Paraben and Breast Cancer: A Stromal Link

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ABSTRACT

Breast cancer, surpassing lung cancer with the highest estimated new cases, is closely associated with obesity. Cancer research continues to divulge the complexity of the disease including the recognition that stromal cells must cooperate with the cancerous epithelium for cancer growth and metastasis.

Parabens are a class of esters of 4-hydroxybenzoic acid that is ubiquitous in the environment, and act as anti-microbial agents that sustain the shelf-life of personal care products, pharmaceuticals, food, etc. Parabens are classified as endocrine disrupting chemicals, are estrogenic, and bioaccumulate within adipose tissue.

Activation of fibroblasts is caused by tissue damage leading to the release of inflammatory cytokines to stimulate wound healing. Low dose (0.02, 0.1, and 1 μM) methyl-, propyl, and butylparaben (MP, PP, and BP) treatment onto murine 3T3-L1 and human primary breast (BRF) fibroblasts led to an inflammatory response by the upregulation of inflammatory cytokines including interleukin-1β (IL-1β) and cyclooxygenase-2 (COX2) via activation of the NF-κB pathway (Chapter III). MP, PP, and BP also upregulated matrix metalloproteinase-9 (MMP9), indicating potential influence in metastasis. Paraben-treated fibroblasts also increased the mRNA expression of CYP19A1 otherwise known as aromatase, which influences the conversion of androgen to estradiol. Secretions from paraben-treated fibroblasts also obtained the ability to enhance proliferation of estrogen receptor positive (ER+) breast cancer cells (MCF7).

To evaluate the contribution of adipocytes, 3T3-L1 and BRF adipocytes were treated with MP, PP, and BP and induced upregulation IL-1β, COX2, MMP9, and CYP19A1 (Chapter IV). The secretions from paraben-treated adipocytes also enhanced the proliferation of MCF7 cells.

In conclusion, we have shown that environmental achievable low doses of parabens enhance the inflammatory response of stromal fibroblasts and adipocytes, and the secretions produced from paraben exposure enhance the proliferation of breast cancer cells. Our results support the existing evidence that paraben exposure may lead to increased risks of breast cancer and suggest the stromal link between parabens and breast cancer. Further

studies are warranted to confirm the effects of parabens on breast stromal cells and carcinogenesis *in vivo* and to establish the role of NF-κB pathway.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

AARSL	Alanyl-tRNA Synthestase Like
AIAN	American Indian/Alaska Native
Akt	Protein Kinase B
ALDH1	Aldehyde Dehydrogenase 1
AM-1	Adipocyte Maintenance Media
ANOVA	Analysis of Variance
API	Asian/Pacific Islander
BCL2	B-Cell Lymphoma 2
BenzP	Benzylparaben
β-gal	β-Galactosidase
BMI	Body Mass Index
BP	Butylparaben
BRCA1	Breast Cancer Type 1
BRCA2	Breast Cancer Type 2
CaBP-9k	Calbindin-D9k
CAPE	Caffeic Acid Phenethyl Ester
cAMP	Cyclic Adenosine Monophosphate
CARΔ1	Coxsackievirus and Adenovirus Receptor Delta 1
CBMN	Cytokinesis-Block Micronucleus
CCD	Charge-Coupled Device
CD-1	Cluster of Differentiation 1
CDK	Cyclin Dependent Kinase
CELF5	CUGBP Elav-Like Family Member 5
ChIP	Chromosome Immunoprecipitation
CLS	Crown-Like Structures
CM	Conditioned Media
COX2	Cyclooxygenase-2
CS-FBS	Charcoal-Stripped Fetal Bovine Serum

CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
DCIS	Ductal Carcinoma In Situ
DEX	Dexamethasone
DM2	Differentiation Media 2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DSB	Double Stranded Break
E2	Estradiol
E2F1	E2F Transcription Factor 1
ECM	Extracellular Matrix
EDC	Endocrine Disrupting Chemicals
EEF	Estradiol Equivalence Factor
EMT	Epithelial to Mesenchymal Transitions
EP	Ethylparaben
ER	Estrogen Receptor
ERK1/2	Extracellularly Regulated Kinase 1/2
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
ETP	Estrogen-Equivalent Total Paraben
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
GI	Gastrointestinal
GPER1	G Protein-Coupled Estrogen Receptor 1
GPR30	G Protein-Coupled Receptor 30
GR	Glucocorticoid Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hormone receptor
HRG	Heregulin
HSD	Hydroxysteroid Dehydrogenase
IBP	Isobutylparaben

Icabp	Intestinal Calcium Binding Protein
ICI182,780	Faslodex
IκBα	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells Inhibitor, Alpha
IKKβ	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta
IL1β	Interleukin 1 Beta
IL17RB	Interleukin 17 Receptor B
Ins	Insulin
IPP	Isopropylparaben
Irf7	Interferon Regulatory Factor 7
Itmap	Integral Membrane-Associated Protein 1
IVIS	*In vivo* Imaging System
LBD	Ligand Binding Domain
LH	Luteinizing Hormone
LOEL	Lowest-Observed-Effect Levels
LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen-Activated Protein Kinase
MCF7	Michigan Cancer Foundation-7
MCF10A	Michigan Cancer Foundation-10A
MLL2	Myeloid/Lymphoid Mixed Lineage Leukemia 2
MIX	Methylisobutylxanthine
MMP	Matrix Metalloproteinases
MMTV	Mouse Mammary Tumor Virus
MP	Methylparaben
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NHB	Non-Hispanic Black
NHW	Non-Hispanic White
NOAEL	No Observed Adverse Effect Level

NOEL	No-Observed-Effect Levels
Oct4	Octamer-Binding Transcription Factor 4
ORO	Oil Red O
OTOF	Otoferlin
PDX	Patient Derived Xenograph
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PM-1	Preadipocyte Media-1
PP	Propylparaben
PR	Progesterone Receptor
PRF	Phenol Red Free
PRLR	Prolactin Receptor
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SD	Sprague Dawley
SE	Standard Error
TCS	Triclosan
tk	Thymidine Kinase

CHAPTER 1 :
INTRODUCTION

Breast cancer is a complex disease with a variety of different classifications, including location of abnormal cells, level of invasiveness, hormone receptor expression, and age of onset (Figure 1.1.) (Harbeck, Penault-Llorca et al. 2019). The breast is composed of a network of alveoli, comprised of milk-secreting epithelial cells, which are connected to form a breast lobule (Macias and Hinck 2012). A multitude of lobules is connected to lactiferous ducts, all of which form the mammary gland (Macias and Hinck 2012). Abnormal cells can be found in either the milk duct epithelium, ductal carcinoma, or lobule epithelium, lobular carcinoma (Hong, McMasters et al. 2018). As the abnormal cells remain within the epithelium, cancer is classified as in situ; however, as they spread into neighboring regions, cancer is classified as invasive (Hong, McMasters et al. 2018). Hormone receptor (HR) expression, particularly estrogen (ER) and progesterone (PR), as well as human epidermal growth factor receptor 2 (HER2) expression, are typical markers to classify breast cancers and provide avenues for treatment (Harbeck, Penault-Llorca et al. 2019). HER2 is a protein that functions as a growth factor activating signaling pathways such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K/Akt), phospholipase C, protein kinase C (PKC), and signal transducer and activator of transcription all leading to cellular proliferation (Harbeck, Penault-Llorca et al. 2019). Breast cancers are often categorized based on patient's age as either pre-menopausal or post-menopausal. Before menopause women produce estrogens primarily from the ovaries. However, ovarian estrogen cessation occurs after menopause causing other tissues to become the major estrogen producers (Amadou, Ferrari et al. 2013, Neuhouser, Aragaki et al. 2015).

Approximately 48,100 cases of non-invasive ductal carcinoma in situ (DCIS) and 268,600 cases of invasive cancer are estimated in 2019 (DeSantis, Ma et al. 2019). The majority of new diagnoses occur in post-menopausal women with HR+/HER2- expression (DeSantis, Ma et al. 2019). Treatments have proven to be effective over time as death rates have been decreasing since 1995 (DeSantis, Ma et al. 2019). However, incidence rates remain constant over the last 15 years (DeSantis, Ma et al. 2019). In attempts to prevent breast cancer occurrence, many factors outside of genetics have been identified to be associated

with breast cancer including obesity, exposure to xenoestrogens, etc. (Bandera, Maskarinec et al. 2015, Neuhouser, Aragaki et al. 2015). Obesity in postmenopausal women has a direct association with the risk of breast cancer (Chan, Vieira et al. 2014, Arnold, Jiang et al. 2016). However, controversially higher adiposity in premenopausal women appears to be protective against breast cancer and associated with lower risk of premenopausal breast cancer (Nichols, Schoemaker et al. 2017).

A breast cancer tumor is comprised of more than abnormal epithelial cells, but a conglomerate of other cell types surround these epithelial cells and have been found to communicate via paracrine factors (Radisky and Radisky 2007, Bussard, Mutkus et al. 2016, Semenza 2016). This surrounding microenvironment, or stroma, of cells consists of fibroblasts, adipocytes, immune cells, macrophage, endothelial cells, mast cells, etc. (Figure 1.2.) (Soysal, Tzankov et al. 2015). Stromal inflammation has been identified in breast cancer patients' biopsies, which are linked with worse prognosis (Radisky and Radisky 2007, Majidinia and Yousefi 2017). Obesity leads to chronic inflammation, which in turn can influence local stromal inflammation permitting breast proliferation and progression (Brown 2014, Allen and Jones 2015, Agresti, Meneghini et al. 2016). Breast cancer occurs in stages with the worst stage being metastasis, and stromal inflammation is closely linked with breast cancer progression to metastasis (Soysal, Tzankov et al. 2015, Suhail, Cain et al. 2019).

Environmental chemicals, such as parabens, found in personal care products, food and household items that have been observed to accumulate in breast cancer tissue and adipose tissue (Barr, Metaxas et al. 2012, Harvey and Everett 2012, Wang, Asimakopoulos et al. 2015). The identification of parabens within breast tissue of cancer patients leads to an increasing intrigue in the characterization of parabens' actions in breast cancer development. Parabens have been shown to be estrogenic in nature and to directly promote epithelial cell growth (Charles and Darbre 2013, Darbre and Harvey 2014). However, it is unclear whether parabens can induce changes in the stromal fibroblasts and adipocytes to produce an inflamed stroma.

It is well recognized that aggressive cancerous epithelial cells must acquire the "hallmarks" of cancer, which include evading suppressors, resisting cell death, replicating, eliciting vascularization, initiating tissue invasion, acquiring unlimited energy, evading immune targeting, and sending proliferation signals to survive (Hanahan 2011, Darbre and Harvey 2014). The stroma must provide the necessary support to the cancerous cells to meet these hallmarks. As parabens are detected in breast cancer tissue, it is conceivable that these chemicals could contribute to this carcinogenic stroma. Our previous studies have demonstrated parabens' ability to create an obesogenic stroma by forcing fibroblasts to differentiate into adipocytes (Hu, Chen et al. 2013). Previous results also suggest that parabens may negatively change stromal cells, creating a carcinogenic stroma that has pro-inflammatory, pro-angiogenic and pro-mitogenic characteristics.

