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TITLE: Targeting WNT5A-Mediated Therapy Resistance Mechanisms and Tumor Genomic Heterogeneity in Lethal Bone-Metastatic Prostate Cancer

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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 models e developed form surgical prostate cancer bone metastases and prostate cancer 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 Y2 of this grant we have built on our work showing 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. To study the mechanisms of WNT5A/ROR1 signaling in CRPC we generated CRISPR/Cas9 ROR1 knock out cell lines which we are testing in vitro and in vivo for responsiveness to combination therapy of docetaxel plus radiation – the standard of care for bone metastatic CRPC. Cirmtuzumab has demonstrated efficacy in our patient derived xenograft tumors in vivo in combination with the taxane chemotherapy, docetaxel. We have performed in vitro and in vivo testing of the anti-ROR1 Cirmtuzumab-based CART cells in NEPC cell lines and in our patient-derived xenograft (PDX) mouse models. Our most important result thus far is that anti-ROR1 Cirmtuzumab-based CART cells can durably eradicate PC3 xenograft tumors in vivo (more than 100 days so far and counting). We obtained an IRB amendment to an existing IRB protocol and have collected archival NEPC specimens for testing ROR1 expression in immunohistochemistry (IHC) assays to detect ROR1. We are working on the HRPO approval for this and for single cell RNA sequencing of cryopreserved surgical prostate cancer bone metastasis specimens. This 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. The pre-clinical studies being performed in this grant will support the pending Phase 1B clinical trial (not part of this grant) and translation of anti-ROR1 Cirmtuzumab-based CART cells into clinical trials to treat neuroendocrine PCa and CRPC.

15. SUBJECT TERMS None listed.

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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)**.

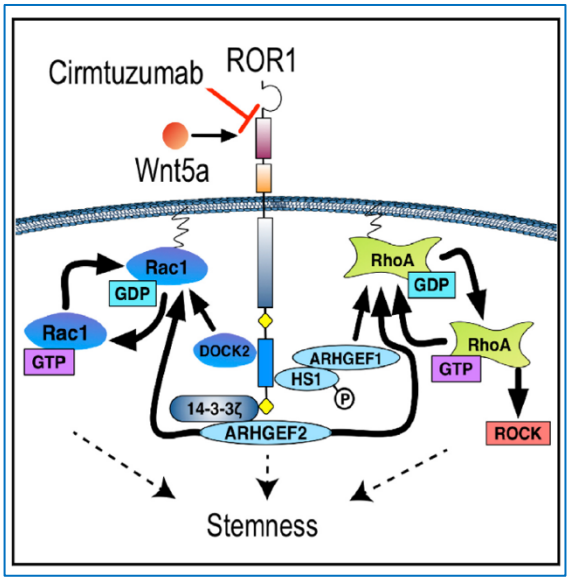


Figure 1. Signaling through the non-canonical Wnt pathway is mediated by Wnt5A binding to its receptor ROR1. Cirmtuzumab is a monoclonal antibody which targets ROR1 (Choi 2018).

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

RESEARCH ACCOMPLISHMENTS – Year 2

New Publications:

1. Ma, JSY, Lee, SC, Kim, MS, Hampton, EN, Laborda, E, Choi, SH, Chavda, B, Muldong, M, Wu, CN, Ma, W, Kulidjian, AA, Kane, CJ, **Jamieson, CAM,** Woods, AK, Joseph, S, Wright, TM, Wisler, J, Schultz, PG, Kim, CH, and TS. Young . A PSMA-targeted bispecific antibody for prostate cancer driven by a small-molecule targeting ligand. Science Advances. 2021 Aug 11;7(33):eabi8193. doi: 10.1126/sciadv.abi8193. Print 2021 Aug. PMID: 34380625

2. Javier-DesLoges, J, … Cancer Stage, Treatment, and Survival Among Transgender Patients in the United States. Journal of the National Cancer Institute JNCI-22-0063 (in press).

3. Lee, S, Mendoza, TR, Burner, DN, Muldong, MT, Wu, CN, Arreola, C, Zuniga, A, Murtadha, J., Koutouan, E, Miakicheva-Greenburg, O, Zhu, WY, Cacalano, NA, Jamieson, CHM, Christopher J. Kane, CJ, Kulidjian, AA, Gaasterland, T and C.A.M. Jamieson. Novel dormancy mechanism of castration resistance in bone metastatic prostate cancer organoids (In press, International Journal of Molecular Sciences).

SPECIFIC AIM1: Determine the mechanism of WNT5A/ ROR1/ ROR2 signaling in bone metastatic CRPC using patient-derived organoids (PDO) and xenograft (PDX) models using CRISPR/Cas9 ROR1 knock out and small molecule inhibitors of WNT-signaling.

1. ROR1

In Year 1, we showed that ROR1 was expressed on the surface of our prostate cancer bone metastasis PDX, PCSD13, and on two neuroendocrine prostate cancer (NEPC) cell lines, PC3 and DU145. We used CRISPR/Cas9 to knock out ROR1 in PC3 and DU145 cells. Loss of ROR1 protein expression was confirmed by FACS profiling (see Figure 5 below and Year 1 progress report in Appendix A).

In Year 2 we used these NEPC cell lines with and without ROR1 KO to compare their growth rate in vitro and in vivo to ROR1 expressing cells.
Hypothesis: WNT5A+ROR1 signaling leads to more stem cell-like state in CRCP bone metastases which enables them to be slower growing, hence, resistant to treatments that target rapid proliferation and cell division such as docetaxel and radiation therapy.

In vitro: We compared the growth of PC3-RFP luciferase to PC3-ROR1_KO-RFP luciferase cells using the live cell microscope imaging using the Incucyte system. Quantitation of phase contrast images of adherent cells and red fluorescent objects was performed on the time course of measurements every 2-4 hours over 3-4 days. Proliferation rates showed a modest increase in proliferation rate in PC3-ROR1_KO cells compared to ROR1+ PC3 cells.

In vivo: We injected PC3-RFP Luciferase and PC3-ROR1_KO RFP Luciferase cells into immunocompromised mice (Rag2-/-gc-/-) and compared the tumor growth rate using in vivo bioluminescence (IVIS) and caliper measurements. The PC3-ROR1_KO tumors showed a modest increase in tumor growth compared the PC3-RFP_Luciefrase (Figure 2). We are currently testing their response to the standard of care treatments for CRPC bone metastases: docetaxel and irradiation.
We performed FACS sorting on the PDX, PCSD13 cells to enrich for ROR1 expressing cells and re-injected the ROR1+ and ROR1- along with the starting cells in parallel into mice to compare growth

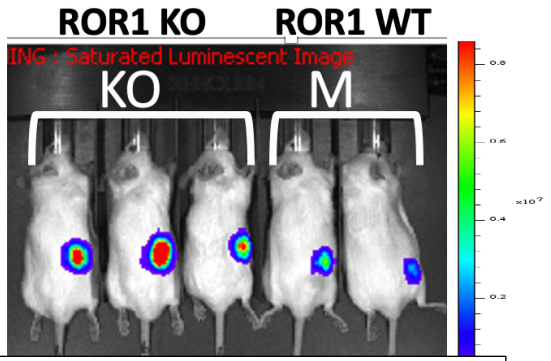
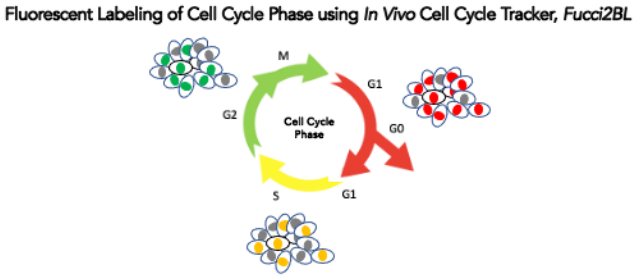
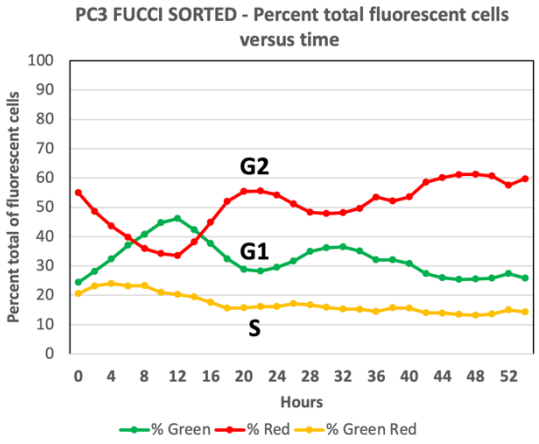


Figure 2. Shows representative IVIS images of sub-cutaneous xenograft tumors of PC3-RFP Luciferase (M or mock) or PC3-ROR1 KO-RFP Luciferase out of 36 mice, n=18 PC3, n=18 PC3-ROR1 KO. PC3 ROR1 KO tumors grew moderately faster.

of the ROR1+ PCSD13 cells to the ROR1- PCSD13 cells. The PCSD13 PDX takes 8-14 weeks to generate tumors. We are currently in the waiting period for tumor growth. We plan to use to the ROR1+ PCSD13 to knock down ROR1 using the same CRISPR/Cas9 system as we used for PC3 and DU145 cells. We will use anti-ROR1 magnetic beads to enrich the PCSD1 ROR1+ cells in the meantime.

Quantification of cell cycle stages using the Live Cell Cycle Fucci Reporter System. Figure 3 Incucyte assay to measure percent PC3 cells expressing Fucci2BL: red, green and yellow fluorescence.





