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13. SUPPLEMENTARY NOTES

14. ABSTRACT Advanced prostate cancer is usually treated with Androgen deprivation therapy (ADT) which can help maintain remission in patients, however, growth and metastatic spread often recur. Treatments with new mechanisms of action are urgently needed. We are using patient-derived xenograft and PCa cell line models to test mechanism of action of a new therapeutic target: the WNT5A/ROR1 signaling pathway in prostate cancer for which a therapeutic ROR1 inhibitor antibody, Cirmtuzumab, has been developed and clinically tested in CLL and metastatic breast cancer patients. In Y1 of this grant we have shown that ROR1 is expressed at high levels on castration resistant small cell PCa and neuroendocrine PCa (NEPC) models; two of the most lethal forms of prostate cancer for which there are no curative treatments in PCa cell lines and PDX models. Cirmtuzumab has demonstrated efficacy in our patient derived xenograft organoid cultures. We are now performing in vitro and in vivo testing in NEPC cell lines and in our patient-derived xenograft (PDX) mouse models. We have developed immunohistochemistry (IHC) assays to detect ROR1 in patient biopsy samples which will allow us to define the prostate cancer patient population that expressed ROR1 and to be able to evaluate the treatment in patients for a future clinical trial. These studies are will inform us about the appropriate patient populations for treatment and clinical trials. These pre-clinical studies being performed in this grant will support the pending Phase 1B clinical trial (not part of this grant).

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1. INTRODUCTION

My research is focused on metastatic prostate cancer, urologic immune-oncology and therapy. My goal is to develop and test new molecularly targeted therapies and immunotherapies to eradicate this lethal disease. The central tenet of my approach is to study and use patient material to generate new models and use them to perform pre-clinical studies for novel treatments as well as to advance understanding of disease mechanisms of resistance. These patient-derived models more accurately replicate and retain the features of the disease tissues, and thus, are more predictive of patient disease responses when used to test therapies. We have developed patient-derived xenograft models for in vivo PDX and in vitro PDX-derived organoids of bone metastatic prostate cancer. We are using these PDX models in this grant to investigate the mechanism of action of WNT5A:ROR1 signaling in metastatic PCa and the anti-ROR1 antibody therapeutic, Cirmtuzumab.

Background-ROR1 as a Target for metastatic prostate cancer

Wnt signaling was originally discovered as a group of signal transduction pathways critical for normal development and physiology (24, 49, 50). Aberrant Wnt signaling and mutations in the pathway were subsequently associated with tumorigenesis, progression, and metastasis in many cancers including prostate cancer (51, 52). Wnt signaling, which is comprised of the canonical (β-catenin dependent) and noncanonical pathways, is frequently altered in prostate cancer(26, 27). Comprehensive sequencing studies in patients with CRPC have identified recurrent molecular alterations in Wnt signaling pathway components in **about 20% of advanced prostate cancer patients (17)**. Analysis of circulating tumor cells (CTCs) from CRPC patients demonstrate **expression of Wnt5A, the prototypical noncanonical Wnt ligand, in >60% of patients with refractory disease (28, 53-56)**.

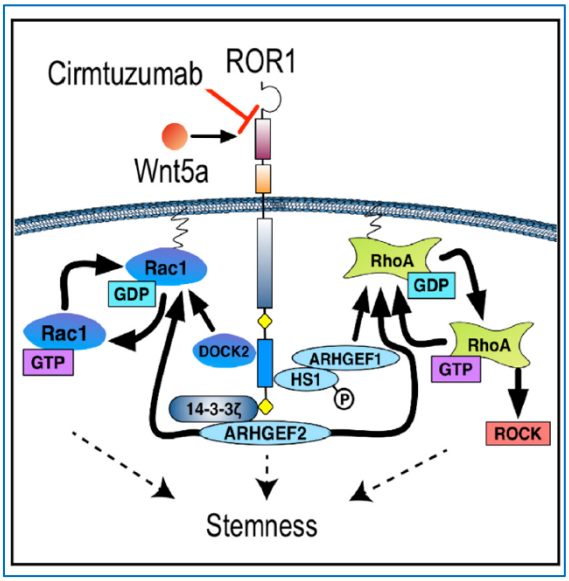


Figure 1. Signaling through the non-canonical Wnt pathway is mediated by Wnt5A binding to its receptor ROR1. Cirmtuzumab is a monoclonal antibody which targets ROR1 (Choi 2018).

The Wnt signaling pathway is complex and context-dependent activities of Wnt signaling are mediated via crosstalk between the canonical and noncanonical Wnt signaling. The Wnt pathway interacts with androgen receptor (AR) signaling, a key pathway in prostate cancer pathogenesis (53). **Noncanonical Wnt signaling is mediated in part through ROR1 tyrosine-kinase-like orphan receptor activation by Wnt5A ligand (Fig 2). Investigating Cirmtuzumab, a cancer stem cell targeting antibody therapeutic, for treatment of lethal metastatic prostate cancer.**

Cirmtuzumab was developed here at UCSD for treating chronic lymphocytic leukemia (CLL). It has successfully passed a Phase 1 safety clinical trial with very few side-effects and is showing efficacy in CLL patients. Cirmtuzumab binds to and inhibits ROR1, an embryonic protein which is not expressed on normal tissues but is upregulated in CLL and some solid tumors. Cirmtuzumab is now in a Phase 1 clinical trial for metastatic breast cancer. We discovered that ROR1 and its partner, WNT5A, are present in some therapy-resistant prostate cancers. We are using our patient-derived models to investigate Cirmtuzumab as a treatment for prostate cancer. We were **awarded the Dept. of Defense Prostate Cancer Research Program Impact Award which now funds this research**.

2. KEYWORDS:

Bone metastatic prostate cancer, WNT5A, ROR1, Patient derived xenograft, organoid, Castration Resistant Prostate Cancer (CRPC), Neuroendocrine Prostate Cancer (NEPC), Docetaxel, Cirmtuzumab

3. ACCOMPLISHMENTS

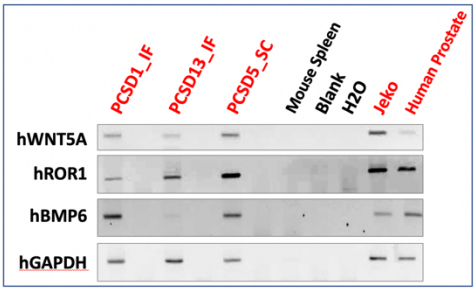
3.1 ACURO approval obtained.
3.2 HRPO not applicable in accordance with ORP_Statement on review of established PDX (Attached as Appendix). The memorandum states: " 2. This memorandum serves to clarify that USAMRDC supported research involving the use of established, existing patient derived xenograft (PDX) models (or the cell lines derived therein) that were established using tissue from deceased donors does not require ORP Human Research Protection Office's review and oversight for cadaver use in accordance with reference (a).on the use of PDX models." The experiments in our proposal Aims 1 and 2 use PDXs that we established between 2011 and 2014 and have published several publications on from now deceased donors. In Aim 3 we propose to do single cell RNA sequencing of frozen samples from donors who are all deceased.

RESEARCH ACCOMPLISHMENTS - Y1

Specific Aim1: Determine the mechanism of WNT5A/ ROR1/ ROR2 signaling in bone metastatic CRPC using patient-derived organoids (PDO) and xenograft (PDX) models using shRNA and small molecule inhibitors of WNT-signaling.

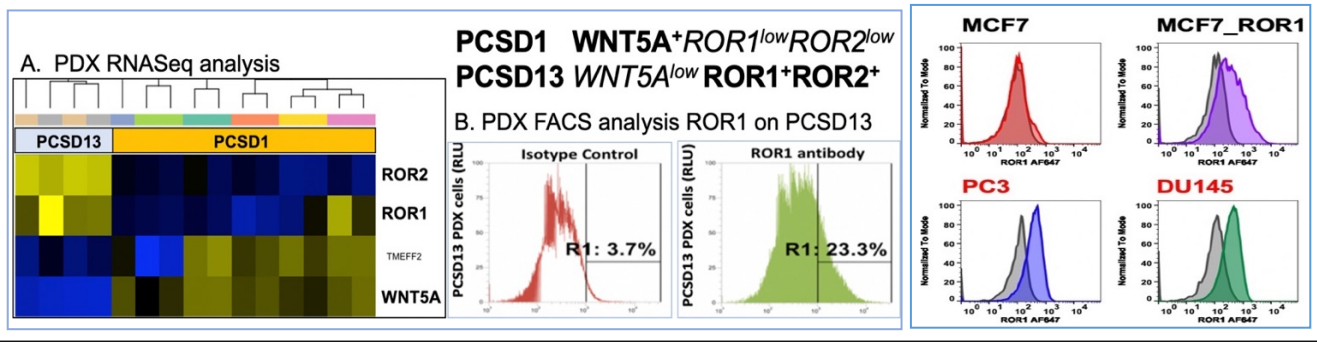
1. **ROR1 mRNA is expressed at high levels on castration resistant small cell PCa and neuroendocrine PCa (NEPC) cell lines and TCGA PCa dataset.**

PCa bone metastases have been challenging to study because they are not typically surgically removed or biopsied; thus, there are relatively few samples profiled and few models to test new therapies(74-76). The **Jamieson Laboratory** has extensive experience in bone metastasis prostate cancer models. In collaboration with our surgical oncology team, we established a Biobank and new patient- derived xenografts (PDX) named the Prostate Cancer San Diego (**PCSD**) series (77-79). These PDXs closely recapitulate the bone metastatic disease and CRPC in the bone of each patient (77-80). *Patient-derived xenograft and organoid models more accurately represent the patient disease, and thus, testing drugs in these pre-clinical models is more predictive of the patient response and drug efficacy*. Employing these models, we demonstrated key hypothesis-generating findings regarding the interplay between Wnt5A, ROR1/2, and bone metastatic prostate cancer (27). Using whole human genome HTA2.0 Affymetrix microarray and RNASeq gene expression profiling, we found that Wnt5A was highly expressed in a patient prostatic adenocarcinoma bone metastasis specimen and in its corresponding PDX, PCSD1. RT-PCR showed expression of Wnt5a and ROR1 in the PCSD series of PDXs (Fig 2).



RNASeq analysis of patient PCa bone metastasis-derived PDX, PCSD13-a small cell prostate cancer, showed significant ROR1 mRNA expression (**Fig 3A**). Employing FACS analysis, **ROR1 protein** was highly

Fig. 3. Bone metastatic prostate cancer patient-derived xenograft models. A: Hierarchical cluster of RNA-seq analysis on PCSD1 and PCSD13. B: Flow cytometry (FACS) of ROR1 protein on the cell surface of PCSD13 cells. C. ROR1 is expressed on the cell surface of prostate cancer cell lines. Antibody Isotype controls are in gray. Negative control cell line: Breast cancer line, MCF7,and ROR1 over- expressing line, MCF7_ROR1. Prostate Cancer lines: PC3 and DU145.



expressed in PCSD13 cells as well as PC3 and DU145 PCa cell lines (**Fig 3B**). We noted the reciprocal expression of Wnt5a and ROR1 in the bone metastases from the prostate adenocarcinoma, PCSD1, compared to small cell prostate cancer, PCSD13. While **PCSD1**, was PSMA+WNT5A+**ROR1^{low}ROR2^{low}**, **PCSD13,** was PSMA^{-} WNT5A+$^{-}$**ROR1^{pos}ROR2^{pos}.** This may reflect a fundamental difference in prostate adenocarcinoma and prostate small cell carcinoma, or NEPC.

Our findings underscore the need to further characterize ROR1 expression at differential stages in prostate cancer development. Such information will be critical for 1) understanding the role of ROR1 in prostate cancer disease progression and the emergence of neuroendocrine prostate cancer (NEPC), and 2) identifying the most suitable patient population for ROR1 targeting with Cirmtuzumab and Cirmtuzumab-CART cells.

We performed a search of TCGA prostate cancer RNA expression for ROR1 expression and found that ROR1 was expressed in metastatic PCa and NEPC which represent a minority of the samples in the TCGA database. Interestingly, we also found ROR1 expression was shown in PC3 and DU145 cell lines which are AR negative, NEPC-like cell lines.

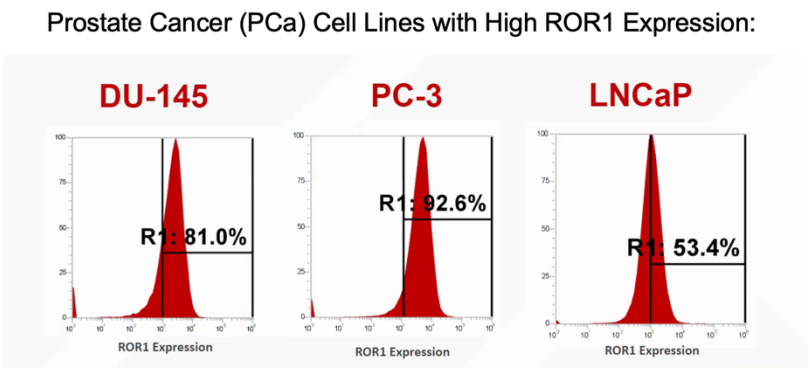
2. **PCa cell lines, PC3 and DU145, express cell surface ROR1 protein**

Next we investigated ROR1 expression on PC3, DU145 and LNCaP cells using flow cytometry and showed high ROR1 cell surface protein expressed on PC3 and DU145 comparable to the MCF7-ROR1 overexpressing cell line (Figure).

Defining WNT5A and ROR1 expression in prostate cancer models

a. ROR1 protein expression in flow cytometry: PCSD13, PC3, DU145, LNCaP

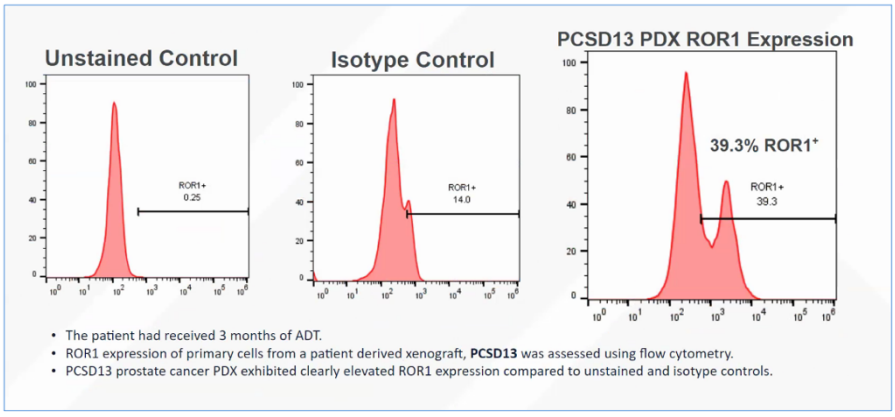
Figure 4.



PCa Cell lines DU145 and PC3 have high levels of ROR1 expression as assessed by flow cytometry (Thermo Fisher Attune NxT Flow Cytometry)

Flow cytometry analysis confirmed ROR1 cell surface protein expression on PDX cells from the small cell prostate cancer bone metastasis, PCSD13:

Figure 5. Flow cytometry analysis of cell surface ROR1 protein showed sub-set of ROR1+ cells in PCSD13 PDX tumor cells.



3. **Generated PC3, DU145, LNCaP RFP-luciferase expressing cells for in vitro 2D and 3D growth and viability assays for testing Cirmtuzumab and WNT5A in vitro (Incucyte) and in vivo xenograft assays** (IVIS).

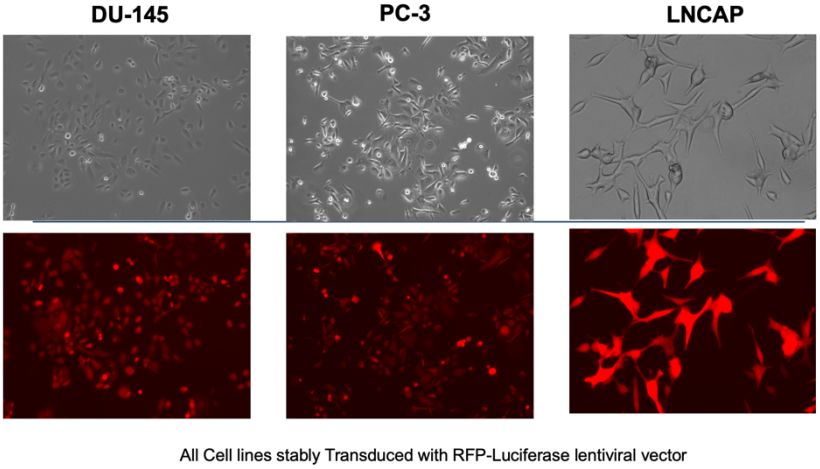
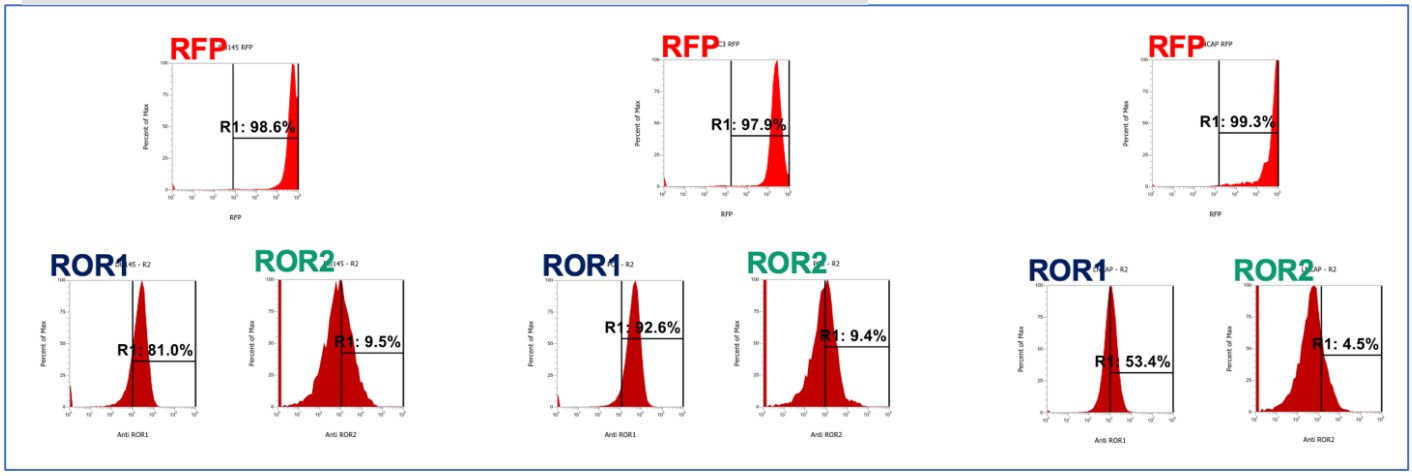


Figure 6. PCa Cell lines were transduced with RFP-Luciferase lentiviral vector particles express RFP as shown in Incucyte microscope.

