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## **Parabens and Human Epidermal Growth Factor Receptor Ligands Cross-Talk in Breast Cancer Cells**

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**Running title:** HER ligands increase potency of parabens

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## Abstract

**Background:** Xenoestrogens are synthetic compounds that mimic endogenous estrogens by binding to and activating estrogen receptors. Exposure to estrogens and some xenoestrogens has been associated with cell proliferation and increased risk of breast cancer. Despite evidence of estrogenicity, parabens are among the most widely used xenoestrogens in cosmetics and personal care products, and generally considered safe. However, previous cell based studies with parabens do not take into account the signaling cross-talk between estrogen receptor (ER $\alpha$ ) and the human epidermal growth factor receptor (HER) family.

**Objectives:** We investigated the hypothesis that the potency of parabens can be increased with HER ligands, such as heregulin (HRG).

**Methods:** The effects of HER ligands on paraben activation of *c-Myc* expression and cell proliferation were determined by real-time PCR, western blots, flow cytometry and chromatin immunoprecipitation assays in ER $\alpha$ - and HER2-positive human BT-474 breast cancer cells.

**Results:** Butylparaben (BP) and HRG produced a synergistic increase in *c-Myc* mRNA and protein levels in BT-474 cells. Estrogen receptor antagonists blocked the synergistic increase in *c-Myc* protein levels. The combination of BP and HRG also stimulated proliferation of BT-474 cells compared to BP alone. HRG decreased the dose required for BP-mediated stimulation of *c-Myc* mRNA expression and cell proliferation. HRG caused the phosphorylation of serine 167 in ER $\alpha$ . BP and HRG produced a synergistic increase in ER $\alpha$  recruitment to the *c-Myc* gene.

**Conclusion:** Our studies demonstrate that HER ligands enhance the potency of BP to stimulate oncogene expression and breast cancer cell proliferation *in vitro* via ER $\alpha$ , suggesting that parabens might be active at exposure levels not previously considered toxicologically relevant from studies testing their effects in isolation.

## Introduction

Xenoestrogens are a class of synthetic estrogens known as endocrine disrupting chemicals that bind to estrogen receptors in cells to mimic or antagonize the action of endogenous estrogens, such as 17 $\beta$ -estradiol (E2) (Zoeller et al. 2012). Numerous xenoestrogens are found in common household products, including plastics, food and soda cans, and personal care products. One class of xenoestrogens that is an increasing public health concern is esters of parahydroxybenzoic acid, commonly known as parabens (Nohynek et al. 2013; Karpuzoglu et al. 2013). They are common ingredients in cosmetics, shampoos, body lotions and sunscreens, where they are used to prevent microbial growth and prolong shelf life (Guo and Kannan 2013; Dodson et al. 2012). Detectable levels of multiple parabens are present in human urine (Calafat et al. 2010; Den Hond et al. 2013; Mortensen et al. 2014) and breast tissue (Darbre et al. 2004; Barr et al. 2012; Darbre and Harvey 2014).

While endocrine disrupting chemicals have been linked to a variety of medical conditions, one of the most troubling is their association with breast cancer (Zoeller et al. 2012; Vandenberg et al. 2012; Darbre and Harvey 2008). Endogenous estrogens promote breast cancer by binding to estrogen receptor  $\alpha$  (ER $\alpha$ ) (Burns and Korach 2012; Sommer and Fuqua 2001), which causes the activation of oncogenes, such as *c-Myc* and *cyclin D1* (Liao and Dickson 2000; Leygue et al. 1995). Cyclin D1 and c-Myc cause cell proliferation by facilitating a G1 to S-phase transition (Foster et al. 2001). Approximately two-thirds of breast tumors express ER $\alpha$ , and therapeutic strategies aimed at preventing and treating ER positive breast tumors are directed at blocking the action of ER $\alpha$ . Parabens are known to bind to ER $\alpha$  (Routledge et al. 1998), promote a G<sub>1</sub> to S-phase cell cycle progression, stimulate the proliferation of MCF-7 breast

cancer cells (Darbre et al. 2003; Wrobel and Gregoraszcuk 2013; Okubo et al. 2001) and activate transcription of cell cycle (Wrobel and Gregoraszcuk 2014) and reporter genes (Darbre et al. 2003; Gomez et al. 2005). These findings indicate that paraben exposure might increase the risk of breast cancer by activating ER $\alpha$  to promote the activation of proliferative genes. However, parabens are considered to be safe due to their weak estrogenic binding affinity, transcriptional activation, and stimulation of cell proliferation, and the dose required for ER $\alpha$  activation often exceeds those found in the body (Lemini et al. 2003; Pugazhendhi et al. 2005). The most estrogenic paraben, butylparaben, was found to be 10,000-fold less potent than E2 (Routledge et al. 1998). However, studies involving xenoestrogens have tested them in the absence of activators of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases (Wrobel and Gregoraszcuk 2013; Wrobel and Gregoraszcuk 2014), a second signaling pathway implicated in breast cancer (Liu et al. 2009).

The HER family comprises four receptors: EGFR/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4 (Davoli et al. 2010). HER2 is a transmembrane protein that is overexpressed in approximately 25% of breast tumors (Davoli et al. 2010). Its presence in human tumors is a negative prognostic indicator, associated with malignant transformation, fast growth, and more aggressive tumors (Barros et al. 2010; Davoli et al. 2010). The association between HER2 expression and breast cancer led to the development of the drug Herceptin (trastuzumab), a recombinant humanized monoclonal antibody against HER2 to treat HER2 positive tumors (Hudis 2007). At least eleven proteins, known as HER ligands, can bind to HER family members to cause dimerization, leading to the activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and other signal transduction pathways (Mosesson and Yarden 2004). Aberrant activation of the PI3K/AKT signaling pathway may increase the risk of cancer by inhibiting

