AWARD NUMBER: **W81XWH-15-1-0058**

TITLE: Factors Essential for Prostate Cancer Metastasis Revealed Through a Novel 3D Microtissue Assay

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REPORT DATE: **June 2016**

TYPE OF REPORT: **Final report**

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

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



**PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| June-2016 | Final report | 1Apr2015 - 31Mar2016 |

**4. TITLE AND SUBTITLE**

Factors Essential for Prostate Cancer Metastasis Revealed Through a Novel 3D Microtissue Assay

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

W81XWH-15-1-0058

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Case Western Reserve University; 10900 Euclid Avenue Cleveland, Ohio 44106

**8. PERFORMING ORGANIZATION REPORT NUMBER**

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

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND
MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

USAMRMC

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

**12. DISTRIBUTION/AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Growing evidence suggest that the micro-dissemination of bone metastatic prostate cancer (BMPCa) to bone marrow (BM) may be facilitated in early-stage of cancer development and could be the origin of future outgrowth and metastasis. Importantly, the osteoblastic niche is known to constitute the sanctuary for bone-homing cancer cells where it confers BMPCa dormancy and therapy-resistance. Under the support of current proposal, we have carefully validated a novel 3D osteoblastic niche (3D-ObN) assay system which recapitulate BMPCa and osteoblastic niche interactions. Throughout the research year we have made following progressions.

1) We verified that the 3D-ObN can recruit BMPCa as well as other metastatic cancers.
2) We conducted a novel application of Hypoxia-sensitive reporters to measure BMPCa invasion ability and to apply for HTS.
3) 3D-ObN recruits cancer cells depending on high metastatic ability, cancer stem-like subsets, and CXCR4-CXCL12 interaction.

**15. SUBJECT TERMS**

Bone metastasis, Prostate cancer, Microenvironment, osteoblast, mesenchymal stem cell, osteoblastic niche, spheroids

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 9 | 19b. TELEPHONE NUMBER *(Include area code)* |

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## Introduction

In the United States, prostate cancer is the most frequently diagnosed cancer in men and the second leading cause of cancer-related death. Skeletal metastasis is a highly common route of prostate cancer dissemination that greatly diminishes the chance of cure by promoting the development of therapy resistance and increases morbidity by elevating the risk for pathological bone fractures[3]. To date, palliative treatments rather than curative therapies are the best-known options for delaying disease progression and for mitigating tumor-derived bone pain and destruction.

Growing evidence suggest that the micro-dissemination of bone metastatic prostate cancer (BMPCa) to bone marrow (BM) may be facilitated in early-stage of cancer development and could be the origin of future outgrowth and metastasis. The BMPCa interactions with the osteoblastic niche have been recognized as a critical step of successful dissemination by which BMPCa outcompete hematopoietic stem cells for the osteoblastic niche. Importantly, the osteoblastic niche is known to constitute the sanctuary for bone-homing cancer cells where it confers BMPCa dormancy and therapy-resistance. Depending upon heterogeneity and molecular repertories active within the niche, the BMPCa could be indolent for months to years prior to the acquisition of an overtly proliferative phenotype. However, the cellular interactions and molecular mechanisms mediating this BMPCa transition in osteoblastic niche represent a gap in our knowledge.

Since the osteoblastic niche was proven as a favorable BM niche for BMPCa, it is reasonable to target BMPCa in the osteoblastic niche as a strategy to enhance therapeutic response. Given the risks associated with metastatic disease, it is imperative to place a high priority on efforts designed to elucidate the regulatory mechanisms of BMPCa dormancy and outgrowth in the context of the osteoblastic niche, as these efforts will enable discovery of molecular targets for niche-directed therapeutics development, as disruption of their function may amplify therapeutic effects of cytotoxic agents.

