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PROJECT: Patient-Derived Organoid Systems for the Study of Castrate-Resistant Metastatic Prostate Cancer

Abstract

Bone metastases of prostate cancer continue to elude effective clinical management world-wide. A newly developed xenograft model, PCSD1, is a pre-clinical therapeutic development and testing platform to understand the mechanisms of resistance to androgen deprivation therapy of bone metastatic prostate cancer. Prostate cancer is a heterogeneous disease for which it is especially important to study models that represent the range of different patients. However, of the patient samples investigated by Christina Jamieson's lab at UCSD, only 5 out of 20 (25%) were able to successfully create xenografts. The hypothesis I tested is that metastatic cells that have failed to xenograft into a patient-derived tumor xenograft mouse model will be able to successfully survive in vitro in an organoid culture system. The goals of my research were twofold: to successfully develop organoid cultures for both cells that have successfully and unsuccessfully xenografted into the mouse model and to perform imaging and genetic analysis on these organoids.

Background

Over 180,000 new cases of prostate cancer are estimated to occur in the United States for the year 2016 [1]. Despite our best efforts to manage the disease, prostate cancer continues to be the second-leading cause of male cancer mortality in the United States [2]. With the advent of recent clinical advancements, more men are being diagnosed at earlier stages of prostate cancer, decreasing the rate of metastatic disease to 4% [3]. However, metastatic disease remains a significant risk factor for patients not cured of organ-confined disease. Treatment of recurrent prostate cancer typically consists of Androgen Deprivation Therapy (ADT), followed by chemotherapy and radiotherapy if the cancer becomes resistant and non-responding (Castrate Resistant) [4, 5]. Unfortunately, many bone metastasized prostate cancer cells become resistant to ADT, eventually exhausting all treatment modalities [4, 5]. Moreover, 100% of men who die from prostate cancer have bone metastases [5].

These bone metastases are linked to both osteoblastic and osteolytic lesions leading to discomfort, pain, and bone fractures. Various models have been developed to study the metastatic disease due to the lack of curative treatments and unknown reasons for which prostate cancers prefer bone.

Cancer models have historically been stratified into animal models and human-derived models. The latter can then be further split into cancer cell lines and patient-derived tumor xenografts (PDTX) [6]. At present, there are several cancer bone metastasis-derived orthotopic bone models including PC3, LAPC9, VCaP, and PCSD1 [4, 5]. PC3 forms purely osteolytic lesions in intra-tibial xenografts, VCaP produces mixed osteoblastic/osteolytic lesions, while LAPC9 forms purely osteoblastic lesions. Lastly, PCSD1 can form both osteoblastic and osteolytic lesions in the murine model [4, 5].

PCSD1 (Prostate Cancer San Diego 1) is a novel, primary prostate cancer bone metastasis-derived xenograft model developed in Dr. Christina Jamieson's laboratory aimed for use as a therapeutic testing modality for developing novel therapies for prostate cancer [4]. A recent study showed that PCSD1 tumors were castrate resistant, lacking the androgen receptor, in the bone-niche, in contrast to development in soft tissues subcutaneously [5]. It was separately discovered that the Wnt5a signaling pathway activates a Wnt5a/BMP-6 loop that enables the proliferation of the cancer cells in an androgen-deprived environment [7]. This was consistent with findings that showed Wnt5a derived from bone stromal cells induced the expression of BMP-6 by prostatic cancer cells, creating a feedback loop that further induced cancer cell proliferation [7].

Wnt5a, a member of the Wnt family, is a secreted glycoprotein which mediates various cell processes. Earlier studies revealed that osteoblastic differentiation mediated by BMP-2 is associated with increased expression of Wnt5a and Ror2 [8]. Other studies have confirmed that Wnt5a has a profound effect on prostate cancer cell migration using the PC3 cell line [9]. Wnt5a has also been shown to be expressed at higher levels in areas of the bone such as growth plates [10]. The discovery of this association between Wnt5a and bone-niche prostate cancer, allows room for potential novel therapeutic agents specific to bone metastases.

