AWARD NUMBER: W81XWH-16-1-0174

TITLE: A Novel Immune-Intact Mouse Model of Prostate Cancer Bone Metastasis: Mechanisms of Chemotaxis and Bone Colonization

PRINCIPAL INVESTIGATOR: Srinivas Nandana

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA, 90048

REPORT DATE: October 2017

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The proposed studies will help dissect the interplay between CXCL12/ CXCR4 and RANKL/ RANK pathways in prostate cancer bone metastasis in the context of a host with an intact immune system.
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>4. Impact</td>
<td>7</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>7</td>
</tr>
<tr>
<td>6. Products</td>
<td>9</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>9</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>N/A</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>N/A</td>
</tr>
</tbody>
</table>
**INTRODUCTION:** Bone metastasis in human prostate cancer remains a major clinical problem since no effective therapy exists. The cascade of events leading to bone metastasis in human prostate cancer can be visualized as a 3 step process: 1) prostate tumor cells undergo epithelial-to-mesenchymal transition, escape the primary tumor and enter the blood circulation, 2) tumor cells travel in the blood stream and lodge in the bone through enhanced chemotaxis induced by soluble factors such as CXCL12 and its cognate receptor CXCR4 on the tumor cell surface, and 3) prostate tumor cells, after homing to the bone, interact with cells in the bone microenvironment and release factors that trigger a vicious cycle by causing further bone destruction and new bone formation, causing excruciating bone pain and bone fractures in prostate cancer patients. The RANKL/RANK pathway plays a predominant role in the interaction between metastasized prostate cancer cells and osteoclasts that increases the bone turnover. The current therapies, including targeting RANKL with denosumab, address the growth of prostate tumor cells that have already colonized the bone, and are largely ineffective in prolonging the survival of human prostate cancer patients with bone metastasis. Further, a major impediment to prostate cancer bone metastasis research is the lack of an animal model that spontaneously recapitulates human prostate cancer bone metastasis in the context of an intact immune system. To overcome this major limitation, we have made a significant advance by developing a novel immune-intact mouse model to study prostate cancer bone metastasis. Both the CXCL12/CXCR4 and RANKL/RANK pathways have been reported to be overexpressed/dysregulated in human prostate cancer bone metastatic samples. Data generated utilizing our immune-intact mouse model shows that the CXCL12/CXCR4 and RANKL/RANK pathways co-operate with each other to drive prostate cancer bone metastasis. Studies have shown that targeting the CXCL12/CXCR4 and RANKL/RANK pathways individually affects the immune system, thereby making our immune-intact mouse model an indispensable tool for studying the critical co-operation between these 2 pathways in the manifestation of human prostate cancer bone metastasis.

**KEYWORDS:** Prostate Cancer, Bone Metastasis, epithelial-to-mesenchymal transition, RANKL/RANK, CXCL12/CXCR4, Immune-intact mouse model

**ACCOMPLISHMENTS:** We were able to accomplish Major Task 1, Subtask 2 of Specific Aim 1 (listed below), i.e. We submitted documents for ACURO and IACUC approvals. We have obtained the IACUC approval, and are awaiting ACURO approval.

| Specific Aim 1: Determine RANKL/RANK and CXCL12/CXCR4 signaling convergence in PCa bone metastasis in the context of an intact immune system. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and validate these results in human PCa bone metastatic specimens. | 1-12 months | Drs. Nandana, Zhau, Shiao, Posadas and Tighiouart |
| Major Task 1: Characterize CXCL12/CXCR4 and RANKL/RANK downstream signaling components in cell lines with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways. Obtain regulatory approvals. | 1-6 months | |
| Subtask 1: | | |
| a) We will characterize all the cell lines for expression of PI3K, AKT and NF-κB, as well as markers of epithelial-mesenchymal transition (EMT) [Twist 1, Slug, Zeb1, Zeb2], stemness [Sox2, Myc, Oct3/4 and Nanog] and NE phenotype [Sox9, HIF1α and FoxA2] by quantitative real-time RT-PCR and Western blotting analysis. | 1-6 months | Drs. Nandana and Tighiouart |
| b) Invasion assays of the cell lines and their respective controls in the presence of CXCL12. | | |
| c) Soft agar colony (SAC) formation assay of the cell lines and their respective control cells with bone marrow cells (obtained from | | |
bone-marrow flush) from wildtype C57/Bl6 or FVB mice respectively.

