Int J Clin Exp Med 2016;9(3):5426-5438
www.ijcem.com /ISSN:1940-5901/IJCEM0022236

Original Article

CCL-4 enhances prostate cancer migration and invasion by modulating integrin expression

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Received December 18, 2015; Accepted March 5, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men in the United States and is the most commonly diagnosed non-cutaneous cancer. CCL4 is a secreted chemokine that is over expressed in patients that show PCa recurrence after prostatectomy. Currently, no reported evidence shows the biological role of CCL4 in PCa progression. We studied the role of CCL4 in PCa progression using *in vitro* and *in vivo* models. PC3 and 22RV1 PCa cell lines were treated with CCL4 at 0.001 ng/mL and 0.1 ng/mL and subjected to migration and invasion assays. Tumor progression was evaluated using a xenograft model in which the anterior prostate lobes of SCID mice were injected with 250,000 22RV1 cells. CCL4 was administered bi-weekly with intraperitoneal injections. Tumor tissue was collected for immunohistochemical and gene expression analysis. *In vitro* studies showed that CCL4 increased invasion and migration of PCa cells. *In vivo* studies demonstrated that CCL4 increased tumor volume. Immunohistochemical analysis showed that these tumors expressed higher levels of desmin and phosphohistone 3 (pH3), and showed increased angiogenesis. Gene expression analysis showed that CCL4 increased the expression of genes associated with metastasis. Western blot analysis showed that CCL4 increased integrin expression and FAK phosphorylation suggesting integrin pathway activation. This suggests that CCL4 is important for negative outcomes such as metastasis and PCa recurrence.

Keywords: Prostate cancer, cytokines, chemokines, CCL4, MIP1β, integrins, migration, invasion

Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous cancer in the United States and Puerto Rico. It is the second leading cause of cancer related deaths among men, in the United States and the principal cause of mortality in Puerto Rico [1-3]. Prostate cancer is often treated via prostatectomy or radiation. However, approximately 15-30% of prostate cancer patients develop biochemical recurrence (i.e. increased serum prostate specific antigen (PSA) levels) following primary treatment, which often results in death [4, 5]. Biochemical recurrence typically occurs due to prostate cancer cells that have metastasized to distant sites after prostate tumor removal [6]. Although the early detection of potential metastatic or recurrent prostate cancer can lead to proactive use of adjuvant therapeutic options, the available biomarkers and (or) clinical information are insufficient to predict recurrence and metastasis [7]. Thus, there is a pressing need for novel diagnostic and prognostic tools for the management of prostate cancer.

Given the important role of inflammation in cancer development, the expression of inflammatory mediators, such as chemokines, is significant during prostate cancer progression. Previous studies have found that the expression of different cytokines and their receptors varies with stage and aggressiveness in many types of cancer [8, 9]. The pro inflammatory chemokine Macrophage Inflammatory Protein-1β (also known as CCL4 or MIP-1b) was found over expressed in patients with biochemical recurrence after radical prostatectomy [10]. This makes CCL4 a possible predictive biomarker for prostate cancer recurrence.

Moreover, chemokines such as CCL4 modulate macrophage movement by increasing the expression of several integrins in the endothelium [11]. Integrins play an important role in promoting cancer cell motility and invasion that lead to metastasis and recurrence [12]. Nevertheless, no previous work has studied the role of CCL4 in prostate cancer progression or integrin modulation.

In this study, we evaluated the role of CCL4 in migration, invasion, proliferation, tumor growth, and angiogenesis using cellular and animal models of prostate cancer. We show that CCL4 increased prostate cancer cell motility, invasion, and integrin expression *in vitro*. In addition, CCL4 increased tumor growth, promoted metastatic potential, increased desmin and phospho-histone 3 expression, and increased angiogenic potential *in vivo*. Thus, our studies provide evidence that CCL4 is relevant to prostate cancer progression and metastasis; suggesting an important role in recurrence. Furthermore, we suggest a possible mechanism of action through which CCL4 promotes cancer progression by modulating integrin expression.

Materials and methods

Cell culture

Androgen receptor negative (PC3) and androgen receptor positive (22RV1) prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in RPMI-1640 medium (Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Waltham, MA, USA) and penicillin-streptomycin complex (1,000 units/mL) (Gibco, Life Technologies, Carlsbad, CA, USA) at 37°C and 5% CO_2 in a humidified incubator. Murine prostate fibroblasts were obtained from wild-type C57BL/6 mice (Taconic, Germantown, NY, USA). Prostates were dissected in our lab and cells were maintained in DMEM medium (Hyclone, Waltham, MA, USA) supplemented with 5% FBS (Hyclone, Waltham, MA, USA), 5% Nu-Serum (Becton Dickinson, Franklin Lakes, NJ, USA) penicillin-streptomycin complex (100 units/mL), 0.1% insulin (Becton Dickinson, Franklin Lakes, NJ, USA), and testosterone 10 nM (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2 in a humidified incubator.

Scratch wound healing assay

PC3 cells (2×10^5 cells/mL) were grown to 95% confluence in 12-well tissue culture plates. Following serum starvation for 24 hours, a scratch wound was made across the center of the monolayer of cells using a 200 µL pipette tip and washed using PBS. Cells were treated with RPMI medium containing CCL4 (Genway, San Diego, CA, USA) (0.001 ng/mL and 0.1 ng/mL), or control (PBS). Cells were photographed using a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan) at 0, 12 and 24 hours of treatment at a 4× magnification. A total of 10 distance measurements within each wound were analyzed using Image Pro Plus Software. The differences in wound closure were normalized and compared to the control using Student's T-test at a 95% confidence interval. All experiments were performed in triplicate, each with 3 wells per group.

Invasion assay

22RV1 and PC3 cells (4×10^4 cells/mL) were serum starved and seeded in laminin/entactin-coated (Becton Dickinson, Franklin Lakes, NJ, USA) 24-well 8.0 µm pore transwell chamber (Corning, Corning, NY, USA). The cell suspension had serum free RPMI 1640 (Hyclone, Waltham, MA, USA). The reservoir well had a monolayer of murine prostate fibroblasts in DMEM medium supplemented with 5% FBS, 5% nu-serum and CCL4 for a final concentration of 0.001 ng/mL or 0.1 ng/mL. The cells were allowed to invade during 24 hours under culturing conditions (37°C and 5% CO_2). Non-invasive cells were removed from the top chamber using a sterile cotton swab and PBS. Invasive cells were fixed to the membrane with buffered formalin 10% (Thermo Scientific Waltham, MA, USA) for 30 minutes, and stained over night with hematoxylin (American Master Tech, Lodi, CA, USA). The membranes were washed with water and mounted on slides. Photographs were captured at a 4× magnification with a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan) and the total number of invasive cells was counted using Image Pro Plus Software. Results were analyzed using the Student's T-test at a 95% confidence interval. All experiments were performed in triplicate with three wells per group.

