

# Expression and Functional Role of CCR9 in Prostate Cancer Cell Migration and Invasion

Shailesh Singh,<sup>1</sup> Udai P. Singh,<sup>1</sup>  
Jonathan K. Stiles,<sup>1</sup> William E. Grizzle,<sup>2</sup> and  
James W. Lillard, Jr.<sup>1,2</sup>

<sup>1</sup>Morehouse School of Medicine, Atlanta, Georgia; and <sup>2</sup>University of Alabama at Birmingham, Birmingham, Alabama

## ABSTRACT

**Purpose:** Metastasis is responsible for most cancer-related deaths; hence, therapies designed to minimize metastasis are greatly needed. The precise cellular and molecular mechanisms used by cancer cells for metastasis are not fully understood; however, the metastatic spread of neoplastic cells is probably related to the ability of these cells to migrate, invade, home, and survive locally. The migration of tumor cells shares many similarities with leukocyte trafficking, which is regulated by chemokine receptor–ligand interactions. The current study evaluates the molecular mechanisms of CCL25 and CCR9 in prostate cancer cell migration and invasion.

**Experimental Design:** In the current study, real-time quantitative polymerase chain reaction, flow cytometry analysis, and *in vitro* migration as well as invasion chamber analysis (with and without antibody-mediated inhibition) were used to ascertain the biological and functional significance of CCR9 expression by normal prostatic epithelial cells (PrEC) or prostate cancer cell lines (LNCaP-10995 and PC3).

**Results:** We report that functional CCR9 is highly expressed by LNCaP cells and modestly, yet significantly, expressed by PC3 cells when compared with PrEC cells. Neutralization of CCL25–CCR9 interactions impaired the migration and invasion potential of the LNCaP and PC3 cell lines. CCL25 differentially modulated the expression of collagenase-1 or matrix metalloproteinase (MMP)-1, collagenase-3 (MMP-13), stromalysin-2 (MMP-10), stromalysin-3 (MMP-11), and gelatinase-A (MMP-2), but not MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, or MMP-14 in prostate cancer cells.

**Conclusions:** These studies suggest that the expression and activation of CCR9 affect cancer cell migration, invasion, and MMP expression, which together may affect prostate cancer metastasis.

## INTRODUCTION

The spread of neoplastic cells and their survival in lymph nodes and distant organs lead to a clinically incurable disease with a poor prognosis. Despite the obvious importance of metastasis, this process remains incompletely characterized at both the cellular and molecular levels (1). Many factors have been implicated in the process of metastasis, but the precise mechanisms for the directional migration of malignant cells into different organs are unknown (2–4). Chemokines, a superfamily of small cytokine-like proteins, induce cytoskeletal rearrangement through binding corresponding G-protein–coupled receptors, adhesion to endothelial cells, and directional migration (5–7).

It has been shown that CXCL12–CXCR4 interactions may play a significant role in the metastasis of prostate cancer to bone (8). It has also been demonstrated that normal breast tissue expresses low amounts of CXCR4, whereas neoplastic breast tissue expresses higher levels of CXCR4 (9). CXCL12 and CXCR4 have been implicated in the pathogenesis and progression of breast cancer, Burkitt's lymphoma, leukemias, and neuroblastomas (9–12). Recently, it has been shown that CXCR4 is expressed by prostate cancer cell lines (PC3, DU145 and LNCaP; ref. 13).

Unfortunately, CXCR4 interactions alone do not completely explain the pattern of metastasis of prostate cancer, nor do they explain the potential for neoplastic cells to migrate and invade other tissues. We have tested the hypothesis that prostate carcinomas use multiple chemokine-dependent mechanisms for migration and invasion. In this study, we have shown that prostate cancer cell lines express high levels of CCR9 when compared with prostatic epithelial cells.

CCR9 expression also correlates with the enhanced migration and invasion of prostate cancer cells when induced by the ligand of CCR9, CCL25. Inhibition of CCL25–CCR9 interactions effectively reduced the migration and invasive competence of LNCaP and PC3 cells. Additionally, we show that CCL25 differentially modulates matrix metalloproteinase (MMP) expression of LNCaP and PC3 cell lines. Taken together, these studies suggest that the expression of functional CCR9 may facilitate tumor cell migration and invasion.

## MATERIALS AND METHODS

**Cell Lines and Cell Culture.** PC3 and LNCaP cell lines were obtained from American Type Culture Collection (Manassas, VA). Normal prostatic epithelial PrEC cells were obtained from Clonetics-Biowhittaker (Walkersville, MD) and cultured in PrEMB medium (Clonetics-Biowhittaker). PC3 cells were cultured at 37°C with 5% CO<sub>2</sub> in Ham's F12K medium with 2

Received 2/10/04; revised 8/10/04; accepted 9/10/04.

**Grant support:** Department of the Army United States Army Medical Research Acquisition Activity grant DAMD17-01-1-0079 and USPHS National Institutes of Health grants RR03034, GM08248, and CA92078. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** James W. Lillard, Jr., Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, 720 Westview Drive, SW, Atlanta, GA 30310. Phone: 404-752-1581; Fax: 404-752-1179; E-mail: lillard@msm.edu.

©2004 American Association for Cancer Research.

mmol/L L-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate (American Type Culture Collection) with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO). After five passages in Ham's F12K media, PC3 cells were switched to RPMI 1640 at 37°C and 5% CO<sub>2</sub> with 10% FBS. LNCaP-10995 cells were cultured in RPMI 1640 with 10% FBS. Before migration and invasion studies, cells were cultured for 24 hours in RPMI 1640 with 2% FBS.

