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13. **SUPPLEMENTARY NOTES**

14. **ABSTRACT**
The main objective of this DOD-supported project is to identify and develop novel therapeutics to target the undifferentiated (PSA-), castration-resistant PCSCs. We proposed to achieve this objective with two Specific Aims: 1) To perform phage display library (PDL) screening in PSA- PCa cells to identify PCSC-specific homing peptides; and 2) To perform unbiased drug library screening to identify novel PCSC-targeting chemicals.

In the past year, we have made good progress in accomplishing the goals for both Aims. For Specific Aim 1, we have further characterized the JRM2 peptide. For Specific Aim 2, we have finished a targeted library screening to identify several compounds that could sensitize the AR-PSA-LNCaP cells. We have also started screening our UNIQUE PSA- CRPC cells against a Protein Kinase Inhibitor Collection that contains >750 compounds that target >140 kinases. In the second year, we shall continue several experiments in both Aims, especially testing the in vitro and in vivo cytotoxocities of the conjugated JRM2 peptide and finishing up screening and validating the kinase inhibitor library screening.

15. **SUBJECT TERMS**
Castration-resistance prostate cancer; cancer stem cells; differentiation; phage display library; drug screening

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1. INTRODUCTION:
The main goal of this project is to identify and develop novel therapeutics to target the PSA-/-LO undifferentiated, and castration-resistant prostate cancer stem cells (PCSCs). We initially proposed two Specific Aims:
1) To perform phage display library (PDL) screening in PSA-/-LO PCa cells to identify PCSC-specific homing peptides; and
2) To perform unbiased drug library screening to identify novel PCSC-targeting chemicals.

2. KEYWORDS:
Prostate cancer; stem cells; cancer stem cells; prostate cancer stem cells

3. ACCOMPLISHMENTS:

Major Goals of the Project for the first year: 1) To better characterize the JRM2 peptide; 2) to establish a PSA-/-LO PCa cell system that allows high throughput drug screening; and 3) to perform pilot screening in a targeted drug library.

What was accomplished under these goals:
A. Identification of JRM2 peptide from the PSA-/-LO LNCaP cell phase display screening.
We first performed PDL (phage display library) screening in LNCaP cells infected with the PSAP-GFP lentiviral reporter (Figure 1). This vector allows the separation of the minor PSA-/-LO (i.e., GFP-) LNCaP cells to be separated from the bulk PSA+ (GFP+) cells (Qin et al., Cell Stem Cell, 2012; Liu X, et al., Oncotarget, 2015). We started by incubating 2 million of LNCaP/PSAP-GFP cells with a mixed (1:1) CX7C (7mer peptide library) and CX8C (8mer library) library at 4x10^12 peptides/million cells (Figure 1, A-B). After a 2-h incubation, LNCaP/PSAP-GFP cells were subject to FACS and 63,424 GFP+ and 32,388 GFP- LNCaP cells were purified out and then lysed to infect bacteria K91, from which 960 and 704 tet/kan-resistant bacterial colonies were generated from GFP+ and GFP- cells, respectively (Figure 1B). The GFP- (i.e., PSA-/-LO) colonies were expanded to generate a new phase library, which was used to infect a new batch of the LNCaP/PSAP-GFP cells (Figure 1B; Round 2). This process was reiterated two more rounds to Round 4 (Figure 1B). With each round we obtained more colonies (Figure 1B) and observed increasing transforming units (TU) per cell (Figure 1C). At Round 3, we observed a higher differential TU/cell between GFP+ and GFP- cell derived bacteriophages (Figure 1C); therefore, we sequenced 287 colonies each from the GFP- and GFP+ populations (Figure 1B). Of the 576 colonies sequenced, 554 sequences were readable of which 3 sequences (YEWDYLF, VEYDAMEL, LEFDLMLV) were dominant and
nearly equally represented in GFP⁺ and GFP⁻ colonies (Figure 1D). 12 GFP⁻ ONLY sequences were obtained and one sequence, GFYVGQR, was recovered twice and named JRM2.

