Toxicology Reports 2 (2015) 817–823

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# Differential effect of estradiol and bisphenol A on *Set8* and *Sirt1* expression in prostate cancer

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ARTICLE INFO

*Article history:*
Received 19 September 2014
Received in revised form
19 December 2014
Accepted 28 January 2015
Available online 17 February 2015

*Keywords:*
BPA
Prostate
Set8
Sirt1

ABSTRACT

Exposure to estrogenic compounds has been shown to epigenetically reprogram the prostate and may contribute to prostate cancer. The goal of this study was to determine the effect of physiological doses of estradiol and bisphenol A (BPA) on the expression of histone modifying enzymes (HMEs) in prostate cancer. Using two human prostate cancer cell lines we examined the expression of *Set8*, a histone methyltransferase, and *Sirt1*, a histone deacetylase, after exposure to estrogen or BPA. These experiments were carried out in the presence of natural hormones to understand the impact of additional exposure to estrogen or BPA on HME expression. We found differential expression of the HMEs in the different models and between the different compounds. Further, we determined that the changes in gene expression occurred via estrogen receptor signaling using the ER antagonist, ICI 182,780 (fulvestrant). Interestingly we found that the combination of ICI with estrogen or BPA greatly affected the expression of *Set8*, even when the hormone alone had no effect. This study demonstrates that the effects of estrogen and BPA on HME expression vary and that the presence of both the estrogen receptor and androgen receptor may be important for therapeutic intervention.

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## 1. Introduction

Prostate cancer affects one in six men in the United States with more than 225,000 new cases each year [1]. There are only a few known risk factors for prostate cancer, including age, family history, and ethnicity. Advances in treatment have led to a five-year survival rate of 100% for men with local and regional disease [2]. For advanced disease, current therapies target the androgen receptor (AR) or androgen production. Treatment causes the tumor to regress, however relapse occurs and the resulting disease is castration resistant prostate cancer (CRPC) [3]. For this advanced stage, there are few treatment options available and the disease eventually causes lethality.

Environmental exposures have been demonstrated to contribute to disease development and progression [4], including prostate cancer [5]. Specifically, bisphenol A (BPA) is a synthetic estrogen widely produced in the United States [6] that has been shown to impact the development of the nervous [7,8] and reproductive systems [9]. The prostate gland in particular has been found to be susceptible to both estrogen and BPA exposure in animal models and this exposure impacts disease development later in life ([10,11]). The mechanism of action does not appear to be mutagenic but rather evidence indicates that in both breast and prostate tissue BPA alters the epigenome [12,13].

Tang et al. [11] studied the impact of estrogen and BPA on the prostate methylome and found differential methylation patterns for multiple genes throughout the life of exposed animals. This study demonstrated that early

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http://dx.doi.org/10.1016/j.toxrep.2015.01.016
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exposure can cause lasting epigenetic changes to the prostate methylome. The goal of the present study was to analyze the expression of histone modifying enzymes (HMEs) that might be altered after exposure to estrogen or BPA in human prostate cancer. We analyzed the expression of two HMEs, SET8 and SIRT1, that have previously been shown to be involved in both estrogen signaling and cancer.

SET8 is the only enzyme to found to monomethylate histone H4 lysine 20 (H4K20me1). In breast cancer, it has been shown to be an essential co-activator of estrogen receptor alpha (ERα) mediated transcription [14]. Furthermore, SET8 plays a role in the epithelial to mesenchyme (EMT) transition in breast cancer by regulating both E and N cadherin [15,16]. In prostate cancer, SET8 has been found to be enriched at the AR target gene PSA and loss of SET8 resulted in a loss of PSA expression. Additionally, SET8 was found to be necessary for AR induced proliferation of prostate cancer cells [17].

SIRT1 is a histone deacetylase (HDAC) that is involved in regulating ERα transcription in breast cancer [18]. Estrogen has been shown to increase the expression of SIRT1 in breast cancer cells and this required the ER. Furthermore, depletion of SIRT1 decreased growth of breast cancer cells [19]. SIRT1 expression is increased in human prostate cancer [20] and also plays a role in EMT in prostate cancer [21]. Specifically, SIRT1 overexpression induced EMT in an epithelial prostate model and knock down of SIRT1 restored cell adhesion in prostate cancer cells. Similar to SET8, this was mediated by regulation of E-cadherin. In addition, *Sirt1* was decreased after exposure to genistein, a phytoestrogen, in human prostate cancer cells [22], and Taken together, these studies indicate that *Set8* and *Sirt1* play important roles in cancer and that their expression may be regulated by estrogenic compounds.