**Cell lines used:** MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal).

| **Subtask 2:** Submit documents for ACURO and IACUC approvals. Submit documents for USAMRMC ORP HRPO and IRB approvals. | 1-6 months | Dr. Nandana |
| **Milestone(s) Achieved:** Characterized signaling convergence in vitro. Characterized signaling convergence in PCa xenograft models and human PCa bone met specimens. Obtained ACURO, IACUC, USAMRMC ORP HRPO and IRB approvals. | Month 6 |

**Major Task 2:** Determine RANKL/RANK and CXCL12/CXCR4 signaling convergence in PCa homing and growth in bone microenvironment through *in vivo* studies. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and in human PCa bone metastasis specimens.

**Subtask 2:** Characterize the expression pattern of RANKL/RANK and CXCL12/CXCR4 in murine models of PCa bone metastasis and in patients with PCa bone metastasis by immunohistochemistry and multiplex quantum dot labeling (mQDL) approach.

**Milestone(s) Achieved:** Characterized signaling convergence in vivo. Characterized gene expression of CXCL12/CXCR4 and RANKL/RANK in mouse models and human samples.
### Specific Aim 1: Determine RANKL/RANK and CXCL12/CXCR4 signaling convergence in PCa bone metastasis in the context of an intact immune system. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and validate these results in human PCa bone metastatic specimens.

#### Major Task 1: Characterize CXCL12/CXCR4 and RANKL/RANK downstream signaling components in cell lines with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways. Obtain regulatory approvals.

**Subtask 1:**

- **d)** We will characterize all the cell lines for expression of PI3K, AKT and NF-κB, as well as markers of epithelial-mesenchymal transition (EMT) [Twist 1, Slug, Zeb1, Zeb2], stemness [Sox2, Myc, Oct3/4 and Nanog] and NE phenotype [Sox9, HIF1α and FoxA2] by quantitative real-time RT-PCR and Western blotting analysis.
- **e)** Invasion assays of the cell lines and their respective controls in the presence of CXCL12.
- **f)** Soft agar colony (SAC) formation assay of the cell lines and their respective control cells with bone marrow cells (obtained from bone-marrow flush) from wildtype C57/Bl6 or FVB mice respectively.

**Cell lines used:** MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal).

**Subtask 2:** Submit documents for ACURO and IACUC approvals. Submit documents for USAMRMC ORP HRPO and IRB approvals.

<table>
<thead>
<tr>
<th>Milestone(s) Achieved: Characterized signaling convergence in vitro. Characterized signaling convergence in PCa xenograft models and human PCa bone met specimens. Obtained ACURO, IACUC, USAMRMC ORP HRPO and IRB approvals.</th>
<th>Month 6</th>
</tr>
</thead>
</table>

#### Major Task 2: Determine RANKL/RANK and CXCL12/CXCR4 signaling convergence in PCa homing and growth in bone microenvironment through *in vivo* studies. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and in human PCa bone metastasis specimens.

**Subtask 1:** Determine RANKL/RANK and CXCL12/CXCR4 signaling convergence in PCa homing and growth in bone microenvironment through *in vivo* studies.

Experimental groups will be injected in wildtype C57/Bl6 or FVB mice, mice will be monitored for 5 weeks by imaging techniques including luciferase, X-ray and micro CT analysis and mice will be harvested. The following studies will be performed:

1. Bone marrow flushes from hind limbs to harvest the GFP-tagged tumor cells and analyze gene expression changes compared with the corresponding cells prior to injection. We will particularly look at CXCR4 and cell signaling network markers relevant to the expression of EMT, stem, and neuroendocrine phenotypes, as well as downstream signaling components including PI3K, AKT, and NF-κB.
2. Analyze the bone marrow flush by flow cytometry for various immune cell populations.
3. Flow sorting of T cells and myeloid cells for *ex-vivo* cytokine analysis and expression analysis to determine the polarization status of these populations.
4. Histology and histomorphometry - in bone metastases that will not be flushed to study the nature of the bone metastases.

