May 2012

Nicole Danielle Jones

Ndjones_26@yahoo.com

EDUCATION

Bachelor of Science

Biology Non- Teaching December 2004

Department of Natural Science, University of Maryland Eastern Shore

Graduate level:

Masters Program in the Department of Medical and Research Technology, School of Medicine

Undergraduate level with labs: General Biology, Genetics, Microbiology, Biochemistry, Independent Studies, Undergraduate Research, Evolution, Parasitology, Comparative Vertebrate Anatomy, General Zoology, General Chemistry

PROFESSIONAL EXPERIENCE:

Graduate Research Fall 2007

University of Maryland, Baltimore

Department of Pharmacology

Principle Investigator: Dr. Laundette Jones

-Research topic: In vitro regulation of human breast cancer suppressor gene 1 (BRCA1) Expression by Bisphenol A

Graduate Research Assistant Fall 2005

University of Maryland, Baltimore

Department of Medical Research and Technology

Principle Investigator: Dr. Silvia Pineiro

-Research topic: *Bdellovibrio* being studied as a potential therapeutic agent and sequencing the bacteria to determine new strains

Post- Baccalaureate Fellow Fall/Summer 2004 2005

University of Maryland Eastern Shore

Department of Natural Sciences

Principle Investigator: Dr. Kelly Mack

- Research topic: Health disparity related breast cancer research using MCF-7 cells determining the direct correlation with genetic polymorphisms, performing basic laboratory techniques such as tissue culture maintenance, reverse transcription, and real time PCR.

Undergraduate Research Assistant Summer 2004

University of Maryland Baltimore

Department of Epidemiology and Preventive Medicine

Principle Investigator: Dr. O Colin Stine

-Identified Methicillin- Resistant *Staphylococcus aurues* strains, performing sequencing PCR to determine point source patients that passed the infection on to other patients on a nursing home ward at the Veterans Administration Hospital

Undergraduate Research Technician Summer 2003

University of Maryland Baltimore

Department of Epidemiology and Preventive Medicine

Principle Investigator: Dr. O. Colin Stine

-Assistant to Masters and Ph.D. candidates performing varies laboratory techniques as gel electrophoresis, PCR, DNA extraction, and purification of DNA strains.

ABSTRACTS & PUBLICATIONS:

"Probable Transmission and Genotyping Methicillin Resistant *Staphylococcus aureus isolates* of Patients in the Brecc II Ward of the Veterans Administration Hospital."

Abstract

Title of Thesis: In vitro regulation of Human Breast Cancer 1 (BRCA1) gene expression by Bisphenol A

Nicole Jones, Master of Science, 2012

Thesis Directed by: Laundette Jones, PhD

Assistant Professor
Department of Pharmalogy
School of Medicine
University of Maryland

Breast Cancer is the second leading cause of death among women. Risk factors for breast cancer include lifetime exposure to estrogen, hereditary genetic mutations and environmental influences. According to researchers, Bisphenol A (BPA), an environmental chemical used in polycarbonate plastics, may be linked to the development of breast cancer. However, it is unclear how this chemical plays a role in breast cancer development.

Recent microarray studies from Singleton *et al*., demonstrated that BRCA1 gene expression is decreased by BPA in MCF7 cells over expressing estrogen receptor. It is known that decreased expression of BRCA1 accelerates growth of mammary epithelial cells and is often decreased in sporadic breast cancer progression. We hypothesize BPA, like estrogen, could alter BRCA1 expression. Our data suggest that treating cells with BPA at levels comparable to human exposures alters BRCA1 expression and the changes

in BRCA1 expression by BPA is concentration dependent. This study highlights the importance of conducting research to understand how BPA alteration of BRCA1 impacts its normal cellular function.

In vitro regulation of Human Breast Cancer 1 (BRCA1) gene expression by Bisphenol A

By

Nicole D. Jones

Thesis submitted to the faculty of the Graduate School
of the University of Maryland Baltimore in partial fulfillment
of the requirements of the degree of
Master of Science
2012

DEDICATION

To the most high, I thank you Lord for being there through every turn and twist of this Master's program.

To my family, I love you and thank you for all your help, support, and prayers.

To my loves, Darren and Nia, Thank you for making it all worth it, I love you.

ACKNOWLEDGEMENTS

I would like to thank Dr. Laundette Jones for being the most awesome advisor in the entire galaxy! You brought me in the lab with hope and a dream and help me create this thesis project. I will forever be in your debt, and I thank you many times over.

To my Thesis committee, Dr. Hamburger, Dr. Squibb, and Dr. Vucenik thank you for your patience. Your knowledge base has helped shape this paper and I appreciate everyone for taking the time out your busy schedule to help with this project.

To the Jones Lab, thank you for educating me on all the lab techniques that shape this paper and my research skills.

And to all the Department of Pharmacology and the Department of Medical Research and Technology, the faculty, staff and students have been so supportive of graduate students and all of our craziness! Thanks!

TABLE OF CONTENTS

Page

DEDICATION…………………………………………………………… iii.

ACKNOWLEDGEMENTS……………………………………………… iv.

TABLE OF CONTENTS…………………………………………………… v.

LIST OF FGURES………….……………………………………………. vi.

LIST OF ABBREVIATIONS……………………………………………….. vii.

SPECIFIC AIMS…………………………………………………………… 1

INTRODUCTION……………………………………………………………

Bisphenol A: The Background……………………………………………..2-6

i. Environmental relevance
ii. Widespread Exposure
iii. Mechanism
iv. BPA and Breast Cancer
v. BPA and Breast Cancer Suppressor Gene 1

Breast Cancer Suppressor Gene1 significance..…………………………6-7

i. BRCA1 and Breast Cancer

Experimental Rational…………………………………………………7-8

MATERIAL AND METHODS……………………………………………..

