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

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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.					
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1. INTRODUCTION:

Androgen deprivation therapy is initially effective for most prostate cancer (PRCA) patients but after several years PRCA recurs and become a castration-resistant prostate cancer (CRPC). The molecular mechanisms of the transition to CRPC are poorly understood. There are numerous mechanisms demonstrating how CRPC continues to activate AR and most CRPC therapies are aimed to deactivate the AR signaling. However, within the CRPC population, there is a subgroup of patients showing loss of AR in the tumor. For this group, current treatments are ineffective since they are lacking the AR but little efforts have been conducted to investigate the pathways that are associated with the AR negative CRPC development. High attention is given to the effects of environmental factors on biological systems and many studies have shown association of environmental toxicant exposure with adverse health outcomes, while little is known of their impacts on PRCA progression. Phthalates are industrial chemicals widely used in plastics and many every day products. In addition, occupational exposure could further increase the phthalate levels in humans. In this regard, the overall goal of this research is to determine whether phthalates reduce AR expression in the prostate epithelium and leads to CRPC and to determine the phthalate-induced molecular mechanisms associated with the CRPC development. Our hypothesis is that phthalate exposure down-regulates AR expression in mouse prostate and in human prostate cancer cells and increase cell proliferation. We also hypothesize that phthalate exposure increases DNMT1 expression and induce hypermethylation on the AR gene promotor region.

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

3.1. Major Goals

Goal 1: Determine if phthalate treatment reduce AR expression in prostate cancer cell lines or in mice tissue. (Months 1-12, 80% completed)

We have subcutaneously treated adult male C57BL/6J mice with mono-butyl phthalate (MBP; the major metabolite of di-butyl phthalate (DBP)) or control for 1 month and determined the AR mRNA levels and AR protein levels in the prostate tissues. We also treated LNCaP, CWP22Rv1, and BPH-1 cells with various concentrations of MBP, which is the major metabolite of DBP for 24h and determined the AR mRNA and AR protein levels. We will continue on the in vitro studies with other prostate cell lines including C4-2B and RWPE-1.

Goal 2: Determine the ability of phthalate treated prostate cancer cells to metastasize in vivo as the basis of CRPC development. (Months 10-12, 20% completed)

We are in the process of selecting the cell line that is suitable for the xenograft experiment. We are conducting multiple dose-response experiments to see whether MBP treatment increase cell proliferation. We are planning to choose the prostate cancer cell line that is most sensitive to MBP treatment for xenograft.

Goal 3: Determine the co-expression of DNMT1 and AR in human prostate tissues and determine the methylation pattern of the AR promotor region in the prostate in vivo and in vitro. (Month 10-12, 30% completed)

To see if MBP treatment increases DNMT1 level to suppress AR expression, mRNA and protein levels of DNMT1 in MBP-treated cell lines (from Goal 1) were measured. We are in the process of measuring the methylation pattern on the AR promotor region in cells through the methylated DNA immunoprecipitation (MeDIP) assay. We are also in the process of optimizing the immunohistochemistry (IHC) condition to determine the co-localization pattern of DNMT1 and AR through the human prostate cancer tissue microarray (TMA).

3.2. Accomplishments under the goals

Impact of phthalate exposure on AR mRNA and AR protein levels in mice tissues

To determine if phthalates exposure leads to reduced AR protein expression, adult male C57BL/6 mice (6-8 weeks) were subcutaneously treated with a slow releasing compressed pellet containing either 25 mg of MBP or cholesterol control for 1 month (N=5 per treatment). After treatment, mice were euthanized and tissues including prostate lobes (dorsal and lateral, ventral, and anterior), bladder, testis, seminal vesicles, liver, and kidney were collected. Half of the set were formalin-fixation for IHC and the other half were snap-frozen for RT-qPCR analysis. In addition, serum (approximately 300 μ L each) from individual mice were collected and stored in -80 $^{\circ}$ C for LC-MS/MS analysis to determine circulating MBP levels and multiple steroid hormone levels.