Orthotopic mouse model

Male ICR-SCID mice (7-8 weeks old) were housed and maintained in a pathogen-free

environment at The University of Puerto Rico Medical Sciences Campus animal facility, under the Institutional Animal Care and Use Committee regulations. Animals received food and water ad libitum with a 12-hour light cycle. An orthotopic xenograft model in which 22RV1 (250,000 cells) were injected in the anterior prostate lobes of ICR-SCID mice (IcrTac: IcrCrl-SCID) (Taconic, Germantown, NY, USA) was used to develop 2 prostate tumors per mice. Cell suspensions in PBS were placed in 30 µL of collagen I (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to partially solidify to hold cells in place and reduce leakage in the peritoneal cavity during surgery. Mice were intraperitoneally injected bi-weekly with CCL4 (0.001 ng/mL) or vehicle (Saline). At 4 weeks of treatment, tumors were collected. Tumor volume was determined using caliper measurements. Results were analyzed using the Student's T-test at a 95% confidence interval. $n_{control}$ = 26 tumors, n_{CCL4} = 21 tumors.

Tissue collection and processing

Tumor samples ($n_{control}$ = 26 tumors, n_{CCL4} = 21 tumors) were collected and divided in two sections. One section was snap frozen in dry ice and stored at -80°C, the other was fixed in 10% buffered formalin. The fixed tissue was processed, embedded in paraffin, and cut in 5 µm sections for hematoxylin and eosin staining (H&E) and immunostaining. The frozen tissue section was used to isolate RNA for gene expression assays.

Hematoxylin-eosin staining

For histological examination by H&E staining, slides with 5 µm sections of paraffin embedded tumor were deparaffinized in xylene, and hydrated using serial descending concentrations of alcohol. Tissue was stained with hematoxylin followed by stain differentiation with 1% v/v acid alcohol (80% ethanol, 19% deionized water, 1% HCl), 0.3% v/v ammonia water (0.3% NH_4OH in de-ionized H_2O) and washing with 70% ethanol. After eosin staining (0.05% Eosin Y in 70% Ethanol-0.005% acetic acid) the tissue was dehydrated with increasing serial dilutions of ethanol and xylene. Slides were mounted using permount mounting medium. n = 5 representative tumors per group.

Immunohistochemistry and immunofluorescence

Formalin-fixed paraffin-embedded tumor samples were dewaxed in xylene and rehydrated in descending concentrations of alcohol and water. Tumor antigens were retrieved using Antigen Unmasking Solution (1:100 dilution) (Vector Laboratories, Burlingame, Ca, USA) followed by quenching of endogenous peroxidase with 3% v/v H_2O_2. The primary antibodies used were the following: phospho-histone 3 (pH3) (1:1000 dilution) (Abcam, Cambridge; MA, USA), CD31 (1:50 dilution) (Abcam, Cambridge; MA, USA), desmin (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), integrin-β1 (1:100 dilution) (Cell Signaling, Danvers, MA, USA), PFAK (1:100 dilution) (Epitomics, Burlingame, CA, USA). All immunohistochemistry was detected by Dako Envision system-HRP (DAB) (anti-rabbit) (Dako; Glostrup, Denmark) or Dako LSAB System-HRP (DAB) (anti-mouse) (Dako; Glostrup, Denmark) according to the manufacturer's instructions. Hematoxylin was used as a counterstain. For immunofluorescence, the secondary antibody used was Alexa-Fluor 594 (anti-rabbit) 1:2000 (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and nuclei were stained with DAPI 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To quantify pH 3 and desmin, a subjective scale from 1-4 was used. In this scale, we gave a score of one (1) if 25% or less of the tumor cells were stained, a score of two (2) if 26% to 50% of the tumor cells were stained, a score of three (3) if 51% to 75% of the tumor cells were stained, and a score of four (4) if more than 75% of the tumor cells were stained. Score was given in a blind manner. n = 10 representative tumors per group. To quantify CD31, a set of 3 random fields were chosen per slide and the total number of blood vessels was counted. Statistical analysis was done using the Student's T-test t a 95% confidence interval. n = 5 representative tumors per group.

Gene expression

To evaluate changes in gene expression at the RNA level, the Qiagen RT^2 PCR arrays (human tumor metastasis and PI3K pathway) were used (Qiagen Inc., Valencia; CA, USA). RNA was extracted and purified from frozen mice tumors using the RNeasy Mini Kit (Qiagen Inc., Valencia;

Table 1. Primer sets used for Real-Time PCR analysis

Primer*	Sense	Antisense
BRMS1	5'-AGACAGAGTCAGAAGAGGAGAG-3'	5'-CTTCTCCTTTAGCTCCGAGAAC-3'
CXCR2	5'-CTCGTGATGCTGGTCATCTTAT-3'	5'-CAAGGTCAGGGCAAAGAGTAG-3'
GAPDH	5'-GGTGTGAACCATGAGAAGTATGA-3'	5'-GAGTCCTTCCACGATACCAAAG-3'
FOS	5'-GGTGCATTACAGAGAGGAGAAA-3'	5'-GTGTGTTTCACGCACAGATAAG-3'
ITGA7	5'- CTCTGCCTGTCCAATGAGAAT-3'	5'- AGGTGCTAAGGATGAGGTAGA-3'
MMP10	5'-GGCCCTCTCTTCCATCATATTT-3'	5'-CCTGCTTGTACCTCATTTCCT-3'
PI3KA	5'-AGAGCCCCGAGCGTTTCTG-3'	5'-CATCAAGTGGATGCCCCACA-3'
PRKCA	5'-CCATCCGCTCCACACTAAAT-3'	5'-GATCCCAGTCCCAGATTTCTAC-3'
RORB	5'-TAAGTCCTCTGGGATCCACTAC-3'	5'-GCCTTGGGCAGGAATAAGAA-3'
RPS6KA1	5'-GAGGGCAAGCTCTATCTCATTC-3'	5'-CTCCGTGAACATCACCTCTTT-3'

*(BRMS1) Breast cancer metastasis suppressor 1, (CXCR2) Chemokine (C-X-C motif) receptor 2, (GAPDH) Glyceraldehyde 3-phosphate dehydrogenase, (FOS) FBJ murine osteosarcoma viral oncogene homolog, (ITGA7) Integrin-α7, (MMP10) Matrix metallopeptidase 10, (PI3KA) Phosphatidylinositol-4,5-bisphosphate 3-kinase, alpha, (PRKCA) Protein kinase C, alpha, (RORB) RAR-related orphan receptor B, (RPS6KA1) Ribosomal protein S6 kinase, polypeptide 1.