Figure 1. Identification of JRM2 peptide.
A. Experimental scheme for PSA⁻/⁻lo PCa cell specific PDL screening strategy. B. Overall screening results with resultant colonies indicated. C. Transforming units (TU) with each round of screening. D. Peptide sequence results.

B. JRM2 peptide preferentially binds to the PSA⁻/⁻lo PCa cells.
Interestingly, a BLAST search shows that the forward sequence of JRM2 (GFYVGQR) has significant similarity to, among others, several isoforms of EGFR substrate 15-like 1, NOTCH-1 preprotein and protocadhrin Fat 1 precursor whereas the reverse sequence (RQGVYFG) shares significant identity with IL-13 receptor subunit alpha-1 precursor and some other proteins (Figure 2A; data not shown). To determine whether JRM2 can preferentially bind to the PSA⁻/⁻lo LNCaP cells, we made several versions of JRM2 conjugates. The FIRST is biotinylated JRM2 (JRM2-biotin). When we incubated freshly purified GFP⁺ and GFP⁻/⁻lo LNCaP cells with JRM2-biotin followed by incubation with streptavidin-Alexafluor 594, we observed significantly higher binding in the PSA⁻/⁻lo cells under fluorescence microscopy (Figure 2B-C).
Figure 2. JRM2 peptide preferentially binds to the PSA\(^{-/lo}\) LNCaP cells.
A. BLAST search results. B. IF images showing JRM2-biotin binding to GFP\(^{-/lo}\) LNCaP cells. C. Quantification of IF results in B. D. Flow histograms showing preferential binding of JRM2 to GFP\(^{-/lo}\) cells. E. Preferential binding of JRM2-AMC to PSA\(^{-/lo}\) (i.e., GFP\(^{-/lo}\)) LNCaP cells. F. Confocal analysis of JRM2-LR binding to PSA\(^{-/lo}\) LNCaP cells.
Flow cytometric analysis also showed preferential binding of JRM2-biotin to GFP-/lo (i.e. PSA-/lo) LNCaP cells in comparison to GFP+ (i.e. PSA+) cells (23.6% vs. 5.2%; Figure 2D). In contrast, the control peptide JRM0 with a sequence of GMAVGKWK that was uncovered from GFP+ and GFP-/lo colonies with equal frequencies indeed showed similar binding to the two subpopulations of LNCaP cells (Figure 2D; left). SECOND, we also labeled JRM2 with aminocoumarin (AMC) and flow analysis showed that JRM-AMC showed significantly higher binding to the PSA-/lo LNCaP cells (Figure 2E). FINALLY, we made JRM2-lasmamine-rhodamine conjugate and confocal microscopy analysis demonstrated preferential binding of JRM2 to the PSA-/lo LNCaP cells (Figure 2F). Dose studies using 0, 25, 50, 100, and 200 nM of JRM-AMC revealed a dose-dependent increase in the peptide binding only PSA-/lo LNCaP cells (data not shown). Interestingly, JRM2-AMC also exhibited dose-dependent increase in binding to the AR-PSA- PC3 cells (not shown).

C. JRM2 peptide becomes internalized in PSA-/lo PCa cells.
In order for JRM2 to become a potential therapeutic against the PSA-/lo PCSCs, the peptide must be internalized. We sought to address this question by incubating freshly purified PSA+ (GFP+) (Figure 3) and PSA-/lo (GFP-) (Figure 4) LNCaP cells with JRM2-AMC for different time points followed by confocal microscopy analysis. Cells were identified by the red fluorescent dye Syto-85. As shown in Figure 3, there was hardly any binding of JRM2-AMC to the GFP+ LNCaP cells during the 15 min – 4 h incubation period. In contrast, there was obvious binding of JRM2-AMC to the PSA-/lo LNCaP cells as early as 15 min (Figure 4). Importantly, bound JRM2-AMC became internalized as evidenced by the accumulation of AMC signals inside the cells including nuclei (Figure 4). For unknown reasons, we noticed some cytotoxicity of JRM2-AMC (Figure 5). These observations suggest that JRM2 peptide can become internalized into the PCa cells.
D. **Longitudinally tracking the responses of LNCaP cells to three regimens of castration.**

