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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

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Table of Contents

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

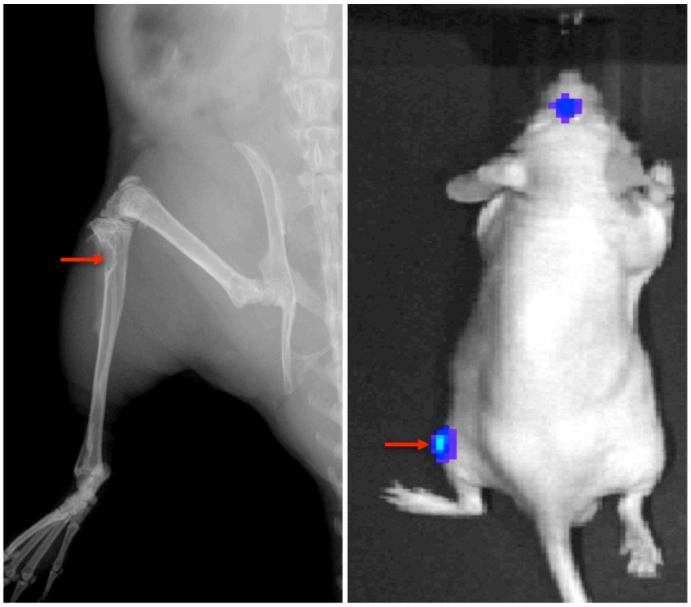
Major goals:

1. Determine the effects of prostate cancer CXCL14 overexpression to reduce skeletal lesions in an animal model of prostate cancer bone metastasis
2. Determine mechanisms of CXCL14 expression regulation in prostate cancer
3. Determine the cellular effects of CXCL14
   a. Determine the effects of CXCL14 on β-arrestin 2 recruitment to CXCR4
   b. Determine the extent to which CXCL14 associates as a dimer with itself and CXCL12
   c. Determine the effects of CXCL14 on *in vitro* prostate cancer migration and invasion
   d. Identify novel physical interactions with CXCL14

Accomplishments

1. Determine the effects of prostate cancer CXCL14 overexpression to reduce skeletal lesions in an animal model of prostate cancer bone metastasis
   Using prostate cancer PC-3 cells that overexpress luciferase, we have generated these PC-3 cells to also overexpress CXCL14 or an empty vector control. After intracardiac inoculation, the development of bone and soft tissue lesions was monitored using *in vivo* bioluminescent imaging. The experiment ended 13 weeks after intracardiac inoculation. An example of the same lesion as seen by X-ray and IVIS is shown (**Fig. 1**). 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 (**Fig. 1**).

The conclusion from the data generated suggests that overexpression of CXCL14 does not alter homing of prostate cancer cells to bone and/or expansion of prostate cancer cells in the skeleton.



	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

Fig. 1. Prostate cancer PC-3 overexpression of CXCL14 in an animal model of prostate cancer bone metastasis

2. Determine mechanisms of CXCL14 expression regulation in prostate cancer
As reported in the proposal, we examined CXCL14 protein expression by immunohistochemistry in a comprehensive human prostate cancer tissue microarray (TMA) containing 30 normal prostate samples, 61 primary prostate tumors, 57 soft tissue metastases and 113 bone metastases. CXCL14 expression was significantly

higher in prostate cancer bone metastases compared to soft tissue metastases, primary prostate tumors and normal prostate (**Fig. 2**).

There are a number of possible reasons for increased CXCL14 expression in bone.
A hypoxic environment of 1-7% O_2 is created within bone [1, 2] that promotes the development of bone metastasis [3-6]. Hypoxia induces activation of HIF-1α signaling that targets genes containing hypoxia response elements.

