AWARD NUMBER: W81XWH-16-1-0246

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

PRINCIPAL INVESTIGATOR: Sarah Neuman, Ph.D.

CONTRACTING ORGANIZATION:
University of Wisconsin System
MADISON, WI, 53715-1218

REPORT DATE: July 2018

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
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.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>4. Impact</td>
<td>10</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>10</td>
</tr>
<tr>
<td>6. Products</td>
<td>10</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>10</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>11</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>11</td>
</tr>
</tbody>
</table>
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. We have found that the RNA helicase DDX3 plays a critical role in translational control of the androgen receptor (AR) in CRPC, highlighting a new mechanism by which prostate cancer becomes castration-resistant. Future efforts will be dedicated to determining if DDX3-dependent translational control plays a role in phthalate-triggered reduction of AR in prostate cancer.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

3.1. Major Goals

Goal 1: Determine if DDX3 levels are altered in CRPC. (Months 1-12, 80% completed)
To determine if DDX3 levels change in CRPC, we first conducted a meta-analysis of gene expression data from the Oncomine database. We then directly tested if DDX3 levels changed in human prostate cancer samples by using immunofluorescent staining with an anti-DDX3 antibody and by utilizing prostate cancer immunohistochemical metadata. Finally, we used western blots to analyze DDX3 expression levels in non-tumorigenic (RWPE-1, BHPRE, BPH1), mid-grade (PC3, DU145, LNCaP), and CRPC (C42B) cell lines.

Goal 2: Determine if DDX3 localization changes in CRPC. (Months 10-12, 80% completed)
We used immunofluorescent staining with an anti-DDX3 antibody to assess DDX3 localization patterns in both normal (NT1) and CRPC (MT10, C42) cell lines. Other antibodies were used to assess for co-localization. We also used an anti-AR antibody to test for changes in AR expression levels in CRPC cell lines.

Goal 3: Determine if DDX3 directly binds AR mRNA to regulate AR translation. (Month 10-12, 80% completed)
To determine if DDX3 directly affects expression of AR, we overexpressed DDX3 in both benign (NT1) and mid-grade prostate cancer (LNCaP) cell lines and used western blots to assess AR expression levels. We also used RNA immunoprecipitations and in situ hybridization to determine if DDX3 bound directly to AR mRNA.
3.2. Accomplishments under the goals

**Analysis of DDX3 expression levels in CRPC.**

Our meta-analysis of DDX3 expression levels in Oncomine revealed that DDX3 does appear to be upregulated in three separate studies (Fig. 1A). Immunofluorescent staining for DDX3 in human prostate tissues also revealed a dramatic increase in diffuse, cytoplasmic staining for DDX3 in prostate cancer tissues compared to benign controls (Fig. 1B). Additionally, our analysis of immunohistochemistry metadata revealed a positive correlation between prostate cancer grade and DDX3 expression levels (Fig. 1C). Finally, we used western blots to analyze DDX3 protein expression levels in cell lines representing different grades of prostate cancer progression. The lowest levels were observed in benign cell lines (RWPE, BHPRE, BPH1), with increased levels in metastatic prostate cancer (PC3, DU145, LnCaP), and the highest levels in CRPC (C42B) (Fig. 1D).

![Figure 1](image)

**Figure. 1. DDX3 expression is significantly increased in prostate cancer and castration-resistant prostate cancer (CRPC).** A. DDX3 meta-analysis in prostate cancer vs. normal tissue from Oncomine. B. DDX3 cytoplasmic localization (immunofluorescence) intensity increases in prostate cancer (vs. normal) (Red = DDX3, Blue = DAPI). C. DDX3 expression is higher in high-grade prostate cancer vs. low grade prostate cancer as determined by immunohistochemistry-metadata. D. DDX3 protein increases in metastatic (M) and CRPC vs. non-tumorigenic prostate cell lines (NT) as detected by western blot analysis. This data suggests that DDX3 expression levels increase in prostate cancer progression and CRPC.