CA, USA). RNA (2 μg per array) was copied to cDNA using the RT^2 First Strand Kit including DNA elimination procedure (Qiagen Inc., Valencia; CA, USA). Results were analyzed using the MS Excel based tool provided by Qiagen (PCRArrayAnalysisV4, available for download at https://www.qiagen.com/us/resources/resourcedetail?id=d8d1813e-e5ba-4d29-8fdf-07a3f4227e0a&lang=en). To validate results, quantitative Real time PCR (qRT-PCR) was performed under standard conditions using the Step One Plus Real-time PCR System (Applied Biosystems, Carlsbad; CA, USA). Real-time PCR was performed using SYBR super mix (Bio-Rad, Hercules, CA, USA) in a total volume of 10 μL. Depending on the gene of interest the cycle was, at 95°C for 15 seconds and 62°C for 1 minute or 95°C for 15 seconds and 56°C for 1 minute. PCR efficiency was examined using serial dilutions of the template cDNA and the melting curve data was collected for PCR specificity. GAPDH was used as housekeeping gene. Results were quantified using the $\Delta\Delta C_t$ method. No PCR product was detected in control samples in which the template was omitted. Primer sequences are listed in **Table 1**. PCR arrays: n = 3 tumors per group, randomly chosen. Real-time PCR confirmation: n = 5 representative tumors per group.

Western blotting

22RV1 prostate cancer cells were co-cultured using 6-well 0.4 μm transwell plates (Corning, Corning, NY, USA) for 24 hours with murine prostate fibroblasts treated with PBS (Control), or CCL4 at 0.001 ng/mL and 0.1 ng/mL. 22RV1 cells were washed and protein was extracted with lysis buffer (HEPES 25 mM, $MgCL_2$ 5 mM, NaCl 300 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, 10% Glycerol, 1% Triton-X, 0.1% Na-deoxycholate, 0.1% SDS, Na_3VO_4 1 mM, p-nitrophenyl phosphate 20 mM, beta-glycerolphosphate 20 mM, Na-pyrophosphate 2 mM, PMSF 1 mM, Microcystin 10 nM, Aprotinin 10 μg/mL, Leupeptin 10 μg/mL) followed by centrifugation at 15,000 g for 10 minutes at 4°C. Supernatants were stored at -20°C. Protein concentration was determined using the detergent compatible (DC) protein determination kit (BioRad, Hercules, CA, USA). Samples (40 μg of protein) were separated on 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated for an hour with 5% w/v BSA in TBST to block nonspecific antibody binding. Membranes were incubated overnight at 4°C with primary antibody, integrin-β1 1:1000 (Cell Signaling, Danvers, MA, USA), PFAK 1:2000 (Epitomics, Burlingame, CA, USA), and β-actin 1:5000 (Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies, goat anti-mouse 1:5000, and goat anti-rabbit 1:5000, (Cell Signaling, Danvers, MA, USA) were incubated for an hour at room temperature. Membranes were developed using ECL (BioRad, Hercules, CA, USA) and radiography films (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Densitometry was determined using the Image J software. Quantification was done using β-actin as a loading control and all ratios were normalized to the control sample. Statistical analysis was performed using the Student's T-test at a 95% confidence interval. All experiments were performed in triplicate with one sample each.

Results

CCL4 increased prostate cancer cell motility and invasion

To study the effect of CCL4 in cell motility we performed a wound healing assay. A confluent

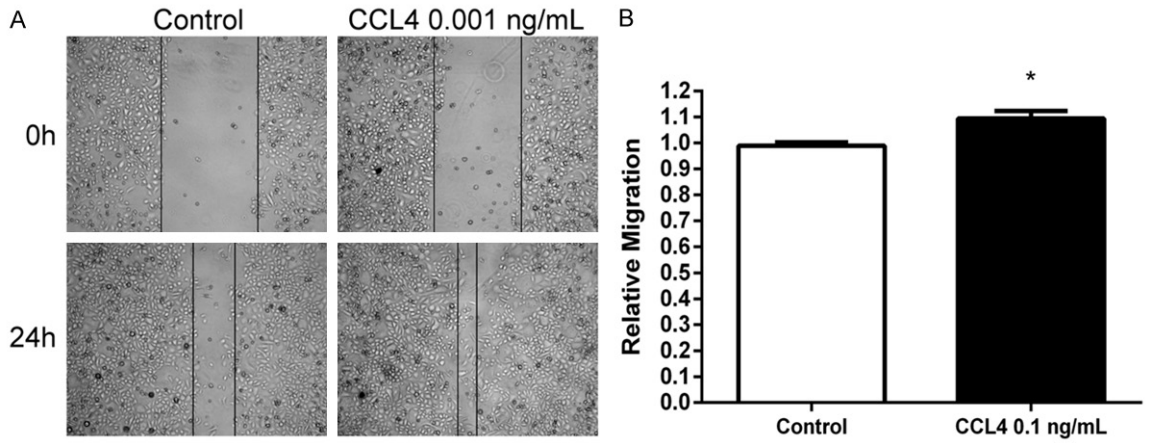

Figure 1. CCL4 increased PC3 cell migration. PC3 cell migration was evaluated using the wound healing method. A. Representative images (4× magnification) of PC3 cells treated with CCL4 0.1 ng/mL at 0 h and 24 h. B. CCL4 treatment caused a significant increase in cell migration, Triplicate experiments, Mean + SEM (*P<0.05).