Figure 7. Flow cytometry sorting and subsequent analysis was performed on PCa Cell lines transduced with RFP-Luciferase showed high RFP expression on almost 100% of cells. Immunostaining of ROR1 and ROR2 showed high ROR1 staining on PC3 and DU145.



4. **Established PC3 PCa cell line organoids (below, left) to study WNT5A:ROR1 signaling and Cirmtuzumab mechanism of action compared to PCSD1 (below, right) and PCSD13 PDX organoids.**

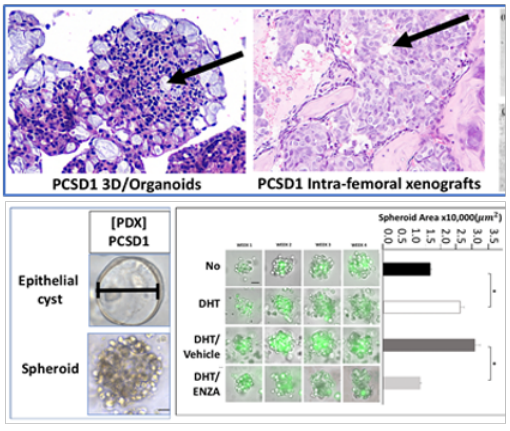


Fig 8. PCSD1 PDX tumors cells form 3D organoids that replicate histomorphology of the xenografts growing in the femur Black Arrows point to these similar structures. Organoids are GFP+,A.PDXs from prostate cancer bone metastases, PCSD1 express GFP-Luciferase B. Enzalutamide PCSD1 reduced lumen size and number of epithelial cysts.

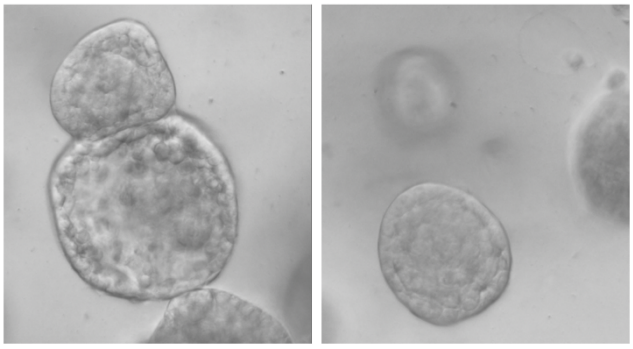


Figure 9. Live microscope images of the PCa Cell line, PC3 organoids grown in 3D optimized PCa organoid conditions (Lee et al 2020).

5. Established Live Cell Cycle Tracking System – The Fucci2BL lentiviral vector for testing cell cycle changes in live organoids was established in PC3, DU145, LNCaP, PCSD1, PCSD13. We are using this to test the cell cycle response to in response to recombinant WNT5A and Cirmtuzumab alone or in combination with This unique system overcomes the limitations of cell cycle analysis as an endpoint and allows for **non-invasive, serial cell cycle analysis in real time in live 3D organoids.**

Standard-of-care therapies: androgen deprivation such as enzalutamide and docetaxel are being tested in our Fucci2BL expressing PCa organoids. The Fucci2BL viral vector and packaged viral particles were obtained as a collaboration with Drs. Gabriel Pineda and Catriona Jamieson, UCSD, and used to transduce PC3, DU145, LNCaP, PCSD1, PCSD13 successfully as shown in **Fig 10** below. PCa cell lines expressing Fucci2BL are shown to have cells in all three of the cell cycle stages: red (G1), yellow (S), and green (G2/M). These cells will be FACS sorted to purify the transduced Fucci2BL cells for use in organoid and xenograft experiments. PCSD1-Fucci2BL cells were used to establish the effects of ADT on cell cycle as shown below and PCSD13-Fucci2BL are now being further FACS sorted and purified for organoids and PDX experiments with WNT5A and Cirmtuzumab alone or in combination with enzalutamide and docetaxel.

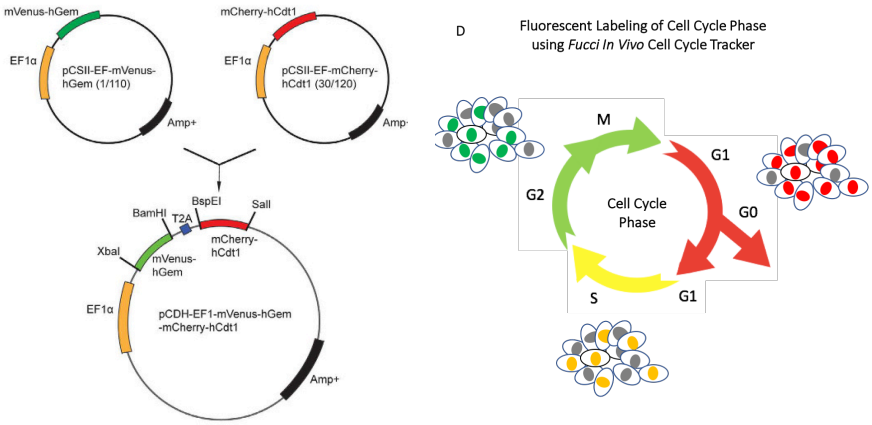
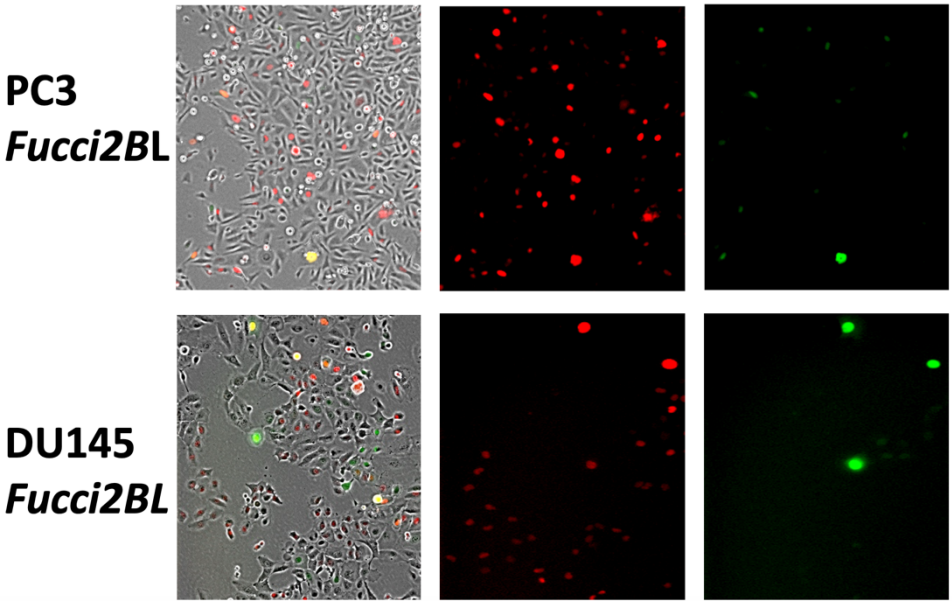
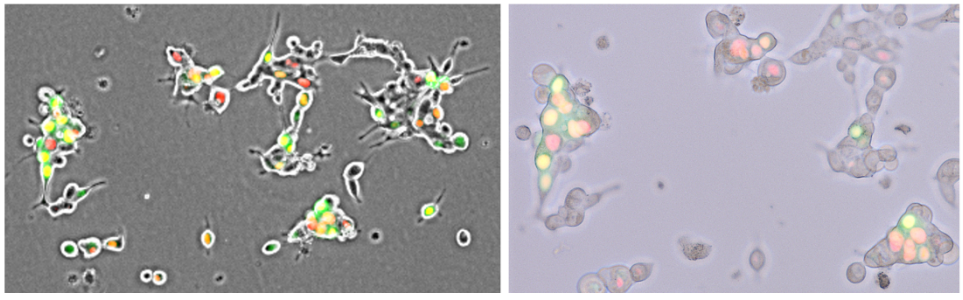


Fig 10. Fucci2BL vector generation and characterization. (a) Diagram and map of construct design and generation. Both mVenus-hGem(1/110) and mCherry-hCdt1 (30/120) were subcloned into a pCDHEF1α-T2A lentiviral expression vector (b) Fluorescent labeling of cell cycle phases using Fucci2BL live cell cycle tracker system (Pineda *et al* 2016 Sci Rep).





6. **Identified a new dormant CRPC basal-luminal hybrid prostate cancer cell and gene signature under standard-of-care enzalutmaide or androgen deprivation in PDX organoids** which may be targeted to eradicate dormant CRPC bone metastases in order to prevent disease recurrence. We will use this experimental paradigm of CRPC to test WNT5A and Cirmtuzumab in these organoids in combination with standard of care androgen deprivation therapy

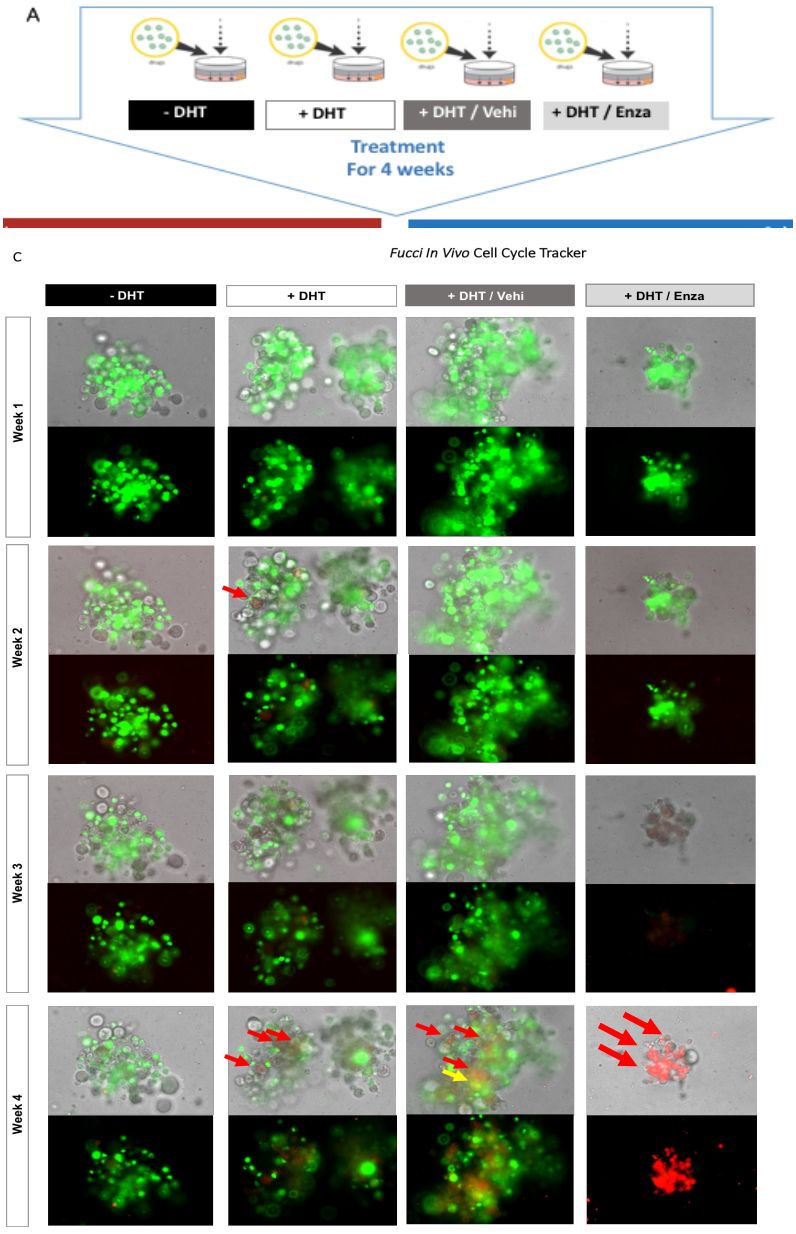
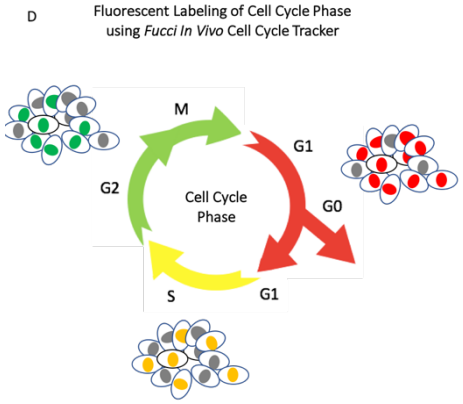


Figure 11. Enzalutamide-Resistant Population of PCSD1 Organoids are Dormant Cells.
Time course of cell cycle stages in live PCSD1 organoids under the four treatment conditions. Representative images in each treatment condition at week 1, 2, 3, and 4 of treatment of PCSD1 organoids stably expressing the *Fucci2 BL* bicistronic fluorescent, ubiquitination-based cell cycle indicator reporter system showing three cell cycle phases: G_1 / G_0 by red fluorescence, G_1/S by yellow fluorescence, and S/G_2/M by green fluorescence. The images of bright-field, red fluorescent channel and green fluorescent channel were taken and merged. (D) The *Fucci2 BL* fluorescent, ubiquitination-based cell cycle indicator reporter system visualizes the phase of cell cycle shown by colorimetric signal of red, yellow and green fluorescence for G_1, G_1/S and S/G_2/M, respectively.



Background: Androgen deprivation therapy (ADT) can help maintain remission in advanced prostate cancer (PCa) patients with bone metastases, however, growth and metastatic spread often recur.
Objective: To address the need for more predictive pre-clinical research platforms and to identify new targets and therapies for bone metastatic castration-resistant prostate cancer (CRPC).
Design, setting, and participants: We used patient-derived xenograft (PDX) tumors from bone metastatic prostate cancer patients to establish three-dimensional (3D) organoids and investigated their response to ADT by either withholding di-hydro-testosterone (no DHT) or treating with enzalutamide.
Outcome measurements and statistical analysis: Cyst/spheroid quantitation, immunohistopathology, cell viability assay, qRT-PCR, RNA sequencing, gene set enrichment analysis (GSEA) and live cell cycle tracking using *Fucci2BL* were performed.

Results and limitations: ADT resulted in CRPC spheroids with $CK5^+$ $CK8^+$ cells, up-regulated stem-cell transcription factors, steroidogenic and neurogenic pathways and down-regulated AR-target genes, interferon, cell cycle, cell division and circadian pacemaker pathways. Enzalutamide-treated spheroids transitioned to G_0 and AR protein was decreased but not AR mRNA. Moreover, ADT decreased both ACE2 and TMPRSS2, host cell viral entry factors for the severe acute respiratory syndrome (SARS) SARS-CoV-2.

Conclusions: This study identified a new dormant CRPC basal-luminal hybrid prostate cancer cell and gene signature which may be targeted to eradicate dormant CRPC bone metastases in order to prevent disease recurrence. The PDX organoids can be used also to screen for therapies that reduce ACE2 and TMPRSS2 expression thus suppressing viral load of SARS-CoV-2 and its variants.

Patient Summary: In organoids, or **mini-tumors**, established from prostate cancer bone metastases, a novel type of dormant ADT-resistant cell with specific gene changes emerged which may be targeted to eradicate dormant metastases before they can progress. ADT also reduced the cell factors required for SARS-CoV-2 or its variants to infect its host cells and thus may reduce COVID-19 disease severity.

HIGHLIGHTS

- Patient-derived xenograft (PDX) models for bone metastatic prostate cancer and three-dimensional (3D) organoids (mini-tumors) more predictive pre-clinical research platforms.
- PDX Organoids used to test androgen deprivation therapy (ADT) resistance mechanisms.
- Novel hybrid basal-luminal cells that were ADT resistant, quiescent prostate stem-cell like gene expression profile.
- ADT decreased expression of the severe acute respiratory syndrome (SARS) SARS-CoV-2 host cell viral entry factors, TMPRSS2 and ACE2 as well as the Middle East respiratory syndrome (MERS) MERS-CoV receptor, DPP4 as well as anti-viral interferon response.
- Therefore, PDX organoids may be used to screen for novel therapies that target mechanisms of ADT resistance in PCa and for therapies that reduce TMPRSS2 and ACE2 expression and thus block SARS-CoV2 infection.

GRAPHICAL ABSTRACT

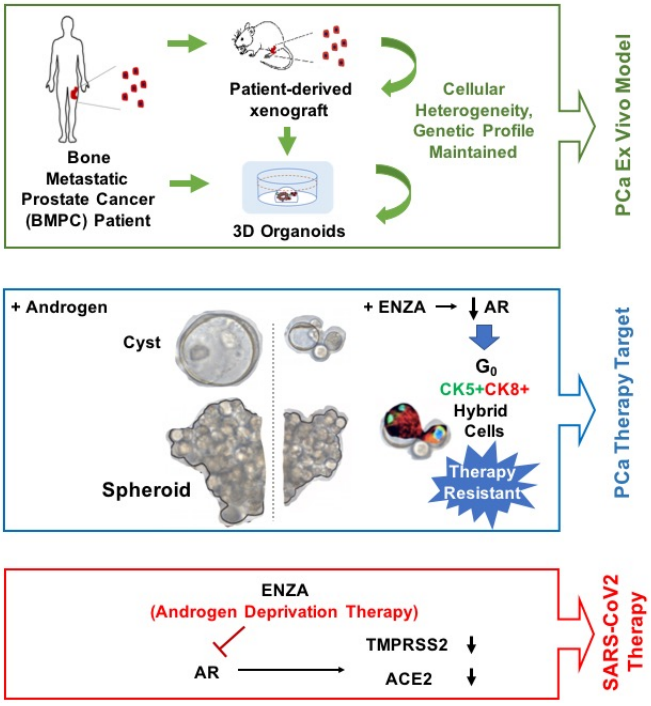


Figure 12. PDX organoids treated with Androgen deprivation therapy were CRPC and Dormant. Pre-clinical drug screening platforms for new CRPC and SARX-CoV2 and variants treatments.

7. Developing ROR1 CRISPR/Cas9 knock out in PCa cell lines and PDX PCSD13

The original plan was to knock down ROR1 using shRNA constructs. However, after discussions with Dr. Kipps lab members we decided to use CRISPR/Cas9 instead since shRNA does not sufficiently . We are using the Synthego Gene Knock Out Kit v2 – Human ROR1 along with the ThermoFischer Neon transfection system. We will perform and optimize CRISPR/Cas9 knock out in the PCa cell lines: PC3, DU145 and LNCaP.
The PDX PCSD13 will be FACS sorted to enrich for ROR1 positive cells and then ROR1 will be knocked out with the Synthego CRISPR/Cas9 ROR1 kit.

