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Research Article

Parabens Promote Protumorigenic Effects in Luminal Breast Cancer Cell Lines With Diverse Genetic Ancestry

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Abstract

Context: One in 8 women will develop breast cancer in their lifetime. Yet, the burden of disease is greater in Black women. Black women have a 40% higher mortality rate than White women, and a higher incidence of breast cancer at age 40 and younger. While the underlying cause of this disparity is multifactorial, exposure to endocrine disrupting chemicals (EDCs) in hair and other personal care products has been associated with an increased risk of breast cancer. Parabens are known EDCs that are commonly used as preservatives in hair and other personal care products, and Black women are disproportionately exposed to products containing parabens.

Objective: Studies have shown that parabens impact breast cancer cell proliferation, death, migration/invasion, and metabolism, as well as gene expression in vitro. However, these studies were conducted using cell lines of European ancestry; to date, no studies have utilized breast cancer cell lines of West African ancestry to examine the effects of parabens on breast cancer progression. Like breast cancer cell lines with European ancestry, we hypothesize that parabens promote protumorigenic effects in breast cancer cell lines of West African ancestry.

Methods: Luminal breast cancer cell lines with West African ancestry (HCC1500) and European ancestry (MCF-7) were treated with biologically relevant doses of methylparaben, propylparaben, and butylparaben.

Results: Following treatment, estrogen receptor target gene expression and cell viability were examined. We observed altered estrogen receptor target gene expression and cell viability that was paraben and cell line specific.

Conclusion: This study provides greater insight into the tumorigenic role of parabens in the progression of breast cancer in Black women.

Key Words: breast cancer, endocrine disrupting chemicals, parabens, health disparities

Abbreviations: ANOVA, analysis of variance; BP, butylparaben; E2, estradiol; EDC, endocrine disrupting chemical; ER, estrogen receptor; EWG, Environmental Working Group; FBS, fetal bovine serum; MP, methylparaben; PP, propylparaben.

Breast cancer is the second leading cause of cancer death in women [1]. Although incidence rates for both Black and White women have converged, Black women are more likely to be diagnosed with breast cancer under the age of 40, and are 40% more likely to die from the disease than White women [2, 3]. The factors underlying breast cancer disparities are multifactorial, including the significant role of sociocultural practices, such as the use of hair and other personal care products containing endocrine disrupting chemicals (EDCs) [4]. EDCs are toxins found in the environment, hair and other personal care products, and food sources. EDCs can disrupt the body's homeostasis through interference with the endocrine system which is responsible for regulation of various biological functions, such as growth and development [5]. EDCs are thought to function by activating and disrupting estrogen receptor (ER) signaling and leading to sustained and aberrant hormone receptor activity [6]. Studies have found associations between exposure to EDCs and increased risk of breast cancer [6-9].

Parabens are a class of EDCs that can mimic estrogen in the body and are used as preservatives in hair and other personal care products. Although there may be additional sources of parabens, hair and personal care products are believed to be the biggest source of exposure to parabens. The Environmental Working Group (EWG) developed a hazardous chemical scale for beauty, personal care, and household product ingredients [10]. Using a scale of 1 (best) to 10 (worst), consumers can determine the hazard level of their products. According to the EWG, products containing butylparaben (BP) and propylparaben (PP) are scored high on the hazardous scale [6], while products containing methylparaben (MP) rank moderately [3, 10]. Hormonally hazardous chemicals have been linked to breast cancer; specifically, studies have found estrogenic properties in paraben-laden hair and

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other personal care products that are heavily marketed to Black women [11-13]. Parabens have been measured in biological samples from the NHANES biomonitoring study; across studies, the median/mean of PP levels is 4 to 15 μg/L [14-20], the range of BP levels is 0.2 to 1240 μg/L [15, 20], and the mean of MP levels is 38 to 63 μg/L [15, 17, 20-22]. There are racial/ethnic disparities in exposure to parabens. For instance, 5 times higher levels of MP, and 3.6 times higher levels of PP were measured in Black women compared with White women [15]. These disparities have been shown to persist into more recent NHANES cycles and are most apparent in children [19]. Although there may be many factors underlying the racial disparity in paraben levels, 1 potential factor is prolonged use of hair and other personal care products that contain high concentrations of parabens [4, 23, 24].

The use of products containing estrogens has been linked to adverse health effects, such as premature sexual development [11]. Estrogen–ER signaling plays a role in breast cancer progression [25]. Estrogenic activity has been detected in personal care products (eg, oil hair lotion, intensive skin lotion) commonly used by Black women, thereby potentially increasing the risk for breast cancer in this under-resourced community [11]. Studies have shown that parabens are present in normal and in tumor breast tissue [26-29]. However, these studies did not include breast tissue from Black women. Furthermore, to the best of our knowledge, studies examining the protumorigenic effects of parabens in vitro have been conducted solely in breast cancer cell lines of European ancestry [30-37]. The lack of studies examining the effects of parabens on breast cancer progression using samples and cell lines from Black women represents a significant gap in knowledge, given that racial disparities in breast cancer risk and mortality exist, and that Black women are disproportionately exposed to hair and other personal care products containing high levels of parabens. We hypothesized that, similar to what has been shown for breast cancer cell lines with European ancestry, parabens promote protumorigenic effects in breast cancer cell lines with West African ancestry. Since parabens are thought to act through ER signaling, we focused on ER+ luminal breast cancer cell lines in this study.

Materials and Methods

Cell Viability

Cell viability of cell lines with European ancestry MCF-7 (ATCC, CAT: HTB-22) and West African ancestry HCC1500 (ATCC, CAT: CRL-2329) was assessed utilizing CellTiter-Glo 2.0 Luminescent Cell Viability Assay (Promega, CAT: G9242). West African ancestry of the HCC1500 cell line was determined using established Ancestry Informative Markers [38]. Approximately 3000 MCF-7 cells and 10 000 HCC1500 cells were seeded in black 96-well optical bottom plates (Thermo Scientific, CAT: 165305) in 100 μL per well, using phenol red–free RPMI medium (Gibco, CAT: 11835030) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals Inc., CAT: S11150) and incubated at 37 °C. Following overnight incubation and cell adherence, the seeding medium was replaced with fresh phenol red–free RPMI medium supplemented with 10% charcoal-stripped FBS (Atlanta Biologicals Inc., CAT: S11650), and cells were incubated at 37 °C for 48 hours to deplete steroid hormone levels in the cells. On day 4, cells were treated with various concentrations of PP (propyl 4-hydroxybenzoate; Acros Organics, CAT: 131591000), MP (methyl 4-hydroxybenzoate; Acros Organics, CAT: 126961000), and BP (butyl 4-hydroxybenzoate; Acros Organics, CAT: 403571000). Chemicals were dissolved in ethanol (Acros Organics, CAT: 615090040), diluted with phenol red–free RPMI supplemented with 10% charcoal-stripped FBS and added to each well. Cells were incubated at 37 °C for 96 hours. On day 8, the medium was replaced with fresh-treated medium. Cells were incubated at 37 °C for 72 hours. On day 11 (after 7 days of treatment), cell viability was measured using CellTiter-Glo 2.0 Luminescent Cell Viability Assay to measure ATP, as an indicator of metabolically active cells, in either the Tecan Infinite M200 Pro or the Biotek Synergy Lx plate reader. Ethanol served as a negative control, and β-estradiol (E2; Acros Organics, CAT: 436320050) served as a positive control. The E2 concentrations used were: 0.01 , 0.1 , 1, 10, and 100 nM. The following concentrations were used for PP, MP, and BP: 0.002 μM, 0.02 μM, 0.2 μM, 2 μM, and 20 μM. These concentrations fall within the range of what has been measured in biological samples in the NHANES biomonitoring study [14-20] and in breast tissue [21, 22, 26-29, 39]. Treatments were done in triplicates, and experiments were repeated 6 times.