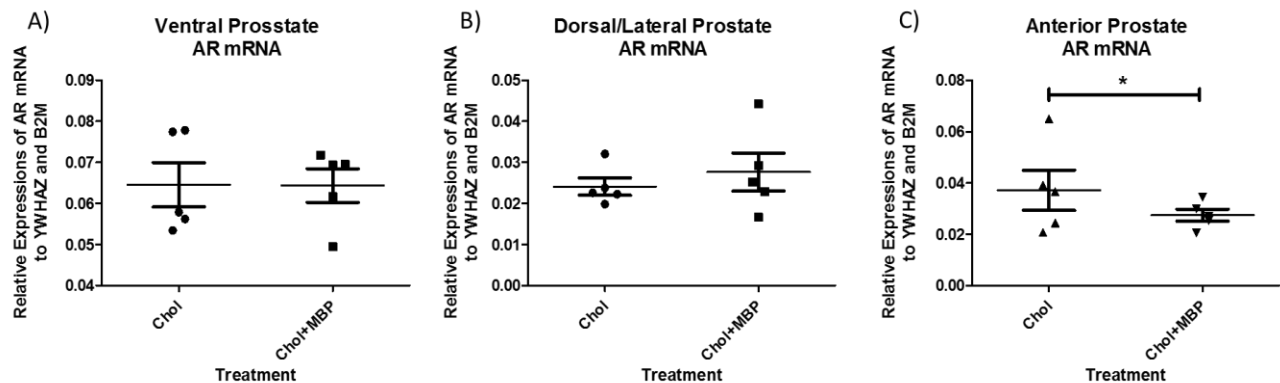


Figure 1. MBP treatment reduced AR mRNA expression in the anterior prostate (AP) Adult male C57BL/6 mice were subcutaneously treated with either compressed cholesterol control pellet or with a cholesterol pellet containing 25mg of MBP for 1 month. There were no significant changes in the AR mRNA in ventral prostates (VP) or dorsal/lateral prostates (DLP) (Figure A and B), while there was a significant decrease ($P < 0.05$) of AR mRNA levels in the MBP treatment group compared to the control in anterior prostates (AP) (Figure C).

Among three different prostate lobes, a significant decrease of AR mRNA levels in the anterior prostate (AP) was found in the MBP treatment group compared to the control. (Figure 1) No differences were found in either ventral prostates (VP) or dorsal/lateral prostates (DLP). (Figure 1). To determine if MBP exposure induce changes in weight of androgen-regulated tissues by lowering the AR expression, the masses of tissues including prostate lobes, seminal vesicles (SV), and testis were measured. No changes were observed between the control group and the MBP-treated group in all measured tissues.

In addition to AR mRNA, we investigated the AR protein expression levels in different cell compartments (epithelial and stromal) in phthalate-treated and control mice tissues. The IHC was conducted on paraffin-fixed mice prostate tissues. Primary antibody used for this analysis was rabbit anti-AR (1:250, Santa Cruz, CA). AR expressions were investigated in three different prostate lobes including AP, DLP, and VP. We are in the process of optimizing the antibody staining condition, and our preliminary data is showing a moderate reduction of AR expression in the DLP of MBP-treated mice (Figure 2).