apoptosis and stimulating cell proliferation (Liu et al. 2009). HER and ER $\alpha$  signaling pathways can cross-talk, as demonstrated by the observation that HER ligands stimulate phosphorylation of the serine 167 (ser167) residue in ER $\alpha$  (Al-Dhaheri and Rowan 2006; Lannigan 2003; Murphy et al. 2011). Eliminating the main source of endogenous estrogens by ovariectomy delays the formation of mammary tumors and increases the lifespan of transgenic mice that overexpress HER2 in the mammary gland (Anisimov et al. 2003). Furthermore, when HER2 transgenic mice are mated to ER $\alpha$  knockout mice, tumor onset is delayed compared to control HER2 transgenic mice (Hewitt et al. 2002). Based on these findings, we hypothesize that activators of the HER2 pathway might cause parabens to be stimulatory of ER $\alpha$  at lower doses than suspected based on studies that examined their effects in isolation. In the present study, we determined the potency of parabens in the presence of the HER ligand, HRG in BT-474 breast cancer cells that express both ER $\alpha$  and HER2.

## **Materials and Methods**

### **Cell culture**

Human BT-474, MCF-7, and SKBR3 breast cancer cell lines were obtained from ATCC and used in these studies because of their differences in expression of HER2 and ER $\alpha$ . BT-474 cells are HER2 and ER $\alpha$  positive, MCF-7 cells are ER $\alpha$  positive and HER2 negative and SKBR3 cells are HER2 positive and ER $\alpha$  negative (Neve et al. 2006). Cells were grown in phenol red-free Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 10  $\mu$ g/ml streptomycin (Life Technologies) under 5% CO<sub>2</sub> at 37°C. Three days prior to treatment, the cells were

incubated with DMEM/F12 supplemented with 10% charcoal-dextran stripped FBS (Gemini Bio-Products). Recombinant human heregulin- $\beta$ 1 (HRG) was purchased from Leinco Technologies and used at a final concentration of 20 ng/ml to activate the HER2 signaling pathway. Estradiol, raloxifene, tamoxifen, methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP) were purchased from Sigma-Aldrich. The estrogen receptor antagonist, ICI 182,780 was purchased from Tocris. The compounds were dissolved in ethanol. The final concentration of ethanol was 0.1%, which had no effect on the cells. An ethanol vehicle was used for the control cells.

### **Real-time RT-PCR**

BT-474 cells (passage numbers 86-95) were grown in 6-well tissue culture dishes to reach 80% confluence and then maintained in DMEM/F12 supplemented with 10% charcoal-dextran stripped FBS for 3 days. The cells were treated with 0.01  $\mu$ M E2 or 10  $\mu$ M MP, EP, PP or BP in the absence or presence of 20 ng/ml HRG for 2 h. The 10  $\mu$ M concentration of parabens was selected by performing preliminary dose-response studies. Total RNA was isolated and purified using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Inc). RNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer. Reverse transcription of total RNA was carried out using iScript (Bio-Rad Laboratories, Inc) as previously described (Paruthiyil et al. 2009). SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc) was used for PCR and DNA amplification of the *c-Myc* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) genes with a Bio-Rad CFX96 Real-Time System. The following PCR primers were used:



GAPDH- Forward 5'-CGATGCTGGCGCTGAGTACGT-3'; GAPDH- Reverse 5'-  
CCTGCAAATGACCCCCAGCCTTC-3'; c-Myc- Forward 5'-  
GGAAAACCAGCAGCCTCCCGC-3'; c-Myc- Reverse 5'-ACGGCTGCACCGAGTCGTAG-3'.

The expression levels *c-Myc* and *GAPDH* were determined by the comparative Ct method as previously described (Paruthiyil et al. 2009).

### **Western blot**

Human BT-474, MCF-7, and SKBR3 cells were grown in 6-well tissue culture dishes in phenol red-free DMEM/F12 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 10 µg/ml streptomycin under 5% CO<sub>2</sub> at 37°C. Three days prior to treatment (80% confluence), the medium was replaced with DMEM/F12 supplemented with 10% charcoal-dextran stripped FBS. The cells were then treated for 2 h with increasing concentrations of BP in the absence and presence of 20 ng/ml HRG. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5 sodium deoxycolate, 0.1% SDS and cOmplete Protease Inhibitor Cocktail (Roche). Total protein concentration was determined with the Coomassie Plus<sup>TM</sup> Protein Assay Reagent (Thermo Scientific). 15 µg of cell proteins from each sample were then separated by SDS-Polyacrylamide Gel Electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk in Tris-Buffered Saline with Tween 20 (TBST) containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% tween 20 and probed overnight with rabbit anti-c-Myc IgG (sc-764, Santa Cruz Biotechnology, Inc.) at 0.5 µg/ml in 1% milk-TBST at 4°C. After washing with TBST for 5 min for three times at room temperature, the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (sc-

2054, Santa Cruz Biotechnology, Inc.) at 1:10,000 dilution in 1% milk-TBST for 1 hour at room temperature. Proteins were visualized using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences). The western blot for ER $\alpha$  phosphorylation was performed as described for c-Myc except that the cell lysis buffer contained a phosphatase inhibitor cocktail (PhosSTOP, Roche) and the primary antibody was anti-phospho-ER $\alpha$  S167 (Bethyl Laboratories).