Cell culture conditions…………………………………………………10

Passage procedures……………………………………………………….11

Western Blot procedures………………………………………………12

RESULTS……………………………………………………………….13-18

DISCUSSION……………………………………………………………19-24

APPENDIX……………………………………………………………….25-26

REFERENCES…………………………………………………………….27-30

LIST OF FIGURES:

FIGURE 1:
Preliminary data of MCF-7 cells using the concentration study in the presence of Bisphenol A………………………………………………………….. Page 12

FIGURE 2:
MCF10A breast epithelial cells transfected with ER alpha IRES cDNA construct expressing high levels of ER alpha protein………………………………Page 13

FIGURE3:
BPA alters BRCA1 protein expression in MCF10A breast epithelial cells transfected with ER alpha cDNA construct ……………………………………………….Page 14

FIGURE 4:
Modulation of BRCA1 by BPA is dependent on ERα……………………..………………………………………………Page 15

FIGURE 5:
Synopsis of the proposed regulation of BRCA1 in the presences of Bisphenol A………………………………………….. ………………Page 17

FIGURE6:
Bisphenol A effect on PPAR gamma expression…………………………. Page 18

LIST OF ABBREVIATIONS

BPA…………………………………......Bisphenol A

BRCA1…………………………………..Breast Cancer 1, early onset

BRCA2…………………………………..Breast Cancer 2

DNA…………………………………... Deoxyribonucleic Acid

EPA……………………………………… Environmental Protection Agency

ER………………………………………Estrogen Receptor

ERIN……………………………………Estrogen Receptor-In (ER positive)

MCF-7…………………………………… Michigan Cancer Foundation-7

MCF10a………………………………..Michigan Cancer Foundation-10a

PPAR gamma……………………………Peroxisome proliferators-activated receptor gamma

Specific Aims

We tested the hypothesis and accomplished the objectives of this application by pursuing the following three specific aims:

1. To determine whether BPA alters BRCA1 expression in mammary epithelial cells.
2. To determine whether BPA alteration of BRCA1 is ER alpha dependent.
3. To determine the mechanism of how BPA regulates BRCA1 expression.

Introduction

What is Bisphenol A?

In the late 1800's, scientists discovered a colorless, solid today known as Bisphenol A. This chemical monomer is used in manufacturing polycarbonate plastics, the majority of epoxy resins, and certain other products such as flame retardants (1). Its first known synthesis occurred in 1905 by the German scientist, Thomas Zincke at the University of Marburg (2). Based on concepts of unpublished research on the Marburg campus, the combined structure of a phenol and acetone would shape the manufacture industry for years to come (Figure 1).

In the 1930's researchers discovered that Bisphenol A could be used as a man-made estrogen (2, 3). Short lived as an estrogen, BPA's ability to retain heat, minimize shatter, and the significant amount of optical clarity opened the door for a different usage. The chemical was used to produce polycarbonate plastic and epoxy resins, making it a one of the most widely used chemicals of its time. From dental products to the inner coating of metal cans, the next 70 years were prosperous for companies producing products that contained BPA. Even though reports started to surface that Bisphenol A

had the ability to percolate from products, there were no regulations sanctions placed on the chemical.

Bisphenol A exposure is widespread

Bisphenol A (BPA) is now one of the highest volume chemicals produced worldwide, with over 6 billion pounds each year and over 100 tons released into the atmosphere by yearly production(4). Reports indicate that over 800 million kg of the plasticizer BPA is generated annually in the United States alone (4). Figure 2. illustrates BPA's manufactured uses in the United States. Apart from food-related uses, BPA is used for automotive and other transportation equipment, optical media such as DVDs, electrical equipment, construction, linings inside drinking water pipes, thermal and carbonless paper coatings (5). In 1982, The National Toxicology Program established a lowest adverse effect level (LOAEL) as 50 milligrams of BPA per kilogram of body weight per day for lab animals (6). This discovery would lead to researchers across the globe questioning if BPA was really as safe and reliable as first suspected.

Regulation of BPA in the United States

In 2007, there was a huge concern about the chemical BPA and the amount of production and usage in the United States. Although BPA is regulated by the

Environmental Protection Agency (EPA), many citizens became alarmed when research panels started to reevaluate the exposure of BPA to humans. Research panels across the globe started to form in order to determine if BPA's exposure to humans was becoming more harmful than helpful although in 2006, it was deemed a safe chemical (7). Currently, the conversation of the safety of BPA and human exposure is still an open one. Researchers and government agencies are at odds on whether research has been exaggerated to make either side look like the winner in this heated battle. Countries other than the United States have taken action; Canada becomes the first country to deem BPA unsafe and start regulation in products that use BPA, including baby bottles. This led to some companies such as Playtex to take a closer look at BPA's safely and the exposure characteristics. The National Toxicology Program issued a final report that changed BPA's status from little or no concern of exposure to "some concern" (2, 8).

In 1991, there was a meeting held called the Wingspread Meeting, a workshop comprised of scientists, philosophers, lawyers, and environmental activists that discussed the widespread man-made chemicals that are having negative effects on the environment. Committee members coined the term "endocrine disruptors" as chemicals that are released into the environment that may have the potential to disturb endocrine systems of animals and potential humans (9). BPA was one of the chemicals named in this category based on its ability to bind to the estrogen receptor alpha and beta (10) and alter normal cellular functions (11). For example, in Maffini et.al (12) outlines BPA as a chemical that effects and alters the brain, mammary gland, increase weight, and disturb the reproduction tract (12)

BPA's mechanism of action

BPA is commonly known as an environmental estrogen due to its ability to bind to estrogen receptors and mimic estrogens' ability to stimulate proliferation of mammary epithelial cells (13). Thus, BPA exposure can have a strong impact on estrogen responsive tissues, like the breast. In particular, BPA has the capabilities to alter responses to estradiol in the mammary gland, increase branching in the gland, and reorganize terminal end buds (12). Numerous research studies have supported the idea that BPA exposure has an effect on cellular proliferation both *in vivo* and *in vitro*. One study investigated the cellular proliferation properties in BPA by taking lactating Sprague Dawley CD rats and placing them on a daily regimen of BPA that included a high dose at 250mg BPA/kg BW and a lower dose one tenth of the high dose 25 mg BPA/kg BW. The study concluded that BPA shifted the natural cell cycle and altered cell proliferation. The low concentration of BPA was found to contribute to chemically induce predisposed diagnoses of mammary cancer (14). Further, a manuscript from our lab, suggested that the loss of BRCA1 function may enhance BPA's proliferative effects in cells via estrogen-related pathways also at doses that reflect the levels of BPA measured in circulating blood of humans (15).

