High C-X-C motif chemokine 5 expression is associated with malignant phenotypes of prostate cancer cells via autocrine and paracrine pathways

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Abstract. The present study aimed to examine the effects and mechanisms of exogenous C-X-C motif chemokine 5 (CXCL5) and lentiviral CXCL5 overexpression on the regulation of malignant behaviors of prostate cancer cells in vitro and in a nude mouse xenograft model. The expression levels of CXCL5 and a number of tumor-related genes were assessed by using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), western blotting, ELISA, or immunohistochemistry in normal and cancerous prostate cells and tissues. Cell proliferation, colony formation, and Transwell assays were performed to determine the effects of exogenous, autocrine, and paracrine CXCL5 on prostate cancer cell proliferative and migratory capacity. The results indicated that CXCL5 expression was upregulated in PC-3 and DU145 prostate cancer cells, in WPMY-1 normal prostate stromal cells, and in RWPE-1 prostate epithelial cells, as well as in prostate cancer tissue specimens. Exogenous CXCL5 exposure resulted in increase in prostate cancer cell proliferation, colony formation, and migration. In cells transfected with a CXCL5 overexpression vector, in cells cultured in conditioned medium from CXCL5-overexpressing WPMY cells, and in cells co-cultured with CXCL5-OE WPMY cells prostate cancer cell malignant phenotypes were induced in an autocrine/paracrine fashion in vitro; similar results were observed in nude mouse xeno-

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grafts. CXCL5 overexpression also regulated expression of tumor-related genes, including BAX, N-Myc downstream-regulated gene 3, extracellular signal-regulated kinase 1/2, C-X-C chemokine receptor type 2, interleukin 18, Bcl-2, and caspase-3. These data demonstrated that CXCL5 expression was upregulated in prostate cancer tissues and that exogenous CXCL5 protein exposure or CXCL5 overexpression promoted malignant phenotypes of prostate cancer cells *in vitro* and *in vivo*.

Introduction

Prostate cancer is the most common male-related malignancy worldwide; it affects 1 in 9 men aged 65 years or older and is the second leading cause of cancer-related mortality in men (1,2). Therefore, identification of novel factors that are involved in prostate cancer development and progression, including tumor cell proliferation, migration and invasion, may provide useful insights into the effective control of prostate cancer progression as well as novel diagnostic and therapeutic approaches. For example, our previous study revealed that N-Myc downstream-regulated gene 3 (NDRG3) was overexpressed in prostate cancer tissue specimens and was able to induce proliferation and migration of prostate cancer cells in vitro and in a xenograft nude mouse model (3). NDRG3 overexpression was also reported to increase the expression of C-X-C motif chemokine 5 (CXCL5; also known as epithelial neutrophil-activating peptide-78) and to promote tumor cell growth (3-5). Therefore, further studies on the role of CXCL5 in prostate cancer may provide valuable information regarding prostate cancer development and progression.

CXCL5 is a member of the CXC family of chemokines and is produced following stimulation by other inflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor- α (6). CXCL5 functions in the cell through interaction with C-X-C chemokine receptor type 2 (CXCR2) on the cell surface (7). The CXCL5 protein contains an ELR motif at the amino terminus, which shares structural homology with IL-8 and, thus, exhibits similar functions as IL-8, such as regulation of cell proliferation or migration (5,8,9). CXCL5 was also reported to serve a role in tumor cell proliferation, metastasis, neutrophil infiltration and angiogenesis in tumor lesions (4,5). Previous studies have demonstrated that CXCL5 is overexpressed in several types of human cancers, including gastric, pancreatic, liver, and prostate cancers (4,10-12), and was associated with poor prognosis (5,10,11,13-15). In addition, CXCL5 has been considered as a crucial chemokine in the promotion of human cancer development and progression (4-15).

The present study investigated CXCL5 expression in normal and cancerous prostate tissues and cell lines, and assessed the effects of exogenous CXCL5 exposure and lentiviral-mediated CXCL5 overexpression on the regulation of malignant behaviors of prostate cancer cells *in vitro* and in a nude mouse xenograft model. In addition, the underlying molecular mechanisms of CXCL5 overexpression in prostate cancer cells were investigated to provide novel insight into the role of CXCL5 in prostate cancer progression, which may aid in the development of future therapeutic strategies.

Materials and methods

Cell lines and culture. Prostate cancer cell lines PC-3, DU145 and LNCaP, the immortalized normal prostate stroma cell line WPMY-1, the immortalized prostate epithelium cell line RWPE-1 and the 293T cells used for lentiviral packaging were obtained from American Type Culture Collection (Manassas, VA, USA). Prostate cancer cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1:100 penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), whereas WPMY-1 and RWPE-1 cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1:100 penicillin/streptomycin in a humidified incubator with 5% CO₂ at 37°C. The medium supplemented with FBS and penicillin/streptomycin was considered as complete medium (CM).