8. **Established ROR1 Immunohistochemical assay (IHC) for measuring ROR1 protein levels on pre-clinical studies in organoids, PDX models and in patient biopsies and tumor tissues.**

Compared specificity and sensitivity of commercially available anti-ROR1 antibodies IHC staining was be performed to determine optimal commercially available anti-ROR1 antibody such as 4A5 (Becton Dickinson) or ProteinTech, a rabbit polyclonal in the UCSD Tissue Technology Shared Resource histology core. IHC optimization was performed by Reveal Biosciences on control tissue formalin-fixed, paraffin-embedded (FFPE) tissue from a patient breast adenocarcinoma as shown in **Fig 13.** ProteinTech anti-ROR1 antibody showed highest specificity and sensitivity in optimization study.

We prepared control FFPE blocks of ROR1 positive control cell lines: Jeko, MCF7-ROR1, PC3, DU145 and negative control cell lines: RAJI, HS5 (WNT5A+) for IHC analysis. IHC analysis of Prostate cancer cell lines, PDXs, organoids and patient prostatectomy sections is currently underway.

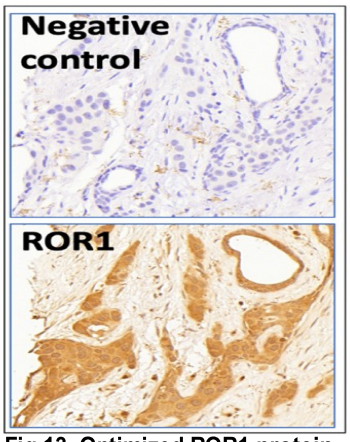


Fig 13. Optimized ROR1 protein IHC analysis. Right panels: ROR1 IHC optimization on patient breast adenocarcinoma formalin-fixed paraffin-embedded sections.

Specific Aim 2. Determine mechanism of action of ROR1-targeting antibody therapeutic, CIRMTUZUMAB (biologic), in pre-clinical trials using PDO and PDX models of bone metastatic prostate cancer. Perform IND-enabling studies and prepare for Phase 1B clinical trial for bone metastatic CRPC.

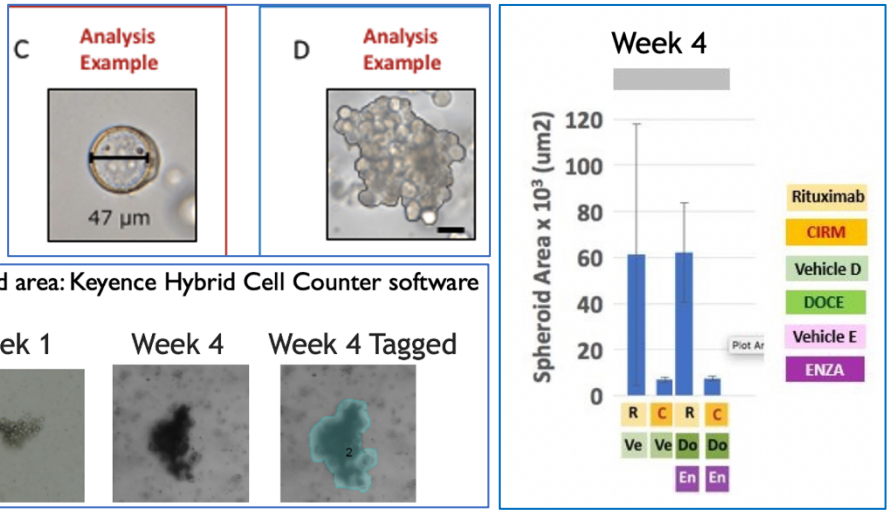
1. **Characterizing docetaxel, cirmtuzumab response in PDX-derived organoids: PCDSD1 and PCSD13**

Performed quantitative analysis of the bone metastatic prostate cancer 3D organoids treated with combinations of the new cancer stem cell antibody therapeutic, Cirmtuzumab, plus standard of care therapies for metastatic prostate cancer: enzalutamide and docetaxel. We showed that Cirmtuzumab reduced the number and growth of the organoids (**Fig 14**). This is a critical result for the pre-clinical evidence supporting the IND-filing for FDA approval for the pending clinical trial of Cirmtuzumab in patients with metastatic prostate cancer.

Fig 14. Cyst and Spheroids in PCSD1 PDX organoids quantitation with Keyence Hybrid Count software. Cirmtuzumab reduced the number and growth of the spheroid organoids

2. **Further Optimization of New Organoid Functional Response Digital Microscope Assay:**

We improved quantitation of digital microscope number and size measurements of Cyst and Spheroid. This approach yields much more comprehensive understanding of the effects of treatments in live cells, over time than the standard viability end-point assays typically used in organoids. It is a key measurement to assess viability and growth in the PDX



organoids which are heterogeneous more like the disease in patients and contain two main types of multi-cellular masses: Cysts and Spheroids. These can respond differently to drug treatments as we have shown in our manuscript Lee et al, Appendix D. The cysts are more differentiated while the spheroids are less differentiated, more cancer stem-like and more therapy resistant. Evaluating them separately allows us to measure the effects on these different types of organoids in order to define the responsive and resistant tumor cells as seen reflected in heterogenous responses of prostate tumors and metastases in patients.

We used the Keyence BZX710 digital microscope to capture images weekly and using the Keyence Hybrid count have measured the size and number of cysts and spheroids. This is an accurate but labor-intensive and time-consuming approach. Recently, we have worked with Reveal Biosciences, 6760 Top Gun St, Suite 100, San Diego, CA to develop more automated digital analysis software to analyze the large number of PDX organoid treatment experiments we have carried out. Analysis is underway for PCSD13 organoids treated with recombinant WNT5A protein plus Cirmtuzumab.

3. **Performing *In vivo* PDX PCSD13 and ROR1+ PCa cell line xenograft experiments to test Cirmtuzumab +/- Docetaxel response and sequencing**

a.) PDX pre-clinical trial for IND-filing for amendment for Phase clinical trial:

Group	Target Cells	Treatment (ROA)	N =
A	PCSD13 SC	Rituximab (control), vehicle	5
B		Rituximab + Docetaxel	5
C		Cirmtuzumab, vehicle	5
D		Cirmtuzumab + Docetaxel	5

b.) *In vivo* experiment using PC3-RFP-Luciferase plus Cirmtuzumab +/- Docetaxel: sub-cutaneous and intra-cardiac models. We have used the PC3-RFP-Luciferase cells we generated to test Cirmtuzumab-CART cells to inhibit sub-cutaneous tumor growth as shown below. We will use PC3-RFP-Luciferase cells to test efficacy of Cirmtuzumab plus Docetaxel as for PCSD13 described above. We will also perform intra-cardiac injection with PCR-RFP-Luciferase as a model of metastatic dissemination to test the ability of Cirmtuzumab to inhibit metastatic tumor growth. Mice will be monitored using in vivo bioluminescence (IVIS) as shown below for CART cell experiments.

Administrative Milestones:

a. ACURO approval received

b. HRPO: PDXs are exempt from HRPO in accordance with DoD Memo () and all samples from deceased subjects.

MILESTONES:

Specific Aim1: Determine the mechanism of WNT5A/ ROR1/ ROR2 signaling in bone metastatic CRPC using patient-derived organoids (PDO) and xenograft (PDX) models using shRNA and small molecule inhibitors of WNT-signaling.

1. ROR1 mRNA is expressed at high levels on castration resistant small cell PCa and neuroendocrine PCa (NEPC) cell lines and TCGA PCa dataset.
2. PCa cell lines, PC3 and DU145, express cell surface ROR1 protein
3. Generated PC3, DU145, LNCaP RFP-luciferase expressing cells for in vitro 2D and 3D growth and viability assays for testing Cirmtuzumab and WNT5A in vitro (Incucyte) and in vivo xenograft assays.
4. Established PC3 PCa cell line organoids (below, left) to study WNT5A:ROR1 signaling and Cirmtuzumab mechanism of action compared to PCSD1 (below, right) and PCSD13 PDX organoids.
5. Established Live Cell Cycle Tracking System – The Fucci2BL lentiviral vector for testing cell cycle changes in live organoids was established in PC3, DU145, LNCaP, PCSD1, PCSD13.
6. Identified a new dormant CRPC basal-luminal hybrid prostate cancer cell and gene signature under standard-of-care enzalutmaide or androgen deprivation in PDX organoids which may be targeted to eradicate dormant CRPC bone metastases in order to prevent disease recurrence.
7. Developing ROR1 CRISPR/Cas9 knock out in PCa cell lines and PDX PCSD13
8. Established ROR1 Immunohistochemical assay (IHC) for measuring ROR1 protein levels on pre-clinical studies in organoids, PDX models and in patient biopsies and tumor tissues.

Specific Aim 2. Determine mechanism of action of ROR1-targeting antibody therapeutic, CIRMTUZUMAB (biologic), in pre-clinical trials using PDO and PDX models of bone metastatic prostate cancer. Perform IND-enabling studies and prepare for Phase 1B clinical trial for bone metastatic CRPC.

1. Characterizing docetaxel, cirmtuzumab response in PDX-derived organoids: PCDSD1 and PCSD13

2. Further Optimization of New Organoid Functional Response Digital Microscope Assay.

3. Performing *In vivo* PDX PCSD13 and ROR1+ PCa cell line xenograft experiments to test Cirmtuzumab +/- Docetaxel response and sequencing

Administrative Milestones:

c. ACURO approval received
d. HRPO: PDXs are exempt from HRPO in accordance with DoD Memo () and all samples from deceased subjects.

4. IMPACT

Advanced prostate cancer is usually treated with Androgen deprivation therapy (ADT) which can help maintain remission in patients, however, growth and metastatic spread often recur. Treatments with new mechanisms of action are urgently needed. We are using patient-derived xenograft and PCa cell line models to test mechanism of action of a new therapeutic target: the WNT5A/ROR1 signaling pathway in prostate cancer for which a therapeutic ROR1 inhibitor antibody, Cirmtuzumab, has been developed and clinically tested in CLL and metastatic breast cancer patients. In Y1 of this grant we have shown that ROR1 is expressed at high levels on castration resistant small cell PCa and neuroendocrine PCa (NEPC) models; two of the most lethal forms of prostate cancer for which there are no curative treatments in PCa cell lines and PDX models. Cirmtuzumab has demonstrated efficacy in our patient derived xenograft organoid cultures. We are now performing in vitro and in vivo testing in NEPC cell lines and in our patient-derived xenograft (PDX) mouse models. We have developed immunohistochemistry (IHC) assays to detect ROR1 in patient biopsy samples which will allow us to define the prostate cancer patient population that expressed ROR1 and to be able to evaluate the treatment in patients for a future clinical trial. These studies are will inform us about the appropriate patient populations for treatment and clinical trials. These pre-clinical studies being performed in this grant will support the pending Phase 1B clinical trial (not part of this grant).

IRB-approved clinical trial:
Co-Investigator, A Phase 1B, Nonrandomized Trial Investigating Docetaxel Combined with Cirmtuzumab in Patients with Metastatic Castration Resistant Prostate Cancer. **PI: Dr. J. Kellog Parsons, UCSD, Co-Investigator: Dr. Rana Mckay.**
We are performing the pre-clinical studies required for filing for an amendment to IND approval at the FDA to perform the clinical trial in prostate cancer patients. I will be guiding the analysis of the Cirmtuzumab target, ROR1, IHC assays on biopsies from patient metastases on the trial to assess the effectiveness of Cirmtuzumab therapy. In parallel we will be performing ROR1 IHC analysis in a cohort of archived pathology patient biopsy and prostatectomy specimens to further define the prostate cancer patients who may benefit from this therapy.

5. CHANGES/PROBLEMS

1. Special Comments about COVID-19

The COVID-19 pandemic threw all of us into survival mode and required us to adapt to a new way to work to ensure the safety and continued productivity of our teams. This gave us all the opportunity to reset and creatively renew our work and assumptions about our lives. I have successfully pivoted my efforts and those of my team to maintain progress and exponentially increase skills, and most notably, our adaptability to our new COVID-19 work-style and our ability and motivation to get work done.

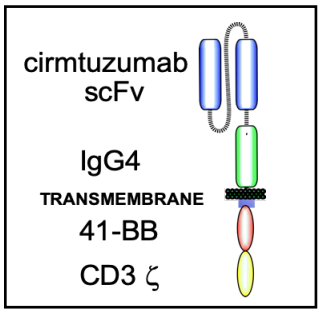
With the lab shut down from March 18th, 2020 to June 30th, 2020 my team and I shifted our efforts to our bioinformatics and digital microscope image analyses which we could do remotely. I upgraded our computer and software for everyone on our team and we were able to work efficiently as a team remotely. I had daily calls with my lab manager and postdoctoral fellow, weekly lab meetings of the whole team and weekly meetings with the undergraduate researchers and Visiting Scholar. We have several experiments already performed for which several hundred images remain to be analyzed. We have also reached out to a local company, Reveal Biosciences, to help our team with independent data analysts.

My lab manager and postdoctoral fellow maintained our irreplaceable patient samples and models. Now that we are allowed 25% lab occupancy time, we are doing new experiments with these models. These pre-clinical experiments are also being used for IND-filing for FDA approval of a new clinical trial for Cirmtuzumab in advanced prostate cancer patients.

We increased our bioinformatics efforts and are performing RNA sequencing of RNA from the organoid and PDX experiments. In collaboration with our bioinformatician, Dr. Terry Gaasterland, PhD, Professor of Computational Genetics, UCSD, we are performing gene expression profiling to identify gene signatures altered by WNT5A and Cirmtuzumab treatment. My undergrads and new graduate student are learning to use publicly available bioinformatics software: Genesis and GSEA to perform directed analysis of gene signatures identified in previous studies of prostate cancer stem cells and Cirmtuzumab treated patients from different cancers. They are reviewing the literature to compile the gene lists and use these to query the RNA sequencing data sets from our organoid experiments. This will help to identify the key genes and signaling pathways that may be used as biomarkers for monitoring effectiveness of Cirmtuzumab in patients in the clinical trial and for designing combination treatment plans with appropriate standard of care regimens.

2. **Recently,** we tested Chimeric antigen receptor **(CAR)-T cells** made with the Cirmtuzumab antibody and have shown that these CART cells completely kill ROR1 expressing PCa cell lines and PDX cells in culture. The Cirmtuzumab-based CAR-T cells can kill cells in culture that are from two of the most lethal types of prostate cancer: castration resistant PCa (CRPC) and NEPC. We are testing these our mouse xenograft in vivo and hope to use these CART cells to see if we can eradicate lethal prostate cancers in a clinical trial. CART cells are killer T cells engineered with a chimeric receptor that combines the known specificity of an antibody with the signaling activator that engages the killing machinery of the T cell. Therefore, the engineered killer T cell can be directed toward a desired target on a cell such as a prostate cancer cell. CART cells are a way to direct the awesome power of the immune system that can truly eradicate cancer as shown in some melanomas and leukemias. We hope to use CART cells that target ROR1 to eradicate two of the most lethal forms of prostate cancer: CRPC and NEPC.

Fig 1 ROR1 targeting Cirmtuzumab based Chimeric Antigen Receptor (CAR) T cell.



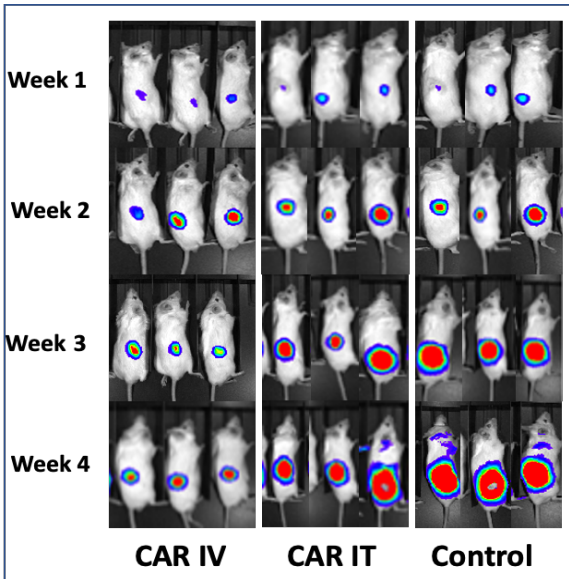


Figure 11-Bioluminescence imaging of mice implanted subcutaneously with PC3 PCa cells and Treated with ROR1 CAR T-cells. Animals were implanted with 2e6 lucifierase expressing PC3 cells on day 1 and were treated with the one-time injection of 3e7 CAR-T cells IV (**CAR IV**) or activated mock transduced cell (**Control) or** 1e7 cells administered intra-tumorly (**CAR IT**) As shown, the CAR-T treated mice that received a single intravenous or intra-tumoral injection had reduced disease burden when compared to control animals, which had to be sacrificed by week 5 The CAR-T IV treated cohort had only minimal amounts of disease at the end of the study, which validates the use of this model for the planned studies detailed in this proposal.

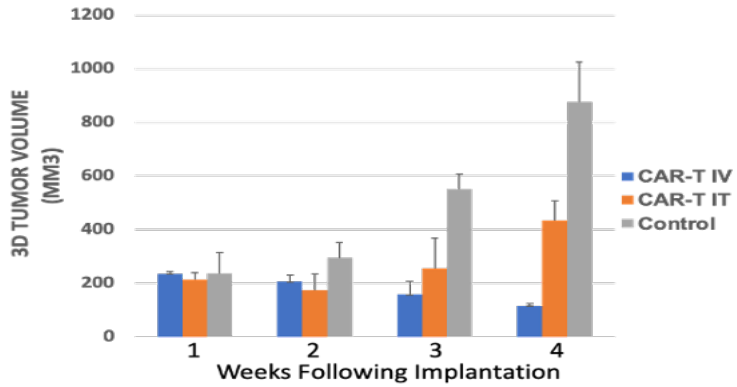


Fig12. Tumor volume measurement of implanted PC3 PCa cells by Caliper measurement. Tumor volumes were measured by weekly caliper measurements for mice treated as described in Fig 11. Results are consistent with the bioluminescent measurements and better demonstrate the activity of the intravenously administered CAR product. Tumor volume was determined with the formulate $mm^2 = (l \times w^2) / 2$. Error bars represent the SD of the mean for the 3 mice.

10. Products

Cirmtuzumab (Oncternal, Inc) therapeutic antibody

To date, no Wnt signaling inhibitors, including ROR1 targeting agents, have been approved for clinical use in the treatment of cancer. Because it has been shown to be expressed on a number of highly malignant hematological and solid tumor cancers, including CRPC and NEPC cells from patient derived samples and has a demonstrated functional role in oncogenesis, ROR1 is an attractive therapeutic target (Table 1). To target this proto-oncogene, cirmtuzumab, a first-in-class ROR1 binding monoclonal antibody (mAb) was developed at the University of California San Diego (UCSD) by Drs. Thomas Kipps, and Charles Prussak with funding support from the California Institute for Regenerative Medicine (45). This humanized mAb has a high affinity for human ROR1 and no apparent off-target activities in in vitro or in vivo test systems.

