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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

Prostate cancer metastasizes preferentially to bone leading to painful, bone destructive lesions and malignant disease progression for which there is no cure. The complexity and heterogeneity of prostate cancer bone metastases make them extremely challenging to treat and new therapies are urgently needed. We developed new patient-derived xenograft (PDX) models using surgical patient prostate cancer bone metastases which were implanted into immunodeficient mice. The resulting xenograft tumors replicated the tumor heterogeneity and bone lesions seen in patients. We are using our patient-derived xenograft models and prostate cancer cell line models to determine the 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.

15. SUBJECT TERMS
None listed.

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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)**.

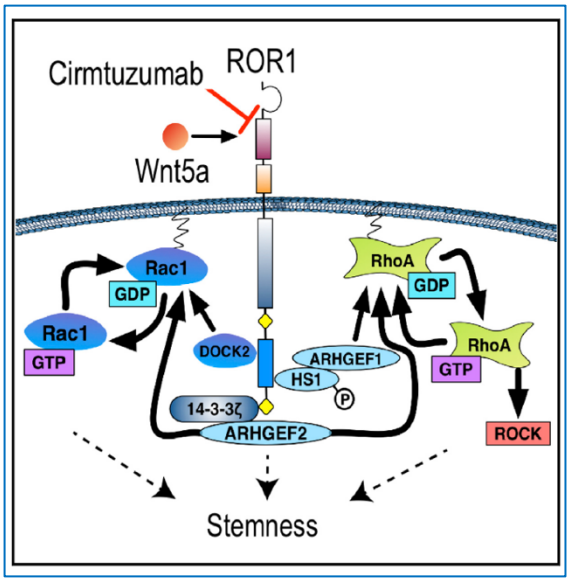


Figure 1. Signaling through the non-canonical Wnt pathway is mediated by Wnt5A binding to its receptor ROR1. Cirmtuzumab is a monoclonal antibody which targets ROR1 (Choi 2018).

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**.

1. KEYWORDS: Bone metastatic prostate cancer, WNT5A, ROR1, Patient derived xenograft, organoid, Castration Resistant Prostate Cancer (CRPC), Neuroendocrine Prostate Cancer (NEPC), Docetaxel, Cirmtuzumab (now called Zilovertamab), Real time cell proliferation assay (RTCA), In vivo bioluminescence system (IVIS),

2. SPECIFIC AIMS

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 increased WNT5A is correlated with bone metastatic PCa. **Hypothesis:** Resistant sub-clones exist that can be distinguished at the single cell RNA expression level. WNT5A may be essential in the resistant sub-clone.

AIM 1. Determine the mechanism of WNT5A signaling in castrate resistant bone metastatic prostate cancer using patient-derived organoids (PDO) and xenograft (PDX) models.

Rationale: We found that WNT5A is overexpressed in patient bone metastasis compared to normal bone. The goal of the following experiment is to examine the potential role of WNT5A signaling in promoting tumor growth and metastasis. We will use small molecules, short-hairpin RNA (shRNA) mediated knockdown and CRSIPR/Cas9-mediated gene knockout. WNT5A, ROR1 and ROR2 will be knocked down using commercially available shRNA lentiviral constructs in our PDX cells then xenografted into mouse femurs to evaluate effect on tumor growth in mice treated with vehicle or the anti-androgen, enzalutamide. RNASeq analysis will be performed in dissociated tumors cells and may expose the ADT-therapy resistant sub-population and its mechanism of resistance. Results of these studies that knock down WNT5A expression will determine if it is a good target for further novel therapy development. If yes, then there are WNT5A small molecule inhibitors, WNT5A inhibiting peptide mimetics and antibody therapeutics for WNT5A and its receptors, ROR1 and ROR2, that could be tested for their ability to inhibit tumor growth in our PDX models of bone metastatic prostate cancer[41-46] .

a.) PDX models from three different patients:

- PCSD1, PSMA (+), mixed osteolytic, osteoblastic bone lesions, adenocarcinoma, CRPC in bone
- PCSD5, PSMA (+), mixed osteolytic, osteoblastic bone lesions, adenocarcinoma, treatment naive
- PCSD13, PSMA (-), osteolytic, some osteoblastic, mixed adenocarcinoma and neuroendocrine

b.) 3D organoid cultures of patient derived xenograft cells

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. Cirmtuzumab is already in a Phase 1b clinical trial for CLL for application in bone metastatic prostate cancer using PDO and PDX bone metastasis models. **ROR1, ROR2 and CIRMTUZUMAB.** Function of ROR1 and ROR2 in bone metastatic prostate cancer, Clinical trial planning, Phase 1B – how much will it cost? Steps toward implementing Phase 1B clinical trial of cancer stem-cell targeting antibody therapeutic, CIRMTUZUMAB, in patients with castrate resistant bone metastatic prostate cancer.

AIM 3. Determine single cell RNA sequencing profiles of our unique series of twenty surgical patient prostate cancer bone metastasis specimens and a longitudinal series of PCa bone metastases of a single patient and his xenografts.

The orthopedic surgical patient bone metastasis samples we have Biobanked and viably cryopreserved single cells consist of a heterogeneous mix of tumor, bone marrow, blood, and stromal cells. Single cell RNASeq will be performed to determine gene expression signatures of the tumor cells and their surrounding cells in the bone microenvironment including bone marrow hematopoietic lineage, bone, and stromal cells in CRPC for precision medicine. The first patient's bone metastasis samples 1, 2, and 3 which were collected at three points over the two-year course of his treatment and will be compared to the RNASeq analysis of his xenografts, PCSD1 grown in the mouse femur and treated with vehicle, bicalutamide or enzalutamide. Results should reveal the signatures present in the tumor and in the bone microenvironment cells from the patient and his intra-femoral xenografts treated with anti-androgen as he was.

3. RESEARCH ACCOMPLISHMENTS – Year 3

SUMMARY

In Year 3, we made significant progress in **AIM 1** in elucidating the mechanism of action of the WNT5A/ROR1 using our models of castration resistant small cell PCa and neuroendocrine PCa (NEPC) models in which ROR1 is expressed at high levels. To study the mechanisms of WNT5A/ROR1 signaling in CRPC we generated CRISPR/Cas9 ROR1 knock out cell lines in PC3, DU145 and LNCaP in which we tested responsiveness to combination therapy of ROR1 inhibition plus the chemotherapy drug, docetaxel, the standard of care for metastatic CRPC. We showed in Incucyte real time cell growth assays that PC3 cells with the ROR1 knock out (KO) were significantly more sensitive to docetaxel than PC3 with ROR1.

In **AIM 2**, We used our PDX models to test a new therapeutic target: the WNT5A/ROR1 cancer stem cell signaling pathway using the ROR1 inhibitory antibody, Cirmtuzumab. Flow cytometry showed heterogeneous expression of ROR1 in the PDX tumor cells as was seen in the patient. In this study the effect of Cirmtuzumab on PDX tumor growth was compared to docetaxel, the standard-of-care chemotherapy drug for these patients. Neither Cirmtuzumab alone nor docetaxel alone lessened PDX tumor growth. However, the combination of Cirmtuzumab plus docetaxel did inhibit tumor growth. In AIM 3, Single cell RNA sequencing of the cryopreserved tumor cells and frozen tissues from this study will allow us to distinguish the molecular phenotypes of the responding and non-responding tumor cell types in the four treatment groups: 1. Vehicle control, 2. Cirmtuzumab alone, 3. Docetaxel alone, 4. Cirmtuzumab + Docetaxel, as well as the effects on the cells of the tumor microenvironment.

In **AIM 3**, we performed single cell RNA sequencing of our PDX PCSD1 cells and are optimizing single live cell enrichment protocols with our cryopreserved PDX PCSD13 tumors from the Cirmtuzumab plus Docetaxel experiment. The results of this single cell RNASeq analysis will enable future scRNASeq analysis of the surgical prostate cancer bone metastasis specimens in our biobank. We obtained an IRB amendment to an existing IRB protocol and have collected archival NEPC specimens for testing ROR1 expression in immunohistochemistry (IHC) and RNAScope assays to detect ROR1.

SUMMARY OF RESULTS:

AIM 1. Determine the mechanism of WNT5A signaling in castrate resistant bone metastatic prostate cancer using patient-derived organoids (PDO) and xenograft (PDX) models.

- ROR1 was expressed at high levels on castration resistant, small cell PCa and neuroendocrine PCa cell lines and bone metastatic prostate cancer PDX models as shown in RNAseq, flow cytometry and Immunoblotting analyses (Figure 5 Year 1 and 2 Progress Reports).
- In real time course cell growth assays (RTCA) using an Incucyte system, the PC3ROR1KO cells were significantly more sensitive to docetaxel induced growth inhibition than PC3 WT ROR1 (IC50 3 nM versus 300 nM, respectively) (**Figure 2**).
- PC3 cells expressing the Fucci2BL live cell cycle fluorescent tracker system showed docetaxel induced G2 arrest, while untreated cells at 100% confluence led to G1 arrest in Incucyte RTCA (**Figure 4**).
- In 3D organoids cultures PC3Fucci treated with docetaxel showed predominant G1 arrest while PC3ROR1KOFucci organoids showed predominant G2 arrest. Passaging of organoids which depends on cancer stem cell/ tumor initiating cells showed significantly fewer organoids in the docetaxel treated PC3ROR1KO organoids than docetaxel treated PC3 ROR1 WT organoids (**Figure 5A**).
- Passaging organoids showed docetaxel significantly decreased the number and size of PC3ROR1KO organoids more than PC3 organoids suggesting loss of tumor initiating cells (**Figure 5B**).
- Generated new PC3Fucci and PC3ROR1KOFucci cells with higher, matched relative fluorescence (**Figure 6**).
- Optimized Incucyte Scratch wound assay as a measure of metastatic potential in PC3 versus PC3ROR1KO cells (**Figure 7**).
- In vivo PC3 RFP Luciferase and PC3ROR1KO RFP Luciferase xenograft tumor growth in vivo. Mice were imaged in the in vivo bioluminescence imaging system (IVIS) (**Figure 8)**.

- We hypothesize that WNT5A/ROR1 signaling mediates a more quiescent cancer stem cell state which allows them to evade therapies that target cell proliferation such as docetaxel. Thus, deletion or inhibition of ROR1 re-sensitizes prostate cancers to chemotherapy.
- These pre-clinical studies supported the Phase 1b clinical trial of Cirmtuzumab (Cirmtuzumab) plus docetaxel in metastatic CRPC patients for which two patients are now on treatment.

Next steps:

- In vivo PC3 RFP Luciferase and PC3ROR1KO RFP Luciferase xenografts treated with vehicle or docetaxel compare growth, transcriptome (RNA sequencing and bioinformatics), protein and signaling analysis.
- In vitro RNAseq analysis of PC3 and PC3ROR1KO -/+ docetaxel time course
- DU145, DU145ROR1KO Fucci 2D and 3D organoids analysis

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.

