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C-C motif ligand 5 promotes migration of prostate cancer cells in the prostate cancer bone metastasis microenvironment

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Chemokines and their receptors have key roles in cancer progression. The present study investigated chemokine activity in the prostate cancer bone metastasis microenvironment. Growth and migration of human prostate cancer cells were assayed in cocultures with bone stromal cells. The migration of LNCaP cells significantly increased when co-cultured with bone stromal cells isolated from prostate cancer bone metastases. Cytokine array analysis of conditioned medium from bone stromal cell cultures identified CCL5 as a concentration-dependent promoter of LNCaP cell migration. The migration of LNCaP cells was suppressed when C-C motif ligand 5 (CCL5) neutralizing antibody was added to cocultures with bone stromal cells. Knockdown of androgen receptor with small interfering RNA increased the migration of LNCaP cells compared with control cells, and CCL5 did not promote the migration of androgen receptor knockdown LNCaP. Elevated CCL5 secretion in bone stromal cells from metastatic lesions induced prostate cancer cell migration by a mechanism consistent with CCL5 activity upstream of androgen receptor signaling.

KEYWORDS
androgen receptor, bone stromal cells, C-C motif ligand 5, migration, prostate cancer

1 | INTRODUCTION

Prostate cancer is among the most frequently diagnosed malignancies in men worldwide.[1] Skeletal metastases occur in approximately 80% of patients with advanced prostate cancer, for which there is no curative treatment.[2,3] Within the tumor microenvironment, chemokines and their receptors influence growth and metastasis.[4,5] For example, C-C motif ligand 2 (CCL2) is present in the microenvironment of many cancers and upregulated in prostate cancer cells.[6] We previously reported that bone stromal cells and SaOS-2 osteoblast-like cells promoted prostate cancer metastasis through activation of transforming growth factor (TGF)-β1,[7] and that CCL2, CCL4 and CCL22 were associated with prostate cancer progression.[8-11] Upregulation of CCL5, a high-affinity ligand of CCR5, can increase the aggressive potential of breast cancer cells[12] and increase the invasiveness of prostate cancer cells.[13,14] CCL5-associated increase in the size of prostate cancer stem cell populations and the metastatic potential of prostate cancer cells have been found to involve

androgen receptor (AR) signaling.[15,16] CCL5 has been shown to mediate prostate cancer progression, but the interactions of CCL5, bone stromal cells and prostate cancer cells in the microenvironment of bone metastases have not been determined.

In this study, bone stromal cells promoted prostate cancer progression through the secretion of CCL5. In vitro co-culture of bone stromal cells with prostate cancer cells induced the expression of CCL5, which promoted prostate cancer cell migration. CCL5 was found to have a key role in the progression of prostate cancer in the bone metastasis microenvironment.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Recombinant human CCL5 (278-RN-050CF) was purchased from R&D Systems (Minneapolis, Minnesota, USA). The primary antibodies used in western blot and immunohistochemistry (IHC) assays included rabbit anti-CCR5 (ab32048; Abcam, Cambridge, Massachusetts, USA), anti-AR (sc-7305; Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-GAPDH (NB300-221; Novus Biologicals, Littleton, Colorado, USA). MAB-678 antihuman CCL5 monoclonal neutralizing antibody was obtained from R&D Systems. Normal rabbit IgG (sc-2027) used as a control in the CCL5 neutralizing antibody assay was obtained from Santa Cruz Biotechnology.

2.2 | Cell culture

LNCaP and PC-3 human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Human SaOS-2 osteosarcoma cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). The LNCaP-SF androgen-independent LNCaP cell line was established in our laboratory by long-term subculture of the parental LNCaP cells in RPMI (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with 5% charcoal-stripped FBS (CCS; Hyclone, Logan, Utah, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, California, USA).[17] Bone-derived stromal cells (BDSC) and bone metastasis stromal cells (BmetSC) were obtained from normal eleventh ribs and left thigh bones with prostate cancer metastases as previously described.[7] The bone stromal cells were cultured in RPMI supplemented with 1% penicillin/streptomycin and 10% FBS (Sigma-Aldrich, St. Louis, Missouri, USA). LNCaP and PC-3 cells were cultured in RPMI supplemented with 5% FBS and 1% penicillin/streptomycin. SaOS-2 cells were cultured in RPMI supplemented with 1% penicillin/streptomycin and 5% FBS. All cells were kept at 37°C in a humidified atmosphere with 5% CO_2.

2.3 | Conditioned medium

Cells were seeded into 6-well culture plates and allowed to adhere overnight. The next day, the medium was aspirated and replaced with 2 mL of medium containing 5% FBS. The supernatant was removed 48 hours later, centrifuged at 300 *g* for 10 minutes and collected as conditioned medium (CM).