In this dissertation, the stromal fibroblasts and adipocytes were subjected to three different types of most common parabens, methyl- (MP), propyl- (PP), and butylparaben (BP) to characterize their inflammatory effects. We hypothesize that at environmentally achievable doses parabens can influence stromal (1) fibroblasts (Chapter 3) and (2) adipocytes (Chapter 4) using both murine 3T3-L1 and human breast stromal cells.

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1.2. Appendix

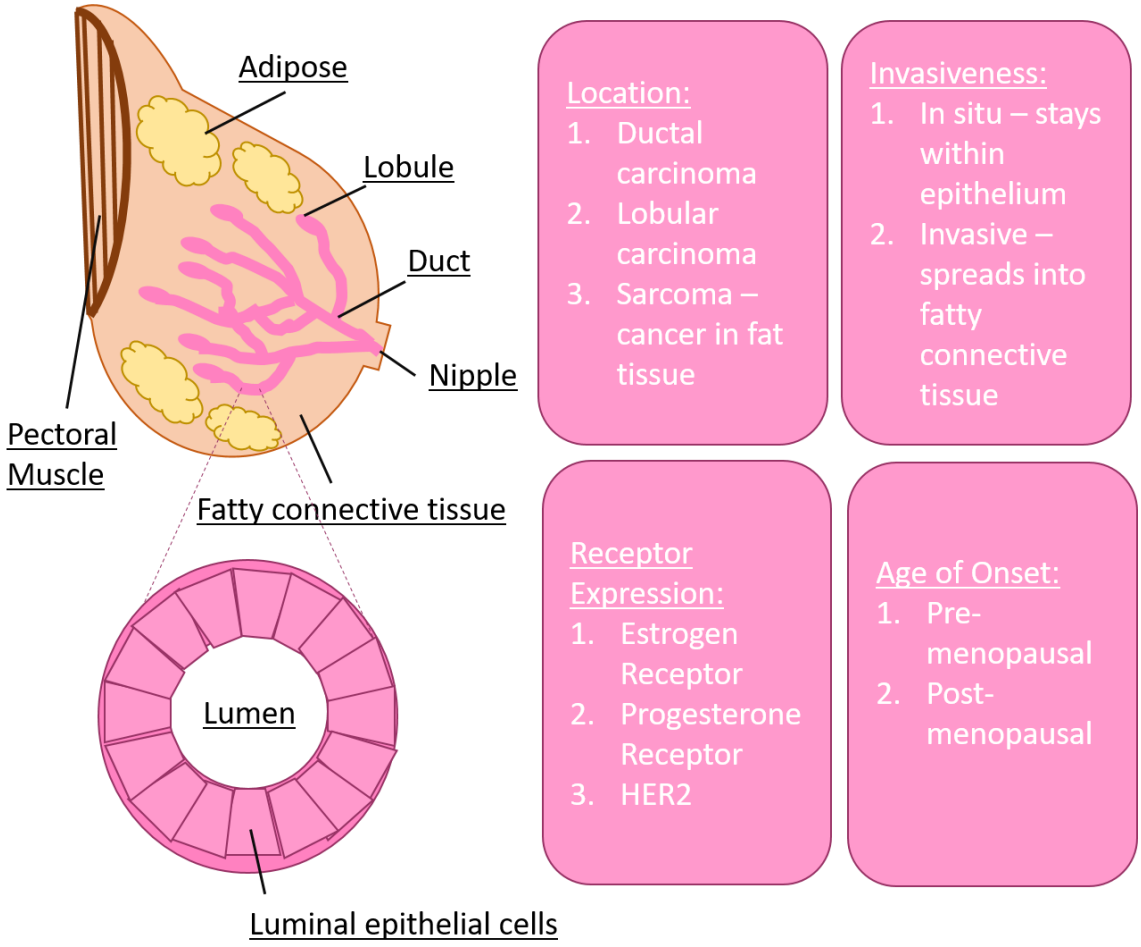

Figure 1.1. Characterization of breast cancer.

Physiology of human breast demonstrating the essential anatomy to understanding breast cancer types. Identifying four common ways of classifying breast cancer based on location, invasiveness, receptor expression, and age of onset. Adapted from: Harbeck N, Penault-Llorca F, Cortes J, et al. Breast Cancer. *Nat Rev Dis Primers*. 2019;5(66):1-31.

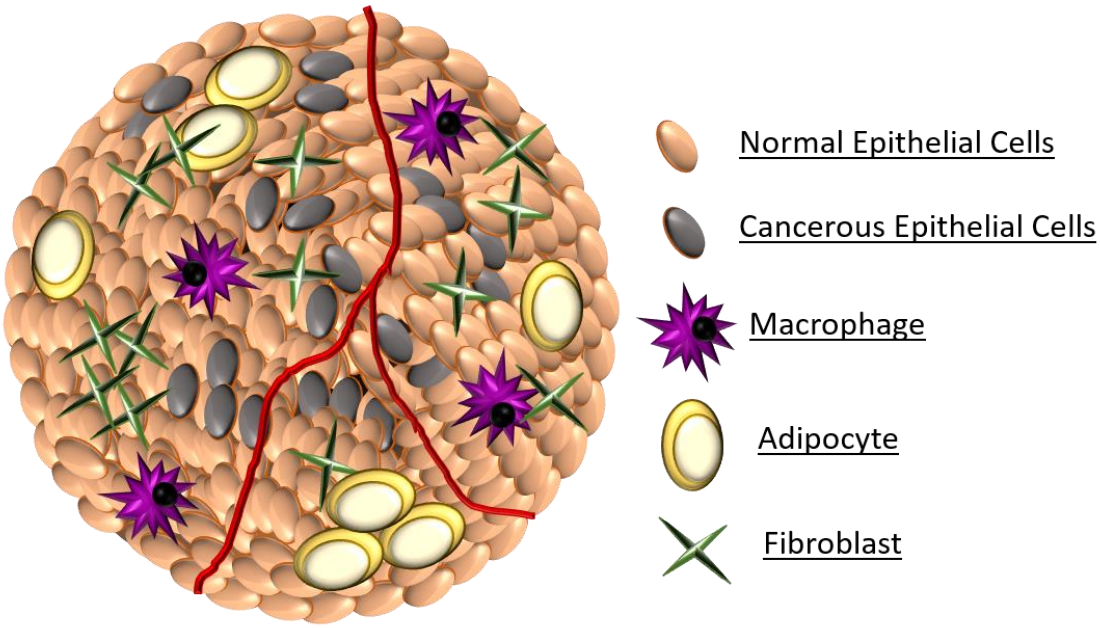

Figure 1.2: Diagram of breast tumor microenvironment.

Demonstration of the cellular connectivity and interactions between cells within the tumor stroma.

CHAPTER 2 :
LITERATURE REVIEW

2.1. Introduction

Breast cancer remains the second leading cause of cancer-related deaths in women in the United States behind lung and bronchial cancers in 2018 (DeSantis, Ma et al. 2019). This trend has occurred since the 1960s when uterine and colon cancer deaths in women began to decline (DeSantis, Ma et al. 2019). However, awareness, screening, and treatment advancements have aided in lowering the death rates of breast cancer by 39% from 1989 to 2015 (DeSantis, Ma et al. 2019). Invasive breast cancer incidence rates have remained stable from 2005 to 2014 (DeSantis, Ma et al. 2019).

The occurrence of breast cancer is a result of the compounding effects of risk factors to which women are exposed. The risk factors for breast cancer can be grouped into non-modifiable factors (family history and hereditary gene) and modifiable (environmental) factors (Darbre 2006). The familial risk factors include a family history of breast cancer, other breast diseases, or high breast density. The hereditary risk factor is the individual that has inherited a *BRCA1*, *BRCA2*, or other breast cancer-related gene mutations. Non-modifiable risk factors only account for approximately 5-10% of breast cancers, whereas 90% of breast cancer occurrence is thought to be related to modifiable, environmental factors (Darbre 2006). There are many environmental risk factors, such as obesity, sedentary lifestyle, oral contraceptives, xenoestrogens, etc., and exposures that are modifiable and are associated with increased breast cancer risk. Additionally, nulliparity, having first child after the age of 30, and postmenopausal hormone use are also considered breast cancer risks (ACS 2018).

It is increasingly recognized that exposure to environmental chemicals is associated with increased risks of developing breast cancer. Many of such environmental chemicals, such as bisphenol A, atrazine, benzophenone-1 and nonylphenol, are reported to be estrogenic and are thought to be implicated in breast carcinogenesis (Burks, Pashos et al. 2017). Parabens are a class of these environmental chemicals, and recent evidence has suggested that parabens are estrogenic and are implicated to impact a variety of physiological abnormalities from reproduction to cancer (Smith, Souter et al. 2013, Darbre and Harvey

2014, Guerra, Sanabria et al. 2017). Recently, significant progress has been made on the relationship between parabens and breast cancer. In this review, a new understanding of parabens'estrogenic effects, mechanisms of action, and their effects on breast cancer development and progression are summarized.

2.2. Parabens, Endocrine Disruptors, and Breast Cancer

2.2.1. Human Paraben Exposure

Parabens, 4-hydroxybenzoic acid alkyl esters, are absorbed or ingested daily from personal care products (such as cosmetics, lotions, deodorants, hair care products, and shaving products), pharmaceuticals and foods (Darbre and Harvey 2014). Parabens enter into the body through dermal or gastrointestinal absorption and are distributed systemically demonstrated by isolation of intact paraben in tissue, blood, breast milk, placenta, and urine (Sandanger, Huber et al. 2011, Kolatorova 2017, Shen, Liang et al. 2017).

The four most common parabens present in biological fluids are methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP), in order of increasing side chain length (Sandanger, Huber et al. 2011, Darbre and Harvey 2014, Zhou, Chen et al. 2018). Parabens can either get metabolized by esterases in the gastrointestinal tract, liver, or dermis for excretion or can travel through circulation to tissues for metabolism or bioaccumulation (Wang, Asimakopoulos et al. 2015, Kolatorova 2017). MP is the most abundant in systemic fluids as the metabolic rate of esterases increases with the size of the paraben's side chain. Additionally, these chemicals have a weak affinity for human serum albumin allowing for greater accessibility to peripheral tissues (Greige-Gerges, Kaissi et al. 2013).

As parabens are ubiquitously utilized in industry as an anti-microbial reagent to preserve products and increase shelf-life, there is an increasing amount of evidence indicating human exposure to parabens are ubiquitous as well. The estimated total intake of parabens from food has been estimated to range from 307 – 940 ng/kg body weight, and absorption differs depending on age with infants having the highest total absorption due to application

of personal care products (Darbre and Harvey 2014). However, the estimated total intake of parabens via personal care product usage ranges from 31 – 766 µg/kg body weight (bw) again with infants being at the upper limit (Darbre and Harvey 2014). Women have higher urine concentrations of parabens as compared to men (Zhou, Chen et al. 2018). The total concentrations of parabens in men's urine ranges from 0.5-79.1 ng/mL, whereas women range from 17-237 ng/mL (Zhou, Chen et al. 2018). As personal care product usage leads to a greater systemic level of parabens, previous research has identified the effect of skin cream usage and systemic paraben levels. The positive correlation between the percentage of skin area creamed per day and median plasma level of MP demonstrates the direct connection between personal care products with paraben absorption and systemic distribution (Sandanger, Huber et al. 2011). This association between cosmetic applications and paraben burden can be confirmed by the disappearance of isopropylparaben and isobutylparaben in all urine samples after a ban was imposed in 2014 on the utilization of these two chemicals in cosmetics (Zhou, Chen et al. 2018).

