCR4, the best characterized chemokines, has been shown to play critical roles in tumor progression and metastasis. Studies demonstrated that hypoxia markedly enhanced CXCR4 expression through HIF-1 in solid tumors. Therefore, to confirm HIF-1 transcriptional activity in hypoxic DU145 cells, we performed real-time PCR to analyze the mRNA expression levels of CXCR4. We found that hypoxia increased CXCR4 mRNA transcript levels in a time-dependent manner (Fig. 3B). Then, we employed siRNA strategy to deplete HIF-1α expression in DU145 cells and re-examined CXCR4 mRNA expression levels under hypoxic condition. The hypoxia-induced HIF-1α protein accumulation in nuclear extracts and CXCR4 mRNA expression were greatly inhibited in the presence of HIF-1α-siRNA compared with scrambled-siRNA (Fig. 3C and D). The results confirmed that HIF-1 is active and could regulate its target gene expression in hypoxic DU145 cells.

NF-κB is another transcription factor that can be activated by hypoxia. As shown in Fig. 4A and B, hypoxia increased p65 protein accumulation in nuclear extracts and induced the mRNA expression of known NF-κB target gene Bcl-2 in DU145 cells. Thereafter, we established p65-siRNA for inhibition of NF-κB activity. Suppression of p65 expression using siRNA blocked hypoxia-induced p65 nuclear translocation and decreased Bcl-2

