AWARD NUMBER: W81XWH-15-1-0703

TITLE: CXXL 14 Blockade of CXCL12/CXCR4 Singnaling in Prostate Cancer Bone Metastasis

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE
CXXL 14 Blockade of CXCL12/CXCR4 Singnaling in Prostate

Cancer Bone Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0703

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Bone metastasis is a common and unfortunate complication of advanced prostate cancer resulting in significant pain and fractures. The most devastating consequence, however, is that once the cancer has metastasized to bone, the disease is incurable. Up to 90% of men who succumb to prostate cancer have bone metastasis, which demonstrates the strong attraction of prostate cancer to bone. Chemokines are essential in cancer progression and metastasis. The chemokine CXCL14 was identified in a screen for factors that support prostate cancer bone metastasis. In a human prostate cancer tissue microarray, CXCL14 expression was found significantly greater in prostate cancer metastasis to bone compared to metastasis to other organs, suggesting that this chemokine has important effects that are specific to bone. The goal of this proposal is to understand the role of CXCL14 in the homing and progression of prostate cancer to bone.

15. SUBJECT TERMS
Prostate cancer, bone metastasis, CXCL14, chemokine signaling, animal models

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	35	**19b. TELEPHONE NUMBER** (include area code)
U	U	Unclassified			

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1. Introduction

Chemokines play critical roles in cancer progression and metastasis. We identified the chemokine CXCL14 in a screen of factors expressed in prostate cancer bone metastasis. In a human prostate cancer tissue microarray, CXCL14 expression was significantly greater in metastasis to bone compared to soft tissue metastasis, primary prostate tumors and normal prostate. CXCL14 expression was also significantly greater in bone xenografts compared to growth outside the skeleton in animal models of bone metastasis. These data suggest bone-specific actions of prostate cancer and CXCL14. The purpose of this proposal is to understand the role of CXCL14 in the development and progression of prostate cancer bone metastasis.

2. Keywords

Prostate cancer, bone metastasis, CXCL14, chemokine signaling, animal models

3. Accomplishments

Major goals:
1. Assess prostate cancer CXCL14 overexpression to reduce CXCL12-mediated homing to bone
2. Assess the effects of CXCL14 knockdown to reduce prostate cancer growth in bone

Accomplishments
1. A manuscript has been completed detailing the molecular actions of CXCL14 on prostate cancer cells. The receptor for CXCL14 had not been clearly defined in the past and controversy existed as to the extent to which CXCR4 acted as the receptor. We have new data showing that CXCL14 does not directly interact with CXCR4, but CXCR4 is dependent on at least some of CXCL14 cellular actions. These data suggest, as has been reported previously, that CXCR4 likely exists as a heterodimer with the unidentified CXCL14 receptor. A draft of this manuscript is attached in the Appendix.
2. We have completed the first round of animal experiments as detailed in the proposal. Using prostate cancer PC-3 cells that overexpress luciferase, we have generated these PC-3 cells to overexpress CXCL14 versus an empty vector control. After intracardiac inoculation, the development of bone and soft tissue lesions was monitored using *in vivo* bioluminescent imaging. This experiment recently ended, 13 weeks after intracardiac inoculation. An example of the same lesion as seen by X-ray and IVIS is shown (Figure). Analyses examining for differences in percent of mice with bone lesions by X-ray, percent of mice with bone lesions on X-ray + IVIS imaging, average number of lesions/mice on X-ray, and average number of lesions on X-ray + IVIS imaging was not different (Figure).
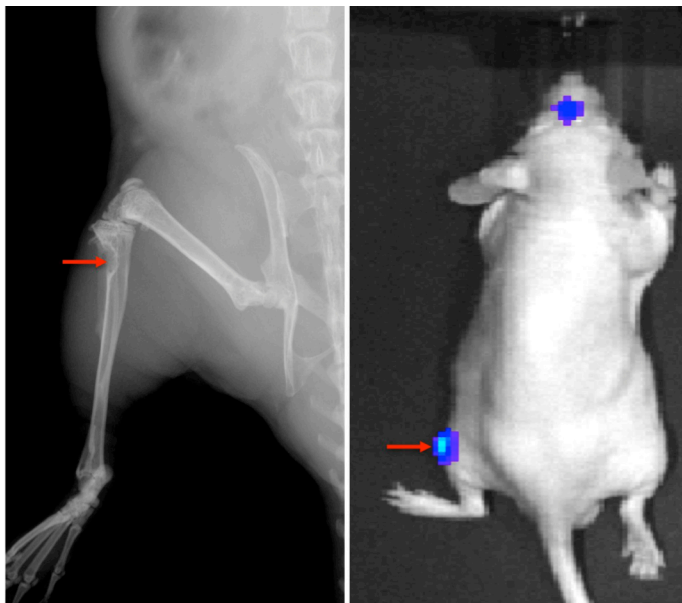
	Empty Vector	CXCL14	p-value
Percent of Mice with Lesions on X-ray	55%	27%	0.2049
Percent of Mice with Lesions/Signals on X-ray/IVIS	67%	45%	0.3110
Average Number of Lesions per Mouse on X-ray (Mean ± SEM)	0.6667 ± 0.2357	0.4545 ± 0.2817	0.5815
Average Number of Lesions/Signals on X-ray/IVIS (Mean ± SEM)	1.333 ± 0.4082	1.273 ± 0.5062	0.9291

Opportunities for training and professional development
Nothing to report

Dissemination of results to the communities of interest
The publication entitled "CXCL14 is a Marker of Prostate Cancer Bone Metastasis: A Pro-Metastatic Chemokine with CXCR4 Dependent and Independent Actions" will be submitted soon.

Plans during the next reporting period
    We will begin work on the following:

Task 1b: Examine competition of CXCL12 and CXCL14 in prostate cancer skeletal invasion
Task 2a: Examine how CXCL14 knockdown in prostate cancer cells, before the arrival to bone, affects the development of bone lesions in a bone metastasis prevention model

4. Impact

Impact on the development of the principal discipline of the project
1. Our recent results confirm that CXCL14 signaling is more complex that once thought and likely involves an unidentified CXCL14 chemokine receptor cooperating with CXCR4.
2. The original hypothesis that CXCL14 is a CXCL12 inhibitor has been modified so that CXCL14 likely has cooperative actions with CXCL12 in prostate cancer bone metastasis.

Impact on other disciplines
Nothing to report

Impact on technology transfer
Nothing to report

Impact on society beyond science and technology
Nothing to report

5. Changes/Problems

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions to resolve them
Nothing to report

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

6. Products

Publications: The paper entitled "CXCL14 is a Marker of Prostate Cancer Bone Metastasis: A Pro-Metastatic Chemokine with CXCR4 Dependent and Independent Actions" will be submitted in the next several weeks.

7. **Participants & Other Collaborating Organizations**

Name:	Gregory A. Clines, MD, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.11
Contribution to Project:	Dr. Clines continues to serve as the PI of the projects
Funding Support:	In addition to the DoD grant, Dr. Clines also receives funding support from the Department of Veteran Affairs and the University of Michigan

Name:	Gary Luker, MD
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.24
Contribution to Project:	Dr. Luker serves as a collaborator on this project and was instrumental in many of the experimental results as detailed in the attached manuscript.
Funding Support:	In addition to the DoD grant, Dr. Luker also receives funding support from the NIH and the University of Michigan

Name:	Katrina Clines, MS
Project Role:	Lab Manager
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4.8
Contribution to Project:	Ms. Clines serves as the lab manager is responsible for animal handling and performance/supervising most of the experiments

Funding Support:	In addition to the DoD grant, Ms. Clines also receives salary support from the Department of Veterans Affairs

Name:	Hyun Sik Moon, BS
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	9.0
Contribution to Project:	Mr. Moon has supported and performed many of the experiments in the DoD grant
Funding Support:	In addition to the DoD grant, Mr. Moon also receives salary support from the University of Michigan

Other organizations
Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

Draft manuscript: "CXCL14 is a Marker of Prostate Cancer Bone Metastasis: A Pro-Metastatic Chemokine with CXCR4 Dependent and Independent Actions"