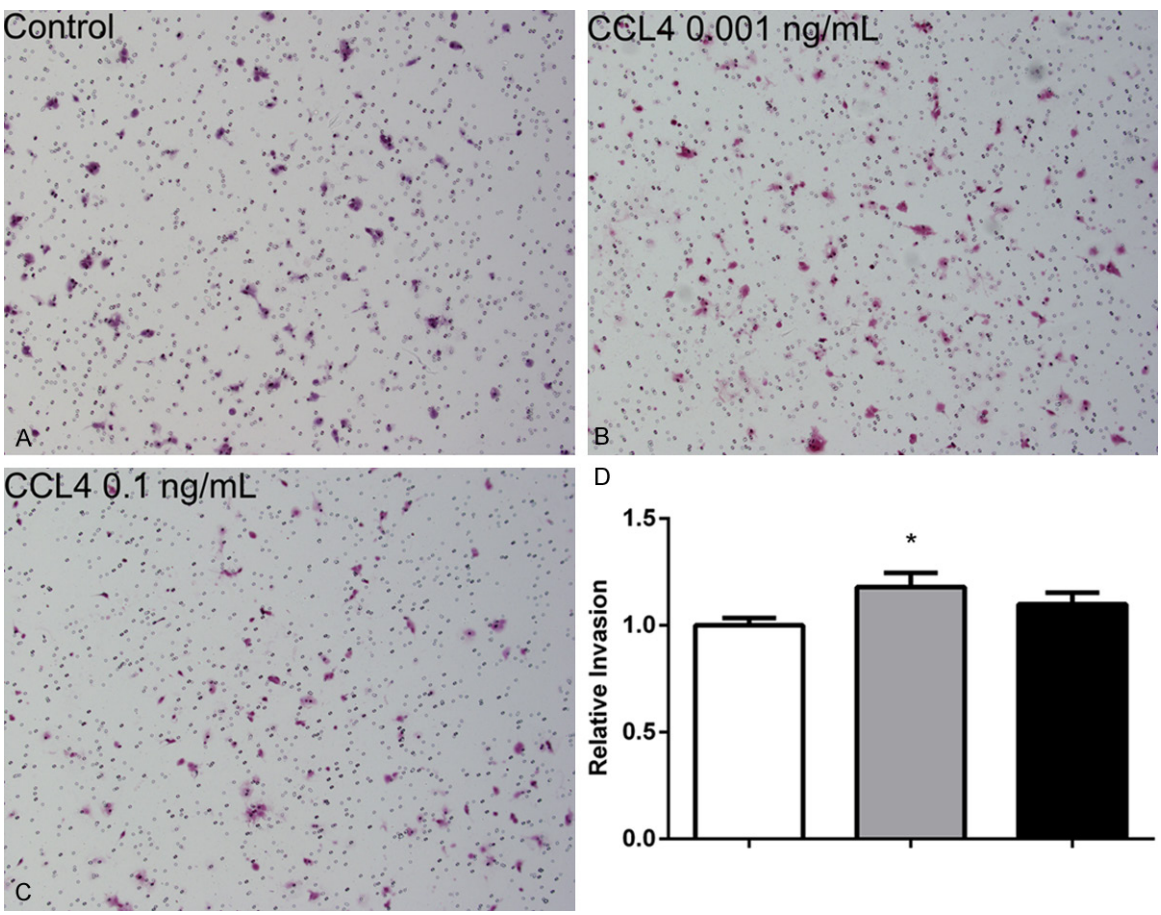

Figure 2. CCL4 increased 22RV1 cell invasion. Cell invasion was evaluated using the boyden chamber method. Representative images (10× magnification) of invasive 22RV1 cells (A) Control (B) CCL4 0.001 ng/mL (C) CCL4 0.1 ng/mL. (D) CCL4 0.001 ng/mL caused a significant increase in invasion. Triplicate experiments, Mean + SEM (*P<0.05).

monolayer of androgen independent PC3 cells was wounded and allowed to migrate for 24 hours with CCL4 at 0.001 ng/mL and 0.1 ng/mL. The migration of PC3 cells treated with

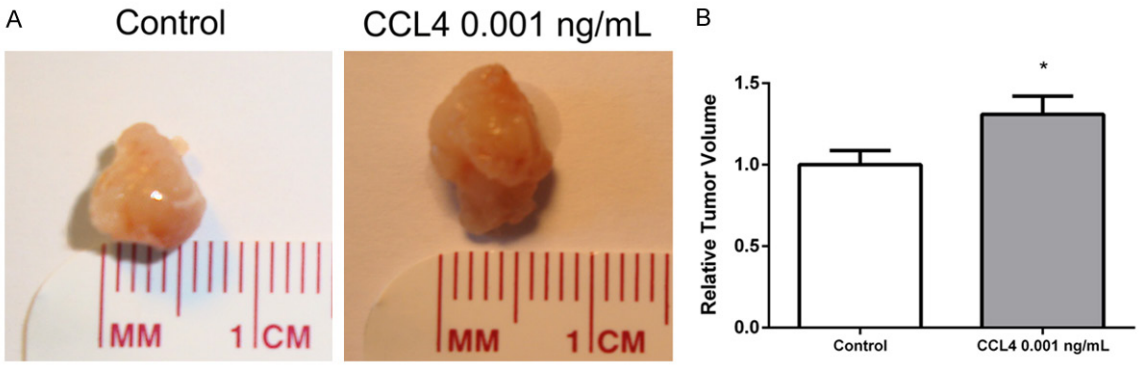

Figure 3. CCL4 increased tumor growth. The role of CCL4 in tumor growth was evaluated with an orthotopic model. 22RV1 cells were xenografted in the anterior prostate lobes. Tumors were allowed to develop for 4 weeks and CCL4 was administered bi-weekly with intraperitoneal injections. The tumor volume was determined with caliper measurements. A. Representative images of tumors show differences in size. B. Relative tumor volume quantification. Mean + SEM (*P<0.05) ($n_{Control}$ = 26 tumors, $n_{CCL4\ 0.001\ ng/mL}$ = 21 tumors).

CCL4 at 0.1 ng/mL increased in 10% when compared to control (P<0.05) (**Figure 1**). 22RV1 cells were not used for the wound-healing assay because these cells do not grow in a confluent monolayer. To study cell invasion, we performed a boyden chamber assay. We found that CCL4 at 0.001 ng/mL increased 22RV1 cell invasion by 10% when compared to the control (P<0.05) (**Figure 2**). Invasion of PC3 cells treated with CCL4 was not significantly different when compared to control (Data not shown). Additionally, we studied cell proliferation using an MTS based assay. We found no significant differences in cell proliferation (both PC3 and 22RV1) with CCL4 treatment (Data not shown). These results show that CCL4 promoted cancer cell motility and invasion in prostate cancer cells while cell proliferation remained unaffected.

CCL4 promoted tumor growth

The effect of CCL4 in tumor growth was studied using an orthotopic model in which the anterior prostate lobes of SCID mice were injected with 250,000 22RV1 cells in a collagen-1/PBS suspension. Chemokine treatment proceeded with bi-weekly intraperitoneal injections of saline solution for the control group and CCL4 0.001 ng/mL solution for the treatment groups. After four weeks of treatment, the mice were euthanized and tumor volume was calculated using caliper measurements. Mice treated with CCL4 intraperitoneal injections (0.001 ng/mL) developed significantly larger tumors when compared to the control (P<0.05) (**Figure 3**). This result suggests that the chemokine CCL4, promotes prostate cancer progression by increasing tumor growth.

CCL4 increased proliferative, metastatic and angiogenic potential in vivo

Pathological, histological and immunohistochemical analysis of collected tumor tissue was used to study the effect of CCL4 in tumor biology. Slides were examined by a pathologist at low, medium and high power under a compound light microscope. Tumor assessment was made as described by Lsaacs and Hukku [13]. Histologically, tumors were classified in four categories: histologically well differentiated, histologically moderately differentiated, histologically poorly differentiated, and histologically anaplastic. A well-differentiated tumor was characterized by the presence of glandular structures or acinic carcinoma with a lumen, basement membrane, and stroma. A moderately differentiated tumor was characterized by smaller glandular structures in which the lumen could be obstructed by tumor cells, but still retained a basement membrane and stroma. A histologically poorly differentiated tumor was characterized by the absence of glandular structures, basement membrane, or consistent relationship between tumor cells and stroma. Individual tumor cells, however, still retained secretory appearance (regular size nucleus, normal nucleus to cytoplasm ratio). A histologically anaplastic tumor lacked all appearance of tissue organization and individual tumor cells were anaplastic in appearance (irregular nucleus size and abnormal nucleus to cytoplasm