BPA is known to increase proliferation as a result of binding to the estrogen receptor alpha (ERα) and disrupting normal endocrine signaling through regulation of ER target genes (15, 16, 17)

Gaps in the knowledge of BPA and breast cancer

The mechanistic link between BPA and breast cancer is still unclear. The present work is a first step towards determining a mechanistic role of BPA in breast cancer. In this study, we focused on a potential link between BPA and the Breast Cancer 1 (BRCA1) gene. Following a brief introduction of BRCA1 and Breast Cancer, the rationale for investigating the link between BPA and BRCA1 will be discussed.

Breast Cancer and the Breast Cancer Susceptibility Gene 1 (BRCA 1)

Breast Cancer is a detrimental disease that affects millions of women per year and has become the second leading cause of death among women (18). According to the National Cancer Institute, the definition of Breast Cancer is a "Cancer that forms on the tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk) (18). It occurs in both men and women, although male breast cancer is rare". In the United States alone for 2011, new cases reported new cases of breast cancer in over 230,000 women; the death totals include 39,520 in females alone (18). It is thought that cancer initiates with a mutation in DNA. Risk factors that increase the chances of DNA damage include heredity, genetic mutations, lifetime

exposure to estrogen, behavioral risk factors that include diet and smoking and environmental influences that include chemicals and viruses.

Breast cancer diagnosis is divided into two categories: Hereditary and Sporadic. Hereditary breast cancers account for 5-10% of all reported cases. Within that 5-10% of reported cases, 40-45% is due to some type of mutations in BRCA1 and/or BRCA2 gene. Sporadic breast cancer cases make up 90-95% of all reported incident of the disease. Thirty three percent of those cases are caused by a decrease in BRCA1 protein expression (18). It has been reported that breast cancers in patients with inherited mutations in BRCA1 breast cancer have tumors that grow more rapidly than comparable sporadic tumors (19).

BRCA 1 has been linked to a diverse array of biological processes. Because BRCA1 has a wide range of functions that include cell cycle checkpoint control, oxidative stress regulator, and DNA repair functions and may play a role in the breast cancer incidents. Research suggests that BRCA1 may normally serve as a negative regulator of mammary epithelial cell growth and that this function is compromised in breast cancer either by direct mutation or by alternations in gene expression (19). In conjunction with these results, other researchers concluded that wild type BRCA1 protein may function, in part, to suppress estrogen-dependent mammary epithelial proliferation by inhibiting estrogen receptor alpha mediated transcriptional pathways related cell proliferation, and the loss of this ability may contribute to tumorigenesis (20). Researchers have study the effects of BRCA1 down regulation in breast epithelial cells and discovered that BRCA1 is expressed at higher levels in normal mammary cells than

in breast cancer cells and that diminished expression of BRCA1 increases the proliferation rate of both benign and malignant breast epithelial cells (20).

Experimental Rational- Estrogen Regulates BRCA1

There has been research that suggests that estrogen has the ability to regulate BRCA1 expression. One study in particular found that estrogen has the ability to alter BRCA1 protein expression in MCF-7 cells. Afshari et.al. investigated the possible relationships between estrogen stimulated cell growth and the expression of BRCA1 in breast cancer cells. They used MCF-7 cells, which express the ERα and performed a western blot analysis using standard techniques (21). Cell proliferation and regulation of BRCA1 was measured based on its effects of being exposed to estrogen. Results indicated that estrogen played a major role on both cell proliferation and regulation of BRCA1 protein expression in MCF-7 cells (22).

Therefore, considering BPA has the ability to act like estrogen and bind to the estrogen receptor, it is very important to determine whether it can also alter BRCA1 expression. Studies have shown that BRCA1 expression levels are directly reduced in sporadic tumors (23). More importantly, emerging evidence has indicated a broader role for BRCA1 in that somatic inactivation perhaps by promoter hypermethylation, may also lead to sporadic breast tumors (22).

The present work is the first step towards determining a mechanistic role of BPA in breast cancer, namely, whether BPA can alter BRCA1 expression. We hypothesized that BPA accelerates mammary epithelial cell proliferation, in part, through the reduction of BRCA1. Studies outlined in this thesis demonstrates (1) BPA induced changes in BRCA1 expression in estrogen receptor dependent and/or estrogen receptor independent cell line; and (2) propose a possible mechanism of BPA regulatory influence on BRCA1 expression. We anticipate that these studies will advance our understanding of the underlying mechanisms behind BPA action as an important determinant of breast cancer risk.

Materials and Methods

The following experimental cell culture models were used to identify variations in gene expression in response to BPA treatment: MCF-7 (a breast cancer cell line expressing estrogen receptor alpha (ERα), MCF10a (non-tumorigentic cells that do not express ERα), and ERIN (non tumorigenic MCF10a cells transfected with ERα).

The MCF-7 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 5% fetal bovine serum (FBS), 100 units/ml of penicillin and 100µg/ml of streptomycin.

MCF-10A (Non- estrogen receptor alpha expressing) and ERIN (Estrogen receptor alpha expressing) were graciously given to us from Ben Ho Park from the Department of Oncology at The Johns Hopkins University.

MCF10a and ERIN cell lines were cultured in DMEM/F-12 Media (Invitogen # 11039047) no phenol red in 2% charcoal stripped dextran treated fetal bovine serum (SH30068.03), Penicillin /Strep (Sigma E -9644 (100ug/ml), acetic acid, Hydrocortisone, 95% Ethanol (Sigma H-0888), EGF (100ug/ml) Cholera Toxin (1mg/ml Sigma C-8052), Insulin (10mg/ml) (Sigma I 9278).

