AWARD NUMBER: W81XWH-16-1-0246

TITLE: Effects of Phthalates on Androgen Receptor Regulation Associated with Castration-Resistant Prostate Cancer Development

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REPORT DATE: July 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2019	Annual	30 June 2018 to 29 June 2019

4. TITLE AND SUBTITLE

Effects of Phthalates on Androgen Receptor Regulation Associated with Castration-Resistant Prostate Cancer Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0246

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Androgen deprivation therapy is initially effective for most prostate cancer patients, but it recurs after several years and becomes resistant, defined as castration-resistant prostate cancer (CRPC). The molecular mechanisms of the transition to CRPC are poorly understood. The androgen receptor (AR) is known to play a key role in the prostate cancer progression. Numerous mechanisms were introduced to show how CRPC continues to grow through AR signaling. Current CRPC therapies are mostly aimed to either inhibit AR expression or block androgen biosynthesis based on those findings. However, there is considerable population of CRPC patients that are typically ignored, showing a loss of AR in the tumor. For this group of patients, current treatments are ineffective. However, little research is conducted to investigate the pathways that are associated with the AR negative CRPC development. Therefore, the overall goal is to elucidate molecular mechanisms involved in the CRPC development specifically in the AR negative cell population. Nowadays, high attention is given to the effects of environmental toxicants on biological systems because we are all exposed to these environmental factors for decades and many studies have shown association of environmental toxicant exposure with adverse health outcomes. Phthalates are widely used in plastics and many everyday products. However, very little is known of their impacts on PRCA progression. The proposed project will investigate the effects of phthalates on AR regulation at molecular levels and determine its role in the CRPC development. This project will use PRCA models both in vitro and in vivo. PRCA xenografts will be implanted into mice and assessed for PRCA-aggressiveness. Additionally, AR levels and their epigenetic alterations will be measured.

15. SUBJECT TERMS
None listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Prostate cancer is one of the leading causes of death for men in the United States. Initially, prostate cancer patients are treated with androgen deprivation therapy. This treatment is usually successful at first; however, prostate cancer can recur years later as castration-resistant prostate cancer (CRPC). CRPC is much more aggressive and is frequently lethal. Despite the immense clinical significance of CRPC, little is known about the molecular mechanisms that cause prostate cancer to become castration-resistant. A subset of patients with CRPC lack expression of the androgen receptor (AR) in the tumor; for these patients, androgen deprivation therapy will not be effective. However, very little is known about how a tumor can become AR-negative. Environmental toxicants, such as phthalates, small molecules found in many plastics, often function as hormone mimetics and may contribute to the development of prostate cancer. These studies are focused on identifying a relationship between phthalate exposure, loss of AR expression, and the development of CRPC. We are investigating two potential mechanisms that may affect AR expression in this context: silencing of AR mRNA expression through DMNT1-mediated methylation, and translational control of AR protein mediated by the RNA helicase DDX3.

2. KEYWORDS:

phthalates, di-butyl phthalate (DBP), castration-resistant prostate cancer (CRPC), androgen receptor (AR), steroidogenesis, translational control

3. ACCOMPLISHMENTS:

3.1. Major Goals

Major Task 3: Determine the relationship between AR mRNA expression and AR protein expression in the prostate. (Months 1-12, 70% completed).
The work during this reporting period has been focused on the regulation of AR mRNA and protein expression in the prostate, specifically during CRPC in response to phthalate treatment. Previously reported work showed that phthalate treatment reduces expression of AR mRNA and AR protein in the prostate. We are exploring two possible mechanisms by which this might happen: silencing of AR mRNA expression through methylation at the promoter, and failure to properly translate AR protein, mediated by DDX3.

3.2. Accomplishments under the goals

Subtask 2: Measurement of the methylation pattern on the AR promotor region in phthalate-treated cells through the methylated DNA immunoprecipitation (MeDIP) assay and analysis of the effect of DDX3 expression levels on AR mRNA and protein expression.