**RNA Isolation and Gene Expression Analysis.** Human mRNA sequences for CCR9, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, membrane type 1 (MT1)-MMP (MMP-14), and 18S rRNA were obtained from National Institutes of Health-National Center for Biotechnology Information GenBank database accession numbers XM003251, NM002421, NM004530, NM002422, XM017384, NM002424, NM004994, NM002425, NM005940, NM002426, NM002427, NM004995, and X00686.1, respectively. These sequences were then used to design primers for reverse transcription-polymerase chain reaction (RT-PCR) analysis, which generated amplicons of 162, 83, 95, 155, 169, 86, 79, 94, 107, 117, 176, 172, and 149 bp in size for CCR9, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MT1-MMP, and 18S rRNA, respectively. Primers were designed using the Primer 3 software program from the Whitehead Institute at the Massachusetts Institute of Technology (Boston, MA). Thermodynamic analysis of the primers was conducted using the following computer programs: Primer Premier (Integrated DNA Technologies, Coralville, IA), and MIT Primer III (Boston, MA). The resulting primer sets for CCR9, MMP-1, MMP-3, MMP-10, MMP-11, MMP-13, and MT1-MMP (MMP-14) were compared against the entire human genome to confirm specificity and ensure that the primers flanked mRNA splicing regions.

Total RNA was isolated from untreated and CCL25-treated prostate cancer cells using Tri-reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocols. Potential genomic DNA contamination was removed from these samples by treatment with RNase-free DNase (Invitrogen, San Diego, CA). RNA was precipitated and resuspended in RNA secure (Ambion, Austin, TX). Complementary DNA was generated by reverse transcribing 1.5 µg of total RNA using TaqMan reverse transcription reagent (Applied Biosystems, Foster City, CA), according to the manufacturer's protocols, and amplified with specific cDNA primers using SYBR Green polymerase chain reaction master mix reagents (Applied Biosystems). The number of copies (>5) of mRNA relative to 18S rRNA copies of these targets was evaluated by RT-PCR analysis using the Bio-Rad Icyler and software (Hercules, CA).

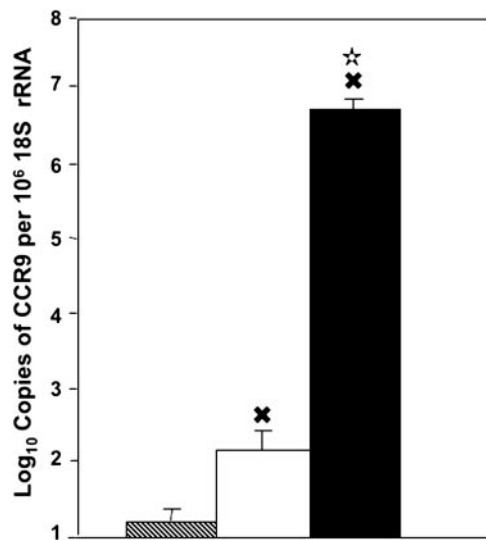
**Flow Cytometry Analysis of CCR9.** Phycoerythrin (PE)-conjugated mouse antihuman CCR9 (clone 12G5) antibody was purchased from R&D Systems (Minneapolis, MN). PE-conjugated mouse IgG2a monoclonal immunoglobulin isotype control was purchased from PharMingen (San Diego, CA). Prostate cancer cells and normal prostatic epithelial cells were washed three times in PBS [supplemented with 0.5% bovine serum albumin (BSA)] and treated with 1.0 µg of Fc Block (PharMingen) per 10<sup>5</sup> cells for 15 minutes at room temperature. Fc-blocked prostate cancer cells and normal prostatic epithelial cells were stained with 1.0 µg of PE-conjugated mouse antihuman CCR9 or PE-conjugated mouse IgG2a isotype control

antibody per 10<sup>5</sup> cells at 4°C for 40 minutes. Subsequently, the cells were washed with 1.0 mL of fluorescence-activated cell-sorting buffer (1% BSA in PBS) to remove unbound antibodies. Next, labeled cells were fixed in 500 µL of 2% paraformaldehyde solution, and 10<sup>5</sup> cells were analyzed by flow cytometry using a FACScan flow cytometer and Cellquest software (BD PharMingen, San Diego, CA).

**Migration and Invasion Assays.** CCL25 was obtained from PeproTech (Rocky Hill, NJ). Unlabeled mouse antihuman CCR9 (clone 112509) antibodies were purchased from R&D Systems. Migration and invasion studies were performed using BD BioCoat migration or Matrigel invasion chambers (Becton Dickinson Labware, Bedford, MA), respectively. RPMI 1640 was added to the interior of the bottom and top chamber of inserts and allowed to hydrate for 2 hours at 37°C with 5% CO<sub>2</sub>. CCL25 or albumin at 100 ng/mL was added to the bottom chamber. Next, 10<sup>4</sup> cells were added to the top chamber of inserts. To block chemokine receptor interaction, 1.0 µg/mL mouse antihuman CCR9 (R&D Systems) was added to the top chamber of Matrigel or control inserts and incubated overnight at 37°C and 5% CO<sub>2</sub>. After incubation, cells at the bottom surface of the insert were fixed with 100% methanol for 2 minutes, stained for 2 minutes in 1% toluidine blue (Sigma) supplemented with 1% borax (Sigma), and rinsed twice with deionized water (distilled H<sub>2</sub>O). Cells were counted by microscopy at ×40 magnification.