MCF-7 and HCC1500 cells were also treated with either E2 (0.01 , 0.1 , 1, 10, and 100 nM) or PP, MP, BP (0.002 μM, 0.02 μM, 0.2 μM, 2 μM) individually or in a mixture of all 3. Ethanol was used as a negative control.

Cell Viability Assay With ER Antagonist

Cell viability was measured as described above. MCF-7 and HCC1500 cells were treated with either E2 (0.1 nM, 1 nM, or 10 nM), or PP, MP, BP (0.002 μM, 0.02 μM, 0.2 μM, or 2 μM), individually with or without the ER antagonist 1 nM fulvestrant (ICI 182780; Tocris Bioscience, CAT: 10-471-0) to determine if the effect of parabens on cell viability is ER mediated. Ethanol was used as a negative control. Experiments were repeated 4 times.

ER Target Gene Expression

Cells were seeded in CytoOne 6-well plates (USA Scientific, CAT: CC7682-7506) at different densities with phenol red–free RPMI medium, supplemented with 10% FBS, and incubated at 37 °C. Cell densities plated per well per cell line are as follows: MCF-7 at 3×10^5 cells/well, HCC1500 at 5×10^5 cells/well, BT-474 (ATCC, CAT: HTB-20; European ancestry) at 4×10^5 cells/well, and MDA-MB-175-VII (ATCC, CAT: HTB-25; West African ancestry) at 5×10^5 cells well. After 24 hours, the medium was replenished to contain fresh phenol red–free RPMI medium supplemented with 10% charcoal-stripped FBS. The cells were incubated at 37 °C for 48 hours. On day 4, the cells were treated with charcoal-stripped phenol red–free RPMI supplemented with the appropriate concentrations of PP, MP, and BP (0.002 μM, 0.02 μM, 0.2 μM, 2 μM, and 20 μM), ethanol, or E2 (10 nM) controls, and incubated at 37 °C. After 6 hours, cells were washed with 1 mL of phosphate-buffered saline (Gibco, CAT: 20012050), and 500 μL prewarmed 0.25% Trypsin-EDTA (Gibco, CAT: 15400054) solution was added. Following incubation and detachment from the plate, 500 mL of phenol red–free charcoal-stripped medium was added. Medium containing the detached cells was collected in 1.5 mL microcentrifuge tubes and centrifuged at 300*g* for 5 minutes. The cell pellets were stored at

Table 1. Oligonucleotide primer sequences for real-time quantitative polymerase chain reaction

Species	Gene	Primer sequence starting at 5′		ER target gene function
Human	*GAPDH*	Forward Reverse	TCG GAG TCA ACG GAT TTG GT TTC CCG TTC TCA GCC TTG AC	Housekeeping gene
Human	*TFF1*	Forward Reverse	GGC CCA GAC AGA GAC GTG TA TGG AGG GAC GTC GAT GGT AT	Stimulates proliferation [40]
Human	*PGR*	Forward Reverse	AGG TCT ACC CGC CCT ATC TC AGT TGT GCT GCC CTT CCA TT	Progresses breast cancer development [41]
Human	*GREB1*	Forward Reverse	GGG ATC TTG TGA GTA GCA CTG T AAT CGG TCC ACC AAT CCC AC	Regulates proliferation [42]
Human	*MYC*	Forward Reverse	TGG AAA ACC AGC CTC CCG TTC TCC TCC TCG TCG CAG TA	Controls cell growth [43]
Human	*CCND1*	Forward Reverse	ATC AAG TGT GAC CCG GAC TG CTT GGG GTC CAT GTT CTG CT	Plays a role in cell cycle progression [44]

−80 °C. Ethanol served as a negative control and E2 (10 nM) served as a positive control.

Total RNA was isolated from each cell line using the RNeasy Mini Kit (Qiagen, CAT: 74106). RNA concentrations were measured using a NanoDrop One (Thermo Fisher, CAT: AZY1810576). RNA (500 ng) of was reverse transcribed to cDNA by using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, CAT: 4388950). The PowerUp SYBR Green Master Mix (Applied Biosystems, CAT: A25777), in combination with primers *hGAPDH*, *hTFF1* [40], *hPGR* [41], *hGREB1* [42], *hMYC* [43], and *hCCND1* [44] (Table 1), was used for gene expression analysis of known ER target genes using a QuantStudio 3 real-time polymerase chain reaction system (Applied Biosystems, CAT: A28567). All primers were purchased from Integrated DNA Technologies. Experiments were repeated 3 times. The housekeeping gene, *hGAPDH* was used for normalization. The delta-delta Ct method was used to analyze the relative changes in gene expression.

ER Target Gene Expression With ER Antagonist

Gene expression was measured as described above. The following concentrations of PP, MP, and BP were used with and without 1 nM ICI 182780, 2 µM, and 20 µM. Ethanol and 10 nM E2 concentrations were used with and without 1 nM ICI 182780. Experiments were repeated 4 times unless otherwise noted. PP- and BP-mediated regulation of ER target gene expression with MCF-7 cells were repeated 3 times.

Time-Dependent ER Target Gene Expression

Gene expression was measured as previously described. MCF-7 and HCC1500 cells were treated with either PP, MP, or BP (2 µM) for 1, 6, and 24 hours. Ethanol was used as a negative control and E2 (10 nM) as a positive control. Experiments were repeated 3 times.

Western Blot

MCF-7 and HCC1500 were seeded in 10-cm dishes (USA Scientific Inc., CAT: CC76823394) at the following densities: 2×10^6 and 3×10^6, respectively. All cells were incubated at 37 °C. After 24 hours of incubation, the seeding medium was replaced with fresh medium containing the addition of 10% charcoal stripped FBS. On day 4, cells were treated with BP (0.002 µM, 0.02 µM, 0.2 µM, 2 µM, and 20 µM), ethanol, or E2 (10 nM) controls. Cells were harvested using cell scrapers (Fisherbrand, CAT: 08100241), centrifuged at 120 rcf for 5 minutes, and stored in −80 °C until they were used for protein extraction and western blot analysis. Total protein was extracted from the cell lines and tissues using NP40 cell lysis buffer (Invitrogen, CAT: FNN0021RIPA) with phenylmethylsulfonyl fluoride (Acros Organics, CAT: 215740010), a Pierce Protease Inhibitor Mini Tablet (Thermo Scientific, CAT: A32955), and a Pierce Phosphatase Inhibitor Mini Tablet (Thermo Scientific, CAT: A32957). Protein concentrations in the lysates were measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, CAT: 23227). Lysate mixed (10-25 µg) with 4 times sample loading buffer (Licor, CAT: 928-40004) and Novex 10X bolt sample reducing agent (Invitrogen, CAT: B0009) were loaded per lane. The proteins in the lysates were separated by gel electrophoresis with Bolt 4% to 12% Bis-Tris plus gels (Invitrogen, CAT: NW04122BOX) and transferred to Immobilon-FL PVDF membranes (MilliporeSigma, CAT: IPFL07810). Membranes were dried overnight, at room temperature, to maximize protein retention. REVERT Total Protein Stain (LI-COR, CAT: 926-11011) was used as a protein loading control. To block nonspecific binding, membranes were incubated with Intercept (Tris-buffered saline) Blocking Buffer (LI-COR, CAT: 92760001) for 1 hour at room temperature, then incubated overnight at 4 °C with the following antibody: ERα (Dilution 1:1 µg/mL; Invitrogen, CAT: MA5-13191). IRDye secondary antibody (Dilution 1:20,000; LI-COR, CAT: 82708364, 92968171, 92632214) was used to visualize the target protein with an Odyssey Classic (LI-COR). Experiments were repeated 6 times.