### **Chromatin immunoprecipitation (ChIP)**

Confluent BT-474 cells were treated with butylparaben in the absence and presence of HRG (20 ng/ml) for 1-3 h. The cells were harvested for ChIP assay as previously described (Cvoro et al. 2006) with some modifications. Briefly, to cross-link proteins to DNA, the cells were fixed by adding formaldehyde to the cell culture medium and incubated at 37°C for 10 min, followed by the addition of glycine for 5 min at room temperature to quench the cross-linking reaction. The cell monolayer was then washed with Phosphate-Buffered Saline (PBS) containing cOmplete Protease Inhibitor Cocktail and collected by scraping. The cells were concentrated by centrifugation and lysed with buffer containing 0.5% of Triton X-100, 50 mM of Tris-HCl, pH 7.4, 150 mM of NaCl, 10 mM of EDTA and cOmplete Protease Inhibitor Cocktail Tablets as previously described (Vivar et al. 2010). The cell lysate was centrifuged at 2000 $\times$  g for 5 min and the pellets were resuspended in RIPA buffer containing cOmplete Protease Inhibitor Cocktail Tablets. Each sample was sonicated on ice to shear genomic DNA, and the samples were incubated with 4  $\mu$ g/ml rabbit anti-ER $\alpha$  IgG (sc-544, Santa Cruz Biotechnology) or the same concentration of normal rabbit IgG (sc-2025, Santa Cruz Biotechnology) at 4°C overnight with rotation. Immunoprecipitation was done with Protein G Mag Sepharose (GE Healthcare) using the manufacturer's instructions. The protein-DNA complex was eluted in 1% sodium

dodecyl sulfate and 0.1 M of NaHCO<sub>3</sub>, and cross-linking of protein bound DNA was reversed by incubating the samples at 65°C overnight. DNA was purified using ChIP DNA Clean & Concentrator kit (Zymo Research). ER $\alpha$  antibody precipitated DNA was amplified by real-time PCR with specific primers for *c-Myc* enhancer region as previously described (Wang et al.).

### **Cell Cycle Analysis using Flow Cytometry**

The effects of different treatments on cell cycle phase was analyzed by flow cytometry based on the method previously described (Wiepz et al. 2006). Briefly, BT-474 cells were plated at 500,000 cells per well in 6-well tissue culture dishes with phenol red-free DMEM/F12 supplemented with 10% stripped FBS. 48 hours later the culture medium was switched to serum-free DMEM/F12. After 24 hours of synchronization with serum-free DMEM/F12, the cells were treated with the indicated concentrations of BP in the presence and absence of HRG (20 ng/ml) for 24 h. The cells were then trypsinized and collected by centrifugation at 1700 rpm for 5 minutes at room temperature. The cell pellets were washed once with ice cold PBS and centrifuged at 1700 rpm for 10 minutes at room temperature followed by resuspension in 500  $\mu$ l propidium iodide solution (PBS containing 0.1% Triton 100, 0.1% sodium citrate, 10  $\mu$ g/ml RNase and 0.05 mg/ml propidium iodide) to stain the cells. The cell suspensions were assayed with a Cytomics FC-500 flow cytometer (Beckman Coulter) using CXP software in the flow cytometry core facility at University of California, Berkeley and the data were then analyzed using FlowJo 7.6.5 (FlowJo).

### **Cell Proliferation Assay**

BT-474 cells were plated in 6-well tissue culture dishes in phenol red-free DMEM/F12 supplemented with 10% charcoal-dextran stripped FBS. The next day, the cells were treated with

increasing concentrations (0.01 - 1  $\mu$ M) of BP in the absence or presence of HRG (20 ng/ml) and incubated for 1 or 5 days without changing the medium. After treatment, the cells were washed with PBS and detached from the plates with trypsin and then placed in ISOTON and counted with a Coulter Counter (Beckman Coulter).

## Statistical Analysis

Data are presented as the mean  $\pm$  SD or mean  $\pm$  SEM as indicated in the figure legend. The statistical significance of differences was examined by one-way analysis of variance or two-way analysis of variance (ANOVA) tests as specified in the figure legend. All ANOVA tests are followed by Tukey's multiple comparisons *post hoc* tests to analyze the difference between different time periods or doses within groups treated with same reagents (BP, HRG or BP plus HRG). Bonferroni's multiple comparisons *post hoc* test was applied to analyze the difference between groups with and without HRG within the same paraben treatment or the same time period. Data analysis was performed by using GraphPad Prism (version 6.01; GraphPad Software Inc.; La Jolla, CA, USA).

## Results

### **Combined effects of parabens and heregulin on *c-Myc* transcript levels in BT-474 breast cancer cells.**

Since BT-474 cells express both ER $\alpha$  and HER2 (Lazaro et al. 2013) they represent a suitable cell model to explore the interactions between ER $\alpha$  and HER2 signaling pathways. BT-474 cells were treated with MP, EP, PP and BP, which are commonly present in cosmetics and personal care products, in the absence and presence of HRG. PP and BP were the most effective

parabens at increasing *c-Myc* mRNA levels in the absence of HRG (Figure 1A). HRG alone produced about a 3-fold increase in *c-Myc* mRNA levels, but a synergistic increase that was greater than additive was observed with PP and BP (Figure 1A). BP was the most effective stimulator of the *c-Myc* mRNA levels in the absence and presence of HRG, and was selected for further studies. The maximal increase of *c-Myc* expression level by BP was observed at 10  $\mu\text{M}$  (Figure 1B). The synergistic effect of HRG was observed when BT-474 cells were treated with BP for 1 hour (Figure 1C). These results demonstrate that HRG decreases the dose required for the BP-mediated increase in *c-Myc* mRNA levels and enhances the magnitude of the BP response.

#### **Combined effects of heregulin and BP on c-Myc protein levels in ER $\alpha$ positive cell lines.**

To determine the effect of HRG on BP stimulation of c-Myc protein production, BT-474 cells were treated with HRG in the absence or presence of increasing concentrations of BP for 2 hours. No increase in c-Myc protein levels were observed with BP or HRG (Figure 2A) alone. In the presence of HRG, with 1  $\mu\text{M}$  and 10  $\mu\text{M}$  BP, the increase in c-Myc protein levels was similar to that induced by 0.01  $\mu\text{M}$  E2 plus HRG. In MCF-7 cells, which express ER $\alpha$ , but not HER2 an enhanced BP induction of c-Myc protein levels were observed with HRG similar to the BT-474 cells (Figure 2B). In contrast, in the SKBR3 cell line, which is HER2 positive and ER $\alpha$  negative, no synergistic increase in c-Myc protein levels was observed. The increase in c-Myc protein levels in BT-474 cells with BP and HRG was blocked by the estrogen receptor antagonists, ICI 182,780, raloxifene and tamoxifen (Figure 3). These results indicate that HRG potentiates BP stimulation of c-Myc only in ER $\alpha$  positive breast cancer cells and that the potentiation requires ER $\alpha$  signaling.