- Cirmtuzumab synergized with docetaxel to inhibit tumor growth in patient derived xenograft PCSD13 in vivo (Figure 5 Year 2 Progress Report).
- RNA sequencing in progress
- Western immunoblotting in progress for ROR1, ROR2 and downstream signaling pathways.
- IHC in progress on FFPE sections for ROR1, ROR2 and TROP2.
- Cirmtuzumab-based CART cells durably eradicated ROR1+ prostate cancer xenograft tumors (**Figure 9**). Mice with PC3 xenografts injected with cirmtuzumab-based anti-ROR1 CAR-T cells showed durable, tumor eradication.

Next Steps:

- PCSD13 Fucci organoids -/+ docetaxel +/- Cirmtuzumab
- Repeat in vivo PDX PCSD13 experiment, treat with Cirmtuzumab and/or docetaxel when tumors are smaller.

AIM 3. Determine single cell RNA sequencing profiles of our unique series of twenty surgical patient prostate cancer bone metastasis specimens and a longitudinal series of PCa bone metastases of a single patient and his xenografts.

- Single cell RNASeq performed on PCSD1.
- Optimizing live, single cell recovery of cryopreserved PCSD13 PDX tumor cells which will be used for patient cells.
- PDX Clinical development of GMP Cirmtuzumab CART cells for a clinical trial in CLL is in progress and may lead to rapid progression to Phase 1 safety clinical trial for metastatic CRPC and NEPC.

New Publications 2022:

1. Javier-DesLoges J, Salmasi A, **Jamieson CAM**, Anger JT. RE: Cancer Stage, Treatment, and Survival among Transgender Patients in the United States. J Natl Cancer Inst. 2022 Feb 22; doi: 10.1093/jnci/djac039. [Epub ahead of print] PubMed PMID: 35191502.
   Role: I contributed to the conception and discussions about the current understanding and state of care for hormone-sensitive cancers in transgender, contributed to manuscript writing, reviewing, and editing.

2. Lee S, Mendoza TR, Burner DN, Muldong MT, Wu CCN, Arreola-Villanueva C, Zuniga A, Greenburg O, Zhu WY, Murtadha J, Koutouan E, Pineda N, Pham H, Kang SG, Kim HT, Pineda G, Lennon KM, Cacalano NA, Jamieson CHM, Kane CJ, Kulidjian AA, Gaasterland T, **Jamieson CAM**. Novel Dormancy Mechanism of Castration Resistance in Bone Metastatic Prostate Cancer Organoids. Int J Mol Sci. 2022 Mar 16;23(6). doi: 10.3390/ijms23063203. PubMed PMID: 35328625; PubMed Central PMCID: PMC8952299.
   Role: Senior author, all experiments were performed in my lab. Initiated and developed the unique patient derived models to create a clinically relevant platform to understand mechanisms of therapy resistance of bone metastatic prostate cancer. In this paper we showed that these three-dimensional cultures (organoids) model platform are mini tumors that closely matched the patient tumor. They can be used to test new therapies for prostate cancer using small amounts of tumor cells and drug and for testing therapies to inhibit SARS-CoV2 infection. I worked closely and synergistically with our bioinformatics co-author (TG) to analyze the transcriptomics data. We developed new ways to interpret and present the bioinformatics results. We used the live cell cycle tracking system developed by CHMJ in solid tumor organoids for the first time. We showed for the first time in live patient derived cells in 3D that they became truly dormant in the presence of the anti-androgen therapy but could be re-awoken when the drug was removed leading to better understanding of cancer therapy resistance and recurrence. In summary, I led this study from start to finish and was primarily responsible for the idea and concept, formulating the hypothesis, methodology development and optimization, validation, formal analysis, and investigation, writing original draft preparation, writing review, and editing, supervision, project administration, all funding acquisition which supported this work.

3. Parsons, JK, Pinto, PA, Pavlovich, CP, Uchio, E, Nguyen, MN, Kim, HL, Gulley, JL, Sater, HA, **Jamieson, CAM**, Hsu, CH, Wojtowicz, M, House, M, Schlom, J, Donahue, RN, Centuori, S, Bailey, S, Bauman, J, Parnes, HL, and H-H. S Chow. Immunotherapy to Prevent progression on Active Surveillance Study (IPASS): A Phase II randomized, double-blind, controlled trial of PROSTVAC in prostate cancer patients on active surveillance. Eur Urol Focus. 2022 Dec 12: S2405-4569(22)00286-3. doi: 10.1016/j.euf.2022.12.002. Online ahead of print. PMID: 36517408.
   Role: Acquisition of data, analysis and interpretation of data, monthly discussions of the project progress and next steps. Drafting of the manuscript.

DETAILED PROGRESS REPORT YEAR 3:

SPECIFIC AIM 1: 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.
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 in Year 1 and Year 2 progress reports).
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.
In Year 3 our results led to the hypothesis that WNT5A/ROR1 signaling leads to more stem cell-like state in CRCP bone metastases which enables them to inhibit their cell cycle and cell division pathways thereby making them resistant to treatments that target rapid proliferation such as docetaxel and radiation therapy.

Detailed Progress and Results:

1. Optimization of Real time Cell-based Incucyte Proliferation Assay
A systematic approach was taken to optimization of the real time, live cell 96-well proliferation assay using the Incucyte S3 as shown in Figure 5A. After the performance of the limited dilution cloning series and the expansion of the clones, we did FACS to select the purest populations of PC3 Fucci cells. Twelve clones of PC3 Fucci were analyzed through FACS. We selected four clones of PC3 Fucci with high red and green fluorescence of the Fucci cell cycle markers as indicated by the red outline in Figure 4. The PC3 Fucci clones were selected for those which had good Fucci2BL expression level and grew well.

Limiting Dilution Cloning and FACS Analysis
After the performance of the limited dilution cloning series and the expansion of the clones, we proceeded onto FACS analysis to select the purest populations of PC3 Fucci and PC3 ROR1KO cells. Twelve clones of PC3 Fucci and five clones of PC3 ROR1KO were analyzed through FACS. After the analysis, we selected four clones of PC3 Fucci and three clones of PC3 ROR1KO as indicated by the red arrows in Figure 1.a. The PC3 ROR1KO clones were selected by looking at the ROR1 expression of the cells. We were interested in having pure PC3 ROR1KO cells, so we selected the clones which did not show any ROR1 expression. Regarding PC3 Fucci clones, we selected the ones which had high ROR1 expression and good Fucci2BL expression level.

2. Effect of Docetaxel on Prostate Cancer Cell Proliferation with ROR1 Knock out.
The impact of Docetaxel on the cell proliferation of PC3 Fucci cells was measured using the live cell imaging of the Incucyte. These observations were consistent among the 3 clones of PC3 Fucci cells used for this experiment. Overall, increasing concentrations of docetaxel produced a decrease in the rate at which the cells were expanding, causing the cells to reach a lower growth plateau. These results show the effectiveness of Docetaxel at inhibiting or slowing down the proliferation of PC3 Fucci cells (Figure 5). The effect of Docetaxel on the cell cycle was measured in real time using the live cell cycle tracker system: Fucci2BL (Figure 6). Green fluorescence is an indicator that the cell is in G2 phase, red indicates that the cell is in G1 or G0, and yellow (green + red) indicates the cell is in S phase (Figure 6). We observed a shift to G2 arrest, that is, increase in the percentage of green (G2 phase indication) fluorescence with Docetaxel compared to G1/G0 arrest in the vehicle treated cells when they reached confluence (Figures 7,8,9).

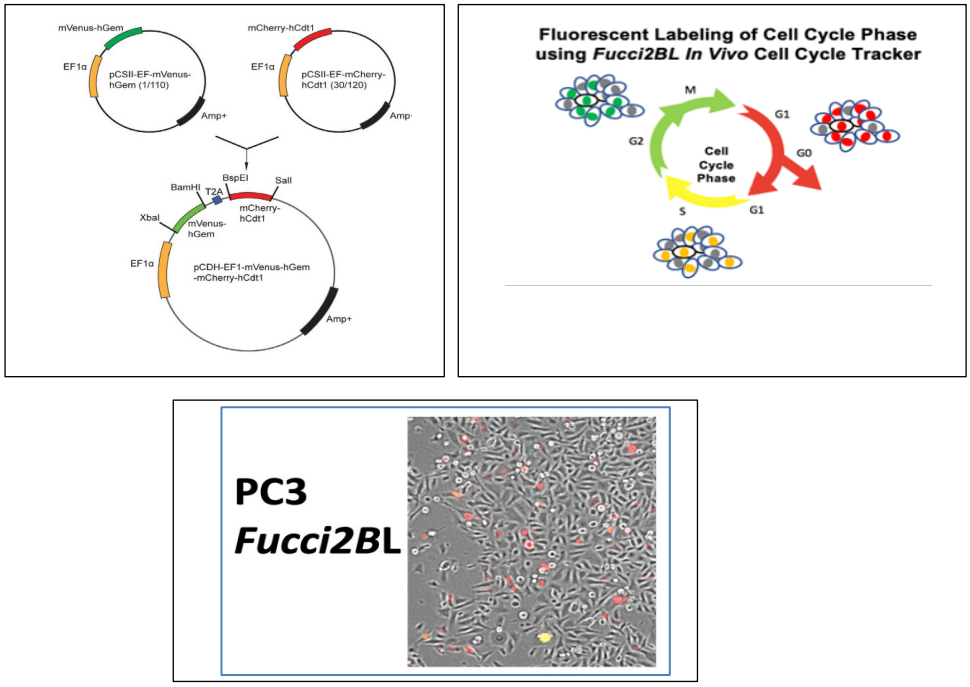
The proliferation of PC3 Fucci cells treated with CIRMTUZUMAB will be tested next with WNT5A, Docetaxel and radiation treatment.