Preparation of CXCL5-lentiviral vector and stable gene transfection. To overexpress CXCL5 in prostate cells, a pReceiver-Lv203 CXCL5 expression vector (cat. no. EX-A1113-Lv203) and a pReceiver-Lv203 empty vector (cat. no. EX-NEG-Lv203) were constructed by GeneCopoeia Inc. (Rockville, MD, USA), and a Lenti-Pac HIV Expression Packaging kit (cat. no. HPK-LvTR-40; GeneCopoeia, Inc. Rockville, MD, USA) was used to produce CXCL5-carrying lentivirus overexpression vector (CXCL5-OE) and mock lentivirus empty vector (mock), according to the manufacturer's instructions. The 293T lentiviral packaging cells were transfected using the transfection reagent provided in the Lenti-Pac HIV Expression Packaging kit, following the manufacturer's protocol. Briefly, 293T cells (1.5x10⁶ cells) were plated in a 10-mm dish in 10 ml of DMEM supplemented with 10% heat-inactivated FBS. Following incubation at 37°C for 48 h, DNA-EndoFectin Lenti complex was directly added to the dish and the cells were incubated at 37°C for 12 h, after which the culture medium that contains the DNA-EndoFectin Lenti complex was replaced with fresh DMEM supplemented with 5% heat-inactivated FBS. A 1/500 volume of the TiterBoost reagent was added to the culture medium and continued incubation at 37°C for 48 h. The lentiviral particle-containing culture medium was collected and centrifuged at room temperature at 1,000 x g for 10 min. Subsequently, PC-3, DU145, or WPMY-1 cells (2.5x10⁵ cells/ well) were incubated in 6-well plates in 1 ml RPMI-1640 or DMEM CM containing lentiviral particle-containing culture medium at a ratio of 1:1 at 37°C for 48 h. To select the stable gene-transfected cells, these cell lines were further cultured and selected by culturing with puromycin (50 ng/ ml, Invitrogen; Thermo Fisher Scientific, Inc.) in RPMI-1640 or DMEM CM. The stable CXCL5-OE-transfected cells and mock-transfected cells were isolated and expanded, and CXCL5 expression was confirmed by ELISA. These isolated cells were used for the subsequent experiments.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from 2x10⁷ cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the Quant Reverse Transcriptase (Tiangen Biotech, Beijing, China), both according to the manufacturers' protocol. cDNA was amplified by PCR to determine the mRNA expression levels of CXCL5 and its receptor CXCR2, as well as the expression levels of BAX, NDRG3, ERK, and β-actin. The primers and PCR thermocycling conditions are provided in Table I and II, respectively. PCR products were visualized by electrophoresis in 1.2% agarose gels stained with ethidium bromide. Relative mRNA expression levels were normalized to β-actin mRNA using Quantity One Software, version 4.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were repeated in triplicate.

ELISA. Cells (1.5x10⁵ cells/well) were seeded into 6-well plates with 1 ml cell culture medium containing 0.5% FBS and incubated at 37°C for 48 h. Cell culture media were collected and the expression levels of CXCL5 protein were measured using a Human CXCL5/LIX/ENA-78 PicoKine ELISA kit (cat. no. EK0728; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Briefly, 96-well ELISA plates were coated with 100 μ l of human recombinant CXCL5 protein from the kit at concentrations of 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.2, and 0 pg/ml to create a standard curve. A total of 100 μ l of the media from the different cell cultures was added to each well and the plates were incubated at 37°C for 90 min. Subsequently, the plates were washed with phosphatebuffered saline (PBS) and incubated with 100 μ l anti-human CXCL5 antibody working liquid (provided in the kit) for 1 h at room temperature. The plates were then washed with PBS again and incubated with ABC working solution at 37°C for 30 min. Following the addition of 100 μ l 3,3',5,5'-tetramethylbenzidine color solution to each well, optical density was immediately detected at 450 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) and the levels of CXCL5 in the media were quantified according to the standard curve. The experiments were repeated at least three times.

Primer sequences $(5' \rightarrow 3')$
F: AGAAAATCTGGCACCACACC
R: CTCCTTAATGTCACGCACGA
F: GCTACCACTTCCACCTTG
R: CCACTATGAGCCTVVTGT
F: CAGGAATGTGGCCAAAAAT
R: GGAAACTCCCTCGTGATG
F: TTGTCGCCCTTTTCTACTTTGCC
R: TCTGAAGATGGGGAGAGGGGCAC
F:GGCGAATTGTCCCCTACCACCAGT
R: CTGCCTCCTGTTCTTACCCACCTA
F: CTTCTCGCCTCAGTTCGC
R: CTCCTGGATGCTTGTCTGGTAA

Table I. Primer sequences used for semi-quantitative reverse transcription-polymerase chain reaction.

CXCL5, C-X-C motif chemokine 5; ERK, extracellular signal-regulated kinase; F, forward; NDRG3, N-Myc downstream-regulated gene 3; R, reverse.

Protein extraction and western blotting. Total protein was extracted from cells (3x10⁶) or 20 mg of xenografted tissues using Pierce Lysis Buffer (Thermo Fisher Scientific, Inc.) on ice for 30 min and centrifuged at 14,000 x g for 15 min at 4°C. Protein concentrations in the supernatant were determined using the Bicinchoninic Acid Protein Assay kit (Wuhan Boster Biological Technology, Ltd.). A total of 30 μ g of each sample was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% skimmed milk in PBS for 2 h at room temperature and incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal anti-BAX (1:1,000; cat. no. TA890001; OriGene Technologies, Inc., Beijing, China), goat polyclonal anti-NDRG3 (1:200; cat. no. sc-19470; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal anti-extracellular signalregulated kinase 1/2 (ERK1/2; 1:1,000; cat. no. AF0155; Affinity Biosciences, Inc., Cincinnati, OH, USA), rabbit polyclonal anti-CXCR2 (1:500; cat. no. sc-30008; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-IL-18 (1:500; cat. no. sc-7954; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-Bcl-2 (1:500; cat. no. sc-492; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-caspase-3 (1:500; cat. no. sc-7148; Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-β-actin (1:1,000; cat. no. TA811000; OriGene Technologies, Inc.). The membranes were subsequently washed with PBS containing 0.05% Tween-20 and incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse secondary antibodies (1:10,000; cat. nos. BA1054, BA1060, and BA1050, respectively; Wuhan Boster Biological Technology, Ltd.) at 37°C for 30 min. Immunoreactive protein bands were detected using the Pierce Enhanced Chemiluminescence solution (Thermo Fisher Scientific, Inc.) and the intensity of each target band was normalized to β -actin and quantified using