Many studies have examined gene expression in response to estrogenic compounds. Typically in these studies, cells are maintained in full media while experiments are carried out after hormone deprivation [23–25]. This approach is utilized since it eliminates confounding issues, such as the presence of other steroid hormones. One caveat to this approach is that it is not representative of physiological conditions, including androgen depletion, as there are still natural and synthetic estrogens present in the system. To understand what might be occurring under more physiological relevant conditions, we analyzed the expression of these genes in the presence of physiological hormone levels rather than after hormone deprivation. Using two different models of human prostate cancer, we found significant changes in the expression of *Set8* but few in *Sirt1*. We also found that the changes in *Set8* expression were dependent on ER as they were reversed by an ER antagonist. Lastly, estrogen and BPA did not elicit the same outcomes with regards to *Set8* and *Sirt1* expression. Taken together this data demonstrates that exposure to estrogenic compounds affects the expression of HMEs differently. Furthermore, there are distinct changes in the different models examined indicating that the regulation of these enzymes may change in different disease states.

## 2. Materials and methods

### 2.1. Cell culture and treatment

The LNCaP and PC3 cell lines were a kind gift from Dr. Shuk-Mei Ho (University of Cincinnati, Cincinnati, OH). The LNCaP cell line was maintained in RPMI/1640 medium (Life Technologies) supplemented with 2 mM/L L-glutamine and 1 mM sodium pyruvate (Gibco). PC3 cells were maintained in DMEM/F12 medium (ATCC). Both medium were supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals) and 100 units/mL of penicillin–streptomycin (Life Technologies). Cells were cultured at 37 °C in a 5% $CO_2$ heated incubator.

17β-Estradiol, bisphenol A, and ICI 182,780 (Fulvestrant) were purchased from Sigma–Aldrich. Cells were treated with 1, 5, or 10 nM estradiol or 10, 25, or 50 nM bisphenol A for 24 h. Where indicated, the ER inhibitor ICI was added at 10 μM at the same time as the estradiol or BPA. All treatments were for 24 h and 95% ethanol (0.1%) was used as the vehicle control.

### 2.2. RNA isolation and PCR

LNCaP and PC3 cells were seeded in six well dishes. After 48 h the cells were treated with either 17β-estradiol or BPA with or without ICI. Total RNA was isolated via Trizol reagent (Life Technologies) as recommended by the manufacturer. Reverse transcription was done using the High Capacity RNA to cDNA kit (Life Technologies) as recommended by the manufacturer. PCR was performed to analyze expression levels of *Sirt1*, *Set8*, and *GAPDH*. The expression level of each gene was normalized to the expression of *GAPDH*. *GAPDH* was chosen as the house-keeping gene because its expression did not change in response to estrogen or BPA. The relative expression was analyzed using Image J software. The experiments were repeated with three individual sets of samples.

### 2.3. Statistical analysis

Data were expressed as mean ± SD. Bonferroni post hoc test (correction test) was performed following ANOVA (Prism v4.0, GraphPad, CA) for multiple comparisons to determine the statistical significance.

## 3. Results

### 3.1. Differential expression of Set8 and Sirt1 in response to estrogen and BPA in LNCaP cells

To understand the effects of estrogen and BPA on the expression of HME genes in human prostate cancer we first utilized the LNCaP cell model. These cells are dependent upon androgens for growth, express a mutant AR and express ERβ. Previous experiments analyzing the effects of estrogen or BPA have been performed under conditions of hormone depletion. To understand changes that occur under physiologically relevant conditions, the treatments

were performed in complete media (FBS) rather than in hormone depleted media (CSS). Three physiologically relevant doses for both estrogen and BPA were chosen to mimic the range of human exposure. The samples were collected 24 later since this time point has been previously shown to allow sufficient time for transcription following hormone treatment [26].