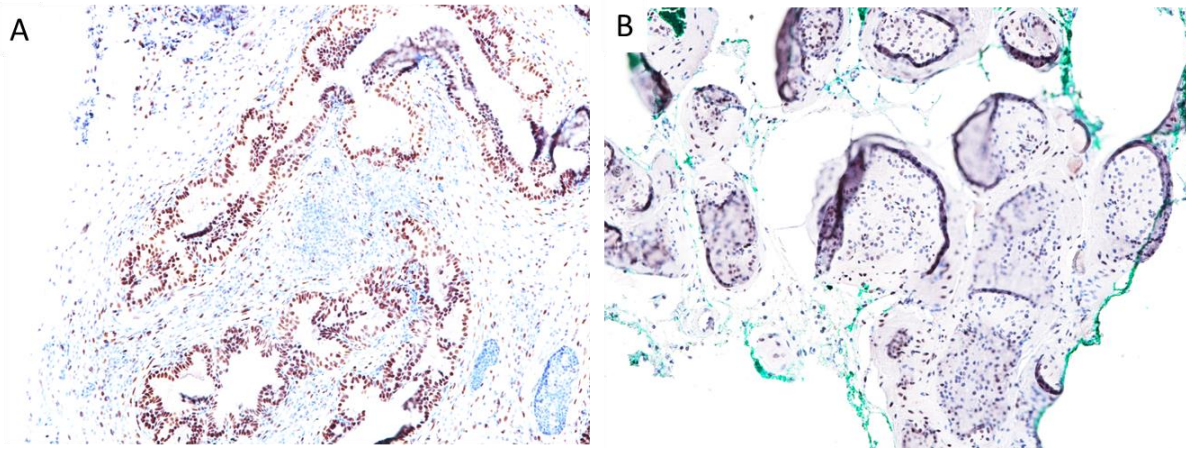


Figure 2. MBP treatment has no effects on AR expression in epithelial or stromal compartments of the dorsal/lateral prostate (DLP) Adult male C57BL/6 mice were subcutaneously treated with either compressed cholesterol control pellet or with a cholesterol pellet containing 25mg of MBP for 1 month. A reduction of AR expression was observed in the MBP-treated tissue (Figure B) compared to the cholesterol control tissue (Figure A). *Dark brown stainings indicate AR positivity.

Impact of phthalate exposure on AR and DNMT1 mRNA levels in prostate cell lines

To determine if MBP treatment induce reduction of AR mRNA levels and induce DNMT1 mRNA levels, various prostate cancer cell lines including LNCaP and CWR22Rv1, as well as non-tumorigenic prostate cell lines including BPH-1. 3×10^5 cells/well were seeded in a 6-well plate with 5% fetal bovine serum (FBS) medium. The next day, media was switched into 5% charcoal-stripped serum (CSS) medium and was incubated for 12-16 hours. After starvation, cells were treated with either dimethyl sulfoxide (DMSO) control or various doses of MBP (100 pM, 1 μ M, and 10 μ M) for 24 hours. After treatment, cells were collected and RNA were isolated with TRIzol® Reagent (life technologies, Carlsbad, CA) followed by cDNA synthesis with Bio-Rad® iScript kit. RT-qPCR analysis was performed for AR and DNMT1 mRNA. We are in the process of analysis and our preliminary data is showing a reduction of AR mRNA in 1 μ M MBP-treated group in CWR22Rv1 cells (Figure 3).

Overall, we investigated the effects of MBP in AR expression both in vitro and in vivo as well as the DNMT1 expression. Our current data shows MBP-induced AR protein reduction in the DLP, while a reduction of AR mRNA was observed in the AP of MBP-treated mice. Currently, little is known about the effects of phthalates on in vitro prostate cancer cells and each cell line has

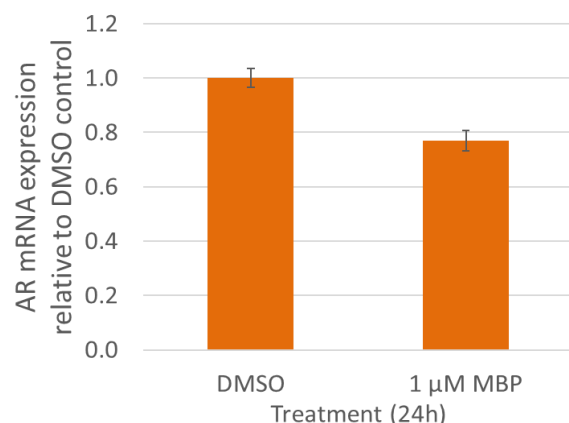


Figure 3. 24h MBP treatment reduces AR mRNA levels in CWR22Ra1 cells in vitro

different AR characteristics such as AR point mutations. Therefore, cell responses to phthalates could differ depending on the cell line characteristics as well as the dose. Thus, we will continue to test a wider range (picograms to micrograms per ml) of dose-response treatments with a different treatment time on various prostate cell lines to find a suitable MBP dose for further mechanism studies.