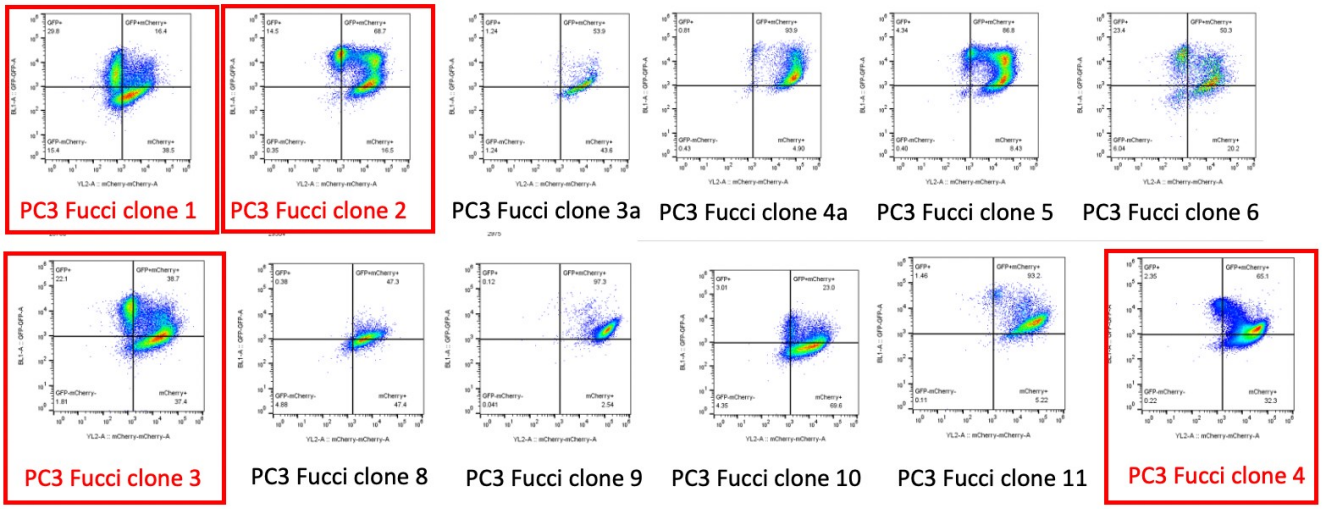
Cell Migration Scratch Assay: PC3 Fucci cells were plated at 10,000, 5,000, 2,500 and cells per well in 96 well plates, incubated for two days, then the Incucyte Scratch assay tool was used to make identical scratches in all 96 wells simultaneously and the plate incubated in the Incucyte for 3 days. Time course of images were collected every 2 hours.

The end point image is shown in Figure 14. The 10,000 cells per well showed the optimal scratch wound and regrowth. Fucci cell cycle analysis showed highly active proliferation.

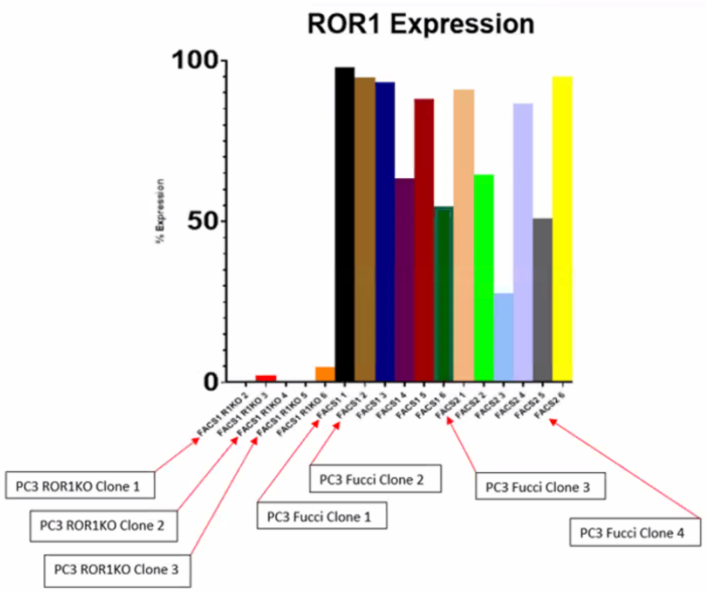
The next steps are to test CIRMTUZUMAB with standard of care drugs which target the cell cycle and cell division in proliferating cells this assay as well as with and without docetaxel and ROR1 inhibition as novel combination therapies.

Figure 2 PC3 and PC3ROR1KO Fucci cell line cloning and characterization. A. 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 (Pineda *et al* 2016 Sci Rep). (**b**) Fluorescent labeling of cell cycle phases using Fucci2BL live cell cycle tracker system. B. Fucci2BL Selection of PC3 Fucci clones after limiting dilution series based on their Fucci red, green fluorescence, ROR1 levels and growth.





Selection of PC3 ROR1KO and PC3 Fucci clones after limiting dilution series based on their ROR1 expression



Selection of PC3 Fucci clones after limiting dilution series based on Fucci2BL expression

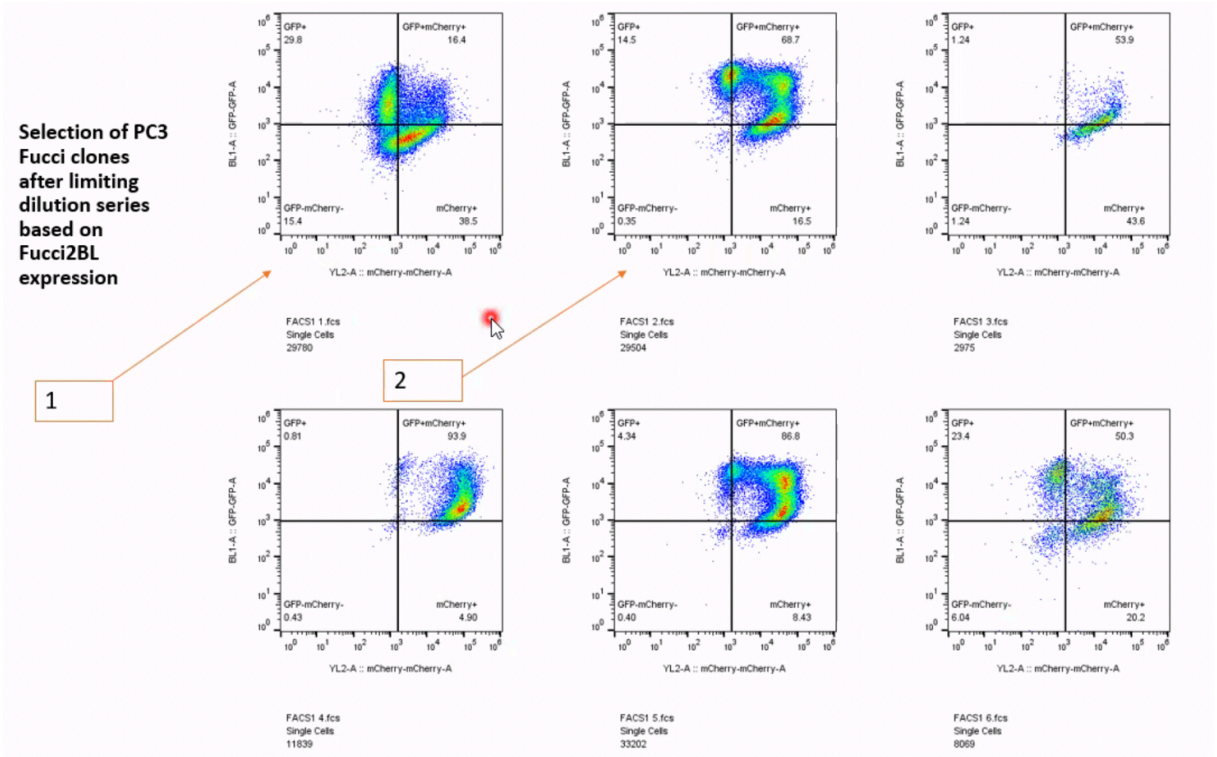


Figure 3A. Treatment with the prostate cancer chemotherapy drug, Docetaxel, resulted in dose-dependent inhibition of proliferation in PC3 cells clone 1, clone 2, and clone 3 in real time cell imaging analysis. After the cells were seeded into the 96 well plate, different condition treatments were added in triplicates. The scans in the Incucyte started about 16 hours after the cells were added into the wells. The images were taken every two-hour s for a total of 154 hours. The percent confluence of each well was generated through the software embedded in the Incucyte. The graphs show the percent confluence of the wells as time advanced in all 96 wells.

	Docetaxel	3. 30, 300 nM										
	1	2	3	4	5	6	7	8	9	10	11	12
A	Fucci-1	untreat.		ROR1KO	-1	untreat.	Fucci-1	vehicle		ROR1KO	-1	vehicle
B	Fucci-2			ROR1KO	-2		Fucci-2			ROR1KO	-2	
C	Fucci-3			ROR1KO	-3		Fucci-3			ROR1KO	-3	
D	Fucci-1	Doc 300		ROR1KO	-1	Doc 300	Fucci-1	-1	Doc 30	ROR1KO	-1	Doc 30
E	Fucci-2			ROR1KO	-2		Fucci-2	-2		ROR1KO	-2	
F	Fucci-3			ROR1KO	-3		Fucci-3	-3		ROR1KO	-3	
G	Fucci-1	Doc 3		Fucci-3	Doc 3		ROR1KO	-1	Doc 3	ROR1KO	-3	Doc 3
H	Fucci-2			media			ROR1KO	-2		media		