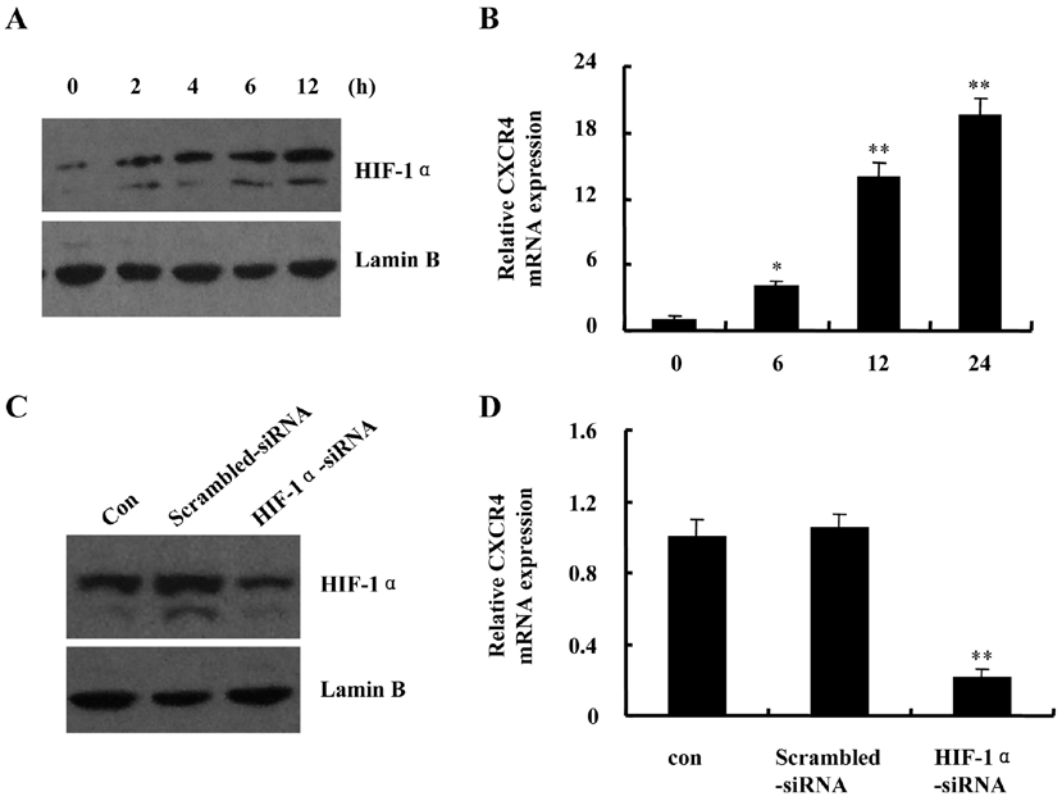

Figure 3. Hypoxia activates HIF-1 in DU145 cells. (A) DU145 cells were exposed to 1% O_2 for different times as indicated. HIF-1α protein accumulation in nuclear extracts was determined by western blot analysis. Lamin B expression was used as a loading control. (B) CXCR4 mRNA expression induced by hypoxia was detected by real-time PCR. DU145 cells transfected with HIF-1α-siRNA or scrambled-siRNA for 48 h were exposed to 1% O_2 for 12 h, then HIF-1α protein level and CXCR4 mRNA expression were detected by (C) western blot analysis and (D) real-time PCR. $^{*}p<0.05$, $^{**}p<0.01$ vs control.

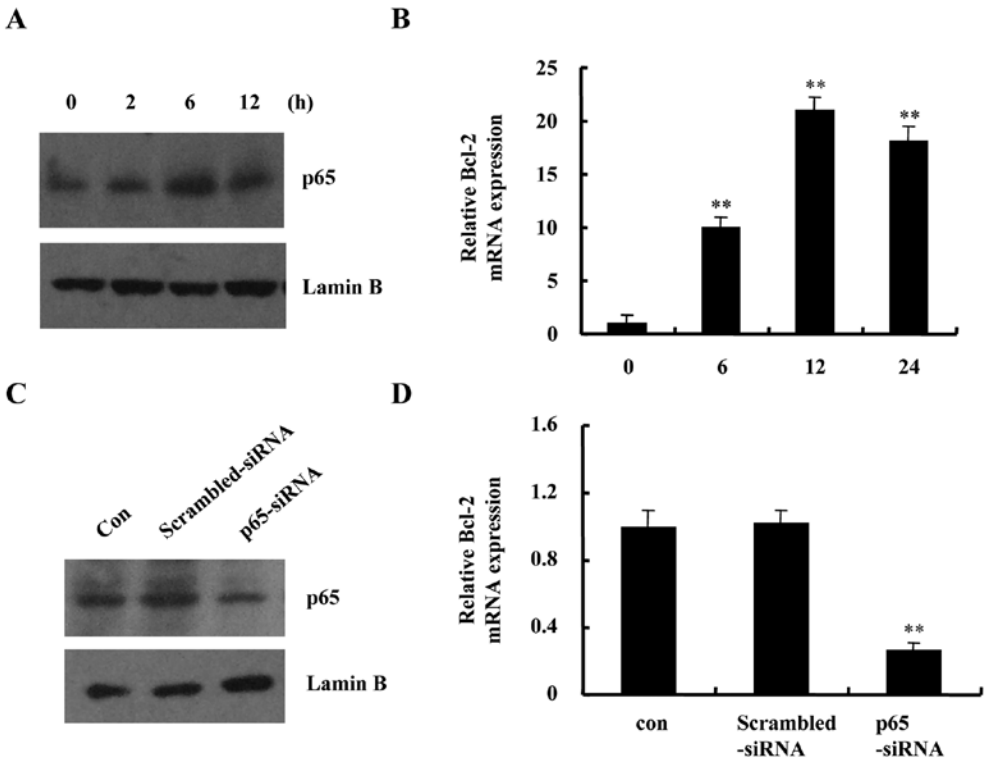

Figure 4. Hypoxia activates NF-κB in DU145 cells. (A) DU145 cells were exposed to 1% O_2 for different times as indicated. NF-κB subunit p65 protein accumulation in nuclear extracts was detected by western blot analysis. Lamin B expression was used as a loading control. (B) Bcl-2 mRNA expression induced by hypoxia was determined by real-time PCR. DU145 cells transfected with p65-siRNA or scrambled-siRNA for 48 h were exposed to 1% O_2 for 12 h, then p65 protein level and Bcl-2 mRNA expression were detected by (C) western blot analysis and (D) real-time PCR. $^{**}p<0.01$ vs control.