2.2.2. Parabens are EDCs

Parabens are endocrine disrupting chemicals (EDCs), which have the ability to alter the normal endocrine function and cause adverse effects in development, reproduction, neurology, and immunity (NIEHS 2018). The most common examples of EDCs are parabens, dioxins, polychlorinated biphenyls, bisphenol A, etc. found in products like pesticides, plastics, detergents, food, toys, cosmetics, and pharmaceuticals (NIEHS 2018). Many EDCs have been identified as possessing estrogenic properties that increase the basal estradiol levels, which in post-menopausal women can lead to exacerbated breast epithelial cell proliferation and tumor growth (Darbre 2006, Yager 2006, Rodgers, Udesky et al. 2017).

Epidemiological evidence directly linking EDC exposure with breast cancer is limited due to the vast amount of exposure variability. As EDCs are commonly used as preservatives in consumables and hygiene productions, absorption occurs through ingestion, dermal contact, and inhalation (Kolatorova 2017). Therefore, increased personal care product

usage leads to greater exposure to EDCs, and women have higher levels of EDCs in bodily fluids as compared to men due to the increased usage of cosmetics and lotions (Zhou, Chen et al. 2018). Previously, individual EDCs are often identified as having a weak estrogenic effect making their association with breast cancer negligible (Pastor-Barriuso, Fernandez et al. 2016). However, prolonged daily exposure, bioaccumulation, and interactions of xenoestrogens can invoke local and systemic endocrine changes (Pastor-Barriuso, Fernandez et al. 2016).

It has been demonstrated that urban communities have a three- to four-fold increased risk of estrogen receptor (ER) positive breast cancer compared to rural communities (Dey, Soliman et al. 2010, Dey, Soliman et al. 2010). In Egypt, more urban areas had a higher incidence of total and ER+ breast cancer from 2001-2006 and uterine cancer from 1999-2002 with little differences in nutritional status, length of breastfeeding, childbearing age, etc. (Dey, Hablas et al. 2010, Dey, Soliman et al. 2010). Female cancers without hormonal etiology, such as leukemia, are less stratified between urban and rural communities, suggesting a hormonal contribution in the urban lifestyle (Dey, Hablas et al. 2010). It is possible that the greater use of plastics, insecticides, detergents, personal care products, and cosmetics in urban as compared to rural communities increases the EDC absorption leading to a greater xenoestrogen burden.

2.2.3. Parabens and Breast Cancer

As parabens have been identified to increase breast cancer growth *in vitro*, extractions of breast tissue from serial locations from the axilla to sternum were analyzed for paraben presence (Barr, Metaxas et al. 2012, Darbre and Harvey 2014). The tissue levels of MP is 16.6 ng/g of tissue, which is greater than previously documented (Darbre, Aljarrah et al. 2004, Barr, Metaxas et al. 2012). Additionally, the concentration of PP in breast tissues is found to be 16.8 ng/g of tissue, higher than MP. BP, EP, and isobutylparaben (IBP) are all present at lower levels (5.5, 3.4, and 2.1 ng/g of tissue respectively) with a total paraben concentration of 85.5 ng/g of tissue (Darbre and Harvey 2014). However, there is no significant association between serial paraben localization and known patient tumor

location (Barr, Metaxas et al. 2012). Parabens have greater bioaccumulation within the outer region (axilla and lateral) of the breast than inner regions (mid and medial) (Barr, Metaxas et al. 2012). The mammary glands are centrally located in the inner regions, while the outer regions consist more of adipose tissue. The lack of association between paraben levels and percent of breast density confirms the lower presence of parabens within the inner regions (Sprague, Trentham-Dietz et al. 2013). Parabens localized in the outer region of the breast is speculated to be caused by the use of deodorants (Barr, Metaxas et al. 2012). However, an alternative hypothesis could be that the parabens are accumulating in the adipose tissue of the breast as parabens are suggested to be the most abundant environmental phenol present in adipose (Wang, Asimakopoulos et al. 2015).

2.3. Estrogenic Effects of Parabens

2.3.1. Estrogenicity

Parabens have been identified to bind ER receptors and affect ER-related gene expression. The estrogenic response is also attenuated with the pre-treatment of an ER inhibitor (ICI 182,780). In addition, parabens increase uterine weight, which is a classic physical outcome that indicates estrogenic activity. The influence of parabens on ER increases with the length of the alkyl chain and with the branching of the side chain. Moreover, MP, EP, PP, BP, and BenzP have been calculated to have an estradiol equivalence factor (EEF) of 1.25(10^{-7}), 6.48(10^{-7}), 2.39 (10^{-6}), 8.09(10^{-6}), and 1.3(10^{-5}) respectively, confirming the increase in estrogenicity with increasing alkyl side chain length (Lange, Kuch et al. 2014). MP and EP produce binding energies of -49.35 and -53.38 kcal/mol when automated docking into human ERα ligand-binding domain (LBD) is conducted (Sun, Yu et al. 2016). This release of energy indicates binding confirming the formation of hydrogen bonds in the LBD at Glu353/Arg394 with the hydroxyl group of the parabens (Sun, Yu et al. 2016).

In addition to cell models demonstrating estrogenic effects, the standard for measuring estrogenic activity in the animal model is determined by evaluating the uterine weight changes upon paraben exposure. Parabens' estrogenicity has been demonstrated in CD-1 mice (Lemini 1997). Recently, the lowest-observed-effect levels (LOEL) for MP and EP

to increase relative uterine weight in Sprague-Dawley (SD) rats was reported to be 20 mg/kg bw/day and 4 mg/kg bw/day (Sun, Yu et al. 2016). The no-observed-effect levels (NOEL) are similar to previous publications at 4 mg/kg bw/day for MP and 0.8 mg/kg bw/day for EP, which is similar to the upper limit of human paraben absorption (Lemini 1997, Sun, Yu et al. 2016). In addition to increases in uterine weight, paraben induced significantly higher levels of estrogen responsive biomarkers compared to control animals including intestinal calcium-binding protein (icabp), integral membrane-associated protein 1 (itmap1), calbindin-D 9k (CaBP-9k), and progesterone receptor (PR) in the rat uterine tissue (Sun, Yu et al. 2016).

2.3.2. Mammary Tissue Development

The mammary gland is an intricate network of ducts that are surrounded by a stroma that changes throughout different stages of life (Macias and Hinck 2012). The mammary gland consists of epithelial ducts expanding from the nipple into a fat pad formed by adipocytes, endothelial cells, fibroblasts, etc. with a function to secrete milk for newborn survival (Macias and Hinck 2012). During the pubertal phase, mammary glands begin to develop with scattered ducts lined with two layers of epithelial cells termed mammary tree (Macias and Hinck 2012, Howard and Lu 2014). The breast epithelium is comprised of luminal cells that line the ducts and of myoepithelial cells (Arendt and Kuperwasser 2015). In humans, the ends of the ducts terminate in a fibroblastic stroma (Howard and Lu 2014). Mammary tissue development in women begins during puberty and continually has morphology changes as a woman enters pregnancy, lactation, and post-lactation (Howard and Lu 2014, Arendt and Kuperwasser 2015). When a woman enters puberty the myoepithelial cells within ducts proliferate in response to estrogen and progesterone (Russo 1999, Arendt and Kuperwasser 2015). Estrogen induces the growth of mammary ducts in cooperation with growth factors by activating the estrogen receptors located in both the epithelium and stromal cells (Silberstein 1982). Pregnancy leads to morphological changes within breasts including epithelial growth to form milk-secreting alveoli and adipose tissue recession (Macias and Hinck 2012). Once lactation is complete, a process

of involution begins when epithelial cells sense stagnant milk and begin to revert back to the pre-pregnancy structure (Macias and Hinck 2012).

Methylparaben exposure at a low dose that was previously determined to produce urinary metabolite concentrations comparable to those found in US population, in the perinatal and prepubertal time periods caused less development of adipose tissue but increased ductal tree expansion within the fat pad and growth of the glandular tissue, respectively, in SD female rats (Gopalakrishnan, Teitelbaum et al. 2017). Female SD rats were treated with MP or triclosan (TCS) at 0.105 and 0.05 (mg/kg/day), representing 1/10,000 and 1/1,000 no observed adverse effect levels (NOAEL) of these chemicals respectively. These low doses were previously determined to produce urinary metabolite concentrations comparable to those reported for the US population (Gopalakrishnan, Teitelbaum et al. 2017). Pubertal MP exposure induced more glandular tissue with a high degree of branching in the SD female rats, which was associated with differential expression of 295 genes (Gopalakrishnan, Teitelbaum et al. 2017). Moreover, it was found that pubertal MP exposure up-regulated or down-regulated subsets of genes that were also up-regulated or down-regulated in breast cancers compared to nearby normal tissues (Gopalakrishnan, Teitelbaum et al. 2017). Paraben exposure, therefore, at a prepubescent age can affect breast tissue development by stimulating epithelial cell growth.

2.3.3. Menstrual Cycles

The menstrual cycles of women are managed by the alterations in hormones that occur in a cyclical pattern. There are both steroidal and non-steroidal hormones secreted that contribute to these changes, and the two most abundant and well-identified steroidal hormones include estradiol and progesterone (Messinis, Messini et al. 2014). Estradiol, secreted from the follicle, plays a primary role in the follicular phase of the menstrual cycle (Messinis, Messini et al. 2014). In the follicular phase, which involves the oogenesis and maturation, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) increase stimulating the release of estradiol (Messinis, Messini et al. 2014). During the peak of these hormone levels, the oocyte is released from the follicle and ovulation occurs. The surge of

LH and FSH elicit higher levels of estradiol and progesterone in the luteal phase. Estrogen and progesterone induce negative feedback on the hypothalamic-pituitary-gonadal axis in which it decreases the production of LH and FSH (Messinis, Messini et al. 2014). Therefore, by the end of the luteal phase, LH and FSH are returning to normal levels along with estrogen and progesterone signaling the beginning of a new cycle and menses.

Parabens can also influence the menstrual cycles in women due to estrogenic properties determined by comparing bleeding records with urinary concentrations of parabens. In 127 premenopausal women of normal body mass index (BMI), the urinary concentrations of parabens were similar to that of US females at 137, 1.3, 29.1, and 0.50 ng/mL for MP, EP, PP, and BP. When estrogen-equivalent total paraben (ETP) of the four parabens was calculated, high urinary ETP and BP concentrations were associated with a shorter menstrual cycle (Nishihama, Yoshinaga et al. 2016). It is suggested that the greater levels of basal estrogen from the simulation by parabens could initiate the negative feedback loop more frequently, thereby inhibiting the amount of LH and FSH production and consequently, the LH surge and ovulation.

2.4. Mechanisms of Action by Parabens

2.4.1. Estrogen Receptor

As discussed in the previous section, parabens have the ability to bind ER and activate ER signaling (Lange, Kuch et al. 2014, Sun, Yu et al. 2016). Additionally, they increase the gene and protein expression of ER (ESR1 and ESR2, their alternative names ERα and ERβ) (Wrobel and Gregoraszczuk 2014). ESR1 functions in cellular proliferation of mammary gland cells, while ESR2 functions by antagonizing ESR1. ESR1 over-expression is linked to breast cancer, while ESR2 expression is lost in breast cancer patients. MP, PP, and BP at 20 nM significantly enhanced ESR1 and ESR2 mRNA expression upon 24 h of treatment in MCF7 cells. MP and PP also increased ESR1 mRNA expression in normal breast epithelial cells (MCF10A) at 6 h (Wrobel and Gregoraszczuk 2014).