Procedure for passaging MCF-7, ERIN, and MCF10a Cells

Cell growth was checked under microscope to confirm that the cells are 90%-100% confluent. Flasks were washed in 1X PBS and trypsin was added to T-75 flasks and placed in the incubator at 37ºC for 3-5 minutes to detach cells. Cells were resuspended, placed in 15 ml centrifuge tube and centrifuged for 5 min at 2000 rpm at 4°*C*.

Procedure for lysis of adherent cells (6 well plate) using RIPA buffer

Growth medium was removed and cells were washed with cold PBS. Ice cold PBS was added and the plate was scraped to remove the cells. Cell lysates were then transferred to a tube on ice. Tubes were spun at 500 rpm in the cold room for 5 minutes. The supernatant was discarded. Cells were resuspended in 100µl of RIPA buffer (Radio Immuno Precipitation Assay) and placed ice for 10 minutes to lyse cells. The tube was centrifuged at 14,000x g for 15 minutes. The supernatant was placed in a newly labeled tube and the pellet was discarded. Protein product was stored at -70° C until further use.

BPA Treatment

For BPA treatment, MCF7, MCF10a and ERIN cell lines were treated with DMEM without phenol-red supplemented were purchased from BioWhittaker, Inc. (Walkersville, MD). The BPA was purchased from Sigma-Aldrich Corp. (St. Louis, MO) and dissolved in ethanol at stock concentration of 10mM.

Cells were treated (for 48 hours) with the following concentrations of BPA dissolved in ETOH 10^{-6} µM, 10^{-8}µM, 10^{-10}µM, 10^{-12}µM, 10^{-14}µM, 10^{-16}µ. Estradiol was used as a

positive control and ETOH was used as a negative control. After 48 hour treatment, cells were harvested, proteins were isolated using standard procedures as described above and quantitated for Western Blot analysis. All experiments were performed in triplicate.

Western Blot analysis

The proteins were separated on SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 1X blocking buffer (Sigma, St.Louis, MO), incubated with primary antibodies, and washed with 1x PBS containing Tween-20.

The membrane was incubated in blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dairy dry milk) at room temperature for 1 hour. The membrane was then washed three times for 5 minutes in TBS/T. The membrane and the anti-BRCA 1 (Santa Cruz) antibody (1:1000) were incubated overnight at 4° C under gentle agitation. The membrane was then washed 3 times for 5 minutes each. Next, the membrane was incubated in a secondary antibody (1:2000) in order to identify biotinylated protein markers for 1 hour at room temperature. After washing the membrane in TBS/T 3 times for 5 minutes each the proteins were detected with LumiGLO and exposed to x-ray film. GAPDH was used as a loading control.

Results

1. **To determine whether BPA alters BRCA1 expression in mammary epithelial cells.**

Since we wanted to establish a direct relationship between BPA exposure and BRCA1 expression, we performed a concentration study using environmental relevant concentrations of BPA and the breast cancer cell line MCF-7 cells. Lysates from MCF-7 cells were transfected with BPA (10^{-5} M, 10^{-7} M, $10^{-9\ M}$, and $10^{-11\ M}$) for 48 hours. Western blot analysis indicates that dose plays a role in the BRCA1 expression and a non-monotonic dose response curve was observed during this experiment.

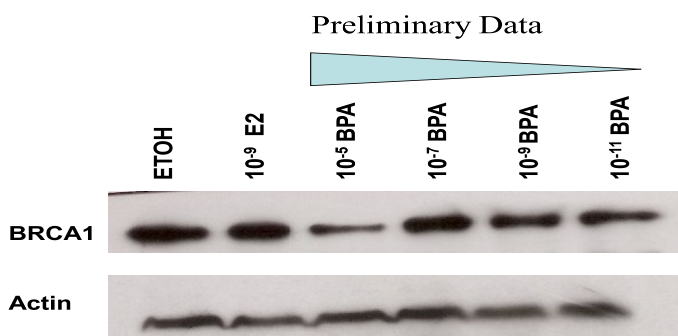


Figure 1: Preliminary Data of MCF-7 cells using a concentration study in the presence of Bisphenol A. Lysates from MCF7 cells were transfected treated with BPA for 48 hours, separated by gel electrophoresis and stained with an antibody against BRCA1. A monoclonal antibody against actin serves as a loading control.

2. **To determine whether BPA alteration of BRCA1 is ER alpha dependent.**

Since we were interested in determining if BPA-induced changes in BRCA1 protein expression and the Estrogen receptor dependency , we decided to select two cell models, MCF10a (ER alpha non-expressing cells) and ERIN (MCF10a cells with ER alpha construct). Western blot analysis was conducted using the lystates of both cell lines and exposed to BPA for 48 hours. Initially, we confirmed the presence of ERα in MCF 10a and ERIN cell lines (Figure 2). Findings indicate that ERIN cells are ER alpha positive and MCF10a cells are ER alpha negative.

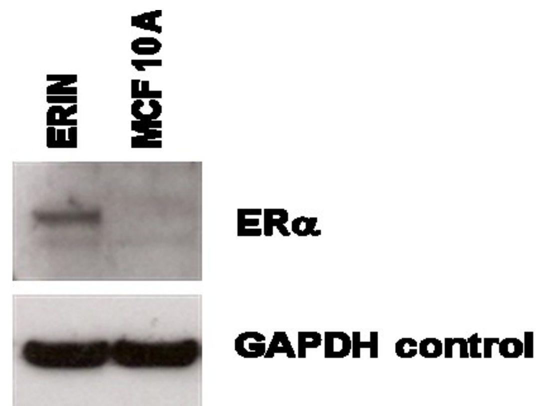


Fig. 2. **MCF10A breast epithelial cells transfected with ERα IRES cDNA construct express high levels of ERα protein**.
Lysates from MCF10A breast epithelial cells transfected with ERα cDNA construct (ERIN cells) were separated by gel electrophoresis and stained with an antibody against ERα. A monoclonal antibody against GAPDH serves as a loading control.