In order for us to be able to longitudinally track the responses of the two subpopulations of PCa cells to castration, we employed a lentiviral-based reporter system in which a PSA promoter (PSAP) drives the expression GFP or RFP (Figure 5A). LNCaP cells regularly cultured in 7% FBS-containing medium and infected with the PSAP-GFP lentiviral reporter contained 5.39 ± 3.18% \((n = 12)\) GFP\(^{-/lo}\) cells (i.e., bottom 6-10% GFP\(^{-/lo}\) population on FACS) (Figure 5B). Freshly purified GFP\(^{-/lo}\) LNCaP cells expressed little AR or its targets PSA and FKBP5, analogous to the AR\(^{-}\)PSA\(^{-}\) PCa cell line, PC3 (Figure 5C). In contrast, the corresponding GFP\(^{+}\) cells (e.g., top 5-10% of GFP-bright cells on FACS) expressed all three proteins (Figure 5C). Neither cell population expressed glucocorticoid receptor (GR) (Figure 5C). As the PSAP-GFP lentiviral system faithfully reports endogenous PSA expression, in foregoing experiments, we often interchangeably use GFP\(^{+}/\)GFP\(^{-/lo}\) and PSA\(^{+}/\)PSA\(^{-/lo}\).

**Figure 4. JRM2 binds to and becomes internalized in the PSA\(^{-/lo}\) LNCaP cells.**

Freshly purified PSA\(^{-/lo}\) (GFP\(^{-/lo}\)) LNCaP cells plated on glass coverslips overnight were incubated with Syto-85 (a general cell labeling dye) or JRM2-AMC (100 nM) for 15’, 1 h, or 4 h. At the end, cells were analyzed by confocal microscopy.
We infected LNCaP cells with the PSAP-GFP at an multiplicity of infection (MOI) of 25, at which virtually all cells were infected, as evidenced by GFP positivity (Figure 5C) and PCR detection of the GFP sequence in genomic DNA of randomly picked clones (data not shown). We then treated the infected LNCaP cells with 3 regimens of castration: charcoal dextran-stripped serum (CDSS), CDSS with bicalutamide (10 mM), and MDV3100 (Enzalutamide, 10 mM) continuously for up to ~2 years (Figure 5D), which resulted in the long-term castration-resistant LNCaP sublines that we termed LNCaP-CRPC cells, i.e., LNCaP-CDSS, LNCaP-CDSS+Bicalutamide [LNCaP-CB], and LNCaP-MDV.

We first characterized the overall growth kinetics of the LNCaP-CRPC sublines (Figure 5E-F). As shown in Figure 5E, infected but untreated parental or “wild-type” (wt) LNCaP-GFP cells...
exhibited steady increases in cumulative population doublings (PDs). The 3 treated LNCaP cell types all grew slower in the beginning and hit a “bump” or “crisis” point around 2-3 weeks when there was little net increase in PDs (Figure 5E; asterisks). Then the treated cells began to grow with a steady increase in PDs, although at slower paces than the untreated LNCaP-GFP cells (Figure 5E). Indeed, after 3 months of treatment, all three LNCaP-CRPC lines showed much lower end-point live cell numbers (Figure 5F, top), suggesting that they were less proliferative and/or more susceptible to cell death. Interestingly, however, at ~4 months (125 days), there was a noticeably significant increase in the growth kinetics in all 3 LNCaP-CRPC sublines, which displayed very aggressive growth patterns thereafter (Figure 5E). In support, all 3 LNCaP-CRPC cultures continuously treated for 10 or 17 months showed significantly more live cell numbers compared to the time-matched control LNCaP-GFP cells (Figure 5F).