**Active Matrix Metalloproteinase Protein Detection.** Prostate cancer (LNCaP and PC3) and PrEC cells (10<sup>5</sup>) were seeded in 24-well plates, and conditioned media were collected from CCL25 (100 ng/mL)-treated or untreated cells. Active collagenases (MMP-1 and MMP-13), gelatinase A and B (MMP-2 and MMP-9), and stromelysin 2 (MMP-10) levels were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems), according to the manufacturer's protocols. Similarly, an ELISA method for active stromelysin-3 (MMP-11) detection was developed to quantify active MMP-11 expression in conditioned media. Briefly, 96-well Falcon ELISA plates (Fisher Scientific, Pittsburgh, PA) were coated with 100 µL of 5 µg/mL rabbit antihuman (hinge region) MMP-11 antibody (Triple Point Biologicals, Forest Grove, OR) overnight at 4°C and blocked with 200 µL of 2% BSA (Sigma) in PBS for 2 hours at room temperature. Serial dilutions of experimental samples and purified human MMP-11 (Triple Point Biologicals), as standard, were added and incubated overnight at 4°C. Plates were washed four times with PBS containing 0.05% Tween 20. Mouse antihuman (active domain) MMP-11 antibody (Calbiochem, La Jolla, CA) diluted in PBS (1:3,000) was added to each well and incubated at room temperature for 2 hours. After washing four times with PBS containing 0.05% Tween 20, 100 µL of goat antimouse IgG-horseradish peroxidase (1:3,000; Southern Biotechnology Associates, Birmingham, AL) were added and incubated at room temperature for 1 hour. After three washes, tetramethylbenzidine substrate (eBioscience, San Diego, CA) was added, allowed to react, and stopped with 50 µL of 2% H<sub>2</sub>SO<sub>4</sub>, and absorbance was read at 450 nm.

**Statistics.** The data are expressed as the mean ± SE and compared using a two-tailed Student's *t* test or an unpaired Mann-Whitney *U* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Inc., Berke-



**Fig. 1** CCR9 mRNA expressed by prostate cancer and normal epithelial cells. Total RNA was isolated from prostate cancer cell lines LNCaP (■) and PC3 (□) as well as from normal PrEC cells (▨). Quantitative RT-PCR analysis of CCR9 mRNA expression was performed in triplicate. The copies of transcripts are expressed relative to actual copies of 18S rRNA  $\pm$  SE. Asterisks indicate statistical significance ( $P < 0.05$ ) between normal and cancer cells (x) and between LNCaP and PC3 cells (☆).

ley, CA) for Macintosh computers and were considered statistically significant if  $P$  values were  $<0.05$ . When MMP levels were below the detectable limit of the ELISA, values were recorded as one-half of the lower detection limit (e.g., 50 pg/mL for MMP-13) for statistical analysis.

## RESULTS

### CCR9 Messenger RNA Expression by Prostate Cells.

PrEC and prostate cancer cell lines previously isolated from bone (PC3) or lymph node (LNCaP) metastases expressed CCR9. However, significantly higher levels of CCR9 were observed in the malignant cell lines when compared with PrEC (Fig. 1). Interestingly, LNCaP cells expressed significantly more copies of CCR9 transcripts than did the PC3 cells. Protein expression of CCR9 by LNCaP, PC3, and PrEC cells was also confirmed by flow cytometry. Consistent with RT-PCR analysis, CCR9 was highly expressed by LNCaP  $>$  PC3  $\gg$  PrEC cells (Fig. 2).

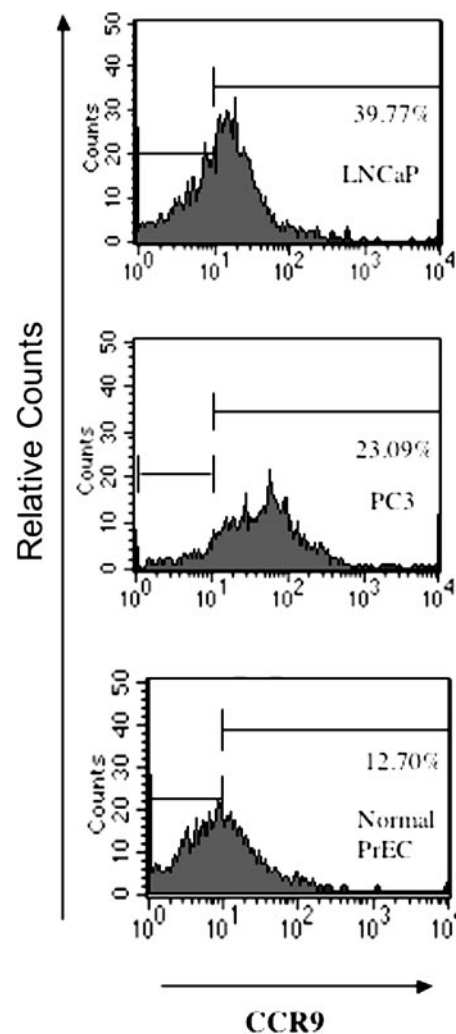
### Migration and Invasion.

The functional significance of CCR9 expression was suggested by the ability of LNCaP and PC3 cells, but not PrEC cells, to migrate toward chemotactic gradients of CCL25 (Fig. 3). The number of LNCaP and PC3 cells that migrated in response to CCL25 was significantly higher than that for unexposed cell lines. This chemokine receptor-dependent chemotaxis was neutralized by treatment with anti-CCR9 antibodies. Additionally, the proportions of LNCaP and PC3 cells that were invasive were accessed, after CCL25 induction. LNCaP cells, which expressed the highest levels of CCR9, were more invasive than PC3 cells when stimulated with CCL25 (Fig. 4). Taken together, both LNCaP and PC3 cells migrate and invade in response to CCL25. In

contrast to the *in vivo* metastatic behavior of these cancer cell lines (14, 15), CCL25 engagement induced higher migration and invasion potentials by LNCaP cells (expressing higher CCR9) than by PC3 cells.