CXCL14 is a Marker of Prostate Cancer Bone Metastasis: A Pro-Metastatic Chemokine with CXCR4 Dependent and Independent Actions

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Abstract

The chemokine CXCL14 has complex effects on inflammation and tumorigenesis. The expression of CXCL14 in prostate cancer bone metastasis was investigated using a human prostate cancer tissue microarray. CXCL14 expression was significantly higher in bone metastases compared to normal prostate, benign prostatic hypertrophy, primary prostate cancer, and metastases to lymph nodes and soft tissues. Investigations were performed to understand the biology of CXCL14 as a pro-metastatic prostate cancer marker. CXCL14 stimulated *in vitro* migration and invasion of the human prostate cancer cell line ARCaP$_M$, and promoted expression of epithelial-to-mesenchymal transition-associated genes in ARCaP$_M$ and PC-3 prostate cancer cells. Moreover, CXCL14 activated MAPK and PI3K/AKT signaling pathway proteins. CXCL14 signaling activation was not due to direct interaction with the chemokine receptor CXCR4, addressing controversy as to the identity of the CXCL14 chemokine receptor. CXCL14 did not activate CXCR4 signaling in a luminescent complementation reporter assay that detects CXCR4 recruitment of β-arrestin 2. Furthermore, an interaction of CXCL14 with CXCR4 or CXCR7 was not detected using a sensitive luminescent complementation assay. Despite the lack of a CXCL14/CXCR4 interaction, CXCL14-activated migration was dependent on CXCR4 as demonstrated using a novel microfluidic cell migration assay. Together, these data suggest that CXCL14 interacts with an unidentified chemokine receptor that may have cooperative actions with CXCR4. This investigation thus identified CXCL14 as a novel prostate cancer pro-metastatic chemokine and may represent an appealing target for the treatment of prostate cancer bone metastasis.

Keywords: Prostate cancer, bone metastasis, CXCL14, CXCL12, CXCR4, chemokine signaling

Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-skin malignancy in men, and is the second highest contributor of cancer-related death in the United States [1]. Bone is a preferred site of metastasis from PCa and is largely responsible for PCa associated morbidity [2-5]. Patients who develop bone metastasis often experience bone pain and pathological fractures. Metastatic spread accounts for the majority of cancer mortalities, highlighting the urgent need to identify factors involved in its progression. The identification of molecular and cellular agents that support bone metastasis would represent therapeutic targets to treat this complication of malignancy.

Chemokines and their receptors were originally identified as mediators of inflammatory disease processes. They also function as crucial links between tumor cells and stromal cells, strongly promoting proliferation, migration and invasion through multiple signaling pathways [6, 7]. The C-X-C chemokine receptor type 4 (CXCR4) is overexpressed in many different cancer types including cancers of the prostate, breast, lung, and pancreas [7, 8]. CXCL12 (stromal cell-derived factor-1/SDF-1), a CXCR4 ligand, is a chemoattractant that facilitates the migration of CXCR4-expressing primary tumor cells towards metastatic sites [9]. Prostate cancers, in particular, utilize the CXCL12/CXCR4 axis to "home" to bone [10, 11]. PCa cells migrate across bone marrow endothelial cell monolayers in response to CXCL12 [12]. Additionally, the invasion of PC-3, LNCaP and C4-2B human PCa cell lines into a reconstituted extracellular matrix was inhibited when CXCR4 expression was decreased [13]. The work by Xing, *et al* confirmed and extended these findings—stable knockdown of CXCR4 blocked CXCL12-dependent PCa cell adhesion to osteosarcoma and endothelial cells, inhibited *in vitro* invasion, and reduced PCa skeletal growth after intratibial inoculation in immunodeficient mice [14].

Although CXCL12 has been a focus in numerous published reports, the related chemokine CXCL14 (earlier designated as BRAK) has been implicated in metastasis as well. CXCL14 is a 77-amino acid secreted protein and member of the C-X-C chemokine family [15]. This chemokine displays activity in immature dendritic cells, monocytes, macrophages and NK cells [16]. CXCL12 and CXCL14 are primordial chemokines and possess close evolutionary sequence conservation with each other as well as orthologous conservation among distantly related species [17]. It has been suggested that CXCL12 and CXCL14 have cooperative actions because of non-overlapping expression in adjacent tissues present during chick and mouse eye development [18].

CXCL14 expression in cancers, compared to normal tissues, had been reported to be both increased and decreased. CXCL14 downregulation had been reported in breast cancer, hepatocellular carcinoma, gastric cancer, colon cancer, mouse acute myeloid leukemia, and neuroendocrine tumors [19-26]. However, malignancy was also reported to be associated with increased CXCL14 expression in gliomas, colon cancer, and BRAF-mutant papillary thyroid [27-29]. Breast cancer tumor-associated mesenchymal cells demonstrated a correlation between CXCL12 and CXCL14 overexpression in bone metastasis tissue as compared to metastases in lung, liver, or brain [30, 31]. It is likely that CXCL14 has pleiotropic effects that are dependent on context and cancer type.

In regards to PCa, CXCL14 has tumor-promoting actions. CXCL14 expression is increased in cancer-associated fibroblasts compared to intermixed PCa cells, and this

expression led to autocrine feedback that promoted PCa growth [32]. Another study reported that CXCL14 expression was increased in PCa and correlated with Gleason score [33]. A genetic approach was utilized to identify PCa susceptibility genes in a genetic cross utilizing the TRAMP mouse model of PCa. CXCL14 was one of five genes identified that correlated with primary tumor burden. Furthermore, a single nucleotide polymorphism within the human CXCL14 gene was found associated with PCa tumor progression [34].

Until now, an association of CXCL14 with PCa bone metastasis was unrecognized. The study reported here was designed to investigate the expression and clinical significance of CXCL14 in PCa bone metastasis. CXCL14 expression was increased in PCa metastatic to bone but unchanged in benign prostatic hypertrophy, primary PCa, lymph node metastasis, and soft tissue metastasis compared to normal prostatic tissue. Furthermore, CXCL14 activated PCa signaling pathways, EMT, and chemotaxis. Luminescent biocomplementation assays revealed the existence of a CXCL14 homodimer and that CXCL14 does not possess a strong affinity for CXCR4 but at the same time is dependent on CXCR4 for chemotaxis. CXCL14 represents an unrecognized factor in the pathogenesis of PCa bone metastasis.

Material and Methods

Human PCa Tissue Microarray

Normal human prostate, and primary and metastatic PCa tissues were obtained through the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program. Samples were obtained from patients who had died of metastatic castrate-resistant prostate cancer (CRPC). Rapid autopsy was performed within 4-10 hours of death, under the support of the Prostate Cancer Donor Program at the University of Washington [35]. The Institutional Review Board of the University of Washington Medical Center approved all procedures involving human subjects, and all subjects signed written informed consent. Tissue samples were collected from bone, metastatic lymph nodes, liver, and lung in a systematic and consistent fashion in every case. A human tissue microarray was constructed from 44 patients with 185 metastatic sites and consisted of the following: 28 histologically normal prostates, 30 benign prostatic hypertrophies (BPH), 52 primary PCa (Gleason grade 3-5), 34 lymph node metastases, 27 soft tissue metastases and 113 bone metastases tissue samples. Each metastatic site was represented by two cores. Five µm tissue sections were probed with a mouse anti-human CXCL14 antibody (MAB866, 5µg/mL; R&D Systems, Minneapolis, MN) and visualized with 3,3'-dioaminobenzidine (DAB). Staining intensity of PCa cells (0-absent; 1+ faint; 2+ moderate; 3+ intense) and percentage of cells at the staining intensity were determined, and the H-score was calculated [H-score = 1X (% 1+ cells) + 2X (% 2+ cells) + 3X (% 3+ cells)].