There exists a novel third category of cancer model: tumor organoids. Organoids have been readily established from surgically resected tissue and biopsies of patients suffering from adenomas and adenocarcinomas of the gut [6]. Colorectal cancer organoids have been described in the literature to grow as irregular, compact structures and can be expanded seemingly indefinitely. Using this type of model should allow for the establishment of multiple organoid lines from the same patient for parallel, controlled experimentation [6].

Organoid culture requires Matrigel (Corning), a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, which makes the culture process more labor intensive than culturing cell lines in two dimensions and adds complexity to potential drug screens. Zhao et al. found that, in contrast to monolayer cultures, organoids responded to therapeutics and behaved more closely to autochthonous prostate tumors [11]. The laminin and collagen IV-rich Matrigel functions as a physiologically relevant basement membrane substitute. On the other hand, maintaining organoid cultures provides the distinct advantages of in vitro models for drug testing and elucidating mechanisms of action compared to in vivo PDTXs. A future collection of organoids, representative of groups of cancers, could help with patient stratification for treatment modalities or oncogenic therapeutics [6].

The labs of Drs. Hans Clevers' at Hubrecht Institute and Charles Sawyers' at Memorial Sloan Kettering have developed a robust protocol for the culturing of 3D organoids for metastatic prostate cancer lesions, amongst other cell types [12]. They found that these cultures mimic the histology of the tumor and only take about 2 weeks to develop, while maintaining genetic stability. In parallel, Dr. Michael Shen of Columbia University has developed a different Matrigel/EGF-based culture system and has reported similar observations with successful long-term culture [13].

Definition

As previously described, bone-metastasized prostate cancer is unpredictable, castrate-resistant, virtually incurable, and causes painful bone lesions in male patients [2, 3, 4, 5].

I am interested in getting a better understanding of metastatic prostate cancer at the molecular level, specifically with respect to the Wnt5a signaling pathway. The goal of my research was to culture organoids using patient tumor-derived cells for the purpose of investigation. My primary hypothesis was that metastatic cells that have failed to xenograft into a PDTX mouse model will be able to successfully survive in vitro as an organoid.

To test my hypothesis, my specific aims were:

1) Develop organoid cultures with PCSD1, PCSD5, and PCSD13 xenograft cells as proof-of-concept systems.
2) Perform immunofluorescence analysis of WNT5A signaling pathway components in organoid cultures.
3) Establish organoid cultures from patient bone metastases specimens beginning with PCSD1, PCSD2, and PCSD8, all derived from the same patient. PCSD2 and PCSD8 previously have not yielded xenografts; I then aimed to establish xenograft tumors from these organoids.
4) Perform RNA sequencing analysis on patient-derived and xenograft-derived organoid cultures.

These specific aims would allow smooth progression through the project and serve as benchmarks for project evaluation.

The innovation of this project lay in creating parallel in vitro/in vivo systems for the study of prostate cancer metastases.

This is medically relevant because development of these sustained organoids will allow for further elucidations to be made in the realm of late-stage metastatic prostate cancer, especially castrate resistant varieties, which do not xenograft successfully. Moreover, creating organoid

repositories for future reference value holds benefits of further developing precision medicine methodology for the participating patients themselves in the future. For this project, I worked with Dr. Christina Jamieson and her lab at Moores Cancer Center. I was be responsible for aims one through four.