2.4 | Co-culture assays

Co-culture experiments were performed using Transwell cell culture inserts (Greiner Bio-One, Monroe, North Carolina) in 6-well or 24-well plates. Briefly, cells were added to the lower compartment and allowed to attach for 12-24 hours. For the migration assay, cells were placed into the upper compartment, the reagents were added to the lower compartment and the plates were cultured for 24-48 hours. For the proliferation assay, cells were placed into the lower compartment and allowed to attach for 12-24 hours. Co-cultured cells were then added to the upper compartment and the plates were cultured for 24-72 hours.

2.5 | Cell migration assay

The cell migration assay was performed using 8.0 μm Transwell inserts in 24-well culture plates. Prostate cancer cells were grown to 80% confluency in an appropriate medium. The cells were synchronized by starvation in serum-free medium containing 0.5% BSA for 16 hours at 37°C in a humidified atmosphere with 5% CO_2. Approximately 2-10 $\times$ 10^4 cells in 200 μL of culture medium supplemented with 1% FBS (0.1% FBS for PC-3 cells) were placed in the upper compartment. The lower compartment was filled with 600 μL of medium containing 1% FBS (2.5% FBS for PC-3 cells). The cells were allowed to attach for 2 hours, and then the lower compartment medium was replaced with 600 μL of medium containing 5% FBS with or without CCL5, or CM, or co-cultured cells, after washing the wells twice with PBS. The cells on the upper surface of the Transwell filter were removed carefully with a cotton swab and those on the lower surface were fixed with 4% paraformaldehyde for 10 minutes, stained with 0.1% crystal violet for 15 minutes, and photographed. The crystal violet dye retained on the filters was extracted into 33% acetic acid. Cell migration was measured by reading the absorbance at 595 nm with correction at 450 nm on a microplate reader, or microscopically assessed by counting stained cells visually. Statistical analysis was performed using Student's *t*-test.

For the anti-CCL5 neutralizing antibody procedures, cancer cells were seeded in the upper Transwell compartment with 1% FBS and the lower compartment was filled with 5% FBS with or without anti-CCL5 antibody at a concentration of 2 μg/mL and/or BmetSC. The plates were incubated at 37°C and 5% CO_2 for 48 hours.

2.6 | Western blot assay

Cell lysates were prepared with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). The soluble lysate (5-10 μg) was mixed with LDS sample buffer and sample reducing agent and was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose

membranes. The membranes were blocked with 1% gelatin in 0.05% Tween in Tris-buffered saline for 1 hour at room temperature (RT) and then incubated overnight at 4°C with anti-CCR5 and anti-AR primary antibodies following the kit manufacturer's instructions. Membranes were washed 3 times before incubation with HRP-conjugated anti-rabbit secondary antibodies for 1 hour at RT. Protein loading was determined with an anti-GAPDH control antibody. Protein bands were read using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

2.7 | Human cytokine antibody array

Cytokines were assayed in CM collected from LNCaP, BDSC and BmetSC cell cultures using a Human Cytokine Array kit (ARY017, R&D Systems) following the manufacturer's instructions.

2.8 | ELISA

Human CCL5 secretion was measured in serum-free CM from LNCaP, LNCaP-SF, BDSC, BmetSC and co-culture CM from BDSC or BmetSC and LNCaP or LNCaP-SF using a Quantikine human ELISA kit (DRN00B, R&D Systems), following the manufacturer's instructions. Absorbance was measured at 450 nm and was corrected at 540 nm on a microplate reader. Statistical analysis was performed using Student's *t*-test.

2.9 | Knockdown of androgen receptor in LNCaP cells

Androgen receptor-specific small interfering (si)RNA, siAR1 (HSS100619) and siAR2 (HSS179972) were synthesized by Thermo Fisher Scientific. A validated nontargeting siRNA (Stealth RNAi siRNA Negative Control Med GC Duplex #2) was also purchased from Thermo Fisher Scientific. For AR knockdown, LNCaP cells were plated into 6-well plates at 1×10^5 cells/well before transfection with 10 n mol L^{-1} of siRNA for 24 hours. Total proteins were extracted at 0, 24, 48, 72 and 96 hours after transfection.

2.10 | Tissue microarray analysis

A human prostate cancer TMA (PR2085b) was purchased from US Biomed (Rockville, MD). A TMA plate contained 20 cores of normal prostate tissue, 184 cores of prostate cancer tissue, and 4 cores of urothelial cancer. IHC staining was performed with an EnVision Detection System Peroxidase/DAB+ Kit (K5007) following the manufacturer's protocol (Dako, Glostrup, Denmark) as previously described by Shin et al[18] Microwave antigen retrieval was performed in Dako Target Retrieval Solution for 10 minutes. The arrays were evaluated by a pathologist blinded to the experimental procedures, and the intensities of the stained epithelial cells were recorded as: −, negative; +, weak; ++, moderate; or +++, strong. Statistical analysis was performed using the χ^2-test for trend.