2. WNT5A

WNT5A Alternatively spliced isoforms:

WNT5A is expressed as two alternatively spliced isoforms, short and long, due to the use of two alternative first exons. The resulting long form protein has an additional 18 amino acids at the N-terminus. In Bauer et al (2013 PLoSOne), Dr. Willert and Gaasterland reported that the long isoform was tumor suppressing while the short isoform promoted oncogenic growth using recombinant long and short WNT5A protein.

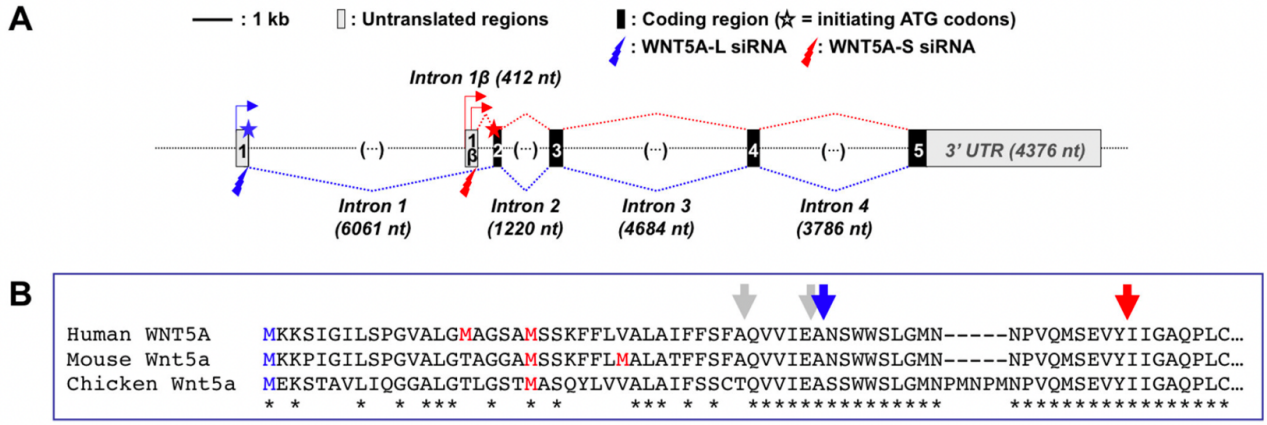
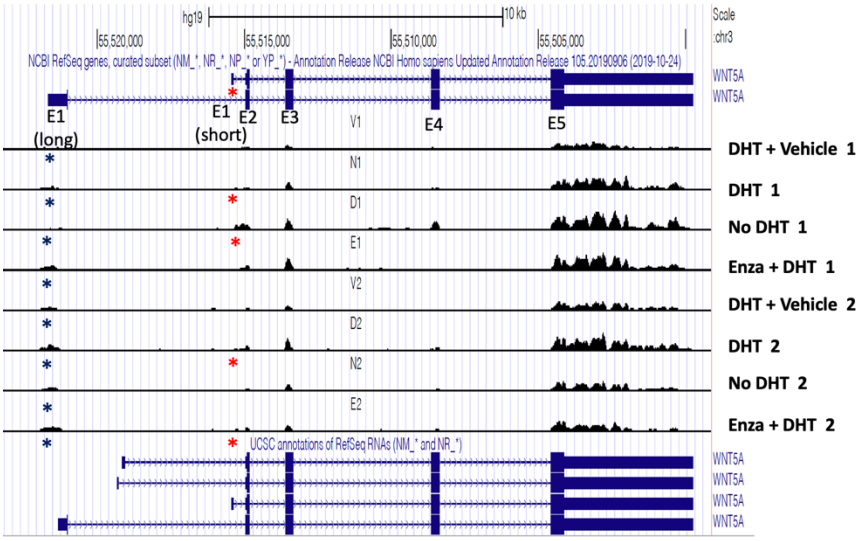
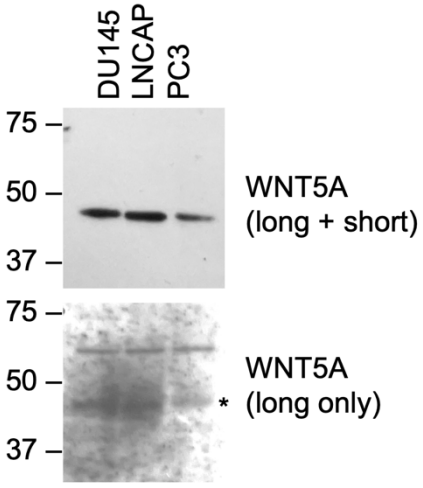


Figure 1 from Bauer et al (2013). Description and biochemical characterization of WNT5A isoforms. A. Structure of the human WNT5A gene and alternative transcripts. Numbered boxes indicate the exons, with 5' and 3' untranslated (UTR) regions in grey and coding regions in black. nt: nucleotides, kb: kilo base. Blue and red arrows and dotted lines respectively indicate the positions of the different transcription initiation sites in exon 1 and exon 1β and splicing of exon 1- and exon 1β-initiated transcripts. Blue and red stars indicate the translation initiation codons for the WNT5A-L and WNT5A-S isoforms located in exon 1 and exon 2, respectively. Blue and red lightning bolt arrows indicate the position of the target sequences of WNT5A-L and WNT5A-S specific short interfering RNA (siRNA). B. Multiple amino acid sequence alignment of the N-terminus of the WNT5A precursor proteins. The blue M denotes the most likely start codon of WNT5A-L while the red Ms denote the most likely start codons of WNT5A-S. The grey arrows indicate the positions of signal peptide cleavage sites for both isoforms as predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Blue and red arrows indicate the position of the observed first amino-acids of the mature WNT5A-L and WNT5A-S proteins, respectively, and thus the position of the observed signal peptide cleavage site for each isoform.

To evaluate the effects of WNT5A isoforms on signaling in prostate cancer we undertook to determine which isoforms were being expressed in our different models.

Figure 3. A. Reads mapped to the genome: both short and long alternatively spliced Wnt5A mRNAs are expressed in our prostate cancer bone metastasis PDX, PCSD1. B. Western blot of WNT5A secreted into media shows that all three prostate cancer cell lines produce WNT5A (Top) and long isoform of the protein (Bottom).





Next steps: Test recombinant WNT5A long and short isoform proteins in PC3-Fucci and PC3-Fucci_ROR1 KO to determine the effects on growth and cell cycle. Perform RNASeq analysis and Western blots on ROR1 and downstream signaling.

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. **New targets for PSMA negative prostate cancer are needed.**

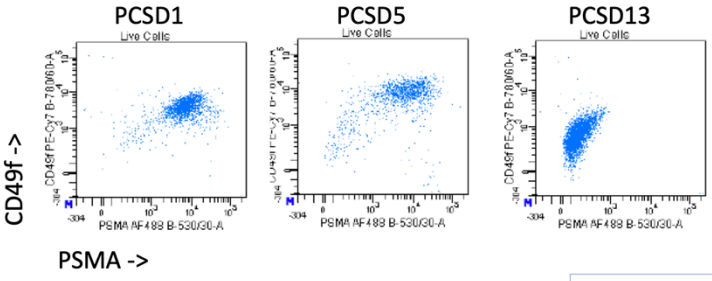
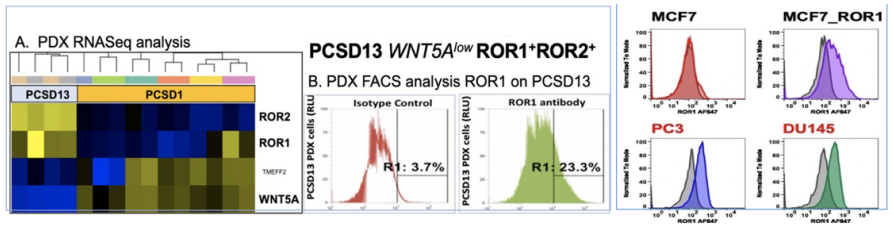


Figure 4. In our PCSD PDX series from prostate cancer bone metastases, the small cell, neuroendocrine PDX, PCSD13, is PSMA negative while the two adenocarcinoma PDXs, PCSD1 and PCSD5 are PSMA+.

Figure 5. In our PCSD PDX series we showed that the small cell, neuroendocrine prostate cancer, PCSD13, is WNT5A-ROR1+ while the adenocarcinoma PCSD1 is WNT5A+ ROR1-. The NEPC cell lines, PC3 and DU145, are also ROR1+.



RNASeq
WNT5A expressed: PCSD1
ROR1, ROR2 expressed: PCSD13

- Patient-derived xenograft, PCSD13. Bone metastasis specimen from Patient with small cell prostate cancer. He had received 3 months of ADT.
- ROR1 expression of PCSD13 cells from intra-femoral xenograft tumor was assessed by flow cytometry.
- PCSD13 prostate cancer PDX cells exhibited a sub-population with increased ROR1 compared to unstained and isotype controls.

PCa Cell lines DU145 and PC3 have high levels of ROR1 expression as assessed by flow cytometry (FACSAria).

MCF7 is a breast cancer cell line engineered to over-express ROR1 (MCF7_ROR1) serves as a positive control (Kipps, Prussak).

UNPUBLISHED

EXPERIMENT 1:
Cirmtuzumab antibody therapy for ROR1 targeting in PSMA negative bone metastatic prostate cancer, PCSD13 PDX

Hypothesis 1a: Inhibition of WNT5A/ROR1 signaling using the anti-ROR1 Cirmtuzumab clinical antibody therapeutic will inhibit growth of bone metastatic neuroendocrine prostate cancer.