Statistical Analysis

GraphPad Prism Software (Dotmatics, Version 8) was used to determine statistical significance. One-way analysis of variance (ANOVA) was performed for cell viability and gene expression assays. Brown-Forsythe and Welch ANOVA tests were used where variances were significantly different. Two-way ANOVA was performed for cell viability and gene expression assays that included cotreatment with ICI 182780. Differences were considered statistically significant when the P value was less than or equal to .05. Error bars represent SD.

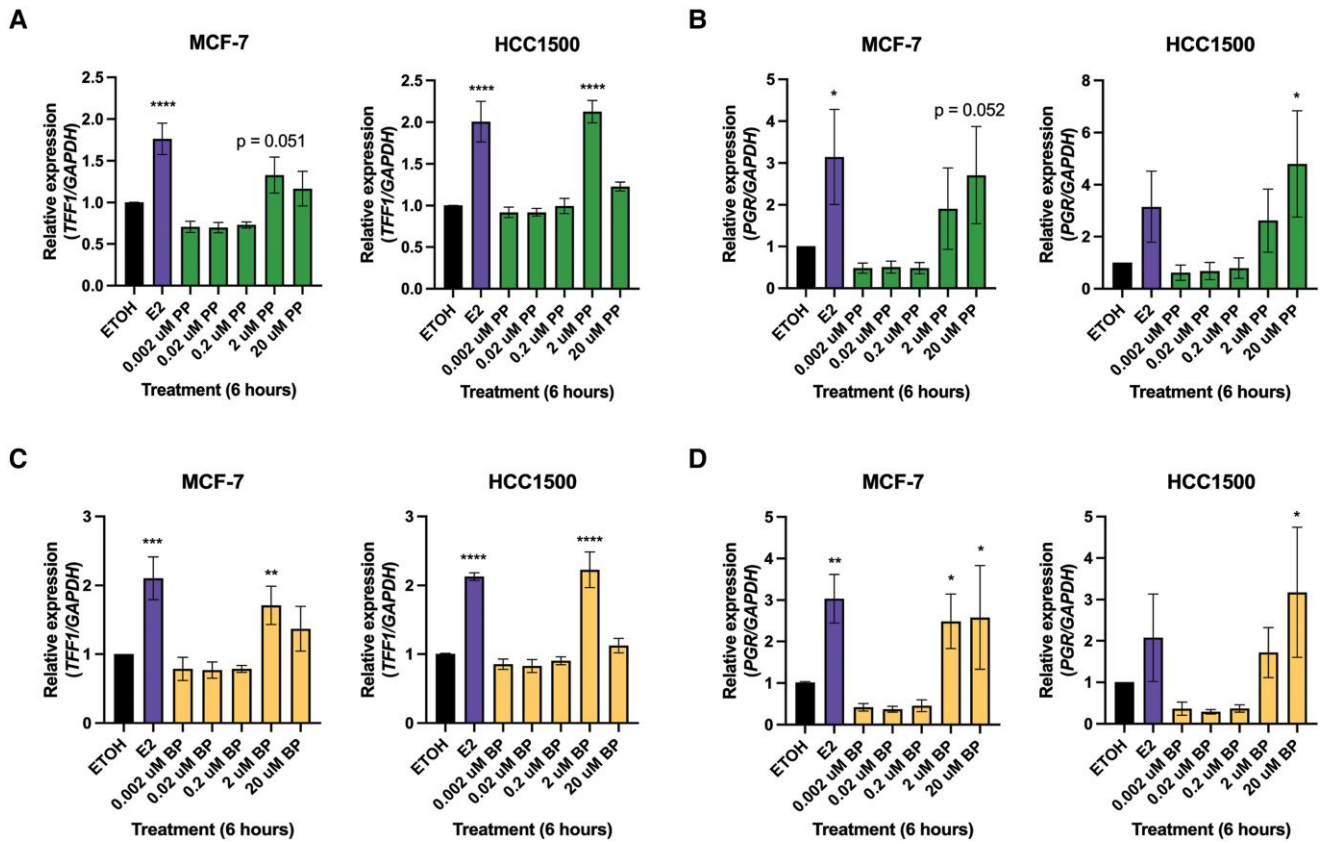

Figure 1. PP- and BP-mediated regulation of ER target gene expression in MCF-7 and HCC1500. MCF-7 and HCC1500 luminal A breast cancer cell lines were treated with the indicated doses of PP or BP for 6 hours. (A-D) Quantification of (A, C) *TFF1* and (B, D) *PGR* gene expression following PP or BP exposure, respectively. Ethanol was used as a negative control, and estradiol (E2, 10 nM) served as a positive control. GAPDH was used as a housekeeping gene. n = 3, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$, 1-way ANOVA.

Results

ER Target Genes are Regulated by Parabens in a Dose-Dependent Manner

To test the effects of paraben treatment on ER target gene expression, MCF-7 (European ancestry) and HCC1500 (West African ancestry) cells were treated with MP, PP, or BP at the doses indicated for 6 hours (Fig. 1). Expression of well-known ER target genes trefoil factor-1 (*TFF1*), progesterone receptor (*PGR*), growth regulating estrogen receptor binding 1 (*GREB1*), *MYC* protooncogene (*MYC*), and cyclin D1 (*CCND1*) was measured via real-time quantitative polymerase chain reaction. Treatment with 2 µM PP significantly increased *TFF1* gene expression in HCC1500, while the observed increase in *TFF1* gene expression did not reach significance in MCF-7 cells (Fig. 1A). Treatment with 20 µM PP significantly increased *PGR* gene expression in HCC1500, while the observed increase in *PGR* gene expression did not reach significance in MCF-7 cells (Fig. 1B). Significantly increased expression of *TFF1* was observed with 2 µM BP treatment in both MCF-7 and HCC1500 cells (Fig. 1C). Treatment with 2 and 20 µM BP significantly increased *PGR* gene expression in MCF-7 cells, while treatment with 20 µM BP significantly increased *PGR* gene expression in HCC1500 cells (Fig. 1D). Paraben- and cell line–dependent results were also observed for *GREB1* (Fig. S1A and D [45]), *MYC* (Fig. S1B and E [45]), and *CCND1* (Fig. S1C and F [45]). Treatment with MP significantly increased *PGR* (20 µM) and *CCND1* (0.002, 0.02, 0.2, and 2 µM) compared with control in HCC1500, but not MCF-7, cells (Fig. S2 [45]). These results suggest that PP and BP may be more estrogenic than MP in luminal breast cancer cells.

To determine whether the estrogenic activity of PP and BP was cell line specific, gene expression was measured in additional luminal cell lines, BT-474 (European ancestry), and MDA-MB-175-VII (West African ancestry) following paraben exposure. Treatment with PP or BP did not increase *TFF1* gene expression in either cell line (Fig. S3A and 4A [45]). Treatment with 20 µM PP or BP significantly increased *GREB1* gene expression in BT-474, but not MDA-MB-175-VII, cells (Fig. S3B and 4B [45]). Treatment with 20 µM PP or 20 µM BP significantly increased *MYC* expression in both cell lines (Fig. S3C and 4C [45]). Treatment with 2 µM BP also significantly increased *MYC* gene expression in BT-474 cells (Fig. S4C [45]). Additionally, treatment with PP, in both cell lines, significantly increased *CCND1* gene expression at all doses except 20 µM (Fig. S3D [45]). Furthermore, BP treatment significantly increased *CCND1* gene expression in both cell lines, except at the 20 µM concentration in the BT-474 cell line (Fig. S4D [45]). Taken together, these results suggest that parabens regulate ER target gene expression in a variety of luminal breast cancer cell lines.

