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TITLE: Discovery of Prostate Cancer Tumor Suppressors and Mediators of MDV3100 Resistance through in Vivo RNA Interference Screen

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 14. ABSTRACT We set out to identify factors that mediate resistance to enzalutamide therapy using a customized shRNA screen in in vivo mice models. During the reporting time (8 months), we had collected and made pools of the required shRNA, mostly of genes that are recurrently deleted in prostate cancer. However, testing of complexity screens using empty vector with random sequences showed that >50% the xenografted tumors to be made of only a few hairpins. The stochastic nature of distribution of cells forming the tumor would confound findings at later stages when we have actual hairpins. Thus, in vitro FACS-based screen was setup to identify factors that lead to formation of NEPC during prolonged enzalutamide treatment. This was carried out in collaboration with Dr. Mark Rubin and Dr. Himisha Beltran at Weill Cornell Medical Center. We were able to devise a construct that would fluoresce as the cell converted towards the NEPC phenotype using a Neuron Specific enolase promoter driven GFP. During my move, we were able to isolate cells in various stages of neuroendocrine differentiation. 							
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The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. INTRODUCTION: Prostate cancer is the second-leading cause of cancer-related deaths in American men. Castration and radical prostectomy comprise the primary line of curative treatment for primary tumors. The metastatic tumors are treated with androgen-lowering agents and androgen-receptor inhibitors. However these develop resistance to lack of androgens and are referred to as castration resistant prostate cancer (CRPC). Our lab identified that reactivation of androgen-receptor (AR) signaling as one way of developing CRPC. This fueled the development of a better androgen-receptor antagonist MDV3100 from the lab. However, only 70% of the patients respond to MDV3100, half of which develop resistance within 2 years due to existing and/or acquired genetic alterations within the tumor. In order to identify these specific drivers of prostate cancer, the lab has profiled transcriptome and copy number alteration (CNA) data from 218 tumors. From the regions of frequent deletions and/or loss of expression, we have identified a core of \sim 800 genes. We propose to generate a custom shRNA library targeting these 800 genes. and carry out an in vivo pooled-shRNA screen using the castrate resistant, but MDV3100 sensitive LNCaP/AR cells (LNCaP overexpressing AR), xenografted in castrated mice. The screen will not only help identify tumor suppressors and biomarkers of castrate resistance, but also identify genes conferring castration resistance, Figure 1. This would provide novel avenues for therapeutic interventions.



Figure 1. Pooled shRNA screen in mouse hepatic cancer model. Enrichment of two *Pten* shRNAs in selected tumors (right) compared to their representation in preinjection plasmid pools (left). Pie graphs show the representation of each *Pten* shRNA in the total shRNA population analyzed by high-throughput sequencing. (From Zender et al, Cell 2008)

2. **KEYWORDS:** Prostate cancer, CRPC, NEPC, ShRNA screen, RNAi screen, Enzalutamide, MDV3100, FACS, neuroendocrine prostate cancer,

3. ACCOMPLISHMENTS:

- What were the major goals of the project?
 - Prostate-cancer deletome-specific shRNA library identification and preparation
 - LNCaP derived tumor complexity determination for pool-size optimization

- In vivo screen for tumor suppressors and MDV3100 resistance conferring factors
- Identification of the mechanism of MDV3100 resistance
- What was accomplished under these goals?

1. Prostate-cancer deletome-specific shRNA library identification and preparation

- a. The identification of all the \sim 800 genes recurrently deleted in PCa was completed which included genes primarily in the regions of blue of Figure 2.
- b. The shRNAs targeting these 800 genes were handpicked. We ensured at least 4 hairpins per gene. A total of ~3200 constructs were accrued.
- c. Pools of random 50 hairpins were cloned and validated using MiSeq into SFFV-GIPZ vector.



Figure 2: Copy number alterations (CNAs) in the prostate cancer oncogenome. Significantly amplified regions (red) and deleted regions (blue) with a false discovery rate of <10% are displayed. The chromosome is depicted within the green bars in the center with their centromeres marked as red. Known genes of interest within the altered region are marked black and those targeted by only CNAs marked gray. (From Taylor et al, Cancer cell, 2010)

2. LNCaP derived tumor complexity determination for pool-size optimization

a. The complexity of the LNCaP-AR xenografted tumor was determined. For this LNCaP_AR cells were infected with a 10 million complex library using a lentivirus system that ensured single integrant per cell as validated using FACS. The cells were puromycin selected and injected into both castrated and non-castrated mice. When tumor developed, DNA from them was isolated and complexity of the library determined using NGS. Figure 3 shows the complexity of the library in cell and that determined from tumors. The tumors showed ~20,000 complex library whereas in the cells it was 120,000. This was well within the realm of 50-hairpin pool.



Figure 3. The diversity of the LNCaP/AR tumors. The plots show the complexity of the preinjected and tumor (large 780mm³ and small 263mm³) samples as determined by next generation sequencing. Total unique sequence counts for pre-injection=120,000, Castrated small=19000, Castrated large=21000.