Hypothesis 1b: Inhibition of WNT5A/ROR1 signaling using the anti-ROR1 Cirmtuzumab clinical antibody therapeutic will improve response of bone metastatic neuroendocrine prostate cancer to therapies targeting cell proliferation and cell division such as the standard of care chemotherapy, docetaxel, Irradiation.

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

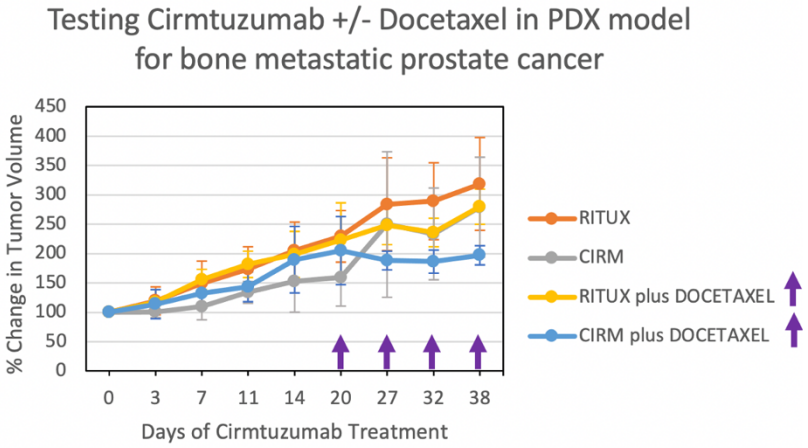


Figure 5. Testing anti-ROR1 antibody therapeutic, Cirmtuzumab, with and without Docetaxel, in the small cell prostate cancer bone metastasis PDX, PCSD13. Clinical grade Control antibody, Rituximab, and Cirmtuzumab antibody were injected intra-venously into mice with established PCSD13 tumors 200-400 mm3 twice a week. At two weeks of treatment half the mice in each group were additionally injected intra-peritoneally with docetaxel (20mg/kg) or with vehicle control. Tumor size was measured using calipers twice weekly.

Results: Pre-clinical trial with anti-ROR1 targeting antibody therapeutic, Cirmtuzumab, inhibited tumor growth in combination with Docetaxel in the ROR1+ bone metastatic prostate cancer PDX: PCSD13.
Flow cytometry showed that approximately 25% of PCSD13 cells in intra-femoral xenograft tumors expressed ROR1 protein. Accordingly, PCSD13 tumor growth was significantly inhibited by combination therapy of Cirmtuzumab plus docetaxel as shown in Figure 5.
These results support **Hypothesis 1b** that Cirmtuzumab inhibition of ROR1 led to sensitization to docetaxel and resulted in tumor growth inhibition in combination with docetaxel.
New immunotherapeutic prostate cancer target, non-canonical WNT5A receptor, ROR1.

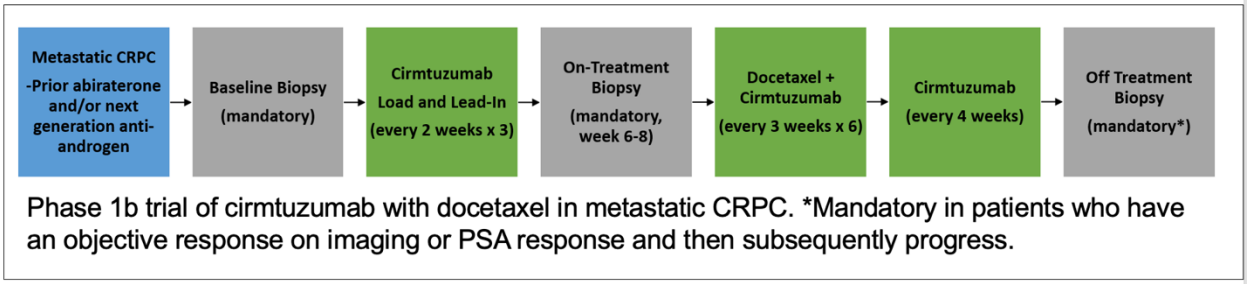
Next Steps:

1. RNA sequencing and transcriptomic analysis of tumors from this study to evaluate stem cell gene expression signatures. We anticipate that Cirmtuzumab will inhibit the stem cell signatures much as it did in the Phase 1 Clinical trial (Choi et al 2018 Cell Stem Cell)
2. ROR1 protein and downstream signaling analysis such as Rac and Rho pathways using immunoblotting and immunohistochemical analyses of the tumors in this study. We anticipate that ROR1 expression will be diminished in Cirmtuzumab treated tumors.
3. Repeat the in vivo testing of Cirmtuzumab in PCSD13 tumors:
a.) simultaneous treatment with Cirmtuzumab plus docetaxel rather than Cirmtuzumab pre-loading in established tumors.
b.) simultaneous treatment with Cirmtuzumab plus docetaxel immediately post tumor cell injection. Expect that Cirmtuzumab inhibition of ROR1 will not only synergize with docetaxel but may also inhibit cancer stem cell pathways and inhibit tumor engraftment and growth at the early stages of tumor establishment.

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

5. These results provided pre-clinical evidence to support the clinical trial (not part of this grant):
Phase 1b clinical trial of cirmtuzumab combined with docetaxel in metastatic CRPC

PI: Rana Mckay, MD



Phase 1b trial of cirmtuzumab with docetaxel in metastatic CRPC. *Mandatory in patients who have an objective response on imaging or PSA response and then subsequently progress.

Enroll 30 patients
Primary Endpoint is safety
Key Secondary Endpoints:

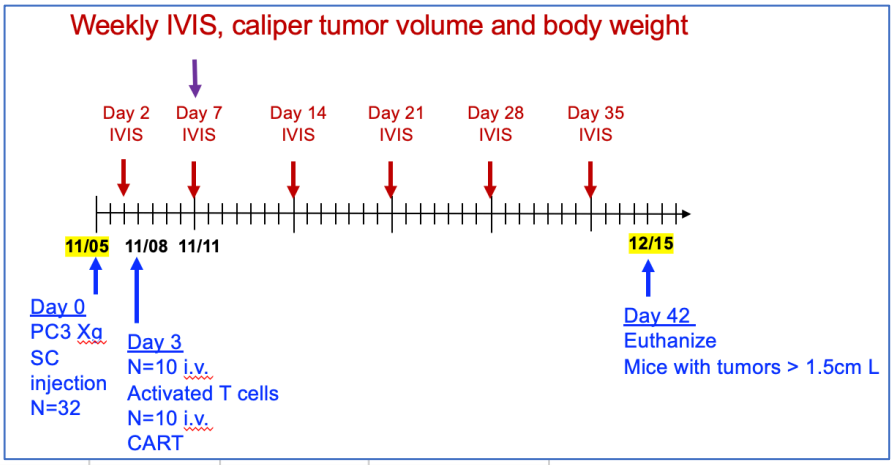
- Response as defined by complete or partial response by RECIST criteria version 1.1
- PSA response PCWG-3: PSA decline > 50% from baseline

EXPERIMENT 2: Cirmtuzumab-based CART cell targeting of ROR1 in PC3 RFP Luciferase Sub-cutaneous Xenograft model for Metastatic Prostate Cancer:

- Mice: 32 mice, *Rag2*$^{-/-}$*g* $c^{-/-}$, 6-8 week old male mice
- Tumor cell: Sub-Cutaneous PC3 RFP Luciferase xenograft:
- 1,000,000 PC3 cells in 100 ul 1:1 media plus Matrigel per mouse. Date: PC3 s.c. injections date: 11/05/21
- IVIS, randomization into 3 groups, i.v. T cell injections: 11/08/21:

1. Untreated
2. Activated T cells alone: 30 x 10^6 cells i.v. 100ul
3. Cirm anti-ROR1 CART cells: 30 x 10^6 cells i.v. 100ul

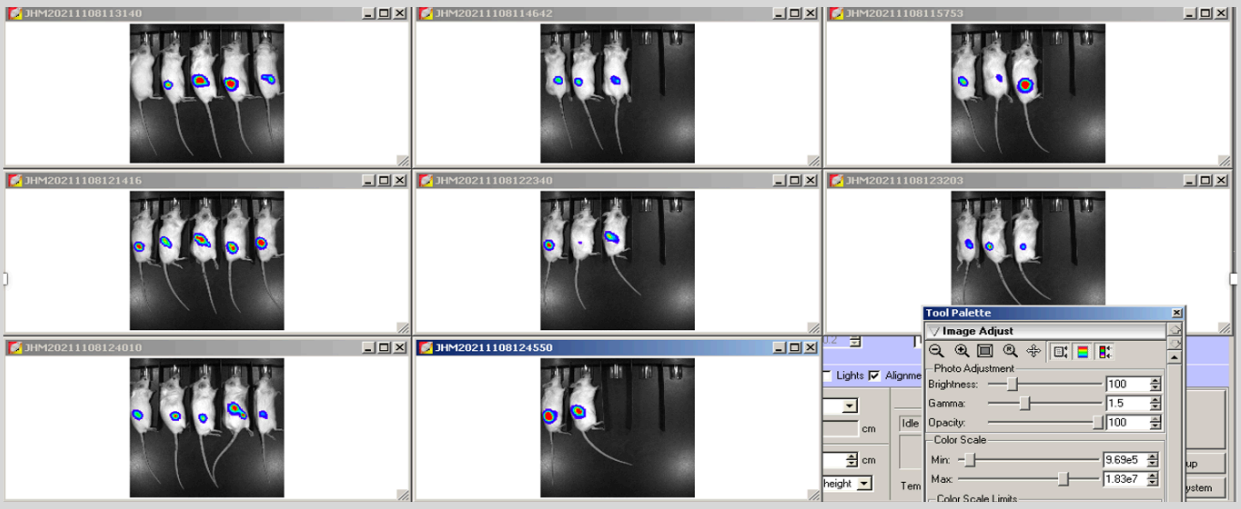
Group	Target Cells	Treatment (ROA)	N =
A	PC3 RFP Luciferase SC	Untreated	9
B		Activated T cells	9
C		Cirm CART	9