Table II. Thermocycling conditions of polymerase chain reaction amplification for each gene.

Gene	Denaturation	Annealing	Extension	Cycles
β-actin	94°C (1 min)	52.9°C (30 sec)	72°C (1 min)	25
CXCL5	94°C (1 min)	64.0°C (30 sec)	72°C (1 min)	38
CXCR2	94°C (1 min)	54.0°C (30 sec)	72°C (1 min)	35
BAX	94°C (1 min)	55.0°C (30 sec)	72°C (1 min)	32
NDRG3	94°C (1 min)	61.7°C (30 sec)	72°C (1 min)	32
ERK	94°C (1 min)	55.6°C (30 sec)	72°C (1 min)	30

CXCL, C-X-C motif chemokine; ERK extracellular signal-regulated kinase; NDRG3, N-Myc downstream-regulated gene 3.

ImageJ software (version 1.45s; National Institutes of Health, Bethesda, MD, USA). Each experiment was repeated at least three times.

Tissue samples and immunohistochemical analysis. Prostate tissue samples were collected from patients (age, 56-72 years) with prostate cancer (n=13), with benign prostate hyperplasia (BPH; n=3), with cystic dilatation prostate (n=3), and with normal prostate (n=3). Of the 13 patients with prostate cancer, 1 had Gleason score 4 tumor, 2 had Gleason score 5, 1 had Gleason score 6, 2 had Gleason score 7, 3 had Gleason score 8, 3 had Gleason score 9, 1 had Gleason score 10, and of the 13 cases, 7 had metastatic tumors. All patients had undergone surgical treatment at The Affiliated Hospital of Jiamusi University (Jiamusi, China) between June 2013 and June 2015. This study was approved by the Ethics Committee of Jiamusi University (approval no. JMSU-195) and written informed consent was provided by all patients prior to enrolment.

A tissue microarray (TMA) comprising 50 prostate cancer, 20 BPH, and 10 normal prostate tissue samples was purchased from Alenabio (Xi'an, China). Among the 50 cancer tissues, patients were aged between 20 and 87 years and had Gleason scores between 4 and 10 (4 with Gleason score 4; 7 with Gleason score 5; 9 with Gleason score 6; 8 with Gleason score 7; 2 with Gleason score 8; 9 with Gleason score 9; 11 with Gleason score 10) and of the 50 cases, 19 with metastatic tumors).

Tissue samples were fixed in 10% buffered formalin at room temperature for 24 h, embedded in paraffin and sectioned into 5 μ m-thick sections. These and the TMA (5 μ m) sections were deparaffinized in xylene at room temperature and rehydrated in a descending ethanol series. The sections were subjected to antigen retrieval in a high-pressure cooker with 0.01 M citric acid buffer for 3 min, and endogenous peroxidase activity was quenched by incubating the sections in 3% H₂O₂ in PBS for 30 min at room temperature. The sections were blocked with 20% normal goat serum (cat. no. AR0009; Wuhan Boster Biological Technology, Ltd.) at 37°C for 1 h, followed by incubation with a mouse monoclonal anti-CXCL5 antibody (1:50; cat. no. sc-73931; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Following this incubation, the sections were washed with PBS and incubated with a HRP-conjugated secondary goat anti-mouse immunoglobulin G (1:200; cat. no. BA1050;

Wuhan Boster Biological Technology, Ltd.) for 30 min at the room temperature. Subsequently, the sections were washed with PBS and incubated with the Avidin-Biotin Enzyme Complex (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at room temperature, followed by incubation with 3,3'-diaminobenzidine solution at the room temperature for 5 min for colorimetric determination of protein expression, and then counterstained with hematoxylin at room temperature for 15 sec. For quantification, Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to determine the average optical density and relative area of total CXCL5 expression in each tissue section within three random fields at x400 magnification under an Olympus BX-60 Epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