Based on a battery of preclinical studies, a pilot phase I study of single agent cirmtuzumab was conducted in patients with relapsed/refractory chronic lymphocytic leukemia. In this clinical study, the anti-ROR1 mAb was well tolerated with no antibody associated serious adverse events noted, had a prolonged half-life no off-tumor normal tissue binding and early evidence of anti-tumor activity (NCT02222688) (44).

Cancer Type	ROR1 Expression (%)
Uterus	96
MCL	>95
CLL	95
Lymphoma	90
Prostate	90
Skin	89
Pancreas	83
Adrenal	83
Lung	77
Breast	75
Testicular	73
Colon	57
Ovarian	54
Bladder	43

Table 1. Cancer type and percentage of ROR1 expression

Because of its favorable therapeutic index, cirmtuzumab has subsequently been studied in a number of clinical trials targeting lymphoid and solid tumor cancers in combination with standard chemotherapies (NCT02776917, 02860676 and 03088878), as the targeting moiety for an antibody drug conjugate (ADC) (NCT03833180) and an IRB-approved Phase 1b clinical trial with this mAb is imminent for patients with metastatic CRPC.

For these reasons, solid tumor cancers of the breast, prostate, lung and colon represent particularly intriguing targets for anti- ROR1 targeting therapies with elevated expression of this proto-oncogene being detected on more malignant forms of these cancers. For example, we have demonstrated elevated expression of ROR1 on highly malignant, taxane resistant, breast cancer cells (Table 1). Because this mAb blocks ROR1 signaling by blocking Wnt5A mediated activation, cirmtuzumab has demonstrated substantial therapeutic activity against these chemotherapy resistant tumors in preclinical models. Based on these studies, a phase Ib clinical trial combining cirmtuzumab with paclitaxel is being tested in patients with advanced breast cancer (NCT02776917)(34). Like breast cancer, a taxane (docetaxel) based chemotherapy regimen is a mainstay for the treatment of patients with advanced CRPC(72). However, when combined with abiraterone and enzalutamide, this docetaxel containing chemotherapy cocktail has minimal activity in treating CPRC, generating a 3- month median time to progression and limited impact on reducing PSA levels (<30% of patients) (73). Additionally, noncanonical Wnt signaling has been hypothesized to be a mechanism of resistance to enzalutamide(2 8) and taxane chemotherapy (63). Available data suggest that Wnt blockade with chemotherapy may be the most effective way to implement Wnt pathway modulation(52).

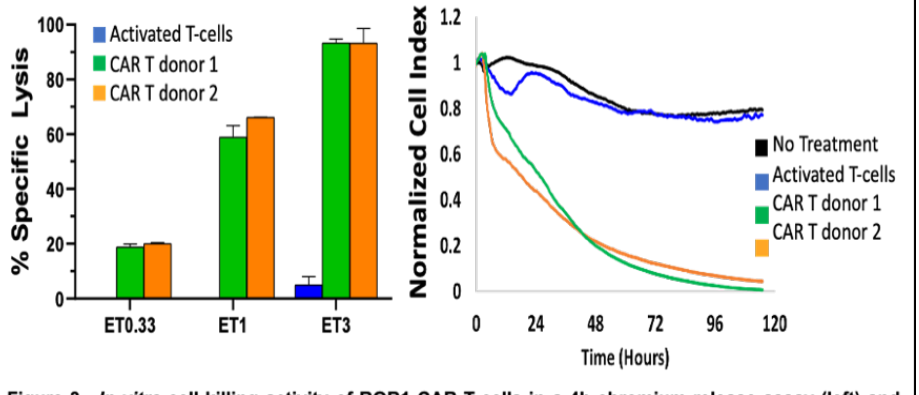


Figure 3. ***In vitro*** **cell killing activity of ROR1 CAR T-cells in a 4h chromium release assay (left) and 120h ACEA impedance assay (right).** ROR1 CAR T-cell products were generated from 2 healthy donors and tested in a chromium release assay at the indicated E:T ratios against leukemic Mec1 ROR1^{pos} cells in the left panel and an ACEA impedance assay against MB 231 ROR1^{pos} breast cancer cells in the right panel. The anti-ROR1 CAR T-cells demonstrated high and specific cytotoxicity without significant killing of ROR1-negative target cells..

11. Participants & Other Collaborating Organizations

Co-investigators:
Dr. Terry Gaasterland, PhD, Professor of Computational Genetics, UCSD, we are performing gene expression profiling to identify gene signatures altered by WNT5A, ROR1 CRISPR/Cas9 knock out, and Cirmtuzumab treatment alone, with standard-of-care: enzalutamide +/- docetaxel, or in combination in PDX organoids and PDX in vivo. Performed expression profiling and GSEA analysis in PDX organoids plus ADT for Lee, Mendoza, Burner et al (manuscript Submitted to European Urology, attached as Appendix D).

Dr. Nicholas A. Cacalano, PhD, Associate Professor, Dept of Radiation Oncology, UCLA, is performing the CRISPR/Cas9 KO, performing ROR1 IHC and IFC on PCa cell lines and PDX Fucci2BL expressing organoids and working on ROR1 expression analysis and western optimization for PCa cell lines, organoids and PDXs.

Dr. Karl Willert, PhD, Associate Professor, Dept of Medicine, UCSD, has provided WNT5A recombinant protein and the Porcupine inhibitor for in vitro assays in PDX and PCa cell line models. Discussions and experiment planning and interpretation.

Dr. Rana Mckay, MD, Associate Professor, Dept of Medicine, UCSD, GU Oncologist, and
Dr. Christopher Kane, MD, Professor, Dept of Urology, UCSD
Cirmtuzumab clinical trial planning and discussion of pre-clinical in vivo trial design to support and inform IND-enabling FDA approval of Phase 1B Clinical trial planning with UCSD Alpha Stem Cell Clinic, identification of target population, wrote and submitted IRB protocol which has been approved.
Co-Investigator, A Phase 1B, Nonrandomized Trial Investigating Docetaxel Combined with Cirmtuzumab in Patients with Metastatic Castration Resistant Prostate Cancer. PI: Dr. J. Kellog Parsons, UCSD, Co-Investigator: Dr. Rana Mckay.

12. Special Reporting Requirements

N/A

13. Appendices

A. OPR Memo on PDX and HRPO

B. Technical Abstract

C. SOW

D. Manuscript: Lee, Mendoza, Burner et al. (pdf Attached)

DEPARTMENT OF THE ARMY
HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MARYLAND 21702-5000

FCMR-RP

MEMORANDUM FOR RECORD

SUBJECT: Clarification of Office of Research Protections (ORP) oversight of the use of existing, established PDX models in USAMRDC supported research.

1. References:

a. Memorandum, SECARMY, 5 November 2019, subject: Army Policy for Use of Human Cadavers for Research, Development, Test and Evaluation, Education or Training.

b. Guidance on HRPO Review Requirements for Research Involving the Secondary Use of Data/Specimens, 8 May 2020

2. This memorandum serves to clarify that USAMRDC supported research involving the use of established, existing patient derived xenograft (PDX) models (or the cell lines derived therein) that were established using tissue from deceased donors does not require ORP Human Research Protection Office's review and oversight for cadaver use in accordance with reference (a).

3. HRPO submission and review remains required when DoD funding is used to combine human specimens (e.g. cell lines, tissue) with non-human material to create a xenograft for research use in accordance with reference (b). In addition, the use of established, existing PDX models established using tissue from living donors and/or the creation of cell lines from established, existing PDX models currently does not require MRDC HRPO review.

4. The research use of established, existing PDX models created without DoD support (e.g., research with mice) does require institutional animal use and USAMRDC ORP Animal Care and Use Review Office (ACURO) review.

Kimberly L. Odam, MS,CIP
Director
Office of Research Protections

Technical Abstract: ***Targeting WNT5A-mediated Therapy Resistance Mechanisms and Tumor Genomic Heterogeneity in Lethal Bone Metastatic Prostate Cancer.***

BACKGROUND: One in six men will be diagnosed with prostate cancer (PCa), making it one of the leading health problems affecting men in today's society. Patients diagnosed during the earlier stages are surviving longer due to improved therapies. However, a growing number of these patients later go on to develop advanced PCa. Since the 2012 USPSTF recommendation against PSA screening there has also been an increase in the number of men presenting with higher grade, metastatic prostate cancer. The main treatment for advanced prostate cancer (PCa) is androgen deprivation therapy (ADT) which targets androgen receptor (AR) signaling. Unfortunately, patients invariably become resistant to ADT, that is, they develop castrate resistant prostate cancer (CRPC) and their cancer metastasizes - most often to bone.

Bone metastatic prostate cancer affects the majority of men with advanced prostate cancer and is present in more than 90% of patients who die of the disease. Bone metastases cause extreme pain and some of the most debilitating effects of cancer in patients including pathologic fractures and spinal compression that require significant and ongoing intervention. It is also associated with increased therapy resistance and lethal, malignant disease progression. There are no curative treatments for bone metastatic prostate cancer. Therefore, development of therapies that increase therapeutic targeting of PCa in the bone is urgently needed.

RATIONALE: Prostate cancer (PCa) is highly heterogeneous making it difficult to treat with targeted therapies. Most bone metastatic PCa develops therapy resistance. We and others showed WNT5A, a Wnt-family ligand, is correlated with bone metastatic, castrate resistant PCa (bmCRPC). Here, we aim to determine the involvement of WNT5A in ADT therapy resistance and to test CIRMTUZUMAB, the WNT5A receptor, ROR1-inhibiting antibody biologic therapy, for its effectiveness against bmCRPC in pre-clinical, IND-enabling studies leading to a Phase 1B clinical trial. Next, we will define the gene expression profiles at the single cell level of ADT therapy resistant metastatic cells in a biobanked series of surgical prostate cancer bone metastasis specimens and matched PDO and PDX models. Single cell RNASeq gene expression profiles will allow us to measure heterogeneity of the patient tumor cells and the non-tumor cells of the bone microenvironment in our collection of twenty surgical patient prostate cancer bone metastasis samples and patient-derived xenografts. Single cell RNA sequencing is best performed using viable, single cells. To the best of our knowledge we are the only group to have a biobank of these rare patient samples cryopreserved as viable, single cells.

HYPOTHESIS: WNT5A and its downstream signaling pathways were strongly up-regulated in the bone-niche in our patient specimens and in their matched xenografts (PDX). We postulate that WNT5A/ROR1/ROR2 signaling supports early stage, androgen-independent prostate development which may be re-accessed in bone metastatic prostate cancer under the selection pressure imposed by anti-androgen treatment. Our model states that anti-androgen suppression of AR may induce the expression of WNT5A and subsequently a prostate stem/progenitor cell-like program that mimics early prostate development in low or no androgen conditions. We propose experiments to test our hypothesis that inhibition of WNT5A expression and/or signaling via blocking its receptor, ROR1, will eradicate the tumor initiating and tumor perpetuating cells in the bone niche.

AIM 1. Determine the mechanism of WNT5A/ ROR1/ ROR2 signaling in bone metastatic CRPC using patient-derived organoids (PDO) and xenograft (PDX) models using shRNA and small molecule inhibitors of WNT-signaling. **AIM 2.** Determine mechanism of action of ROR1-targeting antibody therapeutic, **CIRMTUZUMAB** (biologic), in pre-clinical trials using PDO and PDX models of bone metastatic prostate cancer. Perform IND-enabling studies and prepare for Phase 1B clinical trial for bone metastatic CRPC. Cirmtuzumab is in Phase 1b clinical trials for CLL and metastatic breast cancer. **AIM 3.** Perform single cell RNASeq analysis on our unique series of surgical patient prostate cancer bone metastasis specimens. Determine single cell RNA profiles of tumor and bone environment non-tumor cells in our biobank of twenty viably cryopreserved, surgical patient prostate cancer bone metastases and the organoids and xenografts derived from them. We have used these PDO and PDX models to test standard of care therapies for inhibiting prostate cancer growth. Thus, we are poised to use our PDX and PDO models to test novel WNT5A targeting therapies to overcome bone-niche mediated CRPC. The proposed research addresses two of the PCRP 2018 overarching challenges: 1. to develop treatments that improve outcomes for men with lethal prostate cancer and 2. to define the biology of lethal prostate cancer to reduce death.

STATEMENT OF WORK – 10/10/2018
PROPOSED START DATE Oct 01, 2019

Site 1: **Moores Cancer Center, UC, San Diego (UCSD)**
3855 Health Sciences Drive
La Jolla, CA 92093820
PI: Dr. Christina Jamieson

Site 2: **Dept of Radiation Oncology, UC, Los Angeles (UCLA)**
10833 LeConte Ave.,
Los Angeles, CA 9
PI: Dr. Nicholas A. Cacalano

SPECIFIC AIMS	Timeline	Site 1	Site 2
Specific Aim 1: Determine mechanism of WNT5A signaling in bone metastatic CRPC using PDX and PDO models.			
Major Task 1: Gene silencing (shRNA) of WNT5A, ROR1 and ROR2 in patient-derived xenograft (PDX) models.			
Subtask 1: WNT5A, ROR1 and ROR2 will be silenced in 3 PDXs: PCSD1, PCSD5 and PCSD13 using commercially available shRNA lentiviral particles.	1-6 months	CJ	
Subtask 2: Confirm WNT5A, ROR1, ROR2 silencing in xenografts: Immunoblotting.	3-7	KW	NC
Major Task 2: Determine effect of silencing WNT5A, ROR1 or ROR2 on tumor growth and signaling *in vivo.*			
Subtask 3: Expt. 1. Intra-femoral injection of PCSD1 shRNA control, shRNA_WNT5A, shRNA_ROR1 and shRNA_ ROR2. Establish intra-femoral xenografts in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$, n=10 mice x 4 shRNA stably transduced lines. Measure tumor growth using calipers and IVIS, serum PSA and ALP. Harvest at 8 weeks or max tumor size for qPCR, RNASeq and immunoblotting of WNT5A, ROR1, ROR2 and signaling pathways. MicroCT of bone lesions, IHC, IFC PCa markers.	3-6	CJ KW TG	NC
Subtask 4: Expt. 2. Intra-femoral injection PCSD5 shRNAcontrol, shRNA_WNT5A, shRNA_ROR1 and shRNA_ ROR2. Establish bone metastatic intra-femoral PCa in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. Measure tumor growth and collect tumor tissues as above.	6-10	CJ KW TG	NC
Subtask 5: Expt. 3. Intra-femoral injection PCSD13 shRNAcontrol, shRNA_WNT5A, shRNA_ROR1 and shRNA_ ROR2. Establish bone metastatic intra-femoral PCa in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. Measure tumor growth and collect tumor tissues as above.	9-12	CJ KW TG	NC
Subtask 6. Expt. 4. **Intra-cardiac** injection of PCSD1, PCSD5 and PCSD13 with shRNAcontrol, shRNA_WNT5A, shRNA_ROR1 and shRNA_ ROR2 into 5 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice per shRNA, Total = 60 mice. Measure #, size metastases using IVIS.	12-15	CJ KW TG	NC
Subtask 7: Expt. 5. Intra-femoral PCSD1_shRNA_ROR1 plus ENZALUTAMIDE (ADT). Establish bone metastatic intra-femoral PCa in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. Treat with vehicle or enzalutamide daily, 28 days. Measure tumor growth using calipers and IVIS, Serum for PSA, ALP. Collect tumor tissue at end of treatment for qPCR, RNASeq and immunoblotting of PCa markers and WNT5A/ROR1/ROR2 signaling. MicroCT analysis of bone lesions and IHC, IFC of PCa markers.	16-19	CJ KW TG	NC
Subtask 8. Expt. 6. Intra-femoral PCSD5_shRNA_ROR1 plus ENZA (ADT). Establish intra-femoral PCa in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. Treat and analyze as above.	20-23	CJ	
Subtask 9: Expt 7. Intra-femoral PCSD13_shRNA_ROR1 plus ENZA (ADT). Establish intra-femoral PCa in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$ mice. Treat and analyze as above.	24-27	CJ KW	NC
Subtask 10: Bioinformatics on all RNASeq, qPCR and immunoblotting confirmation	28-34	CJ TG	NC

Milestone #1: *Manuscript on WNT5A, ROR1, ROR2 signaling and role in ADT resistance in bone metastatic PCa.*	32-36	CJ KW TG	NC
Major Task 3: Define proteins interacting with ROR1in PDX intra-femoral tumors using Mass Spectrometry.			
Harvest tumors from mice with PCSD1, PCSD5 and PCSD13 intra-femoral xenografts. Extract protein from fresh tumor tissue, immunoprecipitate with anti-ROR1 or isotype control antibody. Analyze by Mass Spec to identify co-immunoprecipitating proteins.	1-2	CJ	NC
Confirmation of protein-protein interactions by co-ip and immunoblotting for ROR1.	3-9		NC
Milestone #2: *Manuscript ROR1-associating proteins in patient derived bone met PCa.*	9-18	CJ	NC
Major Task 4: Determine effect of silencing WNT5A, ROR1 or ROR2 on viability, growth and signaling *in vitro* using 3D/ patient derived organoid (PDO) models of bone metastatic prostate cancer.			
Establish 3D/organoid cultures from PCSD1, PCSD5 and PCSD13 cells with shRNAcontrol, shRNA_WNT5A, shRNA_ROR1 and shRNA_ROR2.	6-8	CJ	
Treat with vehicle or enzalutamide for 4 weeks then measure viability, cysts, spheroids, PSA (IHC), apoptosis (Cleaved caspase 3 IFC), proliferation (Ki67), Basal cells: CK5, p63, Luminal cells: CK8, FZD, RTK signaling mediators by IFC, confocal microscopy.	6-12	CJ KW	NC
Milestone #3: *Manuscript on mechanism of WNT5A signaling in 3 PDOs of bone met PCa.*			
Specific Aim 2: Determine mechanism of action (MOA) of ROR1-targeting biologic, **CIRMTUZUMAB** in PDO and PDX models of bone metastatic PCa.			
Major Task 1: Use PDOs from PCSD1, PCSD5 and PCSD13 to test Cirmtuzumab alone or in combination with standard of care therapies.			
Subtask 1: PCSD1 in 3D/organoid culture plus Cirmtuzumab -/+ Enza, -/+ docetaxel, -/+irradiation (IR), -/+olaparib, + IR+olaparib, + PORCN inhibitors:. Measure overall viability, size and number of cysts and spheroids, PSA, apoptosis (Cleaved caspase 3), proliferation (Ki67), Basal cells: CK5, p63, Luminal cells: CK8, FZD, RTK signaling.	1-6	CJ KW	NC
Subtask 2: PCSD5 in 3D/organoid culture plus Cirmtuzumab alone, or in combination with enzalutamide, docetaxel, IR, olaparib, IR plus olaparib, PORCN inhibitors.	1-6	CJ KW	NC
Subtask 3: PCSD13 in 3D/organoid culture plus Cirmtuzumab alone, or in combination with enza, docetaxel, IR, olaparib, IR plus olaparib, PORCN inhibitors.	1-6	CJ KW	NC
Major Task 2: Use PDXs from PCSD1, PCSD5 and PCSD13 to test Cirmtuzumab *in vivo* with enzalutamide.			
Subtask 1: Expt. 1. PCSD1-GFP_Luciferase Establish bone metastatic intra-femoral xenografts with PCSD1 in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$, n=10 mice x 4 groups: 1. Vehicle, isotype Ab control/ 2. Enza, isotype Ab control / 3. Vehicle, CIRM/ 4. Enza, CIRM. Measure tumor growth: calipers, IVIS, Serum PSA,ALP. Tumor: qPCR, RNASeq and immunoblotting: WNT5A, ROR1, ROR2 signaling pathways. MicroCT, IFC, IHC.	6-12	CJ, KW TG	NC
Subtask 2: Expt. 2. PCSD5-GFP_Luciferase Establish bone metastatic intra-femoral xenografts with PCSD1 in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$, n=10 mice x 4 groups. Treat and analyze as above.	12-18	CJ, KW TG	NC
Subtask 3: Expt. 3. PCSD13-GFP_Luciferase Establish bone metastatic intra-femoral xenografts with PCSD1 in 40 male *Rag2*$^{-/-}\gamma_c^{-/-}$, n=40 mice. Treat and analyze as above.	18-24	CJ KW TG	NC
Milestone #4: *Manuscript on efficacy of CIRMTUZUMAB in PDX and PDO models of bone metastatic PCa.*	24-28	CJ KW TG	NC