### CCL25-Induced Matrix Metalloproteinase Expression.

The Matrigel system used for the invasion assays consisted of laminin, collagen IV, and entactin; hence, MMP-1, MMP-3, MMP-8, MMP-10, MMP-11, and/or MMP-13 are needed to optimally digest these components. To determine whether the associated increase in cancer cell invasion was due to higher MMP expression, the change in MMP expression by untreated and CCL25-treated LNCaP, PC3, or PrEC cells was characterized by RT-PCR and protein analysis. Neither untreated nor CCL25-treated PrEC, LNCaP, or PC3 cells expressed detectable levels of MMP-3, MMP-7, MMP-8, MMP-12, or MMP-14



**Fig. 2** Changes in CCR9-positive prostate cell populations. In confirmation of RT-PCR analysis of CCR9 mRNA expression, LNCaP, PC3, and normal PrEC prostatic cells were stained with PE-conjugated isotype control or anti-CCR9 monoclonal antibodies and quantified, in triplicate, by flow cytometry. The percentage of positive CCR9 surface expression by LNCaP, PC3, or PrEC cells was repeated three times; representative histograms are shown.

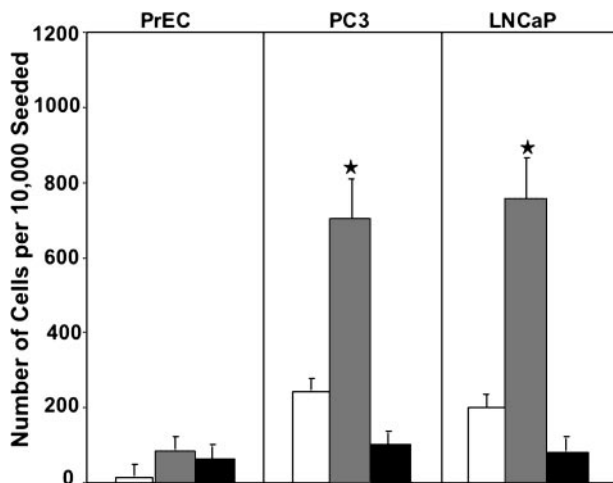


Fig. 3 CCR9-mediated prostate cancer cell migration. LNCaP, PC3, and normal prostatic epithelial PrEC cells were tested for their ability to migrate to 0 ng/mL CCL25 (□), 100 ng/mL CCL25 (■), or 100 ng/mL of CCL25 + 1 µg/mL anti-CCR9 monoclonal antibody (■) in the lower chamber. The number of cells ( $\pm$ SE) that migrated to CCL25, of the initial  $10^4$  cells used to seed the migration chamber, are shown with asterisks that indicate significant differences ( $P < 0.05$ ) between no additions and CCL25-treated cells (\*).

mRNA. Whereas PrEC cells expressed low levels of MMP-1, MMP-2, and MMP-9 mRNA (Table 1) and protein (Fig. 5), transcripts of other MMPs were below the level of detection. MMP-10, MMP-11, and MMP-13 mRNAs were expressed by untreated LNCaP and PC3 cells, but not by PrEC cells. Untreated LNCaP and PC3 cells secreted significantly higher levels of active MMP-13 and moderate to high levels of active MMP-10 and MMP-11 compared with PrEC cells. Specifically, unstimulated PC3 cells produced significantly higher levels of MMP-10 compared with LNCaP and PrEC cells, whereas similarly treated LNCaP cells generated substantially higher levels of MMP-11 compared with PC3 and PrEC cells. Hence, untreated PC3 cells had a higher capacity to generate MMP-1, MMP-10, and MMP-13 mRNA and active protein, whereas untreated LNCaP cells had a greater propensity to produce high levels of MMP-9, MMP-11, and MMP-13 than PrEC cells.

After CCL25 induction, the transcript for MMP-1 was newly expressed by LNCaP cells. Similarly, PC3 cells expressed MMP-11 mRNA and LNCaP cells expressed MMP-1 mRNA only after CCL25 stimulation. Correspondingly, active MMP-1 and MMP-11 were significantly produced by both LNCaP and PC3 cells, but not PrEC cells, after CCL25 stimulation. Interestingly, a significant increase in the level of MMP-13 mRNA expression by PC3 cells was noted after CCL25 treatment. This increase coincided with MMP-13 secretion by similar cells; in fact, both LNCaP and PC3 cells produced significant levels of active MMP-13 after CCL25 treatment compared with untreated cells.

Whereas gelatinase-A (MMP-2) and gelatinase-B (MMP-9) were not needed to degrade the Matrigel system used for our cell invasion assays, these gelatinases are important for prostate cancer cell metastasis (16, 17). MMP-2 and MMP-9 mRNAs were expressed at low levels by untreated PrEC,

LNCaP, and PC3 cells. This trend corresponded to modest MMP-2 and MMP-9 active protein expression by PrEC, LNCaP, and PC3 cells. Interestingly, the level of MMP-2, but not MMP-9, mRNA expression by CCL25-treated LNCaP and PC3 cells was significantly higher than that in similarly treated normal prostatic epithelial cells. This trend partially carried over to active gelatinase expression by cancer cells; CCL25 treatment dramatically increased active MMP-2 and MMP-9 production by LNCaP and PC3 cells. Taken together, these results indicate that CCL25 plays a significant role in enhancing MMP expression by prostate cancer cells.

## DISCUSSION

Most prostate cancer-related deaths are not the result of primary tumor growth but are rather caused by the spread of cancer to other organs. Despite the clinical importance of metastasis, much remains to be learned about the process of metastasis. Many molecular factors have been identified that may contribute to metastasis. In this regard, chemokines are involved in the chemotaxis of lymphocytes and may cause cancer cells to “home” to specific secondary sites to promote organ-specific metastasis (18).