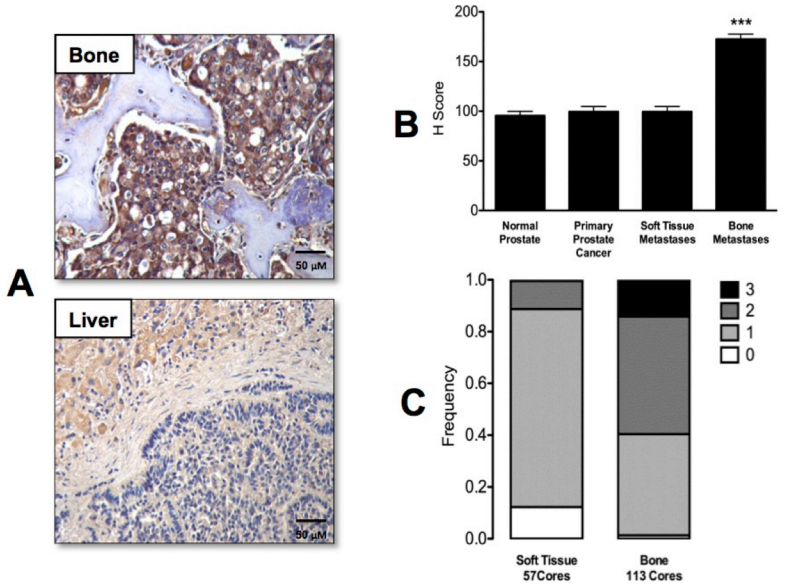


Fig. 2. CXCL14 prostate cancer expression is increased in human bone metastasis compared to soft tissue metastasis, primary tumor and normal prostate. (A) Examples of human bone and liver prostate cancer metastases immunostained for CXCL14. (B) Combined CXCL14 staining score (H-score) of a tissue microarray representing 30 normal prostate samples, 61 primary prostate tumors, 57 soft tissue metastases and 113 bone metastases. (C) Detailed CXCL14 staining intensity in soft tissue metastases vs. bone metastases. Staining intensity (0-absent; 1+ faint; 2+ moderate; 3+ intense) and percentage of cells at the staining intensity was determined. H-score = 1X (% 1+ cells) + 2X (% 2+ cells) + 3X (% 3+ cells). *** = p<0.001.

We examined the effects of hypoxia on CXCL14 expression in the $ARCaP_M$ prostate cancer cell line. The expression of other genes related to CXCL14 was also examined. CXCL14 expression increased in 1% and 5% O2 compared to normoxia (21%) (**Fig. 3**). Despite this relative increase in cellular mRNA concentration, the absolute amount of CXCL14 mRNA remained low. The RT-PCR Ct value of CXCL14 in

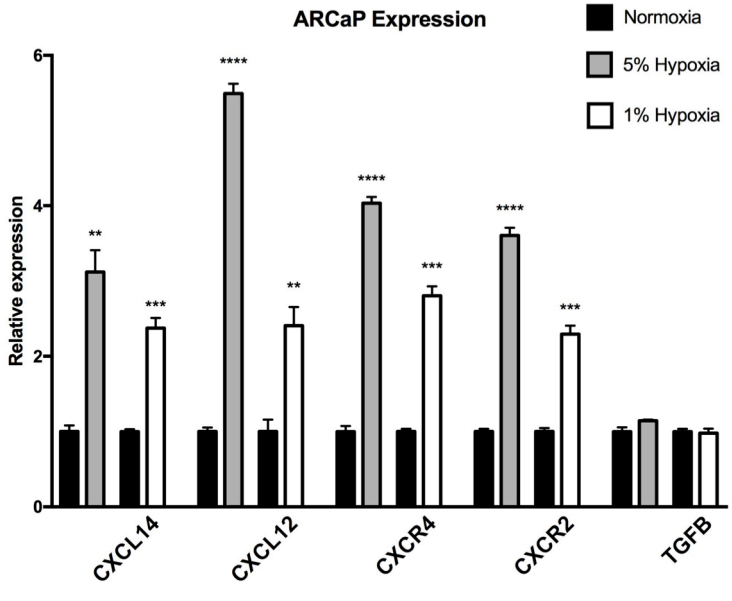


Fig. 3. Expression of select chemokine pathway members. $ARCaP_M$ cells were grown in normoxia (21%), and hypoxia conditions of 5% and 1% O_2. Real-time RT-PCR was performed. The Ct values were normalized to the RPL32 housekeeping gene.

hypoxia was approximately 28 comparted to a Ct value of approximately 15 with the RPL32 housekeeping gene used for normalization.