Cell treatments and proliferation assay. To determine the effects of exogenous, autocrine and paracrine CXCL5 expression on the proliferation of prostate cancer cells, the following three experimental treatments were conducted: i) Exogenous assay, in which PC-3 and DU145 cells were seeded (2x10³ cells/well) into 96-well plates and incubated in CM overnight at 37°C in a 5% CO₂ incubator; following removal of the culture media, 100 μ l of CM containing different concentrations of recombinant CXCL5 (0, 10, 20, 40 or 60 ng/ml; PeproTech, Inc.) was added to each well; ii) autocrine assay, in which the untransfected parental, mocktransfected, and CXCL5-OE-transfected PC-3 and DU145 cells were seeded (2x10³ cells/well) into 96-well plates and incubated in 100 μ l of CM; and iii) paracrine assay, in which parental, mock-transfected and CXCL5-OE-transfected WPMY-1 cells were seeded (5x10⁵ cells/well) into 100 mm cell culture dishes and incubated in CM at 37°C for 48 h. The WPMY-1 CM was collected and subsequently added to PC-3 and DU145 cell cultures (containing 2x10³ cells/well in 96-well plates) at different dilutions (20, 50, 80 or 100%). The three groups of cells were incubated at 37°C in a 5% CO₂ incubator for 72 h without any subsequent medium change. Cell proliferation was then determined by using the Cell Counting Kit-8 (CCK-8; Boster Biotechnology), following the manufacturer's protocol. Briefly, 10 µl CCK-8 reagent was added into each well and cells were incubated at 37°C for 1 h. The optical density was measured at 450 nm using a spectrophotometer (BioTek Instruments, Inc.). Each assay was repeated at least thrice.

Colony formation assay. PC-3 and DU145 cells were seeded (100 cells/well) into 24-well plates and incubated with CM containing 60 ng/ml (for PC-3 cells) or 20 ng/ml (for DU145 cells) CXCL5 for 12 days (according to pre-test experiments; data not shown) at 37°C with 5% CO₂ until the colonies were visible. The aforementioned CXCL5-containing medium was replaced every 3 days. The colonies were stained with 0.01% crystal violet solution at room temperature for 15 min and counted under an Olympus BX-60 Epifluorescence microscope BX-60 (Olympus Corporation).

Transwell migration assay. The Transwell migration assay was performed using 24-well chambers with polycarbonate membrane filters (pore size, 8 μ m; Corning, Inc., Corning,

NY, USA), which was modified from our previously described protocol (16). The effects of exogenous, autocrine, and paracrine CXCL5 exposure on the migratory capcity of prostate cancer cells was determined as follows: i) Exogenous assay, in which PC-3 cells (2x10⁴ cells/well) or DU145 cells $(5x10^3 \text{ cells/well})$ were seeded into the upper Transwell chamber with serum-free RPMI-1640 medium; 600 µl RPMI-1640 medium with 10% FBS and various concentrations of CXCL5 (0, 10, 20, 40, or 60 ng/ml) was added into the lower chamber; ii) Autocrine assay, in which parental, mock-transfected, and CXCL5-OE-transfected PC-3 cells $(2x10^4 \text{ cells/well}) \text{ or } DU145 \text{ cells} (5x10^3 \text{ cells/well}) \text{ were seeded}$ into the upper chamber with serum-free RPMI-1640 medium; 600 µl RPMI-1640 medium with 10% FBS was added into the lower chamber; and iii) Paracrine assay, in which PC-3 cells ($2x10^4$ cells/well) or DU145 cells ($5x10^3$ cells/well) were seeded into the upper Transwell chamber containing serumfree RPMI-1640 medium; 600 μ l of the conditional medium prepared by dissolving different ratios of culture media from parental, mock-transfected, and CXCL5-OE-transfected WPMY-1 cells (20, 50, 80 or 100%) in DMEM containing 10% FBS was added into the lower chamber. In addition, a co-culture system was used in the paracrine assay, in which PC-3 cells ($2x10^4$ cells/well) or DU145 cells ($5x10^3$ cells/well) were seeded into the upper chamber containing RPMI-1640 medium, with parental, mock-transfected, or CXCL5-OEtransfected WPMY-1 cells (3x10⁴ cells/well) were cultured in the lower chamber containing 600 μ l of DMEM containing 10% FBS. For all experimental groups, cells were incubated at 37°C in a 5% CO₂ incubator for 48 h; subsequently, the upper surfaces of the membranes were scraped with a cotton swab to remove the non-migrating cells, and the filters were washed with PBS, fixed and stained with 95% ethanol containing 0.5%crystal violet solution at room temperature for 30 min. The filters were then washed with PBS and the migrating cells were counted in five random fields using a bright field microscope at x50 magnification.

Nude mouse tumor cell xenograft assay. Male nude mice (nu/nu; age, 5 weeks; weight 13-16 g; n=7) were purchased from Charles River Laboratories International, Inc. (Beijing, China). The mice were housed in cages under laminar-flow HEPA-filtered hoods at 25°C with a 12-h light/dark cycle and were provide with sterile rodent chow and sterile water ad libitum. Mock and CXCL5-overexpressing PC-3 cells were cultured and harvested at the exponential growth phase, washed with PBS, and resuspended in serum-free RPMI-1640 medium. The trypan blue exclusion assay was used to ensure that cell viability was >99% prior to injection. PC-3 cells $(1x10^6 \text{ cells in } 100 \ \mu \text{l culture medium})$ were subcutaneously injected into male athymic mice; each mouse was injected with CXCL5-overexpressing cells into one side of the flank, and the mock control cells were injected into the other side for simultaneous comparison. The animals were sacrificed with CO₂ at week 8 post-injetion and the tumor cell xenografts were harvested for analysis and comparison. The experiments were repeated three times.