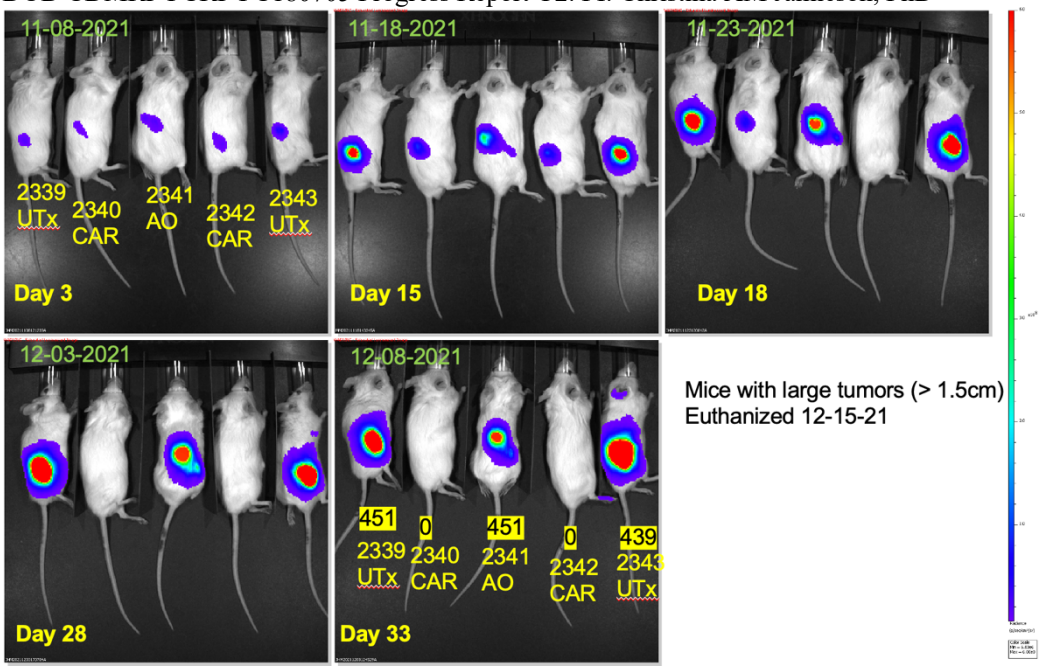


All mice had tumor on Day 3					1/5/21	
		Total	Tumor	No Tumor	Spont. Died	Alive
Untreated	U	10	10	0	0	0
Activated T only	AO	9	7	2	4	0
CART	C	9	3	6	0	7

Day 3
PC3
Tumor
Growth

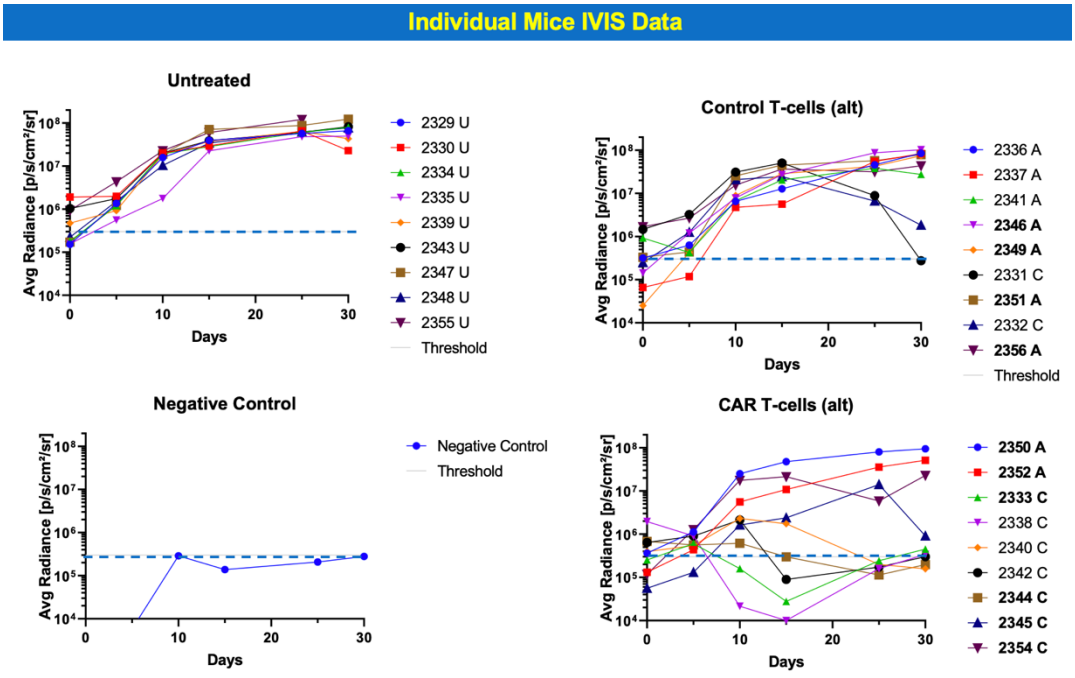
In vivo
Bioluminescence
(IVIS)

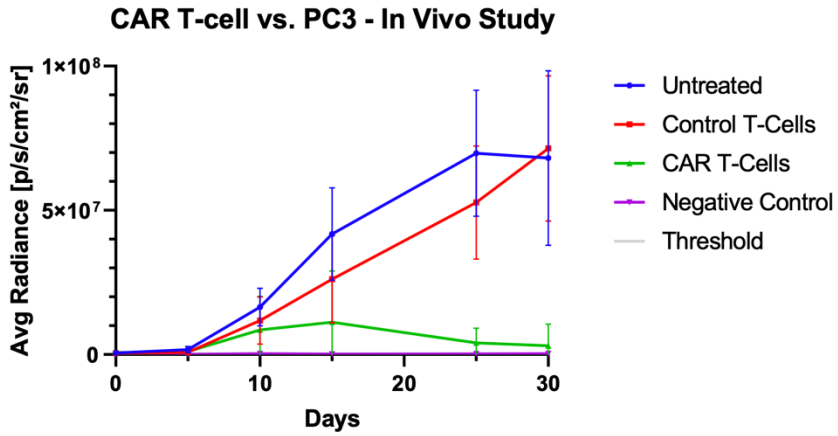




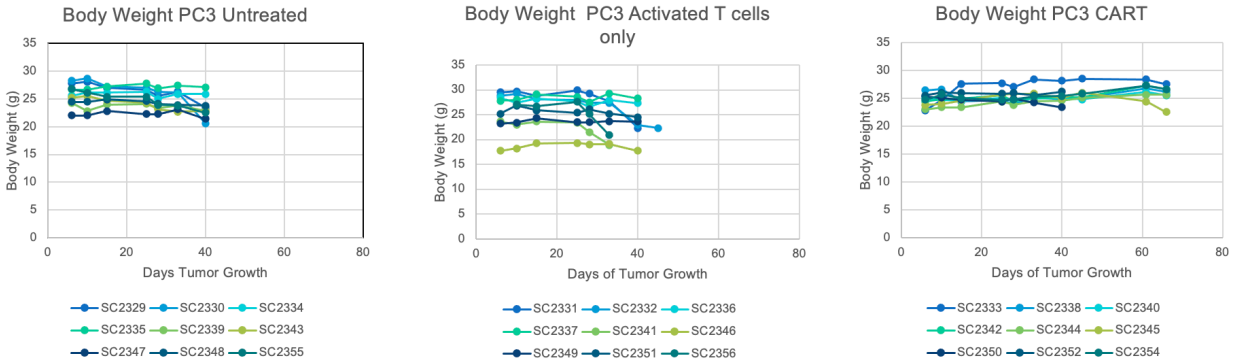
Representative IVIS images of PC3-RFP-Luciferase sub-cutaneous tumors in mice groups injected i.v. with: Untreated (UTx), Activated T cells only (AO) and CART cells (CAR) at Days 3, 15, 18, 28 and 33 post-injection.