We hypothesized that chemokines mediate prostate cancer migration and invasion. To address this hypothesis, we first identified the expression of chemokine receptors by both prostate cancer cell lines (isolated from lymph node and bone) and normal prostatic epithelial cells. To our knowledge, for the first time, *in vitro* migration and invasion studies were used to test the biological and functional significance of CCR9 expression. Whereas these receptors were expressed in normal prostatic epithelial cells, the levels of their expression were significantly higher in prostate cancer cell lines than in normal prostatic epithelial cells. LNCaP cells, which expressed higher levels of

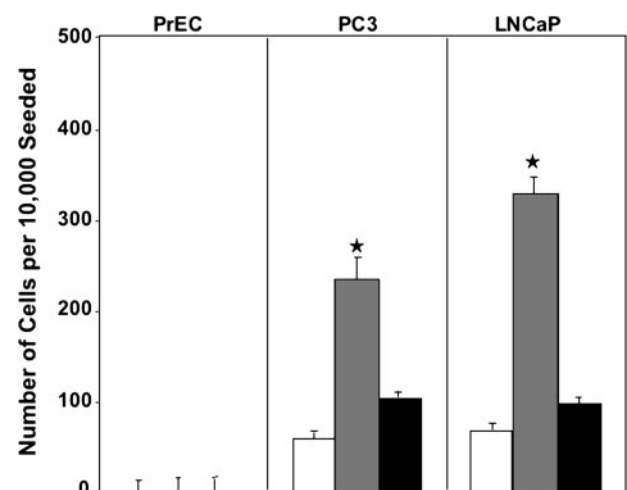


Fig. 4 CCR9-mediated prostate cancer cell invasion. LNCaP, PC3, and PrEC cells were tested for their ability to invade or translocate across a Matrigel matrix in response to chemotactic gradients of 0 ng/mL CCL25 (□), 100 ng/mL CCL25 (■), or 100 ng/mL CCL25 + 1 µg/mL anti-CCR9 monoclonal antibody (■) in the lower chamber. Asterisks indicate significant differences ( $P < 0.05$ ) between no additions and CCL25-treated cells (\*).

**Table 1** MMP expression by untreated or CCL25-treated normal prostatic epithelial cells (PrEC) and prostate cancer cell lines (LNCaP and PC3).

MMP	PrEC		LNCaP		PC3	
	Untreated	CCL25 treated	Untreated	CCL25 treated	Untreated	CCL25 treated
MMP-1	$1.50 \times 10^1$	$1.66 \times 10^2$	BD	$5.23 \times 10^4$ *†	$1.20 \times 10^5$ *	$1.40 \times 10^5$ *
MMP-2	$1.05 \times 10^2$	$1.7 \times 10^2$	$1.50 \times 10^2$	$3.09 \times 10^4$ *†	$1.81 \times 10^2$	$5.03 \times 10^4$ *†
MMP-3	BD	BD	BD	BD	BD	BD
MMP-7	BD	BD	BD	BD	BD	BD
MMP-8	BD	BD	BD	BD	BD	BD
MMP-9	$1.80 \times 10^1$	$3.20 \times 10^1$	$2.85 \times 10^2$	$2.85 \times 10^2$	$1.88 \times 10^2$	$5.53 \times 10^2$
MMP-10	BD	BD	$1.05 \times 10^1$ *	$1.53 \times 10^1$ *	$4.00 \times 10^3$ *	$1.40 \times 10^4$ *
MMP-11	BD	BD	$2.00 \times 10^3$ *	$1.40 \times 10^3$ *	BD	$1.70 \times 10^3$ *†
MMP-12	BD	BD	BD	BD	BD	BD
MMP-13	BD	BD	$2.90 \times 10^2$ *	$4.32 \times 10^2$ *	$6.61 \times 10^2$ *	$1.90 \times 10^5$ *†
MMP-14	BD	BD	BD	BD	BD	BD

NOTE. The number of copies of MMP mRNA per  $10^6$  copies of 18S rRNA was determined by RT-PCR analysis, which was capable of detecting >5 copies of mRNA. Hence, copy numbers < 5 are below detection.

Abbreviation: BD, below detection.

\* Significantly higher ( $P < 0.05$ ) expression of MMP mRNA copies between similarly treated normal prostatic epithelial cells (PrEC) and prostate cancer cells (LNCaP and PC3).

† A significant ( $P < 0.05$ ) increase (or decrease) in MMP expression of the CCL25-treated cell line compared with the untreated cell line.

CCR9 than did PC3 cells, also had the greatest migration and invasion profile toward CCL25, in contrast to their *in vivo* behavior (14, 15). CCR9 blockade, with specific antibodies, remarkably impaired the migration and invasive potential of these cancer cells, which indicates that these responses were dependent on chemokine and chemokine–receptor interactions.

Recently, it has been shown that CXCR4 is expressed by prostate cancer cell lines (PC3, DU145, and LNCaP; ref. 13). Additionally, anti-CXCR4 antibodies block the metastatic spread of breast tumors to the lungs and lymph nodes (9). Unlike CXCR4, very little is known about the expression of CCR9 by carcinomas or normal epithelium. However, it has been shown that CCR9 is expressed by the thymus, lymph nodes, and spleen by hematopoietic stem cells and (mature and immature) T cells (19, 20). CCL25, the receptor for CCR9, is also an activator of dendritic cells and thymocytes, which implicates this chemokine in T-cell development (21).