After confirming that the ERIN cell line was ERα positive we conducted the dose-concentration study with BPA. The concentration studies were conducted on weekly bases and performed in triplets. Concentrations include 10^{-6} M, 10^{-8} M, 10^{-10}M, 10^{-12} M, 10^{-14} M, 10^{-16} M. Estradiol (10^{-9} M) was used as a positive control. Results indicated a non-monotonic dose-response (inverted-U-shaped dose-response curve) relationship between BPA exposure and BRCA1 protein expression in ER alpha expressing cell lines (Figure 3).

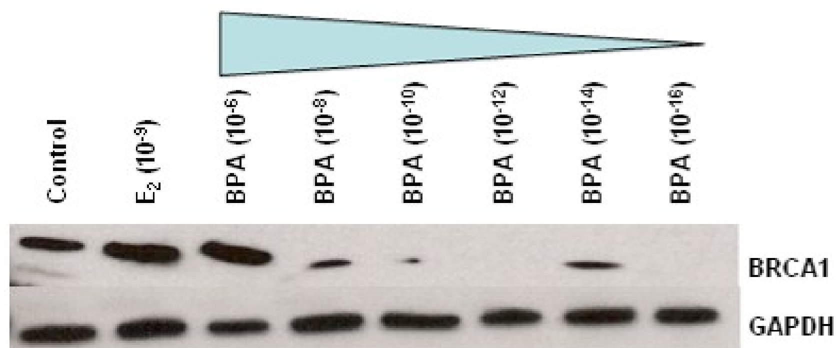


Fig. 3. BPA alters BRCA1 protein expression in MCF10A breast epithelial cells transfected with ER alpha cDNA construct.
Lysates from MCF10A breast epithelial cells transfected with ERα cDNA construct (ERIN) treated with BPA for 48 hours were separated by gel electrophoresis and stained with an antibody against BRCA1. A monoclonal antibody against GAPDH serves as a loading control.

MCF10a cells were also treated with BPA under the same conditions as described for the MCF-7 and ERIN cells. In contrast to the ERα- expressing cells, there was no change detected in BRCA1 expression in the estrogen receptor alpha negative MCF10a cell line (Figure 4).

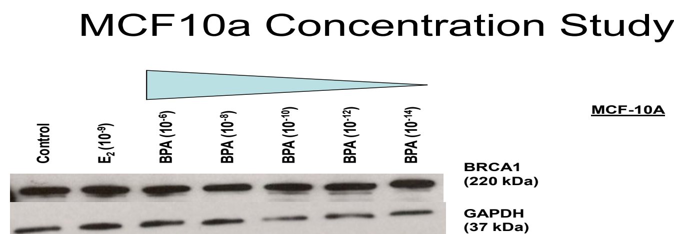


Fig. 4. Modulation of BRCA1 by BPA is dependent on ERα
Lysates from Parental MCF-10A treated with BPA for 48 hours were separated by gel electrophoresis and stained with an antibody against BRCA1. A monoclonal antibody against GAPDH serves as a loading control. BPA does not alter BRCA1 protein expression in parental MCF10A breast epithelial cells (untransfected).

3. **To determine the mechanism of how BPA regulates BRCA1 expression**

This aim sought to determine the mechanism of how BPA regulates BRCA1 expression. These studies focus on explaining the underlying mechanism of how BPA might decrease BRCA1 protein at the low, environmentally relevant doses in our in vitro

assays. Based on available data at the time of these studies, the Jones lab decided to take an indirect approach towards addressing this question. Among the genes that have been linked to the regulation of BRCA1, Peroxisome proliferators-activated receptor gamma (PPAR gamma), was the only one that was also found to be altered by BPA exposure. PPAR gamma, a nuclear hormone receptor, not only plays a role in the coordination of adipocyte gene expression and differentiation, but can also regulate BRCA1 expression (23). In particular, M.Pignatlli et.al treated MCF-7 cells with two PPAR gamma agonists, Ligands, 15dPG-J2 and rosiglitazone, for sixteen hours at different concentrations and recorded BRCA1 gene expression. These studies concluded that the two PPAR gamma agonists could induce the expression of BRCA1 (23). Researchers have also discovered that certain synthetic compounds, in particular an isomer of BPA had the ability to bind to PPARγ specifically, Spiegelman et.al discovered that bisphenol A diglycidyl ether (BADGE), a synthetic isomer of BPA, can bind to the PPAR gamma receptor and act as a pure antagonist for PPAR gamma (24).

BPA Regulation of BRCA1

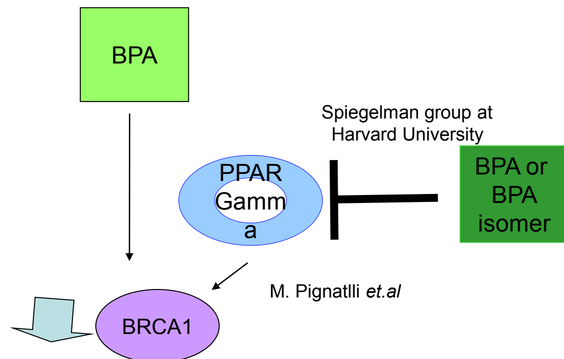


Figure 5. Synopsis of the proposed regulation of BRCA1 in the presences of Bisphenol A.

Taken together, the two previous studies that show that PPAR gamma agonist can increase BRCA1 expression and that BADGE can antagonize PPAR gamma, we hypothesized that BPA may be decreasing BRCA1 protein expression by antagonizing PPAR gamma protein(see figure above). We conducted a dose response experiment that measured PPAR gamma protein expression after 48 hours of BPA treatment at concentrations of 10^{-6}M, 10^{-8}M, 10^{-10}M, 10^{-12}M, 10^{-14}M and 10^{-16} M.