We further characterized LNCaP-GFP and LNCaP-MDV cells at crisis point (3 weeks) and found that MDV3100 treatment led to both increased cell-cycle arrest (Figure 5G) and cell death (Figure 5H). Specifically, more LNCaP-MDV cells remained in the G1 phase compared to LNCaP-GFP cells (87.1% versus 67.7%) and less were at the G2/M phase (27% versus 10.5%) (Figure 5G). With respect to cell death, ~90% untreated LNCaP-GFP cells were viable; in contrast, only 42% LNCaP-MDV cells treated for 3 weeks were viable with 31% of cells being apoptotic and 24% being necrotic (Figure 5H).

E. Chronic castration led to homogenous PSA^–/lo cells.

We next monitored dynamic changes in GFP^+ cells in chronically castrated LNCaP cells (Figure 6). Because castration blocks AR signaling and thus should shut down PSA expression, we predicted that the three regimens of castration should lead to gradual reduction in GFP^+ (i.e., PSA^+ ) cells. Indeed, as early as 1 week after treatment, there was, on average, a ~5-10% decrease in GFP^+ population in all 3 conditions (Figure 6B). By 4-5 weeks, there were 15-25% decreases in GFP^+ cells with concomitant increases in GFP^–/lo (PSA^–/lo ) cells, with MDV3100 showing the strongest effect (Figure 6A-B). The 1-month LNCaP-CRPC cultures displayed much lower cell densities than untreated LNCaP-GFP cells (Figure 6A). By 2 months, the GFP^+ population dropped to ~70% in all 3 LNCaP-CRPC cultures and at 5-7 months the GFP^+ population dramatically decreased (Figure 6, A and C). By 9

Figure 6. Time-dependent decrease in PSA^+ cells in response to castration.
A. Representative phase and GFP images of LNCaP and 3 types of LNCaP-CRPC cells treated for 1, 2, 5, and 9 months. B. Quantification of GFP^+ percentage in short-term treated LNCaP cells. C. Quantification of GFP^+ % in long-term treated LNCaP cells.
months, there were barely detectable GFP*+ cells in the 3 LNCaP-CRPC sublines (Figure 6, A and C). In contrast, untreated LNCaP-GFP cells remained mostly GFP*+ over the 9-month period (Figure 6).

F. Chronic castration led to loss of AR and PSA and other molecular changes. Accomplicying the loss of PSA*+ cells, quantitative RT-PCR analysis revealed a time-dependent decrease in AR and PSA mRNA levels in the three LNCaP-CRPC cultures (Figure 7A). Western blotting showed that at 3 weeks of treatment, the LNCaP-CRPC cells expressed AR protein and its 4 targets, i.e., PSA, FKBP5, PAP (prostate alkaline phosphatase), and PSMA (prostate-specific membrane protein) (Figure 7B). Interestingly, the 3-week treated cultures showed several lower AR bands that might represent the AR splice variants though none of these bands represented AR-V7 since an AR-V7 specific antibody (Figure 7C) failed to detect any products in the LNCaP-CRPC cultures (Figure 7B). Remarkably, at 9-26 weeks, all 3 LNCaP-CRPC lines lost AR and PSA protein expression and also showed decreased expression of other AR targets (Figure 7B). Interestingly, the LNCaP-abl, a castration-resistant LNCaP subline very commonly used to study resistance mechanisms, were AR+/PSA- (Figure 7B).