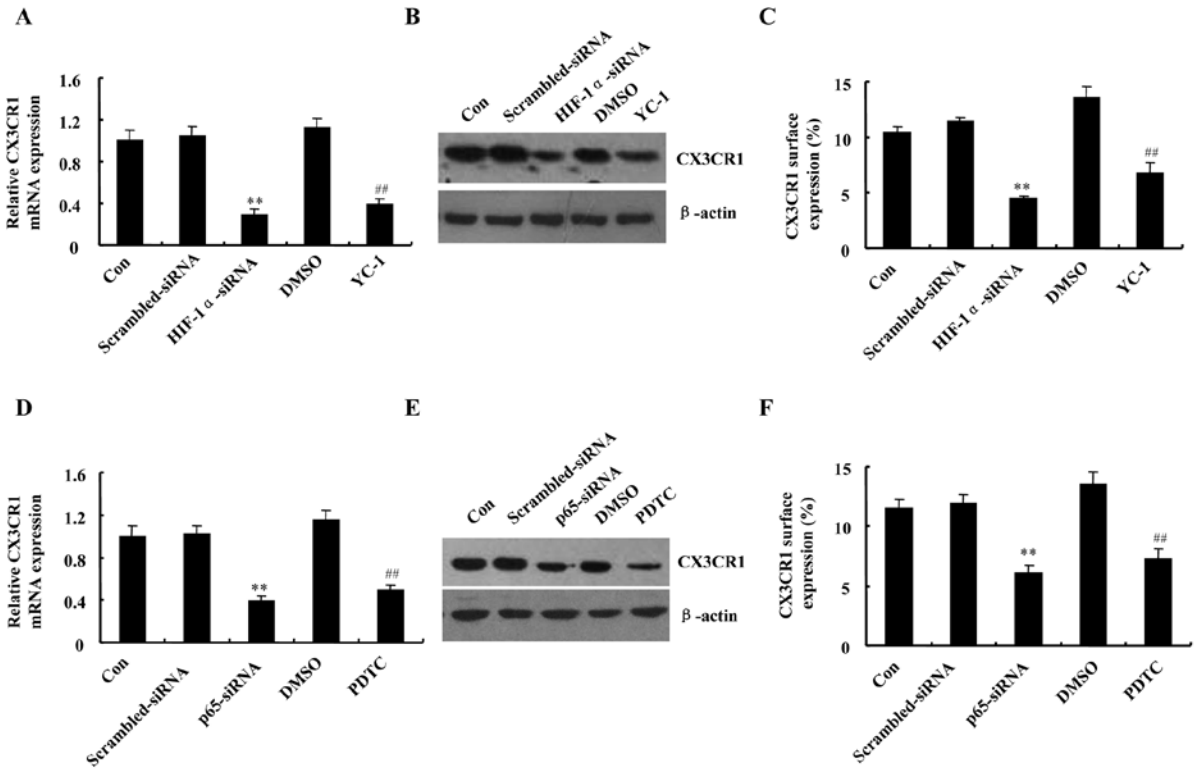

Figure 5. Roles of HIF-1 and NF-κB in hypoxia-induced upregulation of CX3CR1 in DU145 cells. DU145 cells transfected with HIF-1α-siRNA or scrambled-siRNA for 48 h, or pretreated with DMSO or YC-1 for 1 h, were exposed to hypoxia (1% O_2) for another 24 h. CX3CR1 mRNA and protein expression were determined by (A) real-time PCR and (B) western blot analysis. (C) CX3CR1 surface expression of the cells mentioned above in (A) and (B) was detected by flow cytometry. DU145 cells transfected with p65-siRNA or scrambled-siRNA for 48 h, or pretreated with DMSO or PDTC for 1 h, were exposed to hypoxia (1% O_2) for another 24 h. CX3CR1 mRNA and protein expression were determined by (D) real-time PCR and (E) western blot analysis. (F) CX3CR1 surface expression of the cells mentioned above in (D) and (E) was detected by flow cytometry. **p<0.01 vs scrambled-siRNA. $^{\#\#}$p<0.01 vs DMSO.