PPAR gamma Concentration Study

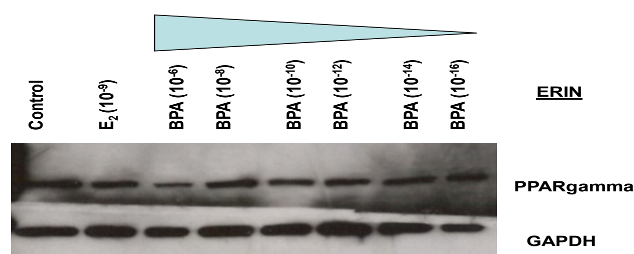


Fig. 6. Bisphenol A effect on PPAR gamma expression

Lysates from ERIN treated with BPA for 48 hours were separated by gel electrophoresis and stained with an antibody against PPARgamma. A monoclonal antibody against GAPDH serves as a loading control.

No significant change in PPAR gamma protein expression was observed in the BPA-treated ERIN cell line (Figure 6).

Discussion

There are many factors that have been associated with breast cancer risk. For example, studies have shown that women with mutations in BRCA1 have an increased risk of developing breast cancer (25). It is also known that BPA exposure-can induce proliferation in human mammary epithelial cells and in mouse models suggesting that BPA may contribute to breast tumorigenesis. Instead of focusing on one risk factor, our lab sought to determine how combinations of risk factors may work together to contribute to breast tumorigensis.

This study examined the mechanism behind BPA exposure and in particular, its effects on breast cancer susceptibility gene (BRCA1). Our data indicate in non-tumorigenic ERIN cells over expressing estrogen receptor (ER) - alpha that an environmentally relevant dose of BPA can decrease BRCA1 protein levels. This decrease in BRCA1 was not observed in non- estrogen receptor expressing, MCF10A cells. Therefore these data suggest that the effects BPA exposures on the BRCA1gene expression are estrogen receptor dependent.

These findings are supported by data reported in articles published by Khan et.al (26) which indicated that xenoestrogens can mimic estrogen in cell lines. This group sought to find out if BPA and estradiol (E_2) regulate similar genes involved in growth and development. They hypothesized that the model systems, C4-12 ER- alpha HA breast cancer cell lines and Yeast strain RS188N, BPA would regulate genes in a manner similar to E_2. Using a microarray approach, results indicated that five genes link BPA and E2 regulation in the ER alpha HA cell line. Results also indicated some cells were regulated by E2 or BPA and that in the yeast; BPA had no effect on ER alpha's

transcriptional activity. There was, however a 100-fold change in mammalian cell expression. Khan et. al (26) concluded by saying that, data suggest that there are genes that are some sensitive to low doses of BPA or other endocrine disruptors. Most notably, their microarray results indicated that BPA- treatment of MCF 7 cells that over expressed estrogen receptor alpha can decrease BRCA1, which is consistent with our results.

Although the work by Khan et. al, supports some of my findings, there are key elements that differ between the studies. Cells were only treated for 3 hours in Khan et. al., however, in comparison, our current study examined cell lines exposed to BPA for 48 hours. Also, in Khan et.al the only concentrations of Bisphenol A tested was 10^{-6} M, which may be viewed as environmentally irrelevant as opposed to the nanomolar concentrations used in our study.

Khan's study concluded that treating MCF-7 cells can transcriptionally down regulate BRCA1 expression; however, the study had some limitations, including the fact that these results are based on microarray studies. Many can argue that microarray analysis can give false positives leading to less than accurate results. Therefore validating microarray with QPCR and/or protein studies is important to confirm actual down regulation of the gene. By conducting protein studies, our work could in fact be a confirmation of Khan's paper. Considering both studies determined that BPA has the ability to down regulate BRCA1 gene expression provides evidence is provided to indicate that BPA may increase breast cancer development in women through a pathway that involves changes in the BRCA1 gene expression.

Non- monotonic dose curves describes the action of some chemicals in which their targeted effects demonstrate graphs that may resemble an inverted U-shaped curve, where the highest levels of the toxicant-induced effects can appear in the middle of the graphed data and the lowest levels are at the beginning and the end of the graph. While it's often understood why a low concentration would generate a minimal effect, it's not clear why the intermediate concentrations would show either the lowest or highest response. Our findings are consistent with other studies that reported a non-monotonic dose-response curve for the effect of Bisphenol A on endpoints such mammary gland morphological parameters (i.e. terminal end buds, ductal extension) in perinatal exposed mice (27).

In our study here we found that a low dose of BPA, unexpectedly, had the greatest ability to decrease BRCA1 protein expression. Our data indicate that in non-tumorigenic cell lines, ERIN cells (over expressing ER) - low dose BPA decreases BRCA1, but this does not occur in MCF10A cells (non-ER expressing). These data suggest that the effects of BPA exposure on the BRCA1 estrogen receptor dependent. Other research articles have found that BRCA1 down regulation can occur in both ER-positive and ER- negative cell lines (27). These results suggest that BPA may alter cell gene expression through multiple pathways (e.g. through ER alpha mediated effects at low BPA, but through other receptors at higher BPA exposure concentrations).

In order to determine the mechanism by which Bisphenol A down regulates BRCA1 expression, we wanted to find a connection between the two. We considered that finding a protein that is both activated by BPA and alters both BRCA1expression would provide clues. Not much is known about a direct connection between these two

factors, so an indirect connection had to be established. The indirect approach to understanding the mechanism started with identifying a protein that had the ability to regulate BRCA1, and also could be altered by BPA exposure the peroxisome proliferator-activated receptor, (PPAR gamma) meets all these criteria.

To justify the use of PPAR gamma as an indirect approach to determining this mechanism, we took a look at two studies before conducting our own study. One study, M. Pignatlli *et.al,* tested the hypothesis that PPAR gamma plays an important role in the BRCA1 regulatory pathway involved in the pathogenesis of sporadic breast and ovarian cancer (23). In order to prove such theory, researchers conducted western blot analysis of the BRCA1 protein in MCF-7 after 16 hours of incubation with various concentrations of 15dPG-J2, which Trans-activate PPAR gamma and Rosiglitazone (an anti diabetic drug in the family of PPAR gamma activators). Research concluded that the endogenous nuclear levels BRCA1 protein increased significantly 16 hours after treatment of MCF7 cells, compared to the untreated control (23).