Paraben-Mediated Effects on ER Target Gene Expression Are ER dependent

To determine whether the observed PP- and BP-mediated effects on ER target gene expression are ER dependent,

MCF-7 and HCC1500 cells were cotreated with PP or BP and ICI 182780 (Fulvestrant) for 6 hours. Cotreatment with ICI 182780 blocked the PP-mediated increase in *TFF1*, *PGR*, *MYC*, and *CCND1* expression in both cell lines ($P = .055$ for TFF1 in MCF-7 cells) and in *GREB1* expression in HCC1500 cells (Fig. 2). Cotreatment with ICI 182780 blocked the BP-mediated increase in *TFF1*, *PGR*, *GREB1*, *MYC*, and *CCND1* in both cell lines (Fig. 3). It should be noted that 1 nM ICI 182780 is not enough to block the increased ER target gene expression observed with 10 nM E2 treatment but is sufficient to block paraben-mediated ER target gene expression. Treatment with 10 nM ICI 182780 is sufficient to block the E2-mediated increase in expression of *TFF1* in both cell lines (Fig. S6 [45]). Collectively, the results suggest that regulation of ER target gene expression by PP and BP is ER dependent.

ER Target Genes Are Regulated by PP and BP in a Time-Dependent Manner

To examine time-dependent regulation of ER target genes by parabens, luminal breast cancer cells (ie, MCF-7 and HCC1500) were treated with PP, BP, or MP (2 µM) for either 1, 6, or 24 hours. As expected, we observed time-dependent expression of ER target genes in both cell lines treated with E2 (10 nM) (Fig. S7 [45]). Treatment with PP significantly increased expression of *PGR* (1 and 6 hours), *GREB1* (6 hours), and *MYC* (1 hour), but not *TFF1* or *CCND1*, in MCF-7 cells (Fig. 4A-4E). Treatment with PP significantly increased expression of *TFF1* (6 and 24 hours), *GREB1* (6 hours), and *CCND1* (1 hour), but not *PGR*, in HCC1500 cells (Fig. 4A-4C and 4E). *MYC* expression was significantly decreased upon treatment with PP at the 6- and 24-hour timepoints in HCC1500 cells (Fig. 4D). Expression of *TFF1* was significantly decreased with BP treatment for 1 hour in MCF-7 cells (Fig. 5A). Treatment with BP increased expression of *PGR* (6 and 24 hours), *GREB1* (6 hours), and *MYC* (1 hour), but not *CCND1*, in MCF-7 cells (Fig. 5B-5E). There was an observed increase in PGR with 1 hour of BP treatment in MCF-7 cells that did not reach statistical significance (Fig. 5B). Treatment with BP increased *TFF1* (6 and 24 hours), *PGR* (6 hours), *GREB1* (6 hours), *MYC* (1 hour), and *CCND1* (1 and 6 hours) in HCC1500 cells (Fig. 5). Expression of *MYC* was significantly decreased upon treatment with BP for 24 hours in HCC1500 cells (Fig. 5D). No significant change in *TFF1* expression was observed following treatment with MP at any timepoint, in both cell lines (Fig. S8A [45]). However, treatment with MP for 1 hour significantly increased expression of *PGR* and *CCND1* in MCF-7 cells (Fig. S8B and E [45]) and *CCND1* in HCC1500 cells (Fig. S8E [45]). Expression of *MYC* was significantly decreased in MCF-7 cells treated with MP for 6 and 24 hours (Fig. S8D [45]). Expression of *PGR*, *GREB1*, and *MYC* was significantly decreased in HCC1500 cells treated with MP for 1, 6, and 24 hours (Fig. S8B-D [45]). Together, the data suggest that parabens alter ER target gene expression in a time-dependent manner.

Butylparaben-Mediated Effects on Cell Viability Are not ER dependent

To test the effects of parabens on cell viability, MCF-7 and HCC1500 cells were treated with PP, BP, or MP at the indicated doses for 7 days. Treatment with either PP or MP did not increase cell viability in either MCF-7 nor HCC1500 cells (Fig. 6A and 6B). Treatment with BP (2 µM) significantly increased cell viability in the HCC1500 cell line but not in the MCF-7 cell line (Fig. 6C).

To investigate whether the observed effects of parabens on cell viability is mediated through ER, MCF-7 and HCC1500 cells were cotreated with either PP, BP, or MP in the presence or absence of 1 nM ER ICI 182780. While we observed significantly decreased BP-mediated cell viability in the presence of ICI 182780 in HCC1500 cells, decreased cell viability in the presence of ICI 182780 was also observed in the absence of BP, suggesting that BP effects on cell viability are not ER mediated (Fig. 6D). As expected, treatment with E2 (10 nM) significantly increased cell viability of MCF-7 and HCC1500 cells at all doses tested (Fig. S9A [45]). Like what was observed with BP-mediated cell viability in HCC1500, treatment with ICI 182780 significantly inhibited both basal cell viability and E2-mediated cell viability (Fig. S9A) [45]). Cotreatment with ICI 182780 did not reverse the decreased cell viability observed in MCF-7 and HCC1500 cells treated with either PP or MP (Fig. S9B and C [45]).

Hair and other personal care products commonly contain more than 1 paraben; therefore, we determined the effect of treatment with a mixture of PP, BP, and MP for 7 days on cell viability. We observed that the BP-mediated increase in HCC1500 cell viability was blunted in the presence of PP and MP at all doses tested (Fig. 6E). In addition, treatment with the mixture significantly decreased HCC1500 cell viability (0.0002, 0.02, and 0.2 µM) (Fig. 6E). Collectively, these data suggest that BP increases HCC1500 cell viability in an ER-independent manner.

Butylparaben Treatment Stabilizes ER Protein Expression in Luminal Breast Cancer Cells

To determine whether butylparaben treatment regulates ER protein expression, we performed Western blot analysis. As expected, treatment with E2 (10 nM) significantly decreased ERα protein expression compared with control in the HCC1500 cell line (Fig. 6F). In contrast, treatment with BP (0.0002, 0.02, and 0.2 µM) significantly increased ERα protein expression compared with control in the HCC1500 cell line (Fig. 6F). Treatment with BP (0.0002, 0.02, 0.2, and 2 µM) also significantly increased ERα protein expression in MCF-7 cells (Fig. S10A [45]). We did not, however, observe a significant decrease in ERα protein expression as expected with E2 treatment (10 nM) in MCF-7 cells due to variability across biological replicates (Fig. S10A [45]). Nonetheless, these data suggest that BP exposure may induce prolonged activation of ERα in luminal breast cancer cells.