In addition to the molecules in the AR signaling axis, we also examined changes in several molecules related to castration resistance (BCL2 and SOX9), epithelial-mesenchymal transition or EMT (E-cadherin, SLUG, and vimentin), and CSCs (i.e., CD44, integrin a2b1, and ABCG2) (Figure 7B). Flow cytometry revealed time-dependent increases in cells expressing high levels of 3 CSC markers in LNCaP-CRPC cultures (data not shown), consistent with the notion that castration enriches for stem-like cancer cells. All other molecules showed variegated changes (Figure 7B). For example, BCL2, an anti-apoptotic molecule shown previously to be upregulated during castration, actually exhibited slight decreases in our LNCaP-CRPC cells (Figure 7B). SOX9, a stem cell molecule recently reported to be regulated by AR, showed rapid downregulation in 3-week castrated LNCaP cells and then slightly increased in long-term LNCaP-CRPC cells (Figure 7B). Castration has been linked to EMT. However, our LNCaP-CRPC cells showed decreased E-cadherin and subtle changes in SLUG and vimentin (Figure 7B).
N-cadherin was not expressed in parental LNCaP cells nor was it induced in LNCaP-CRPC cells (data not shown). These results suggest that castration resistance in LNCaP cells in vitro is not associated with apparent EMT.

**G. The long-term LNCaP-CRPC cells are completely refractory to further castration but are partially sensitive to anti-BCL2 and several kinase inhibitors.** The many molecular changes observed above in addition to alterations in AR signaling pathway suggest that the long-term LNCaP-CRPC cells, which bear many phenotypic properties of CSCs such as being PSA-/- and overexpressing CSC molecules including CD44, a2b1, and ABCG2, would be refractory to ‘therapeutic’ treatments other than antiandrogens. To test this suggestion, we treated 5-mo LNCaP-GFP and the 3 LNCaP-CRPC cell types, for 72 h, with a ‘candidate library’ of 15 compounds that included: 2 antiandrogens (MDV3100 and Bicalutamide), 2 chemotherapeutic drugs (docetaxel and etoposide), 2 plant-derived experimental drugs (Avicin and Oxetane), a telomerase inhibitor (Imetalstat, also called GRN163L), Metformin (an antidiabetic drug shown to inhibit CSCs), selective BCL-2 inhibitor ABT-199, 2 epigenetic inhibitors, i.e., 5-Aza-2’-deoxycytidine (Aza, an inhibitor of DNA methyltransferase) and trichostatin A (TSA, a histone deacetylase inhibitor), and 4 inhibitors of signaling pathways, i.e., XAV-939 that inhibits Wnt/b-catenin; SB431542 that inhibits TGFBR1; SU 5402 that inhibits VEGFR1 and FGFR1; and AEW541 that inhibits the IGF-1R (Figure 8). We used H2O2 as a control, which non-selectively

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**Figure 8. Differential sensitivity of LNCaP-CRPC and LNCaP-abl cells to compounds in the targeted library screening.**

Presented is the relative cell survival (%), as determined by AlmarBlue assay, in LNCaP-GFP (LNCaP; 5-mo), LNCaP-CRPC (5-mo), and LNCaP-abl cells when exposed to 15 compounds in the candidate library for 72 h. Compound concentrations were selected based on the reported IC50 values. The response patterns were classified into 4 categories.
killed all cell types at $\geq 5$ mM, and also compared with the drug sensitivities of LNCaP-abl cells (Figure 8).