With the support of a DOD-PCRP-Exploratory-Hypothesis Development Award (W81XWH-15-1-0058), our laboratory successfully developed a novel three-dimensional osteoblastic niche (3D-ObN) assay system using immortalized human osteoblast (hFOB1.19) and human MSC (HS5) to exploit unidentified targets for disruption and prevention of prostate cancer dissemination to bone. This novel 3D-ObN has the capacity to recruit hematopoietic stem cells (HSCs) through the established molecular mechanisms and ability to maintain HSC stemness for weeks. Most importantly, we have verified that this 3D-ObN has the capacity to mimic BMPCa homing to the osteoblastic niche. Thus, this assay platform is suitable to study the behavior of BMPCa in the osteoblastic niche, as the 3D-ObN models the microenvironment that BMPCa encounter as they home to the osteoblastic niche. We strongly believe that this hypothesis development study would contribute to establishment of an optimized 3D-Ob niche assay platform, which can be utilized in assessing therapeutic targets, and amended for the high throughput drug screening.

Keywords: Bone metastasis, Prostate cancer, osteoblast, mesenchymal stem cell, osteoblastic niche,

## Accomplishment

### 1. Three-dimensional Osteoblastic Niche favorably recruits bone-homing cancer cells

Recently, *3D spheroid model mimicking Osteoblast microenvironment (niche)* was developed which can control hematopoietic stem cell (HSC) homing, anchorage, and proliferation[7]. Since prostate cancer cells compete with and hijack homing of HSCs for Osteoblastic niche, *this Osteoblast niche spheroid model will permit a valuable approach to investigate BMPCa homing, lodging, proliferation, and more significantly, the interplaying mechanisms between BMPCa in Osteoblast niche as well as to define novel key chemo/cytokines and cell-cell interacting molecules critical for homing*. To establish this system for bone-homing cancer studies for the first time, we generated a 3D osteoblastic spheroid model to measure the invasive ability of T-cell leukemia and bone metastatic breast cancer. Osteoblast-like cells are induced from human MSC by culturing them for 10 days in osteoblast differentiating medium (Fig.1A) as described by de Barros et al[7]. Differentiation of osteoblast-like cells is confirmed by staining with Alizarin Red that visualize the presence of mineralized matrix consisting of collagen, calcium, phosphorous and other minerals of new bone. Furthermore, the messenger RNA expression of osteopontin, a marker of osteoblast, is increased during the culture in differentiation medium (Fig. 1A, RT-PCR). We stained cells with cell tracking dyes to clearly demonstrate the reconstituted Ob-niche spheroidal structure and imaged by confocal microscopy (the structure consists of osteoblast-like cells derived from human MSC (red) in central sphere and hMSC (green) in outer layer, Fig.1B, C). Next, we tested *whether the Ob-niche spheroid has a capacity to truly attract GFP-expressing cancer cells* into the sphere by co-culturing for 3 days in ultra-low attachment U-bottom 96 well plate (Sigma) and acquiring images (Z-section) with confocal microscopy (Fig.1D). We show that bone-homing cancer cells ($2x10^4$/well) effectively translocate into the spheroid in a manner similar to hematopoietic stem cell recruitment. This preliminary study suggests that it is feasible to form size-controlled Ob-niche spheroids in multi-well plates that possess the capacity to favorably recruit T-cell leukemia and breast cancer cells. This platform enables us to interrogate the potential utility of 3D-ObN as a surrogating *in vitro* assay system for BMPCa homing into osteoblastic HSC niche.