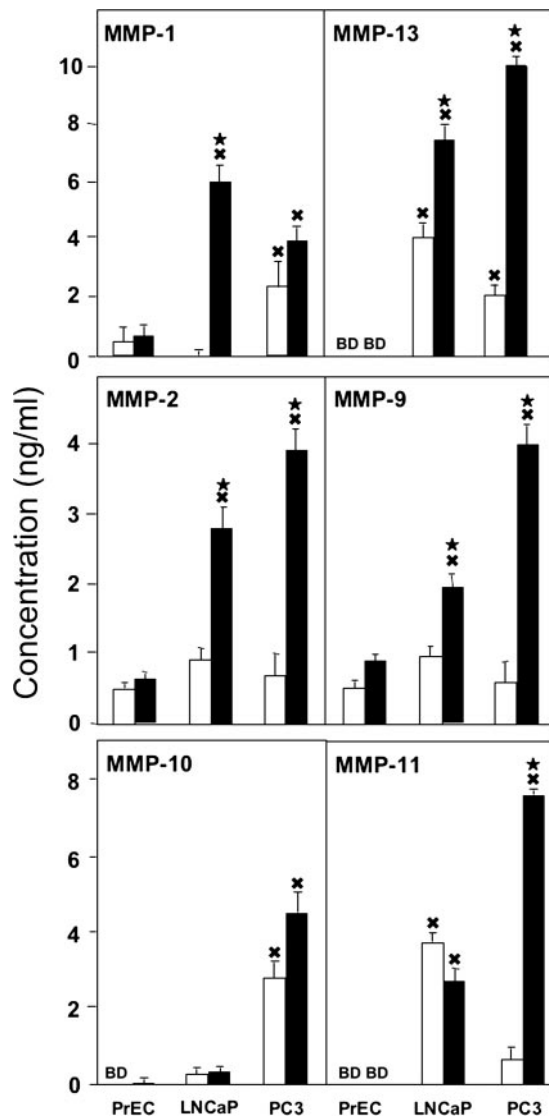
In this study, we have shown for the first time that CCR9 is expressed by prostate cancer cell lines. Moreover, CCR9 expression plays a functional role in migration as well as invasion. We hypothesized that prostate cancer cells with the ability to metastasize to lymph nodes initially might express CCR9. The likelihood of such a CCR9-dependent event is demonstrated by the inability of anti-CCR9 antibody-treated cells to migrate or invade. Selective expression of CCL25 by lymph nodes (22) and the expression of CCR9 by prostate cancer cell lines suggest that CCR9–CCL25 interactions may play a significant role in prostate cancer metastasis. Interestingly, LNCaP expresses higher levels of CCR9 compared with PC3 cells, and this lymph node-derived cell line has higher migration and invasion potentials when stimulated by CCL25, compared with PC3 cells.

Metastasis is enhanced by a highly selective competition favoring the survival of a relatively small subpopulation of metastatic cells (23). Indeed, early in the growth of a primary tumor mass, the metastatic subpopulation of cancer cells is a small component of the tumor (24). Less than 0.01% of the malignant cells entering circulation actually form metastatic

colonies (25). Therefore, metastatic cells must survive in the circulation to and at the metastatic site. Intriguingly, previous studies have shown that CCR9–CCL25 interactions play anti-apoptotic roles under certain biological conditions (26). Therefore, the higher expression of this receptor by prostate cancer cell lines and the presence of CCL25 at potential sites of metastasis may not only attract circulating neoplastic cells but may also enhance their survival by inhibiting apoptosis.

MMPs have been widely studied due to their role in tumor metastasis (27). In this study, we have quantified the modulation of MMP expression by prostate cancer cells after CCR9 activation. MMP-1 expression has been correlated with higher mortality rates in colorectal and esophageal cancers (28, 29). In bladder cancer, it has been shown that tumors that invade adjacent muscle display significantly higher collagenase activity than their noninvasive counterparts (30). Higher urinary MMP-1 expression has also been correlated with disease progression and poor survival in bladder cancer (31). For the first time, we demonstrate that the expression of collagenases MMP-1 and MMP-13 by LNCaP and PC3 cells can be modulated by CCL25–CCR9 interactions. These collagenases degrade several native fibrillar collagens, including types I, II, and III, resulting in their cleavage. Hence, the ability of these chemokines to modulate MMP-1 and MMP-13 mRNA and active protein expression may aid in selective cancer cell migration and, hence, cancer dissemination.

Stromelysins are expressed predominantly by normal epithelial cells but are also found in carcinomas. They can degrade a broad range of substrates, including type IV, V, IX, and X collagens, fibronectins, laminin, elastin, gelatin, and proteoglycan core proteins (32). MMP-10 expression by PC3 cells was elevated after CCL25 activation when compared with similarly treated LNCaP cells. Levels of MMP-11 expression were greater in untreated LNCaP cells when compared with untreated PC3 cells; nevertheless, MMP-10 and MMP-11 mRNA and active protein levels were significantly increased by PC3 cells treated with CCL25. Previous studies have reported higher



**Fig. 5** CCL25-induced active MMP expression by LNCaP, PC3, and PrEC cells. Cells were cultured for 24 hours with (■) or without (□) 100 ng/mL CCL25. MMP-1, MMP-2, MMP-3, MMP-10, and MMP-11 protein levels, in cultured supernatants, were determined by ELISA. x, significantly higher ( $P < 0.05$ ) expression of MMP between similarly treated normal prostatic epithelial cells (PrEC) and prostate cancer cells (LNCaP and PC3). \*, a significant ( $P < 0.05$ ) increase (or decrease) in MMP secretion by a CCL25-treated cell line compared with the untreated cell line.

levels of stromelysin expression in prostate cancer when compared with control tissue (33). Higher levels of MMP-10 expression in head and neck carcinomas relative to matching adjacent tissues have also been observed (34). Whereas MMP-3 expression was not affected in our studies, we show that PC3 and LNCaP differentially express MMP-10 and MMP-11 and that CCL25 increases the expression of MMP-11 by PC3 cells, further suggesting a regulatory role of CCL25 in metastasis.