3 | RESULTS

3.1 | SaOS-2 cells promoted migration of androgen receptor-positive human prostate cancer cells; androgen receptor-positive human prostate cancer cells inhibited migration of SaOS-2 cells

As most prostate cancer bone metastases are osteoblastic, the SaOS-2 osteoblast-like cell line was used in the co-culture experiments. When the AR-positive LNCaP and LNCaP-SF cells were co-cultured with equal numbers of SaOS-2 cells, migration was significantly increased (Figure 1A,B). However, increasing the number of co-cultured SaOS-2 cells to twice that of the prostate cancer cells did not further promote the migration of LNCaP and LNCaP-SF cells (Figure 1A,B). Increase in the number of osteoblasts may, thus, not be needed to promote the migration of AR-positive metastatic prostate cancer cells within the tumor microenvironment. Co-culture with SaOS-2 cells did not increase the migration of AR-negative PC-3 cells (Figure 1C) or the proliferation of prostate cancer cells regardless of AR status for up to 72 hours (Figure 1D-F). AR-positive prostate cancer cells significantly inhibited the migration of SaOS-2 cells (Figure 1G), but AR-negative PC-3 cells did not influence SaOS-2 migration (Figure 1H). AR-positive prostate cancer cells may remain near, and promote changes in, osteoblasts. The study results are consistent with previous in vivo findings that AR-positive and AR-negative prostate cancer cells formed osteoblastic and osteolytic lesions, respectively.[7,17]

3.2 | Co-culture increased migration of both bone stromal and androgen receptor-positive human prostate cancer cells

Bone-derived stromal cells were co-cultured with LNCaP cells to investigate their interactions in the tumor microenvironment,[7] and their effect on the progression of osteoblastic bone metastasis. LNCaP migration was significantly increased by both BDSC and BmetSC; the effect of BmetSC was much stronger than that of BDSC (Figure 2A). LNCaP cells significantly increased BDSC migration but significantly decreased BmetSC migration (Figure 2B,C). The results suggest that prostate cancer cells initially activated stromal cells, leading to cancer cell migration, and that they could subsequently inactivate stromal cells, leading to inhibition of migration and re-initiation of proliferation.[19]

3.3 | Bone stromal cells secreted C-C motif ligand 5

A human cytokine antibody array including of CM from LNCaP, BDSC and BmetSC cultures revealed that CCL5 was secreted by both BDSC and BmetSC and that BmetSC secreted more CCL5 than BDSC (Figure 3A). ELISA determined that the amount of CCL5 was proportionate to the bone stromal cell effect on LNCaP migration and that neither LNCaP nor LNCaP-SF increased CCL5 secretion by bone stromal cells (Figure 3B). To confirm that CCL5 was the only chemokine to induce LNCaP migration, LNCaP cells were cultured with CM from BDSC and BmetSC cultures. LNCaP migration was