Cell Culture

The ARCaP$_M$ cell line (Novicure Biotechnology, Birmingham, AL) was derived by single cell cloning of the parental ARCaP cells [36]. The ARCaP$_M$ cell line was maintained in MCaP growth medium (Novicure Biotechnology) supplemented with 5%

fetal bovine serum (FBS). The PC-3 human PCa cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). PC-3 cells were maintained in F-12K medium supplemented with 10% FBS. C4-2B human PCa cells were obtained from Dr. Theresa Guise at Indiana University School of Medicine and maintained in RPMI medium supplemented with 10% FBS. MDA-MB-231 cells were purchased from ATCC and maintained in DMEM medium supplemented with 10% FBS. All cell lines were grown in a humidified incubator with 5% CO_2 at 37°C and supplemented with 100 I.U./ml penicillin and 100 µg/mL streptomycin.

Scratch Migration Assay

Twelve-well plates were coated with Matrigel (Corning Inc., Corning, NY) overnight, followed by one-hour incubation with 2 mg/ml of bovine serum albumin (BSA). Cells were plated and grown to confluency. Thereafter, a confluent monolayer of ARCaP$_M$ and PC-3 cells were treated with either 0.5% FBS or 0.5% FBS/CXCL14 250 ng/ml overnight. The next day, the bottom of the well was scratched with a 200 µl pipette tip. After several washes with serum-free medium, cells were incubated with 0.5% serum-containing medium with or without CXCL14. Images of cell migration into the scratch were captured at 0, 6, and 24 hour intervals. Analyses of cells that migrated into the scratch area were performed using the ImageJ software package.

Transwell Migration Assay

Transwell inserts with 8-mm pores (Corning Inc., Corning, NY) were coated with Matrigel (Corning) for 2-18 hours at 37°C. Approximately 200,000 cells were added to the transwell inserts in RPMI medium containing 0.5% FBS and allowed to attach overnight. The next day, RPMI medium containing 0.5% FBS, 10% FBS or 0.5% FBS/CXCL14 250ng/ml were added to the bottom of respective wells, and the plates were incubated in a humidified incubator with 5% CO_2 at 37°C. After 72 hours, transwell inserts and wells were washed with PBS, a 4:1,000 ratio of calcein AM (ThermoFisher Scientific) to PBS was placed at the bottom of each well, and plates were incubated for 10 minutes. Next, 10 µl of 10X trypsin was added to each well, and incubated for 10 additional minutes. Transwell plates were gently agitated on the shaker for 1 minute. Contents of each well were plated in a 96-well black-walled plate, and fluorescence was measured using a plate reader (495 (ex)/515 (em) nm).

Real-Time RT PCR

Confluent ARCaP$_M$ and PC-3 PCa cells were treated with various concentrations of CXCL14 (0, 10, 100, and 200 ng/ml) in RPMI medium supplemented with 0.5% FBS for 24 hours in a humidified incubator with 5% CO_2 at 37°C. Cells were washed in ice-cold PBS and RNA was extracted (Zymo Direct-zol kit, Irvine, CA). A DNase digestion step was included during RNA purification. Real-time RT PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA). Changes in mRNA concentration were determined using the $2^{-[\Delta][\Delta]Ct}$ method and *RPL32* was used as the internal control. Amplification was performed using primers specific to human *SNAIL, SLUG,* and *TWIST*. (*SNAIL* forward: gaccccaatcggaagcctaa; *SNAIL* reverse: agggctgctggaaggtaaac; *SLUG* forward: gactaccgctgctccattcc; *SLUG* reverse: actcactcgccccaaagatg; *TWIST* forward: gtccgcagtcttacgaggag; *TWIST* reverse:

atcttgctcagcttgtccga; *RPL32* forward: cagggtgcggagaaggttcaaggg; *RPL32* reverse: cttagaggacacgttgtgagcaat).

Western Blot Analyses

ARCaP$_M$ and PC-3, at 80% confluence, were serum-starved in medium supplemented with 0.1% bovine serum albumin (Probumin, Celliance) for 8 hours. Serum-starved cells were treated with 250 ng/ml of CXCL14 for 0, 10, 30, 60, and 120 minutes. Thereafter, cells were washed in ice-cold PBS. Cells were lysed in mammalian extraction protein reagent (M-PER) lysis buffer (ThermoFisher Scientific) that contained protease and phosphatase inhibitors. Cell lysates were subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked using 5% milk. Antibodies specific for phospho-AKT (Ser473), AKT, Phospho-p38 MAPK (Thr180/Tyr182), Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), Phospho-SAPK/JNK (Thr183/Tyr185) and GAPDH (Cell Signaling, Danvers, MA) were diluted in TBS buffer at a 1:1000 ratio. Membranes were incubated with antibodies overnight at 4°C, followed by a horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (Cell Signaling) and western blot detection reagent. Bands were visualized using a luminescent imager (ChemiDoc Bio-Rad, Hercules, CA). Experiments were performed at least three times.

Luciferase Complementation Reporter Assay for CXCR4 Activation

The generation of MDA-MB-231 human breast cancer cells stably expressing CXCR4-CBRN and β-arrestin 2-CBC was previously described [37]. Cells were plated at 20,000 cells per well in 96-well black-walled plates. Cells were grown in a humidified incubator with 5% CO$_2$ at 37°C for two days. After a two day incubation, medium was replaced with 50 µl phenol red free DMEM (Life Technologies) with 0.2% media grade bovine serum albumin (Probumin, Celliance, Billerica, Ma,) 30 minutes before imaging. Next, 7 µL of a 15 mg/mL luciferin stock was added to cells and incubated for 5 minutes before the addition of chemokine treatments. Immediately before imaging, 14 µL phenol red-free DMEM that contained 0.2% media grade bovine serum albumin was added in increasing concentrations of CXCL12 and/or CXCL14 (Cat# ab174986, Abcam, Chambrige, MA). A series of 60 images with large binning and a 10-second exposure was acquired. Cells were maintained at 37°C and imaged for 120 minutes. To determine relative induction of bioluminescence, bioluminescence was normalized in wells treated with chemokines to cells incubated with vehicle control at each time point (n=4 per condition). Luminescence was measured with an IVIS Lumina (Perkin-Elmer, Waltham, MA).

NanoLuc Luminescence Assays

Plasmids were constructed expressing proteins fusions to NanoLuc luciferase or NanoBit fragments LgBit or SmBit for protein fragment complementation (Promega, Madison, WI). Open reading frames were amplified by PCR from synthetic double stranded templates (gBlocks from IDT) or plasmids containing the reference sequences for human CXCR4 or human CXCR7. Open reading frames for hCXCL14-NanoLuc, hCXCL14-LgBit, hCXCL14-SmBit, and mCXCL12-NanoLuc were cloned into pLVX Puro (Takara-Clontech). mCXCL12-SmBit was cloned into vector pLVX EF1α-ires-mCherry

(Takara-Clontech) and mCXCL12-LgBit was cloned into the same vector with the mCherry replaced with mTagBFP (Evrogen). Fusions LgBit-Lag16, LgBit-hCXCL4 and LgBit-hCXCR7 with a synthetic secretory signal (derived from Gaussia Luciferase pCMV-GLuc (NEB) at the N-terminus of LgBit were cloned into the NheI and EcoRI sites of pEGFP-C1 (Takara-Clontech) in place of EGFP. All open reading frames were fully sequenced.

HEK-293T cells (140,000 cells/well in 12-well plates) were cultured in DMEM with 10% FBS with 1% Pen/Strep/Glutamine. Cells were transfected with 250 ng of each plasmid alone or in pairs using calcium phosphate transfection. Transfected cells were transferred 24 hours later to 96-well black-walled cell culture plates with 10,000 cells/well in 100 µl media. Luminescence assays of cells and media were performed 48 hours after transfection, as indicated. Luminescence was measured with an IVIS Lumina (Perkin-Elmer) in cells or cell supernatants using a final concentration of 1 µg/ml furimazine (Nano-Glo Live Cell Assay, Promega) diluted into media or PBS.