### **Combined effects of heregulin and BP on BT-474 cell proliferation.**

The effect of HRG on BP stimulation of BT-474 cell proliferation was examined by flow cytometry. BT-474 cells were treated with BP alone or with HRG and BP for 24 hours. DNA content in the cells was measured by flow cytometry. BP treatment alone increased the number of cells entering the S-phase at 1  $\mu\text{M}$  (Figure 4A). The addition of HRG resulted in an increased potency of BP. The  $\text{EC}_{50}$  for BP alone was 0.551  $\mu\text{M}$ , whereas the  $\text{EC}_{50}$  for the BP/HRG combination was 0.024  $\mu\text{M}$  (Figure 4A). To compare the results derived from the flow cytometry study, we counted the cells with a Coulter counter after 24 hours treatment with BP in the absence and presence of HRG. BP alone did not stimulate cell proliferation after 24 hours (Figure 4B). A significant increase in cell number occurred with 0.1 and 1  $\mu\text{M}$  BP in the presence of HRG (Figure 4C). The shift in BP potency was more pronounced after treatment for 5 days. In the absence of HRG, 1  $\mu\text{M}$  BP was required to produce a significant increase in cell number (Figure 4D), whereas in the presence of HRG, 0.01  $\mu\text{M}$  BP significantly increased cell number (Figure 4E). These findings indicate that HRG lowers the dose of BP required to stimulate BT-474 cell proliferation.

### **Effect of heregulin on serine 167 phosphorylation of $\text{ER}\alpha$ and the recruitment of $\text{ER}\alpha$ to the *c-Myc* enhancer by the heregulin and BP combination.**

One potential mechanism whereby HRG and BP could cooperate to produce a synergistic activation of *c-Myc* expression is through phosphorylation of  $\text{ER}\alpha$  and subsequent enhanced binding of  $\text{ER}\alpha$  to the *c-Myc* gene. To explore this possibility, the phosphorylation of ser167 in  $\text{ER}\alpha$  was assessed by western blotting in BT-474 cells treated with HRG for increasing times.

HRG caused a detectable increase in the phosphorylation of ser167 in ER $\alpha$  at 30 min with a maximal response obtained at 2 hours (Figure 5A). HRG did not change the level of the unphosphorylated form of ER $\alpha$  or the levels of  $\beta$ -actin. The recruitment of ER $\alpha$  to a known ER binding site in the *c-Myc* enhancer element was examined by CHIP in BT-474 cells after treatment with HRG and BP. A maximal 8-fold enhancement of ER $\alpha$  binding to the *c-Myc* enhancer sequence was observed after 1 hour (Figure 5B). The increase in ER $\alpha$  binding was greater in cells treated with both HRG and BP compared to cells treated with only HRG or BP (Figure 5C). These results demonstrate that the HRG and BP combination increases ER $\alpha$  phosphorylation and the recruitment of ER $\alpha$  to the *c-Myc* enhancer.

## Discussion

HER2 and ER $\alpha$  are components of two major signaling pathways that are often altered in breast cancers (Nair et al. 2012). Xenoestrogens can mimic endogenous estrogens to promote proliferation of breast cancer cells (Jenkins et al. 2012). Most studies have investigated the effects of xenoestrogens alone on endpoints such as cell proliferation (Wrobel and Gregoraszczyk 2013; Wrobel and Gregoraszczyk 2014). However, the biological effects of xenoestrogens, particularly at low doses, might be altered in the presence of factors that activate other signaling pathways that can cross-talk with estrogen receptors (Schiff et al. 2004). For example, growth factors such as HRG and EGF activate downstream Akt signaling, which causes the phosphorylation of Ser167 in ER $\alpha$  (Nagashima et al. 2008; Joel et al. 1998). Phosphorylation of ER $\alpha$  plays a critical role in gene transcription by enhancing ligand binding to ER, nuclear localization, dimerization, DNA binding, and coactivator recruitment (Al-Dhaheri and Rowan 2006; Lannigan 2003; Murphy et al. 2011). Based on these findings, we

hypothesized that studies using parabens and other xenoestrogens alone could underestimate their proliferative effects in breast cells, and potency to promote breast cancer, particularly at lower doses.

A major rationale promulgated in favor of the safety of xenoestrogens in consumer products is that, at biologically relevant concentrations, they bind to estrogen receptors with too low affinity to produce significant biological effects in humans (Golden et al. 2005). For example, BP was found to bind to ER $\alpha$  with about 10,000 fold lower affinity than E2 (Bolger et al. 1998). Similarly, functional assays of ER $\alpha$  such as reporter assay activation and MCF-7 cell proliferation found that physiologically implausible concentrations of parabens are needed for ER $\alpha$  activation (Golden et al. 2005). However, this argument does not take into account the possibility that other signaling pathways in cells, particularly those that promote cell proliferation, might potentiate paraben and other xenoestrogen effects by sensitizing ER $\alpha$  to activation at lower doses. The current studies investigated if parabens are more potent in the presence of HER ligands. We demonstrated that HRG and butylparaben can produce a synergistic increase in mRNA levels of the oncogene *c-Myc*. Increased *c-Myc* mRNA levels is accompanied by a corresponding increase in c-Myc protein levels. The synergy requires the presence of ER $\alpha$ , because the synergy was blocked by estrogen receptor antagonists and no synergy was observed in the ER $\alpha$  negative, HER2-positive SKBR3 cell line. Unexpectedly, we observed that synergistic activation occurred in HER2-negative MCF-7 cells which suggests that other receptors of the HER family can mediate the effect of HRG on estrogenic sensitivity, as discussed below. A HRG-mediated increase in potency of BP was observed in two different



proliferative assays. Our results demonstrated that HRG lowers the dose of BP required to significantly affect proliferation of ER positive breast cancer cells.