ERα protein (ESR1 product) expression was greatly enhanced by MP, PP, and BP at 48 h than by estradiol, the positive control, in MCF7 cells, which function as a model for a ER+ breast cancer tumor. (Wrobel and Gregoraszczuk 2014). MP induced the longest response in MCF7 cells with increasing protein expression at 72 h, similar to estradiol stimulation, which could be due to relatively low MP metabolism rates (Wrobel and Gregoraszczuk 2014). As for comparisons, MP increases the ERα protein expression in normal breast epithelial cell line, MCF10A cells at 48 h, whereas both MP and PP had effects at 72 h (Wrobel and Gregoraszczuk 2014). Moreover, the dependence of ER for the paraben's effects was demonstrated by the co-treatment with ER inhibitor ICI 182,780 on MCF10A, which attenuated the parabens effect. (Wrobel and Gregoraszczuk 2014).

All three parabens increased ERβ protein in MCF7 cells at 48 h and only MP at 72 h (Wrobel and Gregoraszczuk 2014). In contrast, all three parabens increased ERβ protein (ESR2 product) expression after 72 h treatment, but had no effects after 48 h, in MCF10A cells (Wrobel and Gregoraszczuk 2014). ICI 182,780 treatments seemed to have no effect on MCF10A cells (Wrobel and Gregoraszczuk 2014).

2.4.2. Progesterone Receptor

Progesterone receptor is another major receptor marker for patients being diagnosed with breast cancer (Harbeck, Penault-Llorca et al. 2019). Similar to that of estrogen, progesterone is a steroid hormone that influences the reproductive system and breast development, and PR functions to regulate transcription and cell proliferation (Cenciarini and Proietti 2019). PR mRNA expression is significantly increased by PP and BP after 24 h treatments in both MCF7 and MCF10A cells (Wrobel and Gregoraszczuk 2014). Interestingly, however, MP and PP, but not BP, increased protein expression of PR in MCF7 cells, but not MCF10A cells (Wrobel and Gregoraszczuk 2014). (Wrobel and Gregoraszczuk 2014). Similarly, *in vivo*, MP and EP at 20, but not 4 mg/kg/day, induced a significant increase of PR mRNA expression in SD rats' uterine tissue (Sun, Yu et al. 2016).

2.4.3. Human Epidermal Growth Factor Receptor

Parabens have been demonstrated to crosstalk with HER2 to promote oncogenic c-myc gene expression and proliferation of an ERα- and HER2-positive human BT-474 breast cancer cells in an ERα dependent manner (Pan, Yuan et al. 2016). HER is a family of transmembrane proteins that act as receptor tyrosine kinases PI3K/Akt signaling (Pan, Yuan et al. 2016). Activation of PI3K/Akt can lead to the phosphorylation of ERα, which enhances the affinity for binding of ER. Adding to the complexity, ERα has the ability to crosstalk with HER signaling, but the synergy is greatly dependent on the presence of ERα (Pan, Yuan et al. 2016).

Akt activation via HER has the ability to affect c-myc expression. c-myc mRNA expression was enhanced by HER2 agonist heregulin (HRG) upon co-treatment with PP and BP in the BT-474 cells (Pan, Yuan et al. 2016). Interestingly, PP and BP at 10 μM without HRG appeared to induce a 10-20 fold increase of c-myc mRNA expression. Blocking ERα activation with an ER inhibitor, such as ICI 182,780, raloxifene, or tamoxifen, attenuated the synergistic effect of HRG and BP on c-Myc protein expression (Pan, Yuan et al. 2016). Moreover, HRG was demonstrated to lower the dose of BP required to promote the BT-474 cell proliferation. Chromatin Immunoprecipitation (ChIP) assays further demonstrated that BP and HRG synergistically recruit ERα transcription factors to the promoter region of the c-myc gene enhancing cellular proliferation which is attenuated by ER antagonists.

2.4.4. Androgen and Glucocorticoid Receptor

Androgen is another steroid present in women and functions primarily as testosterone, a precursor to estrogen. Glucocorticoids are steroid hormones that function to abrogate inflammation decreasing immune activity, to regulate electrolyte balance, and to regulate glucose metabolism (Kolsek, Gobec et al. 2015, Busada and Cidlowski 2017, Schiffer, Barnard et al. 2019). It has been shown that paraben (at 50 μM, verified as non-toxic to the cells) stimulated glucocorticoid receptor (GR) by activating mouse mammary tumor virus (MMTV) reporter in the MDA-kb2 cells, which are MDA-MB-453 cells that stably express MMTV luciferase reporter gene and high levels of GR and androgen receptor (Kolsek,

Gobec et al. 2015). Moreover, MP and EP showed anti-androgenic activities while BP showed androgenic activities by the MDA-kb2 MMTV reporter assay (Kolsek, Gobec et al. 2015).

2.4.5. G-Protein Coupled Receptor

Parabens have also been suspected to function through other pathways that are non-genomic, which are more rapid. G-protein coupled estrogen receptor 1 (GPER1 or GPR30) is associated with the plasma membrane and acts through protein-kinase cascades such as ERK1/2 (Wrobel and Gregoraszczuk 2015). GPR30 mediated signaling regulates matrix metalloproteinases (MMPs), which are enzymes that function in the rearrangement of the microenvironment and involved in cancer metastasis. GPR30 is highly expressed in cancerous breast epithelial cells and breast tumors, especially those from patients who are resistant to ERα inhibitors, like tamoxifen (Ignatov, Ignatov et al. 2011). MP, PP, and BP at 20 nM upregulated GPR30 mRNA expression greater than estradiol in MCF7 cells after 24 h and in MCF10A cells after 6 and 24 h (Wrobel and Gregoraszczuk 2015). Additionally, MP and PP increased GPR30 protein expression after 48 h in both MCF7 and MCF10A cells and after 72 h in MCF7 cells. Interestingly, only BP, but not MP or PP, increased GPR30 protein levels at 72 h in MCF10A cells (Wrobel and Gregoraszczuk 2015). It is well known that estrogen-GPR30 signaling activates a rapid response through the activation of ERK1/2 and cAMP/PKA. Whereas no tested parabens affected cAMP levels in either cell line, PP and BP increased phosphorylation of ERK1/2 in MCF-7 cells, whereas MP and BP, but not PP, increased phosphorylation of ERK1/2 in MCF-10A cells. This activation GPR30/ERK1/2 signaling pathway by parabens demonstrates an additional mechanism by which breast cancer cells proliferation and progression can be enhanced by parabens.

2.5. Parabens Effect on Breast Cancer Progression

The influence on breast cancer proliferation by parabens have been well-reviewed previously (Darbre and Harvey 2014). However, recent publications confirm previous

findings and elaborate on the mechanism by which parabens affect breast cancer initiation, proliferation, and metastasis.

2.5.1. Mimic Estrogen in Inducing ER+ Breast Cancer Gene Expression

As parabens are estrogenic, it is beneficial to identify if parabens elicit the same genomic response as estrogens. Not only does this confirm the estrogenic nature of parabens, but it could also unveil novel gene targets for breast cancer treatment. Using a set of 120 estrogen responsive genes, paraben (10 μM) induced expression profile was compared to that of estrogen (17-estradiol or E2) used at 10 nM in MCF7 cells. Among MP, EP, PP, and BP, significant correlations of expression profiles to that of E2 were observed for PP and BP (R = 0.74 and 0.60, respectively), indicating estrogenic effects of parabens in a manner that is dependent on alkyl chain length. PP and BP-induced expression profiles correlated significantly with the effects of E2 in genes coding for enzymes, signaling, proliferation, transcription, and transport, and others. However, profiles induced by PP and BP resembled each other more than that of estrogen (Terasaka, Inoue et al. 2006).

To gain a full picture of how paraben regulates genes in comparison with that of E2 in MCF7 cells, the expression profiles of 19881 genes in MCF7 cells following a 7-day exposure to $5(10^{-4})$ M MP, 10^{-5} M n-BP and 10^{-8} M E2 were studied using the GE-Am2ersham CodeLink 20 K human expression microarray system (Pugazhendhi, Sadler et al. 2007). Venn diagram showed that 2963 genes were changed by ≥ 2 fold by one or more treatment. Only 61 genes were induced and 198 genes were downregulated by all three treatments. The majority of genes were regulated by only one or two of the treatments, indicating that significant differences in MCF7 cells' response to paraben and E2. For example, MP and BP upregulates interleukin 17 receptor B (IL17RB), otoferlin (OTOF), and prolactin receptor (PRLR), but suppresses ubiquitin D and adrenomedullin, similar to E2 (Pugazhendhi, Sadler et al. 2007). However, genes such as bruno-like5 RNA binding protein (CELF5), myeloid/lymphoid mixed-lineage leukemia 2 transcription factor (MLL2) and alanyl-tRNA synthetase like (AARSL) were upregulated more with paraben treatments (Pugazhendhi, Sadler et al. 2007). Analyzing the global gene expression

patterns demonstrated that even though some gene expression patterns are similar to E2, the alterations do not match perfectly.

In addition to MCF7 cells, the estrogenic effects of pubertal exposure to 4 mg/kg bw/day MP or EP in a uterotrophic assay similar to human exposure levels in immature SD rats induced an upregulation of 138 genes and downregulation of 157 genes including estrogen-responsive genes in the uteri of the rats by quantitative real-time RT-PCR (Sun, Yu et al. 2016). mRNA expression of icabp, itmap, CaBD-9k, and PR were increased by both MP and EP treatment (Sun, Yu et al. 2016). MP-induced globally upregulated genes include those functioning in DNA replication and regulation of cell cycle such as E2F transcription factor 1 (E2f1) (Gopalakrishnan, Teitelbaum et al. 2017). In contrast, MP globally downregulated genes function in immune response and interferon signaling pathways, including interferon regulatory factor (Irf7) (Gopalakrishnan, Teitelbaum et al. 2017). Therefore, parabens can enhance DNA replication while also diminishing the immune response setting the ideal conditions for proliferation.

Moreover, the effects of paraben on gene expression of select cell cycle/apoptosis genes were compared with those of E2 in MCF7 and MCF-10A (Wrobel and Gregoraszczuk 2014). At mRNA levels, estradiol-induced G1/S phase genes (cyclin D1, D3 and cyclin-dependent kinase (CDK) 2 and 4), suppressed some cell cycle progression inhibitors and also induced pro-survival genes (BCL2) in both cell lines. Parabens did not alter cyclins expression, and MP and BP did not affect the select apoptotic genes in MCF-7 cells (Wrobel and Gregoraszczuk 2014). However, parabens induced a greater response in MCF-10A cells, were MP, PP, and BP all increased cyclin D1, E1, CDK 4, and CDK 2 expression, but suppress p21 expression (Wrobel and Gregoraszczuk 2014).

2.5.2. Induce Genome Instability

Genome instability refers to the genomic alterations cells acquire, leading to carcinogenesis. Genome instability includes base-pair mutations, insertions, deletions, or translocations of whole or fragmented chromosomes. These chromosomal aberrations

hinge upon double-strand breaks (DSB) and often times occur in tandem with mutated DNA repair genes, such as BRCA1 and BRCA2 (Duijf 2019).