Microfluidic Cell Migration Assay

Cell migration assays were performed using a previously published microfluidic migration platform [38, 39]. To achieve higher throughput, the design was modified to have 300 migration channels per device, and the migration channel was designed to be 5 µm in height and 30 µm in width. Before cell loading, PBS was used to prime the device for one hour, and the cell culture medium flowed through the channel for one hour for better cell adhesion and viability. The cells were trypsinized, centrifuged, and then re-suspended to a concentration of 4X 10^5 cells/ml for loading into the device. After cell loading, the cell suspension in the left inlet was replaced with serum-free cell culture media, and 10 nM (94 ng/ml) CXCL14 was applied to the other inlet to induce chemotactic migration. The microfluidic chip was then put into an incubator, and migration distance was measured based on the final cell position after 24 hours of incubation without medium replenishment. The images were analyzed by custom MATLAB code automatically. Cells were identified based on their fluorescence, and debris was ignored by their small size. A comparison between MDA-MB-231 (known to be motile) and T47D (known to be non-motile) breast cancer cells served as the validation of the platform (Supplement Figure 1). For all conditions in this work, 4 replicates (total of 1,200 channels) were performed.

Statistics

In experiments with two experimental groups, a standard t-test was performed. In three or more experimental groups, a one-way ANOVA was performed followed by Tukey's multiple comparison test. Microfluidic migration assay results were analyzed using two-way ANOVA with Tukey's multiple comparison test. A p-value ≤ 0.05 was considered significant. Data were graphed as mean values, using standard deviation (SD) or standard error of the mean (SEM).

Results

Increased PCa CXCL14 Expression in Bone Metastasis

A cohort of patient samples from 28 histologically normal prostates, 30 benign prostatic hypertrophies (BPH), 52 primary prostate cancers (Gleason grade 3-5), 34 lymph node metastases, 27 soft tissue metastases and 113 bone metastases tissue samples were collected into a tissue microarray (TMA). The TMAs were analyzed for CXCL14 protein expression. Immunocytochemical CXCL14 staining was almost exclusively restricted to the cytoplasm (data not shown). CXCL14 quantification was determined by an H-score that encompassed both number and intensity of stain. No significant difference in H-score was detected in normal prostate compared to BPH, primary PCa, lymph node metastasis and soft tissue metastasis. A significant increase in CXCL14 expression was detected in bone metastasis compared to all other groups (Figure 1A). A secondary analysis was performed to assess correlation of CXCL14 H-score with primary PCa Gleason grade. No association was detected (Figure 1B). These data suggested bone-specific actions of CXCL14 after arrival of PCa to bone.

CXCL14 Stimulates Migration and Invasion of PCa

CXCL14 had been implicated in both promoting and inhibiting cancer cell motility, proliferation, invasion, and metastasis in several reported models [32, 40-42]. *In vitro* scratch migration and transwell invasion assays were performed on human PCa cell lines (ARCaP$_M$, PC-3, and C4-2B) to assess the effects of CXCL14. In the scratch assay, ARCaP$_M$, but not PC-3, cells robustly responded to CXCL14 after 24 hours of treatment (Figure 2A).

Transwell invasion assays were then performed. PCa cells were plated on a cell matrix above a porous membrane. The extent to which cells migrated through these barriers towards a CXCL14 gradient on the opposite side of the membrane was examined. After 72 hours, a minimal invasive response was found with PC-3 and C4-2B PCa cells, as compared to no treatment group. However, ARCaP$_M$ cell invasion was significantly increased with CXCL14 treatment (Figure 2B). These data suggested that ARCaP$_M$ cells, but not PC-3 or C4-2B, possess the correct complement of CXCL14 receptor(s) and/or signaling pathways to respond to CXCL14.

CXCL14 Regulates the Expression of EMT-Associated Genes

Activation of epithelial-to-mesenchymal transition (EMT) has been implicated as a contributor to cancer invasion and metastasis [43]. The effects of CXCL14 to regulate EMT-associated gene expression were investigated to further establish that CXCL14 promotes PCa metastatic behavior. Real-time RT PCR was performed on the ARCaP$_M$ and PC-3 PCa cell lines. A dose response of *SLUG*, *SNAIL* and *TWIST* mRNA expression to increasing CXCL14 treatment concentrations was detected in the ARCaP$_M$ PCa cells (Figure 3A).

Interestingly, CXCL14 10 ng/ml treatment down-regulated EMT gene message. However, higher concentrations of CXCL14 restored *TWIST* and *SNAIL* to baseline (Figure 3B). *SLUG* expression was increased with CXCL14 100 and 200 ng/ml treatment. These data suggest a mechanism of increased migration and invasion is due to EMT.

Regulation of Kinase Signaling by CXCL14

CXCL12 activates MAPK and PI3K signaling pathways in a number of different cell types [13, 44-46]. MAPK and PI3K signaling pathways have been linked to the uncontrolled growth and resistance to apoptosis during tumor progression [47]. The extent to which CXCL14 activated similar pathways was examined. PC-3 and ARCaP$_M$ PCa cells were treated with CXCL14 250 ng/ml at 10-120 minutes. Western blot analysis assessed ERK-1/ERK-2, AKT, SAPK/JNK, NF-κB, and p-38 signaling pathway activation upon CXCL14 treatment.

Rapid phosphorylation of AKT, ERK, SAP/JNK, NF-κB and p-38 proteins was observed within 10-30 minutes of CXCL14 treatment in PCa cells (Figure 4A, B). ARCaP$_M$ cells responded with a marked increase in ERK, p38, and SAPK/JNK phosphorylation at 10 and 30 minutes that returned to baseline after 1-2 hours. Persistent NF-κB phosphorylation remained at two hours. PC-3 cells demonstrated AKT and NF-κB activation after 30 minutes of stimulation (Figure 4B). PC-3 cells showed no detectable phosphorylation of SAPK/JNK or p-38 (data not shown). Though an increase in activation of signaling molecules was detected in both cell lines, only ARCaP$_M$ demonstrated a significantly robust increase in phosphorylated proteins that corroborates earlier reported cellular effects of CXCL14.

Lack of CXCL14-Dependent β-Arrestin 2 Recruitment to CXCR4

CXCL14 stimulated migration, invasion, EMT, and MAPK signaling in PCa cell lines, yet the mechanism of signal transduction remained unclear. The structurally similar CXCL12 binds CXCR4, and recruits the cytosolic adaptor protein β-arrestin 2 that functions as a scaffold protein linking GPCR to intracellular signal transduction pathways [48]. To assess CXCL14-dependent recruitment of β-arrestin 2, the validated MDA-MB-231 green click beetle luciferase complementation reporter system was used to examine mechanisms of CXCL14 signaling [37, 49, 50]. In this system, the MDA-MB-231 cell line was transduced with fusion proteins CXCR4-CBRN and β-arrestin 2-CBC. Activation of CXCR4 recruits of β-arrestin 2 and joins the incomplete luciferase products to generate an active luciferase molecule.

As a control for CXCL14 biologic activity, the transduced MDA-MB-231 cells treated with CXCL14 (50 and 250 ng/ml) demonstrated a marked increase in AKT phosphorylation at 10 minutes (Figure 5A). Cells that stably expressed the complementation reporters were treated with CXCL14 and CXCL12 individually and combined at concentrations of 60 ng/ml (Figure 5B) and 250 ng/ml (Figure 5C). Cells were serially imaged for up to 120 minutes. Luciferase activity increased rapidly, in a dose-dependent manner, with CXCL12 but not with CXCL14 treatment. Moreover, the addition of CXCL14 did not lessen the ability of CXCL12 to recruit β-arrestin 2 to CXCR4. The relative increase in CXCL14-dependent recruitment of β-arrestin 2 to CXCR4 was due to the depletion of luciferin substrate over time.