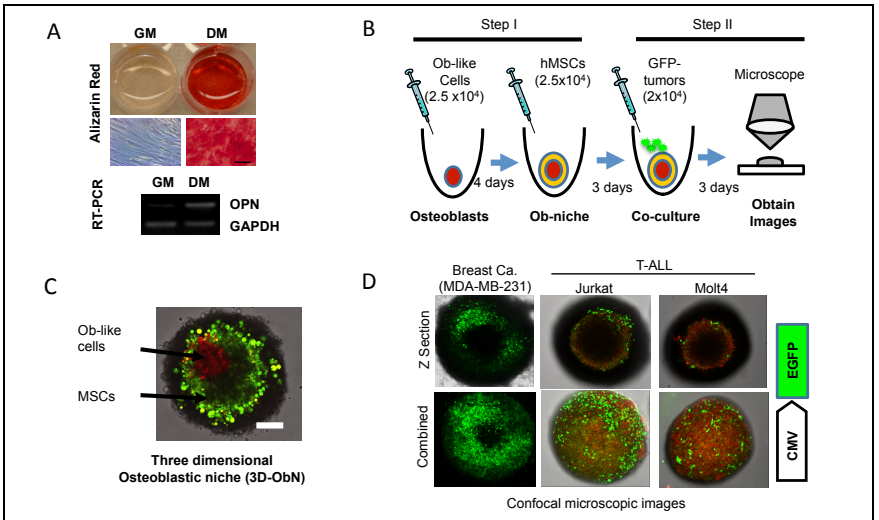


**Figure 1 The 3D-ObN microtissue model** (A) Human MSCs were cultured in osteoblast differentiation medium for 10 days and secreted mineralized matrix and expressed osteopontin gene. (B) Overall procedure for 3D-ObN formation and co-culture. (C) Bi-layered structure of 3D-ObN consists of Ob-like cells in core and MSCs in outer layer. (Bar=100 μm) (D) Z-section and combined confocal microscopic images after co-culture of cancer cells (GFP) with 3D-ObN.

**Hypoxia-dependent reporter activities are inducible in a spheroid-specific manner.** As proof-of-principle experiments, we employed two lentiviral reporter constructs only activated by hypoxic stimulation. First, we examined pGF-HRE4-GFP-fLuc construct containing four tandem repeats of hypoxia response elements ($HRE_4$) and followed by GFP/firefly luciferase (Fig. 2A). The reporter construct introduced into metastatic melanoma (A375), breast cancer (MDA-MB-231 marked as MDA231 hereafter), and prostate cancer (DU145) which were selected by puromycin and then tested the reporter activity in response to $CoCl_2$, a HIF-1 stabilizing agent. A375 and DU145 reporter cells have shown 4 or 5-fold promoter activation whereas MB231 cells have only less than 2-fold induction suggesting that the response to hypoxia could be variable. Thus, the reporter cells have to be defined and optimized prior to the utility in Ob-niche co-culture studies. Next, the reporter cells were co-cultured with Ob-niche spheroid and then conducted confocal microscopic analysis with frozen sections for HRE-mediated GFP expression. The results demonstrated that reporter cells were actively translocated into the spheroids (Fig. 2B) and showed enhanced expression of GFP as compared to the cells residing outside of the sphere (middle). These data indicates that $HRE_4$-GFP reporter system enables us to selectively quantify the amount of cells within spheroids. Secondly, we assessed a new version of reporter construct, pGF-$HRE_4$-secNLuc (Fig. 2C and 2D) harboring secreting form of nano luciferase (secNLuc). The advantage of secNLuc is small and stable nature of the protein as well as at least 200-fold more sensitive than firefly luciferase meaning that it is suitable for 3D spheroid system. The secNLuc activities were markedly increased in the conditioned media stimulated with $CoCl_2$. More importantly, the conditioned media from cancer/3D-ObN co-culture were significantly higher than that of 2D co-culture, indicating that this HRE4-SecNLuc will be excellent reporter system to quantify cancer cells in 3D-ObN.