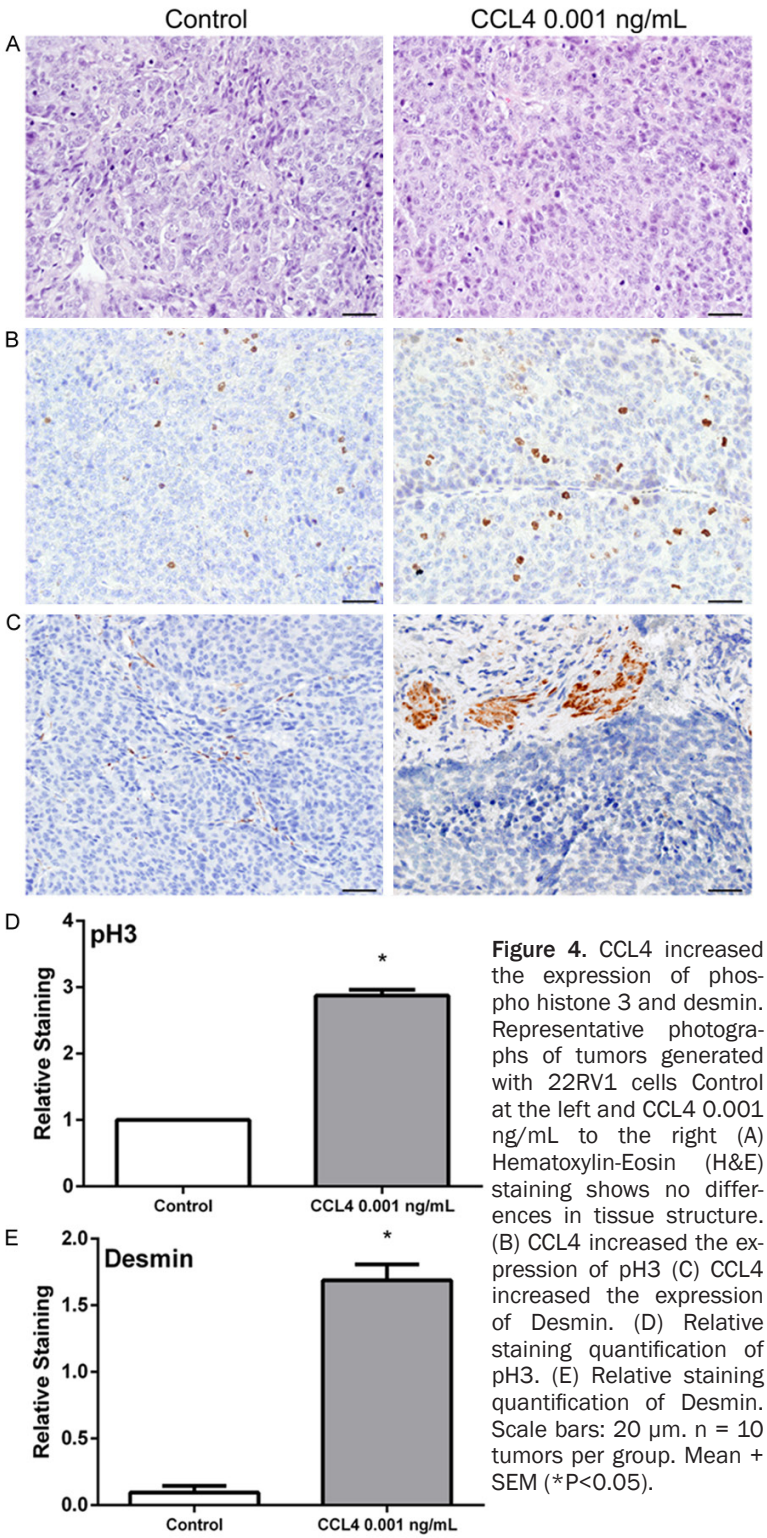

Figure 4. CCL4 increased the expression of phospho histone 3 and desmin. Representative photographs of tumors generated with 22RV1 cells Control at the left and CCL4 0.001 ng/mL to the right (A) Hematoxylin-Eosin (H&E) staining shows no differences in tissue structure. (B) CCL4 increased the expression of pH3 (C) CCL4 increased the expression of Desmin. (D) Relative staining quantification of pH3. (E) Relative staining quantification of Desmin. Scale bars: 20 μm. n = 10 tumors per group. Mean + SEM (*P<0.05).

ratio). All tumor samples, regardless of the treatment, were classified in histologically poorly differentiated or histologically anaplastic showing no significant differences among treatments (**Figure 4A**). To determine if CCL4 affected cell proliferation, the expression of phospho-histone 3 (pH3) was measured in tumors by immunohistochemistry (**Figure 4B**). Tumors developed in mice treated with CCL4 showed a significant increase in pH3 expression suggesting increased proliferation (P<0.05) (**Figure 4D**). To study mesenchymal characteristics and changes in stroma, we measured the expression of desmin in tumors using immunohistochemistry (**Figure 4C**). Our results showed that tumors developed in mice treated with CCL4 had increased expression of desmin, suggesting an increased metastatic potential (P<0.05) (**Figure 4E**).

During gross examination, we observed that tumors developed in mice treated with CCL4 were more vascular than control tumors. Therefore, we evaluated angiogenesis measuring CD31 expression by immunofluorescence (**Figure 5A**). Our results showed that CCL4 significantly increased the number of blood vessels in tumor tissue (P<0.05) (**Figure 5B**). These results suggest that CCL4 treatment affects the tumor development by increasing proliferation, mesenchymal properties, and angiogenesis.

CCL4 altered the expression of genes associated with tumor metastasis and the PI3K pathway

To identify changes in gene expression caused by CCL4, we performed PCR arrays. Given that we focused on prostate cancer recurrence, we specifically tested the tumor metastasis array. Additionally, since CCR5, the CCL4 receptor, is