The findings that parabens promote both MCF7 and MCF10A cell growth promoted the study of genotoxicity studies by parabens. *In vitro* genotoxicity test and *in vitro* screening cytokinesis-block micronucleus (CBMN) tests using human lymphoblastoid TK6, mouse lymphoma L5178Y cells and human lymphocytes were performed (Finot, Kaddour et al. 2017, Guzel Bayulken, Ayaz Tuylu et al. 2019). The use of MCF-10A cells is to model the effect of parabens on cells that are not cancerous, and parabens do increase the proliferation of these cells at 100 nM. However, there has not been much insight into the genotoxic effects of parabens on this cell type. EP, PP, and BP induce a genotoxic effect at high doses (50-500 μg/mL) on lymphocytes, which is associated with worse clinical outcomes in breast cancer patients (Finot, Kaddour et al. 2017, Guzel Bayulken, Ayaz Tuylu et al. 2019).

CBMN are miniature nuclei separate from the primary nucleus that contain desegregated chromosomes where extensive DNA damage occurs and can subsequently be incorporated back into the primary nucleus (Duijf 2019). L5178Y and TK6 cells had increased mutations at the thymidine kinase (tk) locus upon high dose treatment of EP for 3 h indicative resistance to apoptosis and inflicted a greater amount of CBMN at 120 μg/mL (Finot, Kaddour et al. 2017). At 24 h, BP, PP, and isopropylparaben (IPP) all induced greater numbers of CBMN at the highest dose of 100 μg/mL (Guzel Bayulken and Ayaz Tuylu 2019). With a 48 h exposure, PP and BP caused greater CBMN formation at 50 and 100 μg/mL (Guzel Bayulken and Ayaz Tuylu 2019). Shorter treatments were conducted in the presence of S9, a metabolic activator, generating an unknown set of metabolites. TK6 cells still had a significant CBMN presence after 26 h of treatment without S9, indicating EP's genotoxic effect is independent of S9 metabolic conversion (Finot, Kaddour et al. 2017). Metabolite 4-hydroxybenzoic acid does not have the same CBMN formation effect as parabens and only causes the formation of CBMN at 500 μg/mL for 48 h (Guzel Bayulken, Ayaz Tuylu et al. 2019).

Telomere shortening is one determinant to demonstrate parabens' influence in genotoxicity. A telomere is a nuclear protein that caps the chromosomes and prevents degradation or DNA damage. Telomere shortening leads end-to-end joining of adjacent chromosomes causing dicentric chromosomes. When these chromosomes undergo mitosis DNA breaks occur promoting genome instability (Duijf, P. et al. 2019). EP causes a decrease in fluorescence of telomere length in murine lymphoma cells (L5178Y) and human lymphoma cells (TK6) cells as compared to their control cells (murine bone marrow, human lymphoblastoid, and Burkitt lymphoma cells) using telomere staining and comparing total fluorescence intensity of metaphase chromosomes (Finot, Kaddour et al. 2017). Loss of one telomere is the most significant disruption to telomere length upon EP treatment (Finot, Kaddour et al. 2017). Additionally, when controlling for age, the telomere length of paraben-exposed human lymphocytes was significantly shorter by both telomere loss and deletions than the paraben-unexposed lymphocytes (Finot, Kaddour et al. 2017). Therefore, parabens at higher doses (100-350 μg/mL) can affect telomere length.

As mentioned previously, the incorporation of DSB into the genome initiates carcinogenesis if in a vulnerable DNA location (Kidane 2018, Jiang, Panda et al. 2019). γH2AX and 53BP1 foci have been identified as markers of the formation of DSB if localized to intra-chromosomal regions (Finot, Kaddour et al. 2017). Untreated L5178Y and TK6 cells had these markers distributed throughout the nuclei, but EP treatment leads to the localization of these foci at or near shortened or uncapped telomeres, indicating telomere shortening (Finot, Kaddour et al. 2017). Even though there have been no identifiable DSB, EP treatment of L5178Y cells demonstrated both clonal and sporadic dicentric chromosomes (12 and 13) and trisomy chromosomes (15 and 18) (Finot, Kaddour et al. 2017). EP treatment of TK6 cells has a greater amount of acentric chromosomes (Finot, Kaddour et al. 2017). 4-Hydroxybenzoic acid at 250-500 μg/mL dosages and PP, BP, and IBP at 50-100 μg/mL also led to the chromosomal aberrations, most commonly chromatid breaks (Guzel Bayulken and Ayaz Tuylu 2019, Guzel Bayulken, Ayaz Tuylu et al. 2019). Comet assay is the gold standard for analyzing DNA damage where the damaged DNA fragments migrate through electrophoresis faster than the undamaged DNA

developing a tail (Jiang, Panda et al. 2019). The length and intensity of the tail indicate the number of DSB (Jiang, Panda et al. 2019). Lastly, a comet assay was conducted to conclusively identify the presence of DSB in lymphocytes upon a 4-hydroxybenzoic acid treatment, and only the highest concentration (1000 μg/mL) had significant DNA damage compared to the solvent control (DMSO) (Guzel Bayulken, Ayaz Tuylu et al. 2019). However, 100 μM BP appeared to show protective properties against genotoxicity, but not cell proliferation induced by silver nanoparticles (Roszak, Domeradzka-Gajda et al. 2017). Therefore, parabens have the ability to induce genotoxicity, but only at doses higher than those from a typical environmental exposure; however, more studies at environmentally achievable doses are necessary for a realistic perspective on parabens-induced genotoxicity.

2.5.3. Evoke Breast Cancer Proliferation

Parabens at concentrations greater than 10 nM induce higher proliferation of cancerous epithelial cells (MCF7) with attenuation by the inhibition of ERα and ERβ (Okubo, Yokoyama et al. 2001, Lillo, Nichols et al. 2017). As mentioned previously, parabens and their metabolite p-hydroxybenzoic acid bind to ER as compared to other environmental phenols (Okubo, Yokoyama et al. 2001, Darbre, Byford et al. 2002, Pugazhendhi, Pope et al. 2005). The potency of the paraben effect on proliferation and binding competition correlated with their chain length and branching (Okubo, Yokoyama et al. 2001). Long, branched parabens have more potency and have the greatest binding affinity and rank from least to greatest as MP < EP < PP < IPP < BP and IBP. This affinity and potency ranking was confirmed for IBP, benzP, and p-hydroxybenzoic acid by competitive binding assays for ERα and ERβ (Okubo, Yokoyama et al. 2001, Darbre, Byford et al. 2002, Darbre, Byford et al. 2003, Pugazhendhi, Pope et al. 2005).

Parabens also have the ability to enhance cellular proliferation in MCF10A cells, implicating parabens' effects on breast carcinogenesis (Khanna and Darbre 2013, Darbre and Harvey 2014). Butylparaben at 0.5 mM MP, 50 μM PP, and 50 μM produced greater number and size of cell colonies and MP and PP induced significantly greater colony

formations (Khanna and Darbre 2013, Darbre and Harvey 2014). PP and BP maximum colony formation peaked at 5 μM; however, MP had twice this maximum at 0.5 mM, demonstrating that MP has the greatest effect on MCF10A cell growth (Khanna and Darbre 2013).

In vivo, MP significantly increases tumor growth of MCF-7 cells and patient-derived xenograft (PDX) breast tumor in immunocompromised female mice in comparison with the estrogen treatment (Lillo, Nichols et al. 2017). However, MDA-MB-231 mammospheres treated with 10 nM MP for 10 days did not induce proliferation nor increase in tumor size, which confirmed previous reports of the non-prolific effect of parabens on these cells (Lillo, Nichols et al. 2017). MDA-MB-231 cells are representative of triple-negative breast cancer, which has no ER, PR, and HER2 expression, is the most aggressive and is the most difficult to treat. Therefore, this suggests that parabens promote both carcinogenesis and cancer proliferation in a hormone receptor-dependent manner at low doses.

The mechanisms by which parabens promoted cell proliferation in normal or cancerous breast epithelial cells have been explored. It was reported that the paraben activated mitogenic ERK1/2 pathway through the activation of GPR30. PP and BP, but not MP, at 20 nM caused phosphorylation of ERK1/2 upon 5 to 120 minutes of exposure in MCF7 cells (Wrobel and Gregoraszczuk 2015). MP and BP, but not PP, at 20 nM also activated ERK1/2 in MCF10A cells. The phosphorylation of Akt was induced by PP but was significantly suppressed by BP in MCF7 cells (Wrobel and Gregoraszczuk 2015).

Moreover, another study suggested that parabens may enhance proliferation through increasing aromatase (CYP19A1) expression. Aromatase functions in the conversion of androgens to estrogen, which is a hormone that increases cellular proliferation of estrogen responsive normal breast epithelial cells and breast cancer cells. Treatment of 20 nM MP and PP caused a greater expression of CYP19A1 mRNA and protein expression in MCF7 cells; however, MP, PP, and BP decreased this expression in MCF10A cells below the

vehicle control (Wrobel and Gregoraszczuk 2013). Consistently, parabens also increased the production of estradiol in MCF7 cells and decreased MCF-10A cells estradiol production at a low concentration range (0.2 nM-200 nM) as compared to the high-dose (2 μM), which secreted less estradiol (Wrobel and Gregoraszczuk 2013).

2.6. Parabens Effect on Breast Cancer Metastasis

The majority of diagnosed breast cancers are ER-positive and luminal A or B, which have effective adjuvant pharmaceutical treatment (Gao and Swain 2018). Luminal A breast cancers can be ER/PR+, HER2 negative and low levels of Ki67 (a marker of proliferation), and Luminal B is defined as ER/PR+, HER2 +/- and high levels of Ki67 (Gao and Swain 2018). These cancerous epithelial cells can progress to mesenchymal-like cells typically found in basal-like, hormone-resistant, and advanced breast cancers commonly known as triple-negative breast cancer, the most deadly form of breast cancer. This process of dedifferentiation is called epithelial-to-mesenchymal transition (EMT) and also occurs in embryogenesis and wound healing (Macias and Hinck 2012, Lamouille, Xu et al. 2014). The phenotypical alterations demonstrating EMT include anchorage independence, polarization, migration, and invasion (Lamouille, Xu et al. 2014, Huang, Li et al. 2015). Once cells have undergone EMT, they are able to function as mesenchymal cells and more able to migrate through the basement membrane and into circulation leading to metastasis.

2.6.1. Cell-Surface Transformation

To begin the process of metastasis, the cancerous cells lose the expression of basement membrane attachment cell-cell contact proteins, such as E-cadherin and vimentin, characteristic of epithelial cells. Mesenchymal markers, such as fibronectin and N-cadherin begin to be expressed leading to alterations in the extracellular matrix. The shift of E-cadherin to N-cadherin is linked with loss of cell-cell interaction and ultimately poor clinical outcomes (Lamouille, Xu et al. 2014, Huang, Li et al. 2015). MCF7 cells exposed to parabens showed the significant invasion of Matrigel and reduced the protein expression of E-cadherin and β-catenin with multiple treatments, indicative EMT (Darbre and Harvey

2014, Khanna, Dash et al. 2014). The mechanisms by which parabens reduced E-cadherin expression have not been elucidated.