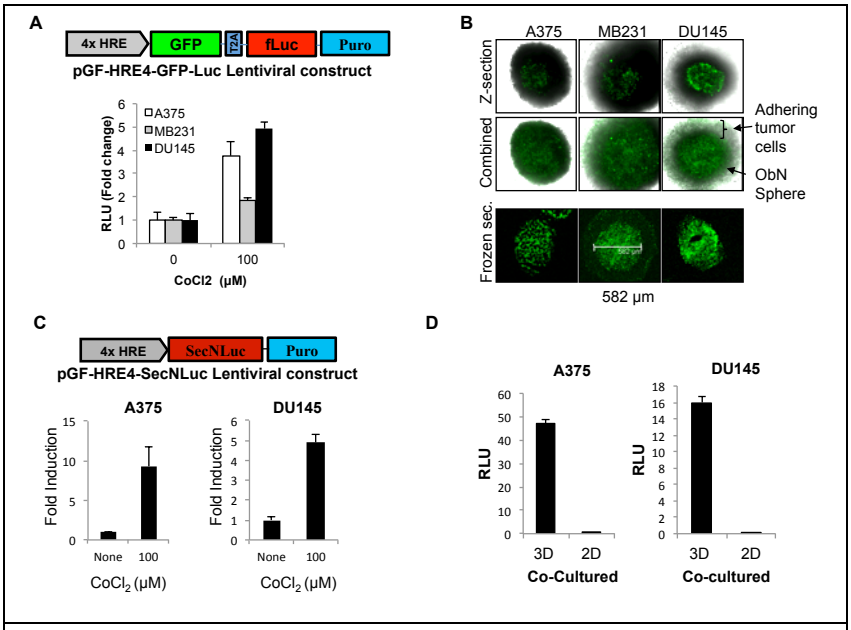


**Figure 2. Hypoxia-dependent reporter system enabled to visualize the translocated cells within 3D-ObN.** (A) The components of lentiviral construct encoding four-tandem repeat of hypoxia response elements in the promoter and followed by GFP-T2A-fLuc-Puro reporters. The cancer cells (A375, MDA-MB-231, DU145) carrying the lentiviral reporter showed inducible luciferase activity in response to $CoCl_2$. (B) The microscopic images demonstrate the HRE-dependent expression of GFP in a spheroid-specific manner. (C) The novel reporter construct encoding 4HRE-SecNLuc-Puro that is inducible by $CoCl_2$. (D) The reporter cells secreted SecNLuc to media as they co-cultured with 3D-ObN, but not in the 2D co-culture condition, which contains reporter cells, FOB, and HS5 as in 3D culture.

**The 3D-ObN prefers to recruit malignant bone-homing cancers than non-metastatic tumor.**

To have insights into the capacity of 3D-ObN favoring malignant cancer cells, we compared three metastatic cancers to non-metastatic cancer cells carrying pGF-HRE4-GFP in co-culture with 3D-ObN. The intensity of GFP expressing cells was recorded in IncuCyte, which was compared by subtracting each values with that of time 0 and initial starting value was set to 1 (Fig. 3A). Bone metastatic cancer cells (Prostate (DU-145, C4-2B) and melanoma (A549)) were more actively migrated into 3D-ObN than non-metastatic cancer cells (MCF10A) as compared for 3 days. The hypoxia-dependent GFP expression was gradually increased in bone metastatic cancer cells and showed maximum intensity around 2-3 days, while non-metastatic MCF10A cells showed almost no change. This observation was further confirmed in luciferase assay using reporter cells carrying hypoxia-dependent SecNLuc reporter gene (Fig. 3B). The hypoxia-dependent luciferase activity increased in highly metastatic cells but not in non-metastatic MCF10A at 3 days post co-culture. Next, we conducted another luciferase assay to examine if this tumor-niche interaction is CXCR4/CXCL12-dependent. During the co-culture, we treated cells with AMD3100 (50 μM), a CXCR4 antagonist or Acroflavine, a HIF-1 inhibitor as a control. The tumor-niche interaction was partially prevented by CXCR4 antagonist, meaning that bone-metastatic cancer interact with 3D-ObN via CXCR4-CXCL12 (Fig. 3C). Moreover, prostate cancer stem-like cells (CD44/CD133$^{high}$, upper 10% of subpopulation) tend to migrate into the 3D-ObN more aggressively than non-prostate cancer stem like cell subpopulation (the bottom 10%)(Fig. 3D). Thus, these results suggest that 3D-ObN has ability to induce homing of malignant cancer cells via cell-cell interaction mechanisms including CXCR4/CXCL12, especially prostate cancer stem-like cells than non-malignant cells which we may explore to identify novel interacting mechanisms between BMPCa/PCSCs and osteoblastic niche.