Major Task 3: Prepare for Clinical trial for CIRMTUZUMAB in bone metastatic CRPC			
Phase 1B Clinical trial planning with UCSD Alpha Stem Cell Clinic, identification of target population, writing and submitting IRB protocol. Informed by ongoing clinical trials in CLL and breast cancer.	12-36	CJ CK RM	
Prepare regulatory documents to obtain CIRMTUZUMAB for clinical trial	12-36	CJ PW	
Milestone #5: IRB approved clinical trial protocol and CIRMTUZUMAB supply for trial.			
Specific Aim 3: Determine single cell RNA profiles and deep RNASeq whole transcriptome profiling of sorted tumor cells and microenvironment cells of patient surgical bone metastasis samples.			
Purify viable, single cells from cryopreserved patient bone metastasis samples PCSD1-PCSD20, and normal bone marrow then immediately transport to Dr. Kristen Jepsen, UCSD IGM for 10XGenomics platform sample preparation and single cell RNA sequencing.	24-28	CJ	
Bioinformatics to identify different cell types from expression profiles and to identify tumor cell specific surface markers for sorting tumor cells from microenvironment cells, eg. PSMA	28-30	TG	
FACS sort tumor cells from microenvironment cells and make RNA, Deep RNASeq of RNA from sorted bone metastatic tumor cells, microenvironment cells and normal bone marrow.	30-33	CJ TG	
Bioinformatic analysis of Deep RNASeq of patient bone met samples and all PDX experiments.	33-36	CJ TG	
Milestone #6: Manuscript of single cell RNA and whole transcriptomic analysis of patient bone metastatic prostate cancer samples and xenografts to distinguish tumor and bone microenvironment profiles for biomarker and prognostic signatures of bone marrow fine needle aspiate/biopsies of bone metastases.			

Title Page

Dormant castration-resistant prostate cancer organoids with hybrid basal-luminal cells and loss of SARS-CoV-2 host factors emerge under androgen deprivation

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Keywords

Androgen Deprivation Therapy, Angiotensin-Converting Enzyme 2 (ACE2), Basal-luminal hybrid, Bone Metastatic Prostate Cancer, Enzalutamide, Patient-derived Organoids, Patient-Derived Xenograft (PDX), SARS-CoV-2, Transmembrane Protease Serine 2 (TMPRSS2)

Word count of the text

Word count of the abstract 300

Abstract (Final Version: 300, updated by CJ)

Background: Androgen deprivation therapy (ADT) can help maintain remission in advanced prostate cancer (PCa) patients with bone metastases, however, growth and metastatic spread often recur.

Objective: To address the need for more predictive pre-clinical research platforms and to identify new targets and therapies for bone metastatic castration-resistant prostate cancer (CRPC).

Design, setting, and participants: We used patient-derived xenograft (PDX) tumors from bone metastatic prostate cancer patients to establish three-dimensional (3D) organoids and investigated their response to ADT by either withholding di-hydro-testosterone (no DHT) or treating with enzalutamide.

Outcome measurements and statistical analysis: Cyst/spheroid quantitation, immunohistopathology, cell viability assay, qRT-PCR, RNA sequencing, gene set enrichment analysis (GSEA) and live cell cycle tracking using *Fucci2BL* were performed.

Results and limitations: ADT resulted in CRPC spheroids with $CK5^{+}$ $CK8^{+}$ cells, up-regulated stem-cell transcription factors, steroidogenic and neurogenic pathways and down-regulated AR-target genes, interferon, cell cycle, cell division and circadian pacemaker pathways. Enzalutamide-treated spheroids transitioned to G_0 and AR protein was decreased but not AR mRNA. Moreover, ADT decreased both ACE2 and TMPRSS2, host cell viral entry factors for the severe acute respiratory syndrome (SARS) SARS-CoV-2.

Conclusions: This study identified a new dormant CRPC basal-luminal hybrid prostate cancer cell and gene signature which may be targeted to eradicate dormant CRPC bone metastases in order to prevent disease recurrence. The PDX organoids can be used also to screen for therapies that reduce ACE2 and TMPRSS2 expression thus suppressing viral load of SARS-CoV-2 and its variants.

Patient Summary: In organoids, or mini-tumors, established from prostate cancer bone metastases, a novel type of dormant ADT-resistant cell with specific gene changes emerged which may be targeted to eradicate dormant metastases before they can progress. ADT also reduced the cell factors required for SARS-CoV-2 or its variants to infect its host cells and thus may reduce COVID-19 disease severity.

Introduction (381 -> 308)

Over 80% of advanced prostate cancer (PCa) patients develop bone metastases. Standard-of-care androgen deprivation therapies (ADTs), such as enzalutamide, can be helpful initially to prolong life, however, the majority of patients with bone metastases inevitably develop ADT resistance known as castration-resistant prostate cancer (CRPC). As a result, many CRPC patients experience significant morbidity including debilitating fractures and severe bone pain. Established prostate cancer cell lines have been used as cost-effective and readily available *in vitro* models of prostate cancer, however, they do not recapitulate the full complexity and heterogeneity of prostate tumors[1-5]. Therefore, we and other investigators have established patient-derived xenograft (PDX) models through the transplantation of patient tumor samples into immunocompromised mice[6-14].

Establishing models from prostate cancer patient tumors has been challenging because cancer cells often become senescent upon isolation[3, 6, 9, 15]. 3D organoid culture systems were developed by the Clevers group for colorectal cancer organoids which transformed the field by enabling reproducible cultures that provide niche-specific growth factors for patient-derived tumor cells[16-19]. Prostate organoids retained the histopathological and functional traits of their origin including gene expression profile, androgen responsiveness, and drug resistance[20-24 25].

Recently a connection between prostate cancer and coronavirus disease 2019 (COVID-19) was revealed via transmembrane protease serine 2 (TMPRSS2). TMPRSS2 occurs as a gene fusion with ETS family transcription factors in over 2/3 of prostate cancer patients[26]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-19, infects cells using its S protein which binds to its receptor, angiotensin-converting enzyme 2 (ACE2), on host cells[27]. TMPRSS2 was shown to be required for proteolytic processing the S protein once it is bound to ACE2 to allow fusion of the virus with the cell[27].

We report the characterization of a new ADT-resistant population and gene signature showing androgen signaling-dependent SARS-CoV-2 host entry factors in *ex vivo* models of advanced prostate cancer organoids.

Methods (596 -> 438)

Patient-derived xenograft model and 3D organoid culture

This study was carried out in strict accordance with the approval by the University of California San Diego (UCSD) Institutional Review Board (IRB). Informed consent was obtained from all human participants. A surgical prostate cancer bone metastasis de-identified specimen was harvested from a patient who progressed to castrate resistant bone metastatic prostate cancer which we called Prostate Cancer San Diego 1 (PCSD1). Intra-femoral injection of PCSD1 was performed to establish a patient-derived xenograft (PDX) model[11, 12, 28]. All animal protocols were performed under a UCSD animal welfare IACUC approved protocol.

Organoid growth

PCSD1 tumor cells were embedded in a dome of growth factor reduced Matrigel (BD Biosciences) at a seeding density of 50,000 cells per 40 µL of Matrigel. 3D Organoid cultures grown in prostate organoid medium[17] with the addition of fetal bovine serum. DHT and Enzalutamide were added to the medium at a final concentration of 1 nM and 10 µM, respectively. The vehicle control was 0.1% (v/v) DMSO in culture medium. The epithelial cyst lumen diameter, cyst counts, spheroid area, and spheroid counts were determined using a Keyence BZ-X710 microscope (Keyence Corporation).

Quantitative RT-PCR

Custom-designed human-specific primers were used for AR, prostate specific antigen (PSA) and PSMA as previously described[11]. Human and mouse-specific ACTB-specific primers were used as an internal reference control for qPCR[12]. Positive control RNA included human prostate RNA (Clontech).

RNA Sequencing Analysis and Bioinformatics

RNA sequencing was performed on bulk RNA from the organoids and sequenced with 100 basepair (bp) paired end reads (PE100) to a depth of approximately 25 million reads per sample on an Illumina NovaSeq 6000. The goal of the RNA sequencing was to identify genes responsive to ADT through analysis strategies that could take into account the heterogeneity across organoid cultures. Recognizing that each organoid potentially represented a different clone within the cancer, a query-based analysis method was developed to accommodate for inter-organoid variability. Genes were first filtered by consistent direction of response across organoid experiments 1 and 2, and then ranked by degree of fold change and consistency of their response to the androgen deprivation treatments.

Immunohistopathology

The PCSD1 organoids were harvested and processed using a spin down method as previously described[29].

Live cell cycle imaging

The cell cycle phase was determined by lentiviral bicistronic florescent, ubiquitination-based cell cycle indicator reporter (*Fucci2 BL*) system. The *Fucci2 BL* expression vector was designed and generated to have mVenus-hGem(1/110) and mCherry-hCdt1(30/120) in Pcdh-T2A-copGFP (SBI Systems Biosciences) as previously reported[30].

Statistical analysis

Statistical significance for all the experiments was determined using the Student's t-test.

Further details for each method are provided in the Supplementary material.

Results (1,188, needs to be 966)

PDX Organoids Recapitulate Heterogeneity and Androgen Deprivation Therapy Resistance of the Patient's Tumor.

Organoids were grown from the PDX, PCSD1, using intra-femoral xenograft tumor cells (Figure 1A). Small gland-like structures were observed in PCSD1 organoids and xenografts (Figure 1.B). These were similar to tumor areas that were Gleason grade 10 (5+5) prostate cancer in the donor patient's prostatectomy specimen. PCSD1 organoids consisted of a heterogeneous mix of multi-cellular epithelial cysts and spheroids (Figure 1C).

Prostate epithelial cells typically require DHT to develop and function properly. Accordingly, the average cyst lumen diameter was significantly greater ($P < 0.05$) with DHT (Figure 2) compared to no DHT and was significantly decreased ($P < 0.05$) with 10 μM enzalutamide compared to vehicle control. The total number of epithelial cysts was significantly increased ($P < 0.05$) plus DHT and decreased by 10 μM enzalutamide ($P < 0.05$). PCSD1 organoid cultures also contained spheroids, the cell-filled clusters which made up the majority of the organoids. By week 4, no DHT ($P < 0.0001$) or enzalutamide decreased the average area of spheroids ($P < 0.0001$). There was no significant difference in total number of spheroids under any of the treatment conditions. ADT-resistance of the spheroids was confirmed in Alamar Blue viability assays (Figure S3).

Androgen Deprivation Increased AR mRNA but Reduced AR protein and PSA in PDX PCa Organoids.

In organoids without DHT or with enzalutamide, AR mRNA levels increased as seen in qPCR assays (Figure 3A). In contrast, AR protein levels were low in the enzalutamide-treated organoids (Figure 3B). As expected, AR protein was in the nucleus with DHT (Figure 3B).

The effect of androgen deprivation on PSA, a transcriptional target of AR, and PSMA were evaluated. Low PSA and high PSMA mRNA expression in the organoids were consistent with the levels in the donor patient and PCSD1 PDX[11, 12]. PSA protein expression was heterogeneous in

PCSD1 organoids as was seen previously in PCSD1 PDX[12]. Androgen deprivation (no DHT or +DHT/Enza) reduced PSA mRNA expression and protein (Figure 3C-E).

Gene Expression Profiles from PDX Organoids under ADT Reveal Down-Regulation of Cell Cycle, Circadian Pathways, and SARS-CoV-2 Host Cell Entry Factors, TMPRSS2 and ACE2.

To define the global gene expression landscape of the organoids under androgen deprivation we performed whole transcriptome RNA sequencing. The bioinformatics analysis strategy was to compare gene expression in organoids with AR signaling, that is grown plus DHT, or in Vehicle plus DHT, to those with no AR signaling, i.e., no DHT or plus Enzalutamide. This strategy yielded an ADT-resistance signature of 787 differentially expressed genes: 312 genes Down-regulated with high average expression, or high RPKM, 206 genes Down-regulated, low RPKM, 171 genes Up-regulated, high RPKM and 98 genes Up-regulated, low RPKM. Hierarchical, unsupervised clustering of genes was performed on the filtered gene lists of the four gene expression patterns (Figures 4, 5 and Figure S4). Inspection of the heatmaps revealed significant down-regulation under ADT of prostate tissue and known AR-target genes such as *TMPRSS2, KLK3(PSA)* (Table 1). Interestingly, *ACE2,* the host receptor for the SARS-CoV-2 “S” protein and DPP4, the MERS-CoV host receptor were also down-regulated by ADT.

ADT significantly down-regulated genes involved in cell cycle, cell division, mitosis and mitotic spindle structure and function (*AURKA, AURKB, CCNA2*), DNA synthesis and repair (*PAPRP9, BRCA1*), circadian clock gene PER1 (Figure 4B), and an increase of two negative regulators of the circadian clock (*BHLHE41, ARNTL2*) (Figure 5B). Notably, the glucocorticoid steroid hormone receptor, *GR,* which can reset the circadian clock, was also down-regulated (Figure 4B).

ADT significantly altered genes in the following signaling pathways: Interferon signaling with down-regulation of *IRF7* and *STAT1,* which are involved in anti-viral response (Figure 4A); WNT

signaling with down-regulation of *WTIP, LBH, WNT3, and* up-regulation of *WNT4* (Figure 4 and 5)*;* epithelial to mesenchymal transition (EMT) with down-regulation of *PARD6* (Figure 4B). Interestingly, ADT led to up-regulation of genes involved in receptor tyrosine kinase (RTK) and serine/threonine kinase signaling and growth such as *EGFR, PIM1*, G-protein coupled receptor *(*GPCR) signaling (*RGS2*) and Cytokine signaling (*TNFRSF21, TNFS8, TGFB3*) (Figure 5). Many of these are actionable targets.

ADT led to significant upregulation of genes involved in lipid metabolism, cholesterol, and steroid hormone biosynthesis including *HSD3B1,* (Figure 5). Intriguingly, there was significant upregulation of genes involved in neuronal development and synaptic function (*SEMA5A, SEMA3E*). Consistent with this was downregulation of *SLITRK5*, an inhibitor of neuronal growth cone migration. Notably, ADT up-regulated *NRP1*, a pro-nociceptive factor and another SARS-CoV-2 receptor. Finally, ADT led to upregulation of developmental transcription factors involved in stem cells and cancer stem (*ETV4, SOX9, FOXO1, FOXO3*).

Gene set enrichment analysis (GSEA) identified significantly down-regulated functional pathways include interferon, cell cycle, cell division, mitosis, DNA damage response and cytokine response. Significantly up-regulated functional pathways include neurogenesis, development and differentiation, lipid metabolic process, partial apoptotic response and signal transduction response to stimulus (Figure 6.A). Enrichment ranged from 1.4-fold to 5.4-fold with p-values ranging from 0.032 (circadian clock) up to 2.6E-09 (interferon signaling) and 5.8E-11 (cell cycle) (Figure 6B-E, Table 1).

Androgen Deprivation Therapy Induced a Novel Population of Dormant $CK5^{+}CK8^{+}$ Basal-Luminal Hybrid Cells in PCSD1 Organoids.

Analysis of prostate luminal and basal cell markers in PCSD1 organoids showed that ADT led to mostly $CK5^{+}CK8^{+}$ cells with large $CK5^{+}$ nuclei and $CK8^{+}$ in the cytoplasm. PCSD1 organoids were viable but Ki67 negative thus not proliferating (SI3 and Figure 7B). To determine the status of the cell cycle in the live organoids over time we used the live cell cycle tracker system, *Fucci2BL* (Figure 7C). The majority of PCSD1 organoids in +DHT/Enza shifted to G_1/G_0 phase while the rest of organoids were green, and thus, in $S/G_2/M$ phase (Figure 7C-D). Taken together, our results are consistent with the live PCSD1 cells shifting to the G0 phase over time, and thus, becoming dormant ADT-resistant bone metastatic prostate cancer cells.

Discussion (1333 except the yellow highlighted statement, needs to be 881)

In this study, we established a new bone metastatic prostate cancer organoid model using tumor cells from the patient-derived xenograft, PCSD1, and investigated their ADT response and mechanisms of resistance to ADT. We captured the ADT-induced transition to castration resistant prostate cancer and identified gene and protein expression changes culminating in dormant CRPC organoids. The dormancy CRPC signature identified here may be targeted for novel combination drug treatments that eradicate these dormant prostate cancer metastases and prevent further progression to recurrent tumor growth and metastatic spread.