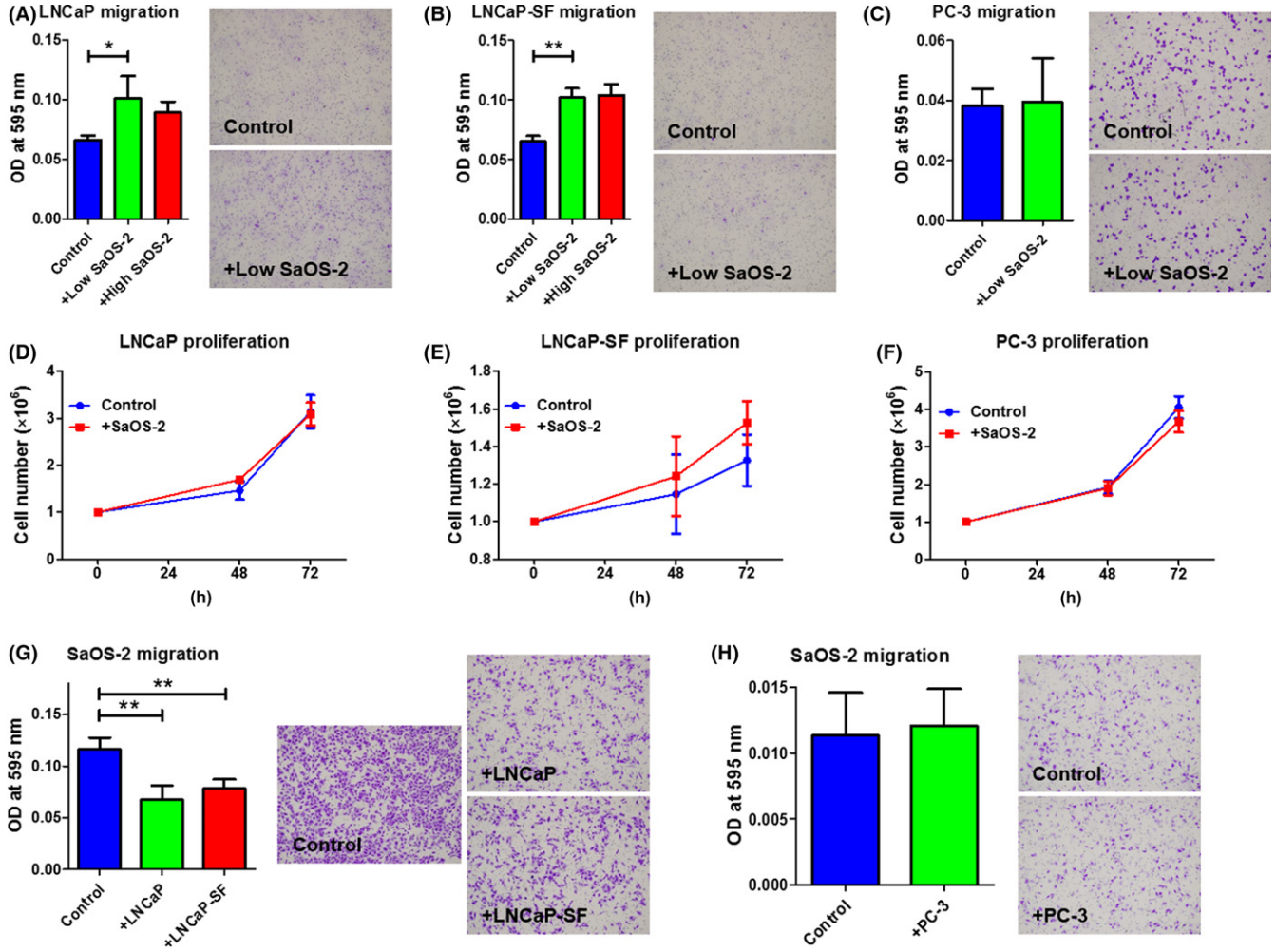

FIGURE 1 Cell migration and proliferation in co-cultures of SaOS-2 and prostate cancer cells. A, LNCaP, B, LNCaP-SF (4×10^4 cell/well) and C, PC-3 (2×10^4 cells/well) were placed in 24-well plates with Transwell inserts and co-cultured with 4×10^4 or 8×10^4 SaOS-2 cells/well (2×10^4 cells/well for PC-3 cells). Cell migration was assayed after 24 h by staining with 0.1% crystal violet and reading the absorbance at 595 nm with correction at 450 nm with a microplate reader. D, LNCaP, E, LNCaP-SF and F, PC-3 cells (1×10^6 cells/well) were placed into the lower Transwell compartment in 6-well plates, and SaOS-2 cells were placed on the inserts. After co-culture for 24-72 h, prostate cancer cells were harvested and the cells were counted using a hemocytometer. G,H, SaOS-2 cells (4×10^4/well) were placed on Transwell inserts in 24-well plates and co-cultured with 4×10^4 cells/well of LNCaP, LNCaP-SF G, and PC-3 H, cells. Migration was assayed after 24 h by staining with 0.1% crystal violet. Data are means ± SD. All experiments are performed in triplicate. $^*P < .05$, $^{**}P < .01$

increased in proportion to CCL5 concentration, as determined by ELISA (Figure 3C).

3.4 | C-C motif ligand 5 promoted LNCaP migration in a dose-dependent manner

C-C motif ligand 5, at concentrations of 0.5 to 5.0 ng/mL, increased LNCaP migration in a dose-dependent manner (Figure 4A), but it did not increase LNCaP cell proliferation (Figure 4B). Western blot assays confirmed that CCL5 receptor was constitutively expressed in LNCaP cells, not only in cultures with CCL5, but also in control cultures with only FBS (Figure 4C). Inhibition of BmetSC-induced LNCaP migration in cocultures including CCL5 neutralizing antibody confirmed CCL5 as a cause of BmetSC-induced LNCaP migration. (Figure 4D).