Phthalates alter enzymatic activities and influence androgen levels among adolescents (aged 12-20) and young adults (aged 20-30) [1]. In addition, phthalates inhibit testes steroidogenesis and decrease testosterone levels both in humans [2] and in rodents [3].

Intratutural steroidogenesis is one possible mechanism that contribute to the CRPC progression. Previous studies have shown that alteration in testosterone, DHT, or estradiol levels could affect the AR expression rodent male reproductive tracts [4]. Therefore, it is possible that phthalates alter the steroidogenesis pathway by either increasing or decreasing androgen levels that further influences the AR expression in the prostate tissues. Thus, through collaborations with the WI primate center and the analytical instrument core in the school of Pharmacy at the UW-Madison, we are developing a method to quantitatively measure 12 steroid hormones (Figure 4) in mice serum (300 μL) and in cell culture media by LC-MS/MS. This method will enable to monitor the changes in steroidogenesis induced by phthalates treatment.

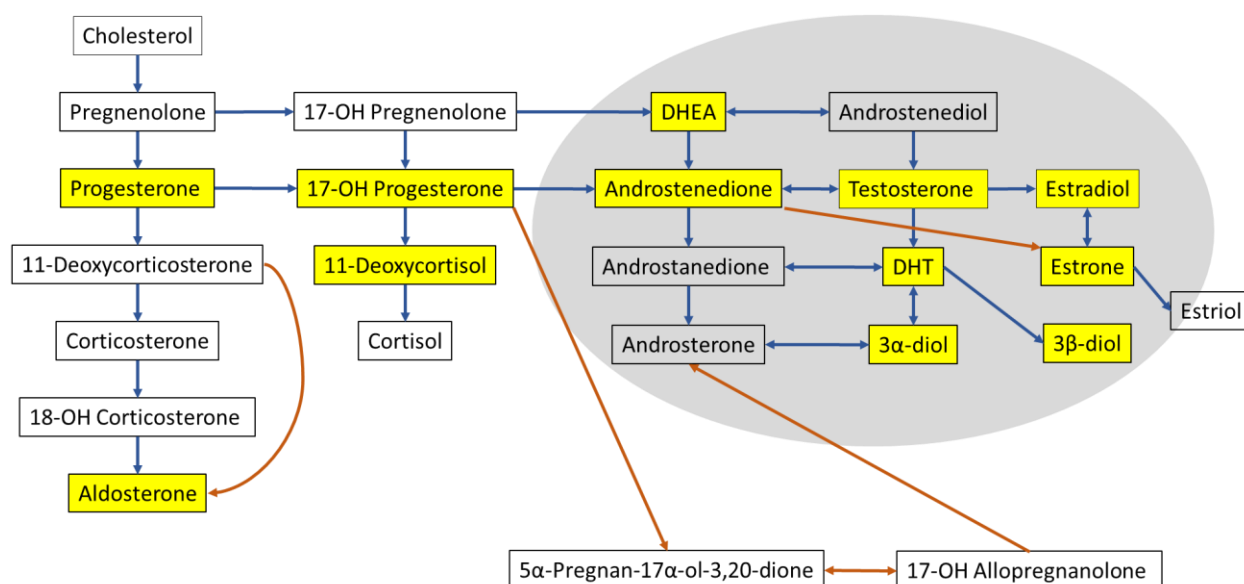


Figure 4. Steroidogenesis pathway and 12 steroid hormones of interest (highlighted in yellow) for the LC-MS/MS analysis The gray area indicates the major pathway related to intratumoral steroidogenesis.

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, and LC-MS/MS.
- b) Developed knowledge of cell biology and oncology by auditing Oncology 703, “Carcinogenesis and Tumor Cell Biology” and by attending weekly “Cancer Biology” seminars provided by the UW-Madison.
- c) Developed grant writing skills by attending the workshops provided by the Korean-American Scientists and Engineers Association (KSEA) and by the UW’s Institute for Clinical and Translational Research (ICTR)-writing center.
- d) 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.
- e) Presented (one oral presentation and two poster presentations) at the UW O’Brien Center 2017 Symposium to share my results with others and build my scientific network.
- f) Mentored three undergraduates to guide them with their own projects and developed mentoring skills.