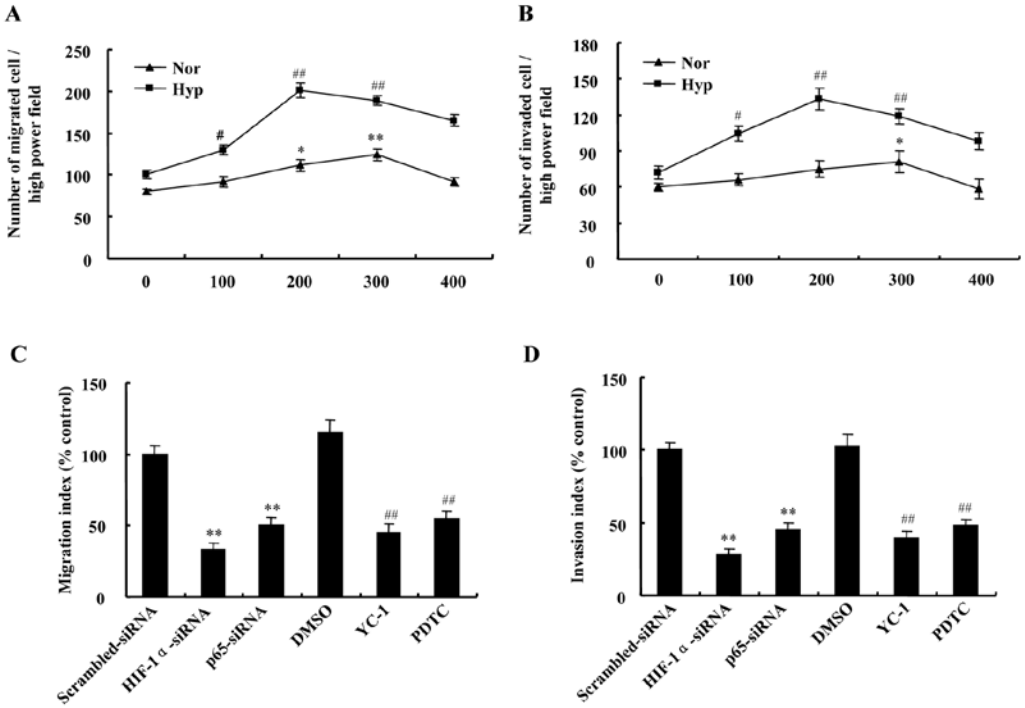

Figure 6. HIF-1 and NF-κB are involved in hypoxia-induced metastasis response to CX3CL1 in DU145 cells. (A) The chemotactic response of DU145 cells to CX3CL1 was assessed by migration assay in normoxic or hypoxic conditions (1% O_2). (B) Invasive activity of the cells mentioned above was also analyzed by invasion assay. *p<0.05, **p<0.01 vs normoxic control, $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs hypoxic control. (C) DU145 cells transfected with HIF-1α-siRNA, p65-siRNA or scrambled-siRNA for 72 h, or pretreated with DMSO, YC-1 or PDTC for 1 h, were subjected to migration assay in hypoxia as described in Material and methods. (D) Invasive activity of the cells mentioned above was also analyzed by invasion assay. **p<0.01 vs scrambled-siRNA; $^{\#\#}$p<0.01 vs DMSO.