2.6.2. Stem Cell Markers

EMT can also be confirmed by examining the markers of stem cells which indicate the dedifferentiation of cancerous cells to the mesenchymal state. A physiological level of MP (4.4 μg per day) caused an increase of tumor size from MCF-7 xenografts and ER+ PDX tumors. MP induced a shift to mesenchymal by upregulating mRNA expression of *Nanog*, *Oct4*, and *ALDH1* in both MCF7 and PDX mammospheres (Lillo, Nichols et al. 2017). Interestingly, the pre-treatment of MCF7 cells with ER antagonist tamoxifen, a common therapy for ER (+) breast cancers, does not impede the MP induction of mammosphere size or NANOG protein expression (Lillo, Nichols et al. 2017). Therefore, it is likely that MP impacted mammosphere formation and NANOG protein expression through ER-independent signaling pathways signaling.

2.6.3. Cellular Migration and Invasion

Disruption of tight junctions between the cells allows for tumor progression and metastasis, cellular migration and invasion (Lamouille, Xu et al. 2014). These structural changes induce stress and the formation of stress fibers, which provide structural strength to the malformed cell. A migratory property allows the cancerous cells to traverse the extracellular matrix for proliferation and nutrient acquisition. Once cells develop an invasive characteristic, they progress to intravasation where the cells are able to pass through the basement membrane and to survive in circulation (Semenza 2016).

It has been reported that 50 μM BP induced matrix degradation and invasion of MCF7 in a 7-day treatment, whereas 0.5 mM MP, 50 μM PP and 50 μM BP promoted MCF7 cell migration and invasion in 20 weeks of treatment (Darbre and Harvey 2014, Khanna, Dash et al. 2014). Migratory effects by parabens also were demonstrated in other two estrogen-responsive human breast cancer cells T-47-D and ZR-75-1 cells (Khanna, Dash et al. 2014).

2.7. Conclusion

Humans are exposed to parabens throughout life from personal care products, food, pharmaceuticals, industrialization, etc., and these parabens can be absorbed into circulation. Parabens can negatively affect many different hormone and endocrine-related processes. Specifically, parabens can influence breast cancer progression by binding and activating ERs and modulating the expression of ERs and other steroid-related receptors, such as PR, AR, and GPR30. Consequently, parabens can activate various signaling pathways ultimately leading to breast epithelial cell proliferation. Moreover, parabens have also been shown to encompass the ability to induce EMT, increase cell migration and invasion, characteristics critical for breast cancer metastasis. However, many of the studies tested parabens at very high doses, such as 50 μM. Therefore, further research is necessary to understand the full extent of parabens toxic effects at environmental achievable doses.

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CHAPTER 3 :
PARABENS INDUCE INFLAMMATION IN STROMAL FIBROBLASTS LEADING TO BREAST CANCER CELL PROLIFERATION

3.1. Abstract

Background: It is increasingly realized that breast stroma plays a critical role in breast carcinogenesis and breast cancer progression. Understanding the alterations that occur in the stromal cells in breast carcinogenesis can lead to novel and effective therapeutic or preventative strategies. Parabens are a group of 4-hydroxybenzoic acid esters, commonly found in pharmaceuticals, food, personal care products, and paper as anti-microbial agents. Studies have shown that parabens are estrogenic, act as endocrine disrupting chemicals in experimental animals, and can accumulate in higher amounts in the breast cancer samples. Therefore, we hypothesize that parabens may promote breast carcinogenesis by negatively modulating breast stroma nearby breast epithelial cells.

Methods: 3T3-L1 murine and BRF human fibroblasts were exposed to the three most common parabens, methyl-, propyl-, and butylparaben (MP, PP, and BP) at environmentally relevant doses (0.02, 0.1, and 1 μM) for 6, 12, and 24 hours. Gene expression was determined by RT-PCR. 3T3-L1 CARΔ1 cells, which are engineered to express adenovirus receptors, were employed to study NF-κB activation followed by Western analysis of p-p65 and IκBα degradation, two critical events for NF-κB signaling pathways. Lastly, paraben-treated fibroblast conditioned media was tested for their abilities to promote the proliferation of autobioluminescent MCF7 breast cancer cells.

Results: A single exposure to all three parabens at the tested doses induced inflammatory gene *IL-1β*, *MMP9*, *COX2*, and *CYP19A1* in both murine 3T3-L1 fibroblasts and human BRF cells. Consistently, all three parabens activate NF-κB in both 3T3-L1 and BRF cells as demonstrated by the activation of NF-κB reporter and phosphorylation of p65 and IκBα degradation. Moreover, the conditioned media from paraben-treated 3T3-L1 fibroblasts and human BRF cells induced proliferation of breast cancer MCF7 cells.

Conclusion: Parabens may play important roles in breast cancer progression by inducing inflammation in breast stromal fibroblasts.

3.2. Introduction

Breast cancer remains the second leading cause of cancer mortality in women within the US behind lung and bronchial cancers (ACS 2017). The tumor microenvironment or stroma impacts breast cancer progression by enabling proliferative signaling, activating angiogenesis, allowing invasion and metastasis via extracellular matrix (ECM) remodeling, etc. through the communication of secreted chemokines and cytokines (Hanahan 2011). Tumor stroma is composed of a variety of cell types, including adipocytes, fibroblasts, macrophage, neutrophils, mast cells, and endothelial cell, all of which cross-talk with each other and with nearby breast epithelial cells to promote carcinogenesis and cancer progression (Soysal, Tzankov et al. 2015, Lee, Lee et al. 2018).

Chronic inflammation has been recognized for its association with an increased risk of cancer development. Stromal inflammation is thought to activate dormant tumors or initiate tumorigenesis in transplanted primary epithelial cells, a process called stromal induction (Radisky and Radisky 2007). The stroma is densely populated with fibroblasts, and these fibroblasts can enable tumor proliferation, sustain elevated pro-inflammatory gene expression, accommodate metastasis, etc. (Lim and Moon 2016). Fibroblasts are typically activated by wound healing or tissue injury, where they begin to play a role in inflammation in the recruitment of immune cells to repair the damage (Kalluri 2016). A tumor comprises a similar response to that of tissue damage or an injury with the activation of stromal fibroblasts that release inflammatory cytokines for healing (Kalluri 2016). However, the stroma consists of many other different cell types, including adipocytes, macrophage, mast cells, endothelial cells, etc., and each cell type begins secreting different factors into the stroma which are all able to have autocrine or paracrine action (Semenza 2016). Somehow, the communication between these cells begins to permit the growth of cancerous cells and begin to work in collaboration with cancer. Therefore, it is critically important to understand the complex interactions between these stromal cells and the epithelial cells.

The inflammatory response by fibroblasts is critical in the communication with the cancerous epithelial cells (Lim and Moon 2016, Balachander, Talukdar et al. 2018).

Cyclooxygenase-2 (COX2) has been implicated in the development of various cancers, including gastric, breast, lung, esophageal, and colon cancer (Li, Zheng et al. 2016, Sano, Kogashiwa et al. 2018). COX2 is overexpressed in aggressive breast tumors associated with poor prognosis and survivability, and knockout of COX2 in MCF7 breast cancer epithelial cells attenuates proliferation (Denkert, Winzer et al. 2004, Brown, Subbaramaiah et al. 2008, Han, Yang et al. 2014). Prostaglandin E2 (PGE2), a downstream product of COX2, enhances tumor growth through inflammation, leading to angiogenesis, invasiveness, and evading cell death (Li, Shan et al. 2015). Interleukin-1 beta (IL1β), one prominent cytokine released into the inflamed stroma, has been shown to increase invasiveness and metastasis of breast tumors and correlates with worse prognosis (Brown 2014, Stender, Nwachukwu et al. 2017). Additionally, IL1β contributes to endocrine and chemotherapy resistance in ER+ breast cancers through activation of IKKβ, upstream of NF-κB signaling (Stender, Nwachukwu et al. 2017).

Moreover, proteases within the stroma have been recognized as influential in the growth of tumors. Matrix metalloproteinases (MMP) are enzymes that breakdown the ECM, providing space for tumor growth, passages for invasion and metastasis, and are overexpressed in many breast cancers (Zaremba-Czogalla, Hryniewicz-Jankowska et al. 2018). MMP-9 functions in digestion of ECM proteins, including collagen, fibronectin, and laminin; in addition, MMP-9 can also degrade other non-matrix signaling proteins within the stroma (Radisky and Radisky 2007). Interestingly, MMP-9 is a target of NF-κB activation and is associated with COX2 activity via PGE2 production (Li 2012, Chimal-Ramirez, Espinoza-Sanchez et al. 2013).

It is well recognized that environmental exposures contribute to the development of many chronic diseases, including cancer (Cui, Balshaw et al. 2016). Endocrine disrupting chemicals (EDC) are industrial chemicals utilized to enhance product sustainability and extend shelf-life but have been identified to dysregulate endocrine systems, causing adverse health outcomes (Darbre 2006, Burks, Pashos et al. 2017). Parabens are alkyl esters of *p*-hydroxybenzoic acid incorporated into personal care products, pharmaceuticals, food

packaging, and paper products for their anti-microbial and anti-fungal properties. The most common parabens used in the commercial products and therefore often being detected in human biological samples are MP, PP, and BP (Figure 3.1.) (Giulivo, Lopez de Alda et al. 2016).

Even though parabens have been classified as weak EDCs, previous studies have demonstrated their ability to bind hormone receptors at low-dose exposures, enhancement of estrogenicity, and promotion of proliferation, invasion, and migration of breast epithelial cells (Darbre and Harvey 2014). Moreover, total parabens have been detected within normal breast tissue at an average concentration of 85 ng/g of tissue, indicating parabens proximity to potentially cancerous epithelial cells (Barr, Metaxas et al. 2012). Furthermore, parabens have been detected in higher amounts in breast cancer samples than their normal breast tissue controls, ranging from 17.3 ng/g of tissue to 115 ng/g of tissue (Darbre, Aljarrah et al. 2004). To this end, parabens have been shown to promote breast epithelial cell growth; however, it is not clear whether parabens also negatively affect breast stroma (Darbre and Harvey 2008).

The objective of this study was to investigate the inflammatory effects of common parabens (MP, PP, and BP) at environmentally achievable doses (0.02, 0.1, and 1 μM) on the stromal fibroblasts from both murine and human origins and the ability of exposed fibroblasts to enhance breast cancer cell proliferation. We show that parabens induce the upregulation of inflammatory genes (*COX2* and *IL1β*), ECM remodeling gene *MMP-9* and aromatase gene *CYP19A1* by activating the NF-κB signaling pathway. We further demonstrate the conditioned media (CM) collected from paraben-exposed fibroblasts enhanced MCF7 breast cancer cell proliferation. Our study demonstrates that common paraben at environmentally achievable doses promotes pro-inflammatory gene expression in the fibroblasts of the stroma and leads to enhanced breast cancer proliferation.

3.3. Materials and Methods

3.3.1. Reagents

Dimethyl sulfoxide; methyl-, propyl-, and butylparaben were from Acros Organics (>99%, Thermo Fisher Scientific, Pittsburg, PA). Caffeic acid phenethyl ester (CAPE) was ordered from Tocris Bioscience (Minneapolis, MN). Ultra-pure lipopolysaccharide (LPS) was from List Biological Laboratories (Campbell, CA). Phospho-NF-κB p65 (Ser536) (no. 3033) and NF-κB -p65 (D14E12) XP (no. 8242) were purchased from Cell Signaling Technology. IκBα (C-21) (sc-371) was purchased from Santa Cruz Biotechnology. Anti-β-actin (AC-15) (no. A1978) was from Sigma-Aldrich. Neomycin (G-418) was purchased from Research Products Incorporated (Mount Prospect, IL).