Average Radiance of tumors

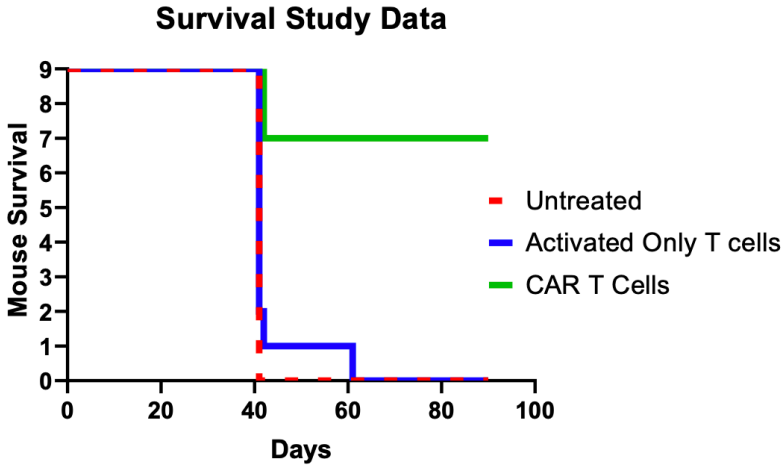




Body Weights of mice with PC3 Xenografts

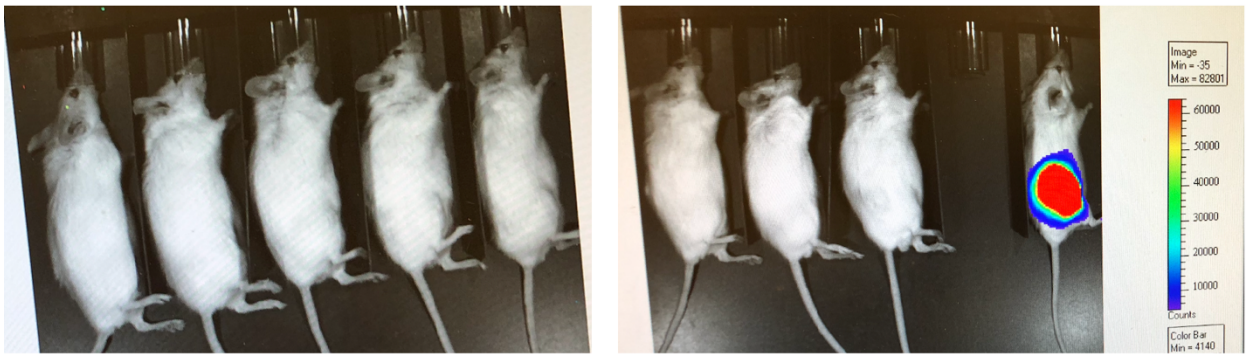


Significantly increased survival in mice with Cirmtuzumab-based CART cells



Mice with large tumors (>1.5cm L) were euthanized on Day 42

PC3 + CART mice on 1-5-2022



Cirmtuzumab-based CART cells produced durable eradication of NEPC tumor, PC3, in 7/10 mice which persists past 110 days thus far.

Conclusions

- New immunotherapeutic prostate cancer target, **non-canonical WNT5A receptor, ROR1**: Pre-clinical studies with anti-ROR1 targeting antibody therapeutic, Cirmtuzumab, showed tumor growth inhibition in ROR1+ bone metastatic prostate cancer PDX: PCSD13.
- *In vitro* cytotoxic killing studies showed promising results for anti-ROR1 CAR-T cell treatments as an effective treatment against prostate cancer.
- Cirmtuzumab-based anti-ROR1 CAR-T cells provide highly potent, prolonged and specific activity against prostate cancer models *in vivo* in PCa xenograft studies in mice thus encourage the advancement of CAR-T cell immunotherapies as a potential therapeutic product for PCa patients.
- Further in vivo studies in PCa PDX models and human clinical trials are necessary to develop the clinical product with determined dosing and toxicity profile.
- Cirmtuzumab Anti-ROR1 CAR-T cells have the potential to provide curative treatment against the most aggressive forms of prostate cancer: NEPC and CRPC.

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 Y2 of this grant we have built on our work showing 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. To study the mechanisms of WNT5A/ROR1 signaling in CRPC we generated CRISPR/Cas9 ROR1 knock out cell lines which we are testing in vitro and in vivo for responsiveness to combination therapy of docetaxel plus radiation – the standard of care for bone metastatic CRPC. Cirmtuzumab has demonstrated efficacy in our patient derived xenograft tumors in vivo in combination with the taxane chemotherapy, docetaxel. We have performed in vitro and in vivo testing of the anti-ROR1 Cirmtuzumab-based CART cells in NEPC cell lines and in our patient-derived xenograft (PDX) mouse models. **Our most important result thus far is that anti-ROR1 Cirmtuzumab-based CART cells can durably eradicate PC3 xenograft tumors in vivo (more than 100 days so far and counting).** We obtained an IRB amendment to an existing IRB protocol and have collected archival NEPC specimens for testing ROR1 expression in immunohistochemistry (IHC) assays to detect ROR1. We are working on the HRPO approval for this and for single cell RNA sequencing of cryopreserved surgical prostate cancer bone metastasis specimens. This 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 will inform us about the appropriate patient populations for treatment and clinical trials. The pre-clinical studies being performed in this grant will support the pending Phase 1B clinical trial (not part of this grant) and translation of anti-ROR1 Cirmtuzumab-based CART cells into clinical trials to treat neuroendocrine PCa and CRPC.

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

Problem: Matrigel shortage delayed organoids and PDX studies by several months. Matrigel-like replacements were tested from other suppliers, but these did not support organoid or PDX growth. New Matrigel stock received mid-November and PDX and organoid studies has resumed.

Studies using NEPC cell lines were performed to establish parameters and conditions for follow up experiments using our PDXs.

COVID-19 regulations have been in place all through 2021 until now. More people were allowed in the lab and in the mouse facility, but numbers were still restricted by spatial and social distancing rules.

6. PRODUCTS:

a.) 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).
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(28) and taxane chemotherapy (63). Available data suggest that Wnt blockade with chemotherapy may be the most effective way to implement Wnt pathway modulation(52).

b.) **Cirmtuzumab-based CART cells (Oncternal, Inc)** These studies are highly innovative due to the focus on using a safe and clinically active antibody for generating an anti-ROR1 CAR T cell to target ROR1 which is highly expressed on neuroendocrine prostate cancer. Although other investigators are targeting ROR1 with anti-ROR1 T-cell CAR T-cells (NCT02706392 and NCT02194374) (68-70), these ROR1 CAR T cells have had minimal activity in treating patients with ROR1^{pos} hematological or solid tumor cancers(30, 63, 66, 68-74). The lack of therapeutic effect in these other studies may be attributable to the rabbit/human chimeric ROR1 targeting domain employed in these T-cell CARs, which have the potential for off target binding and for recognition as foreign antigens resulting in the rapid inactivation of the CAR T product(68). To improve the clinical activity of a ROR1 targeting CAR product, we have created a series of anti-ROR1 T-cell CARs that employ an scFv antigen targeting domain generated from fully humanized cirmtuzumab (**Fig 2**)(1, 2, 53, 56). In generating our anti-ROR1 T-cell CAR, we employed the same iterative processes that we used to generate the **anti-ROR1 mAb cirmtuzumab.** This high-affinity, human ROR1-specific antibody, currently in advanced clinical trials, is has been used to treat over one hundred patients with advanced hematological malignancies and has been **well-tolerated with no evidence of off-target toxicity**(2). More importantly, cirmtuzumab, when combined with standard chemotherapies or combined with a cellular toxin to create an ADC, has shown substantial clinical activities in patients with treatment resistant cancers including a high percentage of durable, complete responses (NCT03420183, NCT02776917)(56, 57). We have developed a CAR employing cirmtuzumab as the antigen binding component that we expect to have greater activity in treating ROR1^{pos} cancers than existing anti-ROR1 CAR T cells. These anti-ROR1 CAR T cells are innovative because they employ the binding domain of cirmtuzumab, which has proven to be well-tolerated and non-immunogenic in multiple clinical trials, Moreover, a cirmtuzumab-based CAR T cell is expected to have the same high-specificity for ROR1 expressed on neoplastic cells without the normal tissue cross-reactivity noted for other anti-ROR1 human mAbs that have been tested in in vivo and in vitro test systems. Finally, the generation and clinical use of a cirmtuzumab directed CAR T will be expected to have enhanced activity when compared to previously tested anti-ROR1 CAR T cells (68).

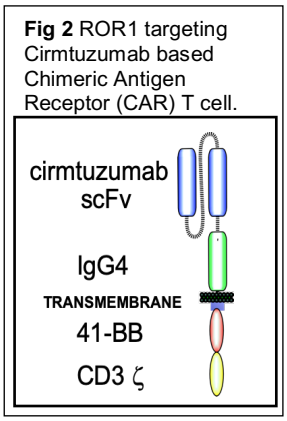


Fig 2 ROR1 targeting Cirmtuzumab based Chimeric Antigen Receptor (CAR) T cell.

10. 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 (in press, International Journal of Molecular Sciences). Performed gene expression profiling on PCSD1 patient, PDX and PCSD13 PDX in vivo experiments with and without enzalutamide treatment to identify bone-niche signatures correlated with enzalutamide-resistant growth.

Dr. Nicholas A. Cacalano, PhD, Associate Professor, Dept of Radiation Oncology, UCLA, is performing the CRISPR/Cas9 KO on PCSD13 ROR1 enriched cells, 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. Cacalano is co-author on our manuscript (Lee et al. IJMS) which is now in press.

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. Performed Western Immunoblots on WNT5A isoforms.

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.

Collaborator:

Dr. Charles Prussak, PharmD, PhD, director of the Cell Therapy Translational Laboratory at UCSD Moores Cancer Center. Dr. Prussak's team led the development of the Cirmtuzumab-based CART cells, made and provided expertise in the use of the Cirmtuzumab-based CART cells in our study to eradicate PC3 xenograft tumors. We have weekly meetings with Dr. Prussak's team and are working to test the next generation of Cirmtuzumab CARTs and on translation of the CARTs into clinical trial. Dr. Prussak is Co-I on a Phase 1 clinical trial of these CARTs for CLL patients which is approved and funded. Dr. Prussak is overseeing the production of the CARTs for the trial. The safety results of this CLL trial should greatly accelerate the translation of the CARTs into prostate cancer patients just as the clinical trial of the Cirmtuzumab antibody therapeutic itself showed its safety and tolerability in CLL patients which has led to clinical trials for metastatic breast and prostate cancer.