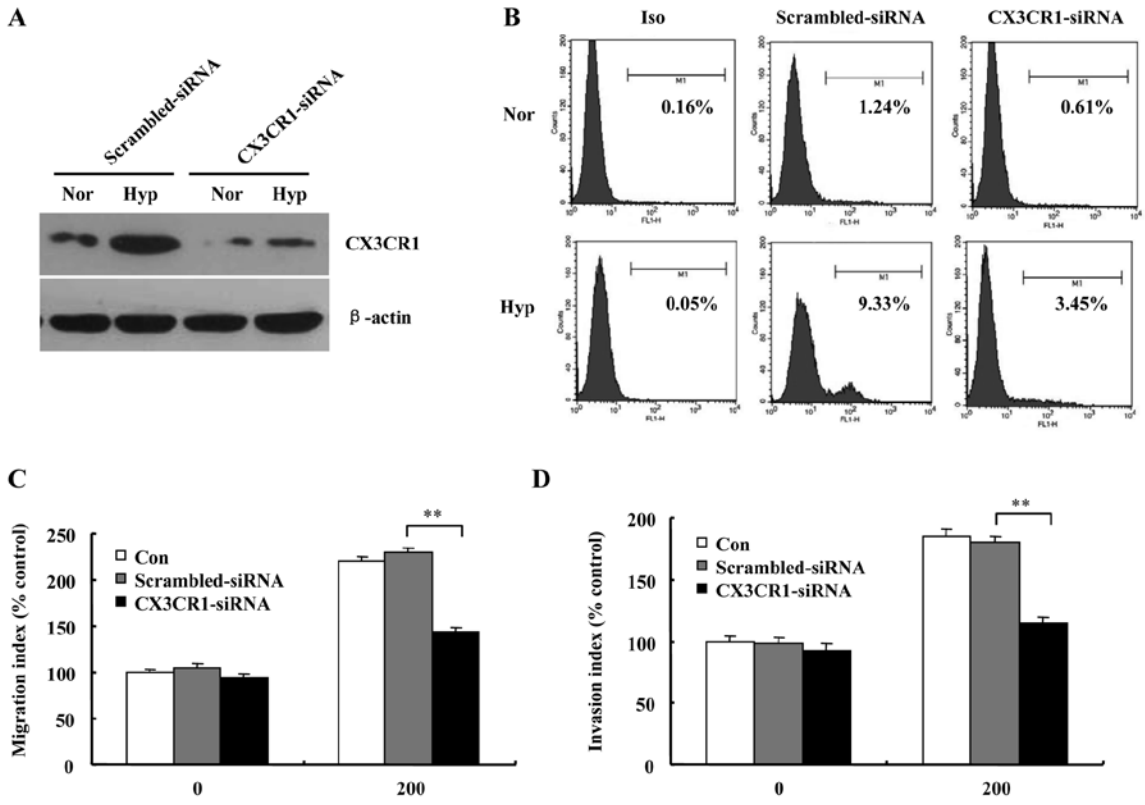

Figure 7. Inhibition of CX3CR1 by siRNA decreases cell migration and invasion of DU145 cells. DU145 cells transfected with CX3CR1-siRNA or scrambled-siRNA for 72 h were exposed to either normoxia or hypoxia (1% O_2) for 24 h. CX3CR1 (A) protein expression and (B) surface expression were determined by western blot analysis and flow cytometry. (C) The chemotactic response of the cells mentioned above to CX3CL1 was assessed by migration assay. (D) Invasive activity of the cells was also analyzed by invasion assay. $^{**}p<0.01$ vs scrambled-siRNA.

mRNA transcription (Fig. 4C and D). Taken together, the results confirmed that the transcription factors HIF-1 and NF-κB are all active in DU145 cells under hypoxic conditions.

Hypoxia increases CX3CR1 expression by HIF-1 and NF-κB. Next, we investigated the roles of HIF-1 and NF-κB in the hypoxia-induced CX3CR1 upregulation in DU145 cells. As demonstrated in Fig. 5A and B, CX3CR1 mRNA and protein expression levels were reduced greatly in HIF-1α-siRNA transfected cells, compared with scrambled-siRNA. Accordingly, preincubation of DU145 cells with HIF-1α inhibitor YC-1 drastically decreased hypoxia-induced CX3CR1 expression both at mRNA and protein levels. The inhibitory effects of HIF-1α-siRNA and YC-1 were also confirmed on CX3CR1 surface expression level (Fig. 5C). Similar responses in attenuating CX3CR1 mRNA, protein and surface expression levels were obtained following pretreatment of DU145 cells with p65-siRNA or NF-κB inhibitor PDTC, respectively (Fig. 5D-F). Taken together, these data suggest that HIF-1 and NF-κB are involved in CX3CR1 upregulation induced by hypoxia.

HIF-1 and NF-κB regulate CX3CL1-induced metastasis of DU145 cells under hypoxic conditions. Migration and invasion assays were performed to test the effect of hypoxia on metastasis of DU145 cells in response to CX3CL1. The data demonstrated chemotaxis toward CX3CL1 was greatly changed in a dose-dependent manner and was increased under hypoxic conditions compared with normoxic. The number of migratory cells was the highest at the concentration of 300 ng/ml CX3CL1 in normoxia, but in hypoxia at the concentration of 200 ng/ml (Fig. 6A). In accordance with migration assay, number of invaded cells was the highest at the concentration of 300 ng/ml and 200 ng/ml CX3CL1 in normoxic and hypoxic conditions, respectively (Fig. 6B).