We next measured CXCL14 protein in conditioned media of the $ARCaP_M$ and PC-3 prostate cancer cell lines in normoxia and 5% hypoxia. Using an ELISA assay with a sensitivity of ~31 pg/ml, we were unable to detect CXCL14 protein in the conditioned media of the conditions described. Western blots were then performed on cell lysates using a CXCL14 antibody. Unfortunately, the quality of commercial antibodies are poor and were unable to detect CXCL14 in prostate cancer cell lysates.

3. Determine the cellular effects of CXCL14

a. Determine the effects of CXCL14 on β-Arrestin 2 Recruitment to CXCR4

Controversy has existed as to the identity of the CXCL14 receptor. 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 [(7)]. Moreover, CXCL14 was reported to co-immunoprecipitate with CXCR4. The conclusions of this report were disputed by Otte *et al* that asserted that CXCL14 is not a CXCR4 ligand and that CXCL14 did not modulate CXCL12-mediated CXCR4 receptor phosphorylation, GPCR Ca^{2+} signaling, ERK phosphorylation, or CXCL12-mediated CXCR4 receptor internalization [(8)]. In a more recent report, Collins *et al* propose a mechanism in which CXCL14 binds to a CXCR4 homodimer that displaces CXCL12 from the adjacent CXCR4 dimer and effectively downregulates CXCL12 activation of CXCR4 [(9)].

The availability of tools to adequately study CXCL14 and CXCR4 signaling in prostate cancer has been lacking. We therefore utilized research resources currently available to better understand mechanisms of CXCL14 cellular action. This work was performed in collaboration with Dr. Gary Luker, a collaborator in this proposal. To assess CXCL14-dependent recruitment of β-arrestin 2, the validated MDA-MB-231 click beetle green luciferase complementation reporter system was used to examine mechanisms of CXCL14 signaling [(10-12)]. 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 β-arrestin 2 and joins the incomplete luciferase products to generate an active luciferase molecule.