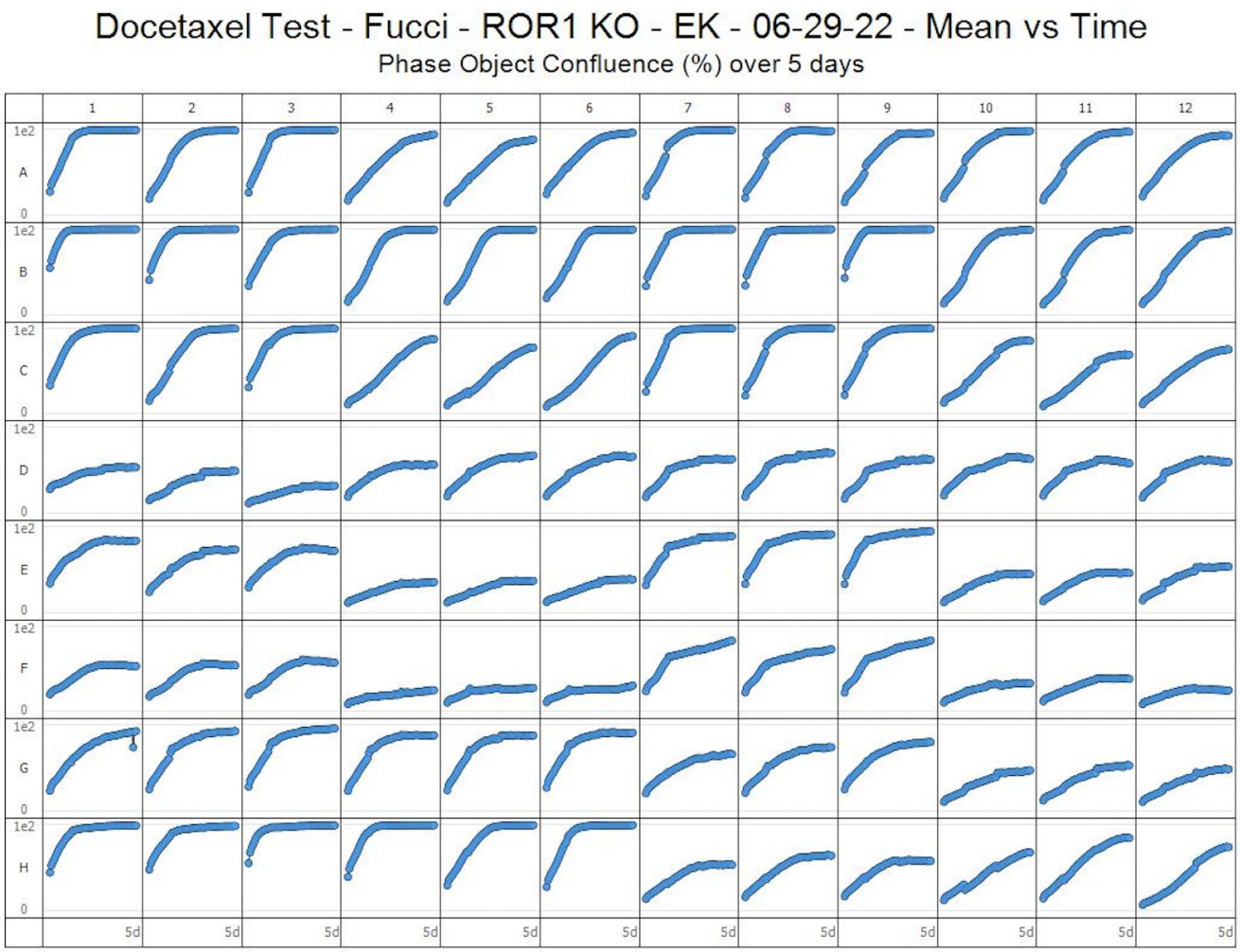


Figure 3B. Treatment with the prostate cancer chemotherapy drug, Docetaxel, resulted in greater dose-dependent inhibition of proliferation in PC3 ROR1KO clones than in PC3 WT clones in real time cell proliferation assays (RTCA). Percent Confluence was determined using Incucyte Real time cell proliferation assay. Growth plateau of PC3 cells vs PC3 ROR1KO when treated with chemotherapy drug, Docetaxel shows greater sensitivity of PC3 ROR1KO cells to Docetaxel treatment. The graphs (left panels) show the percent confluence of the wells as time advanced of PC3 Fucci and the graphs (right panels) shows the change in the percent confluence of PC3 ROR1KO clones 1, 2 and 3 with increasing doses of docetaxel PC3ROR1KO cells were significantly more sensitive to docetaxel induced growth inhibition than PC3 WT ROR1 (IC50 3 nM versus 300 nM, respectively).

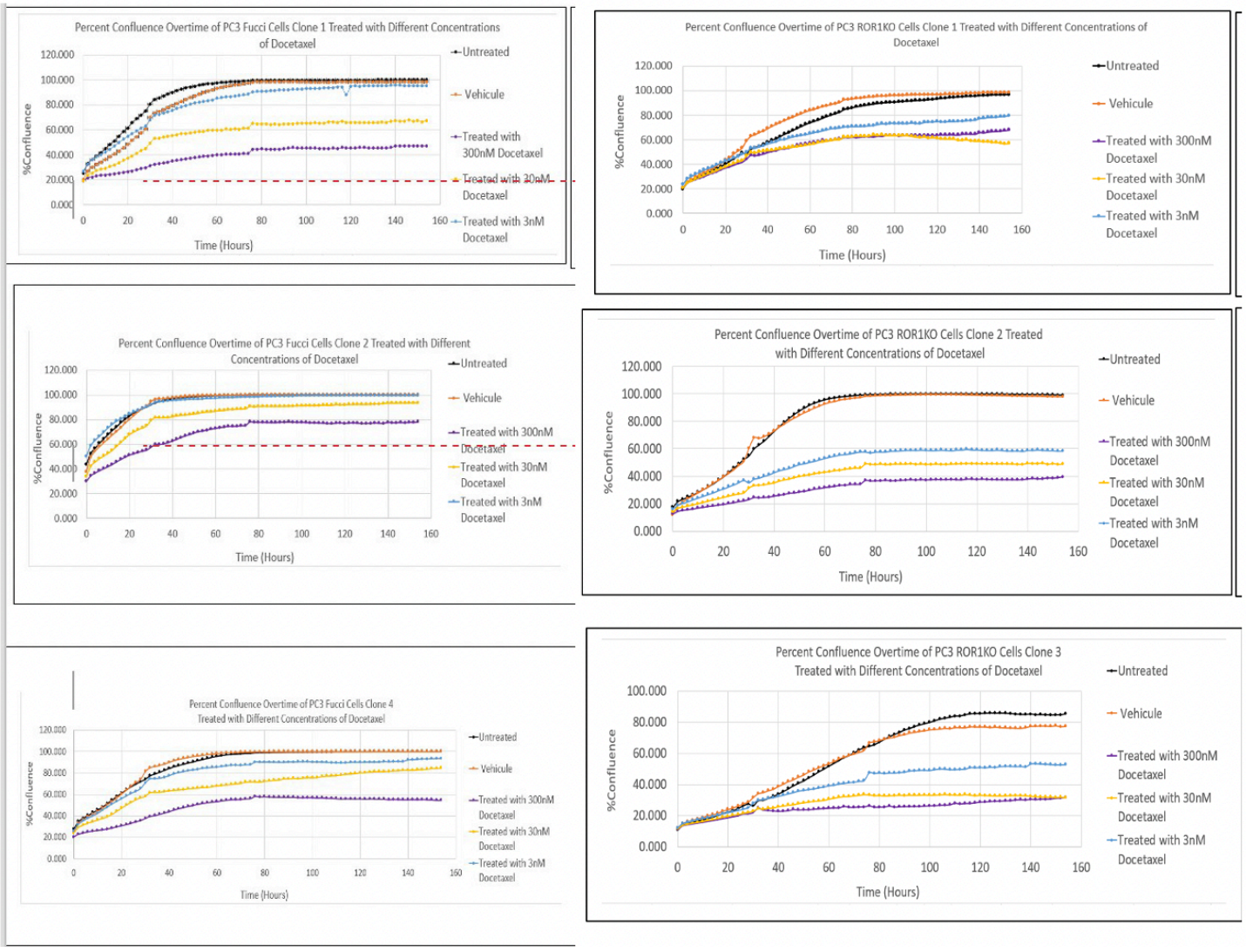
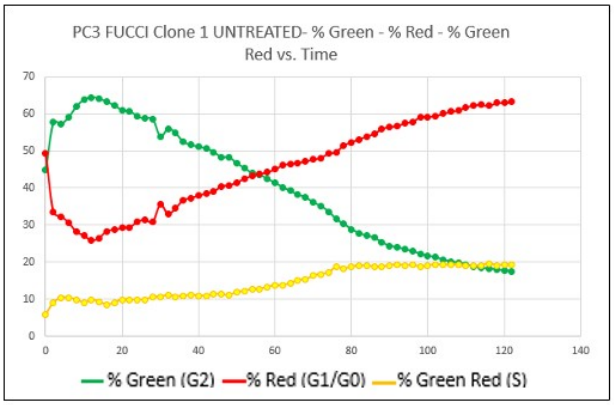
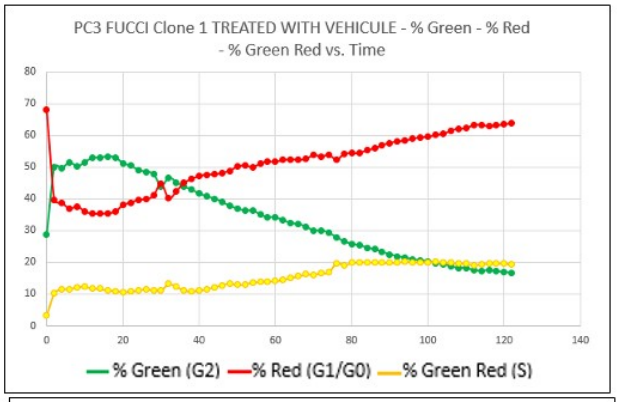
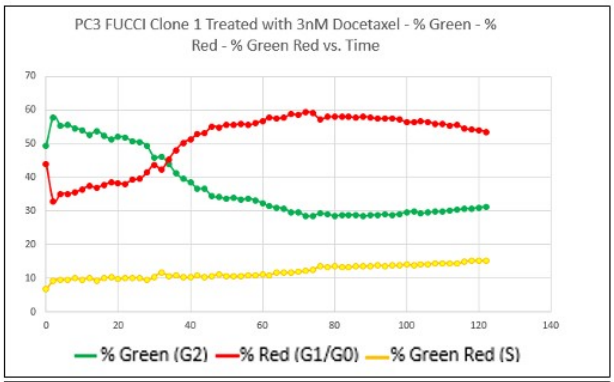
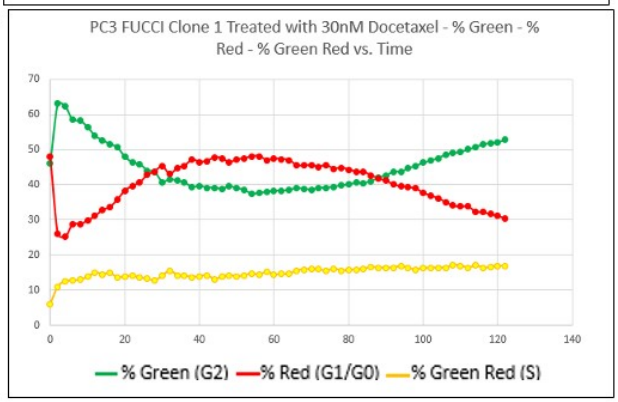


Figure 4. Docetaxel impeded the cell cycle in PC3 Clone 1 cells expressing the Fucci live cell cycle tracking system, resulting in inversion of final G1:G2 ratio to show a G2 arrest. The green (G2), red (G1 or G0), yellow (S, green + red) counts were measured through the software embedded in the Incucyte. From these numbers, the percent green, red, and yellow count were calculated. The graphs show the percent green, red, and yellow count as time advanced and the corresponding cell cycle stage based on the fluorescent label from Fucci2BL Cell Cycle tracker. The images were taken every two hours.









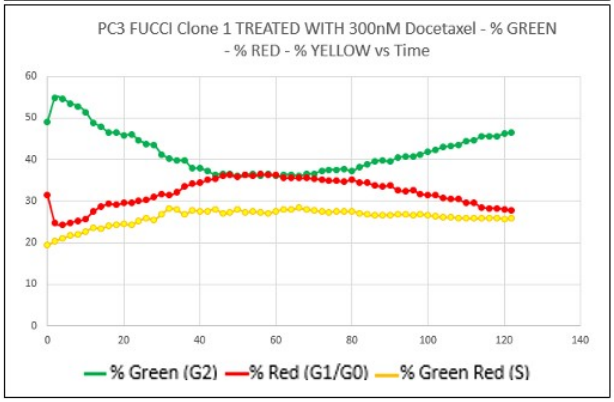


Figure 4A. Docetaxel impeded the cell cycle in PC3 Clone 1 cells expressing the Fucci live cell cycle tracking system resulting in inversion of final G1:G2 ratio.

The green (G2), red (G1 or G0), yellow (S, green + red) counts were measured through the software embedded in the Incucyte. From these numbers, the percent green, red, and yellow count were calculated. The graphs show the percent green, red, and yellow.
count as time advanced. The images were taken every two hours.