Professional Development:

- a) Memberships in professional and scientific societies
 - Society for Basic Urologic Research (SBUR), 2015-present
 - Korean-American Scientists and Engineers Association (KSEA), 2015-present
 - Society of Toxicology (SOT), 2014-present
- b) Journal Review
 - Scientific Reports, IBM Journal of Research and Development
- c) Workshops and Services
 - American Urological Association (AUA) Early-Career Investigators Workshop Organized by the AUA Office of Research, Linthicum, MD, 2017 (scheduled).
 - Pedagogy Course on “Teaching in Science and Engineering: The College Classroom” DELTA program, University of Wisconsin-Madison, Madison, WI, 2017 (scheduled).
 - Scientists and Engineers Early-Career Development (SEED) Workshop Organized by the KSEA Office of Research, Vienna, VA, December, 2016.
 - Served as a moderator at the O’Brien Research Center Spring Symposium, 2016
 - Served as a moderator for the journal club in Dr. Ricke group, 2016 – present
- d) Invited Lectures and Talks
 - MET 606: Colloquium in Environmental Toxicology UW-Madison, Spring 2016
 - Guest Lecturer, Title: Impact of endocrine disrupting chemicals on prostate disease

3.4. Dissemination of the results to communities of interest

Nothing to Report.

3.5. Plan during the next reporting period

Quantitatively measure multiple steroid hormone levels in mice serum and cell culture media using LC-MS/MS

Using the recently developed LC-MS/MS method, we will quantitatively measure multiple steroid hormones in MBP-treated mice serum and MBP-treated cell culture media to determine if MBP treatment have altered the steroidogenesis leading to an indirect AR regulation.

Determine the ability of phthalate-treated prostate cancer cells to metastasize in vivo as a basis of CRPC development

We will utilize prostate cancer cells xenografts to determine if MBP treatment enhances the ability of cells to metastasize into lymph nodes. Numerous animal models incorporating cell xenografts have been developed for a wide range of human disease studies to promote cell growth in an in vivo environment. We choose the renal capsule site due to its high graft take rate, abundant blood supply, and ability to implant a greater number of xenografts into one confined site and the ability of the xenograft to metastasize [5]. Prostate cancer cell xenografts containing 250,000 cells will be inserted either with or without MBP treatment under the kidney capsule of immunocompromised male nude mice and be monitored for 8 weeks. Previously, LNCaP and BCaPT10 xenografts were shown to metastasize into lymph nodes which will serve as appropriate models for metastasis models [6]. BCaPT10 is a primary metastatic prostate cancer cell line derived from BPH-1 xenograft [6]. We are planning to choose one cell line among these cell lines that is more sensitive to MBP treatment based on the in vitro proliferation assay results. After 8 weeks of monitoring, lymph nodes will be collected and will be analyzed to determine if MBP treatment enhanced metastatic activity of cells by Ki67 proliferation marker staining. Change in the AR status of the cell xenografts will also be measured. All procedures will be performed with approval of institutional animal care.

Compare the methylation pattern on the AR promoter region in cells between DMSO treated prostate cancer cells and MBP-treated cells through the MeDIP assay

We hypothesize that MBP treatment will induce hypermethylation in the AR gene promotor region to reduce AR expression. Thus, MeDIP qPCR will be used to assess the relative abundance of DNA methylation across the AR gene promoter according to the method described previously [7, 8]. The MeDIP assay will be performed with DNA from control- and MBP-treated prostate cell lines and mouse prostates.

Determine the co-expression of DNMT1 and AR through the human prostate cancer TMA

We will use a human PRCA progression human TMA and Vectra analysis to conduct multispectral imaging and determine whether DNMT1 protein abundance inversely correlates with AR protein abundance in human PRCA patient tissues. Valdez et al. showed that DNMT1 knockdown increase AR protein abundance in human prostate epithelial cells and demonstrated increased DNMT1 expression with advancing prostate cancer stage [9].