Protein extracts were obtained from mouse prostate tissue (dorsolateral prostate and anterior prostate lobes) of mice treated with a cholesterol pellet and mice treated with an MBP-containing

pellet. An immunoprecipitation was performed using the MeDIP assay, as previously published [1,2]. qPCR was then used to assess for the levels of methylation at the AR promoter. Our preliminary studies did not show a significant increase in methylation at the AR promoter in MBP-treated cells compared to cholesterol controls, suggesting that phthalate treatment may not have a significant effect on DNA methylation at the AR promoter (Figure 1).

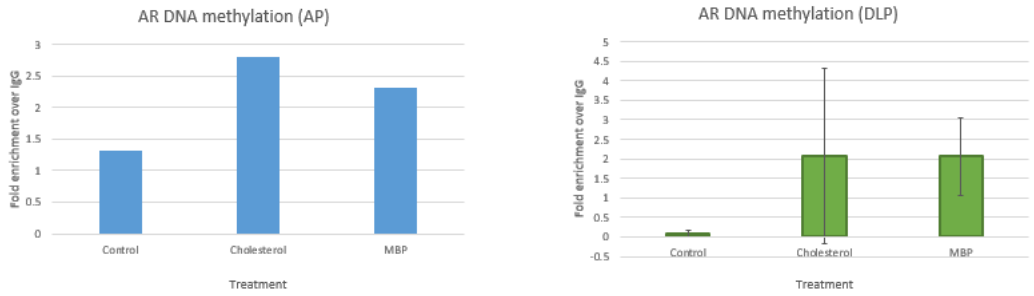

Figure 1. MeDIP assay to measure methylation at the AR promoter in response to phthalate treatment. An immunoprecipitation for methylated DNA was performed, and qPCR was used to measure methylation at the AR promoter. Control represents IgG control, cholesterol is a vehicle control, and MBP is the treatment sample. No significant enrichment of DNA methylation was detected in MBP-treated cells compared to cholesterol controls in either the anterior prostate (AP) or dorsolateral prostate (DLP).

We also analyzed the effects of DDX3 expression on AR mRNA and AR protein expression in CRPC tissues and cell lines. Pharmacological inhibition of DDX3 using the small molecule inhibitor RK33 significantly reduces expression levels of DDX3, and also significantly increases expression of AR (Figure 2). This data suggests that DDX3 an inverse relationship between DDX3 expression levels and AR expression levels in DDX3. This data also suggests that RK33 treatment might be a new way to alter AR expression levels and restore androgen sensitivity in CRPC.

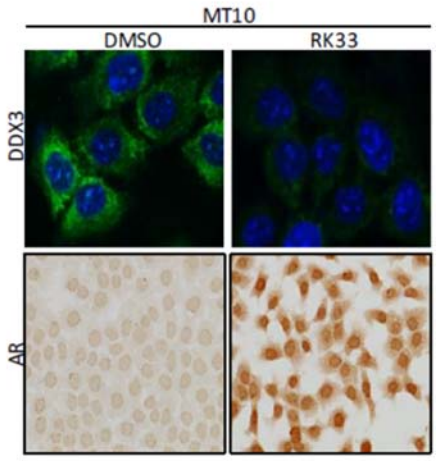

Figure 2. RK33, a small molecule inhibitor of DDX3, decreases DDX3 expression and increases AR expression in CRPC. Immunocytochemistry for DDX3 (green, top panels) and AR (brown, lower panels) was performed on MT10 CRPC cells. Note decrease of

DDX3 and resolution of stress granules, along with increased AR expression with addition of RK33.

Subtask 3: Determine the co-localization pattern of DNMT1/DDX3 and AR in CRPC mice tissue through IHC.

We used a xenograft model to test the localization of DDX3 and AR in CRPC tissues *in vivo*. C42 xenografts (CRPC model) grown *in vivo* for two months showed two distinct populations of cells, consistent with the heterogeneity found in human CRPC (Figure 3). One population had high expression levels of DDX3 and low expression levels of AR, while a second population had low expression levels of DDX3 and high expression levels of AR. This data supports the concept that AR protein expression is inversely related to DDX3 protein expression.