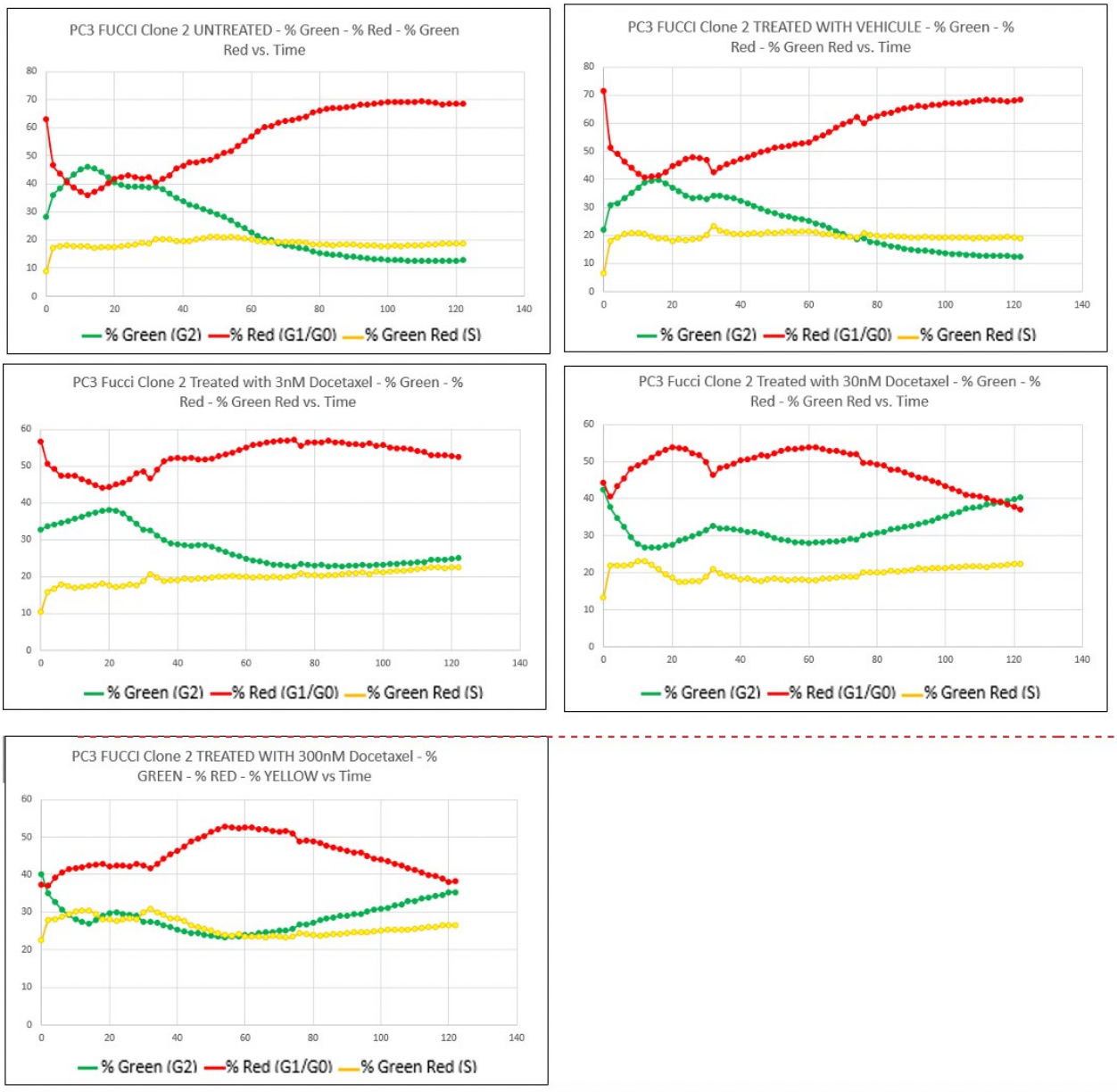


Figure 4B. Docetaxel impeded the cell cycle in PC3 Clone 2 cells expressing the Fucci live cell cycle tracking system resulting in inversion of final G1:G2 ratio.

The green (G2), red (G1 or G0), yellow (S, green + red) counts were measured through the software embedded in the Incucyte. From these numbers, the percent green, red, and yellow count were calculated. The graphs show the percent green, red, and yellow
count as time advanced. The images were taken every two hours.

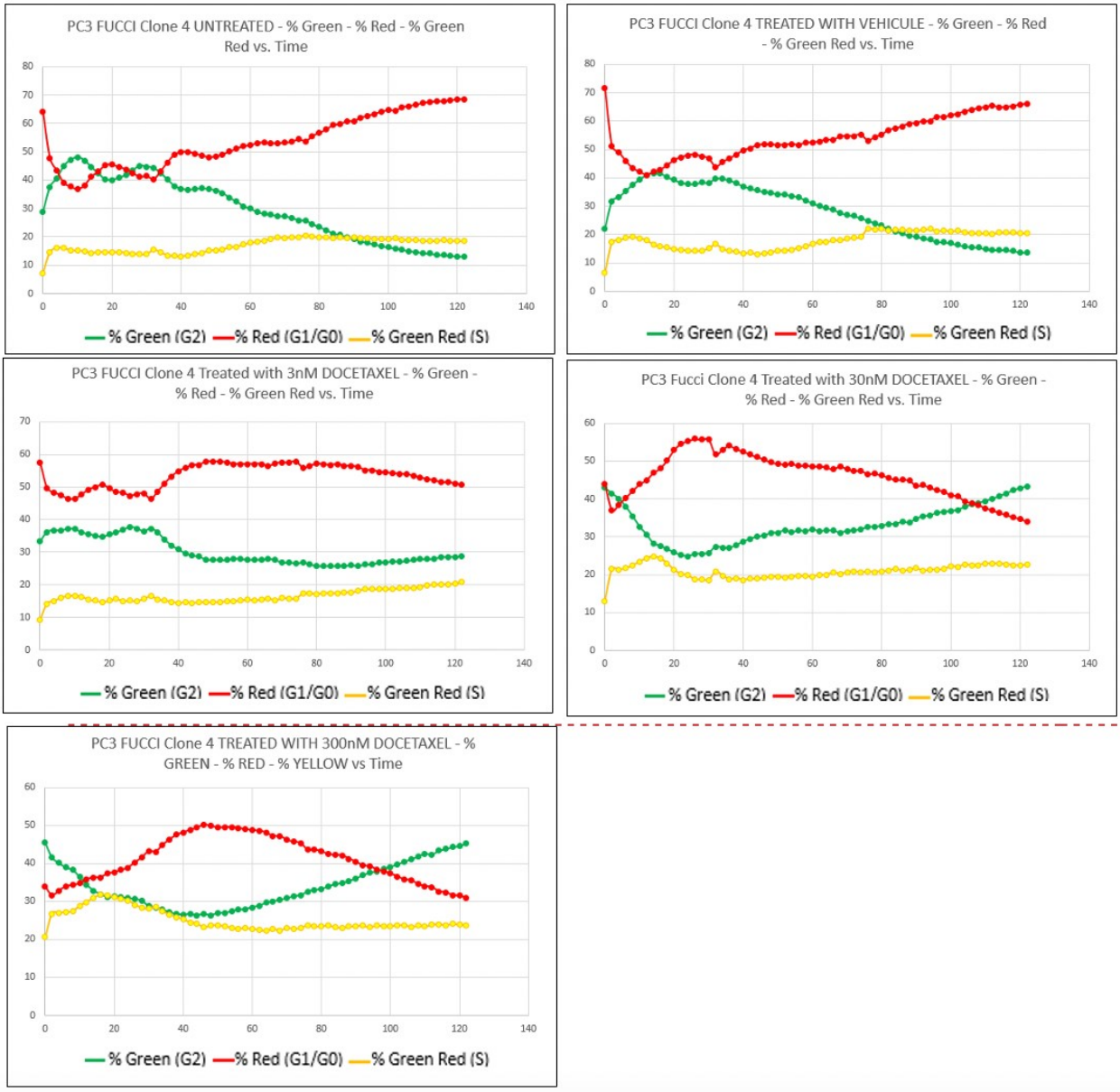


Figure 4C. Docetaxel impeded the cell cycle in PC3 Clone 4 cells expressing the Fucci live cell cycle tracking system resulting in inversion of final G1:G2 ratio.

The green (G2), red (G1 or G0), yellow (S, green + red) counts were measured through the software embedded in the Incucyte. From these numbers, the percent green, red, and yellow count were calculated. The graphs show the percent green, red, and yellow
count as time advanced. The images were taken every two hours.

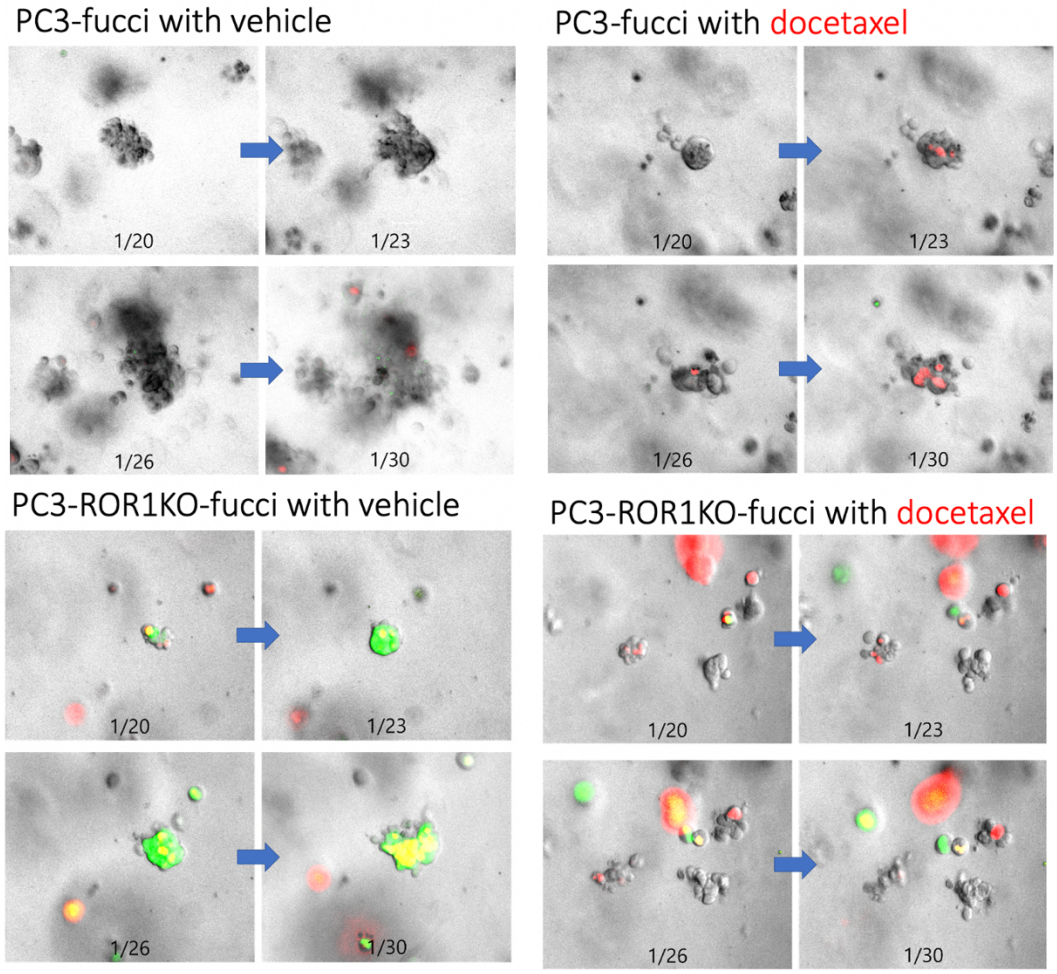
Specific Aim1 Organoids:

Three Dimensional (3D) Organoid Cultures as mini tumor models to test cancer stem cell properties of ROR1 signaling in prostate cancer cell models. Organoid, 3D cell cultures form mini tumors which replicate more closely the growth of prostate cancer cell in patients. We showed in our recent publication (Lee et al 2022 IJMS) that patient derived xenograft organoids from our PDX, PCSD1, treated with the standard of care anti-androgen, enzalutamide, resulted in quiescent, dormant state. Gene expression profiling showed a quiescence cancer stem cell signatures also seen in vivo and, in the patient, (Jamieson et al, manuscript in preparation). Dormancy was reversible and the organoids re-entered the cell cycle when enzalutamide was removed. Thus, we and others have shown that organoids are good models for understanding cancer stem cell pathways involved in therapy resistance, dormancy, and disease recurrence.

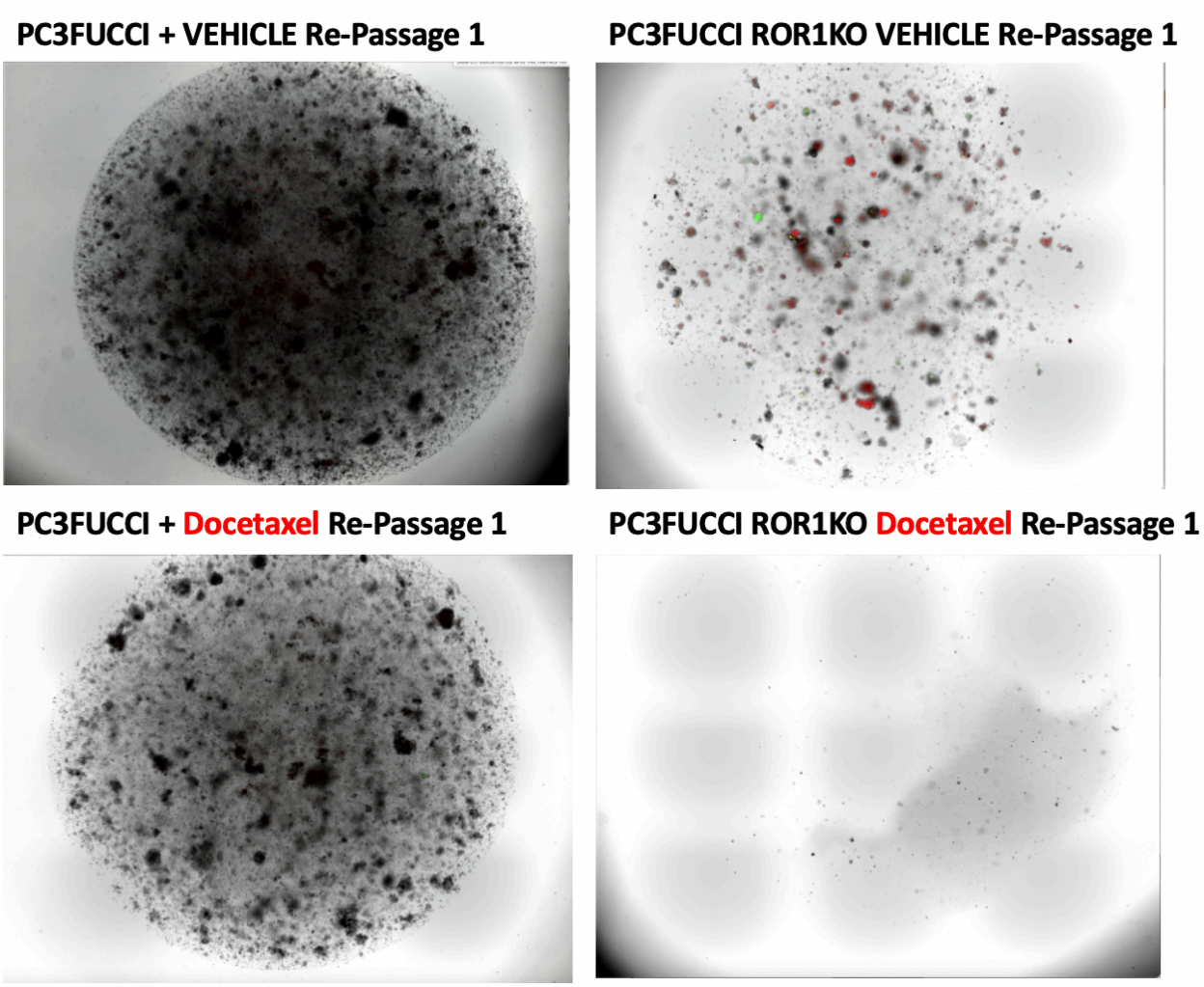
We established 3D organoids using PC3Fucci and PC3ROR1KO Fucci cells treated with docetaxel or vehicle control. Docetaxel (100nM) treatment of established PC3 organoids resulted in decrease in size and number of organoids and an apparent difference in the cell cycle response in PC3Fucci versus PC3ROR1KOFucci organoids. PC3Fucci showed more G1 arrest (red) organoids while PC3ROR1KOFucci showed more proliferating cells in vehicle treated than PC3Fucci and G2 arrest (green) in docetaxel treated organoids (Figure 5A). Passaging of the organoids showed that docetaxel reduced the number of organoids in re-passaged PC3ROR1KO organoids more than PC3 which rely on cancer stem/tumor initiating cells (Figure 5B).

Figure 5 Prostate cancer cell line, PC3, Organoids A. PC3 Fucci and PC3ROR1KO Fucci cells were grown in 3D prostate cancer organoid conditions (Lee et al Jamieson 2020 JoVE). Keyence microscope was used to capture images twice a week. 10X Magnification. B. Organoids in A. were Passaged and re-domed in new Matrigel Re-Passage 1. Cells were maintained in vehicle or 100nM Docetaxel. Stitched Whole domes are shown at one and a half weeks post-re-passaging.

A.



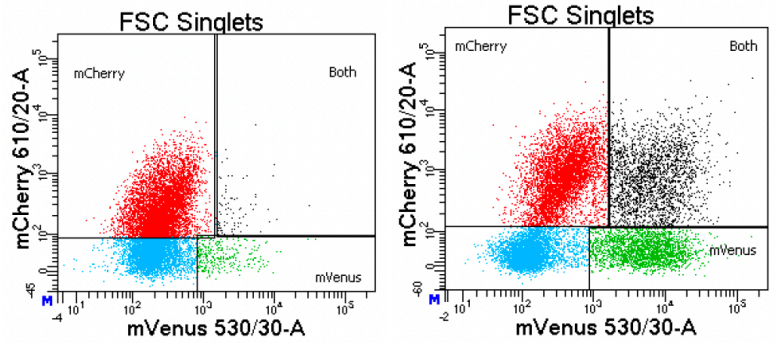
B.



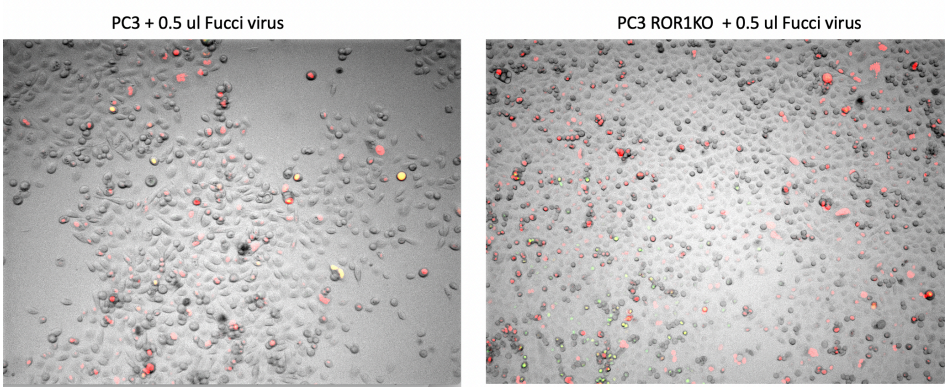
We compared the levels of relative fluorescence intensity (RFI) in the PC3Fucci and PC3ROR1KOFucci using FACS and noticed that the RFI of the PC3Fucci was lower than PC3FucciROR1KO. We made newly transduced PC3Fucci and PC3ROR1KO Fucci cell lines with the same aliquot of Fucci2BL non-replicating lentivirus particles. We analyzed the new cell lines using FACS to select those with matched Fucci fluorescence intensity which we are using for continuing studies of ROR1 signaling in cancer stem cell and cell cycle regulation in 2D Incucyte and 3D organoids. Cells will be FACS sorted on Fucci and ROR1 levels.

Figure 6. Fucci fluorescence levels analysis of PC3 Fucci and PC3ROR1KO Fucci clones by FACS and microscope imaging. A. FACS analysis of Original PC3Fucci clone 1 and PC3ROR1Fucci clone 2E7, B. Microscope imaging and new PC3 and PC3ROR1KO transduced with new Fucci2BL non-replicating lentiviral particles. Cells were plated at 50,000 cells per 96 well then 0, 0.5 ul, 0.75 ul, 1.0 ul and 2.0ul of Fucci2BL virus was added per well in 100ul FK12 media plus 10% FBS and pen-strep. After 72 hours virus containing media was removed and replace with new media. Cells were transferred to 24 well plates the next day. Microscope images were taken on the Keyence microscope. C. Cells were analyzed on the FACS Aria for mCherry fluorescence levels (Y-axis) and mVenus fluorescence levels (X-Axis).