We

b. Poor distribution of clones in the tumors that are formed during xenograft experiments. Analysis of the distribution of clones within the 120,000 complexity is quite good for the LNCaP-AR cells. However, when the same cells form tumor, though they have ~20,000 individual cells (determined by tag, 1 for each cell), the distribution was very heterogenous; less than 10 cells formed the bulk of the tumor, Figure 4. The stochastic distribution of the tags is a major concern. How would one identify actual enrichment of driver hairpin? It might require expansion of the experiment (already at 360 mice) and extensive validations to meet statistical requirements.



Figure 4. Pie chart shows the relative abundance of the tags identified from two xenografted tumors. Each tag represents around 1 cell and except for red pie, colors do not represent each tag. Ideally, there should have been an equal distribution of the tags, but two representative tumors show that few cells form the bulk of the tumor. Furthermore, the most abundant clones do not match within each tumor. The most abundant clone within the cells (C) is not the most abundant within one tumor (B) or 6th abundant (red pie, C).

decided to carry out the experiment in vitro with emphasis on selection of MDV resistant clones that might develop neuroendocrine prostate cancer (NEPC) phenotype, in collaboration with Mark Rubin and Himisha Beltran at Weill Cornell Medical Center. This was based on the hypothesis that prolonged treatment of LNCaP cells would lead to appearance of neuroendocrine positive population. Upto 30% of patients who die of CRPC have NEPC. The rationale governing the in vitro screen was based on the fact that markers of NEPC (Neuron Specific

Enolase (NSE), Synaptophysin, etc) would start to appear as the LNCaP differentiated towards the NEPC phenotype. Analogously, expressing GFP using the NSE promoter would show increased GFP expression as the cell differentiated into the NE phenotype.



Figure 5: Rational of the FACS-based screen. Left, the concept of increase of NSE and decrease of PSMA with NED. Right, the quadrants of a hypothetical FACS-plot after Enzalutamide treatment from which cells with different characteristics will be acquired for analysis.

Loss of AR dependency is associated with increase in Prostate specific membrane antigen (PSMA) expression. Thus, we also decided to sort for cells that have decreased PSMA expression with prolonged Enzalutamide treatment. Using the above markers and FACS we attempted to sort out 4 populations of cells with prolonged Enz treatment:

Of these, we were primarily interested in AR-dependent and AR-independent NEPC phenotype and identify factors that drive their differentiation using genomic and expression based studies.

Calibration and acquisition of the various cell populations:

NSE-GFP: the construct was introduced into LNCaP cells using lentivirus. Half of the cells under the bell-shaped curve formed after FACS analysis was collected and recultured for 2 more weeks. This was repeated to get very high GFP expressing cells, till there was almost plateauing of the GFP signals, Figure 6.



Figure 6: Isolation and subculturing of NSE-driven GFP population of cells over 2 months.

Similarly, populations of PSMA positive populations were also isolated, figure 7:



Figure 7: FACS based isolation of PSMA positive and negative population, which are NSE-GFP positive.

Each of the above fractions was then cultured independently for characterization.

c. What opportunities for training and professional development has the project provided?

The training provided opportunity to learn in depth about pertinent questions related to prostate cancer. It helped me in interacting with

people who have contributed enormously towards this field. Finally, it helped me in getting a job.

d. How were the results disseminated to communities of interest?

- i. "Nothing to Report." Negative results were not published from the in vivo study. Study is still ongoing for the FACS based screen.
- e. What do you plan to do during the next reporting period to accomplish the goals? i. "Nothing to Report."

4. IMPACT

f. What was the impact on the development of the principal discipline(s) of the project?

A viable in vivo RNAi (shRNA-based) screen cannot be carried out using LNCaP.

- g. What was the impact on other disciplines?
 - i. "Nothing to Report."
- h. What was the impact on technology transfer? i. "Nothing to Report."
- i. What was the impact on society beyond science and technology? i. "Nothing to Report."

5. CHANGES/PROBLEMS:

• Changes in approach and reasons for change

The screen was altered from in vivo mouse-based to in vitro FACS-based. Though the final outcome would be to identify factors mediating enzalutamide resistance.

- Actual or anticipated problems or delays and actions or plans to resolve them
- Changes that had a significant impact on expenditures
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
- Significant changes in use or care of human subjects
- Significant changes in use or care of vertebrate animals.
- Significant changes in use of biohazards and/or select agents
- 6. **PRODUCTS**:
 - Publications, conference papers, and presentations
 - Journal publications.
 - Books or other non-periodical, one-time publications.
 - Other publications, conference papers, and presentations.

Yadav K.K., Phil Watson, Ling Cai, Rohit Bose, Kenneth Chang, Simon Knott, Stephanie Shaw, Minna Balbas, Vivek Arora, Yu Chen, Taslima Ishmael, Silvia Fenoglio, Amy Valentine, Xin Zhou, Christine Peterson, YoungJoo Yang, Krista Marran, Gregory J Hannon, Charles L Sawyers. *Discovery of prostate cancer tumor suppressors and treatment biomarkers through rna interference screens*, STARR consortium retreat, CSHL, NY

- Website(s) or other Internet site(s)
- Technologies or techniques
- Inventions, patent applications, and/or licenses
- Other Products

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?
- 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
 - COLLABORATIVE AWARDS:
 - QUAD CHARTS:
- 9. **APPENDICES:**