This ‘targeted’ drug screening effort revealed that the 5-mo LNCaP-CRPC cells that lacked AR and PSA expression, within 72 h, were resistant to antiandrogens, chemotherapeutic drugs (docetaxel, etoposide, Avicin, and Oxetane), and TSA (Figure 8A-B), non-responsive to Imetastat, Metformin, Aza, and XAV-939 (Figure 8C) but sensitive to ABT-199, SB431512, SU 5402, and AEW541 (Figure 8D). Specifically, MDV3100 dose-dependently inhibited LNCaP-GFP cells but the LNCaP-CRPC cells showed resistance to even 200 mM of MDV3100 (Figure 8A). Bicalutamide, known to be less potent that MDV3100 in antagonizing AR, did not affect LNCaP-GFP or LNCaP-CRPC cells, even at 200 $\mu$M. Interestingly, LNCaP-CRPC cells demonstrated most prominent resistance to docetaxel, Avicin, Oxetane, and TSA compared to LNCaP-GFP cells (Figure 8A-B). On the other hand, both LNCaP-GFP and LNCaP-CRPC cells did not appreciably respond to Imetalstat, metformin and Aza and both showed only slight response to XAV-939 (Figure 8C), suggesting that either these inhibitors were ineffective or needed $>72$ h to manifest effects. Importantly, however, the 5-mo LNCaP-CRPC cells, like LNCaP-GFP cells, responded, in a dose-dependent manner, to ABT-199 and 3 kinase inhibitors, i.e., SB431542, SU 5402, and AEW541 (Figure 8D), implicating potentially critical roles of Bcl-2 and TGFBR1, VEGFR1/FGFR1, and IGF-1R signaling in the survival of LNCaP-CRPC cells.

Interestingly, LNCaP-abl cells, which were AR$^+$PSA$^-$, showed both similar and different responses compared to the 5-mo LNCaP-CRPC (AR$^+$PSA$^-$) cells and to LNCaP-GFP (AR$^+$PSA$^+$) cells (Figure 8) as well. For example, LNCaP-abl cells exhibited a dose-dependent sensitivity to both MDV-3100 and Bicalutamide (Figure 8A). LNCaP-abl cells also demonstrated exquisite sensitivity to docetaxel and Avicin (Figure 8A-B). On the other hand, LNCaP-abl cells were reproducibly more resistant to 10 mM of ABT-199 (Figure 8A; data not shown). These results highlight differences in various castration-resistant LNCaP sublines such as observed here between our LNCaP-CRPC vs. LNCaP-abl cells.

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
1) We are currently testing whether JRM2 peptide can bind the PSA-/lo PCa cells in vivo;
2) The JRM2 peptide has been conjugated to the (KLAKLAK)$_2$ ‘killer’ peptide, which will be used to test it specific cytotoxicity to the PSA-/lo PCa cells in vitro and in vivo;
3) We are also attempting the PDL screening in vivo in a reporter tumor model (LAPC9);
4) We have started characterizing the 3 new peptides, JRM 3-5, that were uncovered in similar PDL screenings in the LNCaP system.
5) To perform candidate ‘targeted’ library screening in 10.5 month LNCaP-CRPC cells;
6) To further study the effects of AEW541 on prostate cancer cells;
7) To perform candidate library screening in patient tumor-derived cells;
8) To perform medium-throughput screening against a collection of kinase inhibitors.
4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
For the first time, we have generated AR-PSA- LNCaP cell sublines that are amenable for high-throughput drug library screening. Importantly, we have identified JRM2 peptide, and, potentially several other peptides, that can specifically bind to the PSA-\textsuperscript{lo} PCa cells. These new developments should greatly facilitate our functional characterizations of the CSC-enriched PSA-\textsuperscript{lo} cells as well as develop novel inhibitors and therapeutics that can target these cells. This should in turn greatly impact the clinical management of prostate cancer patients.

What was the impact on other disciplines?
The basic principles utilized herein to study PCa cell subpopulation dynamics and to develop novel therapeutics targeting the undifferentiated CSC populations should be readily applicable to other tumor systems.

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:
Nothing to Report

6. PRODUCTS:
The current project intersects with several other projects in the lab, all of which have a common goal, i.e., to dissect PCa cell heterogeneity and to elucidate the role of different subpopulations of PCa stem/progenitor cells in tumor initiation, maintenance, progression, drug resistance, and metastasis. The following published manuscripts have cited the partial support of the DOD grant.


7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

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<th>Kiera Rycaj</th>
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<th>Name:</th>
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Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:
N/A

9. APPENDICES:
N/A