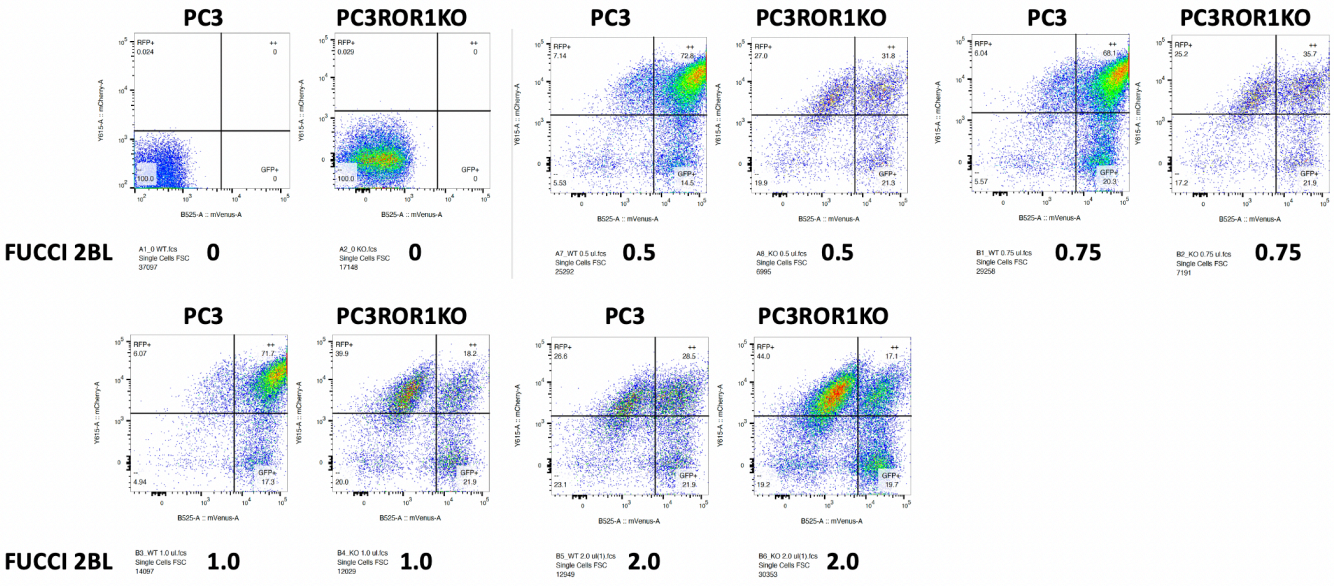
A. PC3Fucci clone1 **PC3ROR1KOFucci clone2E7**



B. NEW PC3Fucci clone1 **NEW PC3ROR1KOFucci clone 2.**



C. NEW PC3Fucci clones and NEW PC3ROR1KOFucci clones



PC3 Fucci cell number optimization in Incucyte Scratch wound migration assay.

Metastatic migration capability of cells can be measured using the scratch wound assay. We will compare the migration capabilities of PC3 Fucci and PC3ROR1KO Fucci cells in addition to their proliferation characteristics. We will perform the Scratch wound assay using the Incuctye and the Incucyte Scratch wound 96 well tool. This will enable us to obtain highly reproducible scratch wounds for quantitative time course measurements and time lapse videos of the cell migration and growth to fill the wound space. We have optimized the PC3Fucci cell numbers for this assay as shown in Figure 7 below.

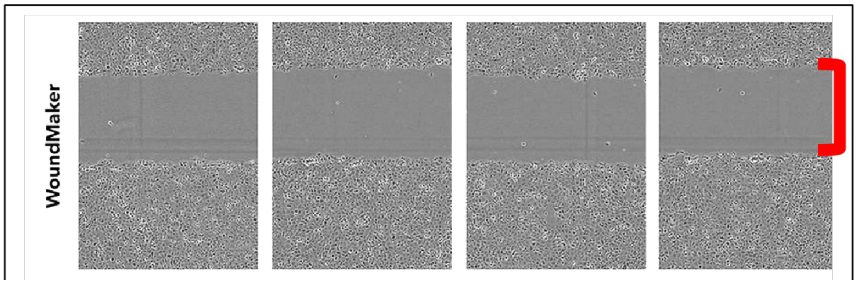
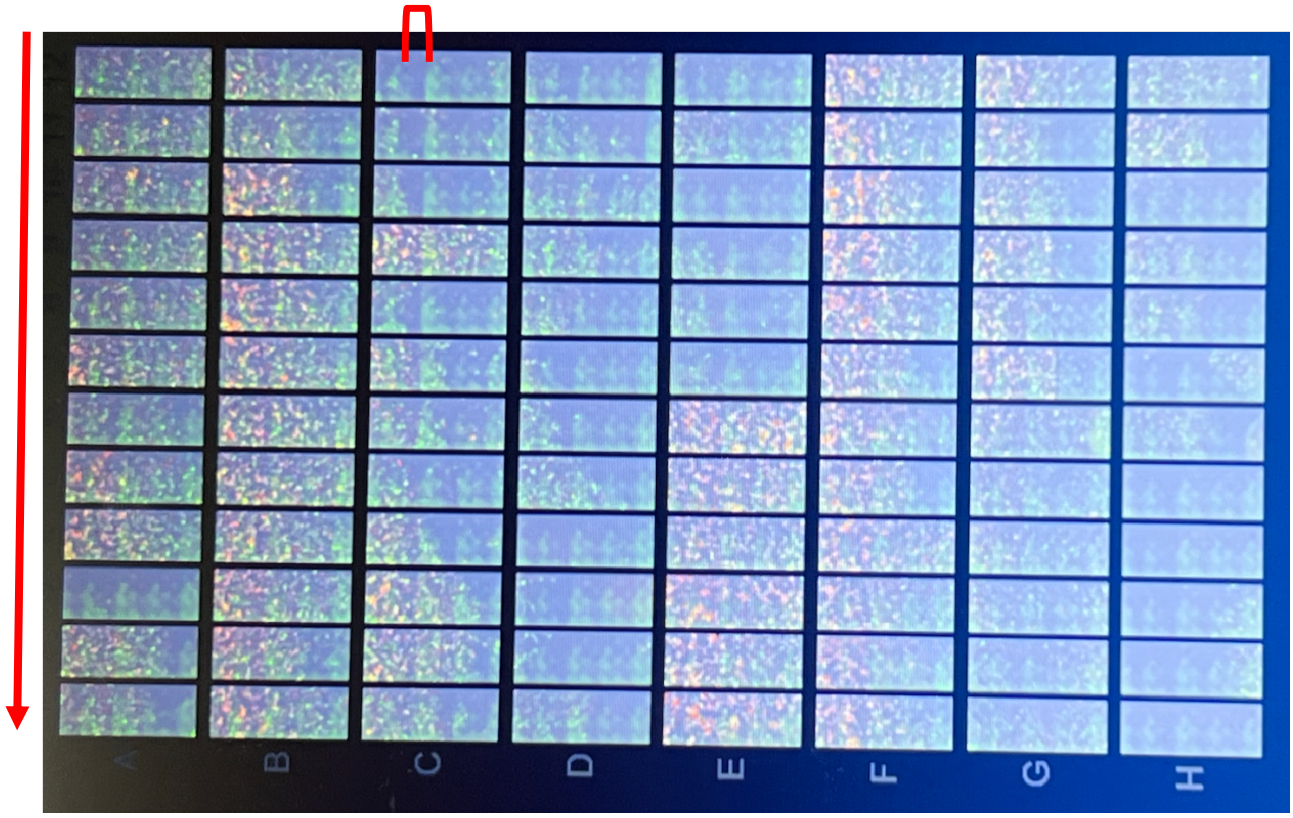


Figure 7. A. The Wound Healing Scratch assay using the 96-well Incucyte Woundmaker Tool: a 96-pin mechanical device designed to create homogeneous scratch wounds in each of the 96 wells simultaneously, rapidly, and reproducibly. The scratch path through the confluent monolayer of cells is shown by the red bracket.

Figure 7B. PC3 Fucci cells clones 1,2 and 4 were plated in triplicate with decreasing number of cells from 100, 000, 50,000, 25,000, 5,000 and 2,500 per well from A1 to D12 and again in E1 to H12. The scratch tool pins made 96 scratches going from top to bottom as shown by the arrow to the left of the image below. This representative image is from a 72-hour time point.



PC3 Xenografts in NSG mice ROR1+ and ROR1- (KO) with anti-ROR1 CART cells

PC3 (Mock) and PC3ROR1KO xenografts were implanted sub-cutaneously in the immunodeficient mouse strain, NOD-SCID-IL2Rcommon gammachain knock out (NSG) male mice. Both grew well and were challenged with 10 million anti-ROR1 CART cells. The dose was not high enough to control the PC3 tumor cells. The xenograft tumors were harvested and are being used to optimize anti-ROR1 Western blots, IHC in formalin fixed paraffin embedded sections (FFPE) as well as for RNAScope in situ hybridization.

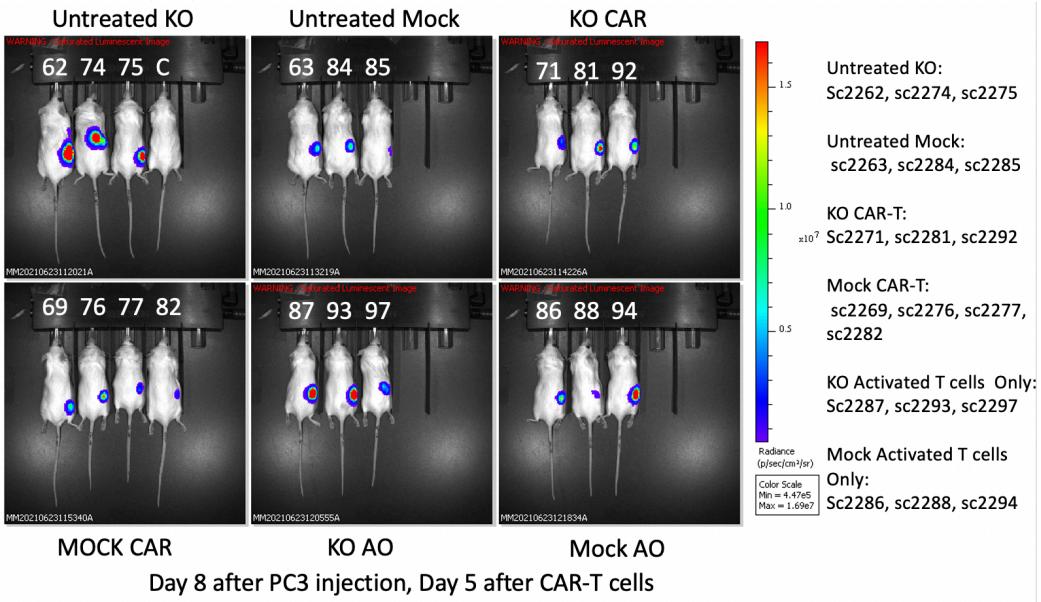
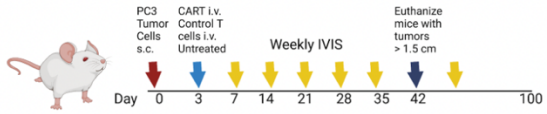


Figure 8. In vivo PC3 RFP Luciferase and PC3ROR1KO RFP Luciferase xenograft tumor growth in vivo. Mice were imaged using the Perkin Elmer Spectrum In vivo bioluminescence imaging system (IVIS).

Cirmtuzumab-based CART cells durably eradicated ROR1+ prostate cancer xenograft tumors.