A second research paper focusing on PPAR gamma examined a purified compound that exhibited PPAR gamma binding activity. Using high pressure liquid and gas chromatograph/mass spectrometry, the compound was found to be Bisphenol A diglycidyl ether (BADGE), a synthetic substance used in polycarbonate and industrial plastic production (23). Because of its inability to bind to Rosiglitazone, BADGE is a perfect antagonist for PPAR gamma. Researchers used 3T3-Lli and 3T3 F442A cells to conduct a ligand binding assay. Results indicated that BADGE is a pure antagonist for PPAR gamma. Based on the two papers, we hypothesized that BPA may be altering BRCA1 protein expression by changing the activity PPAR gamma protein.

In our study, we also examined protein expression western blotting using ERIN cells exposed to BPA. Our results indicated that BPA regulation of BRCA1 is not mediated through reduction of PPAR gamma protein. This may have occurred because we made the assumption that like BADGE, BPA may also have some antagonist activity towards PPRA gamma.

One key next step in the research would include understanding and determining if BPA alters BRCA1 on a transcriptional level. Although microarray studies were conducted in the initial stages of this research, they were not used in the results of this project. Confirming experiments using mRNA could have solidified these experiment results (29).

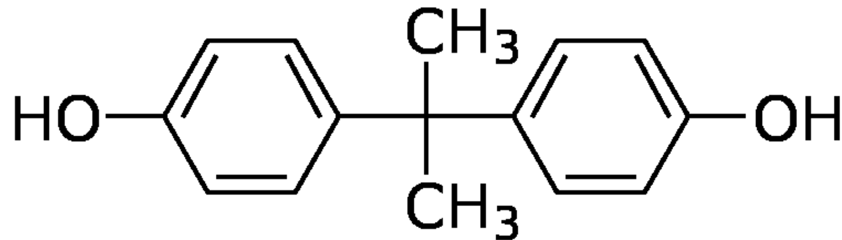
Further experiments can also be conducted to determine if reduced BRCA1 caused by BPA affects any of BRCA1's normal cellular functions such as DNA repair, cell cycle regulation, and protein degradation. Many of these pathways are co-regulated and their regulation can differ based on the level of stress within the cell. The dose response curve leaves unresolved questions in this research. In most toxicology studies, as the dose increases so does the response but in this case different concentrations cause responses a lower than excepted doses. Because of this, the known environmentally relevant doses regulated by the EPA should be revaluated. Experiments like this one shed the light on standards set to protect the human population.

In summary, this study helped to determine that treating cells with BPA at levels comparable to human exposures, alters BRCA1 expression, and changes in BRCA1 expression by BPA is concentration- and ER alpha dependent. In mammary gland

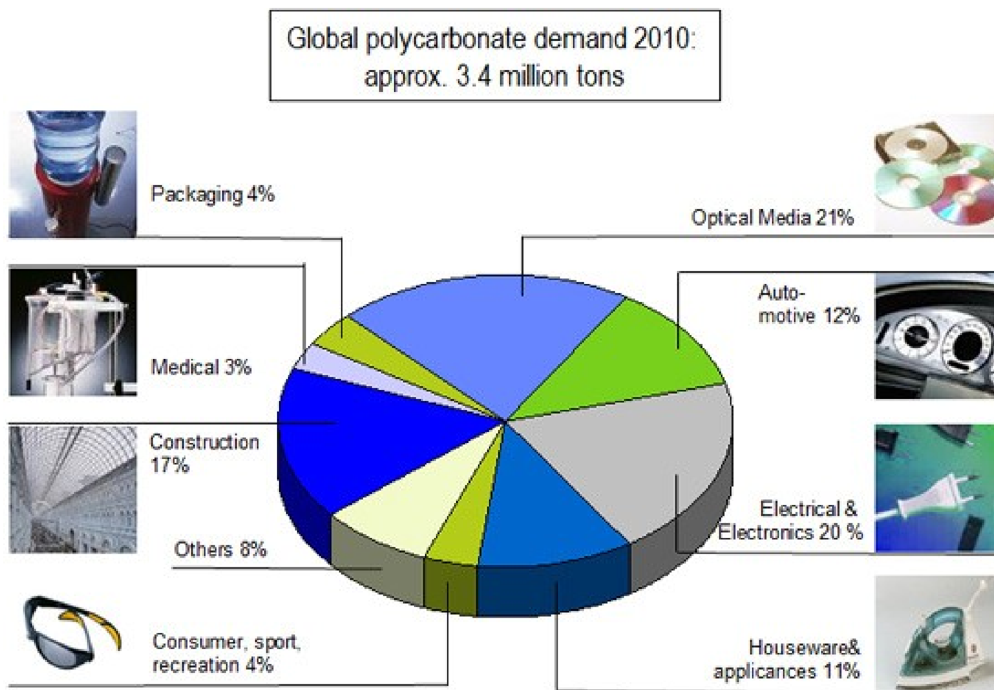
epithelial cells (30) BRCA1 expression is normally induced in periodic waves that correspond to defined developmental periods in which intense proliferation, morphogenesis and/or apoptosis take place. Given our observations, it is not unreasonable to expect that unintended exposure to BPA may alter the normal periodic waves of induced changes in BRCA1 expression during mammary development (31). Therefore based on our results, the timing and level of human BPA exposure is indeed important particularly during critical windows of mammary gland development where levels of BRCA1 normally expected to be high.

Appendix

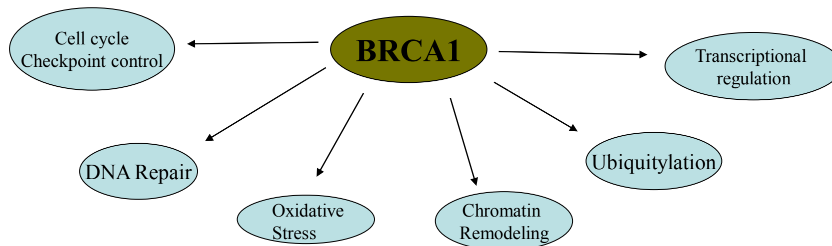
A. Bisphenol A



B: Bisphenol A distribution
http://www.bisphenol-a-europe.org/index.php?page=polycarbonates



C: Breast Cancer 1 (BRCA 1) involvement in cellular processes.