In our study, several features confirmed that the different 3D multi-cellular PCSD1 organoids all arose from the purified PDX tumor cells. First, the PCSD1 PDX tumor cells had been engineered in previous studies to stably express green fluorescent protein (GFP) and luciferase for *in vivo* imaging[12]. Accordingly, all of the PCSD1 organoids expressed GFP and luciferase. Second, PSA was expressed in PCSD1 organoids but at low levels which was consistent with the donor patient and PCSD1 in vivo PDXs. [11, 12]. Third, the PCSD1 organoids expressed AR mRNA which increased in the CRPC organoids under androgen deprivation conditions previously seen in the PCSD1 PDX treated

with anti-androgen in vivo. Fourth, the organoids expressed the prostate marker proteins, PSA, the prostate basal cell marker, CK5, and the prostate luminal cell marker, CK8. Fifth, whole genome RNA sequencing showed the expression of prostate-specific genes such as KLK3 (PSA) and NKX3.1 and decreased expression of well-known AR-target genes in the absence of AR-signaling, that is, in the organoids No DHT or plus enzalutamide. (references Raheem 2011, Godebu 2014, Toth 2019)

We and others have established 3D organoid models in culture from patient tissues which retain the stem cell and their progenitors and differentiated cells[16-19, 22, 24, 25, 29, 31-35]. The hollow prostate epithelial cysts and cell-filled, acinar spheroids which were observed in the PCSD1 organoids have also been seen in organoids from other epithelial tissues such as breast, lung and colon[18, 19, 36]. In breast cancer 3D culture models cancer cells which had less malignant properties tended to form epithelial cysts or acini with a hollow lumen while more malignant cells tended to form cellular aggregates or filled acini reminiscent of their growth as tumors in patients[36, 37]. We postulated that the epithelial cysts may come from a less malignant sub-population of tumor cells, whereas, the spheroids may arise from more malignant cancer stem-like cells in the heterogeneous PDX tumor.

Strikingly, we found that while enzalutamide-treatment of organoids led to an increase in AR mRNA there was a concomitant decrease in AR protein. This may have been due to direct binding and inhibition of AR by enzalutamide which triggered AR protein degradation. A similar response was described in Giridhar et al in which AR protein was continuously translated in prostate stem cells treated with the anti-androgen, flutamide, but it was continuously degraded by the E3 ligase, MDM2, and proteasome. Thus, the cells had no or low steady-state AR protein which was required to maintain an AR negative, prostate cancer stem cell phenotype [38]. Interestingly, our preliminary data show that PCSD1 PDX and patient tumor cells have increased copy number of the MDM2 gene and thus may have up-regulated this proteolytic pathway.

The novel ADT-resistant, dormant CRPC $CK5^+CK8^+$ hybrid cells induced in no DHT or enzalutamide-treated organoids may be developmental intermediates or transition states that were stably captured under ADT in the organoid culture conditions. In previous studies, prostate cells with both basal and luminal markers $CK5^+CK18^+$ Intermediate cells have been observed in prostate inflammatory atrophy (PIA)[39]. Recently, PIA-like, low CD38-expressing, inflammation-associated luminal cells are known to initiate prostate cancer[40]. Such intermediates, or transition states, are found in other cancers as well. Kroger *et al.* showed a hybrid epithelial to mesenchymal (E/M) transition state which down-regulated both CK5 and CK8 which was highly tumorigenic in breast cancer cell lines suggesting a model in which these transition states represent a spectrum in tumor lineage plasticity, regeneration in response to damage and tumorigenicity[41, 42]. Transcriptomic and protein analyses in IHC and IFC revealed that the ADT resistant $CK5^+CK8^+$ hybrid epithelial cells had up-regulated stem-cell transcription factors, steroidogenic and neurogenic pathways while down-regulating cell cycle, cell division and circadian pacemaker machinery, as well as the SARS-CoV-2 host cell entry factors, TMPRSS2 and ACE2. The *Fucci2BL* system live cell cycle tracker system allowed us for the first time to follow the time course of ADT-induced changes in the cell cycle in live cells while maintaining critical cell-cell interactions in the three-dimensional organoid structure. We postulate that this may more accurately reveal the dynamic effects of ADT on the cell cycle *in vivo*. We showed that in the presence of androgen such as DHT, the majority of the cells in the organoids were in S/G2/M and not actively proliferating. With Enzalutamide, the organoids transitioned from S/G2/M to G0. This lack of proliferation was confirmed by lack of Ki67 protein as shown by immunostaining of the organoids. Therefore, enzalutamide treatment led to the emergence of a dormant, ADT-resistant hybrid basal-luminal prostate cancer cell type in the organoids. The findings here tie together previous studies on a rare population of prostate epithelial cells which were suggested to be an antecedent in the events leading to prostate cancer with a new mechanism of castration resistance in patient-derived prostate cancer organoids. The newly identified, castration-resistant, basal-luminal hybrid prostate cancer cells

may represent a type of treatment-emergent, transitional state between ADT-resistant dormancy and re-current growth.

The dormancy CRPC gene signature identified in these PDX organoids revealed specific genes and pathways that may be targeted alone or in combination to eradicate dormant CRPC before further progression and recurrence to a more malignant, harder to treat disease.

Interestingly, we found that ADT down-regulated both of the SARS2-CoV-2 host factors, TMPRSS2 and ACE2, which are required to mediate viral entry. This is the first study directly demonstrating that a therapeutic anti-androgen down-regulated ACE2 in human cells. Androgen regulation of ACE2 has been implicated in studies showing that male alveolar lung cells have higher expression of AR, TMPRSS2 and ACE2 compared to female. It has been observed in studies from multiple COVID-19 pandemic epicenters including Wuhan, China, Veneto, Italy and New York, USA, that men have a worse COVID-19 disease course and higher mortality compared to women[43-49]. We and others have postulated that the increased COVID-19 disease severity and death rate in men may be due in part to increased TMPRSS2 and ACE2 expression in men. Furthermore, prostate cancer cells which often have high levels of TMPRSS2 and co-express ACE2 on prostate tumor cells may represent an additional male cancer vulnerability to SARS-CoV-2. Prostate cancer cells with high TMPRSS2 and ACE2 may have a high level of SARS-CoV2 infection thus, acting as a SARS-CoV-2 infection accelerator and/or reservoir. In addition, cancer cells including prostate cancer cells down-regulate HLA and evade immune surveillance. Therefore, SARS-CoV-2 infection of cancer cells may allow the virus to evade the immune system especially prostate cancer cells which have high levels of SARS2-CoV-2 host factors, TMPRSS2 and ACE2 which mediate viral entry. Therefore, prostate cancer patients may not be able to clear the virus and may constantly shed virus as was reported recently in another immunocompromised patient with CLL[50]. Interestingly, analysis of prostate cancer patients in Veneto, Italy who became infected with COVID-19 showed that patients on androgen

deprivation therapy (ADT) had overall less severe disease than prostate cancer patients not on ADT[51]. They had fewer hospitalizations and a shorter disease course. These observations have led to three currently ongoing clinical trials in which a short course of ADT is being given to patients who test positive for SARS-CoV-2. Anti-androgen therapy may be used as a treatment to reduce TMPRSS2 and ACE2 expression and inhibit SARS-CoV-2 virus levels in both male and female patients. Furthermore, ADT to reduce TMPRSS2 and ACE2 would be effective for reducing viral load of variants of SARS-CoV2 and, therefore, could be a crucial weapon against the new variants that are arising that can evade immune-based therapies.

Conclusions (77 words)

Androgen deprivation or enzalutamide treatment of PCa PDX organoids led to the emergence of newly identified dormant castration resistant hybrid basal-luminal prostate cancer cells and dormant CRPC gene signature showing decreased expression of both SARS-CoV-2 host cell viral entry factors, TMPRSS2 and ACE2. PDX prostate cancer organoids can be used as models that more accurately represent the tissue in vivo to screen for therapies that target dormancy in this new mechanism of ADT resistance and also for therapies that reduce TMPRSS2 and ACE2, thus suppressing viral load of SARS-CoV-2 and its variants.

Take Home Message (40 words)

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generous access to their microtome. We would also like to thank the following people from the Moores Cancer Center shared resources teams for their assistance with our experiments: Kersi Pestonjamasp, Microscopy; Alfredo Molinolo, Sharmeela Kaushal, and Jim Salinas, Biorepository; Valeria Estrada and Laarni Gapuz, Tissue Technology. Tissue Technology Shared Resource is supported by a National Cancer Institute Cancer Support Grant (CCSG Grant P31CA23100). The Keyence Model microscope was supported by the UCSD Cancer Center Microscopy Shared Facility Specialized Support Grant P30 CA23100-28. This publication includes data generated at the UC San Diego IGM Genomics Center utilizing an Illumina NovaSeq 6000 that was purchased with funding from a National Institutes of Health SIG grant (#S10 OD026929). We especially thank Dr. Kristen Jepsen, Director, of the IGM Genomics Center for their work to run our RNASeq samples. We are grateful for the technical support of Jamieson lab members Jamillah Murtadha, Johnathan Hsu and Rejina Roufegarinejad.

Author Contributions

S.L, T.R.M, D.N.B and C.A.M.J conceived and designed the experiments and wrote the manuscript. S.L, T.R.M, D.N.B and W.Y.Z established the 3-dimensional (3D) culture of patient-derived organoids. C.A.M.J. and C.N.W established the patient-derived xenograft (PDX) model of bone metastatic prostate cancer. C.N.W. and M.T.M performed intra-femoral injections of PCSD1 for PDX tumor cells to make organoids. C.N.W, M.T.M., T.R.M., C.A. and A.Z maintained the PDX model and performed tumor harvest. S.L, T.R.M, and D.N.B performed the ex vivo study to determine dihydrotestosterone (DHT) and enzalutamide response. T.R.M, D.N.B and C.A developed and performed quantitative cyst and spheroid analysis. S.L, D.N.B and M.T.M performed histological analysis. S.L, M.T.M, C.A, O.M.G. and A.Z performed immunohistochemistry and immunofluorescent-chemistry. G.P and K.M.L developed *Fucci2 BL* and provided lentiviral particles, expertise and advice. T.R.M and D.N.B performed an enzalutamide dose response test. D.N.B and T.R.M. performed qRT-PCR. N.A.C and T.R.M performed statistical analysis. C.H.M.J, C.J.K, A.A.K and C.A.M.J participated

in discussions and prepared IRB approval and human subject recruitment. A.A.K. performed orthopedic surgery and provided the surgical specimens of bone metastatic prostate cancer. S.L, T.R.M, D.N.B, M.T.M, N.A.C and C.A.M.J participated in the discussion of the results and manuscript editing.

Declaration of Interests

CJ reports investigator-initiated project funding from Calibr, Inc., Astellas, Medivation, and Pfizer outside the submitted work. CK reports having stock ownership in Stratify genomics outside the submitted work. All other authors declare no competing interests related to this study.

Figure Titles and Legends

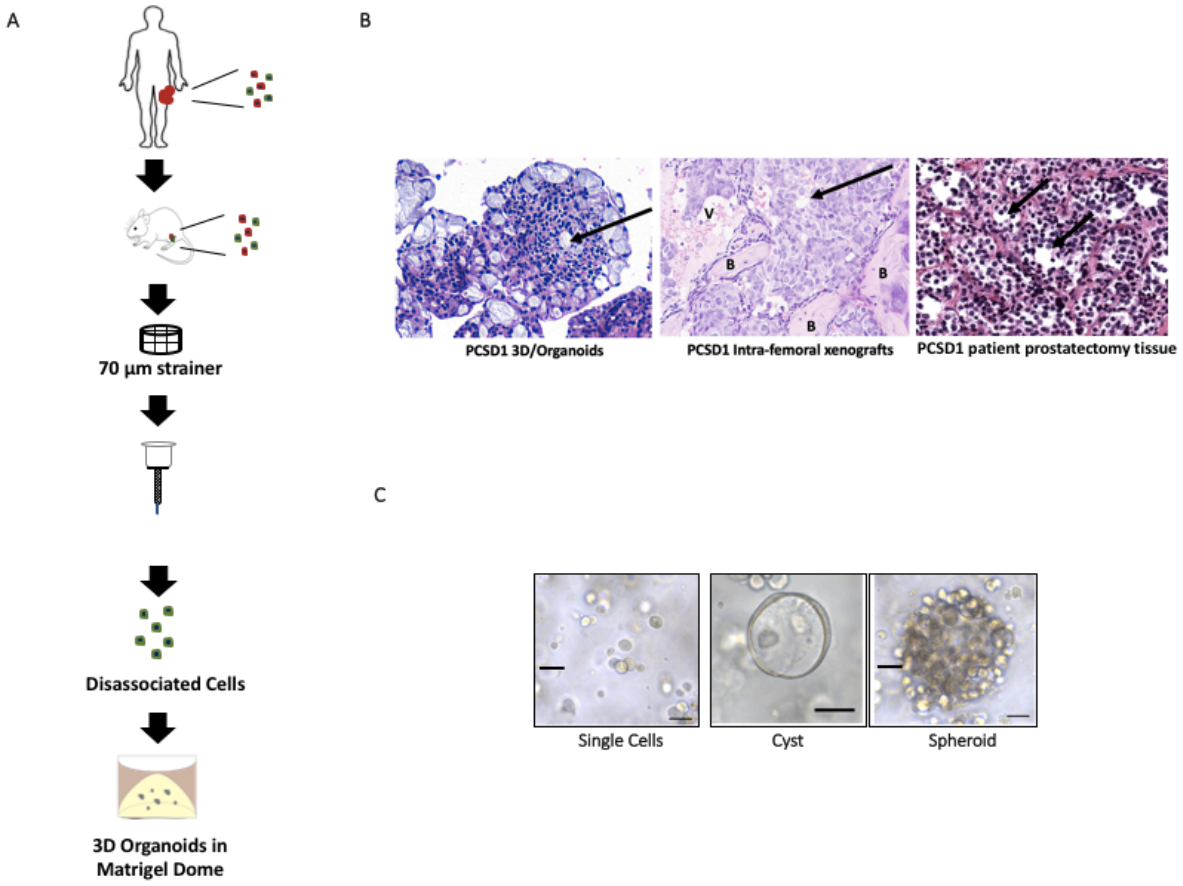
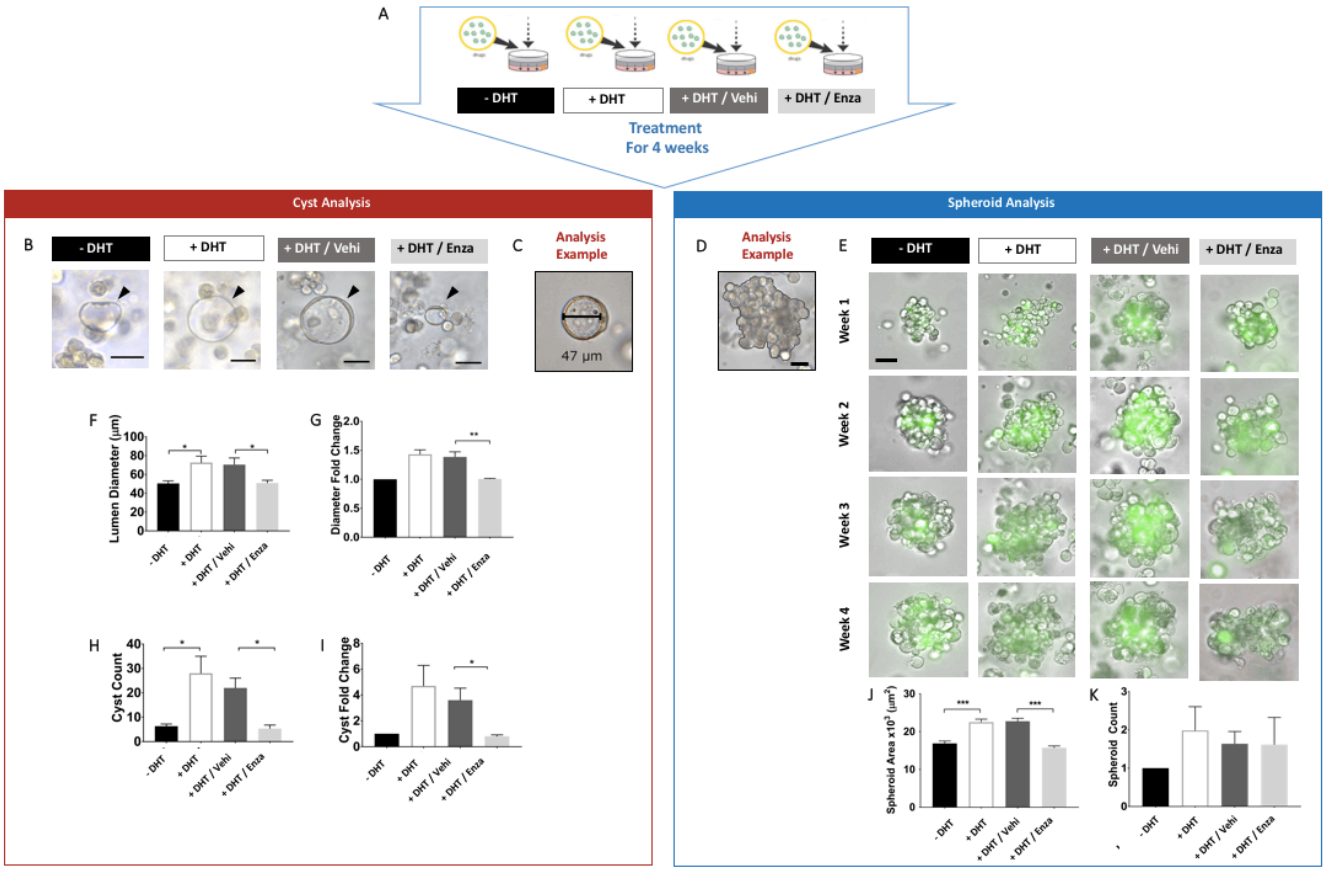


Figure 1. PDX Organoids Recapitulate Cellular Heterogeneity and Histopathology of the Originating Patient's Prostate Cancer.

(A) Workflow to establish and optimize 3D organoids from patient-derived xenograft (PDX) model of prostate cancer bone metastasis. (B) Representative images of hematoxylin and eosin (H&E) stained samples of PCSD1 organoids vs PCSD1 intra-femoral xenografts. Black arrows point to small gland-like structures seen in both the organoids and xenografts growing in the femur. B indicates bone, V indicates blood vessel. H&E images are shown at 10X magnification. (C) A heterogeneous mix of PCSD1 tumor cells grown in 3D organoid cultures consisting of single cells, hollow epithelial cysts, and cell-filled spheroids. Brightfield microscope images of single cells and the spheroid are at 10X magnification and the cyst is at 20X magnification. Scale bars represent 50 µm.