Discussion

To our knowledge, this is the first study to examine the effects of paraben treatment on luminal breast cancer cells of West African ancestry in vitro. We know that Black women are at higher risk for developing breast cancer at younger ages, are more likely to be diagnosed with more aggressive disease, and are more likely to die from breast cancer than their White counterparts [2, 46], yet previous studies examining the protumorigenic effects of parabens solely focused on breast cancer cell lines of European ancestry. This lack of inclusion in breast cancer research hinders advances in

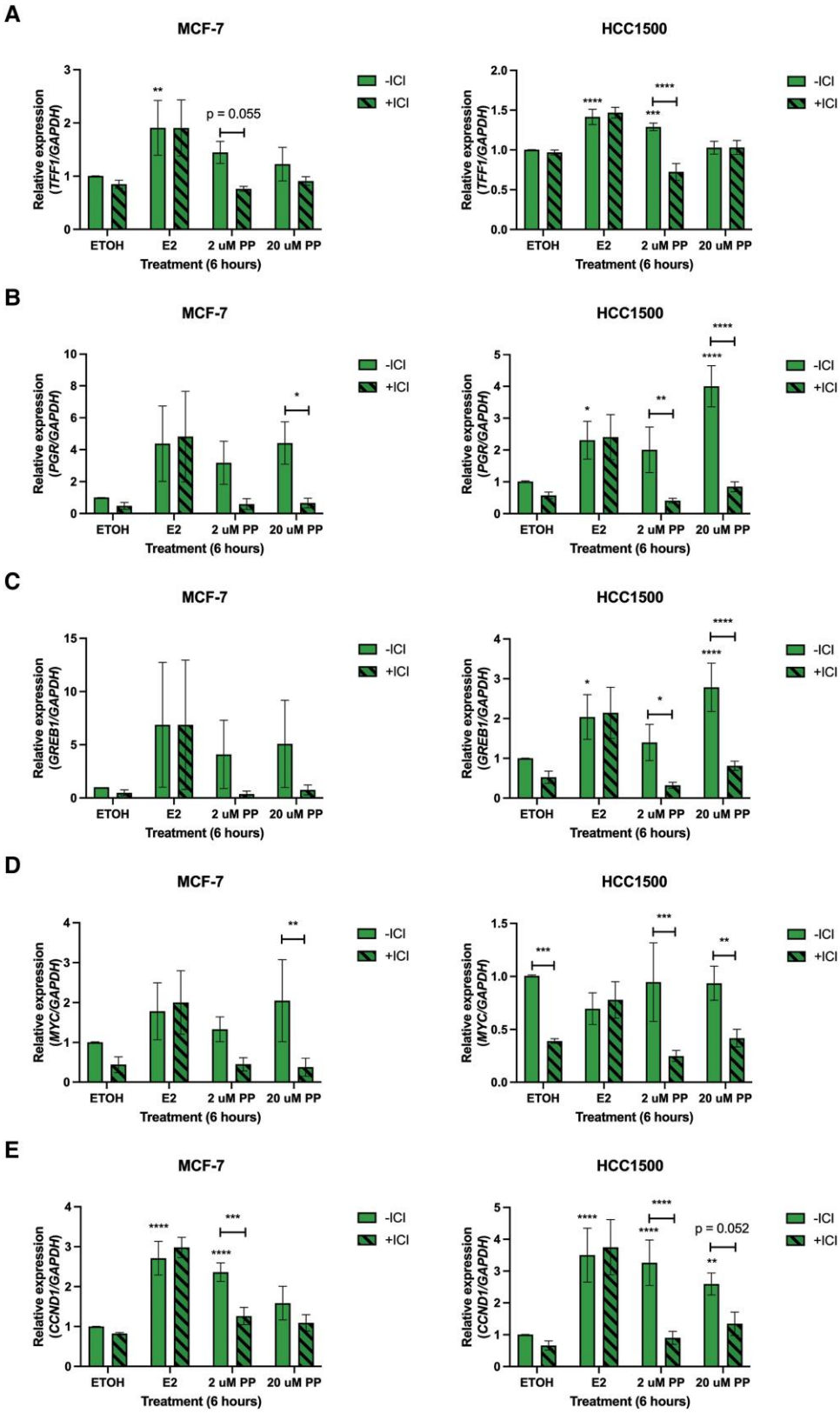

Figure 2. PP-mediated regulation of ER target gene expression is ER-dependent. MCF-7 and HCC1500 luminal A breast cancer cell lines were treated with 2 μM or 20 μM PP for 6 hours in the presence or absence of ER-antagonist, ICI 182780 (1 nM). (A-E) Relative gene expression of (A) *TFF1*, (B) *PGR*, (C) *GREB1*, (D) *MYC*, and (E) *CCND1* was assessed by real-time qualitative polymerase chain reaction. Ethanol was used as a negative control, and E2 (10 nM) served as a positive control. GAPDH was used as a housekeeping gene. n = 3 for MCF-7 and n = 4 for HCC1500, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$, 2-way ANOVA.