11. Special Reporting Requirements

N/A

12. Appendices

A. Year 1 Progress Report

3. ACCOMPLISHMENTS

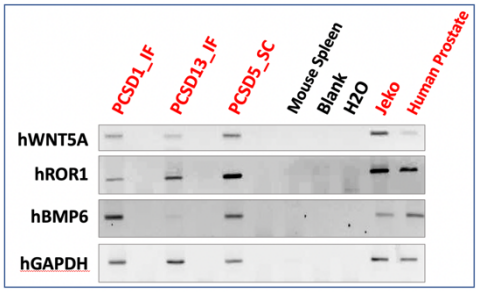
3.1 ACURO approval obtained.
3.2 HRPO not applicable in accordance with ORP_Statement on review of established PDX (Attached as Appendix). The memorandum states: " 2. This memorandum serves to clarify that USAMRDC supported research involving the use of established, existing patient derived xenograft (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 – Year 1

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.

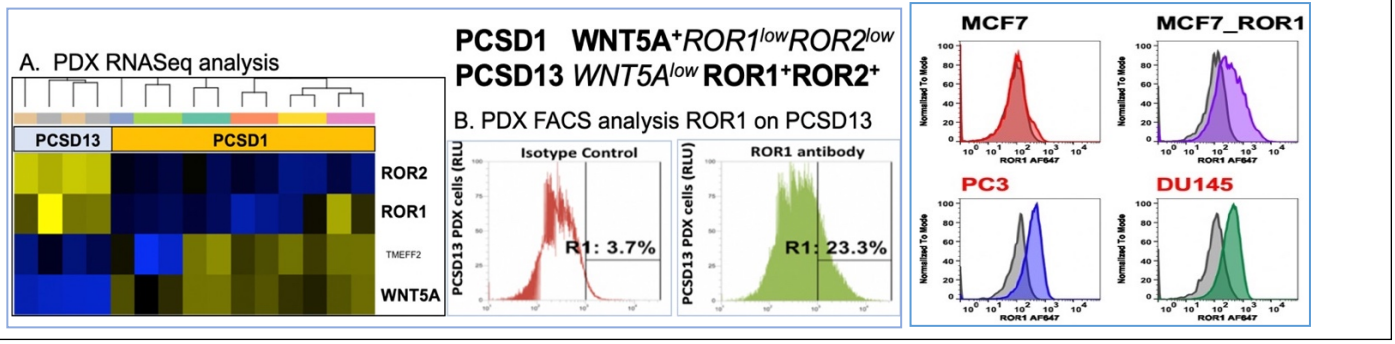
1. ROR1 mRNA is expressed at high levels on castration resistant small cell PCa and neuroendocrine PCa (NEPC) cell lines and TCGA PCa dataset.

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 expressed

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.



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.

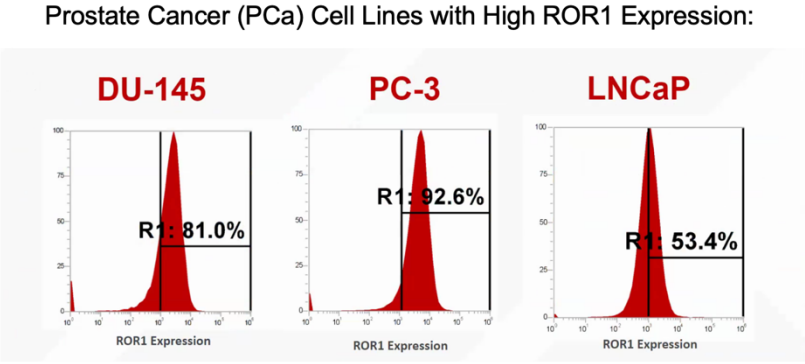
2. PCa cell lines, PC3 and DU145, express cell surface ROR1 protein

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

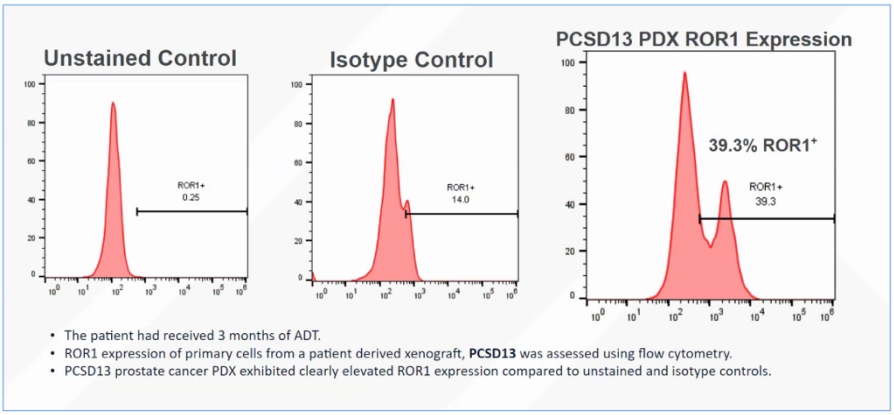
Figure 4.



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).

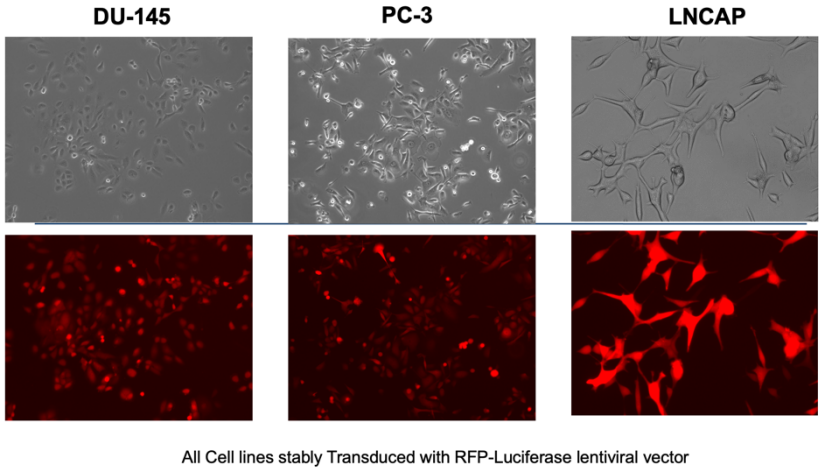
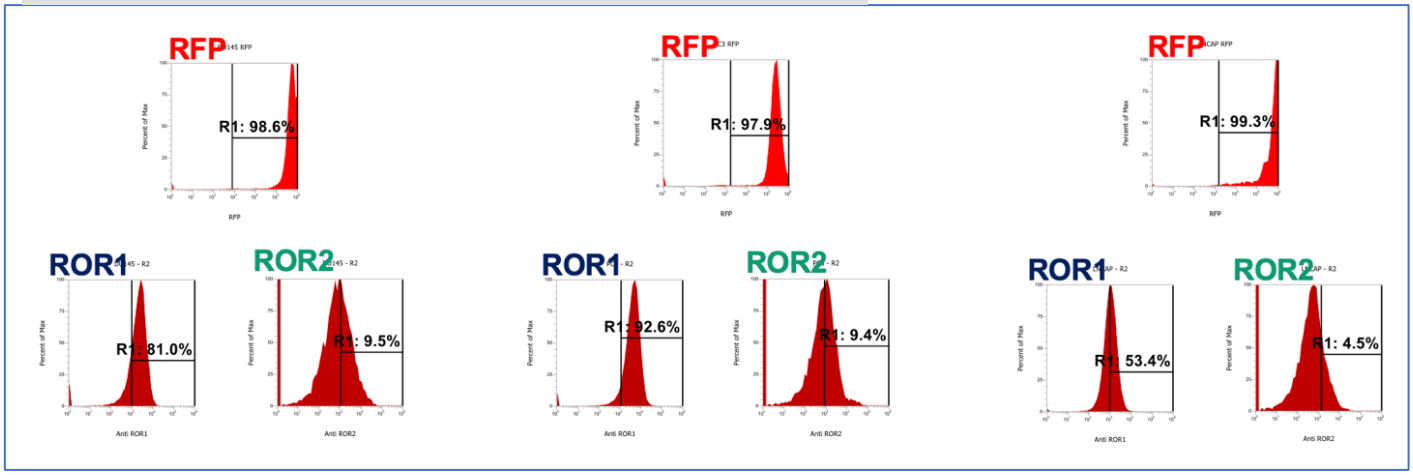


Figure 6. PCa Cell lines were transduced with RFP-Luciferase lentiviral vector particles express RFP as shown in Incucyte microscope.

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.

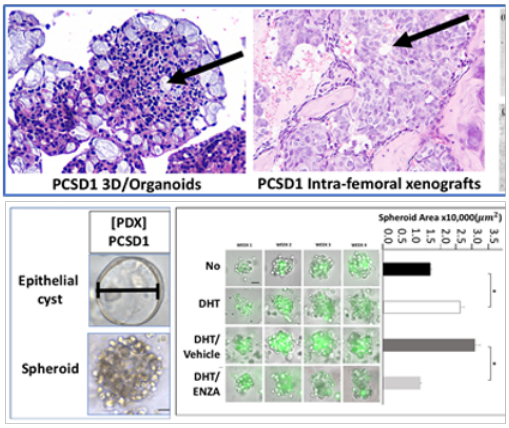


Fig 8. PCSD1 PDX tumors cells form 3D organoids that replicate histomorphology of the xenografts growing in the femur Black Arrows point to these similar structures. Organoids are GFP+,A.PDXs from prostate cancer bone metastases, PCSD1 express GFP-Luciferase B. Enzalutamide PCSD1 reduced lumen size and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

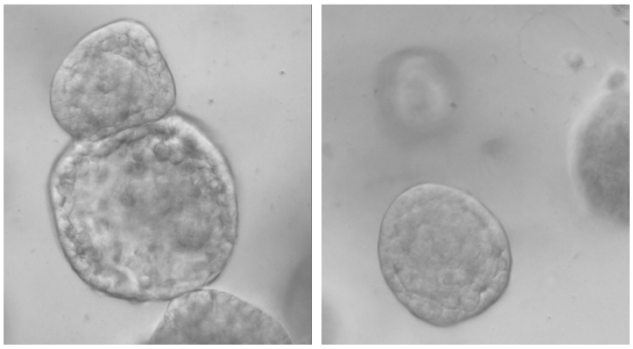


Figure 9. Live microscope images of the PCa Cell line, PC3 organoids grown in 3D optimized PCa organoid conditions (Lee et al 2020).