3.5 | C-C motif ligand 5 did not induce the migration of LNCaP cells after androgen receptor knockdown

C-C motif ligand 5 is known to inhibit AR nuclear translocation and to downregulate androgen signaling in prostate cancer cells.[15] Recent studies clarified that downregulation of AR not only inhibited cell proliferation but also promoted cell migration through epithelial-mesenchymal transition.[9,15] To examine whether CCL5 acted upstream of AR signaling, AR expression in LNCaP cells was silenced by siRNA. AR protein expression in SiAR1 and SiAR2 cells was measured by western blot. Both SiAR1 and SiAR2 cells expressed low AR levels for up to 96 hours after transfection (Figure 5A). SiAR1 inhibited proliferation more strongly than SiAR2 (Figure 5B), suggesting that the effectiveness of AR knockdown by SiAR1 was better than that by SiAR2. SiAR1

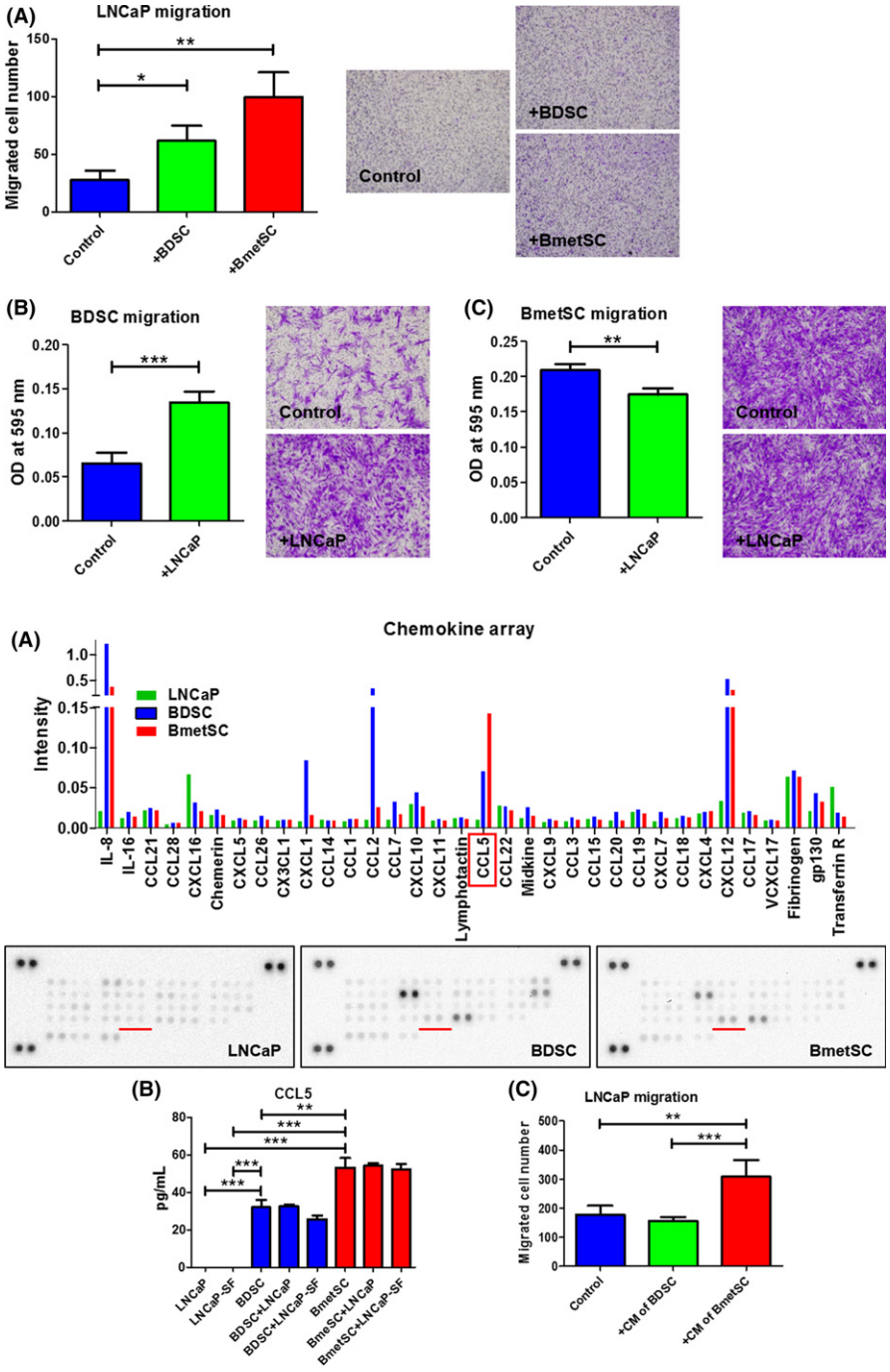

FIGURE 2 Cell migration in co-cultures of bone-derived stromal cells (BDSC) or bone metastasis stromal cells (BmetSC) and LNCaP cells. A, 8×10^4 LNCaP cells/well were placed in Transwell inserts in 24-well plates and co-cultured with 8×10^4 BDSC or BmetSC cells/well. Cell migration was assayed after 24 h. B, 2×10^4 BDSC cells/well C, BmetSC were placed in Transwell inserts in 24-well plates and co-cultured with 2×10^4 LNCaP cells/well. Cells migration was assayed at 24 h by 0.1% crystal violet staining. Data are means ± SD. All experiments are performed in triplicate. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