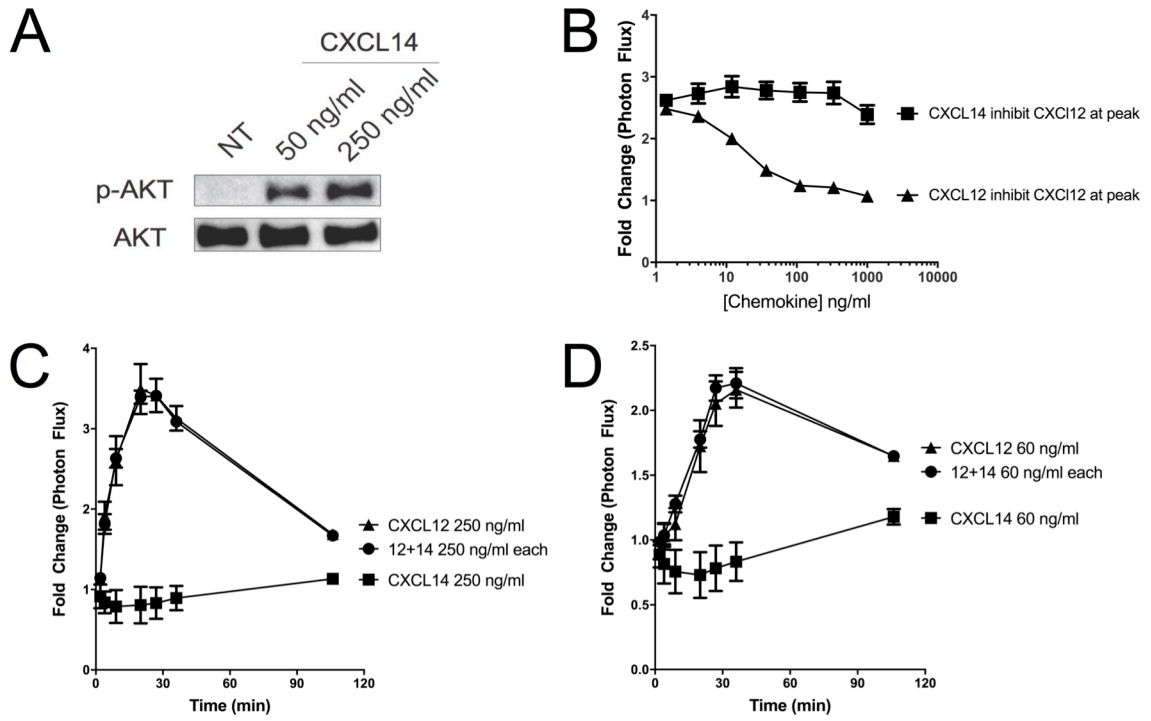


Fig 4. CXCL14 does not recruit β-arrestin 2 to CXCR4. (**A**) MDA-MB-231 cells that stably expressed CXCR4-CBRN/β-arrestin 2-CBC were treated with 50 or 250 ng/ml CXCL14 for 10 minutes then subjected to western blot analysis. Blots were analyzed for phosphorylation of AKT, as a positive control for CXCL14 activity. 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, at 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 200 ng/ml CXCL12. 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).

As a control for CXCL14 biologic activity, the transduced MDA-MB-231 cells were treated with CXCL14. A marked increase in AKT phosphorylation at 10 minutes was observed (**Fig. 4A**). Cells that stably expressed the complementation reporters were treated with CXCL14 and CXCL12 individually and combined at concentrations of 60 ng/ml (**Fig. 4B**) and 250 ng/ml (**Fig. 4C**). 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 to CXCL12 did not lessen the ability of CXCL12 to recruit β-arrestin 2 to CXCR4.

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 availability of surface CXCR4 following CXCL12 treatment was determined using the MDA-MB-231 CXCR4-CBRN/β-arrestin 2-CBC reporter system. The reporter cells

were pretreated with either CXCL12 or CXCL14 in increasing concentrations for one hour followed by a subsequent CXCL12 200 ng/ml treatment at which time luminescence was monitored (**Fig. 4D**). 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 showed concentration-dependent decreases in further luminescence produced by adding additional CXCL12 that is consistent with receptor internalization and desensitization of signaling. These data suggested that CXCL14 does not promote CXCR4 receptor internalization and that CXCR4 is not a CXCL14 receptor.

b. Determine the extent to which CXCL14 associates as a dimer with itself and CXCL12

CXCL12 exists in both monomeric and dimeric forms at physiologic concentration [13, 14]. Each of these CXCL12 species activates CXCR4 but differs in the downstream signaling pathways activated [14]. The extent to which CXCL14 exists as a homodimer or heterodimer with other chemokines such as CXCL12 was investigated. A similar complementation strategy as reported above, using the NanoLuc luminescent complementation reporter system, was utilized to examine these interactions. CXCL14, CXCL12, and an irrelevant control protein were cloned in-frame, each receiving adjacent NanoLuc large fragment (LgBit) or the NanoLuc small fragment (SmBit). The combination of the LgBit and SmBit results in a complete and active luminescent protein. HEK-293T cells were co-transfected with various combinations of CXCL14, CXCL12, and the irrelevant protein, followed by luminescent imaging of intact cells and of the conditioned media. This represented a strategy to detect homo- and heterodimer species located within the cellular fraction and in the media. Increased luminescence above what was detected in the chemokine/irrelevant protein combinations was evidence for an interaction.