Upon chemokine ligand binding to CXCR4, the CXCR4/β-arrestin 2 complex is internalized resulting in less cell surface receptor available for ligand binding. The next experiment evaluated the availability of surface CXCR4 following CXCL12 treatment. The MDA-MB-231 CXCR4-CBRN/β-arrestin 2-CBC reporter cell line was treated with either CXCL12 or CXCL14 in increasing concentrations for one hour. A subsequent CXCL12 200 ng/ml treatment followed. The peak signal was plotted for each chemokine concentration. Pretreatment with increasing concentration of CXCL14 did not alter

CXCR4-CBRN/β-arrestin 2-CBC association. However, pretreatment with CXCL12 did reduce further luminescence with subsequent CXCL12 treatment (Figure 5D). These data suggested that CXCL14 does not promote CXCR4 receptor internalization and that CXCR4 is not a CXCL14 receptor.

CXCL14 Ligand Dimerization and Chemokine Receptor Association

CXCL12 exists in both monomeric and dimeric forms at physiologic concentration [51, 52]. Each of these CXCL12 species activates CXCR4, but differs in the downstream signaling pathways activated [52]. The extent to which CXCL14 exists as a homodimer or other chemokines such as CXC12 was investigated. A similar complementation strategy, using the NanoLuc luminescent complementation reporter system, was utilized to examine these interactions. CXCL14 and CXCL12 were cloned in-frame with adjacent intact NanoLuc, the NanoLuc large fragment (LgBit), and the NanoLuc small fragment (SmBit). HEK-293 cells were co-transfected with combinations of CXCL14 and CXCL12, along with an irrelevant protein as a control, followed by luminescent imaging of intact cells and of the conditioned media. This represented a strategy to detect homo- and heterodimer species located in the cell-associated and extracellular compartments. Increased luminescence, above any signal detected of combination chemokine and an irrelevant protein, was evidence for an interaction.

In the cell-associated space, both CXCL12 and CXCL14 homodimers were detected (Figure 6A). A CXCL12/CXCL14 heterodimer was also present, at least at lower concentrations. Luminescence was also detected in the condition media of cells but at a lower concentration compared to the cell-associated fraction. As with the cell-associated fraction, evidence of CXCL14 and CXCL12 homodimers was detected. However, the CXCL14/CXCL12 heterodimer group displayed signal at the same low intensity as the irrelevant products.

CXCR4 and CXCR7 are receptors for CXCL12 [53, 54]. Using the NanoLuc complementation assay, the physical association of CXCL14 with CXCR4 and CXCR7 was investigated. As expected and previously reported, CXCL12/CXCR4 and CXCL12/CXCR7 interactions were detected (Figure 6A). However, an interaction between CXCL14 and the two tested chemokine receptors was not detected.

Dependence of CXCR4 on CXCL14-Mediated Cellular Migration

A novel microfluidic migration platform has been developed to measure the cellular response to a chemotactic agent [38]. This platform consists of 300 microfluidic channel devices. Single cells enter the channels and responsive cells migrate toward the end of the channel containing the chemoattractant. At treatment completion, the final location of individual cells is determined by microscopic imaging.

To assess the chemotactic effects of CXCL14, the microfluidic cell migration assay was employed. In conjunction, a previously developed resource for studying CXCR4 biology was utilized and consisted of 1) the breast cancer cell line SUM159 (WT) that expresses CXCR4, 2) WT cells that underwent CRISPR/Cas9-mediated CXCR4 knockout (KO), and 3) KO cells that underwent re-expression of CXCR4 (Add-Back). The three cell populations were loaded in quadruplicate devices. No treatment or CXCL14 10 nM (94 ng/ml) was used as the chemoattractant and the microfluidic devices were imaged 24 hours later. In the absence of CXCL14, the migratory

distribution of WT, KO and Add-Back cells were unchanged (Figure 7). However, in the presence of CXCL14, CXCR4 KO resulted in fewer cells migrating compared to WT; the add-back of CXCR4 restored migratory potential. These data supported that CXCL14 is, at least partially, dependent on CXCR4 for cell migration.

Discussion

CXCL14 is associated with cancer metastasis in *in vitro*, pre-clinical, and clinical studies, but a unifying mechanism for CXCL14 as a regulator of PCa bone metastasis function had not been clearly defined [28, 32-34, 40, 41, 55, 56]. This report is the first to identify CXCL14 as a PCa pro-bone metastatic factor. In a PCa tissue microarray, CXCL14 expression was significantly higher in PCa cells that had metastasized to bone compared to normal prostate and extra-skeletal PCa metastasis. This increase in expression was not simply explained by the Gleason grade of the primary tumor. The bone microenvironment is therefore either regulating PCa CXCL14 expression, or rare PCa stem cells within the primary tumor overexpress CXCL14 render a selective advantage to metastasize to bone. The mechanism of increased PCa expression in bone is an area of future study.

CXCL14 may, in fact, reinforce the well-known biological effects of CXCL12, such as invasiveness and EMT, and exhibit at least a partial overlap in function during PCa bone metastasis. Normally, CXCL12 is secreted by bone marrow stromal cells in perivascular spaces [57] and directs the migration of CXCR4-expressing hematopoietic precursors to the bone marrow microenvironment [6, 58-61]. Prostate cancers exploit and hijack this signaling pathway to direct CXCR4-expressing PCa cells to the skeleton. PCa CXCR4 activation also increases EMT, via expression of *TWIST*, *SNAIL* and *SLUG* [62-65]. CXCL14 exhibited a similar response in the PCa cell line ARCaP$_M$, but not PC-3 and C4-2B PCa cells, with increased invasiveness and an EMT response. The differential effects of CXCL14 are aligned with other studies reporting CXCL14 pleiotropic effects that are dependent on context and cell type. One notable example is a study that examined two non-small lung cancer cell lines. A high-affinity CXCL14 receptor was present in both, but CXCL14 treatment elicited unrelated effects between the two cell lines [40].

Initiation of signaling pathways by CXCL14 is likely occurring through chemokine receptor activation resulting in activation of kinase signaling pathways that include ERK, AKT, SAPK/JNK, and NF-κB. The elusive CXCL14 receptor had been reported to be CXCR4. Tanegashima, *et al* reported that a high affinity binding site or sites exists on the human monocytic cell line THP-1 and that knockdown of CXCR4 in this cell line reduced CXCL14 high-affinity binding [66]. Moreover, CXCL14 was reported to co-immunoprecipitate with CXCR4. The conclusions of this report were disputed by Otte, *et al* asserting that CXCL14 is not a CXCR4 ligand [67]. In this subsequent report, CXCL14 did not modulate CXCL12-mediated CXCR4 receptor phosphorylation, GPCR Ca2+ signaling, ERK phosphorylation, or CXCL12-mediated CXCR4 receptor internalization. In the study reported here, CXCL14 did not interact with CXCR4 in a luminescent complementation assay, and CXCL14 was unable to recruit β-arrestin 2 to CXCR4. However, in the microfluidic migration assay, CXCL14 was clearly dependent on

CXCR4 for the migratory response.

These apparent inconsistencies as to whether CXCL14 is a true CXCR4 ligand or not can be reconciled with a model in which the putative CXCL14 chemokine receptor heterodimerizes with CXCR4. Although CXCL14 may not directly bind CXCR4, its actions may be dependent on CXCR4. Precedence for CXCR4 heterodimers with CXCR7, CCR2, CCR5, the cannabinoid receptor 2 (CB2), and the opioid receptor delta have been reported [68-72]. The previously published reports of Tanegashima, *et al* and Otte, *et al* would be consistent with this hypothesis. Another consideration is that CXCL14 forms a heterodimer with a chemokine other than CXCL12, and that crosstalk within these multimers allows for regulation of chemokine receptors in response to stimuli. Because CXCL14 demonstrated no significant direct interaction with CXCR4, the effects on CXCR4 signaling pathway may occur through multimers in complex with the CXCL14 receptor or receptors. Future work is needed to identify the true CXCL14 receptor.

Bone metastasis is a painful consequence of PCa in men with advanced disease. The chemokine CXCL14 has been identified as a marker of PCa bone metastasis. CXCL14 may in fact reinforce CXCL12-mediated events only after arrival of PCa to bone, promoting invasion, metastasis, and residency of tumor cells in bone. The identification of the CXCL14 receptor, combined with understanding of the shared and dissimilar actions with CXCL12, is a new research avenue that may lead to a novel therapeutic target in men with metastatic PCa.