MMP-2 and MMP-9 belong to the gelatinase family of MMPs. MMP-2 overexpression has been reported in many

neoplasms including ovarian (35, 36), urothelial (37, 38), gastric (39), breast (40), and cervical cancer (41). In this study, we observed MMP-2 expression by normal prostatic epithelial cells and prostate cancer cell lines; however, expression of MMP-2 was higher in prostate cancer cell lines when compared with normal prostatic epithelial cells. Increased levels of MMP-2 mRNA and active protein expression by prostate cancer cells were also observed after CCL25 induction. Studies have also shown that increased expression of MMP-2 by prostate cancer cells correlates with prostate cancer progression (42, 43).

Expression of MMP-9 has been shown to affect metastasis, angiogenesis, and tumor progression and is frequently up-regulated in cancer and adjacent tissue (44). Inhibition of MMP-9 expression by a ribozyme has been shown to inhibit metastasis by prostate cancer cell lines without affecting tumorigenesis (17). Our results show that CCL25–CCR9 induction increased active MMP-9 release, but not MMP-9 mRNA expression, by prostate cancer cells. Hence, these interactions might play an as yet undefined role in tumor cell metastasis.

Our current study suggests that differential expression of CCR9–CCL25 interactions may determine the migratory and invasive potential of prostate cancer cells. Expression of functional CCR9 by prostate cancer cells, together with selective ligand expression by bone marrow and lymph nodes, supports our hypothesis that the migration and invasion of metastatic cancer cells may be chemokine-mediated. Other tumor entities of hematopoietic and nonhematopoietic origin, including acute myeloid and lymphoblastic leukemia (45), chronic lymphocytic leukemia (11), non-Hodgkin's B-cell lymphoma (46), and pancreatic cancer (47), also express functionally active chemokine receptors, which might mediate cellular migration. Importantly, the effect of CCL25 on the expression of MMP also suggests that chemokines play important roles in invasion via the modulation of MMPs. This may be increasingly important when one considers that MMP-1, MMP-2, MMP-9, and MMP-13 cleave CXCL12, which results in a loss of binding to CXCR4 that is also expressed on prostate cancer cells (48). Hence, CCL25, which does not contain a MMP cleavage site, may play a major role in prostate cancer cell metastasis.

The metastatic spread of prostate cancer cells is affected by many variables, including (but not limited to) growth factors as well as chemoattractants, hormones, and the ability of tumor cells to survive on reaching the environment of the distal metastatic site. Whereas it is still not certain why PC3 cells are more metastatic than LNCaP cells, when using *in vivo* mouse models, our *in vitro* experiments suggest that the environment (*e.g.*, the presence of CCL25) around PC3 and LNCaP cells might allow these cells to metastasize at higher rates. For example, our results show that LNCaP cells, which express more CCR9 than PC3 cells, were more invasive than PC3 cells after CCL25 induction. It is tempting to speculate that prostate cancer cells (*e.g.*, LNCaP) that differentially express CCR9 might be more inclined to metastasize to distal sites that express CCL25, such as lymph nodes and spleen. Whereas additional studies will be necessary to evaluate the precise mechanisms of chemokine-mediated migration and invasion of prostate cancer cells, these studies show that chemokine and chemokine receptor interactions might play significant roles in prostate cancer metastasis.

Thus, CCL25- or CCR9-directed interventions may be of future benefit to combat this disease.

## ACKNOWLEDGMENTS

This work benefited from many fruitful conversations with investigators at the Morehouse School of Medicine and the Wallace Tumor Institute at the University of Alabama at Birmingham via the National Cancer Institute sponsored "Comprehensive Minority Institution/Cancer Center Partnership."