3.3.2. Cell Culture

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (HyClone). Murine 3T3-L1 coxsackievirus and adenovirus receptor (CARΔ1) cells were a gift from Dr. Orlicky (Orlicky DJ 2001) and were grown in DMEM containing 10% fetal bovine serum (FBS) (Atlanta Biological, Flowery Branch, GA). Human subcutaneous breast fat preadipocytes (BRF) (Zenbio, NC) were grown in Preadipocyte Medium (PM-1) according to the manufacturer's instructions (Zenbio, NC). Human Autobioluminescent MCF7 cells were a gift from Dr. Ripp (490 BioTech, Inc., Knoxville, TN) (Xu, Ripp et al. 2014) and were grown in DMEM 10% FBS with G-418. All are grown in 5% CO_2, 37^{o}C conditions.

3.3.3. Immortalization of Primary BRF Cells

Immortalization of primary BRF cells was achieved by infecting the cells with lentivirus encoding the SV40LT antigen. SV40LT antigen was first cloned into lentivirus expressing vector carrying the neomycin phosphotransferase gene (Alwin Prem Anand, Gowri Sankar et al. 2012). To generate lentivirus particles, SV40LT vector was co-transfected with packaging (psPAX2) and envelope (pMD2.G) vectors (Addgene; Boston, MA) in

HEK293T cells (GeneCopoeia, Inc; Rockville, MD) using FuGENE® HD transfection reagent (Promega Corporation; Madison, WI) following manufacturer's guidelines and then used to infect BRF cells. Stable cell lines were selected using G-418 antibiotic (300 μg/ml) for two weeks.

3.3.4. Paraben Treatment

To detect the inflammatory effects of low-dose paraben on 3T3-L1 or BRF fibroblasts, a single exposure of 0.02, 0.10, or 1.0 μM methyl-, propyl-, or butylparaben was administered in low-serum conditions, 0.25% calf serum (HyClone) for 3T3-L1 cells and 1% charcoal-stripped fetal bovine serum (CS-FBS) (HyClone) for BRF cells. DMSO and LPS were used as vehicle control and positive control, respectively.

3.3.5. RNA and Quantitative Real-Time PCR

Total RNA was prepared from fibroblasts using TRIzol (Invitrogen Coorporation, Carlsbad, CA) following the manufacturer's protocol. Total RNA quantity was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was conducted utilizing High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) following the manufacturer's protocol. mRNA expression of fibroblast inflammation markers and housekeeping gene 36B4 (Table 1) were measured quantitatively utilizing PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and were analyzed in a 96-well template via an ABI 7300 HT Real-Time PCR System with cycle conditions of 50°C 2 min, 95 °C 2 min, and 40 cycles of 95°C for 15s/60°C for 1 min. The relative gene expression was calculated utilizing a $2^{(-\Delta\Delta Ct)}$ method and normalized against 36B4.

3.3.6. Reporter Gene Assays

3T3-L1 CARΔ1 and BRF fibroblasts were seeded in a 24-well plate format and infected with adenovirus encoding NF-κB-Luc reporter gene and adenovirus encoding β-

galactosidase (β-gal) control plasmid for 15 h, as described previously (Purohit, Hu et al. 2013). The cells were probed with various parabens for 12 h. The cell lysate was prepared utilizing Reporter Lysis 5X Buffer (Promega). Reporter luciferase activity was measured using GloMax Luminometer (Promega, Madison, WI) and normalized by β-gal expression.

3.3.7. Western Blot Analysis

Total cell lysates were prepared and protein concentrations measured by Pierce BCA protein assay kit (Thermo Fisher Scientific). 20-40 μg of total cell lysate were run through a polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk or 5% bovine serum albumin (BSA). The membrane was immunoblotted with primary antibodies at 4 ºC overnight followed by secondary antibody conjugated with horseradish peroxidase. The signal was detected utilizing SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), HyBlot CL autoradiography film (Denville Scientific), and a Konica SRX-101A film processor. Quantification by densitometry was conducted using ImageJ software.

3.3.8. Conditioned Media Collection

3T3-L1 and BRF fibroblasts were seeded into 60 mm dishes. Each plate of cells was treated with MP, PP, BP (each at 1 μM) or controls (DMSO or LPS) in 5% CS-FBS. After 72 h or 10 days, the treatment media was removed, and the cells were washed twice with phenol-red free (PRF) media (HyClone) to remove residual parabens. To collect CM, cells were then cultured for 48 h in PRF media with 5% CS-FBS and penicillin and streptomycin. After 48 h, the media was collected and centrifuged at 600 rpm for 5 minutes. The supernatant was transferred into a fresh tube and stored at 4ºC before use.

3.3.9. Bioluminescent Imaging

Autobioluminescent MCF7 cells were plated at 30% confluency in quadruplicate wells of a 24-well opaque, clear bottom plate (Labnet/Corning Inc., Cary, NC). After attachment overnight, MCF7 cells were washed with PRF media and treated with CM: fresh media in a 1:1 ratio. Treatment persisted for 7 days with changing CM at day 4. On day 7, plates were transferred to CCD-based IVIS Lumina imaging system (PerkinElmer). Autobioluminescent readings were acquired 3 times at 10 min intervals according to the manufacturer's instructions.

3.3.10. Statistical Analysis

All data are presented as means $\pm$ SE. Measurements were performed in triplicates unless otherwise indicated Statistical analysis was performed using SigmaPlot 14.0 (Systat Software). One-way ANOVA on ranks or one-way ANOVA with repeated measures followed by multiple comparison tests (Student-Newman-Keuls method) were used to determine the differences between treatment groups or time points. Student's *t*-tests were conducted to evaluate LPS compared to the control. The level of significance was set at $P < 0.05$. The effects of treatment and plate location on fold change of average radiance were analyzed using two-way ANOVA analysis for each time point in 3T3-L1 and human BRF fibroblasts.Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M4 for Windows 64x (SAS institute Inc., Cary, NC).

3.4. Results

3.4.1. Parabens Induce mRNA Expression of Inflammatory Genes and CYP19A1 in Murine and Human BRF Fibroblasts

3T3-L1 fibroblasts were exposed to MP, PP, or BP or their vehicle control (DMSO) at 0.02, 0.1, or 1 µM for 6, 12, or 24 h. LPS was included as a positive control. The inflammatory response to parabens differs depending on the length of the side chain, dose,

and time of exposure. All three tested parabens significantly upregulated inflammatory *Il1β* mRNA expression in 3T3-L1 fibroblasts. Compared to DMSO, 0.1 μM MP increased the maximal 2.5 fold of *Il1β* mRNA expression at 6 h ($p<0.05$) but had minimal effects at 12 or 24 h of exposure (Figure 3.2.A), where 0.1 μM PP increased ~ 2 fold of *Il1β* mRNA expression at 6 h ($p<0.05$), increased to ~ 3 fold at 12 h and maintained at 1.5 fold at 24 h ($p<0.001$) of exposure (Figure 3.2.A). Butylparaben increased greater than 5 fold *Il1β* mRNA expression at 0.1 and 1 μM at 6 h. The increased levels were maintained at 12 h, but dropped at 24 h by 0.1 μM whereas gradually increased and reached the maximal 12 fold at 24 h of exposure by 1 μM ($p<0.05$) (Figure 3.2.A). In contrast, the maximal 7 fold *Il1β* mRNA level by LPS was observed at 12 h and decreased to 3.5 fold at 24 h of exposure (Figure 3.2.A).

In addition to *Il1β*, we also examined *Mmp-9* mRNA expression, a key MMP in matrix remodeling in breast cancer growth. Even though LPS significantly upregulated *Mmp-9* mRNA expression at 6, 12, and 24 h exposure, there is not as great of an influence by parabens except that MP increased 1.5 fold at 6 h by 0.1 μM and PP increased 1.5 fold at 12 h by 1 μM compared to the vehicle control (Figure 3.2.B). Butylparaben had increased *Mmp-9* expression 2 fold at 6 h by both 0.1 and 1 μM ($p<0.01$). We examined the effects of parabens on *Cox2* mRNA expression in fibroblasts as COX2 upregulation is greatly associated with the progression of many cancers, including breast cancer. All three paraben significantly upregulated *Cox2* mRNA expression at 24 h ($p<0.05$) with BP reaching a maximum of 4.5 fold at 1 μM ($p<0.05$) compared to the control. In contrast, LPS increased 1.8 fold of *Cox2* mRNA expression at 12 h and maintained the level at 24 h ($p<0.05$) (Figure 3.2.C).

Lastly, we analyzed mRNA expression of *Cyp19a1*, the enzyme responsible for the conversion of androstenedione and estrone to estrogen (Gerard and Brown 2018). All parabens tested, PP and BP in particular, significantly upregulated *Cyp19a1* mRNA expression. MP at 0.1 μM significantly induced at 6 h ($p<0.05$). PP at concentrations greater than 0.1 μM significantly induced *Cyp19a1* mRNA at all tested time points

($p<0.01$), and induced significant upregulation by all tested concentrations at 24 h ($p<0.05$). All BP concentrations significantly upregulated *Cyp19a1* mRNA expression at 6 h ($p<0.05$), and 12 h. At 24 h, BP greater than 0.1 µM concentrations increase expression ($p<0.01$) with maximal expression of 13 fold 1 µM compared to the vehicle control. In contrast, LPS only increased ~ 2-3 fold of *Cyp19a1* mRNA expression at 12 and 24 h exposure ($p<0.001$) (Figure 3.2.D).

Similarly to murine fibroblasts, immortalized BRF also responded to all three tested parabens, depending on the length of the side chain, dose, and time of exposure (Figure 3.3.). MP induced maximal ~ 2 fold of *IL1β* mRNA expression at 12 h at 0.1 and 1 µM ($p<0.05$) and had no effects at 24 h in human immortalized BRF fibroblasts (Figure 3.3.A). Interestingly, low doses (0.02 and 0.1 µM), but not a high dose (1 µM), of PP induced ~ 2 fold of *IL1β* mRNA at 6 h ($p<0.01$) and had lesser effects at 12 or 24 h of exposure (Figure 3.3.A). However, at 24 h high dose PP induced the greatest response ($p<0.01$). Butylparaben only induced ~ 1.6 fold of *Il1β* mRNA at 12 h at the low dose of 0.02 µM ($p<0.05$) (Figure 3.3.A). In contrast, LPS induced a maximal 3.5 fold of *Il1β* mRNA expression at 12 h and a lesser fold increase at 24 h (Figure 3.3.A).

In contrast, all three parabens induced more robust responses of *MMP-9* in human BRF cells. Compared to the DMSO, MP induced ~ 2.5 fold of *MMP-9* mRNA expression at 6 h by 0.02 µM and ~ 4 fold by 0.10 µM ($p<0.05$), reaching 5 fold at 12 h at 0.1 µM. At 1 µM, MP induced ~ 4 fold of *MMP-9* mRNA expression at 12 h and maintained the level at 24 h (Figure 3.3.B). Propylparaben dose-dependently increased *MMP-9* mRNA, reaching 4.5 fold at 1 µM, at 6 h ($p<0.05$). This dose-dependent response subsided at 12 and 24 h. Butylparaben had an inverse dose-dependent response with 0.02 µM increasing gene expression the most ($p<0.01$) at 6 h. Low dose BP maintained the most significant response at 12 h, but this enhancement subsided by 24 h. LPS only induced a minimal response of *MMP-9* in human BRF cells (Figure 3.3.B).