As mentioned above, we have verified the roles of HIF-1 and NF-κB in regulation of CX3CR1 expression induced by hypoxia in DU145 cells. Based on these results, we continued to explore the contribution of HIF-1 and NF-κB to the chemotactic response of DU145 cells towards CX3CL1 in hypoxia. The results showed that hypoxia-induced migration and invasion of DU145 cells at 200 ng/ml CX3CL1 were attenuated by downregulating HIF-1α or p65 expression, which was more significant in HIF-1α knockdown cells than in those of p65 knockdown (Fig. 6C and D). In addition, HIF-1α inhibitor YC-1 and NF-κB inhibitor PDTC were also included in this experiment. As expected, treatment with YC-1 or PDTC prevented the migratory and invasive responses to CX3CL1 of DU145 cells under hypoxic conditions (Fig. 6C and D). The results showed that HIF-1 and NF-κB play important roles in metastasis of hypoxic DU145 cells.

The involvement of CX3CR1 in hypoxia-induced migration and invasion of DU145 cells. In order to clarify the role of CX3CR1 in prostate cancer cells metastasis, siRNA transfection of DU145 cells for downregulating CX3CR1 was performed, and the transfection efficiency was tested by western blot and flow cytometry analysis. Results in Fig. 7A and B show that both

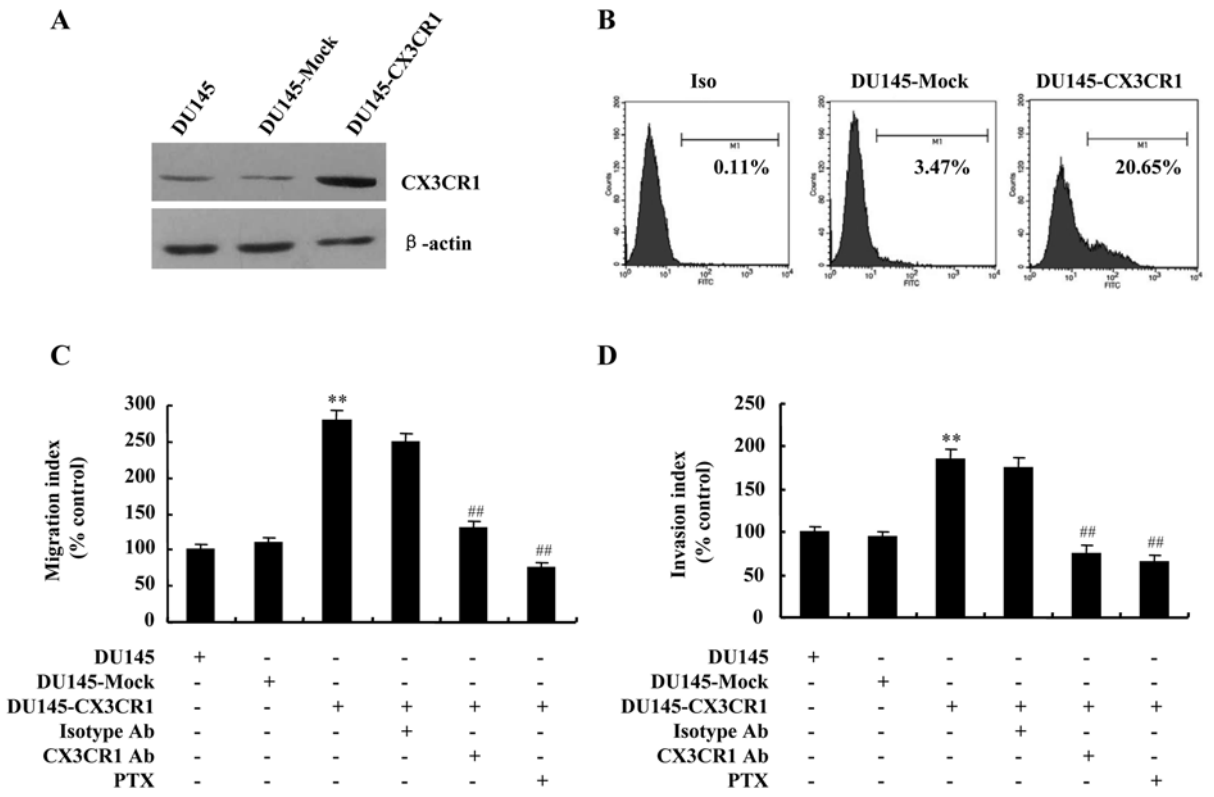

Figure 8. Overexpression of CX3CR1 increases cell migratory and invasive ability in DU145 cells. DU145 cells were transfected with pcDNA3.1-CX3CR1 or pcDNA3.1 empty vector, after selection the G418 resistant cells were subjected to (A) western blot and (B) flow cytometry analysis. (C) The chemotactic response of DU145, DU145-CX3CR1 and DU145-Mock cells to CX3CL1 was assessed by migration assay. (D) Invasive activity of the cells mentioned above was analyzed by invasion assay. $^{**}p<0.01$ vs DU145-Mock; $^{\#\#}p<0.01$ vs DU145-CX3CR1.

protein and cell surface levels of CX3CR1 in CX3CR1-siRNA cells were downregulated significantly compared with scrambled-siRNA cells. Migration and invasion assays demonstrated that chemotaxis toward CX3CL1 was greatly decreased in CX3CR1-siRNA cells compared to scrambled-siRNA cells under hypoxic environment (Fig. 7C and D).