FIGURE 3 Identification and quantification of secreted proteins that induced prostate cancer migration. A, The graph shows chemokine expression in arrays comparing conditioned medium (CM) from LNCaP cells, bone-derived stromal cells (BDSC) and bone metastasis stromal cells (BmetSC) cultures. Underline indicates C-C motif ligand 5 (CCR5) spots. The mean values of 2 spots are shown. B, Prostate cancer cells were co-cultured with or without BDSC or BmetSC for 24 h. Cell culture inserts (1.0-μm pore size) and 24-well plates were used, and CCL5 in the CM was determined by ELISA. C, 10×10^4 LNCaP cells/well were cultured in Transwell inserts in 24-well plate with or without ×2 diluted CM from BDSC or BmetSC cultures and migration was assayed at 24 h. Data are means ± SD. All experiments are performed in triplicate. $^{**}P < .01$, $^{***}P < .001$

cells were, thus, used in the migration assay. Although the migration of SiAR1 cells was strongly promoted by AR knockdown as previously shown (Figure 5C, control vs 0 g/mL), the addition of CCL5 no longer promoted the migration of SiAR1 cells (Figure 5C).

3.6 | C-C motif ligand 5 expression was stronger in prostate cancer than in normal prostate tissue

CCL5-CCR5 signaling in prostate cancer tissue was investigated by IHC staining of CCR5 in TMA plates. Of 184 prostate cancer cores, 2 were peeled off, and 14 diagnosed as Gleason pattern 1 prostate cancer were removed because Gleason score of clinically significant prostate cancer is scored from 6 to 10.[20] The remaining 168 cores were evaluated. Of 20 normal prostate tissues, 1 was peeled off and the remaining 19 cores were evaluated. IHC staining revealed a significant increase in CCR5 expression in prostate cancer cells compared with epithelial cells in normal prostate tissue (Figure 6), indicating that CCL5-CCR5 signaling was increased in prostate cancer cells.

4 | DISCUSSION

The AR is a primary driver of prostate cancer progression, and it is strongly expressed before development of castration resistance.[21]