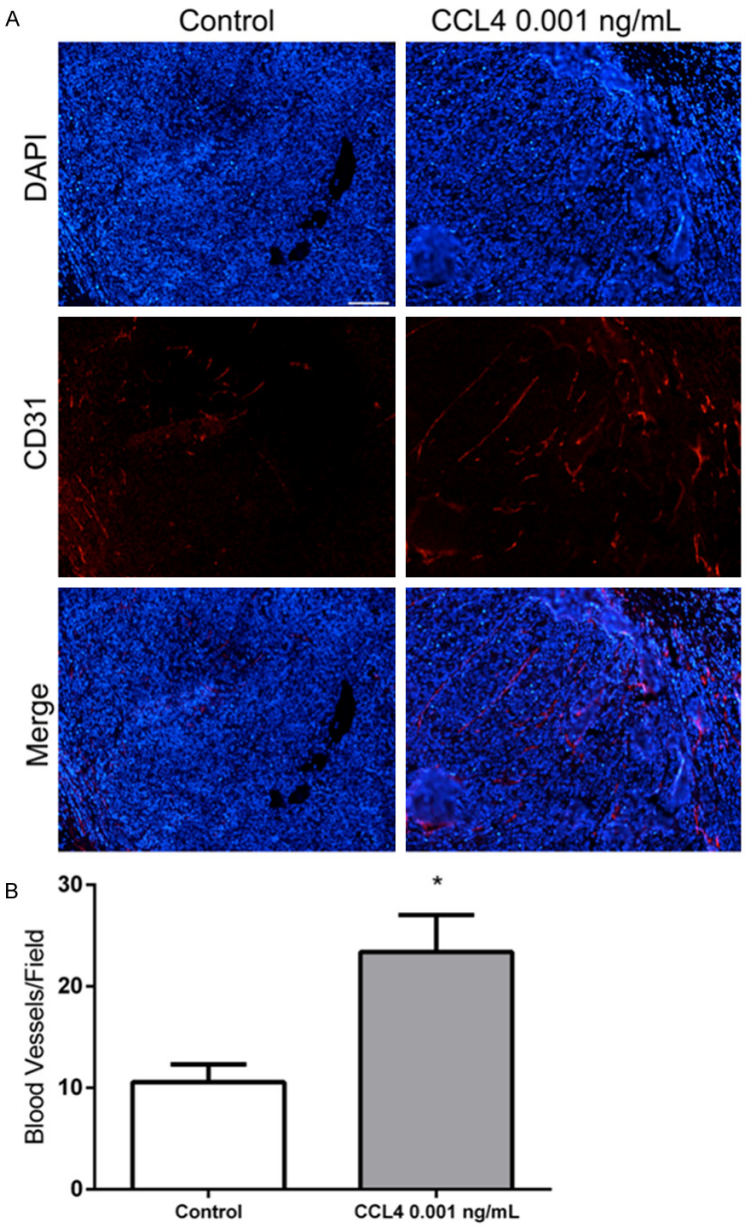

Figure 5. CCL4 increased angiogenesis *in vivo*. A. Representative Immunofluorescence images of 22RV1 tumors, Control and CCL4 0.001 ng/mL. Nuclei are stained in blue and blood vessels are stained in red. B. Blood vessel counts per field are significantly increased with CCL4 treatment.

a G-protein coupled receptor (GPCR), we studied the PI3K pathway; which is activated downstream. Results showed that CCL4 treatment significantly increased the expression of 5 genes associated with tumor metastasis: breast cancer metastasis suppressor 1 (BRMS1), Chemokine (CXC Motif) Receptor 2 (CXCR2), Integrin-α7 (ITGA7), matrix metalloproteinase 10 (MMP10), and RAR-related orphan receptor b (RORB) (**Table 2**). In the context of prostate cancer BRMS1 is a tumor suppressor gene, CXCR2 is the receptor of IL-8, ITGA7 is associated with tumor suppressor activity, MMP10 overexpression is associated with metastasis, and RORB, a member of the hormone nuclear receptor family, is deregulated in prostate cancer [14-19]. In addition, CCL4 increased the expression of 4 genes associated with the PI3K pathway: FBJ murine osteosarcoma viral oncogene homolog (FOS), phosphatidylinositol 3-kinase alpha (PI3KA), protein kinase C-alpha (PRKCA), and Ribosomal Protein S6 Kinase (RPS6KA1) (**Table 2**). The over expression of these genes was confirmed by real time PCR (**Figure 6**). These results show that CCL4 modulated the expression of genes associated with tumor metastasis and PI3K pathway.

Cell motility is associated with integrin expression via FAK phosphorylation

The gene expression analysis showed an over expression of FOS, a constituent of the AP-1 transcription factor, which is regulated by integrin signaling. As integrins mediate intracellular signals in response to the extracellular matrix, we performed co-culture experiments and murine fibroblasts treated with PBS (control), CCL4 at 0.001 ng/mL or 0.1 ng/mL for 24 hours to mimic the tumor microenvironment *in vitro*. Given that integrin signaling is modulated by the phosphorylation of focal adhesion kinase (FAK), we assessed the expression of integrins and phospho-FAK using western blotting (**Figure 7A**). We observed increase of FAK phosphorylation in 22RV1 treated with 0.001 ng/ml

Table 2. Differentially expressed genes

Gene Bank Accession Number	Gene Symbol	Description	Fold Change	*P* Value
NM_015399	BRMS1	Breast cancer metastasis suppressor 1	2.08	0.0004
NM_001557	CXCR2	Chemokine (C-X-C motif) receptor 2	2.88	0.0001
NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog.	1.55	0.0264
NM_001144997	ITGA7	Integrin alpha 7	2.05	0.0002
NM_002425	MMP10	Matrix metallopeptidase 10	2.56	0.0167
NM_006218	PI3KA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, alpha	1.61	1.5×10^{-6}
NM_002737	PRKCA	Protein kinase C, alpha	2.21	0.0043
NM_006914	RORB	RAR-related orphan receptor B	1.27	0.0176
NM_002953	RPS6KA1	Ribosomal protein S6 kinase, polypeptide 1	2.85	0.0415