**Analysis of DDX3 localization in CRPC.**

Given that DDX3 expression levels changed in CRPC, we next tested if DDX3 localization changed in CRPC. To do this, we used immunofluorescent staining with an anti-DDX3 antibody to look at where DDX3 localized within a variety of benign and CRPC cell lines. We found that DDX3 localization was diffuse and cytoplasmic in the benign cell line NT1 (Fig. 2A, green staining). However, in the CRPC lines MT10 and C42, DDX3 localized in bright punctate structures within the cytoplasm (Fig. 2A, green staining). We also found that these bright puncta colocalized with eIF4E, a known marker for cellular stress granules (Fig. 2A, red staining). Stress granules are small, specialized structures where cells can sequester and “protect” critical mRNAs and proteins during stressful conditions. To test if this stress granule localization of DDX3 is important for CRPC development and/or progression, we tested if androgen deprivation caused DDX3 to localize to stress granules. We tested this idea in two ways. First, we treated the androgen-responsive prostate cancer cell line LNCaP with...
the androgen deprivation therapeutic bicalutamide (1µM), and second, we grew the CWR22Rv1 cell line in media that was charcoal-stripped of all androgen. In both cases, we saw an increase in DDX3 puncta, indicative of increased stress granule formation (Fig. 2B, top row). We also saw increased staining for HYOU1, a marker for hypoxic cellular stress (Fig. 2B, middle row). Finally, we saw a decrease in androgen receptor (AR) protein with bicalutamide treatment or growth in charcoal-stripped media (Fig. 2B, bottom row).

Figure 2. DDX3 function and localization during stress. A. Immunocytochemistry: DDX3 (green) co-localizes (orange) with stress granule marker eIF4E (red) primarily in MT10 and C42 CRPC models. B. Immunocytochemistry: DDX3 (green) upper panel, hypoxia marker HYOU1 (green) middle panels, and AR (green) lower panel in LNCaP (column 1) LNCaP + bicalutamide (column 2), CWR22Rv1 + full growth media (column 3), and CWR22Rv1 grown in charcoal-stripped media (CSS) media (column 4) which models a castrate environment. Note androgen “deprived” environments (columns 2 and 4) have induced punctate DDX3 staining (indicative of localization to stress granules), increased HYOU1 expression (indicative of hypoxia), and decreased AR expression.

Analysis of DDX3 binding to AR mRNA and effects on AR translation.

DDX3 is a known regulator of translation for specific mRNA targets. We first wanted to test if DDX3 expression levels directly affected AR expression levels. To do this, we used transient transfection to overexpress DDX3 in the benign cell line NT1 and the androgen-responsive prostate cancer cell line LNCaP. In both cases, overexpression of DDX3 caused an increase in stress granule formation (Fig. 3A). Additionally, immunoblot analysis showed that overexpression of DDX3 reduced AR expression levels by about 25% in both cell lines (Fig. 3A). We then tested if DDX3 bound directly to AR mRNA using an RNA immunoprecipitation (RIP) in the mid-grade prostate cancer cell line LNCaP and the CRPC cell line C42. DDX3 bound to CCNE1 mRNA, a known DDX3 target, serving as a positive control for the RIP experiment (Fig. 3B, blue bars). Importantly, DDX3 bound to AR mRNA in both LNCaP and C42 cells (Fig. 3B, red bars). We also tested for DDX3 binding to AR in the benign cell lines BPH1 and NT1, as well as the CRPC lines MT10 and C42B. Interestingly, DDX3 only bound to AR in the CRPC lines and not the benign lines (Fig. 3B). We also used RNA in situ hybridization to look for colocalization of DDX3 protein with AR mRNA in prostate tissue from human patients. We found that there was little colocalization.
between DDX3 and AR mRNA in benign patient tissue, but strong colocalization in prostate cancer patient tissue (Fig. 3C). Finally, we further tested the relationship between DDX3 expression levels and AR expression levels by using immunoblots to assay expression levels of both proteins in cell lines that represent the progression from benign prostate tissue to CRPC. We saw a striking inverse relationship between DDX3 and AR protein in this assay: the benign cell lines had low expression levels of DDX3 and high levels of AR, while the CRPC lines exhibited high expression levels of DDX3 and low expression levels of AR (Fig. 3D).