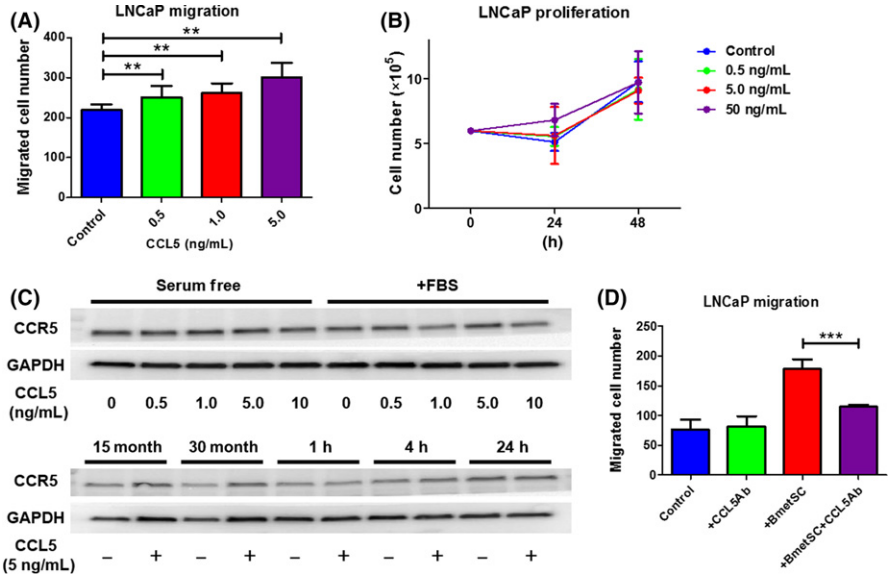

FIGURE 4 Effect of the C-C motif ligand 5 (CCR5) -CCR5 axis on LNCaP cells. A, 8×10^4 LNCaP cells/well are placed in Transwell inserts in 24-well plates with or without 0-5.0 ng/mL human recombinant CCL5 and cell migration was assayed at 48 h. B, 6×10^4 LNCaP cells/well were placed in 6-well plates with or without 0-50 ng/mL human recombinant CCL5 for 24-48 h. Prostate cancer cells were harvested and the cells were counted using a hemocytometer. C, CCR5 protein expression in LNCaP cells was assayed by western blot. LNCaP cells were incubated in serum-free medium or 5% FBS with or without human recombinant 0-10 ng/mL CCL5 (upper panel). LNCaP cells were incubated for 15 min to 24 h with or without 5 ng/mL human recombinant CCL5 (lower panel). D, 5×10^4 LNCaP cells/well were placed in Transwell inserts in 24-well plate with CCL5 neutralizing antibody or normal rabbit IgG (2 μg/mL) or 5×10^4 BmetSC/well. Cells migration was assayed by count at 48 h. Data are means ± SD. All experiments were performed in triplicate. $^{**}P < .01$, $^{***}P < .001$

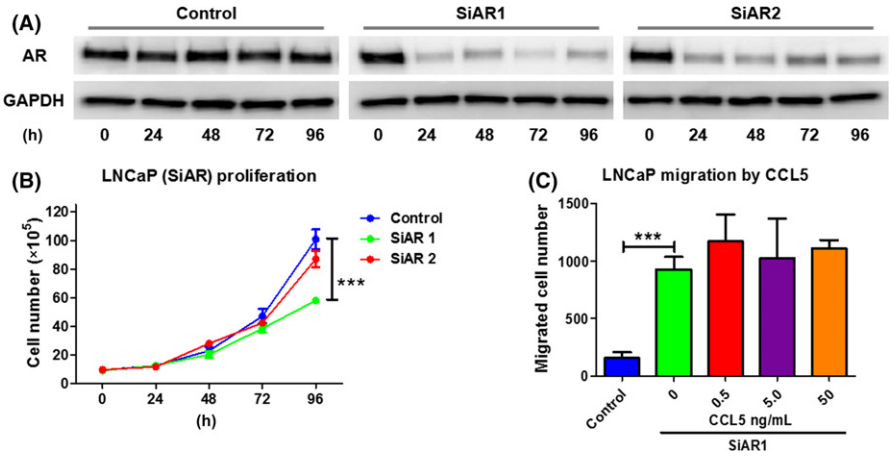

FIGURE 5 Effect of the C-C motif ligand 5 (CCR5) -CCR5 axis on siAR LNCaP cells. A, 1×10^5 LNCaP cells/well were placed into 6-well plates and transfected with 10 nmol L^{-1} of SiAR1 or SiAR2 for 24 h. Total protein was extracted at 0, 24, 48, 72 and 96 h after transfection. B, 1×10^5 LNCaP cells/well were plated into 6-well plates and transfected with 10 n mol L^{-1} of SiAR1 or SiAR2 and cultured for 96 h. Cells were harvested and counted at 0, 24, 48, 72 and 96 h after transfection using a hemocytometer. C, Control and SiAR1-transfected LNCaP cells were placed in Transwell inserts (5×10^4 cells/well in 24-well plates) with 0-50 ng/mL human recombinant CCL5 and cell migration was assayed at 48 h by counting. Data are means ± SD. All experiments were performed in triplicate. $^{***}P < .001$

Androgen-sensitive prostate cancer cells tend to metastasize to bone, with 80%-90% of distant metastases found in bone.[22,23] Once in bone, prostate cancer cells interact with a variety of cells, mainly bone stromal cells, and activate osteoblasts, resulting in formation of osteoblastic lesions.[24] Subsequently, prostate cancer cells in the tumor microenvironment containing bone stromal cells and osteoblasts become migratory cells that are able to metastasize to other sites.[25] However, the mechanism of cancer cell activation in bone metastatic lesions is not clear. Co-cultures of SaOS-2 cells and bone stromal cells with prostate cancer cells found that both cell types induced the migration of AR-positive cancer cells. Bone tissue is rich in cytokines that are known to affect the behavior of cancer cells.[10,26] CCL5 was consistently secreted by BDSC and BmetSC and, as previously shown,[13] directly increased prostate cancer cell