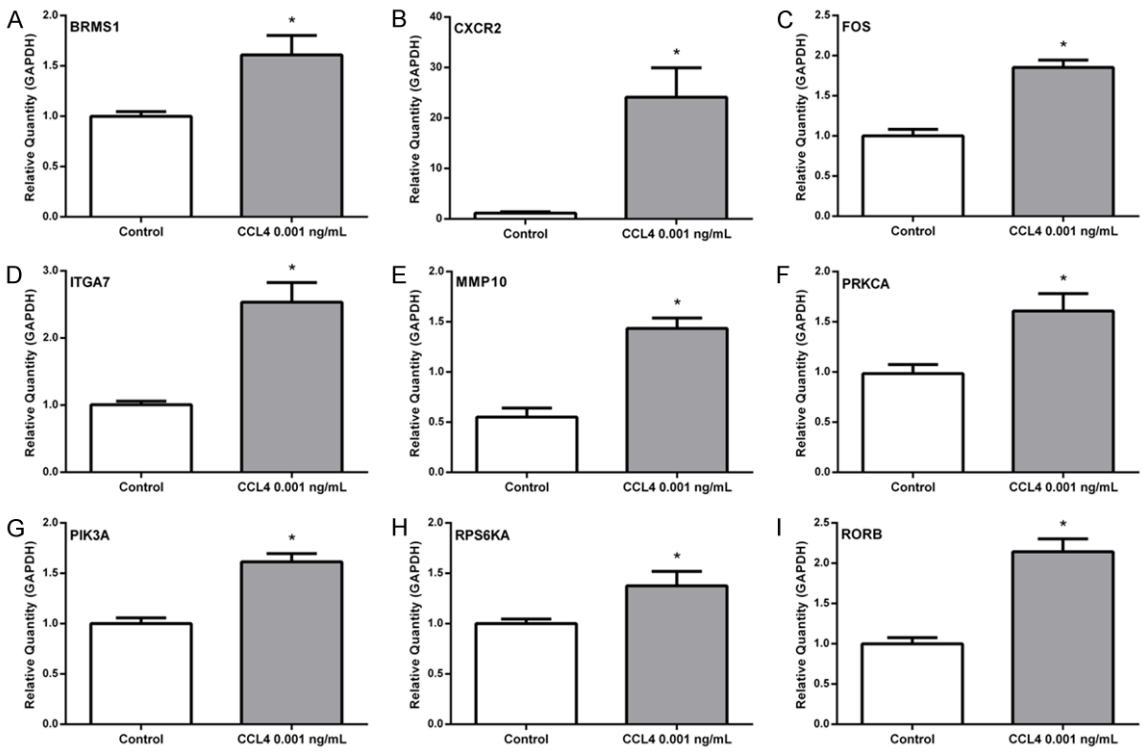

Figure 6. Real-time PCR confirmation of differentially expressed genes. Real time PCR was quantified using the $\Delta\Delta C_t$ method. A. BRMS1; B. CXCR2; C. FOS; D. ITGA7; E. MMP10; F. PI3KA; G. PRKCA; H. RORB; I. RPS6KA1. Values normalized to GAPDH and relative to the control, Mean + SEM (*P<0.05). n = 5 tumors per group.

CCL4 and in integrin-β1 expression in 22RV1 treated with 0.1 ng/ml and 0.001 ng/ml CCL4, although these changes did not reach statistical significance (**Figure 7B**). However, in 22RV1 treated with CCL4 0.1 ng/mL FAK phosphorylation was significantly increased (**Figure 7C**). To determine if these changes were translated *in vivo,* we performed immunostaining against PFAK and ITGB1 in mouse tumor samples. Our results showed that CCL4 caused a significant increase of FAK phosphorylation *in vivo* (**Figure 7D-F**). We observed a trend of increasing ITGB1 expression *in vivo* (**Figure 7G-I**). These results suggest that the presence of CCL4 in the microenvironment increased integrin expression via FAK phosphorylation.

Discussion

Currently, prostate cancer diagnosis and risk stratification of patients is achieved via a combinatorial assessment of PSA value, clinical

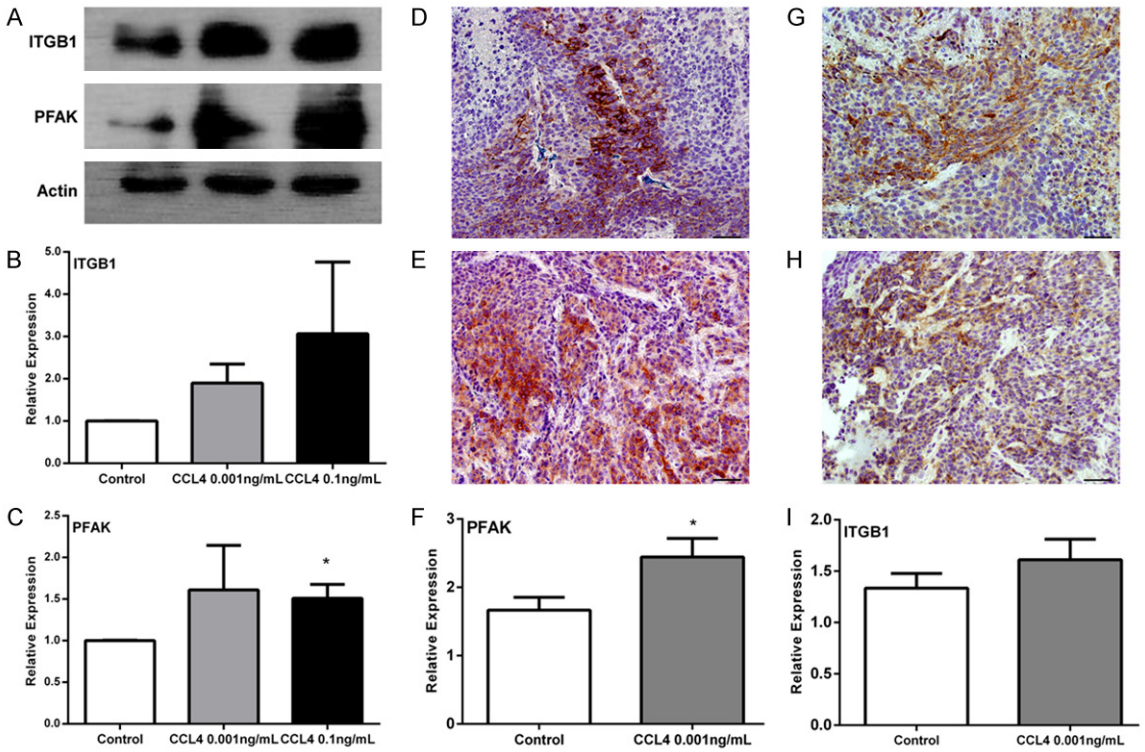

Figure 7. CCL4 increased FAK phosphorylation and integrin-β1 expression. (A) Representative images of western blot films. (B) CCL4 treatment showed a trend of increasing ITGB1 expression *in vitro*. (C) CCL4 treatment significantly increased FAK phosphorylation *in vitro*. Protein expression normalized to β-actin and relative to the control. (D) Representative image (40×) of PFAK immunostaining for Control. (E) PFAK immunostaining for CCL4 0.001 ng/mL. (F) CCL4 treatment significantly increased FAK phosphorylation *in vivo*. (G) Representative image (40×) of ITGB1 immunostaining for Control (H) ITGB1 immunostaining CCL4 0.001 ng/mL (I) CCL4 treatment showed a trend of increasing ITGB1 expression *in vivo*. n = 5 tumors per group. Scale bar: 50 μm. Mean + SEM (*P<0.05).

tumor stage, and biopsy Gleason score. However, these methods are unable to predict biochemical recurrence, which occurs in 15-30% of patients with early-stage prostate cancer after definitive local therapy [5, 20]. Determining which patients can be managed with active surveillance and which require aggressive therapy is a challenging task. Even though prompt anti-androgenic and chemotherapeutic treatment of metastatic cancers has shown benefits, these are administered after serum PSA is detectable in the context of an ablated prostate. It is critical to determine candidates that would benefit from adjuvant therapy because patients that experience biochemical recurrence may probably have metastatic spread of the disease [21]. This highlights the pressing need for novel diagnostic and prognostic tools that can better aid in the identification of prostate cancer.

Over-expression of CCL4 has been associated with prostate cancer recurrence within five years of a radical prostatectomy [10]. This suggests that CCL4 can be a possible predictive biomarker for prostate cancer recurrence.