Statistical analysis. All data are presented as the mean ± standard deviation. Comparisons between two groups were analyzed



Figure 1. CXCL5 expression in prostate cancer cells. (A) Reverse transcription-polymerase chain reaction results and semi-quantitative analysis of CXCL5 mRNA expression levels in PC-3, DU145, LNCaP, WPMY-1, and RWPE-1 cell lines; β -actin was used to normalize the data; *P<0.05 and **P<0.01 vs. RWPE-1 cells. (B) ELISA analysis of the concentrations of secreted CXCL5 proteins 48 h post-treatment in untransfected parental, mock-transfected, and CXCL5-OE-transfected PC-3, DU145 and WPMY-1 cells, as well as LNCaP and RWPE-1 cells. **P<0.01 vs. respective parental or mock cells. CXCL5, C-X-C motif chemokine 5; CXCL5-OE, lentiviral-CXCL5-overexpression vector-transfected cells; mock, lentiviral empty vector-transfected cells.

using Student's t-test, and comparison among multiple groups was analyzed by analysis of variance followed by Student-Newman-Keuls post hoc test or by Kruskal-Wallis test with a Nemenyi test pairwise comparison post hoc test. All statistical analyses were performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered statistically significant.

Results

Upregulation of CXCL5 in vitro and ex vivo. The expression levels of CXCL5 mRNA were examined by RT-PCR in the prostate cancer cell lines PC-3, DU145 and LNCaP, in the normal prostate stroma cell line WPMY-1, and in the normal prostate epithelium cell line RWPE-1. The results demonstrated that CXCL5 mRNA expression was high in PC-3, DU145, WPMY-1, and RWPE-1 cells and expression was low in LNCaP cells (Fig. 1A). ELISA was used to determine whether CXCL5 protein was produced and secreted by these cell lines, and the results revealed that each of these cell lines secreted CXCL5 protein (Fig. 1B), which suggested that CXCL5 may function through the autocrine and paracrine systems in prostate cells. In addition, a significantly increased level of CXCL5 secretion was observed in CXCL5-overexpressing PC-3, DU145, and WPMY-1 cells compared with their respective parental and mock controls (Fig. 1B), which may lay the foundation for subsequent autocrine and paracrine assays. Our previous study demonstrated a high expression level of CXCR2 protein in DU145, LNCaP, and RWPE-1 cells and low CXCR2 expression in PC-3 and WPMY-1 cells (16), which suggested that CXCL5 and CXCR2 expression may lack specificity in prostate cells.

Furthermore, CXCL5 protein expression levels were also examined in tissue specimens from patients with prostate cancer, BPH or cystic dilatation prostate, as well as in normal prostate tissue. CXCL5 expression was significantly higher in prostate cancer tissues (Fig. 2A-E; Table III), compared with compared with expression in the non-cancerous tissue specimens, including cystic dilatation prostate tissues (Fig. 2F), normal prostate tissues (Fig. 2G-H), and BPH tissues (Fig. 2I). The average optical density and relative area data indicated that the levels of CXCL5 protein expression in both non-metastatic cancer tissues (non-METs) and METs were significantly higher compared with non-prostate cancer tissues (non-PCa), which indicated that the increased expression of CXCL5 mayb be associated with prostate cancer development and progression (Table III). However, CXCL5 expression was not associated with Gleason scores or age of the patients (data not shown).

Effects of exogenous CXCL5 exposure on tumor cell proliferation, colony formation, and migration. Owing to the tendency of LNCaP cells to form colonies in culture instead of a cell monolayer, these cells were not used in subsequent experiments. Tumor cell proliferation, colony formation and migratory ability were assessed in PC-3 and DU145 cell lines following treatment with various concentrations of exogenous recombinant CXCL5 protein (0, 10, 20, 40



Figure 2. Expression levels of CXCL5 protein in prostate cancer, cystic dilatation prostate, normal prostate and BPH tissue specimens. (A-I) Immunohistochemistry was performed to assess CXCL5 protein expression in the TMA and freshly collected tissue specimens; representative images are presented. (A-E) CXCL5 expression in TMA prostate cancer tissues with (A and B) Gleason grade 6, (C) Gleason grade 9, and (D and E) Gleason grade 10. (F) CXCL5 expression in fresh cystic dilatation prostate tissue. (G and H) CXCL5 expression in TMA normal prostate tissue. (I) CXCL5 expression in TMA BPH tissue. BPH, benign prostate hyperplasia; CXCL5, C-X-C motif chemokine 5; TMA, tissue microarray.

Table	III.	Levels	of	CXCL5	protein	expression	in	human
prosta	te tis	sues as	det	ermined	by immı	inohistochei	nis	try.

		CXCL5 expression		
Tissue	n	Average optical density	Relative area	
Non-PCa ^a	39	0.303±0.018	8,4487.05±7,1405.03	
Non-METs	37	0.329±0.049 ^b	133,185.24±74,874.95 ^b	
METs	26	0.322 ± 0.026^{b}	159,082.46±67,890.32 ^b	

^aNon-PCa tissues, including benign prostate hyperplasia, cystic dilatation prostate, and normal prostate tissues. ^bP<0.05 vs. non-PCa. CXCL5, C-X-C motif chemokine 5; METs, metastatic cancer tissues; PCa, prostate cancer.

and 60 ng/ml). A significant increase in proliferation was observed following treatment with 60 ng/ml (PC-3 cells) or with 10 and 20 ng/ml (DU145 cells) of exogenous CXCL5 (Fig. 3A), although DU145 cells exposed to higher concentrations of exogenous CXCL5 (for example, 60 ng/ml) had a significant decrease in proliferation compared with untreated control cells. As the 60 and 10-20 ng/ml doses of exogenous CXCL5 were indicated as the optimal concentrations for Table IV. Effects of exogenous CXCL5 expression on colony formation of prostate cancer cells.