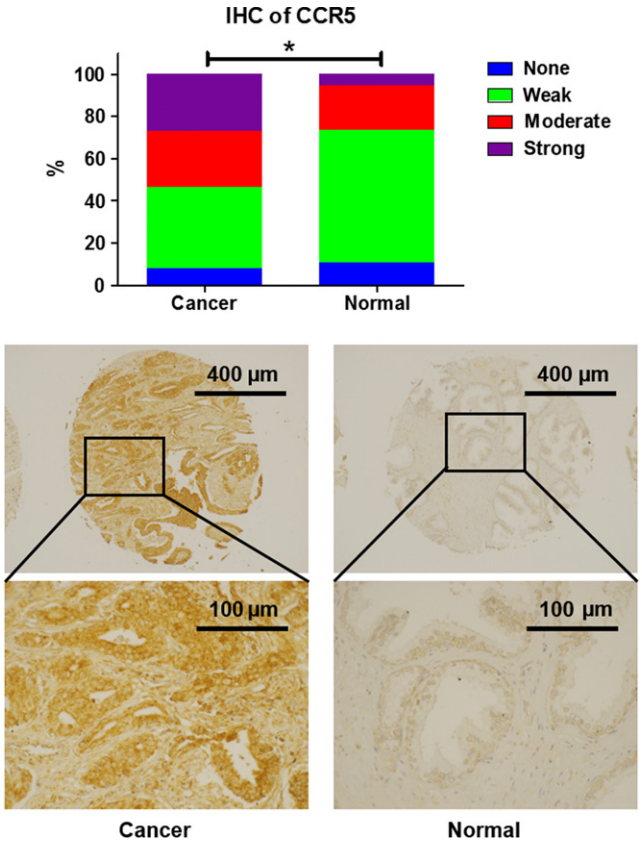

FIGURE 6 Immunohistochemistry staining of C-C motif ligand 5 (CCR5) in normal prostate and prostate cancer tissues. TMA plates with normal prostate or prostate cancer tissues were stained with anti-CCR5 antibody. Of the 208 tissue samples that were stained, 168 prostate cancer and 19 normal prostate cores were evaluated. Staining intensity was scored as: −, negative; +, weak; ++, moderate; or +++, strong. *$P < .05$ by the χ^2-test for trend

migration but not proliferation. The study results are consistent with those of Luo et al who found that upregulation of CCL5 expression in bone marrow mesenchymal stem cells increased the metastatic potential of prostate cancer cells, and that upregulation of CCL5 subsequently downregulated AR signaling, because of inhibition of AR nuclear translocation.[15] CCL5 has also been found to suppress expression of prolyl hydroxylases and lead to suppression of VHL-mediated HIF2α ubiquitination and suppression of AR signaling.[16] AR signaling has been shown to promote proliferation and inhibit migration of prostate cancer cells.[9,27,28] The results obtained with AR-silenced LNCaP cells indicate that CCL5 activity was located upstream of AR signaling. Moreover, SaOS-2 did not promote the migration of PC-3 AR-negative prostate cancer cells. These results suggest that the migratory potential of AR-positive prostate cancer cells in bone metastases was increased by CCL5 secreted by bone stromal cells through the suppression of AR signaling. This novel mechanism of the metastatic cascade is a potential target to prevent secondary metastases. Expression of CCR5 and its receptor were significantly higher in prostate cancer cells than in normal prostate tissues, suggesting that CCL5 specifically acts on cancer cells. Therefore, targeting CCR5 might be a novel treatment for prostate cancer bone metastasis as well as CCL5. Furthermore, the CCL5-CCR5 axis may be a novel biomarker for prostate cancer. The limitations of using a prostate-specific antigen alone as a biomarker for prostate cancer, and the need for more specific and effective biomarkers, have been discussed elsewhere.[29,30] Previously, we reported that the serum CCL2 level in patients with prostate cancer was predictive of the time taken by tumors to become castration-resistant and of overall and prostate cancer-specific survival.[31] Additional clinical evidence is needed to show the superiority of the CCL5-CCR5 axis over the CCL2-CCR2 axis as a prostate cancer biomarker.

There are some points to be discussed carefully in this study. Chemokine array showed high intensity in IL-8 and CXCL12. Although we did not focus on these chemokines because no significant difference between BDSC and BmetSC were observed on array, they may exert some effects on prostate cancer cells cooperating with other functional molecules. For instance, CXCL12-mediated activation of ERK or AKT pathways is modulated by PTEN status.[32] Next, the effect of CCL5 is less than SiAR. As Figures 4A and 5C show, the effect of CCL5 on migration was less than SiAR. Moreover, CCL5 could not inhibit LNCaP proliferation, as shown in Figure 4B. These results indicate that CCL5 can increase LNCaP migration through inhibition of AR signaling, but its effect is not perfect: CCL5 cannot completely (only partially) inhibit AR signaling. In contrast, because high concentration of CCL5 was reported to increase prostate cancer proliferation, there may be different mechanisms not associated with AR signaling.[13]

To our knowledge, this is the first study to show that CCL5 was expressed in bone stromal cells and that the CCL5-CCR5 axis contributed to prostate cancer cell migration. The CCL5-CCR5 axis, which is activated in prostate cancer bone metastasis, may be a novel therapeutic target and a potential biomarker for prostate cancer.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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