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.

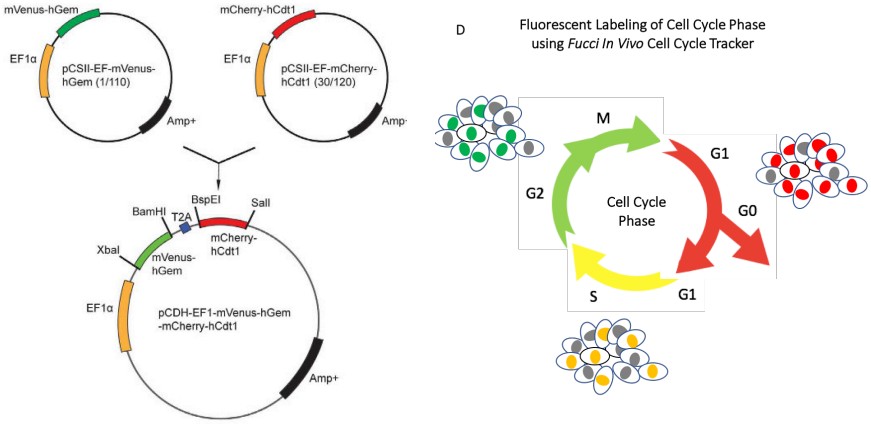
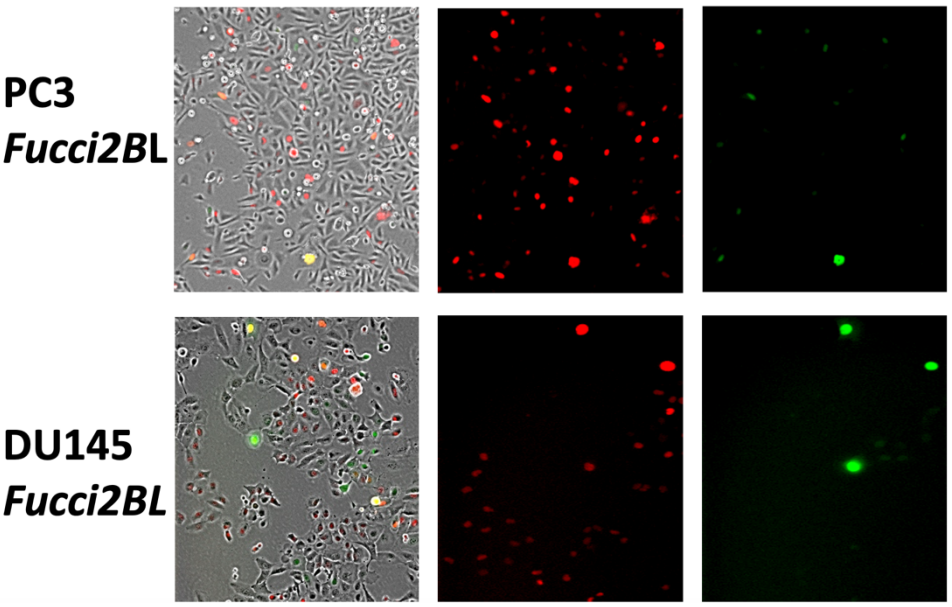
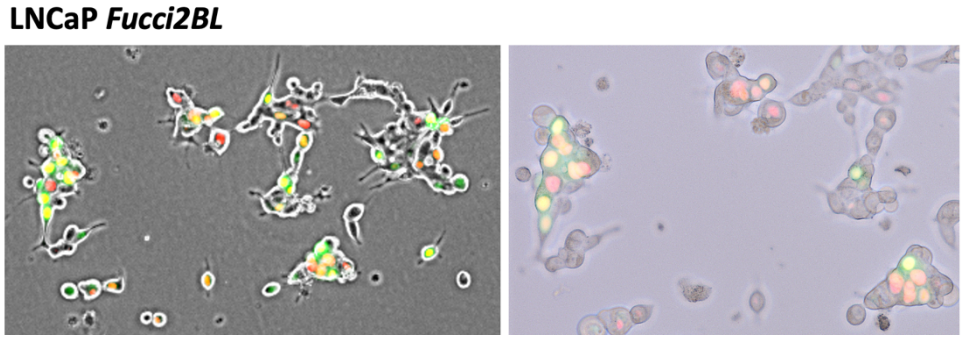


Fig 10. Fucci2BL vector generation and characterization. (a) Diagram and map of construct design and generation. Both mVenus-hGem(1/110) and mCherry-hCdt1 (30/120) were subcloned into a pCDHEF1α-T2A lentiviral expression vector (b) Fluorescent labeling of cell cycle phases using Fucci2BL live cell cycle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


PC3
Fucci2BL
DU145
Fucci2BL


LNCaP Fucci2BL

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

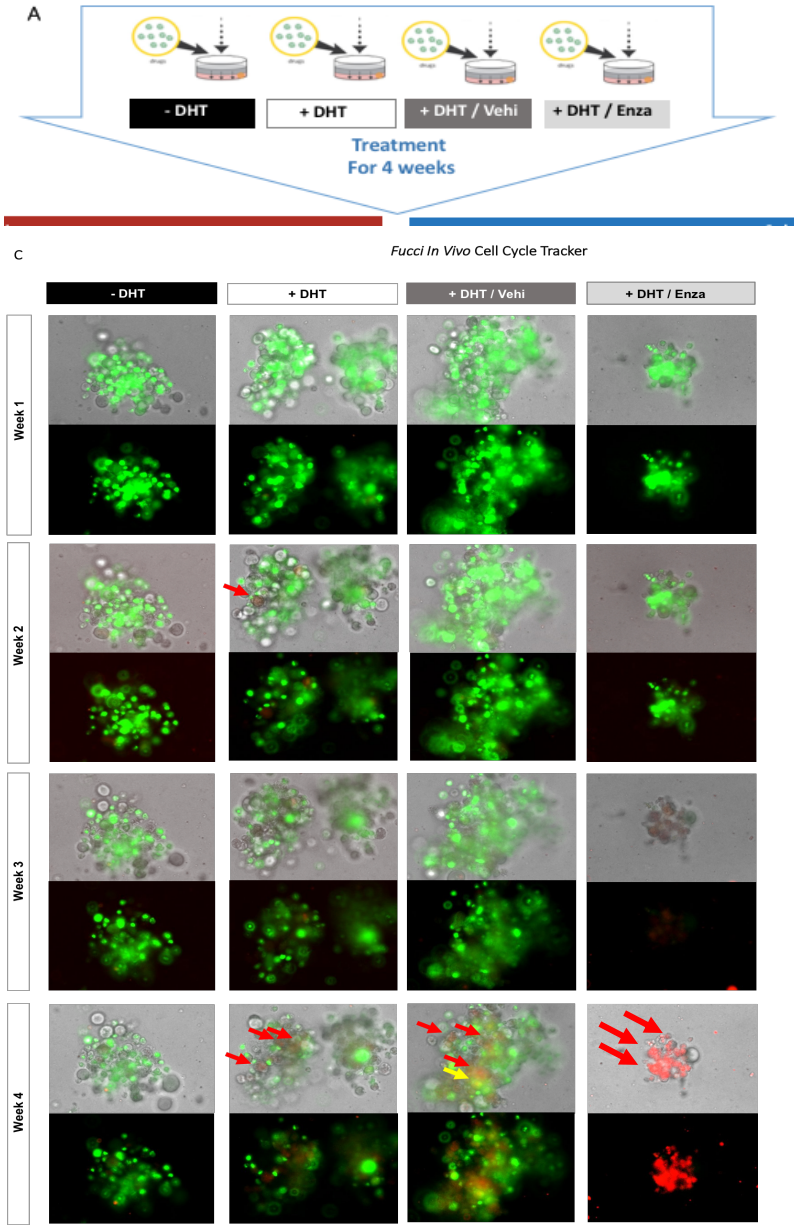
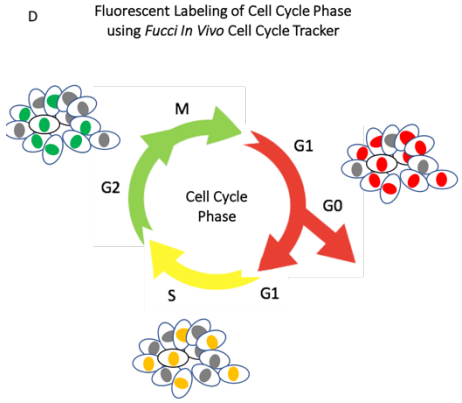


Figure 11. Enzalutamide-Resistant Population of PCSD1 Organoids are Dormant Cells.
Time course of cell cycle stages in live PCSD1 organoids under the four treatment conditions. Representative images in each treatment condition at week 1, 2, 3, and 4 of treatment of PCSD1 organoids stably expressing the *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