While it is clear that endogenous estrogens increase the risk of breast cancer, the role of parabens in breast cancer is controversial, in part due to uncertainty about whether concentrations of parabens present in the body are sufficient to mimic the effects of endogenous estrogens on breast cells (Karpuzoglu et al. 2013; vom Saal et al. 2007; Harvey 2003). Our studies demonstrated that even in the presence of HRG, higher concentrations of parabens than E2 were needed to stimulate *c-Myc* expression and cause proliferation of BT-474 cells. However, the propylparaben and butylparaben concentrations at which we observed estrogenic effects in the presence of HRG are within the range of concentrations previously reported in human breast tissue (Barr et al. 2012). Large-scale biomonitoring studies have reported urinary paraben levels from 0.001 to 1  $\mu$ M (Calafat et al. 2010; Frederiksen et al. 2011), although the relationship between urine and tissue levels remains uncertain. Furthermore, breast tissues may contain multiple parabens (Darbre et al. 2004), and combinations of different parabens can produce additive effects on proliferation (Charles and Darbre 2013). We found that HRG acted synergistically with PP and BP to increase *c-Myc* gene expression. Further studies will be needed to determine the effect of HRG in the presence of combinations of parabens or other xenoestrogens.

The presence of multiple HER receptors and ligands in breast tissues may affect the activity of parabens. The known eleven endogenous HER ligands can bind to one or more of the HER receptors (Mosesson and Yarden 2004) with the notable exception of HER2, for which there is no known ligand (Harari and Yarden 2000). However, the binding of ligands to HER1,

HER3 or HER4 leads to a preferential dimerization and activation of HER2 (Rubin and Yarden 2001). Further work will be needed to determine if other HER ligands potentiate the effect of parabens, and their relative potency compared to HRG. Interestingly, breast cancer cells are autocrine producers of HER ligands. In a study of 363 breast tumors it was found that 80%–96% of the tumors expressed at least one of ten tested HER ligands (Revillion et al. 2008). Similarly, another study found that 48% of breast tumors express HRG (Esteva et al. 2001). Breast tumors may therefore potentiate their own response to estrogenic compounds by producing HER ligands.

## **Conclusion**

Our data showing that lower doses of butylparaben are required to stimulate breast cancer cell proliferation in the presence of HRG together with the observations that breast tumors are exposed *in vivo* to both HER ligands (Revillion et al. 2008) and parabens (Darbre et al. 2004) indicate a potential synergy relevant to proliferation of tumor cells in humans. Further work is needed to assess if indeed HER ligands enhance the potency of parabens in the normal human breast cells and breast tumors. We suggest that reevaluation of the potency of other xenoestrogens in the presence of HER ligands is warranted in the light of our findings.

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## Figure Legends

**Figure 1.** Effect of parabens and heregulin on *c-Myc* transcript levels in BT-474 breast cancer cells. (A) Human BT-474 breast cancer cells were treated with 10  $\mu\text{M}$  methylparaben (MP), 10  $\mu\text{M}$  ethylparaben (EP), 10  $\mu\text{M}$  propylparaben (PP) or 10  $\mu\text{M}$  butylparaben (BP) for 2 hours in the absence and presence of HRG (20 ng/ml). (B) BT-474 breast cancer cells were treated with 0.01  $\mu\text{M}$  E<sub>2</sub> or increasing concentrations (0.1  $\mu\text{M}$  to 100  $\mu\text{M}$ ) of BP in the absence and presence of HRG (20 ng/ml) for 2 hours. (C) BT-474 breast cancer cells were treated with 10  $\mu\text{M}$  BP in the absence or presence of HRG (20 ng/ml) for indicated time periods. Relative mRNA levels for *c-Myc* were determined by real-time PCR and normalized to *GAPDH* using the comparative CT method. The fold changes were obtained by comparing the treated to the control values. Each data point is the average of triplicate samples  $\pm$  SEM. The figures are representative of three experiments with similar results. The statistical significance of means was analyzed by two-way ANOVA followed by a Tukey's multiple comparisons *post hoc* test for analyzing the significance of difference between control and various parabens (A), various doses of BP (B), and various time periods (C) in the presence of HRG. The asterisk \* represents  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ . Differences in *c-Myc* expression comparing cells receiving the same paraben treatment (A), same dose of BP (B) and same time period (C), but with and without HRG, were tested using Bonferroni's multiple comparisons *post hoc* test, ++  $p < 0.01$  and +++  $p < 0.001$ .

**Figure 2.** Effect of heregulin on BP stimulation of c-Myc protein levels in breast cancer cell lines. (A) BT-474 and (B) MCF-7 breast cancer cells were treated with indicated concentrations of BP without or with HRG (20 ng/ml) for 2 hours. (C) SKBR3 cells were treated with 10  $\mu\text{M}$  BP in the absence and presence of HRG (20 ng/ml) for 2 hours. The treated cells were then lysed and cellular lysates were prepared for western blots using antibodies against c-Myc as described in Materials and Methods. Cells treated with E<sub>2</sub> (0.01  $\mu\text{M}$ ) simultaneously were also included for comparison. After exposure to an X-ray film, the membranes were washed and reprobed with an antibody against  $\beta$ -actin as loading control. This figure is representative of two independent experiments with similar results.

**Figure 3.** Effect of estrogen receptor antagonists on the synergistic increase of c-Myc protein levels. BT-474 cells were treated with 10  $\mu$ M BP in the absence or presence of 1  $\mu$ M ICI 182,780, 1  $\mu$ M raloxifene, 10  $\mu$ M tamoxifen and 20 ng/ml HRG for 2 hours. The treated cells were then lysed and cellular lysates were prepared for western blots using antibodies against c-Myc as described in Materials and Methods. After exposure to an X-ray film, the membranes were washed and re probed by antibody against  $\beta$ -actin as loading control. This figure is representative of two independent experiments with similar results.