The effects of E2 and BPA on Set8 and Sirt1 expression in LNCaP cells were examined 24 h after exposure. The expression of *Set8* was unaffected by estrogen treatment (Fig. 1A left panel). In contrast, estrogen (5 nM) induced a 1.4-fold increase in *Sirt1* expression (Fig. 1A). Next we examined the effect of BPA on gene expression in LNCaP cells. *Set8* expression showed a trend of increased expression following BPA exposure, with an average of 1.7-fold increase (25 nM), approaching statistical significance. There was however no significant change in the expression of *Sirt1* (Fig. 1B). This was slightly different than the effect of estrogen implying that different exposures to estrogenic compounds have distinct impacts on gene expression. Furthermore, these experiments show that in the presence of natural levels of hormones, BPA or additional estrogen exposure can significantly change the expression of HMEs.

### 3.2. Differential expression of Set8 and Sirt1 in response to estrogen and BPA in PC3 cells

There are dramatic changes in prostate cancer as the disease progresses. Initially it is androgen dependent but progresses to CRPC. To aid our understanding of the differences between androgen dependent and CRPC we analyzed the expression of *Set8* and *Sirt1* in response to estrogen and BPA in PC3 cells, a castration resistant model. PC3 cells lack the AR but express both ERs. Exposure to estrogen caused a 30% reduction in *Set8* expression at all three doses but had no effect on *Srit1* expression (Fig. 2A). Similarly, BPA also caused a 20% reduction in *Set8* expression but only at the 25 nM dose and had no effect on *Sirt1* expression in PC3 cells (Fig. 2B). While both estrogen and BPA caused a decrease in *Set8*, this is different from the results in LNCaP cells, demonstrating different outcomes in different prostate cancer models.

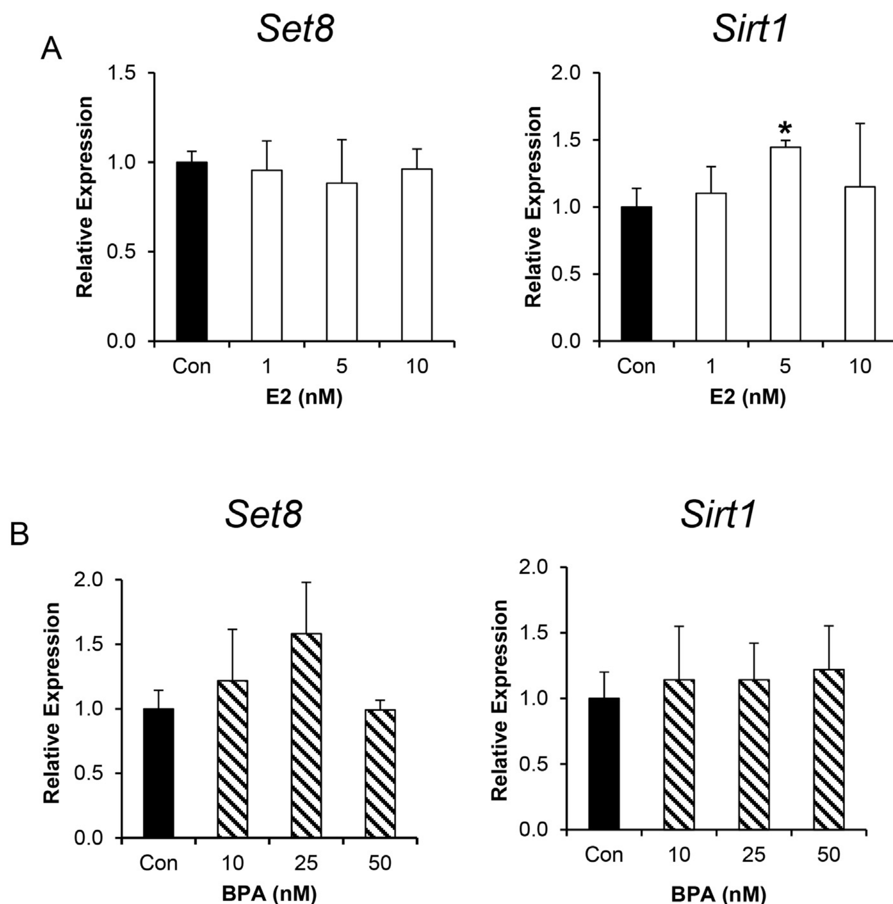


**Fig. 1.** Effect of estrogen or BPA on gene expression of HMEs in LNCaP cells. (A) LNCaP cells were treated with the indicated doses of estrogen for 24 h and the expression of *Set8* (left panel) or *Sirt1* (right panel) was analyzed. (B) LNCap cells were treated with the indicated doses of BPA for 24 h and the expression of *Set8* (left panel) or *Sirt1* (right panel) was analyzed. Image J was used to quantitate the results and the expression of each gene was normalized to *GAPDH* from the same sample. Results are from three independent experiments (*$p < 0.05$).