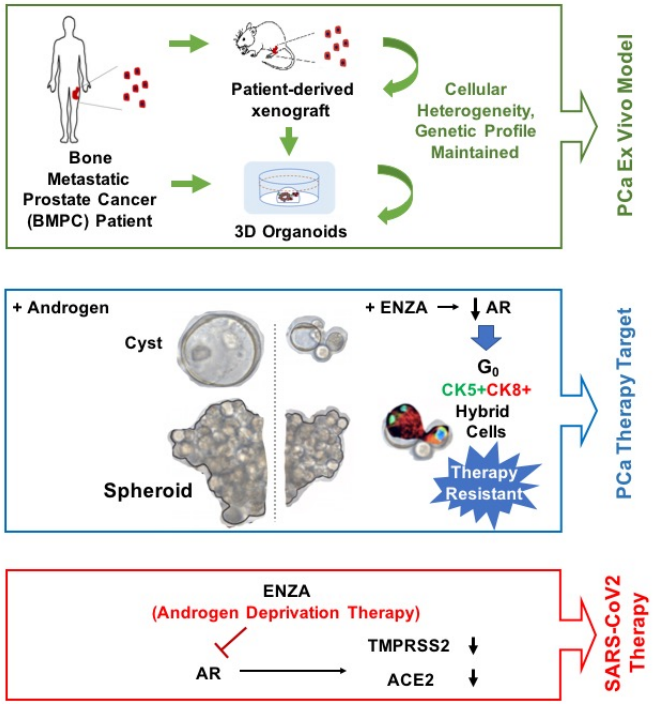


Figure 12. PDX organoids treated with Androgen deprivation therapy were CRPC and Dormant. Pre-clinical drug screening platforms for new CRPC and SARX-CoV2 and variants treatments.

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.

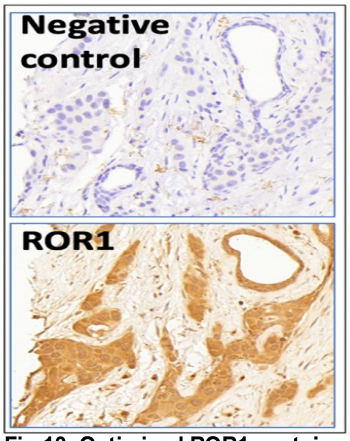


Fig 13. Optimized ROR1 protein IHC analysis. Right panels: ROR1 IHC optimization on patient breast adenocarcinoma formalin-fixed paraffin-embedded sections.

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.

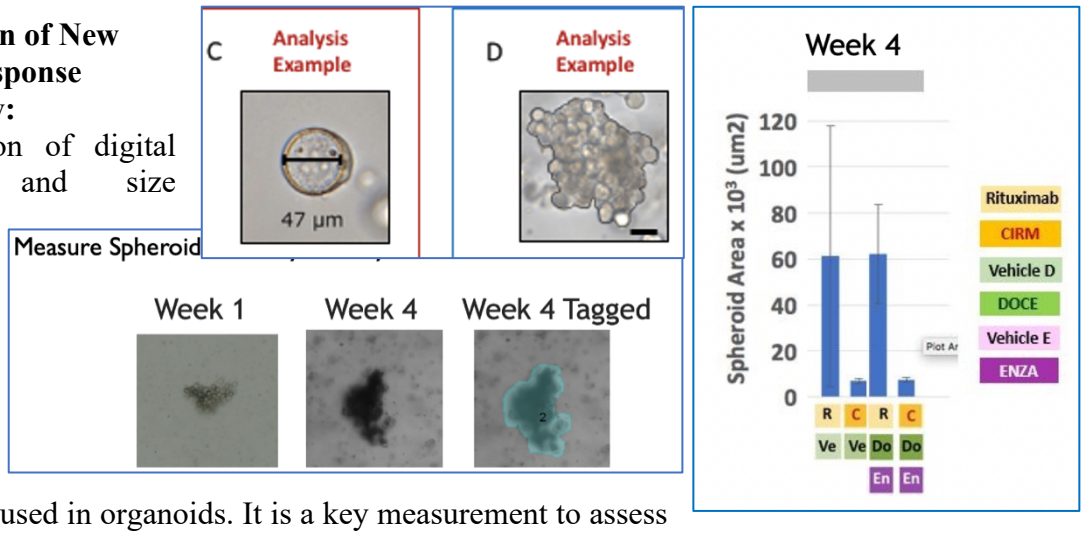
1. Characterizing docetaxel, cirmtuzumab response in PDX-derived organoids: PCDSD1 and PCSD13

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.

Year 1

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.

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

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

9. 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.

10. 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.

11. 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.

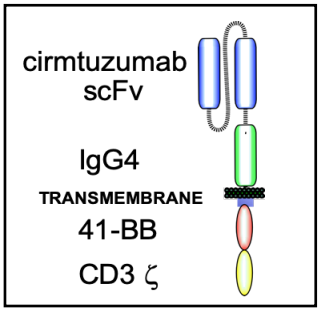
With the lab shut down from March 18th, 2020 to June 30th, 2020 my team and I shifted our efforts to our bioinformatics and digital microscope image analyses which we could do remotely. I upgraded our computer and software for everyone on our team and we were able to work efficiently as a team remotely. 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.



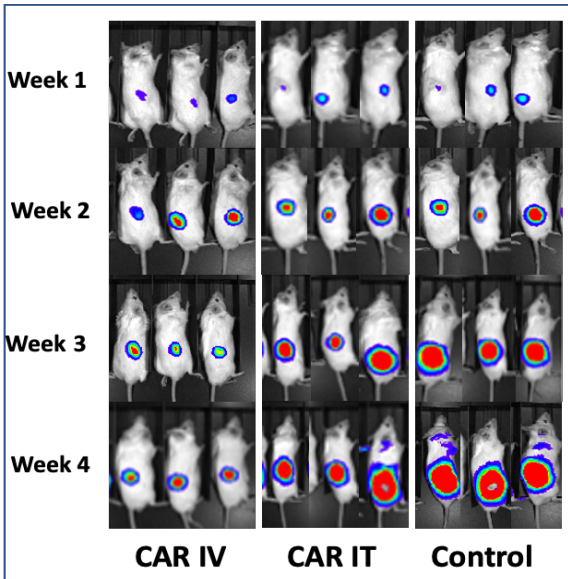


Figure 11-Bioluminescence imaging of mice implanted subcutaneously with PC3 PCa cells and Treated with ROR1 CAR T-cells. Animals were implanted with 2e6 lucifierase expressing PC3 cells on day 1 and were treated with the one-time injection of 3e7 CAR-T cells IV (**CAR IV**) or activated mock transduced 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.

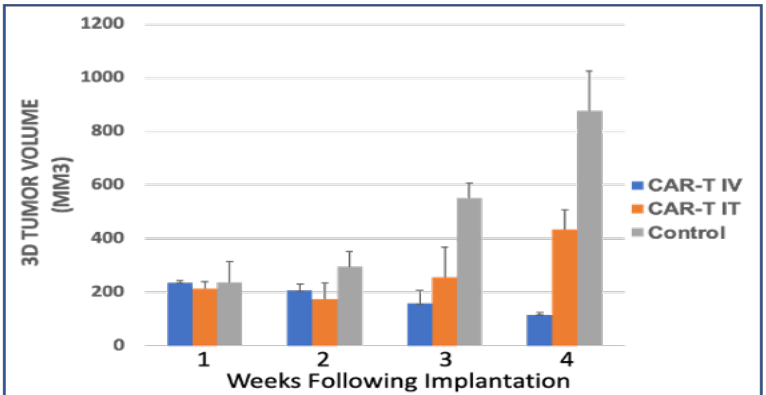


Fig12. Tumor volume measurement of implanted PC3 PCa cells by Caliper measurement. Tumor volumes were measured by weekly caliper measurements for mice treated as described in Fig 11. Results are consistent with the bioluminescent measurements and better demonstrate the activity of the intravenously administered 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.

13. 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(28) and taxane chemotherapy (63). Available data suggest that Wnt blockade with chemotherapy may be the most effective way to implement Wnt pathway modulation(52).

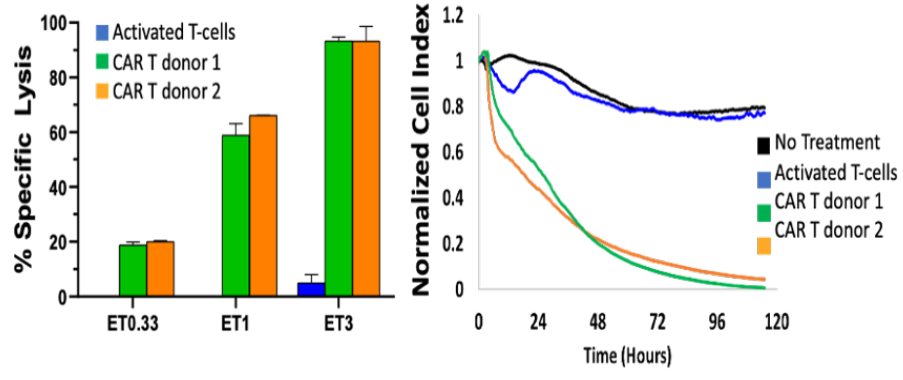


Figure 3. ***In vitro*** **cell killing activity of ROR1 CAR T-cells in a 4h chromium release assay (left) and 120h ACEA impedance assay (right).** ROR1 CAR T-cell products were generated from 2 healthy donors and tested in a chromium release assay at the indicated E:T ratios against leukemic Mec1 ROR1^{pos} 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..

14. 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.

15. Special Reporting Requirements

N/A

16. Appendices

A. OPR Memo on PDX and HRPO

B. Technical Abstract

C. SOW