**Figure 4.** Effect of heregulin on the potency of BP stimulation of BT-474 cell proliferation. (A) BT-474 cells were treated with the indicated concentrations of BP alone or BP and HRG for 24 hours. Changes in cell cycle distribution were then analyzed by flow cytometry as described in Materials and Methods. The percentage of S-phase cells was plotted for different BP concentrations in the absence and presence or HRG. The plotted values are the means ( $\pm$ SD) of biological triplicates, which represents three independent experiments with similar results. (B, C) BT-474 cells were plated in 6-well dishes at 250,000 cells/well. The culture medium was then switched to serum-free media 24 h later and treated with indicated concentration of BP in the absence (B) or presence (C) of HRG (20 ng/ml). The cells were harvested after 24 h of treatment and then counted with a Coulter counter. The figures are representative of three experiments with similar results. The data are expressed as means ( $\pm$  SD) obtained from biological triplicates. Statistical significance was analyzed by one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test to compare difference between control and each dose. (C) \*  $p < 0.05$  and \*\*  $p < 0.01$  compared with HRG alone. (D, E) BT-474 cells were plated in 6-well dishes at 50,000 cells/well and treated with indicated amounts of BP in the absence (D) or presence (E) of HRG. The cells were harvested after five days of treatment and then counted with a Coulter counter. The data are expressed as means ( $\pm$ SD) obtained from biological triplicates and are representative of three experiments with similar results. The statistical significance was analyzed with one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test to compare difference between control and each dose of BP with or without HRG. (D) \*\*\*  $p < 0.001$  compared with the untreated control. (E) \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  compared with cells treated with HRG alone.

**Figure 5.** Effect of heregulin on the phosphorylation of Ser167 in ER $\alpha$  and the recruitment of ER $\alpha$  to the *c-Myc* enhancer. (A) BT-474 cells were treated with HRG (20 ng/ml) for 0.5, 1, 2, 3 and 4 hours. The treated cells were lysed and subjected to Western Blot using an anti-ER $\alpha$  Ser167 phosphorylation antibody to probe phosphorylated ER $\alpha$ . Total ER $\alpha$  expression in the treated cells was also determined by Western Blot with anti-ER $\alpha$  monoclonal antibody. (B) BT-474 cells were treated with BP (10  $\mu$ M) in the presence of HRG (20 ng/ml) for 1, 2 and 3 hours. The ER $\alpha$  recruitment on *c-Myc* enhancer was examined by ChIP assays with anti-ER $\alpha$  antibody. (C) BT-474 cells were treated with HRG (20 ng/ml), BP (10  $\mu$ M), or BP plus HRG for 1 hour and then subjected to ChIP assays with anti-ER $\alpha$  antibody. The data (B and C) shown are derived from quantitative real-time PCR analysis of an ER $\alpha$  binding site in *c-Myc* enhancer region. The Ct values of ER $\alpha$  antibody precipitated DNA were adjusted by non-specific immunoprecipitated DNA. The fold changes were obtained by comparison of adjusted Ct values of treated samples with those of a non-treated sample (control). The results are expressed as mean  $\pm$  SD from triplicate experiments. The statistical significance was analyzed with one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test to compare difference between control and each time period (B) and HRG, BP and HRG plus BP. (C) \*\*\*  $p < 0.001$ .