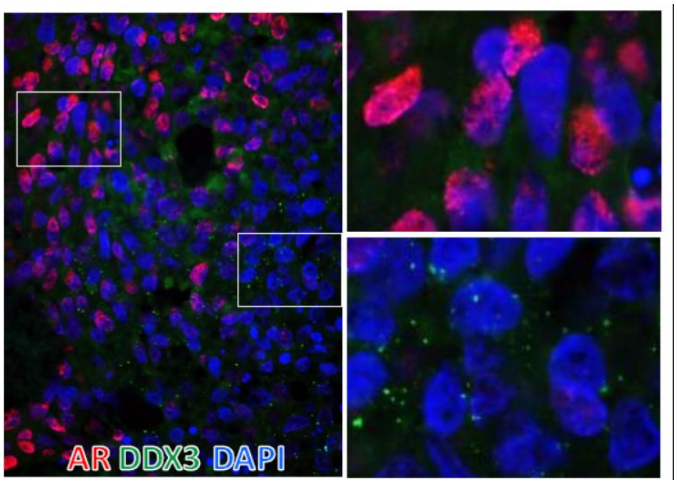

Figure 3. Using dual IHC we co-localized AR (red) and DDX3 (green) protein in FFPE CRPC model C42B xenograft grown *in vivo* for 2 months. We observed two distinct populations of cells: population 1 consists of AR negative and DDX3 puncta positive cells (bottom right portion of micrograph), the second population was AR positive cells which were observed with little and diffuse DDX3 expression patterns (i.e. no stress granules; upper left portion of micrograph).

3.3. Opportunities for training and professional development

Training Activity:

a) Developed and learned lab techniques required for the research project described above including RT-qPCR, Western blot, IHC, *in situ* hybridization and RIPs.

b) Attended weekly group lab meetings, relevant Cancer Biology seminars, monthly Prostate Center of Research Excellence seminar series, and monthly UW O'Brien Center seminar series.

c) Mentored three undergraduates to guide them with their own projects and develop my mentoring skills.

d) Taught two lab modules for undergraduate Pharmacology-Toxicology (PharmTox) majors.

Professional Development:

a) Memberships in professional and scientific societies
- Genetics Society of America (2012-present)
- American Association for the Advancement of Science (2016-present)

b) Journal Review

FASEB Journal, Development, Genesis

c) Workshops and Service
- Volunteer for Science Expeditions and the Wisconsin Science Festival to engage the public in science
- Invited speaker for the School of Science, Technology, and Education Colloquium at Lakeland University, my undergraduate alma mater

d) Invited Lectures and Talks
- PharmTox 558: Cell-based assays: an introduction to qPCR for gene expression analysis

3.4. Dissemination of the results to communities of interest

Nothing to Report.

3.5. Plan during the next reporting period

Determine the ability of phthalate-treated prostate cancer cells to metastasize in vivo as a basis of CRPC development (Major Task 2)

We are utilizing prostate cancer cell xenografts to determine if phthalate exposure affects the metastatic potential of CRPC cells. These studies are in progress, and lymph nodes will be collected and analyzed for proliferation, indicating potential metastasis, 8 weeks after xenografting.

Analysis of DDX3/DMNT1 expression levels in cells after phthalate treatment (Major Task 3, Subtask 1)

We will treat prostate cancer cell lines with phthalates and use qPCR to measure DMNT1 and DDX3 mRNA expression levels and western blots to measure DMNT1 and DDX3 protein expression levels to determine if phthalate exposure alters expression levels of either of these proteins.

Determine the co-localization pattern of DNMT1/DDX3 and AR through the human prostate cancer tissue microarray (TMA) (Major Task 3, Subtask 4)

We will use a human PRCA progression human TMA and Vectra analysis to conduct multispectral imaging and determine whether DNMT1/DDX3 protein abundance inversely correlates with AR protein abundance in human PRCA patient tissues. The construction of the prostate TMA (IRB M-2007-1100-CP003) and detailed information of the PRCA progression array is described in Warren et al. [3]. Briefly, the formalin-fixed, paraffin-embedded tissues were the archive of the Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, collected by prostatectomy and transurethral resection. The TMA consists of 288 duplicates cores from prostate tissues of four different disease groups including benign prostate tissue (BPT; N=48), high-grade prostatic intraepithelial neoplasia (HGPIN; N=50), prostate cancer (PRCA; N=146), and metastatic specimens (MET; N=44). The size of each core is 0.6 mm in diameter and arranged 0.2 mm apart vertically and horizontally using a Manual Tissue Arrayer (Beecher Instruments, Sun Prairie, WI; Model MTA-1). The staining protocol with the Vectra platform (Perkin Elmer, Waltham, MA) will be performed according to the method previously described [4].