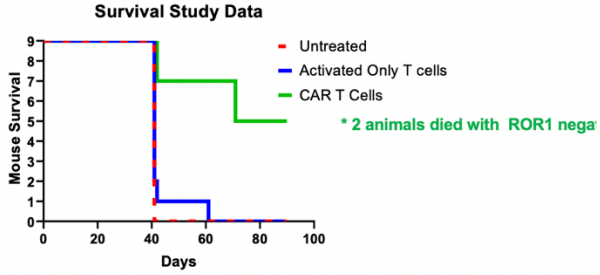
These studies supported the recently launched Phase 1b clinical trial of Zilovertamab plus docetaxel in metastatic CRPC patients. Clinical development of GMP Zilovertamab CART cells for a clinical trial in CLL is in progress and may lead to rapid progression to a clinical trial for metastatic CRPC and NEPC.

Cirmtuzumab CAR-T cells improved survival and completely eliminated tumors in some mice.



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

Fluorescent live cell imaging with Incucyte 53 Live-Cell Analysis System was performed with increasing Effector: Target (E:T) ratios of ROR1 CAR-T cells to PDX PCSD13 cells from patient-derived xenograft model.



Tumor Bioluminescence from individual live mice with PC3 xenografts

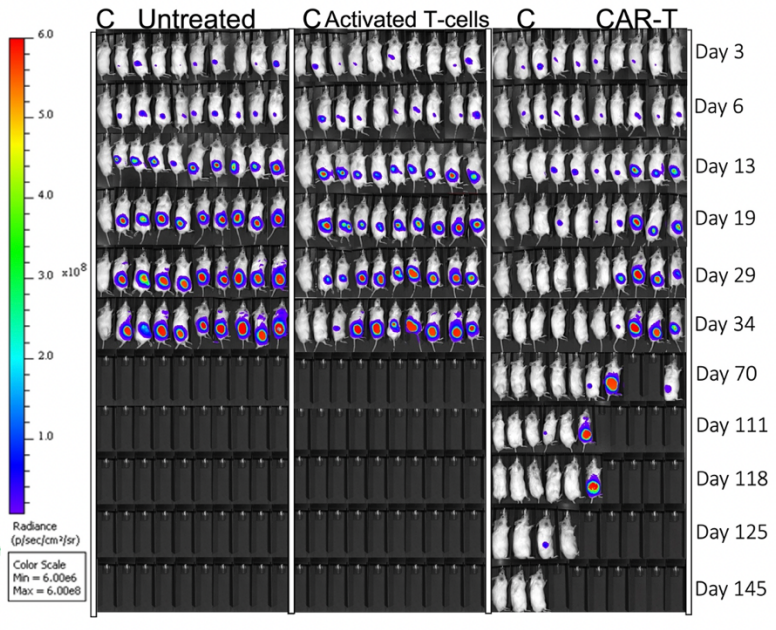


Figure 9. Mice with PC3 xenografts injected with cirmtuzumab anti-ROR1 CAR-T cells showed durable, tumor eradication. Tumor ablation by CART cells occurred in 67% of mice compared to 22% of mice injected with activated T cell only (AO) from the same donor and 0% of untreated mice. Only the PC3 tumor engrafted mice which received anti-ROR1 CART survived past 145 days.

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. Prostate cancer metastasizes preferentially to bone leading to painful, bone destructive lesions and malignant disease progression for which there is no cure. The complexity and heterogeneity of prostate cancer bone metastases make them extremely challenging to treat and treatments with new mechanisms of action are urgently needed. We developed new patient-derived xenograft (PDX) models using surgical patient prostate cancer bone metastases which were implanted into immunodeficient mice. The resulting xenograft tumors replicated the tumor heterogeneity and bone lesions seen in patients. We are using our patient-derived xenograft models and prostate cancer cell line models to determine the mechanism of action of a new therapeutic target: the WNT5A/ROR1 signaling pathway in prostate cancer. A therapeutic ROR1 inhibitor antibody, Cirmtuzumab, has been developed and clinically tested in CLL and metastatic breast cancer patients thus translation to the clinic for mCRPC can be rapid.

In Year 3, we made significant progress in **AIM 1** in elucidating the mechanism of action of the WNT5A/ROR1 using our models of castration resistant small cell PCa and neuroendocrine PCa (NEPC) models in which ROR1 is expressed at high levels. To study the mechanisms of WNT5A/ROR1 signaling in CRPC we generated CRISPR/Cas9 ROR1 knock out cell lines in PC3, DU145 and LNCaP in which we tested responsiveness to combination therapy of ROR1 inhibition plus the chemotherapy drug, docetaxel, the standard of care for metastatic CRPC. We showed in Incucyte real time cell growth assays that PC3 cells with the ROR1 knock out (KO) were significantly more sensitive to docetaxel than PC3 with ROR1. In **AIM 2**, We used our PDX models to test a new therapeutic target: the WNT5A/ROR1 cancer stem cell signaling pathway using the ROR1 inhibitory antibody, Cirmtuzumab. Flow cytometry showed heterogeneous expression of ROR1 in the PDX tumor cells as was seen in the patient. In this study the effect of Cirmtuzumab on PDX tumor growth was compared to docetaxel, the standard-of-care chemotherapy drug for these patients. Neither Cirmtuzumab alone nor docetaxel alone lessened PDX tumor growth. However, the combination of Cirmtuzumab plus docetaxel did inhibit tumor growth. In AIM 3, Single cell RNA sequencing of the cryopreserved tumor cells and frozen tissues from this study will allow us to distinguish the molecular phenotypes of the responding and non-responding tumor cell types in the four treatment groups: 1. Vehicle control, 2. Cirmtuzumab alone, 3. Docetaxel alone, 4. Cirmtuzumab + Docetaxel, as well as the effects on the cells of the tumor microenvironment. In **AIM 3**, we performed single cell RNA sequencing of our PDX PCSD1 cells and are optimizing single live cell enrichment protocols with our cryopreserved PDX PCSD13 tumors from the Cirmtuzumab plus Docetaxel experiment. The results of this single cell RNASeq analysis will enable future scRNASeq analysis of the surgical prostate cancer bone metastasis specimens in our biobank. We obtained an IRB amendment to an existing IRB protocol and have collected archival NEPC specimens for testing ROR1 expression in immunohistochemistry (IHC) and RNAScope assays to detect ROR1.

An alternative immunotherapy approach for targeting WNT5A/ROR1 in mCRPC we have also pursued is 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. Anti-ROR1 Cirmtuzumab-based CART cells durably eradicate PC3 xenograft tumors in vivo more than 140 days. Taken together the results of the studies in **AIMs 1 &2** lead us to the **hypothesis** that inhibition of ROR1 signaling altered the cell cycle and inhibited the cancer stem cell state of the tumor cells which sensitized them to docetaxel. We anticipate the results of scRNASeq analysis in **AIM 3** of this study may reveal the genes and pathways involved in sensitizing the tumor to chemotherapy. Understanding the mechanism of action will enable us to target the appropriate patient population and combination therapies with this novel treatment.

These pre-clinical studies performed in this grant led to FDA approval of an IND amendment to support the now active Phase 1B clinical trial (not part of this grant) for translation of anti-ROR1 Cirmtuzumab-based CART cells into clinical trials to treat neuroendocrine PCa and CRPC. 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.

The clinical trial has opened and is now recruiting patients (**NCT05156905)**:
Co-Investigator, A Phase 1B, Nonrandomized Trial Investigating Docetaxel Combined with Cirmtuzumab in Patients with Metastatic Castration Resistant Prostate Cancer. **PI: Investigator: Dr. Rana Mckay.**

We are performed 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. 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. Continuing these studies to elucidate the mechanism of action of inhibiting the WNT5A/ROR1 pathway in our models of bone metastatic prostate cancer will allow us to use this novel therapy more effectively in the best patient population and in the most effective combination with other therapies.

5. CHANGES/PROBLEMS/ALTERNATIVE APPROACHES

Due to COVID-related negative impacts on our research we were approved for a no cost extension to continue this work for a fourth year.

Problem: Matrigel shortage delayed organoids and PDX studies by several months.
Solution: 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 as shown above in AIM 1 results.

Problem: Due to COVID-related staff shortages and high turnover in our animal care program there was an outbreak of *C. bovis* in our ABSL2 room in which we house our PDX mice.
Solution: We had to halt our in vivo PDX mouse experiments until the facility was de-contaminated. Studies using established NEPC cell lines were performed to establish parameters and conditions for follow up experiments using our PDXs. The PDX experiments are expected to resume at the end of March 2023.

Problem: Experienced lab personnel, namely, Lab manager, Michelle Muldong, and Project Scientist, Sanghee Lee, took positions in biotechnology companies at the end of fall 2021 as we were ramping back to fully in-person lab work.
Solutions: I became fully active in the lab and took over the research projects. I trained two junior graduate students and the undergraduate students who had been doing remote lab research to do in-person wet lab research. Hiring replacement technicians took six months but I now have two SRAs doing fully in-person research. We were joined in August 2022 by three Visiting Scholar Urologists from South Korea whom I trained in the organoid methods. We are now back to full productivity.

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(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).

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).

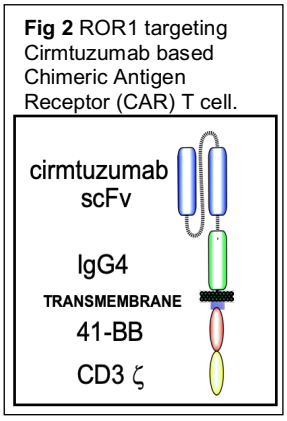


Fig 2 ROR1 targeting Cirmtuzumab based Chimeric Antigen Receptor (CAR) T cell.

7. 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 2022 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. Performing bioinformatics analysis of serial patient PCa bone metastasis specimens and their PDX. Analysis includes copy number variation, whole exome sequencing and transcriptome analysis to show congruence of the PDX model and tumor metastasis evolution. Will perform bulk and single cell RNA sequencing analyses of the PC3 and PC3ROR1 KO +/- docetaxel, and the PDX, PCSD13, Cirmtuzumab plus Docetaxel in vivo experiments.

Dr. Nicholas A. Cacalano, PhD, Associate Professor, Dept of Radiation Oncology, UCLA, is performing the protein level analysis of PCSD13 ROR1 enriched cells, and PCa cell line ROR1Kos. His lab is performing ROR1 IHC and ROR1 and ROR1 downstream signaling using Western blotting on PCa cell lines, organoids and PDXs. Dr. Cacalano is co-author on our manuscript (Lee et al. IJMS) which is now in published.

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. Submitted IND-amendment to the FDA which was approved.
The clinical trial has been opened and is now recruiting patients: A Phase 1B, Nonrandomized Trial Investigating Docetaxel Combined with Cirmtuzumab in Patients with Metastatic Castration Resistant Prostate Cancer. PI: Dr. Rana Mckay, MD, Co-PI: Christina Jamieson, PhD.

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.

8. Special Reporting Requirements N/A