Methods

To establish organoids, I followed the Gao/Clevers protocol [12] in which the tumor tissue is digested first with collagenase type II (Thermo Fisher) with Y-27632 dihydrochloride (Abmole Bioscience), and then TrypLE (Thermo Fisher) with Y-27623 dihydrochloride (washing with adDMEM/F12 +/+/+ (containing penicillin/streptomycin, HEPES, and GlutaMAX) (Thermo Fisher)). The cells were then be suspended in Matrigel (Corning) and incubated in CO_2. Prostate culture medium (B27, N-acetylcysteine, EGF, Noggin, R-spondin 1, A83-01, FGF10, FGF2, Prostaglandin E2, Nicotinamide, SB202190, DHT) with Y-27632 dihydrochloride was used as the initial culture medium; and prostate culture medium was used after the first week of culture. Organoid passaging was accomplished by dissociating the cells using TrypLE with Y-27632 dihydrochloride; adDMEM/f12 +/+/+ containing 5% Fetal Bovine Serum (FBS) (Sigma-Aldrich) will be used to inactivate the TrypLE. The cells were spun down and resuspended in new Matrigel, splitting at a 1:2 ratio, and incubated on CO_2. Human prostate culture medium will be added and subsequently replaced every 2-3 days.

Histological sectioning and staining was done according to manufacturer specifications for various antibodies. Preserved and fixed organoid samples were subjected to either direct

labeling using a known fluorophore-labeled antibody or an indirect staining methodology using a labeled anti-antibody conjugate.

Imaging was performed with confocal microscopy or light sheet fluorescence microscopy or other available fluorescence microscopy modalities, appropriate to the type of technique required for the reporter molecule.

Investigation of transcriptome of the organoids will be analyzed via single cell RNA sequencing. Prostate cancer phenotype will be confirmed using quantitative PCR will be performed on prostate cancer markers including PSA, PSMA, NKX3.1, AR, Cytokeratins 5,8,14, and 18, as well as neuroendocrine markers, syntaptophysin and chromogranin A (Raheem et al 2011, Godebu et al 2014).

Evaluation

The progress of project was evaluated at weekly lab meetings and individual meeting with Dr. Jamieson.

Part 1 was evaluated by presentation of xenograft-derived organoid growth data.

Part 2 was evaluated by presentation of imaging from the immunofluorescence analysis.

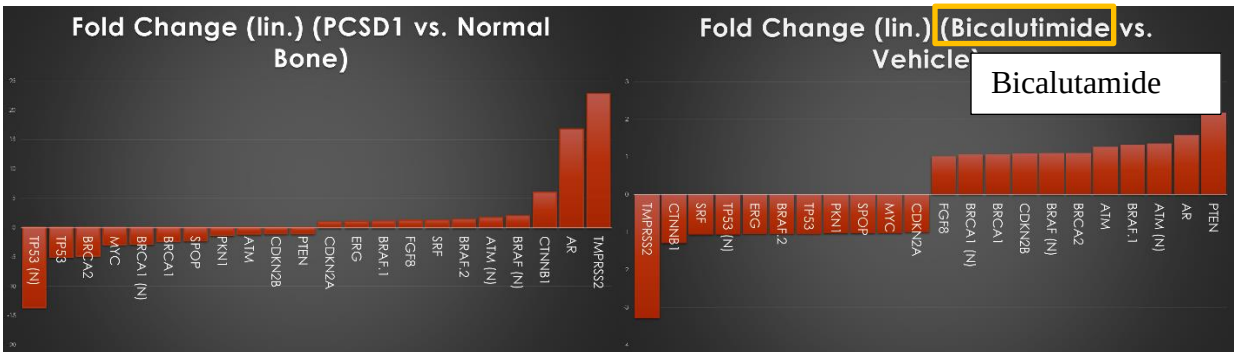
Part 3 was evaluated by presentation of patient bone metastases specimen-derived organoid growth data.

Part 4 will be evaluated by the presentation of the RNA sequencing data.

The project will be terminated when the organoids fail to culture, or are no longer representative of the original cell population. I will be contributing to a manuscript on the results of this study, which will be submitted for publication, with Dr. Jamieson.