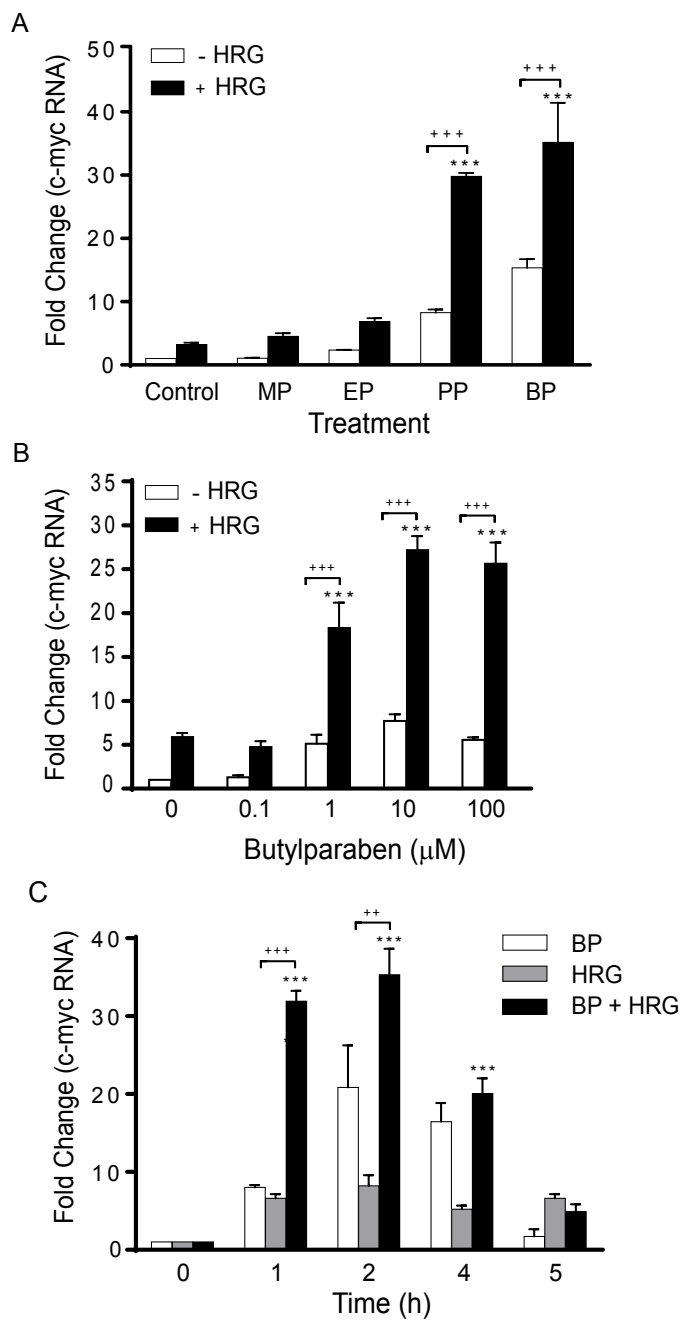


Figure 1

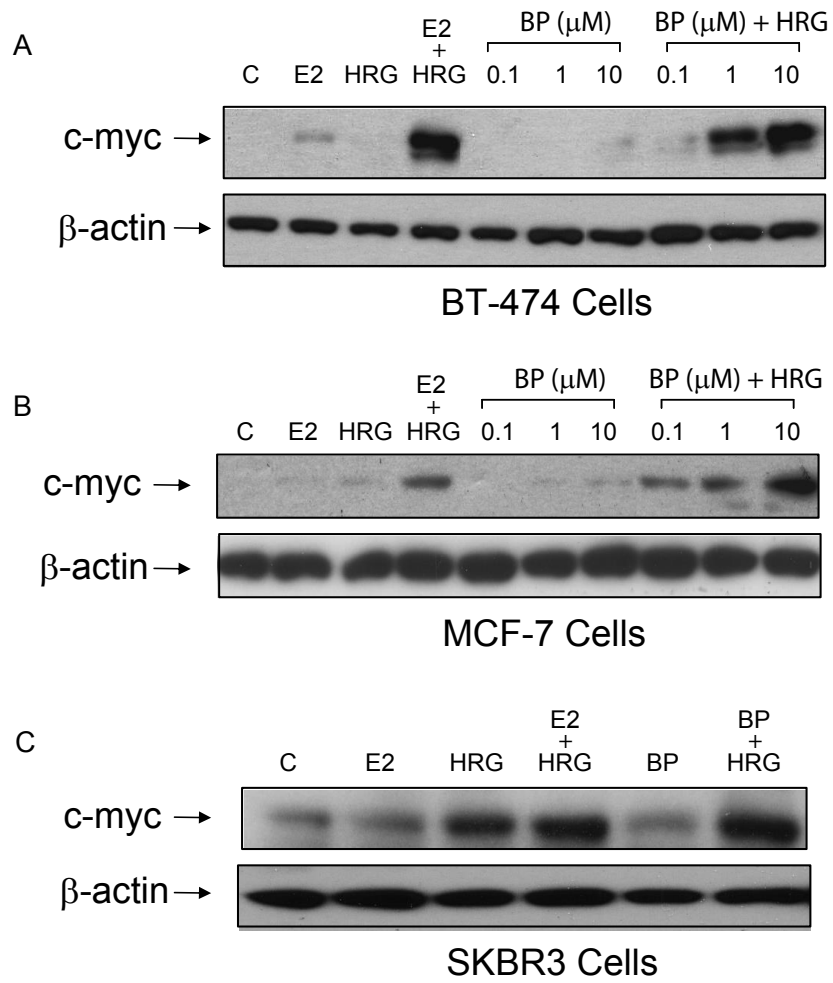


Figure 2

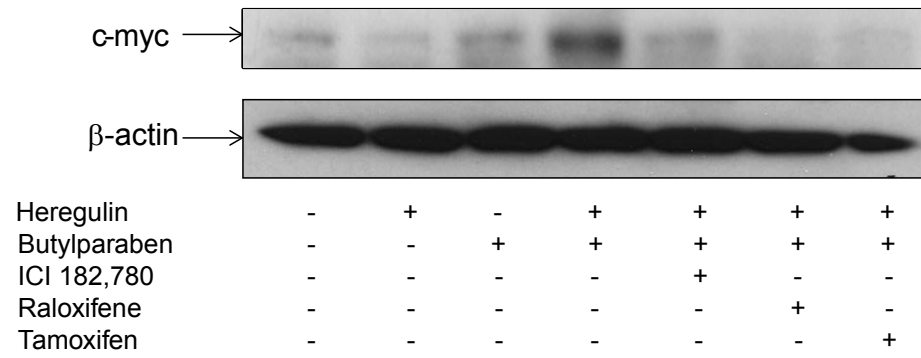
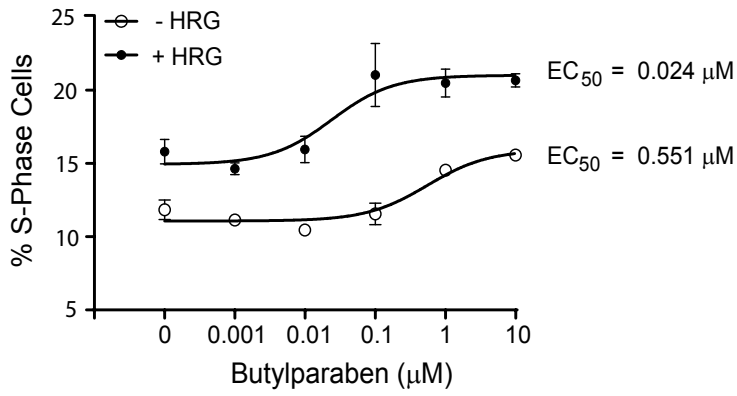
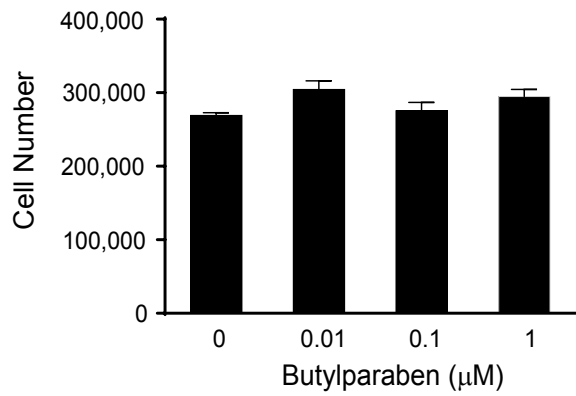