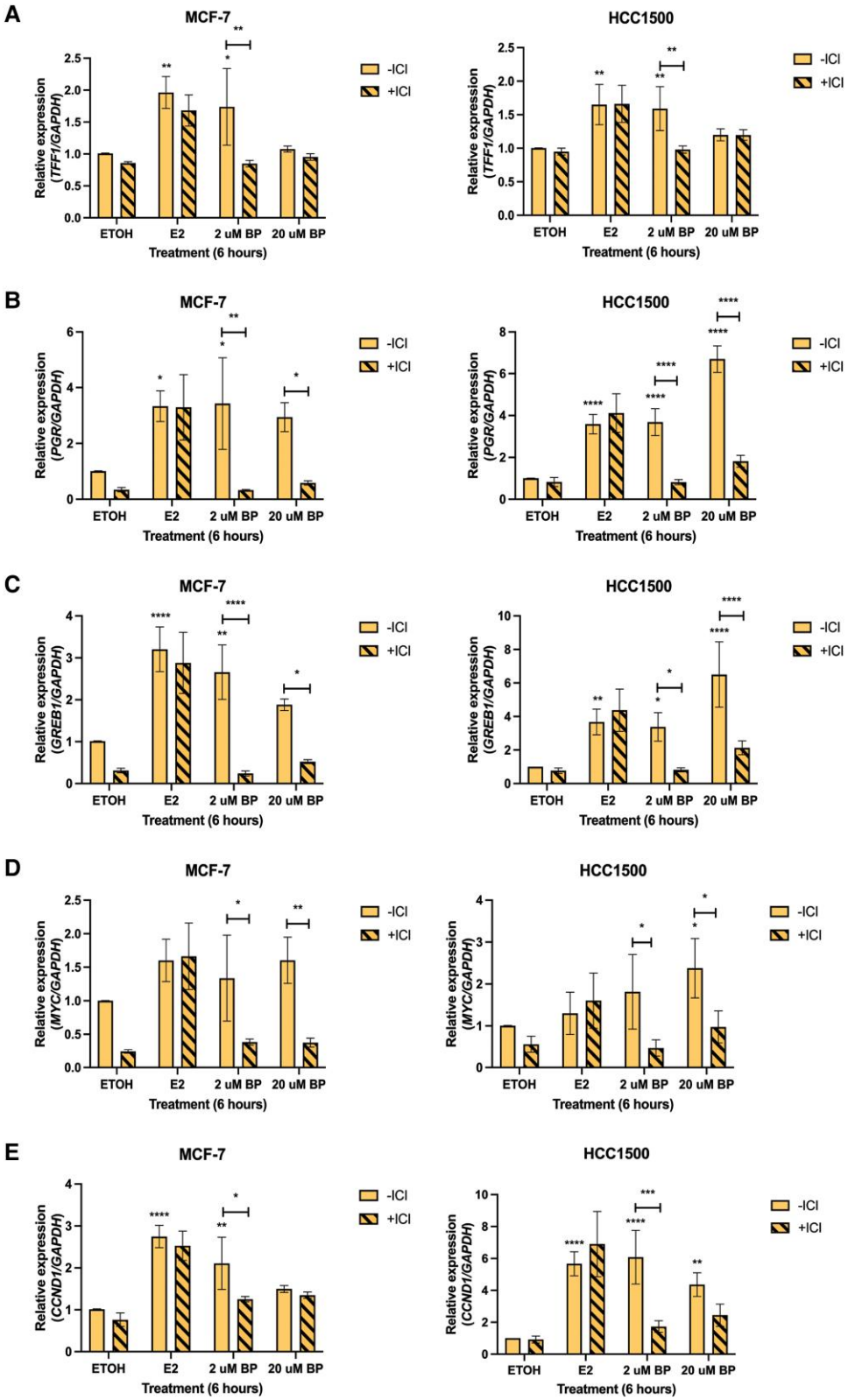

Figure 3. BP-mediated regulation of ER target gene expression is ER dependent. MCF-7 and HCC1500 luminal A breast cancer cell lines were treated with 2 μM or 20 μM BP for 6 hours in the presence or absence of ER-antagonist, ICI 182780 (1 nM). (A-E) Relative gene expression of (A) *TFF1*, (B) *PGR*, (C) *GREB1*, (D) *MYC*, and (E) *CCND1* was assessed by real-time qualitative polymerase chain reaction. Ethanol was used as a negative control, and E2 (10 nM) served as a positive control. GAPDH was used as a housekeeping gene. n = 3 for MCF-7 and n = 4 for HCC1500, $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$, 2-way ANOVA.

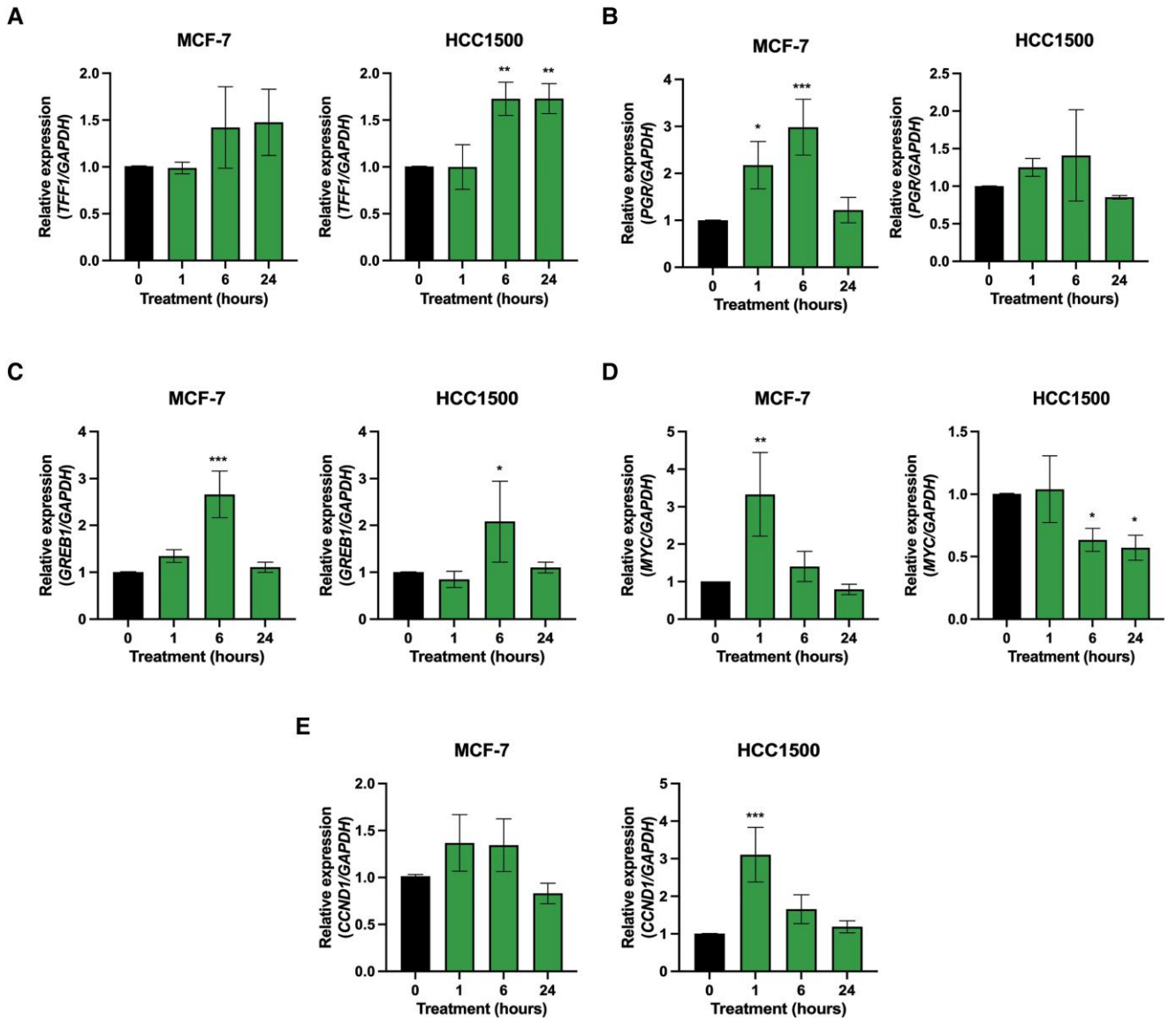

Figure 4. PP-mediated regulation of ER target gene expression is time dependent. MCF-7 and HCC1500 luminal A breast cancer cells were treated with 2 μM PP for 1, 6, or 24 hours. (A-E) Relative gene expression of (A) *TFF1*, (B) *PGR*, (C) *GREB1*, (D) *MYC*, and (E) *CCND1* was assessed by real-time qualitative polymerase chain reaction. Ethanol was used as a negative control, and E2 (10 nM) served as a positive control. GAPDH was used as a housekeeping gene. n = 3, $*P < .05$, $**P < .01$, $***P < .001$, 1-way ANOVA.

prevention and therapeutic strategies that will eliminate breast cancer disparities [6]. Therefore, the goal of this study was to examine protumorigenic effects of parabens in breast cancer cell lines with West African ancestry. Our data provide the foundation for further studies examining the mechanism of paraben action in Black breast cancer cells, as well as for intervention strategies for reducing exposure to hair and other personal care products that contain parabens and other harmful EDCs in Black women.

It is known that estrogens play a role in breast cancer development and progression [25]. Since parabens are thought to act as estrogens, we examined the effects of paraben treatment on ER target gene expression in diverse luminal breast cancer cell lines. ER regulates expression of its target genes in a variety of mechanisms, including through direct binding to DNA at estrogen response elements or via tethering to other transcription factors, such as AP1 and Sp1 [47-49]. We examined the expression of genes that are regulated by direct binding to estrogen response elements (*TFF1*, *PGR*, and *GREB1*) or via tethering (*MYC* and *CCND1*) and found that parabens regulate ER target gene expression in a paraben-specific manner. In general, treatment with PP and BP for 6 hours exerts a greater effect on expression of ER target genes than MP in all cell lines tested. Interestingly, MP treatment for 6 hours increased *CCND1* expression in HCC1500 (West African ancestry), BT-474 (European ancestry), and MDA-MB-175-VII (West African ancestry) cells, suggesting that MP may be as estrogenic as PP and BP in some contexts. These results are supported by other studies that found PP and BP to be more estrogenic than their paraben counterparts in murine, and in breast tumors in women of European ancestry [26, 50-52]. These data also parallel the EWG hazardous chemical score for the individual parabens, where PP and BP are higher on the hazardous scale than MP [53-56].