Plan for Training Activity

I will continue to learn new laboratory techniques as they arise in carrying out this project. I will also continue to mentor undergraduate students in the laboratory to improve my mentoring skills. I will also be working with my advisor to assist with writing grant proposals, which will help improve my grantsmanship and scientific writing skills. Finally, I will continue to attend relevant seminars and conferences and present my work when possible to learn about advances in the field and get feedback on my work. I am planning to submit an abstract with the hope of attending and presenting at the Society for Basic Urologic Research (SBUR) conference in November.

4. IMPACT:

4.1. The impact on the development of the principal discipline of the project

Environmental toxicants often function as steroid hormone mimetics and thus may play important roles in the development of hormone-related cancers, including aggressive castration-resistant prostate cancer. The mechanism by which prostate cancer becomes castration-resistant has long been thought to be understood, and most studies point to a gain-of-function of AR as the molecular mechanism by which prostate cancer becomes resistant to androgen deprivation therapies. However, this work highlights an entirely new mechanism by which prostate cancer can become castration-resistant: through translational inhibition of AR expression. This finding represents an important new advance in prostate cancer research and may spur future studies that more carefully analyze the penetrance and importance of loss of AR expression in prostate cancer progression.

4.2. The impact on other disciplines

Nothing to Report.

4.3. The impact on technology transfer

Nothing to Report.

4.4. The impact on society beyond science and technology

Prostate cancer is the second leading cause of death for men in the United States. Early stages of cancer can be treated with high success rates; however, these cancers can eventually recur and become castration-resistant prostate cancer, which is very difficult to treat and often results in death. Exposure to environmental toxins, including phthalalates, may enhance the risk of developing CRPC. Therefore, the development of new therapeutics for CRPC is absolutely essential. This work has identified a new mechanism by which prostate cancers become castration-resistant, opening the door for development of novel therapeutic strategies to treat this deadly disease.

5. CHANGES/PROBLEMS:

Nothing to Report.

6. PRODUCTS:

6.1. Publications, conference papers, and presentations/Journal publications

Conference Presentations:

1. Neuman, S.D., Vellky, J.E., Ihry, R.J., Bashirullah, A., Ricke, W.A. Novel models to dissect the role of DDX3-dependent translational control in the prostate. Collaborating for the Advancement of Interdisciplinary Research in Benign Urology (CAIRIBU) Conference,

Ellicott City, MD. December 2018. (poster presentation)
2. Neuman, S.D., Vellky, J.E., Ihry, R.J., Bashirullah, A., Ricke, W.A. Translational control in steroid hormone signaling. University of Wisconsin-Madison O'Brien Center Symposium. Madison, WI. April 2019. (oral and poster presentation)

6.2. Books or other non-periodical, one-time publications

Nothing to Report.

6.3. Other publications, conference papers, and presentations

Other publications:

1. Neuman, S.D., Bashirullah, A. (2018). Reconsidering the Passive Diffusion Model of Steroid Hormone Cellular Entry. Developmental

7. APPENDICES

References

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2. Kinoshita, H., et al., *Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer.* Cancer Res, 2000. **60**(13): p. 3623-30.
3. Warren, M., et al., *Protein expression of matriptase and its cognate inhibitor HAI-1 in human prostate cancer: a tissue microarray and automated quantitative analysis.* Appl Immunohistochem Mol Morphol, 2009. **17**(1): p. 23-30.
4. Huang, W., K. Hennrick, and S. Drew, *A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays.* Hum Pathol, 2013. **44**(1): p. 29-38.