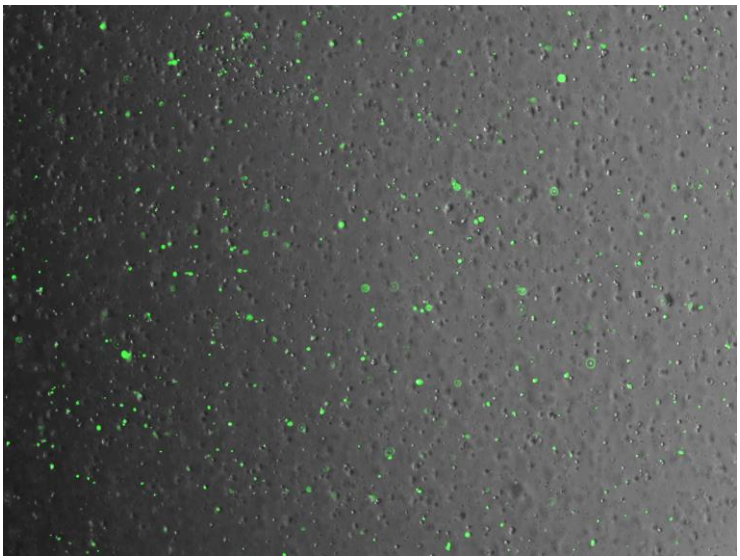
Results

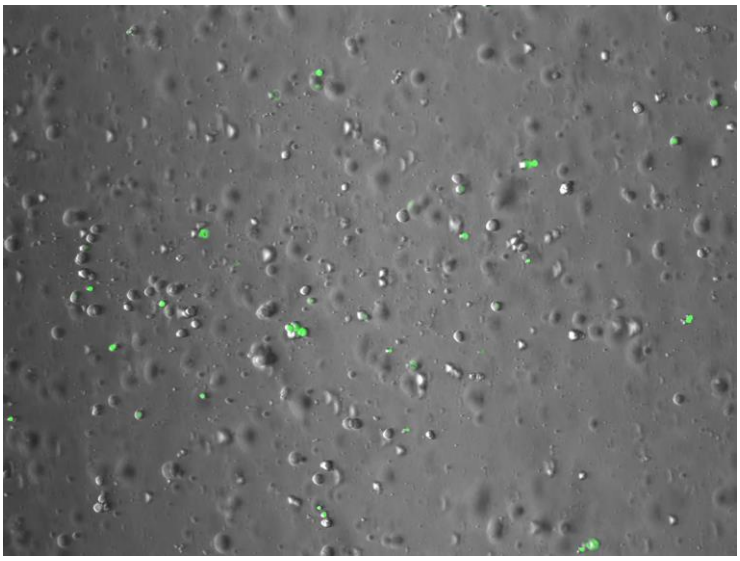
The expression profiles of genes with noted variants in PCSD1, discovered upon previous exome sequencing, and genes with known associations with prostate cancer were compared between PCSD1 and normal bone; as well as between Bicalutamide-treated and Vehicle-treated to look for differences in expression levels.