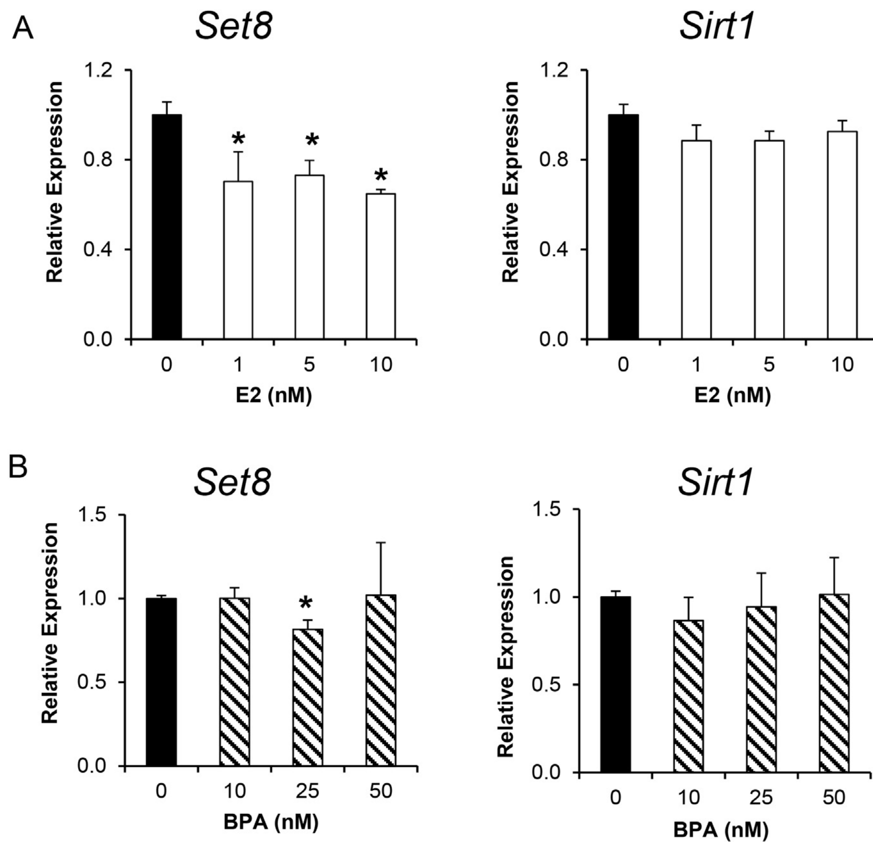


**Fig. 2.** Effect of estrogen or BPA on gene expression of HMEs in PC3 cells. (A) PC3 cells were treated with the indicated doses of estrogen for 24 h and the expression of *Set8* (left panel) or *Sirt1* (right panel) was analyzed. (B) PC3 cells were treated with the indicated doses of BPA for 24 h and the expression of *Set8* (left panel) or *Sirt1* (right panel) was analyzed. Image J was used to quantitate the results and the expression of each gene was normalized to *GAPDH* from the same sample. Results are from three independent experiments ($^*p < 0.05$).

### 3.3. Differential expression of Set8 and Sirt1 by estrogen and BPA is dependent on ER signaling

To determine if estrogen and BPA are altering the expression of *Set8* and *Sirt1* through ER signaling, we utilized the ER antagonist, ICI 182,780 (fulvestrant). The treatments were carried out as described above in the presence of estrogen or BPA in combination with ICI.

As expected, in LNCaP cells the expression of *Sirt1* was reversed in the presence of ICI (Fig. 3A). Interestingly, even though neither estrogen nor ICI alone affected the expression of *Set8*, we found that the combination of estrogen and ICI decreased *Set8* expression. Specifically the combination resulted in approximately a 30% decrease in *Set8* expression at all three doses of estrogen in combination with ICI, which was significant at the 5 nM dose (Fig. 3B left panel). This unexpected result implies that even though estrogen alone did not impact regulation, when there is a certain amount of estrogen present and the estrogen receptor is inhibited, gene expression may be affected.