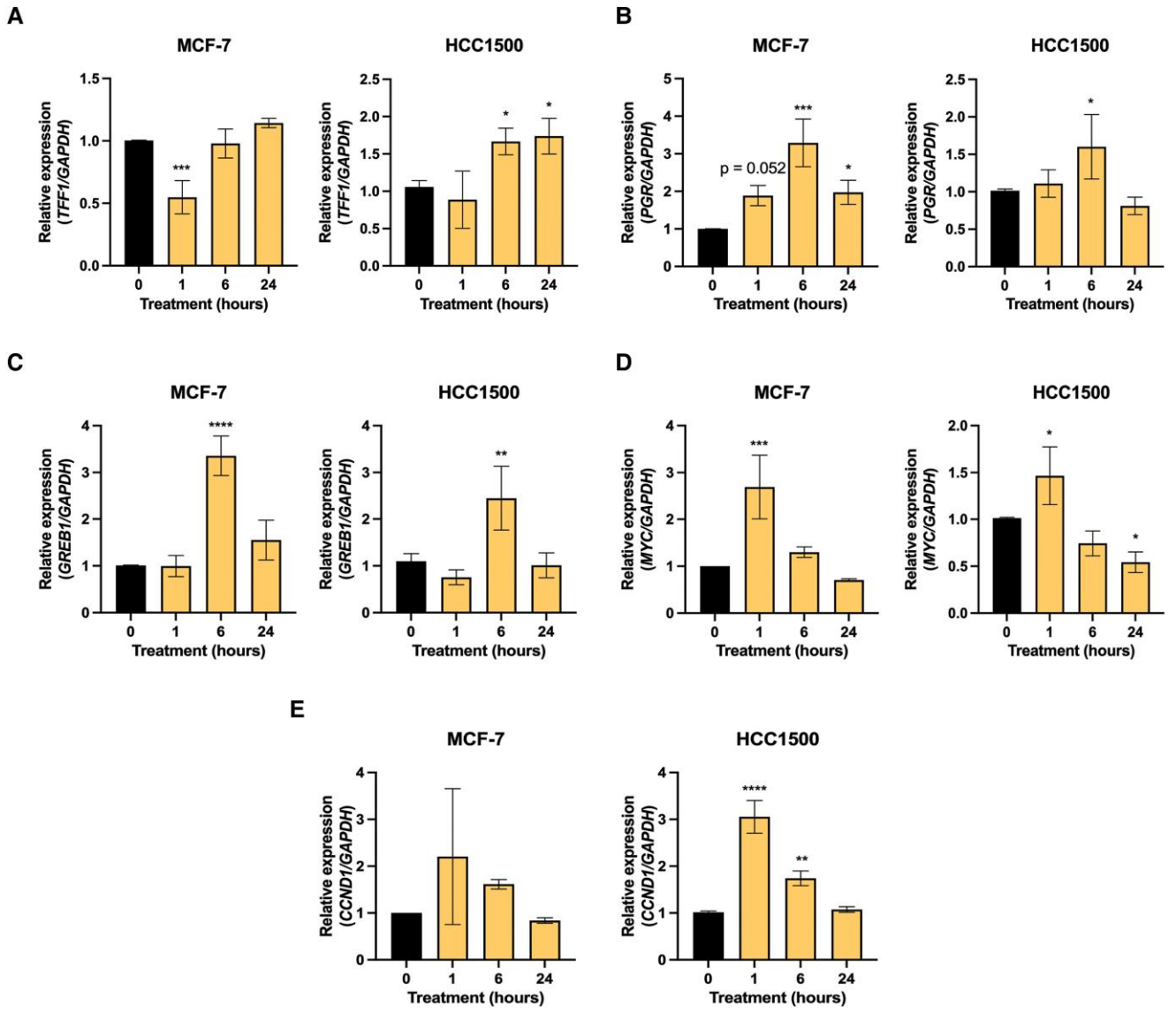

Figure 5. BP-mediated regulation of ER target gene expression is time dependent. MCF-7 and HCC1500 luminal A breast cancer cells were treated with 2 μM BP for 1, 6, or 24 hours. (A-E) Relative gene expression of (A) *TFF1*, (B) *PGR*, (C) *GREB1*, (D) *MYC*, and (E) *CCND1* was assessed by real-time qualitative polymerase chain reaction. Ethanol was used as a negative control, and E2 (10 nM) served as a positive control. GAPDH was used as a housekeeping gene. n = 3, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, 1-way ANOVA.

E2 regulates gene expression with distinct time-course patterns in human breast cancer cells [57-59], so we examined time-dependent regulation of ER target gene expression by parabens to determine whether paraben-mediated effects are time dependent. In general, direct ER targets (*TFF1*, *PGR*, and *GREB1*) are induced by PP or BP with either 6 or 24 hours of treatment. Indirect ER targets (*MYC* and *CCND1*) are induced by PP or BP (and MP for *CCND1*) with 1 hour of treatment. Rapid induction of *MYC* expression by PP (MCF-7) and BP (MCF-7 and HCC1500) treatment for 1 hour is consistent with another study that reported induction of *MYC* at ~30 minutes following E2 exposure and regulation of expression via tethering to AP-1 [47]. The observed paraben-mediated effects on ER target gene expression are consistent with the pattern seen with E2 treatment and the respective mechanisms of regulation for either direct or indirect ER target genes. Future studies should examine the effect of parabens on ER and coregulator recruitment to direct target genes, as well as activation of cell signaling pathways and coregulator recruitment to indirect target genes.

We also observed that parabens regulate ER target gene expression in a cell line–specific manner. In general, the HCC1500 (West African ancestry) luminal A breast cancer cell line seems to be more sensitive to parabens than the MCF-7 (European ancestry) luminal A breast cancer cell line. We observed increased expression of *TFF1*, *PGR*, and *CCND1* with PP (6 hours), increased expression of *GREB1* with BP treatment (6 hours), and increased expression of *PGR* and *CCND1* with MP treatment (6 hours) in HCC1500, but not MCF-7, cells. Paraben-mediated regulation of ER target gene expression is not limited to MCF-7 and HCC1500 luminal A breast cancer cells. We also observed altered ER target gene expression in the luminal B breast cancer cell lines, BT-474 (European ancestry) and MDA-MB-175-VII (West African ancestry). In general, regulation of ER target gene expression by PP or BP was similar in