Dose of CXCL5 (ng/ml)	PC-3	DU145
0 20	35.50±6.80 ND	13.63±6.00 32.63±5.42ª
60	66.38±9.41ª	ND

^aP<0.01 vs. 0 ng/ml. CXCL5, C-X-C motif chemokine 5; ND, not done.

regulation of PC-3 and DU145 cell proliferation, respectively, 60 ng/ml was used for PC-3 and 20 ng/ml for DU145 cells in the subsequent colony formation assays; the treated PC-3 and DU145 cells exhibited a notable increase in colony formation (Fig. 3B and Table IV).

Transwell migration assays were conducted on PC-3 and DU145 cells following treatment with various concentrations of exogenous CXCL5 (0, 10, 20, 40 and 60 ng/ml). The results demonstrated that CXCL5 at a dose of 20 or 40 ng/ml for PC-3 cells or 10, 20 and 40 ng/ml for DU145 cells significantly promoted tumor cell migration capacity compared with respective cells treated with 0 ng/ml of CXCL5 (Fig. 3C and D).



Figure 3. Effects of exogenous CXCL5 protein exposure on prostate cancer cell proliferation, colony formation, and migration *in vitro*. (A) PC-3 and DU145 cells were treated with various concentrations of recombinant CXCL5 (0, 10, 20, 40 and 60 ng/ml) for 72 h and proliferation was assessed using the CCK-8 assay. *P<0.05 and **P<0.01 vs. untreated (0 ng/ml) control cells. (B) PC-3 and DU145 cells were treated with 60 ng/ml or 20 ng/ml recombinant CXCL5, respectively, for 12 days and colony formation was examined. (C) Transwell migration assay for PC-3 and DU145 cells treated with various concentrations of CXCL5 (0, 10, 20, 40, and 60 ng/ml) for 48 h. (D) Results from statistical analysis of Transwell tumor cell migration assay from (C). **P<0.01 vs. 0, 10 or 60 ng/ml; **P<0.01 vs. 20 ng/ml; **P<0.01 vs. 0 or 60 ng/ml; **P<0.01 vs. 40 ng/ml. CCK-8, Cell Counting Kit-8; CXCL5, C-X-C motif chemokine 5.

Autocrine/paracrine effects of CXCL5 overexpression on regulation of prostate cancer cell malignant phenotypes. Results from CCK-8 assays demonstrated that the proliferation of CXCL5-overexpressing PC-3 and DU145 cells was increased compared with that of parental or mock control cells (Fig. 4A). CXCL5 overexpression also significantly increased the migratory capacity of PC-3 and DU145 cells compared with that of parental and mock cells (Fig. 4B and C).

Furthermore, a stable CXCL5-overexpressing WPMY-1 prostate stromal cell line was established and various

concentrations (20, 50, 80 and 100%) of the conditional medium from parental, mock or CXCL5-overexpressing WPMY-1 cells was subsequently prepared and added to the PC-3 and DU145 cell cultures. PC-3 cells cultured with 100% and DU145 cells cultured with either 80 or 100% CXCL5-overexpressing WPMY-1 conditioned medium exhibited a significant increase in tumor cell proliferation compared with the proliferation of cells cultured with similar concentrations of conditional medium from parental or mock WPMY-1 cells (Fig. 5A); no significant differences in tumor



Figure 4. Effects of CXCL5 overexpression on prostate cancer cell proliferation and migration *in vitro*. (A) Cell proliferation of untransfected parental, mocktransfected, and CXCL5-OE-transfected PC-3 or DU145 cells was assessed using the CCK-8 assay. (B) Representative images of Transwell migration assay for parental, mock, and CXCL5-OE PC-3 and DU145 cells. (C) Analysis of cell migratory ability of PC-3 and DU145 cells from (B). *P<0.05 and **P<0.01 vs. parental or mock. CCK-8, Cell Counting Kit-8; CXCL5, C-X-C motif chemokine 5; CXCL5-OE, lentiviral-CXCL5-overexpression vector-transfected cells; mock, lentiviral empty vector-transfected cells.

cell proliferation were identified for cells culutred with 20, 50 or 80% of the WPMY-1 CM. Similar phenomena were also observed in prostate cancer cell migration assays; for example, PC-3 and DU145 cells cultured with 100% CXCL5-overexpressing WPMY-1 conditional medium exhibited a significant increase in migration capacity compared with cells cultured with similar concentrations of conditioned medium from parental or mock WPMY-1 cells (Fig. 5B and C). In addition, PC-3 and DU145 cells that were co-cultured with CXCL5-overexpressing WPMY-1 cells that were co-cultured with CXCL5-overexpressing WPMY-1 cells compared with the number of migrating cells compared with the number of migrating tumor cells co-cultured with parental or mock WPMY-1 cells (Fig. 5D and E).