<table>
<thead>
<tr>
<th>Milestone(s) Achieved: Characterized signaling convergence in PCa bone metastasis specimens.</th>
<th>Drs. Nandana, Zhau, Shiao, Tighiouart</th>
</tr>
</thead>
</table>

---
### IMPACT:
Nothing to Report

### CHANGES/PROBLEMS:
The institutional IACUC approval for this project was delayed, and we are still awaiting ACURO approval. I talked to Dr. Nrusingha Mishra on the phone, and he suggested that I go ahead and submit the Annual Report describing the problems encountered, and submit a Revised SOW with the new start date (please see below). Since the money for the grant has not been spent – the Grant Award was activated by Cedars-Sinai Medical Center only on Sep 18th, 2017 instead of the original start date Sep 30, 2016 – Dr. Mishra suggested that it would be possible to acquire an extension to the contract period originally ending on Sep 29 2020.

### Revised Statement of Work (SOW):

#### PROPOSED START DATE Sep 30, 2016
A
LTUAL START DATE – Sep 18, 2017

| Site: | Uro-Oncology, Dept of Medicine  
Cedars-Sinai Medical Center  
8700 Beverly Blvd, Los Angeles, CA, 90048 |
|---|---|
| PI: | Srinivas Nandana, PhD  
Consultant: Leland Chung, Ph.D.  
Co l’s: Haiyen Zhau, Ph.D.,  
Stephen Shiao, MD, Ph.D.,  
Edwin Posadas, MD,  
Mourad Tighiouart, Ph.D. |

### Specific Aim 1: Determine RANKL/RANK and CXCL12/CCR4 signaling convergence in PCa bone metastasis in the context of an intact immune system. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and validate these results in human PCa bone metastatic specimens.

**Drs. Nandana, Zhau, Shiao, Posadas and Tighiouart**

| 1-12 months |  |

**Milestone(s) Achieved:** Characterized signaling convergence in vivo. Characterized gene expression of CXCL12/CXCR4 and RANKL/RANK in mouse models and human samples.

**Month 12**

### Major Task 1: Characterize CXCL12/CXCR4 and RANKL/RANK downstream signaling components in cell lines with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways. Obtain regulatory approvals.

**Drs. Nandana, Zhau, Shiao, Posadas and Tighiouart**

| 1-6 months |  |

### Subtask 1:

**g)** We will characterize all the cell lines for expression of PI3K, AKT and NF-κB, as well as markers of epithelial-mesenchymal transition (EMT) [Twist 1, Slug, Zeb1, Zeb2], stemness [Sox2, Myc, Oct3/4 and Nanog] and NE phenotype [Sox9, HIF1α and FoxA2] by quantitative real-time RT-PCR and Western blotting analysis.

**Drs. Nandana and Tighiouart**

| 1-6 months |  |
h) Invasion assays of the cell lines and their respective controls in the presence of CXCL12.

i) Soft agar colony (SAC) formation assay of the cell lines and their respective control cells with bone marrow cells (obtained from bone-marrow flush) from wildtype C57/Bl6 or FVB mice respectively.

Cell lines used: MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal).