401

402 **Figure 2. PDX Organoids Recapitulate the Androgen Deprivation Therapy Resistance of the**
403 **Patient.**

404 (A) Experimental design of 3D cultures of PCSD1-GFP/Luciferase+ organoids under four treatment
405 conditions: -DHT, +DHT (1 nM), +DHT/Vehicle (1 nM/0.1% DMSO), and +DHT/Enzalutamide (1 nM/10
406 µM). PCSD1 organoids in each treatment group were imaged weekly with a Keyence digital
407 microscope for four weeks. (B) A representative image of cyst in each treatment condition after 4
408 weeks of treatment. (C) A measurement example of cyst lumen diameter using the Hybrid Cell Counter
409 software. Scale bar for spheroid image is 50 µm. After measurement of cyst lumen diameter, the
410 number of epithelial cysts greater than 50 µm in size was quantified in each culture. (D) An analysis
411 example of spheroid area using the Hybrid Cell Counter software. Scale bar for spheroid image is 50
412 µm. (E) A representative image of spheroid in each treatment condition at week 1, 2, 3, and 4 of
413 treatment. (F) Average lumen diameter of cysts, (E) Fold changes in lumen diameter, (H) number of

cyst count, (I) Fold changes in cyst count. (J) Average spheroid area, (K) The number of spheroids under four treatment conditions: -DHT, +DHT (1 nM), +DHT/Vehicle (1 nM/0.1% DMSO), and +DHT/Enzalutamide (1 nM/10 μM). For fold change analysis in (G), (I) and (K), quantified values were normalized to the DHT- condition. Data represent the mean from four independent (n=4) experiments performed in triplicate ± SEM. A student's t-test was used to determined statistical significance (*indicate $P < 0.05$, ** indicates $P < 0.01$ *** indicated $P < 0.0001$).

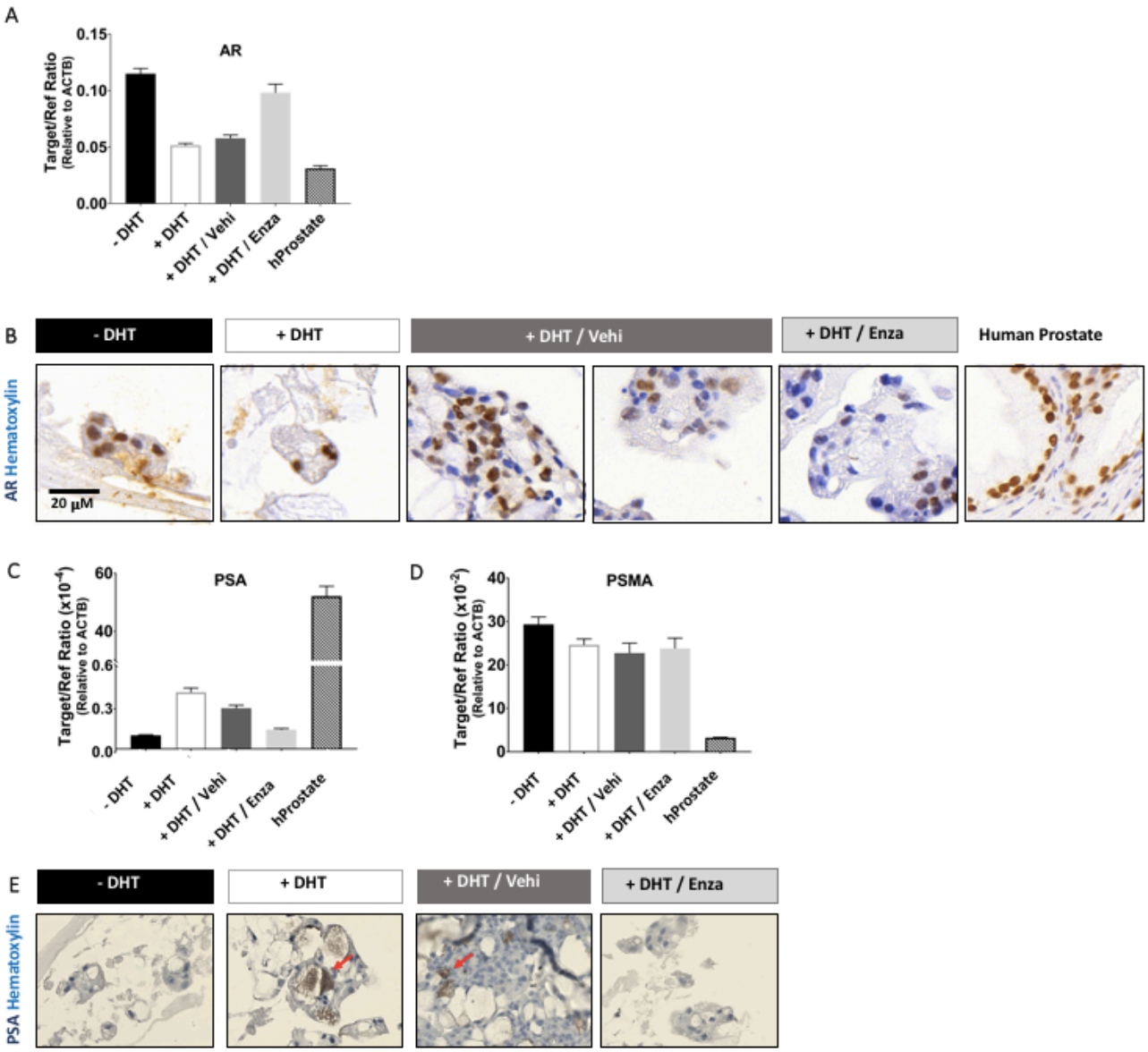


Figure 3. Androgen Deprivation Reduces Levels of AR protein and PSA gene expression in PDX Organoids.

(A) Quantitative RT-PCR analysis for AR level in PCSD1 3D organoids under the four treatment conditions: -DHT, +DHT (1 nM), +DHT/Vehicle (1 nM/0.1% DMSO), and +DHT/Enzalutamide (1 nM/10 µM). Human Beta Actin was used as the internal control, reference gene (delta delta C_t Target / Reference). (B) IHC analysis of AR expression in PCSD1 3D organoids. Representative digital microscope images are shown of AR IHC staining performed on 4% paraformaldehyde fixed, paraffin

embedded 5 µm sections from four culture conditions. Human prostate tissue was used as a positive control. (C) Quantitative RT-PCR analysis for PSA level in PCSD1 3D organoids. (D) Quantitative RT-PCR analysis for PSMA level in PCSD1 3D organoids under the four treatment conditions. Graphs represent one experiment performed in duplicate. (E) Immuno-histochemical (IHC) analysis of PSA expression in PCSD1 3D organoids. Red arrows point to PSA positive staining. Representative digital microscope images are shown of PSA IHC staining performed on 4% paraformaldehyde fixed, paraffin embedded 5 μm sections from four culture conditions.

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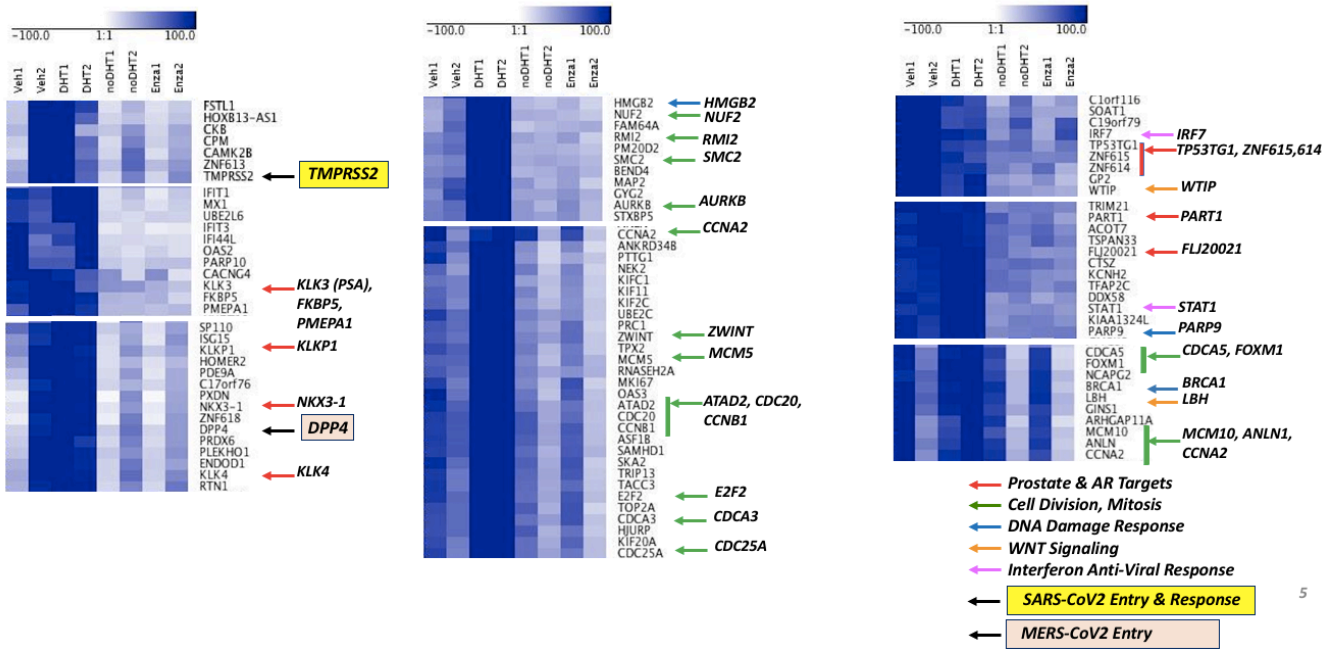
A. Down-Regulated Genes, High RPKM



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B. Down-Regulated Genes, Low RPKM

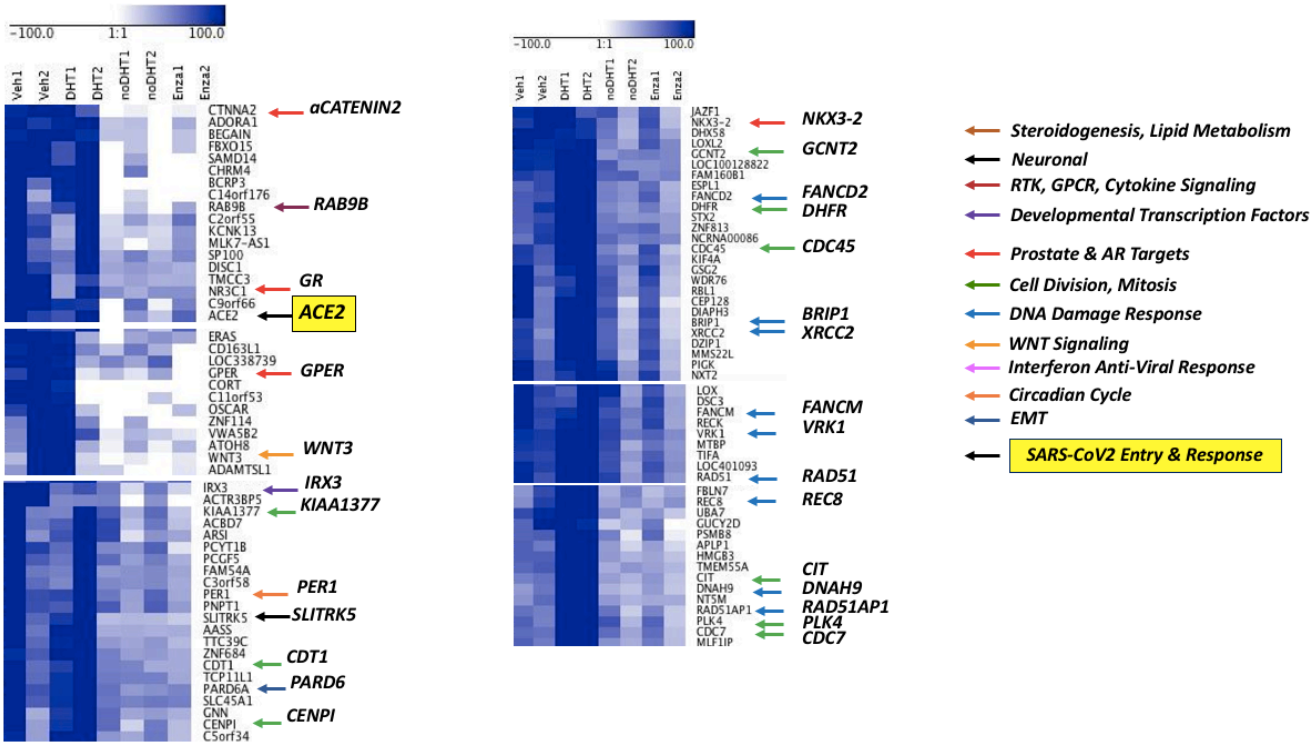

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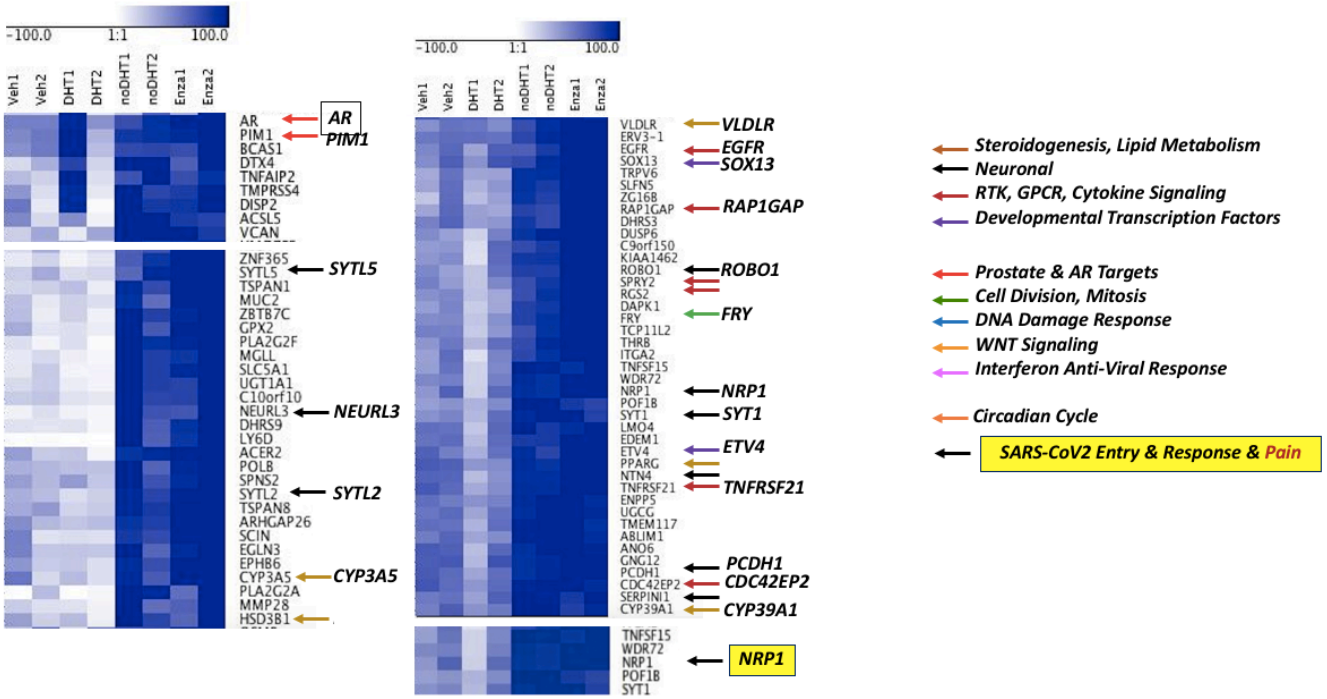
Figure 4. Gene Expression Profiles from PDX Organoids under ADT Reveal Down-Regulation of Cell Cycle and Circadian Pathways, and SARS-CoV-2 Host Cell Entry Factors, TMPRSS2 and ACE2. Heatmaps organized by hierarchical clustering for significantly down-regulated genes in PCSD1 organoids under two androgen treatment conditions: (1) Veh (+DHT/Vehicle – 1 nM/0.1% DMSO), (2) DHT (+DHT – 1 nM) and PCSD1 organoids under two androgen deprivation treatment conditions: (3) noDHT (-DHT) and (4) Enza (+DHT/Enzalutamide – 1 nM/1uM). (A) Significantly down-regulated genes with high normalized read counts (RPKM). Genes from functional categories enriched in the differential genes marked with arrows: prostate & AR targets (red); cell division, mitosis (green); DNA damage response (blue); WNT signaling (orange); interferon anti-viral response (pink); SARS-CoV-2 entry & response (black arrow with yellow box) and MERS-CoV entry (black arrow with beige box). (B) Significantly down-regulated genes with low overall RPKM. Genes from functional categories enriched in the differential genes marked with arrows: steroidogenesis, lipid metabolism (brick red); neuronal (black); RTK, GPCR, cytokine signaling (red); developmental transcription factors (purple); prostate & AR targets (bright red); cell division, mitosis (green); DNA damage response (blue); WNT signaling (light orange); interferon anti-viral response (pink); circadian cycle (orange); EMT (dark blue) and SARS-CoV-2 entry & response (black arrow with yellow box).