Next we examined if the increased expression of *Set8* by BPA was mediated via ER signaling by treating LNCaP cells with BPA and ICI. BPA increased *Set8* expression more than 1.5-fold at the 10 and 25 nM doses and this induction was reversed by ICI. Even though there was no induction of *Set*8 at the 50 nM BPA dose, the combination of BPA and ICI significantly reduced the expression of *Set8* at all three doses (Fig. 3B right panel). The greatest reduction occurred at the 25 nM dose, decreasing *Set8* expression by 55% compared to the untreated (vehicle) control. These data show that the combination of an ER antagonist and an estrogen (E2 or BPA) alter the expression of *Set8* even when there was no observed effect with the hormone.

The same experiments were carried out in PC3 cells to determine if the changes in gene expression were mediated by ER signaling. Treatment with ICI reversed the effect of estrogen on *Set8* expression clearly demonstrating that ER signaling is the mechanism by which estrogen regulates *Set8* expression (Fig. 4A). BPA alone induced a decrease in *Set8* expression at 25 nM. Treatment with ICI reversed this effect (average expression was 1.1), although it did not reach statistical significance (Fig. 4B). Taken together, these data indicate that both estrogen and BPA are regulating gene expression in prostate cancer via ER signaling.

## 4. Discussion

We set out to determine if estrogen and the endocrine disruptor, BPA, affect the expression of HMEs in human prostate cancer in the presence of physiological hormones. We found that both estrogen and BPA regulate the expression of *Set8* and *Sirt1* and that there are distinct effects with

**Fig. 3.** Estrogen and BPA signal through the estrogen receptor to alter Set8 expression in LNCaP cells. (A) LNCaP cells were treated with estrogen alone or in presence of ICI (10 μM) for 24 h and Sirt1 expression was analyzed. (B) LNCaP cells were treated with estrogen (left panel) or BPA (right panel) alone or in the presence of ICI (10 μM) for 24 h and *Set8* expression was analyzed. Image J was used to quantitate the results and the expression was normalized to *GAPDH* from the same sample. Results are from three independent experiments (*$p$<.05).

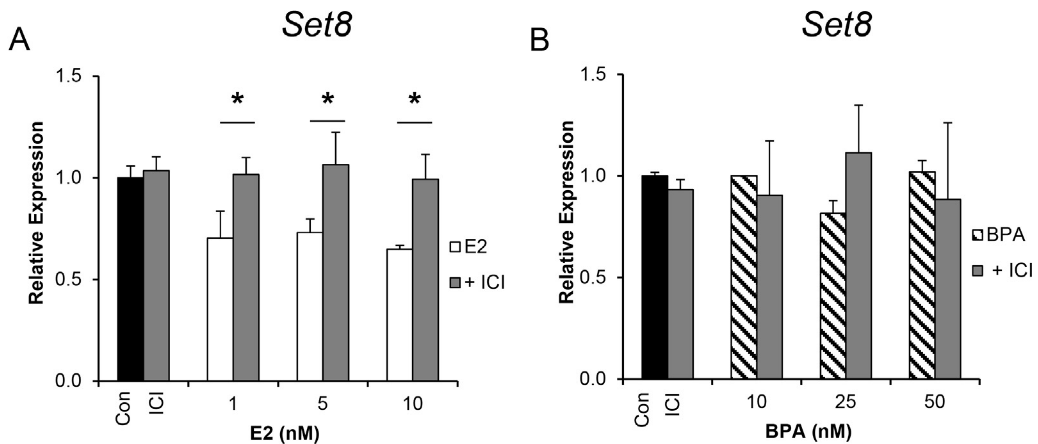


**Fig. 4.** Estrogen and BPA signal through the estrogen receptor to alter Set8 expression in PC3 cells. (A) PC3 cells were treated with estrogen alone or in the presence of ICI (10 μM) for 24 h and *Set8* expression was analyzed. (B) PC3 cells were treated with BPA alone or in the presence of ICI (10 μM) for 24 h and *Set8* expression was analyzed. Image J was used to quantitate the results and the expression was normalized to *GAPDH* from the same sample. Results are from three independent experiments (*$p$ < .05).

each compound. Furthermore, we found that the effects exerted by these compounds differ between the two models of human prostate cancer analyzed. Additionally, we showed that the regulation of gene expression was via ER, as the effects were reversed in all cases by the ER antagonist ICI.