Effects of CXCL5 overexpression on BAX, NDRG3, ERK, and CXCR2 expression in prostate cancer cells. To explore the underlying gene expression of the CXCL5-mediated prostate cancer cell malignat pheotypes, the mRNA and protein expression levels of known tumor-related genes were examined. The results demonstrated that the expression levels of NDRG3,

ERK1/2, and CXCR2 mRNA and protein were significantly increased, whereas the expression levels of BAX mRNA and protein were significantly reduced in CXCL5-overexpressing PC-3 cells compared with those of parental and mock control cells (Fig. 6A-D).

Effects of CXCL5 overexpression on prostate cancer cells in vivo. To assess the *in vivo* effects of CXCL5 overexpression on prostate cancer cells, a nude mouse xenograft assay was performed by subcutaneous injection of CXCL5-overexpressing and mock-treated PC-3 cells into nude mice. Eight weeks following tumor cell injection, tumor xenografts were resected from the mice; weight of tumor cell xenografts was significantly higher in mice implanted with CXCL5-overexpressing PC-3 cells compared with those implanted with mock PC-3 cells (Fig. 7A and B). Western blot analysis of the tumor xenografts revealed that the levels of IL-18 and Bcl-2 proteins were increased, whereas caspase-3 levels were decreased in CXCL5-overexpressing PC-3 xenografts compared with mock PC-3 xenografts (Fig. 7C and D).



Figure 5. Effects of CXCL5-overexpressing WPMY cells on prostate cancer cell malignant potential are paracrine in fashion. (A) PC-3 and DU145 cells were cultured for 72 h in CM (20, 50, 80 or 100%) obtained from untransfected parental, mock-transfected, and CXCL5-OE-transfected WPMY cells and proliferation was analyzed by CCK-8 assay. (B) Representative images of Transwell migration assay for PC-3 and DU145 cells incubated with 100% parental, mock or CXCL5-OE WPMY-1 CM. (C) Analysis of migratory ability in PC-3 and DU145 cells from (B). (D) Representative images of Transwell migration assay for PC-3 and DU145 cells that were co-cultured with parental, mock, or CXCL5-OE WPMY cells for 48 h. (E) Analysis of cell migratory ability of PC-3 and DU145 cells from (D). *P<0.05 and **P<0.01 vs. parental or mock. CCK-8, Cell Counting Kit-8; CM, conditioned medium; CXCL5, C-X-C motif chemokine 5; CXCL5-OE, lentiviral-CXCL5-overexpression vector-transfected cells; mock, lentiviral empty vector-transfected cells.



Figure 6. Effects of CXCL5 gene expression in PC-3 prostate cancer cells. Untransfected parental, mock-transfected, or CXCL5-OE-transfected PC-3 cells were used to determine the effects of CXCL5 on the (A and B) mRNA and (C and D) protein expression levels of BAX, NDRG3, ERK, and CXCR2. *P<0.05 and **P<0.01 vs. parental or mock. CXCL5, C-X-C motif chemokine 5; ERK, extracellular signal-regulated kinase; NDRG3, N-Myc downstream-regulated gene 3. CXCR2, C-X-C chemokine receptor type 2; CXCL5-OE, lentiviral-CXCL5-overexpression vector-transfected cells; mock, lentiviral empty vector-transfected cells.



Figure 7. Effects of CXCL5 overexpression on PC-3 prostate cancer cell-derived xenograft formation and growth in nude mice. Mock-transfected or CXCL5-OEtransfected PC-3 cells (1x10⁶) were subcutaneously injected into nude mice; the mice were sacrificed after 8 weeks and the tumors were analyzed for (A) sizes and (B) weight. (C) Tumor cell xenografts were resected and subjected to western blot analysis for IL-18, Bcl-2 and caspase-3 protein expression levels. (D) Quantified data from the western blot analysis in (C). *P<0.05 and **P<0.01 vs. mock. CXCL5, C-X-C motif chemokine 5; CXCL5-OE, lentiviral-CXCL5overexpression vector-transfected cells; IL, interleukin; mock, lentiviral empty vector-transfected cells.

Discussion

Prostate cancer is the most frequently diagnosed malignancy in men in the United States, accounting for >35% of all male cancers diagnosed (17). Thus, research on the molecular mechanisms of prostate cancer development and progression may identify novel strategies to effectively control prostate cancer. To this end, chemokines, their receptors, and cancer-related inflammation serve important roles in cancer development, including prostate cancer (18-20), and cancer initiation (21). Human cancer development may be initialized through crosstalk between tumor cells and the adjacent microenvironment, where a number of chemokines are continuously secreted by other cancer cells and/or stromal cells (22-24). Many types of cancer cells are able to manipulate the microenvironment to optimize growth and metastatic advantages, whereas autocrine/paracrine signaling leads to communication of tumor cells with the neighboring non-tumor cells (25-27). Thus, chemokines and their receptors appear to serve vital roles in formation of peri- and intratumoral infiltrations (23,28). Such intercellular crosstalk between tumor and neighboring cells through chemokines in the extracellular environment may serve crucial roles in the establishment of mesenchymal status and tumor progression and metastasis (8,22,29).