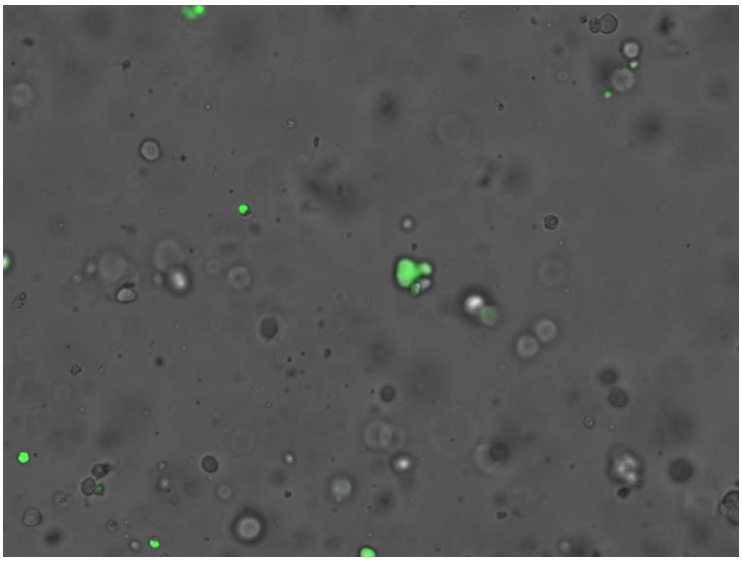


Organoid structures were successfully developed from PCSD1 Xenograft cells using the Clevers/Sawyers protocol. This cell population had previously been transfected with a GFP-Luciferase reporter molecule. This allowed for the use of fluorescence microscopy to image the organoid cells, confirming their identity as separate from the stromal, supportive cells that may have been in the heterogenous seeding cell mixture.

Presented here are 3 images taken of the organoids. The images are taken at 4x, 10x, and 20x objective lens magnification, respectively. The green fluorescence signals have been overlaid upon the light micrograms.