Previous studies have looked at the effect these compounds have on the rat prostate methylome and the enzymes that modify the methylome. It is understood from these studies that BPA alters the prostate methylome in animal models. To further our understanding of the disease state we sought to determine if estrogen and BPA altered HMEs in human prostate cancer since histone modifications are another key epigenetic mechanism altered in cancer. Additionally, drugs targeting HMEs are more readily tolerated by patients than compounds that impact DNA methylation, therefore identifying which enzymes are altered by endocrine disruptors may be informative for therapeutic intervention.

Both *Set8* and *Sirt1* have been found to play a role in EMT and so understanding how exposure to endocrine disruptors affects their expression could aid in understanding disease progression. While we identified very few changes in *Sirt1* expression, we found that *Set8* was altered under multiple conditions and that its regulation differed between the two models of prostate cancer. While estrogen had no effect on *Set8* in LNCaP cells, it reduced the expression of *Set8* in PC3 cells, demonstrating that the molecular status of prostate cancer influences the outcome of *Set8* expression. Unlike the results with estrogen, BPA induced an increase in *Set8* expression in LNCaP cells but only reduced the expression of *Set8* at one dose in PC3 cells. In both models then, BPA had a distinct outcome with regards to *Set8* expression.

Interestingly we found that the combination of estrogen or BPA with ICI decreased the expression of *Set8* in LNCaP cells. It is important to note that ICI alone had no effect on *Set8* expression, but only reduced *Set8* expression when in combination with an estrogenic compound. The fact that this only occurred in LNCaP cells and not PC3 cells may be explained by studies showing that ICI inhibits prostate cancer progression both by down regulating the androgen receptor (AR) [27] and through inhibition of ERβ [28]. LNCaP cells express AR and only ERβ, while PC3 cells are AR negative and express both ERs [29]. This has relevance for treatment because a decrease in *Set8* expression could result in an inhibition of EMT in patients. However, in the PC3 cells, ICI in combination with estrogen or BPA restored the expression of *Set8*, which may or may not promote EMT and therefore have a negative clinical outcome. There have been recent investigations into the use of ICI as a treatment for CRPC with conflicting outcomes. In a small clinical study of CRPC patients, ICI decreased PSA levels without any reports of toxicity when a loading dose was given in the first month of treatment [30], however there was no discernible affect in a phase II clinical trial [31]. The utility of ICI as a therapy for prostate cancer, therefore, may in part depend on both the AR and ER status of the tumor.

There has been some investigation into the role of GPR30 in prostate cancer and its potential as a therapeutic target. GPR30 is an orphan G-protein coupled receptor with high affinity for estrogen. It rapidly activates Erk1/2 signaling upon stimulation [32]. It is possible that GPR30 is playing a role in response to either estrogen or BPA, however, given that ICI, the estrogen receptor antagonist, reversed the effects of both estrogen and BPA, GPR30 most likely has a minimal role in this study. Additionally, ICI has been found to be an agonist for GPR30 [33] and we did not observe and significant changes in gene expression when the cells were treated with ICI alone. Taken together, our data indicates that GPR30 is not the main mechanism by which estrogen or BPA regulate SET8 or SIRT1.

Many studies have been performed to show that HMEs play a role in estrogen signaling. We set out not only to determine if estrogen and BPA regulate the expression of HMEs but whether they do so in the presence of physiological hormones. This is an important distinction as it more closely resembles the disease state because even with androgen deprivation, there is still a plethora of hormones, including natural and synthetic estrogens that could impact gene expression. It would be of interest to examine the effects of estrogen and BPA on SET8 and SIRT1 enzyme activity as our study solely analyze gene expression. Additionally, investigating what occurs at later time points as well as with prolonged exposure to BPA would be of interest as humans are constantly exposed to BPA. Lastly, understanding how current therapies are impacted by exposure to endocrine disruptors like BPA could inform treatment.

## 5. Conclusion

It is clear from our study that with a very short exposure time (24 h), changes in HME gene expression were observed in response to E2 and BPA. Perhaps most interestingly, we found that the combination of an ER antagonist and an estrogenic compound had distinct effects on HME expression than either compound alone. This is relevant as estrogens and estrogen receptor inhibitors are being investigated, alone or as adjuvants, as potential therapies for prostate cancer.

## Transparency document

The Transparency document associated with this article can be found in the online version.

## Acknowledgements

The authors would like to thank the Canisius Earned Excellence Program for supporting part of this work.

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