In the cellular fraction, both CXCL12 and CXCL14 homodimer luminescence was significantly greater than the chemokine/irrelevant heterodimer control indicating the existence of CXCL12 and CXCL14 homodimers (**Fig. 5A**). A CXCL12/CXCL14 heterodimer was also detected, albeit at levels lower than either homodimer. Luminescence was also detected in the conditioned media of cells but at a lower concentration compared to the cell-associated fraction. In the secreted fraction, the luminescence level trended higher for both the CXCL12 and CXCL14 homodimer group but the level was not statistically different compared to the chemokine/irrelevant heterodimer controls. Similarly, the existence of a CXCL12/CXCL14 heterodimer was not detected in the secreted fraction. These data indicate that CXCL12 and CXCL14 homodimers, and a CXCL12/CXCL14 heterodimer exist at higher concentrations within secretory vesicles, but that once secreted from the cell the chemokines revert to monomers.

CXCR4 and CXCR7 (also known as ACKR3) are receptors for CXCL12 [15, 16]. 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 (**Fig. 5B**). However, an interaction between CXCL14 and the two tested chemokine receptors was not detected.

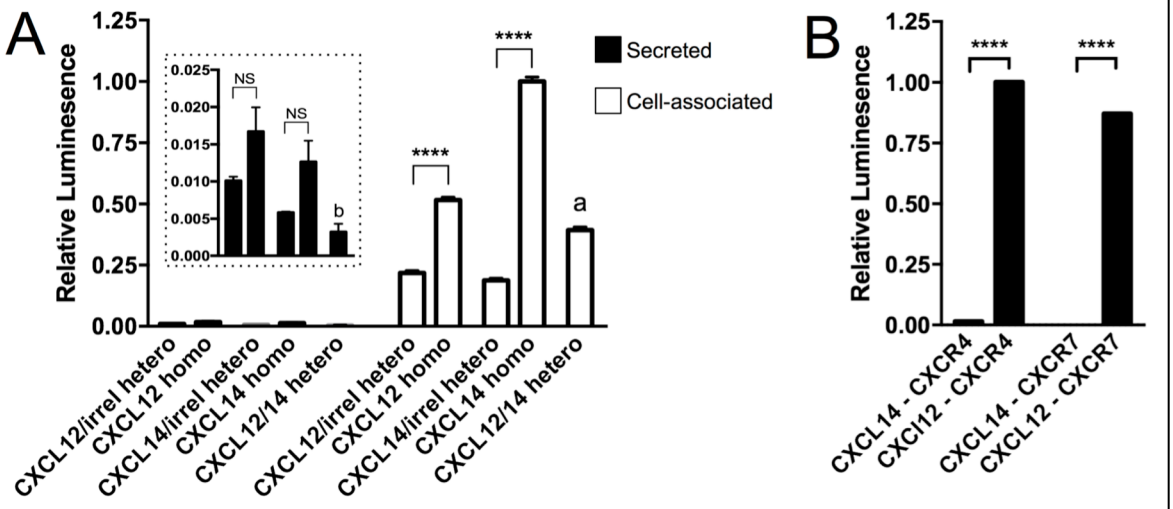


Fig. 5. CXCL14 forms a homodimer, but does not interact with CXCR4 or CXCR7. CXCL14 luciferase complementation fusion genes were constructed to investigate the ligand and receptor physical interactions. (**A**) HEK-293T cells were transfected with combinations of CXCL14 or CXCL12, fused to either the LgBit or SmBit of NanoLuc luciferase. Both secreted and cell-associated fractions were tested for bioluminescence. 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 cell-associated but not the secreted fractions. The existence of a CXCL12/CXCL14 heterodimer (hetero) was detected in the cell-associated fraction. (**B**) HEK-293T cells transfected with CXCL12 or CXCL12 fused to SmBit were co-cultured with equal numbers of cells expressing LgBit-CXCR4 or -CXCR7. As has been previously reported, CXCL12 associated with CXCR4 and CXCR7. A similar interaction was not detected with CXCL14. Graphs show mean values ± SEM for fold change in NanoLuc luciferase activity relative to background control. **** $p \leq 0.0001$; NS = not significant; a = $p \leq 0.0001$ compared to CXCL12/irrel hetero and CXCL14/irrel hetero; b = NS compared to CXCL12/irrel hetero and CXCL14/irrel hetero

c. Determine the effects of CXCL14 on *in vitro* prostate cancer migration and invasion