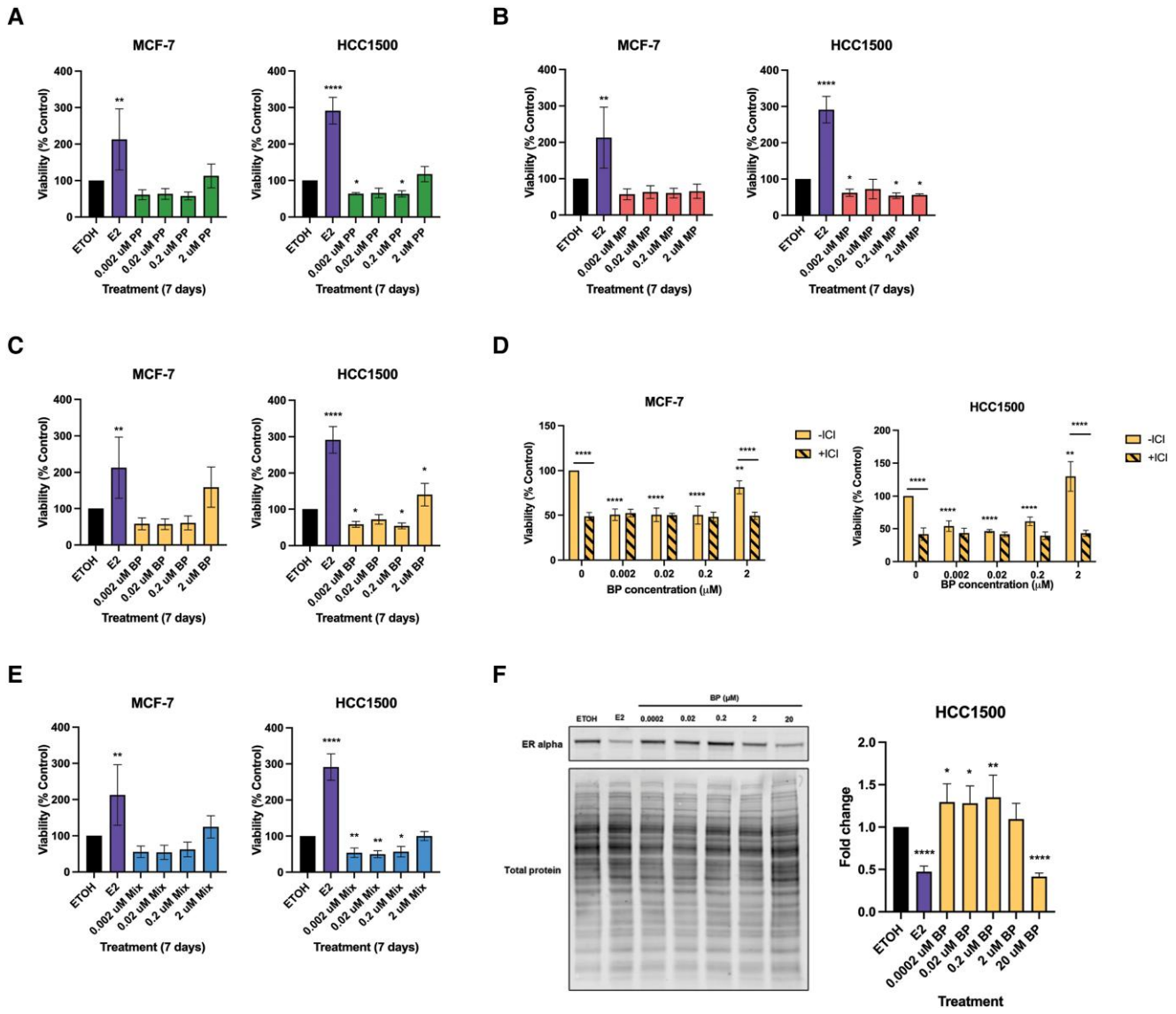

Figure 6. Effect of paraben exposure on cell viability is not ER dependent. MCF-7 and HCC1500 luminal A breast cancer cells were treated with the indicated doses of (A) PP, (B) MP, or (C) BP for 7 days (n = 4). (D) Effect of BP on cell viability with cotreatment with ER antagonist, ICI 182780 (fulvestrant, 1 nM) for 7 days (n = 4). (E) Effect of a mixture of PP, MP, and BP at the indicated doses for 7 days on cell viability (n = 4). (F) HCC1500 cells were treated with the indicated doses of BP for 24 hours. Representative western blot image, and quantified bar graph displaying BP-mediated effect on ERα protein expression in the HCC1500 cell line (n = 6). Ethanol was used as a negative control, and E2 (10 nM) serves as a positive control. n = 4, $*P < .05$, $**P < .01$, $****P < .0001$, (A-C, E) 1-way ANOVA and (D, F) 2-way ANOVA.

BT-474 and MDA-MB-175-VII cells, except for *GREB1* and *MYC*, which are not upregulated by E2 treatment (6 hours) in MDA-MB-175-VII cells. Treatment with PP or BP increased expression of *GREB1* in BT-474, but not MDA-MB-175-VII, cells. *MYC* gene expression was significantly increased in both BT-474 and MDA-MB-175-VII cell lines with PP (20 μM) or BP treatment (2 μM or 20 μM). It should be noted that *PGR* is not expressed in MDA-MB-175-VII; therefore, results for *PGR* expression in BT-474 are not shown.

Estrogens are known to regulate cell proliferation in ER+ breast cancer cells [30, 42, 60]. Studies have found that parabens interact with ER to regulate *MYC* and *CCND1* gene expression in breast cancer and nonmalignant breast cells [33, 61]. *MYC* controls cell growth by promoting the cell cycle and activating cyclins, while *CCND1* plays a role in cell cycle progression [43, 44]. Parabens increase cell growth in breast cancer cell lines with European ancestry [31, 62]; therefore, we also measured the effects of parabens on cell viability in a luminal A breast cancer cell line with West African ancestry (HCC1500). We observed a paraben- and cell line–specific effect on cell viability. Specifically, BP increased cell viability in HCC1500, but not MCF-7, cells. There was no effect of PP and MP on cell viability in either cell line. Once again, this is in line with previous studies that reported BP is more estrogenic compared to the other 2 parabens [50, 51, 63]. Interestingly, the BP- mediated effect on cell viability in HCC1500 is not ER-mediated, suggesting that an alternative mechanism is responsible for this effect. These alternative mechanisms include, but are not limited to (1) through interaction with the membrane-bound G-protein coupled ER, (2) crosstalk with growth factor signaling pathways such as

human epidermal growth factor receptor 2, or (3) by inducing aromatase through regulation of local estrogen levels [61, 63-65]. For instance, Pan et al found that breast cancer cell (BT-474: European ancestry) proliferation was stimulated when BP and heregulin were combined [34]. Whether these alternative mechanisms are responsible for the BP-mediated effects on cell viability in breast cancer cell lines of West African ancestry remains to be further investigated.

We utilized luminal A (MCF-7 and HCC1500) and luminal B (BT-474 and MDA-MB-175-VII) breast cancer cell lines for these experiments. The diversity of cell lines in this study was limited by the number of commercially available breast cancer cell lines of each tumor subtype with confirmed West African ancestry [66]. This limited resource presents a challenge when examining genes and pathways involved in adverse EDC exposures in the context of health disparities [38, 66]. Therefore, examining the ancestry of commonly used cell lines is warranted to adequately model the disparity of breast cancer risk and outcomes within laboratory studies [6]. Prior to this study, West African ancestry of the HCC1500 cell line was confirmed using established Ancestry Informative Markers [38]. Another limitation of this study is the lack of a detailed mechanism underlying the observed effects on ER target gene expression and cell viability due to paraben treatment. We observed that paraben-mediated effects on ER target gene expression, but not cell viability, are ER dependent. We also observed that BP stabilizes ER protein expression in luminal A breast cancer cell lines, suggesting prolonged and abnormal activation of ER upon exposure. However, the exact mechanisms of paraben action are beyond the scope of this paper and will be explored in future studies.

Conclusion

In this study, we observed paraben-mediated changes in ER target gene expression and cell viability that are comparable with those changes observed with E2 treatment. The parabens tested in this study are all commonly used in hair and personal care products marketed to Black women. Given that Black women exhibit higher burden of parabens (and EDCs in general), our results demonstrating protumorigenic effects of parabens in luminal breast cancer cell lines of West African ancestry have translational relevance. It is important to note that this laboratory study exists within the larger context of cultural normative beliefs linked to beauty, individuality, and identity for Black women. Our data highlight the need for culturally relevant intervention strategies to reduce adverse exposure to parabens and other EDCs in hair and other personal care products, thus addressing and reducing known breast cancer disparities in Black women at higher risk for developing the disease.

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Disclosure Statement

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Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

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