References

1. Mannsville. 2008a. Chemical Products Synopsis: Bisphenol A, Mannsville Chemical Products Corp., January 2008

2. www.ewg.org/reports/bpatimeline- Timeline: BPA from Invention to Phase-Out

3. Dodds, EC and W Lawson "Molecular structure in relation to oestrogenic activity. Compounds without a phenanthrene nucleus". Proceedings of the Royal Society. London B. 1938 **125**:222-232.

4. Soto, A. et.al, "An evaluation of evidence for the carcinogenic activity of bisphenol A". Reproductive Toxicology, 2007. **24** : p.240-252

5. EPA review 2010- Agency, U.S. Environmental Protection Agency. [Website] 2010 March 29, 2010; Available from www.epa.gov/oppt/existingchemicals/pubs/.../bpa_action_plan.pdf

6. The National Toxicology Program 1982 http://ntp.niehs.nih.gov/ntp/htdocs/.pdf

7. www.epa.gov

8. http://cerhr.niehs.nih.gov/evals/bisphenol/bisphenol.pdf

9. www.ourstolenfuture.org. Our Stolen Future: New Science: Basic Mechanisms: Non-monotonic curves.

10. Markey, C.M., Rubin, B.S. Soto, A.M., Sonnenchein, C., “Endocrine disruptors: from wingspread to environmental developmental biology”. J. Steroid Biochem. Mol.Biol. 2002 **83**, 235-244

11. Soto, A.M., Sonnenschein, C., Chung K.L., Fernandez, M.F., Olea, N., Serrano, F.O., “The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environment pollutants”. Environ. Health Perspect. 1995 **103**, 113-122

12. Maffini, M.V., Soto, A.M., Sonnenschein, C., “Neoplasia as development gone awry: the role of endocrine disruptors”. Int. J. Androl. 2008 **31**, 288-293

13. Vandenberg, L.N., Maffini, M.V., Sonnenschein, C., Rubin, B.S., Soto, A.M., “Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption”. Endocr. Rev.2009 **30**, 75-95

14. Lamrtiniere, Jenkins, Bentancourt, Wang, Russo “Intro exposure to the Endocrine Disruptor Bisphenol A alters susceptibility for mammary cancer”. Horom Mol Biol Clin Investig 2011 March 1; **5** (2) 45-52

15. Jones, L., Sampson, A., Kang, H.J., Kim, H.J., Yi, Y.W., Kwon, S.Y., Babus, Wang, A., Bae, I. “Loss of BRCA1 leads to an increase sensitivity to Bisphenol A”.2010 Toxicology Letters **199**. 261-268

16. Welshons, W.V.et.al, “Large effects from small exposures.III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure”. Endocrinology, 2006. **147**: p S56-S69

17. Vom Saal et.al Chapel Hill bisphenol A expert panel consensus statement: integration of mechanism, effects in animals and potential impact human health at current levels of exposure. Reproductive Toxicology. **24** : p 131-138

18. www.cancer.gov

19. Holt, J.T. et.al, "Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression". Nature Genetics, 1995. **9** : p.444-450

20. Fan, S., et.al, "BRCA1 inhibition of estrogen receptor signaling in transfected cells". Science,1999 **284:** p.1354-1356

21. Afshari et.al, "Estrogen Up regulation of BRCA1 Expression with No Effect on Localization". Molecular Carcinogenesis 1998 **22**: 102-109

22. Mueller, C. And Roskelley, C. **"**Regulation of *BRCA1* expression and its relationship to sporadic breast cancer" Breast Cancer *Res* 2003, **5**:45-52

23. Pignatelli, M. Claudia, C., Santos, A. Perez-Castillo, A. "Enhancement of BRCA1 gene expression by the peroxisome proliferators-activated receptor gamma in the MCF-7 breast cancer cell line". Nature-Oncogene. 2003 **22**, 5546-5450

24. Spiegelman, B. et.al" A synthetic Antagonist for the Peroxisome Proliferator-activted Receptor gamma Inhibits adipocyte differentiation". The Journal of Biolo. Chem. 2000. **275, 3** 1873-1877

25. Friedman LS, Ostermeyer EA, Szabo CI, Dowd P, Lynch ED, Rowell SE, and King MC. "Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families". Nat.Genet. 1994, **8**:399-404.

26. Khan et.al. "Gene expression profiling reveals novel regulation by Bisphenol A in estrogen receptor alpha positive human cells" Environ Res. 2005 **100**(1):86-92

27. Conolly RB, Lutz WK 2004. “Nonmonotonic dose-response relationships: mechanistic basis, kinetic modeling, and implications for risk assessment”. Toxicol Sci **77**:151–157

28. Khan et.al. “Gene expression profiling reveals novel regulation by Bisphenol A in estrogen receptor alpha positive human cells” Environ Res. 2005 **100**(1):86-92

29. Dov Greenbaum**,** Christopher Colangelo**,** Kenneth Williams and Mark Gerstein “Comparing protein abundance and mRNA expression levels on a genomic scale” Genome Biology 2003**, 4:**117
30. Galizia E, Giorgetti G, Piccinini G, Santinelli A, Loretelli C, Bianchi F, Gagliardini D, Carbonari G, Pisa, E, Belvederesi L, Bracci R, Ferretti C, Corradini F, Cellerino R. “BRCA1 expression in triple negative sporadic breast cancers”. Anal Quant Cytol Histol. 2010, **32**(1):24-29.
Jing F, Yuping W, Yong C, Jie L, Jun L, Xuanbing T, Lihua H. “CpG island methylator phenotype of multigene in serum of sporadic breast carcinoma”. Tumour Biol. 2010, **31**(4):321-331.

31 Jing F, Yuping W, Yong C, Jie L, Jun L, Xuanbing T, Lihua H. “CpG island methylator phenotype of multigene in serum of sporadic breast carcinoma”. Tumour Biol. 2010, **31**(4):321-331.

31.