An inverse relationship between DNMT1 and AR expression was observed from a TMA analysis composed of prostate tissue from a prostate cancer mouse model [9]. We hypothesize that DNMT1 and AR will inversely correlate in the human PRCA TMA as well. We also hypothesize

that MBP exposure increases DNMT1 expression in prostate cancer cells and reduce AR expression in vitro. 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. [10]. 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 [11].

Plan for Training Activity

I have been selected to attend the AUA Early Career Investigator Workshop in September, 2017. I am also planning to gain teaching experiences by giving several lectures in chemistry to undergraduate students during the semester.

4. IMPACT:

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

This project is determining if environmental toxicants-induced biological modifications are involved in AR reduction that further leads to a transition to CRPC with elevated rate of tumor proliferation. These findings will bring awareness and provide better guidelines for patients and communities to lower the cancer recurrence risk. This research project addresses the mechanisms of resistance in CRPC, especially in the sub-population of CRPC patients with AR negative cells. This is a unique opportunity to study the role of AR negative cell population in CRPC development. Findings from this research will lead us to develop new therapy for CRPC patients who received no advantages from current drugs due to the lack of target AR.

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

Nowadays, high attention is given to the effects of environmental toxicants on biological systems because we are all exposed to these environmental factors and many studies have shown association of environmental toxicant exposure with adverse health outcomes, while little is known of their impacts on cancer progression, especially in prostate cancer. Phthalates are industrial chemicals widely used in plastics and many products. In addition, occupational exposure could further increase the phthalate levels in humans. In this regard, this project will provide an awareness and insights on how environmental exposures could contribute to disease progressions or treatments in general.

5. CHANGES/PROBLEMS:

Nothing to Report.

6. PRODUCTS:

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

Conference Presentations:

1. Jeong, C. H.*, Thomas, S., Ricke, W.A. Multi-Steroid Hormones in Mice Serum by Liquid Chromatography-Tandem Mass Spectrometry. George M. O'Brien Spring Symposium, Madison, WI, May, 2017.
2. Jeong, C. H., Bae, S., Ricke, W.A. Characterization of protease-activated receptor-3 (PAR3) in prostate cancer. George M. O'Brien Spring Symposium, Madison, WI, May, 2017.
3. Jeong, C. H., Sun, C., Ricke, W.A. Effects of Phthalates on Steroidogenesis in Prostate Cancer Cells. George M. O'Brien Spring Symposium, Madison, WI, May, 2017.

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

Nothing to Report.

6.3. Other publications, conference papers, and presentations

Other publications:

1. Jeong, C. H.*, Machek, E. J., Shakeri, M., Duirk, S. E., Ternes, T. A., Richardson, S. D., Wagner, E. D., and Plewa, M. P. The impact of iodinated X-ray contrast agents on formation and toxicity of disinfection by-products in drinking water, J. Environ. Sci. 2017, (in press)
2. Jeong, C. H., Gao, G., Dettro, T., Wagner, E. D., Ricke, E. A., Plewa, M. J., Flaws, J. A. Monohaloacetic acid drinking water disinfection by-products inhibit follicle growth and steroidogenesis in mouse ovarian antral follicles in vitro, Reprod. Toxicol. 2016, 62, 71-76.

Other Conferences:

1. Jeong, C. H., Wagner, E. D., Siebert, V. R., Richardson, S. D., Plewa, M. J. Occurrence and Toxicity of Disinfection Byproducts in European Drinking Waters in Relation with the HIWATE Epidemiology Study, US-Korea Annual Conference, Dallas, TX, August, 2016.

Other book chapters:

1. Jeong, C. H., Zhou, C., and Flaws, J. A., Environmental Toxicants and Reproduction. In: R. Farquharson & M. Stephenson (Eds.), Early Pregnancy. Second Edition, Cambridge University Press. pp. 64-76, 2017.

7. APPENDICES:

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11. 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.