Previous work has not studied the role of CCL4 in prostate cancer progression. In this study, we examined the role of CCL4 in prostate cancer using both *in vitro* and *in vivo* models. We evaluated different hallmarks of cancer such as cell motility, invasion, tumor growth, proliferation, and angiogenesis. Our data showed that the presence of CCL4 in the micro environment enhanced prostate cancer cell migration and invasion, increased tumor growth, increased proliferation, and increased blood vessel formation. Additionally, we found that CCL4 altered the expression of several genes associated with tumor metastasis such as integrins, MMPs, and transcription factors that promote cell proliferation.

CCL4 is a secreted chemokine involved in pro inflammatory processes. Some of the main

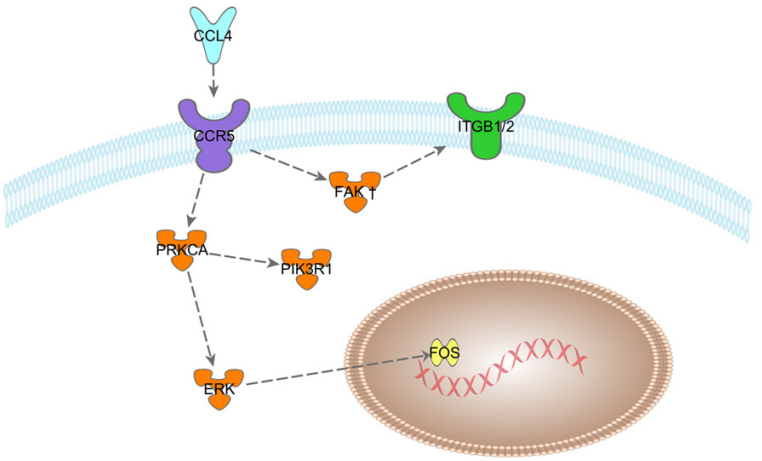

Figure 8. Proposed mechanism of action. CCL4 binds its receptor CCR5. This GPCR activates protein kinase C (PRKCA) and promotes focal adhesion kinase (FAK) phosphorylation that activates integrin-β1 (ITGB1) pathway. Downstream of PRKCA, we activate PI3K and MAPK pathways that promote the internalization of FOS to form the AP-1 transcription complex.

functions of CCL4 are: the attraction of macrophages to infection sites, the promotion of regulatory T cell (Treg) migration, and the induction of mast cell degranulation [22]. In the context of cancer, the over expression of CCL4 and its receptor, CCR5, has been associated with breast cancer metastasis [23]. Furthermore, due to Treg recruitment, CCL4 expression in solid tumors promotes immune system evasion; which increases tumor growth [24]. CCR5 is a G-Protein coupled receptor (GPCR) that can activate several pathways, including: the MAPK pathway, the PI3K pathway, and the integrin signaling pathway [25].

This study showed that CCL4 increased the expression of integrin-β1 via phosphorylation of focal adhesion kinase (FAK). Integrin-β1 is up-regulated in progressive prostate cancer and allows cells to grow in an anchorage-independent manner. In turn, this increases interactions with the extracellular matrix (ECM) and promotes cell invasion [12]. Moreover, integrin-β1 signaling can increase the expression of chemokine receptors; allowing a positive feedback loop that promotes prostate cancer progression [26]. Further studies are needed to investigate if CCR5 expression is increased by integrin-β1 signaling.

In addition to its association with cell invasion, integrin-β1 expression is associated with tumor growth via increased IGF1R signaling [12]. Our data showed that CCL4 increased tumor volume and as shown by increased pH3 staining. Therefore, we can conclude that cell proliferation was promoted *in vivo* with CCL4 treatment [27]. Further studies are necessary to verify if IGF1R signaling was increased.

We also found that CCL4 treatment increased angiogenesis. As shown by immunostaining, CD31 and desmin expression was increased with CCL4 treatment. Increased expression of CD31 and desmin is associated with increased angiogenesis and invasive carcinoma [28]. Furthermore, CD31 expression in blood vessels increases integrin-β1 signaling and promotes T-cell, eosinophil, and macrophage infiltration [29]. In addition, integrin-β1 has been shown to be involved in the modulation of blood vessel formation by promoting endothelial cell proliferation, cell migration, and vascular patterning [30]. This suggests an explanation for the increased metastatic potential in CCL4 treated tumors.

As shown by PCR array analysis, CCL4 altered the expression of several genes involved in tumor metastasis and the PI3K pathway. Given that CCR5 is a GPCR, we expected the increased PRKCA mRNA expression obtained though qRT-PCR. PRKCA is activated downstream of GPCRs [31] and is responsible for the activation of the MAPK pathway, which is associated with increased cell proliferation [32]. Additionally, the over expression of PI3KA suggests activation of the PI3K pathway downstream of GPCR. This pathway is associated with deregulated cell cycle, increased cell proliferation, and invasion [33]. The over expression of this gene may aid in explaining the increased tumor volume and metastatic potential observed *in vivo*. The ribosomal protein S6 kinase polypeptide 1 (RPS6KA1) gene, also up-regulated in prostate cancer tumors treated with CCL4, has been found to be activated downstream of the MAPK pathway and involved in the promotion of mRNA translation into protein [34]. FOS, another gene up-regulated in prostate cancer tumors treated with CCL4, can be found downstream of the

MAPK pathway. It is a constituent of the AP-1 transcription factor, which promotes cell proliferation and increases the expression of proinflammatory cytokines [35]. CXCR2, also up-regulated in prostate cancer tumors treated with CCL4, is likewise a GPCR that binds interleukin 8 or CXCL8; a pro inflammatory cytokine associated with aggressive prostate cancer and regulated by AP-1 [36]. The MMP10 gene, which was likewise over expressed in prostate cancer tumors treated with CCL4, is associated with high grade prostate cancer and increased angiogenesis [37]. This correlates with the increased angiogenesis observed *in vivo.* Further studies are needed to explain the increased expression of ITGA7 and BRMS1, since both genes are associated with metastasis suppression.

In this present study, we showed that CCL4 plays a critical role in prostate cancer by increasing cell motility, invasion, tumor growth, angiogenesis, and metastatic potential. With these data, we propose a mechanism of action in which CCL4 binds its receptor CCR5 and activates PRKCA, promoting FAK phosphorylation and, hence, integrin over expression. Additionally, we have activation of the PI3K and MAPK pathways downstream of PRKCA. These pathways promote the internalization of FOS to form the AP-1 complex (which in turn promotes cell growth and survival (**Figure 8**)). This suggests that CCL4 is important in promoting negative outcomes such as metastasis and recurrence in prostate cancer. CCL4 expression should therefore be considered as part of the combinatorial assessment to better identify and classify patients at risk of developing prostate cancer recurrence.

Acknowledgements

This work was funded by NIH grant K01 CA140711, MBRS-RISE grant R25GM061838, MARC grant 5T34GM007821-35 and Institutional funds from the University of Puerto Rico Comprehensive Cancer Center and the School of Pharmacy. We thank Némesis Merly, and Yarimar Padín for technical assistance. We also thank Dr. Navone and Dr. Montaner for revision of the manuscript.

Disclosure of conflict of interest

None.

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