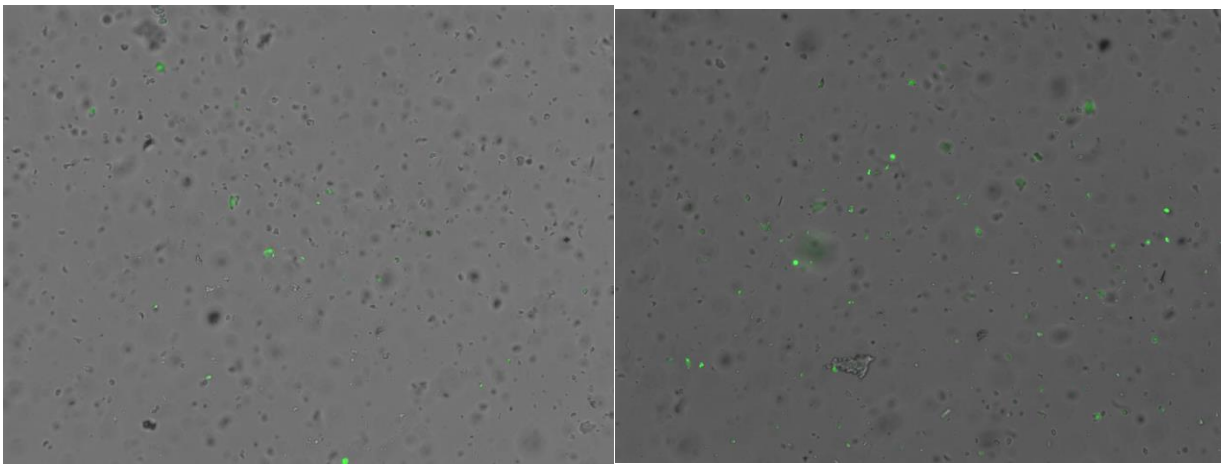




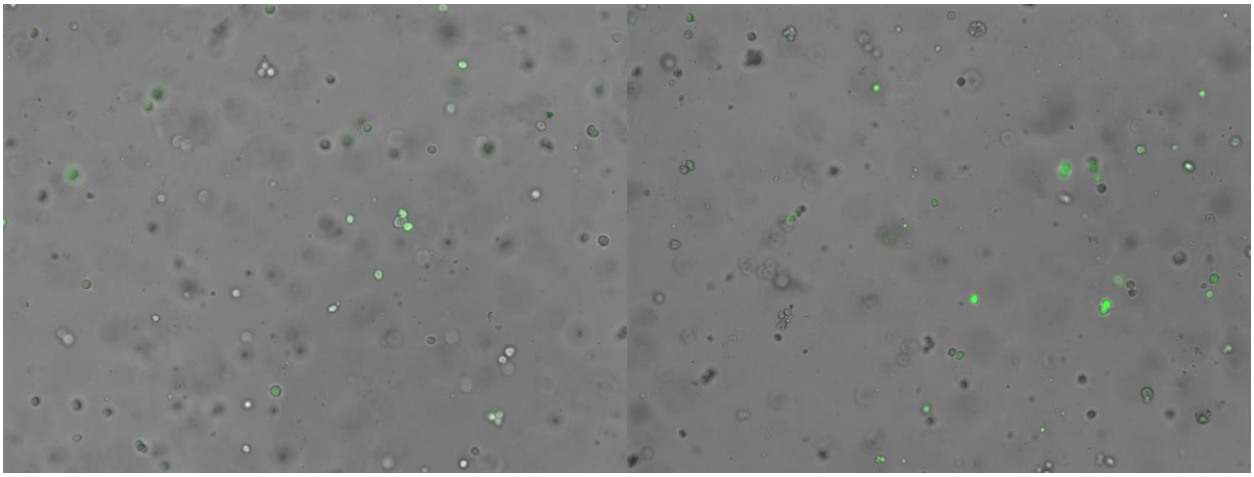


I postulated that various signal artifacts were occurring due to the three-dimensional matrix in which these cells are growing, the non-uniform distribution of the cells, and the unpredictable lensing at the surface of the Matrigel dome. The lack of signal in many of the organoids is a property of our imaging methodology. Black balance was performed to reduce noise from the autofluorescence of dying cells, thus many smaller signals were lost in the noise reduction process. Moreoever, due to optical dispersion, in any given plane, there are signals that exist, that do not correlate with a visible organoid structure. However, one can clearly appreciate that the coincident fluorescence signal with organoid structures exists.

Organoids of PCSD13 Xenograft cells were also successfully established using a modified prostate organoid growth media in dihydrotestosterone positive and negative conditions. Images are shown here. (DHT positive and negative at 10x objective lens magnification, respectively)



A small experiment was set up using the PCSD1 Xenograft organoids to test the effects of Enzalutamide (Astellas), an AR-binding anti-androgen used in the treatment of metastatic castration-resistant prostate cancer. Presented here are before and after pictures of 34 days of Enzalutamide as well as an image of a control organoid culture on the same plate.



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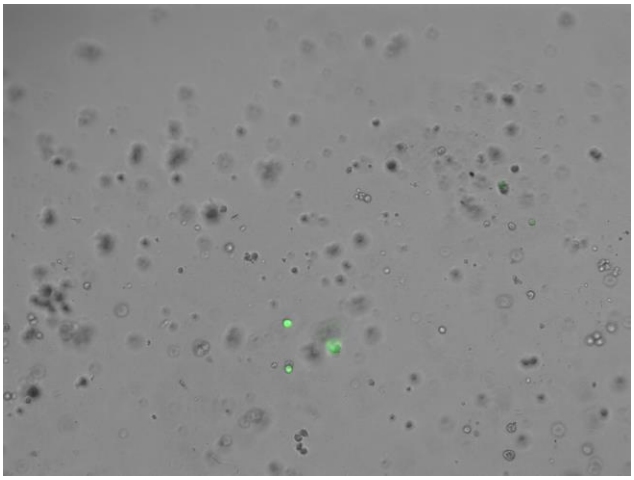

Control

Fluorescence and bright field microscopy were used to investigate changes in cell morphology. The organoids appear to be cystic-type in both the before and after treatment images, as well as in the control. The distribution of sizes was also similar, as both larger and smaller organoids persist. Cell counts were not obtainable by microscopy. Thus, microscopy could not be immediately used as a technique for quantitative evaluation of treatment effect due to the aforementioned optical effects. Attempts were made to analyze to averaged luminence values from a low power fluorescence micrsocopy scan of the 24-well plate. However, due to black balance, optical effects, and microscopy limitations, this yielded limited and ineffective data. Other methods such as Luciferase assays against PCSD1 cells could potentially offer robust quantitative data on the proliferation of the organoids in this experiment.

The results of this Enzalutamide treatment recapitulate the results of an in vivo experiment by Dr. Jamieson's lab, in which xenograft PCSD1 cells resist treatment by Enzalutamide.

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