Conflict of Interest
The authors disclose no potential conflicts of interest.

Acknowledgements

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Figure Legends

Figure 1. CXCL14 PCa expression is increased in human bone metastasis. A PCa tissue microarray was immunostained for CXCL14. A) The combined CXCL14 staining H-score was determined. Representative examples of CXCL14 stained samples along with secondary antibody-only controls are shown: normal prostate, benign prostatic hypertrophy (BPH), primary PCa (1° PrCa), lymph node metastasis (LN), soft tissue metastasis (Soft Tiss) and bone metastasis. B) CXCL14 H-score of primary PCa subdivided into Gleason stage. Error bars indicate standard deviation (SD) from the mean. **** $p \leq 0.0001$, NS = not significant.

Figure 2. CXCL14 promoted *in vitro* migration and invasion of human PCa cell lines. (A) Representative images from a scratch migration assay using ARCaP$_M$ and PC-3 PCa cells following treatment with CXCL14 (250 ng/ml) or no treatment (NT). (B) Invasion assays of ARCaP$_M$, PC-3, and C4-2B PCa cells were performed using transwell cell culture chambers coated with a cellular matrix. Cancer cells were plated above the membrane and CXCL14 250 ng/ml was present in the bottom chamber. Results are shown are after 72 hours of treatment. *** $p \leq 0.001$, NS = not significant

Figure 3. CXCL14 regulated the expression of EMT-associated genes. RT-PCR was performed to assess EMT-regulated genes modulated by CXCL14 (10, 100, and 250 ng/ml) in (A) ARCaP$_M$ and (B) PC-3 cells. After 24 hours of treatment, RNA was subjected to RT-PCR using specific primers for human *SLUG*, *SNAIL*, and *TWIST*. Messenger RNA concentration was normalized to expression of the *RPL32* housekeeping gene. * $p<0.05$, *** $p <0.001$, **** $p <0.0001$, NT = no treatment.

Figure 4. CXCL14 increased MAPK and PI3K pathway phosphorylation of PCa cells. (A) PC-3 and (B) ARCaP$_M$ PCa cells were serum starved for 8 hours. PCa Cells were treated with CXCL14 250 ng/ml and compared to no treatment (NT) for the indicated time (10, 30, 60, and 120 minutes) before cells were lysed. Cell lysates were subjected to western blotting. Lysates were examined for pErk1,2, pAKT, pNF-kB and pSAPK/JNK; total protein corresponding to each phosphoprotein was used as a loading control.

Figure 5. CXCL14 does not recruit β-arrestin 2 to CXCR4. (A) MDA-MB-231 cells that stably expressed CXCR4-CBRN/β-arrestin 2-CBC were treated with CXCL14 50 and 250 ng/ml for 10 minutes then subjected to western blot analysis. Blots were analyzed for phosphorylation of AKT, as a control for CXCL14 activity. (B) Bioluminescence assays were performed. Cells were treated with increasing concentrations of CXCL14 alone, CXCL12 alone, or in combination at (B) 60 ng/ml and (C) 250 ng/ml for one hour. Photon flux values for each time point then were normalized to values obtained for control cells not incubated with chemokine at each time point through 90 min. CXCL12, but not CXCL14, 60 and 250 ng/ml increased CXCR4/β-arrestin 2 recruitment. The addition of CXCL14 did not alter CXCL12-mediated CXCR4/β-arrestin 2 recruitment. (D) The availability of surface CXCR4 after chemokine treatment was determined. MDA-MB-231 CXCR4-CBRN/β-arrestin 2-CBC cells were treated with CXCL12 or CXCL14 at

increasing concentrations for one hour. Then, all groups received CXCL12 200 ng/ml. Bioluminescence was assessed and the peak signal was plotted for each chemokine concentration. CXCL14 pretreatment did not alter CXCL12-mediated recruitment of β-arrestin 2 to CXCR4. However, increasing CXCL12 treatment concentrations reduced β-arrestin 2 recruitment to CXCR4. Data are expressed as mean values ± SEM for fold change relative to control (n=4 per point).

Figure 6. CXCL14 forms a homodimer, but does not interact with CXCR4 or CXCR7. CXCL14 luciferase complementation fusion genes were constructed to investigate the ligand and receptor physical interactions. HEK-293 cells were transfected with combinations of CXCL14, CXCL12, CXCR4 and CXCR7, each fused to either the LgBit or SmBit of NanoLuc luciferase. Both secreted and cell-associated fractions were tested for bioluminescence. (A) The absolute amount of luminescence was altogether less in the cell-associated compared to the secreted fraction. The inset box is an expanded view of the secreted data from below. Compared to CXCL14 or CXCL12 co-transfected with an irrelevant protein (irrel), CXCL14 and CXCL12 homodimers (homo) were detected in the secreted and cell-associated fractions. The existence of a CXCL12/CXCL14 heterodimer (hetero) was detected in the cell-associated fraction. (B) As has been previously reported, CXCL12 associated with CXCR4 and CXCR7. A similar interaction was not detected with CXCL14.

Figure 7. CXCL14 is dependent on CXCR4 for cellular migration. A microfluidic cell migration assay was utilized to assess the dependence of CXCR4 in CXCL14-mediated cellular migration. For all conditions, four replicates were performed. The breast cancer cell line SUM159 (WT) underwent CXCR4 knockout (KO); KO cells also underwent re-expression of CXCR4 (Add-Back). SUM159 WT, KO, and Add-Back were applied to microfluidic devices with the addition of CXCL14 10 nM (94 ng/ml) at the end of 1000 µm channels. Cells were imaged 24 hours after the addition of CXCL14. Microfluidic devices contained 300 channels and a scatter plot indicating the final location of individual cells is shown. The mean is shown as horizontal lines within each scatter plot. Statistical analyses were preformed using two-way ANOVA of the means with a Tukey's multiple comparison test. Significant differences were detected between the CXCL14 WT vs. KO, and KO vs. Add-Back.