<table>
<thead>
<tr>
<th>Subtask</th>
<th>Description</th>
<th>Timeframe</th>
<th>Responsible PI(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtask 2:</td>
<td>Submit documents for ACURO and IACUC approvals. Submit documents for USAMRMC ORP HRPO and IRB approvals.</td>
<td>1-6 months</td>
<td>Dr. Nandana</td>
</tr>
<tr>
<td>Milestone(s) Achieved:</td>
<td>Characterized signaling convergence in vitro. Characterized signaling convergence in PCa xenograft models and human PCa bone met specimens. Obtained ACURO, IACUC, USAMRMC ORP HRPO and IRB approvals.</td>
<td>Month 6</td>
<td></td>
</tr>
<tr>
<td>Major Task 2:</td>
<td>Determine RANKL/RANK and CXCL12/CCR4 signaling convergence in PCa homing and growth in bone microenvironment through in vivo studies. Characterize the expression of the two signaling pathways in murine models of PCa bone metastasis and in human PCa bone metastasis specimens.</td>
<td>7-12 months</td>
<td>Drs. Nandana, Zhau, Shiao, Tighiouart</td>
</tr>
</tbody>
</table>
| Subtask 1: | Determine RANKL/RANK and CXCL12/CCR4 signaling convergence in PCa homing and growth in bone microenvironment through in vivo studies. Experimental groups will be injected in wildtype C57/Bl6 or FVB mice, mice will be monitored for 5 weeks by imaging techniques including luciferase, X-ray and micro CT analysis and mice will be harvested. The following studies will be performed:  
3. Bone marrow flushes from hind limbs to harvest the GFP-tagged tumor cells and analyze gene expression changes compared with the corresponding cells prior to injection. We will particularly look at CXCR4 and cell signaling network markers relevant to the expression of EMT, stem, and neuroendocrine phenotypes, as well as downstream signaling components including PI3K, AKT, and NF-κB.  
8. Analyze the bone marrow flush by flow cytometry for various immune cell populations.  
9. Flow sorting of T cells and myeloid cells for ex-vivo cytokine analysis and expression analysis to determine the polarization status of these populations.  
10. Histology and histomorphometry - in bone metastases that will not be flushed to study the nature of the bone metastases. | 7-12 months | Drs. Nandana, Zhau, Shiao, Tighiouart |
| Cell lines used: | MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal). \([(15 mice for 6 groups) + (6 mice for 4 groups) = Total 114 mice]\) | |
| Subtask 2: | Characterize the expression pattern of RANKL/RANK and CXCL12/CXCR4 in murine models of PCa bone metastasis and in patients with PCa bone metastasis by immunohistochemistry and multiplex quantum dot labeling (mQDL) approach. | 7-12 months | Drs. Nandana, Zhau, Tighiouart |
| Milestone(s) Achieved: | Characterized signaling convergence in vivo. Characterized gene expression of CXCL12/CXCR4 and RANKL/RANK in mouse models and human samples. | Month 12 | |
| Specific Aim 2: | Determine the efficacy of targeting the RANKL/RANK and CXCL12/CXCR4 pathways individually or in combination for treatment of PCa bone metastases. | 13-36 months | Drs. Nandana, Zhau, Shiao, |
## Major Task 1: Determine the optimal concentration of RANK-Fc and AMD3100 for treatment of cells and mice. Use RANK-Fc and AMD3100 for treatment in vitro and in vivo in prostate cancer bone metastasis.

Subtask 1: Determine the *in vitro* efficacy of targeting the RANKL/RANK and CXCL12/CXCR4 signaling pathways individually or in combination to treat PCa bone metastases.

- a) Invasion assays of treated and untreated (control) cells in the presence of CXCL12.
- b) Soft agar colony formation assay of the treated and untreated (control cells) with bone marrow cells from wildtype C57/Bl6 or FVB mice respectively.

Cell lines used: MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal).

Subtask 2: Determine the *in vivo* efficacy of targeting the RANKL/RANK and CXCL12/CXCR4 signaling pathways individually or in combination to treat PCa bone metastases.

Experimental groups will be injected in wildtype C57/Bl6 or FVB mice, mice will be monitored for 5 weeks by imaging techniques including luciferase, X-ray and micro CT analysis and mice will be harvested. The following studies will be performed:

1. Bone marrow flushes from hind limbs to harvest the GFP-tagged tumor cells. We will particularly look at CXCR4 and cell signaling network markers relevant to the expression of EMT, stem, and neuroendocrine phenotypes, as well as downstream signaling components including PI3K, AKT, and NF-κB.
2. Analyze the bone marrow flush by flow cytometry for various immune cell populations.
3. Flow sorting of T cells and myeloid cells for *ex-vivo* cytokine analysis and expression analysis to determine the polarization status of these populations.
4. Histology and histomorphometry.

Cell lines used: MPC3 and Myc-CaP mouse prostate cancer cells with genetic modulation of CXCL12/CXCR4 and RANKL/RANK pathways (as detailed in Table 1 of proposal). [10 mice per group x 10 groups x 4 treatments = Total 400 mice]

Month 36

**Milestone(s) achieved:** Determined the efficacy of targeting the CXCL12/CXCR4 and RANKL/RANK pathways in prostate cancer bone metastasis *in vitro* and *in vivo* in the context of an intact immune system.

**PRODUCTS:** Nothing to Report

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:** No Change