To further verify the contribution of CX3CR1 to DU145 cells invasiveness, stable transfection of the cells with a plasmid for overexpression of human CX3CR1 was also employed in this study to establish high levels of CX3CR1. As shown in Fig. 8A and B, stable CX3CR1 transfectant, namely DU145-CX3CR1, was detected to have increased protein and cell surface levels of CX3CR1 compared with control vector transfectant, the DU145-Mock. Migration and invasion assays were also performed to determine if CX3CR1 upregulation increased the migratory and invasive ability of DU145 cells. The results showed that the number of migrated and invaded cells increased significantly in DU145-CX3CR1 cells compared to that of DU145-Mock (Fig. 8C and D).

Since CX3CR1 is a Gi-coupled receptor, and inhibitory effect of pertussis toxin (PTX), the Gi-protein-specific inhibitor, was observed in the migration of HUVECs stimulated by CX3CL1, we preincubated DU145-CX3CR1 cells with PTX for 1 h before chemotaxis in a further test of specificity. As expected, the migratory and invasive responses to CX3CL1 were significantly blocked by treatment with PTX. Similarly, neutralizing anti-CX3CR1 antibody also attenuated CX3CL1-induced migration and invasion of DU145-CX3CR1 cells (Fig. 8C and D).

Discussion

Chemokines are secreted chemoattractants that induce cell migration by activating chemokine receptors. Migrating cells follow a chemokine gradient that is thought to form through chemokine diffusion and binding to extra-cellular glycosaminoglycans of the seven transmembrane G-protein coupled receptors (39). These signaling proteins regulate leukocytes accumulation and activation in normal inflammatory processes, but are also implicated in many disease states, including HIV-1/AIDS, arthritis, asthma and Crohn's disease (40). Furthermore, the broader impact on tumor growth and metastasis of chemokines has been appreciated in the last few years. A number of chemokine receptors have been detected in haematological and solid malignancies. As a result, chemokines and their receptors are extensively studied as an area for pharmaceutical intervention. Although the importance of CX3CR1 has been shown in tumor metastasis, the regulation of CX3CR1 expression in epithelial-derived cancers is poorly understood. In the present work, we have investigated the effect of hypoxia on CX3CR1 expression in androgen-independent prostate cancer cells and the role of CX3CL1/CX3CR1-axis in cancer cells metastasis under hypoxic environment.