Supplement Figure 1. Microfluidic migration assay validation. Comparison between motile MDA-MB-231 and non-motile T47D breast cancer cell lines. In these conditions, 4 replicates were performed. The box graphs were plotted using Origin 9.0.
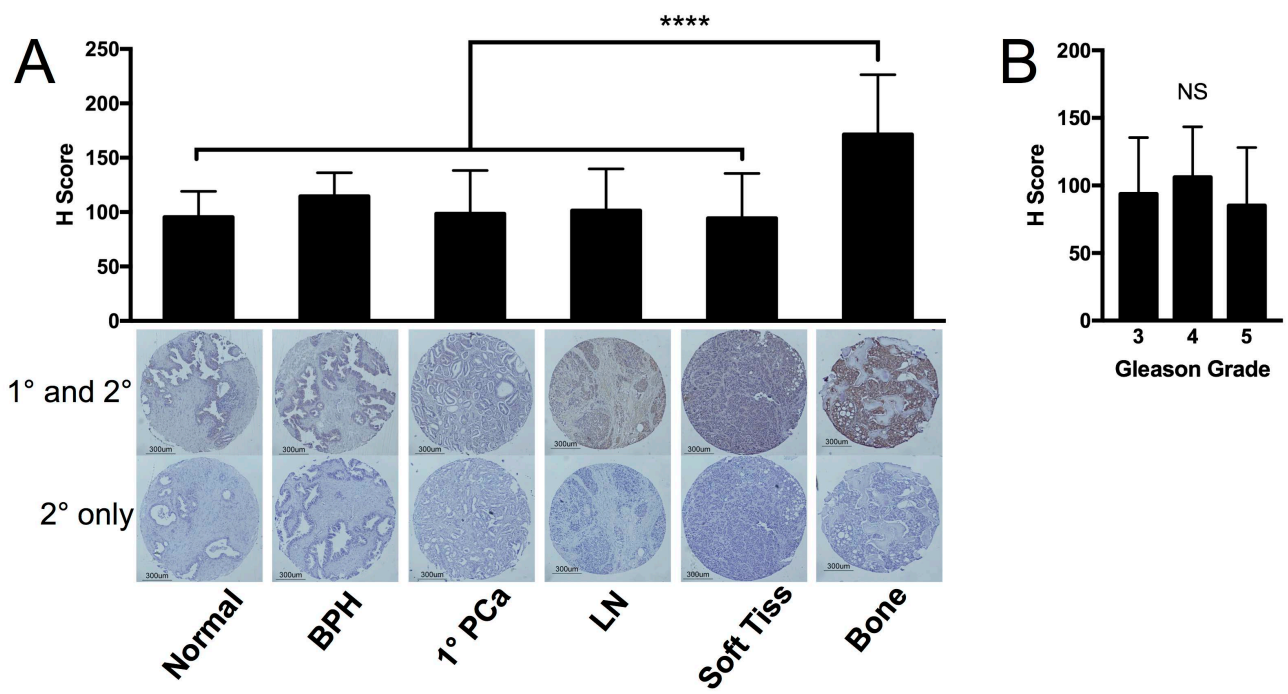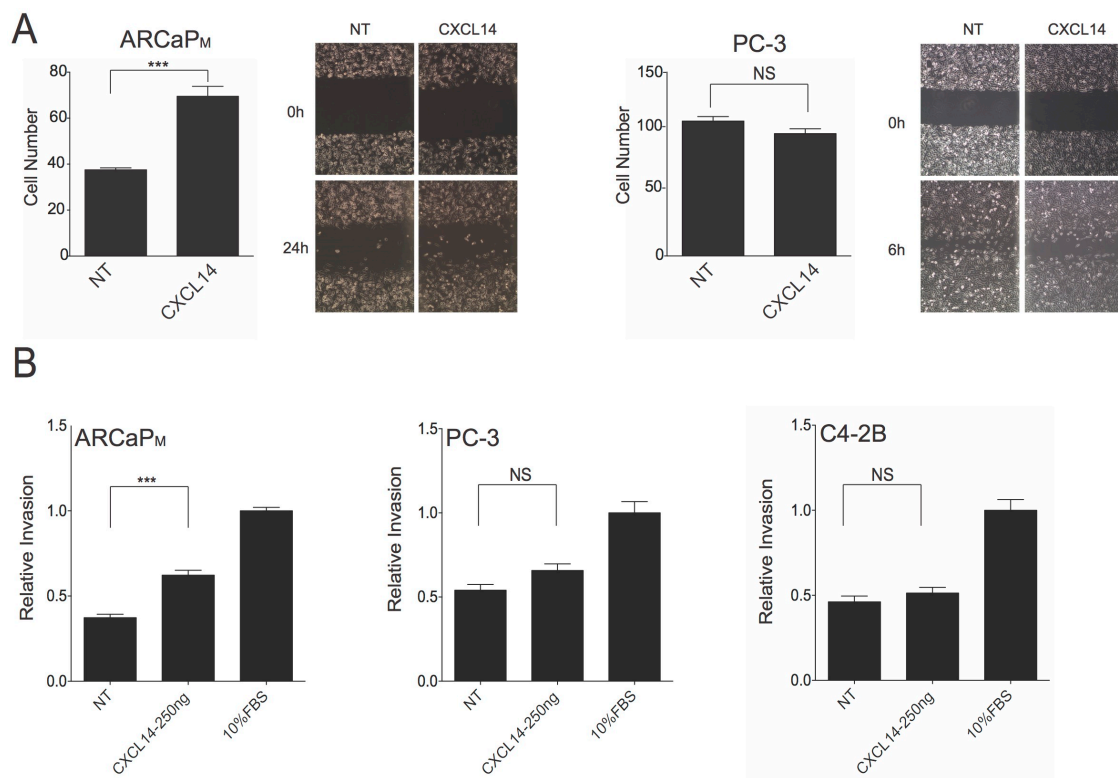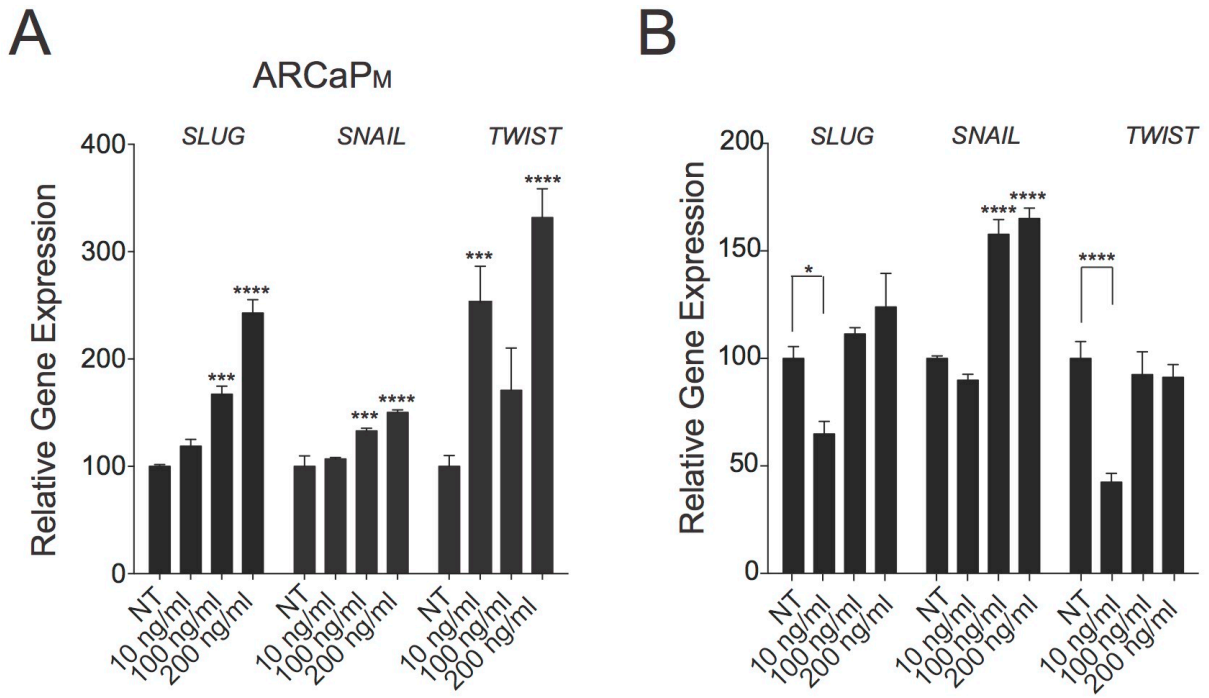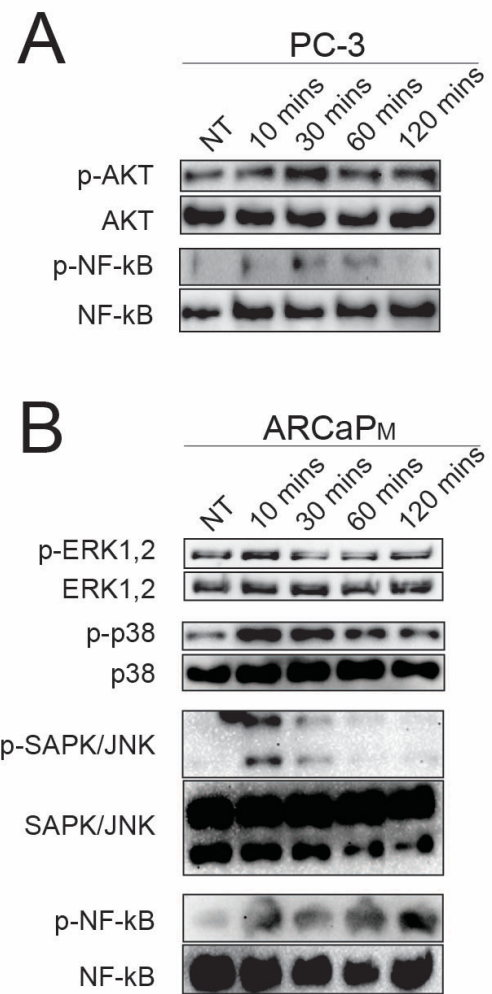
Figure 1.