## REFERENCES

- Fidler IJ. Critical determinants of metastasis. *Semin Cancer Biol* 2002;12:89–96.
- Nicolson GL. Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. *Cancer Metastasis Rev* 1993;12:325–43.
- Wang JM, Deng X, Gong W, Su S. Chemokine and their role in tumor growth and metastasis. *J Immunol Methods* 1998;220:1–17.
- Yeatman TJ, Nicolson GL. Molecular basis of tumor progression: mechanisms of organ-specific tumor metastasis. *Semin Surg Oncol* 1993;9:256–63.
- Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–7.
- Campbell JJ, Butcher EC, Bowman EP. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing: developmental switches in chemokine response profiles during B cell differentiation and maturation. *Curr Opin Immunol* 2000;12:336–41.
- Butcher EC, Willima M, Youngman K, et al. Lymphocyte trafficking and regional immunity. *Adv Immunol* 1999;72:209–53.
- Taichman RS, Cooper C, Keller ET, et al. Use of stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* 2002;62:1832–7.
- Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature (Lond)* 2001;410:50–6.
- Sehgal A, Keener C, Boynton AL, et al. CXCR-4, a chemokine receptor, is overexpressed in and required for proliferation of glioblastoma tumor cells. *J Surg Oncol* 1998;69:99–104.
- Mohle R, Failenschmid C, Bautz F, Kanz L. Overexpression of the chemokine receptor CXCR4 in B cell chronic lymphocytic leukemia is associated with increased functional response to stromal cell-derived factor-1 (SDF-1). *Leukemia (Baltimore)* 1999;13:1954–9.
- Geminder H, Sagi-Assif O, Goldberg L, et al. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J Immunol* 2001;167:4747–57.
- Sun YX, Wang J, Shelburne CE, et al. Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo. *J Cell Biochem* 2003;89:462–73.
- Nemeth JA, Harb JF, Barroso UJ, et al. Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. *Cancer Res* 1999;59:1987–93.
- Thalmann GN, Anezinis PE, Chang SM, et al. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res* 1994;54:2577–81.
- Lichtinghagen R, Musholt PB, Lein M, et al. Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue. *Eur Urol* 2002;42:398–406.
- Sehgal G, Hua J, Bernhard EJ, et al. Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. *Am J Pathol* 1998;152:591–6.
- Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature (Lond)* 2001;411:375–9.
- Wright DE, Bowman EP, Wagers AJ, et al. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J Exp Med* 2002;195:1145–54.
- Zaballos A, Gutierrez J, Varona R, et al. Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. *J Immunol* 1999;162:5671–5.
- Vicari AP, Figueroa DJ, Hendrick JA, et al. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity* 1997;7:291–301.
- Carramolino L, Zaballos A, Kremer L, et al. Expression of CCR9 beta-chemokine receptor is modulated in thymocyte differentiation and is selectively maintained in CD8(+) T cells from secondary lymphoid organs. *Blood* 2001;97:850–7.
- Fidler IJ, Hart IR. Biological diversity in metastatic neoplasms: origins and implications. *Science (Wash DC)* 1982;217:998–1003.
- Kerbel RS. Growth dominance of the metastatic cancer cell: cellular and molecular aspects. *Adv Cancer Res* 1990;55:87–132.
- Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994;79:185–8.
- Youn BS, Yu KY, Oh J, et al. Role of the CC chemokine receptor 9/TECK interaction in apoptosis. *Apoptosis* 2002;7:271–6.
- Chambers AF, Matrisian M. Changing views of the role of matrix metalloproteinases in metastasis. *J Natl Cancer Inst (Bethesda)* 1997;89:1260–70.
- Murray GI, Duncan ME, O'Neil P, et al. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. *Nat Med* 1996;2:461–2.
- Murray GI, Duncan ME, O'Neil P, et al. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol* 1998;185:256–61.
- Wirl G, Frick J. Collagenase: a marker enzyme in human bladder cancer? *Urol Res* 1979;7:103–8.
- Durkan GC, Nutt JE, Tadjayabun PH, et al. Prognostic significance of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in voided urine samples from patients with transitional cell carcinoma of the bladder. *Clin Cancer Res* 2001;7:3450–6.
- Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;10:602–8.
- Lein M, Nowak L, Jung K, et al. Metalloproteinases (MMP-1, MMP-3) and their inhibitors (TIMP) in blood plasma of patients with prostate carcinoma. *Urologe A* 1998;37:377–81.
- Birkedal-Hansen B, Pavelic ZP, Gulckman JL, et al. MMP and TIMP gene expression in head and neck squamous cell carcinomas and adjacent tissues. *Oral Dis* 2000;6:376–82.
- Livermore DM, Afzal S. MT1-MMP and MMP-2 mRNA expression in human ovarian tumors: possible implications for the role of desmoplastic fibroblasts. *J Antimicrob Chemother* 1998;41:576–7.
- Davidson B, Goldberg I, Gotlieb WH, et al. High levels of MMP-2, MMP-9, MT1-MMP and TIMP-2 mRNA correlate with poor survival in ovarian carcinoma. *Clin Exp Metastasis* 1999;17:799–808.
- Davies B, Waxman J, Wasan H, et al. Levels of matrix metalloproteinases in bladder cancer correlate with tumor grade and invasion. *Cancer Res* 1993;53:5365–9.
- Kanayama H, Yokota K, Kurokawa Y, et al. Prognostic value of matrix metalloproteinase-2 and tissue inhibitor or metalloproteinase-2 expression in bladder cancer. *Cancer (Phila)* 1998;56:1359–66.
- Mori M, Mimori K, Shirachi T, et al. Analysis of MT1-MMP and MMP2 expression in human gastric cancer. *Int J Cancer* 1997;74:316–21.
- Pacheco MM, Mourao M, Mantovani EB, et al. Expression of gelatinase A and B, stromolysin-3 and matrylysin gene in breast carcinomas: clinico-pathological correlations. *Clin Exp Metastasis* 1998;16:577–85.
- Nuovo GJ, MacConnell PB, Simsir A, et al. Correlation of the in situ detection of polymerase chain reaction-amplified metalloproteinase complementary DNAs and their inhibitors with prognosis and cervical carcinoma. *Cancer Res* 1995;55:267–75.

42. Still K, Robson CN, Autzen P, et al. Localization and quantification of mRNA for matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in human benign and malignant prostatic tissue. *Prostate* 2000;42:18–25.
43. Stearns M, Stearns ME. Evidence for increased activated metalloproteinase 2 (MMP-2a) expression associated with human prostate cancer progression. *Oncol Res* 1996;8:69–75.
44. Himelstein BP, Canete-Soler R, Bernhard EJ, et al. Metalloproteinases in tumor progression: the contribution of MMP-9. *Invasion Metastasis* 1994;14:246–58.
45. Mohle R, Schittenhelm M, Failenschmid C, et al. Functional response of leukaemic blasts to stromal cell-derived factor-1 correlates with preferential expression of the chemokine receptor CXCR4 in acute myelomonocytic and lymphoblastic leukaemia. *Br J Haematol* 2000;110:563–72.
46. Arai J, Yasukawa M, Yakushijin Y, et al. Stromal cells in lymph nodes attract B-lymphoma cells via production of stromal cell-derived factor-1. *Eur J Haematol* 2000;64:323–32.
47. Koshiba T, Hosotani R, Miyamoto Y, et al. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor. *Clin Cancer Res* 2000;6:3530–5.
48. McQuibban GA, Butler GS, Gong JH, et al. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 2001;276:43503–8.