Three different strategies were employed to determine the effects of CXCL14 to regulate prostate cancer migration and invasion: 1) scratch assays, 2) slide-based chemotaxis assays, and 3) slide-based invasion assays. The strategies were used to test the effects of both CXCL14 overexpression and CXCL14 treatment in the ARCaPM and PC-3 prostate cancer cell lines. We were unable to detect differences with CXCL14 overexpression or treatment on *in vitro* migration or invasion.

d. Identify novel physical interactions with CXCL14

Based on our preliminary data and hypothesis that CXCL12 and CXCL14 compete for the same receptor, the new data above suggests that this is not the case. CXCL14 does not compete with CXCL12 to bind with the chemokine receptor

CXCR4. We therefore currently do not know the receptor for CXCL14 or the downstream signaling pathways activated. Instead of assessing competition between CXCL12 and CXCL14, we instead focused on identifying the CXCL14 receptor. This was performed by identifying CXCL14 binding partners using chemical-modifying linkers and mass spectroscopy. The proteins identified (below) belong to the desmosomal complex. It is unclear to us at this time how a chemokine ligand would interact with the desmosome and such interaction has not been reported. However, this is an area of future study.

Keratin, type II cytoskeletal 1
Keratin, type II cytoskeletal 2 epidermal
Keratin, type I cytoskeletal 10
Keratin, type I cytoskeletal 9
Keratin, type II cytoskeletal 5
Keratin, type I cytoskeletal 14
Keratin, type II cytoskeletal 6C
Keratin, type I cytoskeletal 16
Keratin, type I cytoskeletal 17
Keratin, type I cytoskeletal 19
Keratin, type I cytoskeletal 28

Desmoglein-1
Desmoplakin
Junction plakoglobin
Keratinocyte proline-rich protein
Vimentin

Actin, cytoplasmic 1
Actin, cytoplasmic 2

Opportunities for training and professional development
Nothing to report

Dissemination of results to the communities of interest
We are currently preparing a manuscript that details much of the negative work reported here. It is however important to contribute to the understanding of CXCL14 with data presented here that CXCR4 is unlikely the receptor for CXCL14.

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
Based on our preliminary data and hypothesis that CXCL12 and CXCL14 compete for the same receptor, we have new data that this is not the case. CXCL14 does not compete with CXCL12 to bind with the chemokine receptor CXCR4. We therefore currently do not know the receptor for CXCL14 or the downstream signaling pathways activated. Because of this new data, we proposed a change to Task 1b. Instead of assessing competition between CXCL12 and CXCL14, we will instead focus on identifying the CXCL14 receptor. We have already begun this task by identifying CXCL14 binding partners using chemical-modifying linkers and mass spectroscopy. An initial screen identified the desmosomal complex.

Actual or anticipated problems or delays and actions to resolve them
Difficulties in the CXCL14 shRNA lentivirus were encountered. Despite another report of the successful mRNA downregulation of CXCL14 using a similar sequence, we have been unable to demonstrate a significant decline in mRNA concentration after transduction of the CXCL14 shRNA lentivirus in the prostate cancer cell line $ARCaP_M$. With the assistance of the University of Michigan Viral Vector Core, we generated an additional four CXCL14 shRNA lentivirus constructs that again were unsuccessful in CXCL14 mRNA knockdown. Because were unable to identify an shRNA that successfully knocked down CXCL14, we were unable to complete the experiments of Aim 2a.

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: We are currently preparing a manuscript that details much of the negative work reported here. It is however important to contribute to the understanding of CXCL14 with data presented here that CXCR4 is unlikely the receptor for CXCL14.

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

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