Figure 3. DDX3 binds AR mRNA within putative stress granules to inhibit AR translation. A. Over expression (vs vector control) of DDX3 in NT1 and LNCaP cells. Note increased stress granule formation in DDX3-OE cells. Over expression of DDX3 (vs vector control) in NT1 and LNCaP cells results in ~25% reduction of AR protein expression by protein immunoblot. B. RNA-IP (RIP) of DDX3 pulls down positive control CCNE1 mRNA (blue bars) and AR mRNA (red bars) in LNCaP and CRPC derivative (C42) cell lines. Similarly, RNA-IP of DDX3 pulls down positive control CCNE1 mRNA in benign (BPH1, NT1) and CRPC (MT10, C42B) cell lines. DDX3 RIP pulls down AR mRNA in CRPC (MT10, C42B) cells, but not benign (BPH1, NT1) cells. Negative control β-tubulin and TBP mRNAs were not detected in DDX3 RIPs, data not shown. C. RNAscope\textit{-in situ} hybridization shows AR mRNA (green) co-localizing (orange) with DDX3 (red) in aggressive cancer, but not benign patient prostate tissue. Note cytoplasmic puncta of DDX3-AR mRNA double localization (orange) in cancer. D. DDX3 expression increases in cell line models of PC progression (NT1, T1, T10, MT10), and in castration resistance (LNCaP-C4 series). Note the inverse relationship of DDX3 and AR.
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 Molecular and Environmental Toxicology Center (METC) seminar series, monthly Prostate Center of Research Excellence seminar series, and monthly UW O’Brien Center seminar series.

c) Mentored four 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

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

*Analysis of DDX3 expression and localization in phthalate-treated tissue.*

We have previously found that treatment with phthalates, industrial chemicals used in many plastics, resulted in reduced AR expression levels in prostate cancer. In future experiments, we will test if DDX3 expression levels are altered and/or if DDX3 localization changes in phthalate-treated prostate cell lines and mouse tissue. We will use immunofluorescent staining and immunoblotting with an anti-DDX3 antibody for these assays. We will also co-stain for the stress granule marker eIF4E as appropriate. We will also assay DDX3 expression levels and localization in human prostate tissue samples.
Test if pharmacologic inhibition of DDX3 affects prostate cancer progression.
A small molecule inhibitor of DDX3, called RK33, has previously been identified. We will test if RK33 treatment affects AR expression levels and prostate cancer progression in both cell lines and animal models of CRPC. In cell lines, we will test if RK33 treatment affects proliferation and survival. We will also test if RK33 affects AR expression levels in CRPC cell lines. In animal models, we will use a tumor xenograft model established by our lab to test if RK33 treatment affects tumor growth in vivo.

Further analysis of DDX3 binding to AR mRNA and identification of other DDX3 target mRNAs.
Our results indicate that DDX3 can bind directly to AR mRNA; however, the mechanism by which this occurs is unclear. We will use RNA immunoprecipitation coupled with next-generation sequencing (RIP-seq) to identify the specific sequence where DDX3 binds to AR mRNA. This will help us further understand how DDX3 is regulating AR translation. Additionally, there are likely many other mRNAs that are also bound by DDX3 in CRPC. We will therefore use RIP-seq to identify these other mRNAs to gain new insights into other genes whose translation is regulated by DDX3.

Establish a new genetic model to dissect the function of DDX3 in translational control.
DDX3 is highly conserved, with orthologs present from yeast to humans, including the fruit fly Drosophila melanogaster. Belle, the fly ortholog of DDX3, has also been implicated in steroid hormone-dependent translational control. This provides us with an ideal genetic system to analyze how DDX3/Belle expression and activity regulate translational outcomes.

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.

4. IMPACT:

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

6.2. Books or other non-periodical, one-time publications
Nothing to Report.

6.3. Other publications, conference papers, and presentations

*Other publications:*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Dr. Clara Jeong was the previous Principal Investigator on this project. She left the University of Wisconsin-Madison. Dr. Sarah Neuman is now the Principal Investigator. A formal PI change request has been submitted.
8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

None.