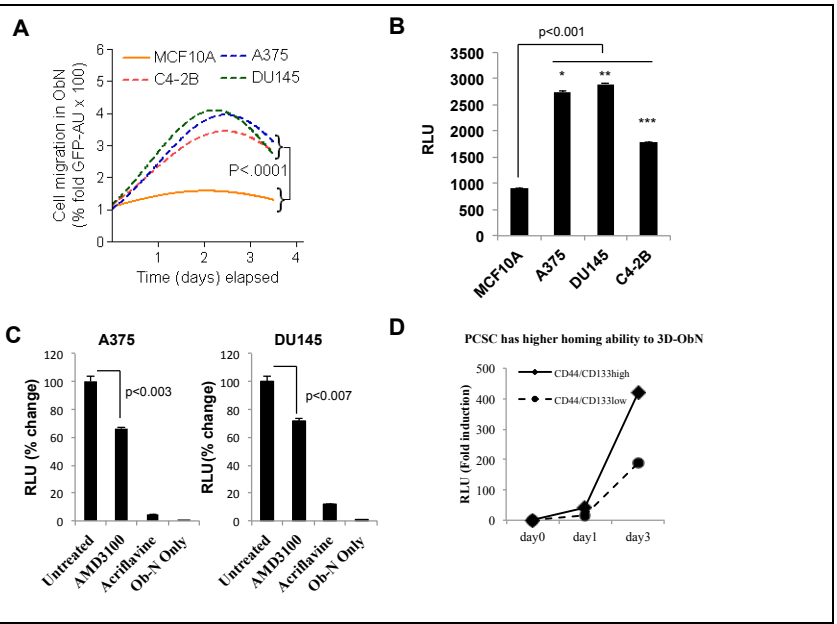


Figure 3. **Hypoxia-dependent reporter system enabled to quantify the translocated cancer cells within 3D-ObN.** (A) The malignant cancer cells tend to migrate aggressively in to the 3D-ObN spheroid compared to non-metastatic MCF10A cell line. (B) The 4xHRE promoter-driven secreting form of luciferase reporter was sensitively activated to $CoCl_2$ in both A375 and DU145. (C) Tumor-niche interaction is mediated in part by CXCR4/CXCL12. A375 and DU145 reporter cells were co-cultured with 3D-ObN for 3 days in the presence of DMSO, AMD3100 (50 μM), or Acriflavine (1μM). (D) The PSCS-like cells (CD44/CD133$^{high}$) of C4-2B showed higher migration ability to 3D-ObN spheroid than the non-PCSC subpopulation (CD44/CD133$^{low}$).

**Impact**

During the research year, we successfully developed a novel 3D-ObN assay platform with human immortalized osteoblasts and MSCs. For the first time, we have employed hypoxia-dependent reporter systems in order to quantify the relative amount of translocated cancer cells in 3D-ObN, which work in a spheroid-specific manner. We verified the capacity of 3D-ObN for inducing BMPCa and other malignant cancer homing via well-known cell-cell interaction mechanism (e.g. CXCR4-CXCL12) and preference of PCSC subsets. We carefully verified that this 3D-ObN also has capacity to recruit hematopoietic stem cells and maintain their stemness (data not shown). Thus, the 3D-ObN with spheroid-specific reporter system has an exceptional capacity to mimic in vivo osteoblastic niche, which would contribute to identifying novel mechanisms of osteoblast and BMPCa interaction, and possibly for further characterization of tumor dormancy and niche-directed drug-resistance mechanisms. In addition, this 3D-ObN is feasible assay system to easily convert to niche-targeted drug screening in a high-throughput format.