A. Up-Regulated Genes, High RPKM



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B. Up-Regulated Genes, Low RPMK

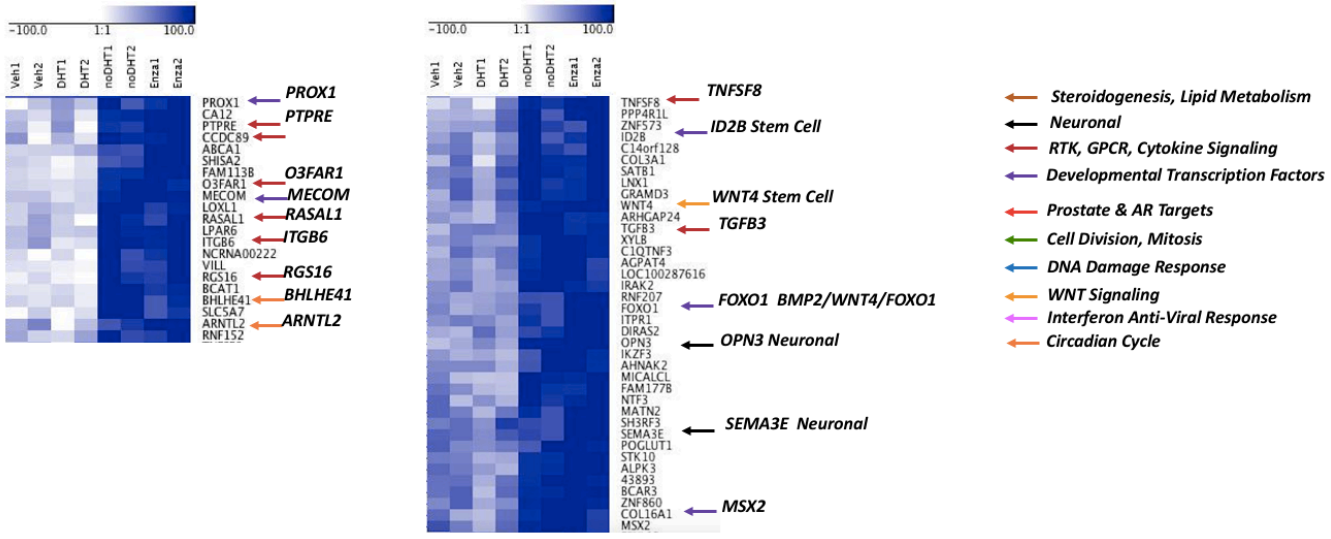

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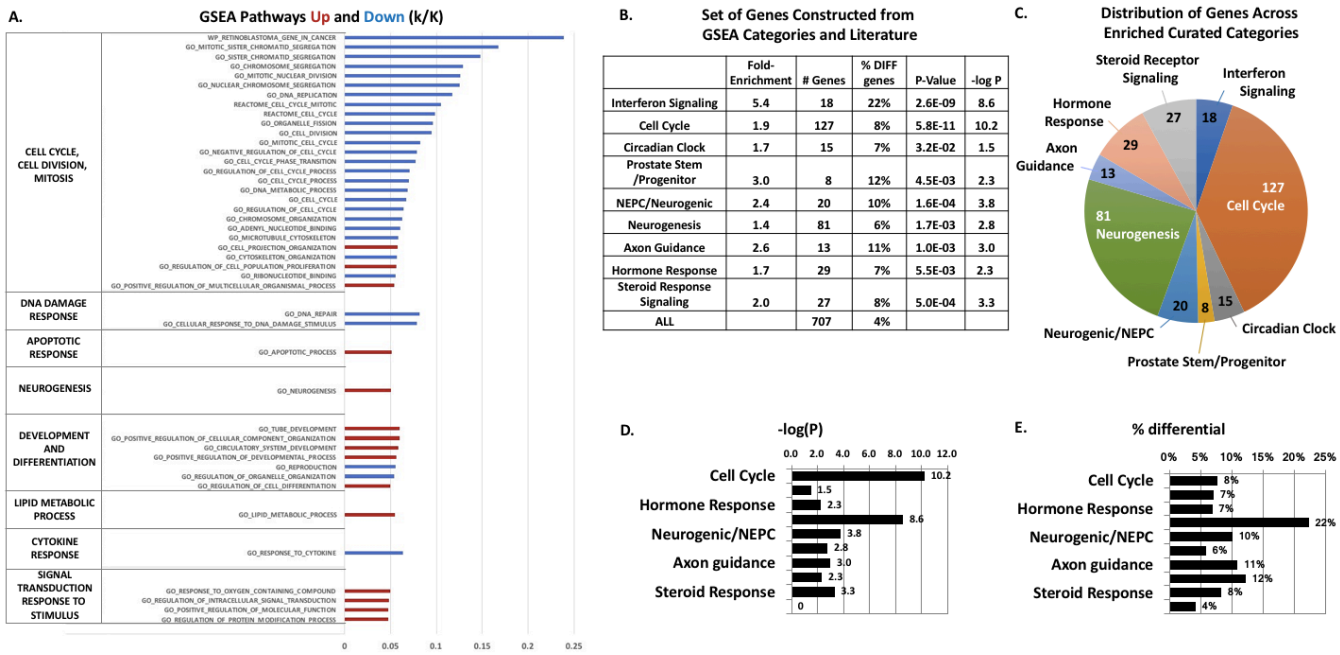
Figure 5. Gene Expression Profiles from PDX Organoids under ADT Reveal Up-Regulation of Neurogenic, Steroidogenic Genes and Stem Cell Transcription Factors.

Heatmaps organized by hierarchical clustering for significantly up-regulated genes in PCSD1 organoids under two androgen treatment conditions: (1) Veh (+DHT/Vehicle – 1 nM/0.1% DMSO), (2) DHT (+DHT – 1 nM) and PCSD1 organoids under two androgen deprivation treatment conditions: (3) noDHT (-DHT) and (4) Enza (+DHT/Enzalutamide – 1 nM/1uM). (A) Significantly up-regulated genes with high normalized read counts (RPKM). Genes from functional categories enriched in the differential genes marked with arrows: steroidogenesis, lipid metabolism (brick red); neuronal (black); RTK, GPCR, cytokine signaling (brown); developmental transcription factors (purple); prostate & AR targets (red); cell division, mitosis (green); DNA damage response (blue); WNT signaling (orange); interferon anti-viral response (pink); circadian circle (dark orange) and SARS-CoV-2 entry & response & pain (black arrow with yellow box). (B) Significantly up-regulated genes with low overall RPKM. Genes from functional categories enriched in the differential genes marked with arrows: steroidogenesis, lipid metabolism (brick red); neuronal (black); RTK, GPCR, cytokine signaling (dark brown); developmental transcription factors (purple); prostate & AR targets (bright red); cell division, mitosis (green); DNA damage response (blue); WNT signaling (light orange); interferon anti-viral response (pink) and circadian cycle (orange).



	Fold-Enrichment	# Genes	% DIFF genes	P-Value	-log P
Interferon Signaling	5.4	18	22%	2.6E-09	8.6
Cell Cycle	1.9	127	8%	5.8E-11	10.2
Circadian Clock	1.7	15	7%	3.2E-02	1.5
Prostate Stem /Progenitor	3.0	8	12%	4.5E-03	2.3
NEPC/Neurogenic	2.4	20	10%	1.6E-04	3.8
Neurogenesis	1.4	81	6%	1.7E-03	2.8
Axon Guidance	2.6	13	11%	1.0E-03	3.0
Hormone Response	1.7	29	7%	5.5E-03	2.3
Steroid Response Signaling	2.0	27	8%	5.0E-04	3.3
ALL		707	4%		

Figure 6. Gene Set Enrichment Analysis (GSEA) Shows Differentially Expressed Genes in Interferon Signaling, Cell Cycle, Circadian Clock, Prostate Stem/Progenitor, NEPC/Neurogenic, Neurogenesis, Axon Guidance, Hormone Response and Steroid Receptor Signaling. (A) List of up-regulated (red) and down-regulated (blue) pathways evaluated using GSEA. (B) Enrichment scores for unified gene sets constructed from overlapping GSEA functional categories and literature. (C) Pie chart showing distribution of genes across enriched categories. (D) Bar graph showing –log(P) for each category, P calculated using Fisher's exact test. (E) Bar graph showing fold-enrichment for each category.

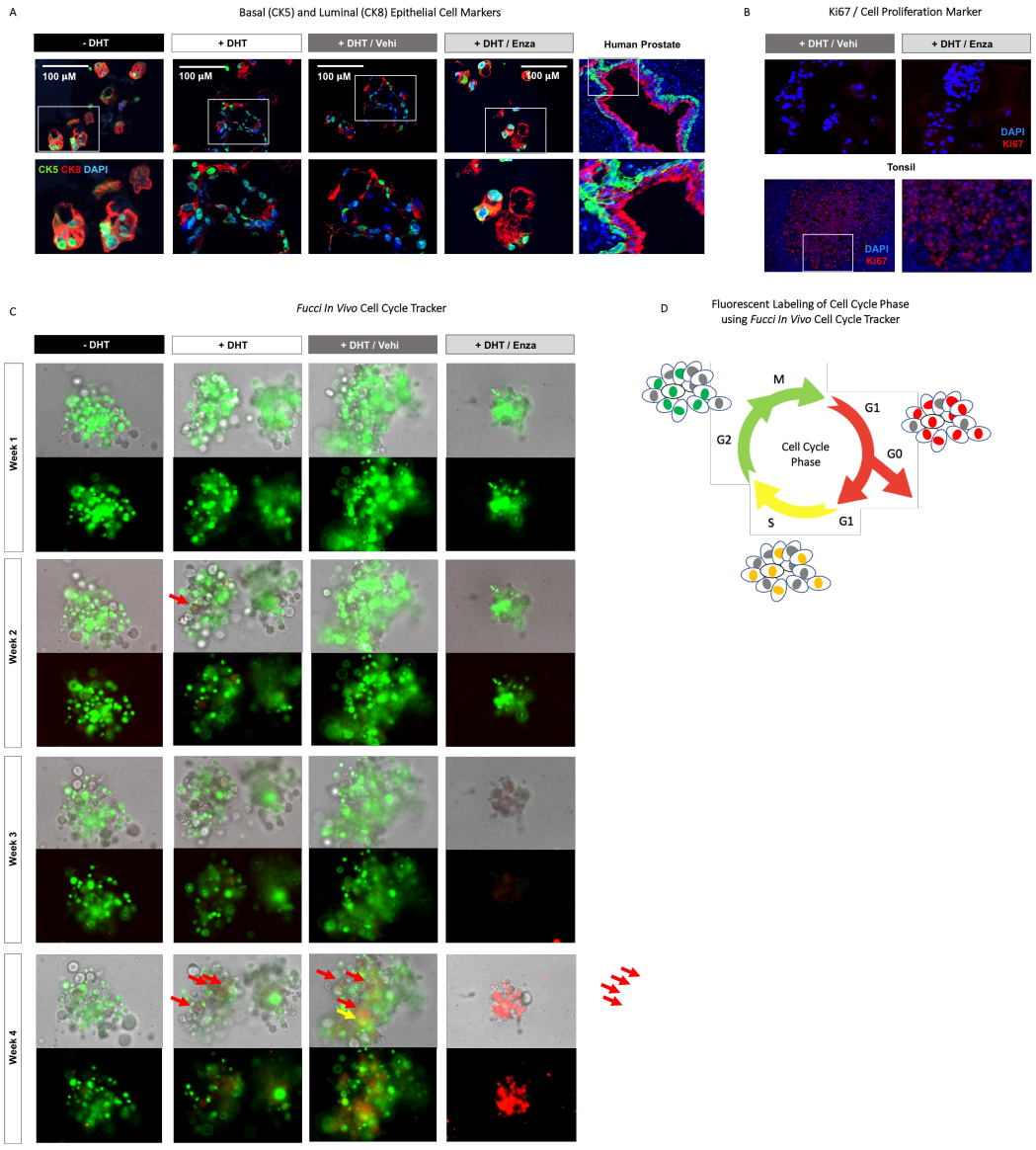

A
Basal (CK5) and Luminal (CK8) Epithelial Cell Markers
- DHT
+ DHT
+ DHT / Vehi
+ DHT / Enza
Human Prostate
100 µM
CK5 CK8 DAPI
B
Ki67 / Cell Proliferation Marker
+ DHT / Vehi
+ DHT / Enza
DAPI
Ki67
Tonsil
C
Fucci In Vivo Cell Cycle Tracker
Week 1
Week 2
Week 3
Week 4
D
Fluorescent Labeling of Cell Cycle Phase
using Fucci In Vivo Cell Cycle Tracker
M
G1
G2
Cell Cycle
Phase
G0
S
G1

Figure 7. Androgen Deprivation Therapy-Resistant Population of PCSD1 Organoids are $CK5^{+}CK8^{+}$ Basal-Luminal Hybrid and Dormant Cells.

(A) Confocal FV1000 images of IFC that was performed on PCSD1 3D organoids and normal human prostate cancer control tissue to visualize the prostate epithelial cell markers: Cytokeratin 8 (CK8), a luminal epithelial cell protein, and Cytokeratin 5 (CK5), a basal epithelial cell protein along with DAPI nuclear stain. Top five panels: PCSD1 3D organoids and patient prostate cancer tissue showed cytoplasmic CK8 protein (red), and perinuclear and nuclear CK5 protein (green). DAPI stained the nuclei (blue). Inset (white square) shows further magnified image of organoids with cytoplasmic CK8 and perinuclear CK5 in the same cells. In the PCSD1 organoids, immunofluorescent cytochemical (IFC) analysis of CK5 and CK8 revealed heterogeneous expression patterns. Confocal microscope imaging of the control tissue, normal human prostate, showed the expected pattern of $CK5^{+}$ basal cells and $CK8^{+}$ luminal cells in glandular structure. (B) Confocal FV1000 images of IFC that was performed on PCSD1 3D organoids and normal tonsil positive control tissue to visualize the cell proliferation marker, Ki67, in two treatment conditions of +DHT/Vehi and +DHT/Enza at week4. Red color indicates Ki67 protein present. (C) Time course of cell cycle stages in live PCSD1 organoids under the four treatment conditions. Representative images in each treatment condition at week 1, 2, 3, and 4 of treatment of PCSD1 organoids stably expressing the *Fucci2 BL* bicistronic fluorescent, ubiquitination-based cell cycle indicator reporter system showing three cell cycle phases: G_1 / G_0 by red fluorescence, G_1/S by yellow fluorescence, and S/G_2/M by green fluorescence. The images of bright-field, red fluorescent channel and green fluorescent channel were taken and merged. (D) The *Fucci2 BL* fluorescent, ubiquitination-based cell cycle indicator reporter system visualizes the phase of cell cycle shown by colorimetric signal of red, yellow and green fluorescence for G_1, G_1/S and S/G_2/M, respectively.

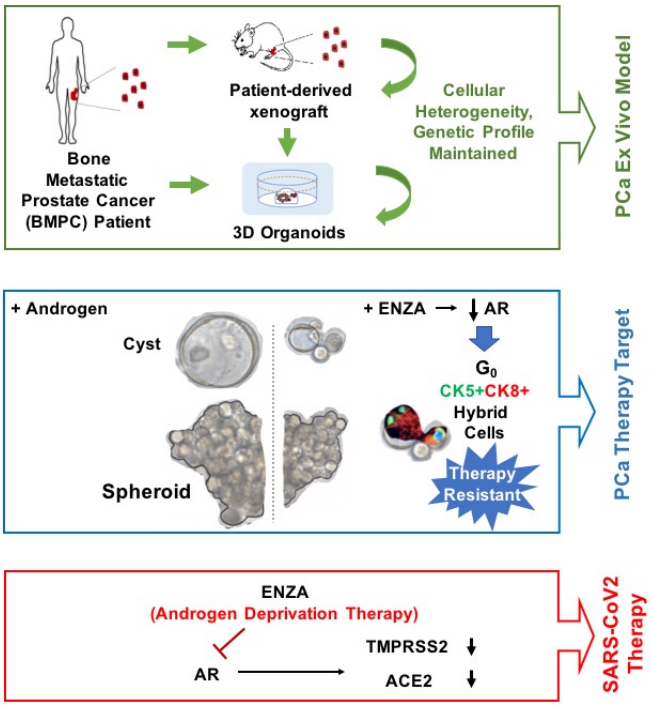
545

HIGHLIGHTS

- Patient-derived xenograft (PDX) models for bone metastatic prostate cancer and three-dimensional (3D) organoids (mini-tumors) more predictive pre-clinical research platforms.
- PDX Organoids used to test androgen deprivation therapy (ADT) resistance mechanisms.
- Novel hybrid basal-luminal cells that were ADT resistant, quiescent prostate stem-cell like gene expression profile.
- ADT decreased expression of the severe acute respiratory syndrome (SARS) SARS-CoV-2 host cell viral entry factors, TMPRSS2 and ACE2 as well as the Middle East respiratory syndrome (MERS) MERS-CoV receptor, DPP4 as well as anti-viral interferon response.

- Therefore, PDX organoids may be used to screen for novel therapies that target mechanisms of ADT resistance in PCa and for therapies that reduce TMPRSS2 and ACE2 expression and thus block SARS-CoV2 infection.

GRAPHICAL ABSTRACT



547 **Table 1. Selected genes of interest in ADT-signature from PDX PCSD1 organoids**
548

Direction of Gene Expression Change with ADT	Number of Genes (787 Total)	Category	Selected Genes of Interest
Down	518		
		Prostate and AR-target genes	*TMPRSS2, KLK3(PSA), KLK4, KLKP1, FKBP5, PMEPA1, NKX3-1, TP53TG1, ZNF614, ZNF615, PART1, FLJ20021, GR, GPER, NKX3-2*
		Cell cycle, cell division, mitosis and mitotic spindle structure and function	*NUF2, RMI2, SMC2, AURKA, AURKB, CCNA2, ZWINT, MCM5, ATAD2, CDC20, CCNB1, E2F2, CDC3A, CDC25A, KIA1377, CDT1, CENPI, GCNT2, DHFR, CDC45, CIT, PLK4,* CDC7
		DNA synthesis and repair	*HMGB2, PAPRP9, BRCA1, FANCD2, BRIP1, XRCC2, FANCM, VRK1, RAD51, REC8, DNAH9, RAD51AP1*
		Circadian clock	*PER1*
		Interferon signaling	*IRF7, STAT1*
		WNT signaling	*WTIP, LBH, WNT3*
		Epithelial to mesenchymal transition (EMT)	*PARD6*
		SARS-CoV2 Host Viral Entry factors	*TMPRSS2, ACE2*
Up	269	Serine/threonine kinase signaling and growth	*EGFR, PIM1, RAPGAP1, SPRY2, CDC42EP2, PTPRE, CCDC89, O3FAR1, RASAL1, ITGB6, RGS16*
		G-protein coupled receptor *(*GPCR) signaling	*RGS2*
		Cytokine signaling	*TNFRSF21, TNFS8, TGFB3*
		Lipid metabolism, cholesterol, and steroid hormone biosynthesis	*CYP3A5, HSD3B1, VLDLR, PPARG, CYP39A1*
		Neuronal function and development	*NRP1, ROBO1, SYT1, NTN4, PCDH1, SERPINI1, SYTL5, SYTL2, SEMA5A, OPN3, SEMA3E*
		Developmental transcription factors in stem cells and cancer stem cells	*ETV4, SOX13, MSX2, SOX9, FOXO1, FOXO3, PROX1, MECOM, ID2B, GATA2*

549

550

			Differential (# Genes)				NO CHANGE (% Genes)				
Category	**Fold Enrichment**	**P-value**	**UP**	**DOWN**	**Total**	**%**	**HIGH**	**LOW**	**Total**	**%**	**Total # Genes with Reads**
Interferon Signaling	4.9	2.0E-08	0	18	18	22.8	40	21	61	77.2	79
Cell Cycle	1.7	1.5E-07	18	109	127	7.8	985	519	1,504	92.2	1,631
Circadian Clock	1.6	9.3E-02	7	8	15	7.2	113	79	192	92.8	207
Prostate Stem /Progenitor	2.6	1.1E-02	5	3	8	12.1	22	36	58	87.9	66
NEPC/ Neurogenic	2.2	1.9E-03	6	14	20	10.0	85	95	180	90.0	200
Neurogenesis	1.3	3.3E-02	41	40	81	5.9	627	655	1,282	94.1	1,363
Axon Guidance	2.4	3.2E-03	12	1	13	11.1	50	54	104	89.9	117
Hormone Response	1.5	3.3E-02	16	13	29	7.0	149	236	385	93.0	414
Steroid Receptor Signaling	1.8	3.2E-03	14	13	27	8.4	146	148	294	91.6	321
ALL	1.0	na	269	518	787	4.6	7,581	8,63	16,21	95.4	17,004

553 **Table 2. GSEA Functional Categories Enriched in Differentially Expressed Genes with**
554 **Enrichment Scores and Numbers of Up- or Down-Regulated Genes.** Enrichment scores were
555 calculated for each merged functional category using the ratio of the percent of differential genes in
556 the category compared to the percent of differential genes overall. (Overall 4% of 17,004 genes were
557 identified as differentially expressed using the filtering queries.)
558
559

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