Figure 3

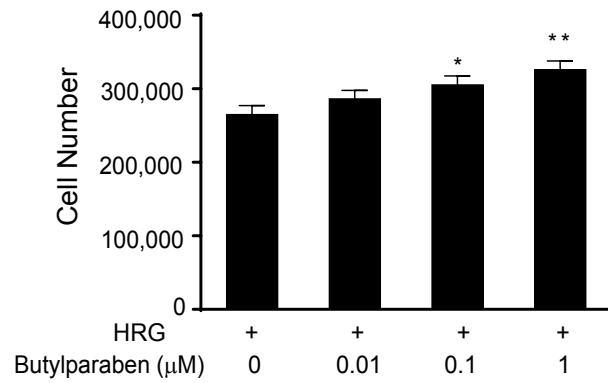
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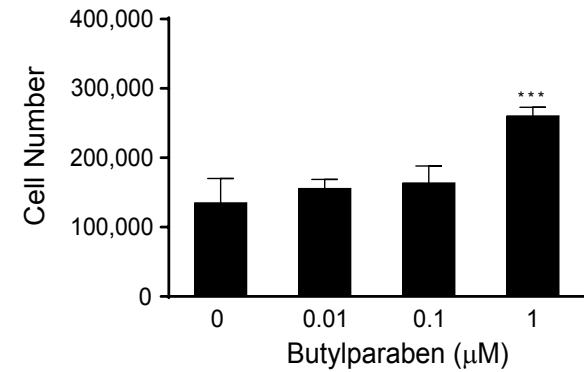
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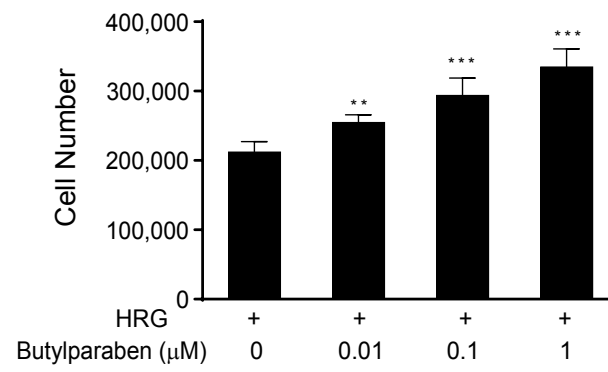


Figure 4

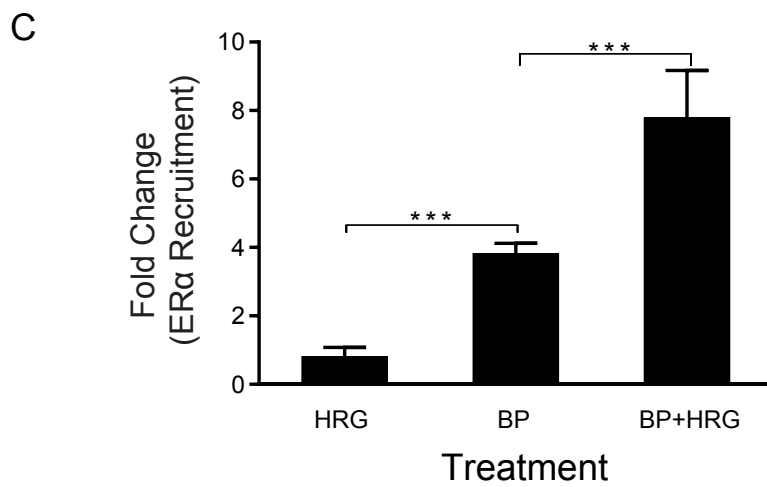
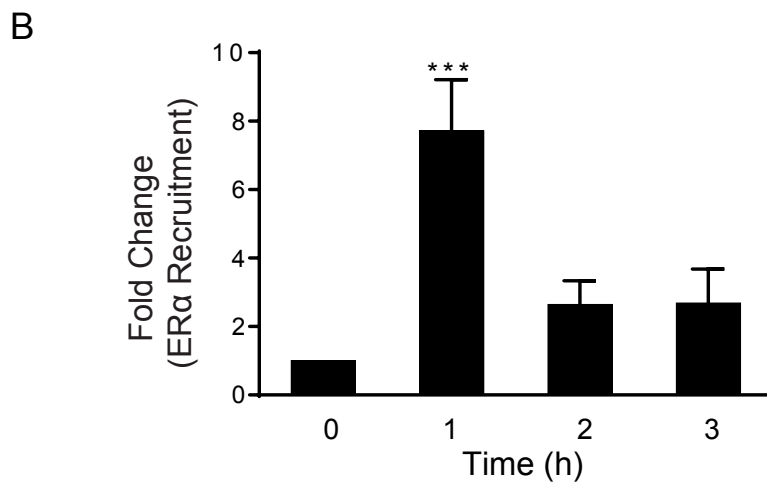
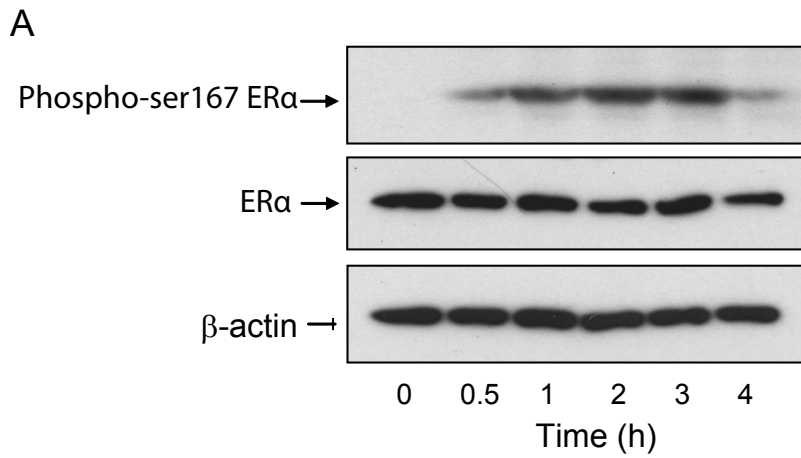


Figure 5