**Products**

Throughout the 3D-ObN development and optimization study, we have following products.

1. A method of novel 3D-ObN spheroid generation with human immortalized osteoblast and MSCs
2. BMPCa and other reporter cell lines (DU145, C4-2B, PC3, A375, A549, MCF10A, MDA-MB-231)
3. Established SecNLuc luciferase assay, real-time acquisition of GFP imaging and quantification of intensity using IncuCyte microscope.

**Participants and other collaborating organizations**

This 3D-ObN development and BMPCa-niche interaction studies have collaborative team efforts. Drs Letterio, Lui, and Wang contributed to optimization of 3D-ObN assay and BMPCa interaction studies by providing their expertise on prostate cancer biology (Dr. Wang), prostate cancer stem cell preparation and FACS (Dr. Letterio), and incuCyte image acquisition and analyses (Dr. Liu). This team effort would have a great value for competitive national grant applications (DOD-PCRP, R01, R21 etc.)

**Changes/Problems**

We have requested and approved for "No cost extension" which extended research period until March 31$^{st}$, 2017. By the time, we will validate 3D-ObN assays further by modifying expression of metastatic regulators as described in task 2 of SOW.

**Special Reporting Requirements**

None

**Appendices**

**Poster Presentation (A31) at AACR meeting-Tumor metastasis 2015 at Austin, Texas (11/30-12/3, 2015)**

**Title: A Novel *3D osteoblastic niche Assay* designed to assess the ability of bone-metastatic Cancer homing to Bone Marrow Niche**

**(Background)** Many patients with metastatic cancer (i.e. Prostate, breast, lung, kidney, and melanoma) struggle with skeletal metastases which is frequently causing cancer-related death. Thus, there is an imminent demand for a better understanding of how bone-homing cancers communicate and physically interact with the bone microenvironment. It is known that cancer-stromal cell interaction in bone marrow is a pivotal step in determining bone metastasis and tumor behavior and is seen in the events of competition with HSC for the osteoblastic niche, entering dormant status, and enhanced tumor growth. However, the lack of biologically relevant assay platforms has hampered interrogation of the mechanism of cancer-bone interaction as well as the development of bone-targeted anti-metastatic drugs. **(Methods)** Here, we present a novel method that can measure the bone-homing ability of cancers by co-culturing 3D osteoblastic niche (3D-ObN) spheroids derived from human mesenchymal stem cells along with reporter cell lines carrying hypoxia response element (HRE)-regulated reporter genes (pGF-HRE4-GFP/SecNLuc). First, the migration ability of metastatic cancers into the 3D-ObN was examined by analyses of O.C.T. frozen section images using confocal microscopy. Next, the metastatic ability of highly metastatic cancers were compared to normal epithelial cells and assessed by using either fluorescent microscopy (IncuCyte) or a luminometer. **(Results)** The HRE-driven reporter was activated in a 3D-ObN spheroid-specific manner in response to the nature of the hypoxia gradient within the spheroid. While the 3D-ObN provided signals that suppressed cancer cell proliferation, highly metastatic cancer cells displayed higher homing ability in the 3D-ObN assay relative to the normal epithelial cells. Prostate cancer cells expressing putative cancer stem cell markers (CD44/CD133$^{high}$) showed higher homing ability compared to CD44/CD133$^{low}$ populations. **(Conclusion)** This assay platform is the first of its kind through the fact that it mimics cancer-osteoblastic niche interactions and allows for quantification of the ability of cancer cells including cancer stem-like cells to invade and anchor in osteoblastic niche. Furthermore, this assay system can be readily amended to an efficient and effective high throughput screening system useful for identifying molecules potentially preventive to critical process of bone metastases.

**List of Personnel receiving pay from the research effort**

1. Dr. Huiping Liu