We firstly examined the expression levels of CX3CR1 in six prostate cancer cell lines. We found the expression of CX3CR1 is a common feature of prostate cancer cells. This is in accordance with previous research (12). Then we observed that CX3CR1 expression can be regulated by hypoxia in prostate cancer cells.

CX3CR1 mRNA and protein expression were strongly upregulated in DU145, PC-3 and PC-3M cells treated with 1% O_2, 100 μM $CoCl_2$ or 500 μM DFX for 24 h, as well as CX3CR1 surface expression level. The results showed that hypoxia could upregulate CX3CR1 expression in prostate cancer cells.

Many cellular responses to hypoxia are mediated by HIF-1 and NF-κB. We found hypoxia increased the activity of HIF-1 and NF-κB by analyzing the transcript levels of the known target genes of HIF-1 and NF-κB, respectively. Previous studies showed that NF-κB and HIF-1 play important roles in the regulation of CXCR1 and CXCR2 in response to hypoxia in prostate cancer cells (41). Therefore, we investigated whether HIF-1 and NF-κB contribute to the regulation of CX3CR1 expression in prostate cancer cells under hypoxic conditions. Our studies showed that CX3CR1 protein and surface expression were greatly reduced when HIF-1 and NF-κB were downregulated by siRNAs specific for HIF-1α and p65, respectively. In addition, YC-1 and PDTC, inhibitors for HIF-1 and NF-κB activity, also decreased hypoxia-induced CX3CR1 expression. The results indicated that hypoxia regulates CX3CR1 expression via both HIF-1 and NF-κB in DU145 cells.

Experimental and clinical studies pointed to the important roles of HIF-1 and NF-κB in tumor invasion and metastasis (42-44). In the current study we investigated whether HIF-1 and NF-κB are involved in migration and invasion of DU145 cells under hypoxic conditions. Previous study showed that prostate cancer cells migrate toward a medium conditioned by osteoblasts, which secrete the soluble form of CX3CL1 (12). Therefore, we tested the impact of HIF-1 and NF-κB activity on the chemotactic response of hypoxic prostate cancer cells towards CX3CL1. Our results showed hypoxia increased greatly the migration and invasion of DU145 cell response to CX3CL1. With the increasing concentration of CX3CL1, the migration and invasion also increased. When the expression of HIF-1α and p65 were downregulated by siRNAs, the above phenomena including migration and invasion were also reduced, respectively. This chemotaxis was also sensitive to inhibitor YC-1 or PDTC. Collectively, HIF-1 and NF-κB are essential for CX3CL1-induced metastasis in DU145 cells under hypoxic environment.

Studies have demonstrated that hypoxia increases CXCL12-induced metastatic ability of breast cancer cells via upregulation of CXCR4 (45). Consistently, hypoxia promotes CCL21-induced metastasis of lung cancer cells by inducing the expression of CCR7 (46). Based on these results, we presumed that hypoxia increases migration and invasion of prostate cancer cells through upregulating CX3CR1 expression. We found CX3CR1 mRNA, protein, and surface expression were significantly increased in DU145, PC-3 and PC-3M cells by exposure to hypoxia or reagents that mimic the response to hypoxia. However, inhibition of CX3CR1 expression by siRNA attenuated the hypoxia-induced migration and invasion of DU145 cells indicating that hypoxia promotes CX3CL1-induced metastasis of prostate cancer cells via upregulation of CX3CR1. To further verify the contribution of CX3CR1 to cell metastasis, we performed stable transfection of DU145 cells for CX3CR1 overexpression. The stable CX3CR1 transfectant exhibited high level of CX3CR1 expression, increased cell migration and invasion response to CX3CL1 were also detected, indicating that CX3CR1 expression levels are correlated with invasive ability of DU145 cells. Thus, our results showed that HIF-1 and NF-κB are prerequisite for migration and invasion through upregulation of CX3CR1 in hypoxic prostate cancer cells.

In summary, the study presented here demonstrated that hypoxia increased CX3CL1-induced migration and invasion of prostate cancer cells by upregulating CX3CR1 expression via HIF-1 and NF-κB. We also showed that CX3CR1, as well as HIF-1 and NF-κB might represent suitable targets for therapies aiming at metastatic prostate adenocarcinoma.

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