Figure 2.

Figure 3.

Figure 4.

A PC-3

	NT	10 mins	30 mins	60 mins	120 mins

p-AKT

AKT

p-NF-kB

NF-kB

B ARCaP$_M$

	NT	10 mins	30 mins	60 mins	120 mins

p-ERK1,2

ERK1,2

p-p38

p38

p-SAPK/JNK

SAPK/JNK

p-NF-kB
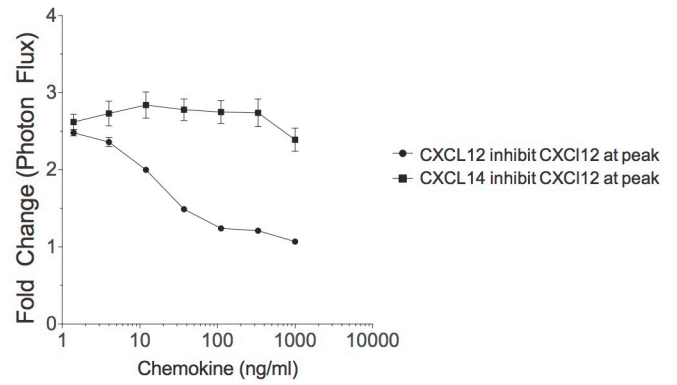
NF-kB

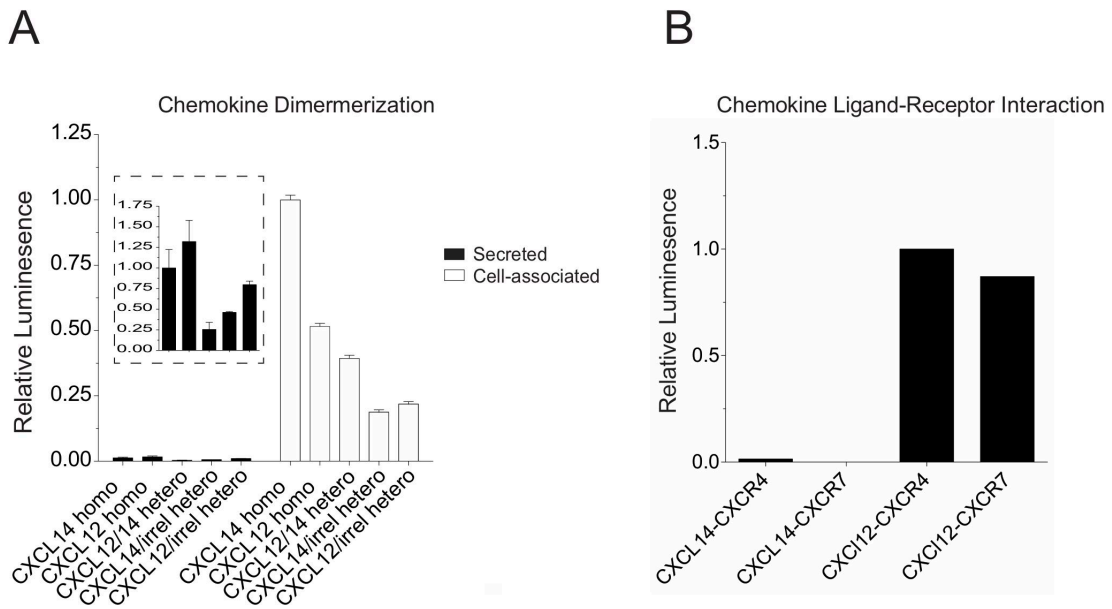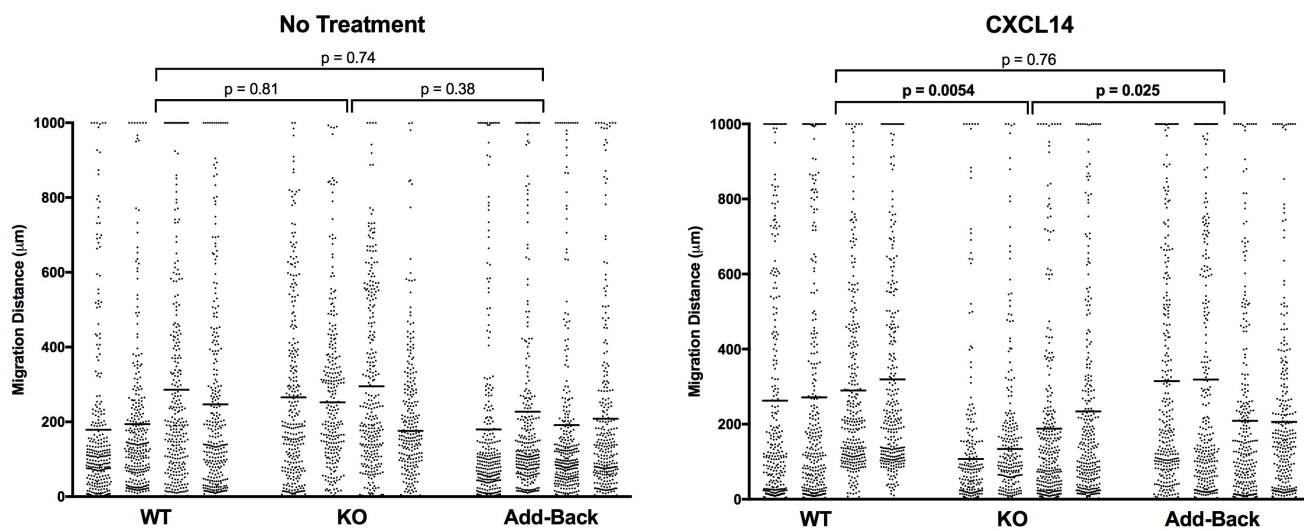Figure 5.

Figure 6.

A

Chemokine Dimermerization

B

Chemokine Ligand-Receptor Interaction
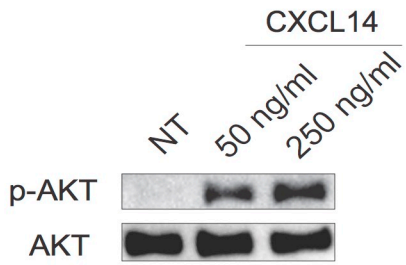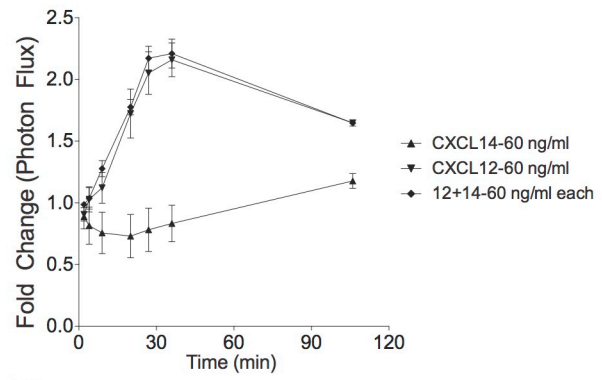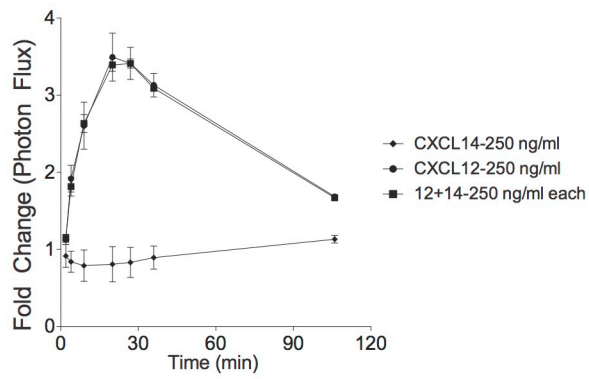
Figure 7.

Supplement Figure 1.