In this regard, the present study assessed the effects of CXCL5 on prostate cancer cells. CXCL5 mRNA was demonstrated to be highly expressed in prostate cancer cells and tissues, in normal prostate epithelial cells, and in prostate stromal cells, and exogenous delivery of CXCL5 protein resulted in increased proliferation and migration of prostate cancer cells. A previous study revealed that proliferation of prostate cancer LNCaP and 22Rv1 cells was significantly induced in response to very low levels of exogenous CXCL5 protein, whereas higher levels of exogenous CXCL5 reduced tumor cell growth (4). Although different prostate cancer cell lines were used, data from the present study demonstrated a similar phenomenon in which low doses of CXCL5 induced tumor cell proliferation, whereas higher doses of CXCL5 inhibited tumor cell proliferation. Taken together, these data suggested that a high level of ligands may reduce the affinity of the receptors to their ligands. Results from the present study demonstrated that 10-20 ng/ml doses of exogenous CXCL5 were the most optimal concentrations for regulation of DU145 cell proliferation and migration; however, the data also indicated that treatment of PC3 cells with a higher concentration of exogenous CXCL5 resulted in an increase in tumor cell proliferation, whereas lower concentrations contributed to tumor cell migration. Indeed, cell proliferation and migration are two phenotypic traits of all tumor cells, and although they may be related, their mechanisms usually differ. Therefore, the present study hypothesized that different concentrations of exogenous CXCL5 may promote PC3 cell proliferation and migration through different signaling pathways, but further investigations are needed to confirm this.

RT-PCR and ELISA data from the present study demonstrated that CXCL5 is expressed in prostate cancer cells, normal prostate epithelium, and stromal cells, and it is acknowledged that various types of cells in the microenvironment continuously secrete abundant chemokines to promote cancer development through their ability to serve as autocrine or paracrine signals with crosstalk between tumor cells and neighboring cells (22-24); therefore, whether CXCL5 was able to modify prostate cancer cell malignant behaviors was examined by focusing on its role in autocrine/paracrine mechanisms. The data indicated that CXCL5 overexpression in PC-3 or DU145 cells, culture of these cells in conditional medium from CXCL5-overexpressing WPMY cells, or co-culture of these cells with CXCL5-overexpressing WPMY cells all induced prostate cancer cell malignant phenotypes in an autocrine/paracrine fashion in vitro. Moreover, nude mouse xenograft assays demonstrated that CXCL5 overexpression promoted prostate cancer cell xenograft formation and growth in nude mice, which further confirmed our *in vitro* data supporting CXCL5 induction of prostate cancer cell proliferation. However, future research is needed to assess whether knockdown of CXCL5 expression can inhibit tumor formation and growth. Although a previous study indicated that exogenous CXCL5 promotes malignant behavior of prostate cancer cells (4), the present study demonstrated the autocrine and paracrine effects of CXCL5 on the malignant behavior of prostate cancer in CXCL5-overexpressing prostate adenocarcinoma cells and stromal cells, which may secrete CXCL5 protein in the tumor microenvironment. The novelty of this study is in the examination of the source of CXCL5, the CXCL5-producing cells, in the tumor microenvironment.

In addition, CXCL5 overexpression was able to regulate the expression of CXCL5-related signaling pathway genes in prostate cancer cells, such as BAX, NDRG3, ERK1/2, CXCR2, Bcl-2, IL-18, and caspase-3 proteins. The interferon y-inducing factor IL-18 not only enhances cytokine production by T and natural killer cells, but also induces their proliferation and cytolytic activity (30,31). Previous studies reported that engineering tumor cells to produce IL-18 resulted in reduced tumorigenicity and that systemic administration of recombinant IL-18 led to notable tumor inhibition (32,33). Another study reported that leptin-induced IL-18 expression was regulated via nuclear factor (NF)-KB/NF-KB1 signaling in tumor-associated macrophages, whereas it was regulated through phosphoinositide 3-kinase (PI3K)/RAC-α serine/ threonine-protein kinase (AKT) signaling in breast cancer cells, which led to tumor invasion and metastasis (34). Furthermore, the ERK signaling cascade serves a crucial role in development, progression, metastasis, and angiogenesis of various types of human cancers (35-38). In addition, BAX is a pro-apoptotic factor and caspase-3 is an apoptosis executor, whereas Bcl-2 is an anti-apoptotic protein that promotes cell survival (39-42). These proteins in the BAX/Bcl-2/caspase-3 signaling pathway interact with each other to regulate apoptosis in various normal and tumor cells (39-42). The present study data on CXCL5 regulation of these gene expressions indicated that the expression of these proteins may be important in mediating CXCL5 activity in prostate cancer progression. However, further studies are needed to determine the importance of each of these proteins in prostate cancer. In addition, our previous study revealed that NDRG3 overexpression significantly increased CXCL5 expression in prostate cancer cells (3), and the present study data demonstrated that CXCL5 overexpression was able to induce NDRG3 expression in PC-3 prostate cancer cells, which suggested a potential positive-feedback loop of CXCL5 and NDRG3 to promote prostate cancer progression. Future studies will investigate the underlying gene regulations involved in the regulation of CXCL5 in prostate cancer development and progression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ and YL performed cell culture and cell experiments. SG, JL, and XJ conducted animal experiments. YO, ML, and DJ collected tissue samples and drafted the manuscript. MS, HY and WS performed reverse transcription-polymerase chain reaction, ELISA and western blot analyses. JW and SW performed immunohistochemistry. HS performed all statistical analyses. WW and PZ conceived and designed the study. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The Medical Ethics committee at Jiamusi University (Jiamusi, China) approved all procedures performed in the present study involving animals and human participants (approval no. JMSU-195), which were in accordance with ethical standards, and all patients provided written informed consent prior to participation in this study.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no conflict of interest with regard to this work.

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