Propyl- and butylparaben also significantly induced *COX2* mRNA expression (Figure 3.3.C). MP induced ~1.5 fold of *COX2* mRNA expression at 0.02 µM at 6 h ($p<0.01$) and induced similar increases at 0.1 and 1 µM at 12 h (Figure 3.3.C). PP induced a maximal 2.5 fold of *COX2* mRNA at lower doses of 0.02 ($p<0.05$) and 0.1 µM ($p<0.01$) at 6 h, which were not maintained at 12 and 24 h. At 1 µM, PP induced a maximal 2.3 fold at 24 h ($p<0.05$) (Figure 3.3.C). BP induced a ~ 2.3 fold of *COX2* mRNA at 1 µM at 6 h and 24 h ($p<0.05$). BP also induced a maximal of 2 fold at 0.02 µM at 24 h ($p<0.01$). In comparison, LPS induced a modest increase of *COX2* mRNA at 6 h and reached the maximal 2 fold at 24 h in human BRF cells ($p<0.05$) (Figure 3.3.C).

Lastly, *CYP19A1* mRNA expression was significantly upregulated by all three tested parabens at 6 h. However, at 12 h only MP induced significant upregulation and PP at 24 h ($p<0.05$). Propylparaben increased *CYP1A1* to ~ 2 fold at 0.1 µM at 12 h compared to the DMSO ($p<0.05$). Propylparaben induced most significant response with a ~ 12 fold increase at 0.02 µM at 6 h ($p<0.05$). All doses of BP increased *CYP19A1* mRNA expression at 6 h with 0.1 µM reaching ~ 10 fold ($p<0.05$). Similarly, LPS induced a ~ 2 fold increase of *CYP19A1* mRNA expression at 6 h which subsided over 12 and 24 h in human BRF fibroblasts (Figure 3.3.D).

3.4.2. Parabens Activate NF-κB Signaling in Murine and Human Fibroblasts

Next, we investigated the effects of paraben on the NF-κB signaling pathway, which is critical signaling that drives inflammatory gene expression (Li and Verma 2002). First, we investigated NF-κB activation using NF-κB-responsive luciferase reporter assays where fibroblasts were transiently infected with adenovirus encoding an NF-κB-Luc receptor. At 1 µM, all three parabens activated NF-κB in 3T3-L1 cells with increasing potency, correlating with the length of the linear alkyl chain (MP < PP ≅ BP) (Figure 3.4.A, left panel). To confirm the findings of the reporter assays, we also tested LPS activation with or without pre-treatment of an NF-κB inhibitor CAPE. LPS induced ~3 fold of NF-κB activation, which was significantly attenuated by CAPE (Figure 3.4.A, right panel). Similar to the gene expression results, MP and PP induced significant NF-κB activation in human

BRF fibroblasts at a lower dose of 0.02 μM ($p<0.05$ for MP and $p<0.05$ for PP), compared to murine 3T3-L1 cells (Figure 3.4.B). In contrast, LPS induced 3.5 fold of NF-κB activation ($p<0.01$) in human BRF fibroblasts.

We further confirmed paraben-induced NF-κB activation in 3T3-L1 fibroblasts by analyzing the phosphorylation of p65 and degradation of IκBα, two key events leading to NF-κB activation (Li and Verma 2002) with treatment of DMSO (Figure 3.5.A), 1 μM BP (Figure 3.5.B), or LPS (Figure 3.5.C) in a time-dependent manner. Quantification comparing 1 μM BP to its own time 0 shows the degradation of IκBα (Figure 3.5.D) and phosphorylation of p65 (Figure 3.5.E). At 15 minutes, 1 μM BP significantly degraded IκBα compared to the zero time point ($p<0.001$) and p65 phosphorylation was enhanced at 30 minutes ($p<0.001$). When normalizing the BP NF-κB activation to the associated DMSO, similar trends in IκBα and p-p65 protein expression occurred with degradation beginning at 15 minute and phosphorylation at 30 minutes (Figure 3.5.F). Moreover, paraben induced NF-κB activation as measured by these two events in the BRF cells with treatment of DMSO (Figure 3.6.A), 1 μM MP (Figure 3.6.B), or LPS (Figure 3.6.C) in a time-dependent manner. Quantification of IκBα degradation showed LPS induced NF-κB activation within 5 minutes of treatment, and 1 μM MP induced NF-κB activation at 30 minutes (Figure 3.6.D), similar to the murine response. Additionally, phosphorylation of p65 quantification demonstrates a drastic NF-κB activation by LPS beginning at 15 minutes and minimal response to 1 μM MP beginning at 5 minutes in human BRF fibroblasts (Figure 3.6.E). When normalizing MP NF-κB activation with DMSO, a change in degradation profile occurred, but IκBα still degraded at 30 minutes and p65 had greatest phosphorylation at 90 minutes (Figure 3.7.F).

3.4.3. Conditioned Media from Parabens-Treated Fibroblasts Regulates Breast Cancer Cell Proliferation

To explore the effects of paraben exposure on stromal induction and cancer progression, we evaluated the effects of the conditioned media (CM) collected from paraben-treated fibroblasts on the proliferation of human breast cancer MCF7 cells. We evaluated CM

collected after short-term (72 h) and long-term (10 day) exposure of parabens on both 3T3-L1 and BRF fibroblasts. The CM was then used to induce the proliferation of autobioluminescent MCF7 cells without the presence of paraben. At 72 h of paraben exposure to 3T3-L1 cells, MP induced greater proliferation of MCF7 cells compared to that of the other parabens; however, parabens elicit less MCF7 proliferation than the vehicle control (DMSO) (Figure 3.7.A). However, CM from 10-days exposure to PP led to significantly increased proliferation of MCF7 cell ($p < 0.01$) compared to DMSO (Figure 3.7.B). Interestingly, MCF7 cells have significantly increased proliferation after exposure to the CM collected from 72 h exposure to all three parabens in human BRF fibroblast ($p < 0.001$) (Figure 3.7.C). None of the CM from 10 days exposure in human BRF cells significantly induced MCF proliferation compared to DMSO, although the CM from BP induced more proliferation than that from MP, but not PP($p<0.01$) (Figure 3.7.D).

3.5. Discussion and Conclusion

To our knowledge, our study is the first study that demonstrates parabens at environmental achievable doses activate the NF-κB signaling pathway and induce inflammatory gene expression in both murine and human stromal fibroblasts derived from breast fat. Using conditioned media, we demonstrate that the inflammatory response stimulated by low-dose parabens in stromal fibroblasts promotes breast cancer cell growth. Our results, therefore, suggest that typical exposure to parabens from the environment can elicit a pro-inflammatory response in the breast stromal fibroblasts, which then stimulate nearby cancerous breast epithelial cells to proliferate.

Parabens are ubiquitously present in the environment due to their properties as an anti-microbial chemical and their preservative nature. These EDCs are absorbed dermally or ingested daily from a variety of different sources, including personal care products such as lotions, cosmetics, deodorants, and hair products; pharmaceuticals; food; etc. (Darbre and Harvey 2014). Once absorbed, parabens are distributed throughout the body and have been detected intact in blood, breast milk, placenta, and urine (Sandanger, Huber et al. 2011, Kolatorova 2017, Shen, Liang et al. 2017). The intakes of parabens from food range from

207-940 ng/kg body weight. However, absorption from personal care products ranges from 31-766 µg/kg body weight (Darbre and Harvey 2014). An individual is exposed to parabens multiple times per day through every consumption or application of product containing the chemical. Unconjugated parabens are present in human serum within 5 h of absorption, and conjugated parabens can remain in human serum for 24 h (Shin, Shin et al. 2019). Therefore, we tested the effects of parabens at 6 h of exposure finding that they induce an inflammatory response. Additionally, we found that the inflammatory response is sustained as the fibroblasts are exposed to parabens for 12 and 24 h. Therefore, continual exposure to parabens throughout a day maintaining a free paraben presence in the serum for 12 or 24 h can also lead to stromal inflammation.

Parabens have been implicated in a variety of different diseases, including obesity, fertility, menstruation, and cancer (Hu, Chen et al. 2013, Smith, Souter et al. 2013, Darbre and Harvey 2014, Nishihama, Yoshinaga et al. 2016). Many of these diseases could be a result of parabens' estrogenic properties, which have been well characterized (Lange, Kuch et al. 2014). More specifically, parabens utilize a variety of signaling pathways to elicit these estrogenic effects including ERK1/2 and Akt signaling associated with ERα, ERβ, HER, GR, and other receptors (Wrobel and Gregoraszczuk 2014, Sun, Yu et al. 2016) (Kolsek, Gobec et al. 2015, Wrobel and Gregoraszczuk 2015, Pan, Yuan et al. 2016). Through these pathways, parabens have been reported to directly promote breast cancer cell proliferation, mammosphere formation, epithelial to mesenchymal transitions, and metastasis (Khanna and Darbre 2013, Darbre and Harvey 2014, Khanna, Dash et al. 2014, Lillo, Nichols et al. 2017).

As it is well recognized that breast cancer survival requires cooperation between breast cancer cells and the surrounding stroma (Hanahan 2011). Our findings add to the current literature by demonstrating parabens' abilities to induce an inflammatory response in stromal fibroblasts, leading to a toxic microenvironment that can stimulate breast cancer cell growth. The inflamed stroma is thought to play critical roles in the breast cancer

development as it is shown that the tumor cells cannot survive if a tumor is removed from inflammation translocated to a normal stroma (Bussard, Mutkus et al. 2016).

The stroma is composed of a variety of different cell types, including fibroblasts, adipocytes, immune cells, endothelial cells, etc.; each plays different roles in tissue homeostasis (Bussard, Mutkus et al. 2016). Stromal fibroblasts are the most abundant cell type in the stroma and are versatile as they act as precursors to various mesenchymal cell types, chondrocytes, adipocytes, myocytes, and endothelial cells (Kalluri 2016). These cells are activated during the wound healing response as well as response in tissue damages caused by tumors (Kalluri 2016, da Cunha, Domingos et al. 2019). Once activated, fibroblasts begin to secrete growth factors, microRNAs, cytokines and chemokines among other factors in both autocrine and paracrine fashion, leading to tumor-stromal cell and stromal cell-stromal cell crosstalk resulting in cancer progression and epithelial-mesenchymal transitions (da Cunha, Domingos et al. 2019). Additionally, fibroblasts release of MMPs promoting the restructuring of the extracellular matrix proteins, including collagens, hyaluronic acid, laminins and fibronectin enabling cancerous cells growth and metastasis (da Cunha, Domingos et al. 2019). Therefore, the secretome contributions from activated fibroblasts can function to promote cancer progression.

In this study, we have attempted to address the inflammatory effects of common parabens at environmentally achievable doses on stromal fibroblasts and the subsequent ability of exposed fibroblasts to enhance breast cancer cell proliferation. Our results indicate that parabens at low concentrations upregulate the mRNA expression of *Cox2*, *Il1β*, and *MMP-9* dependent on the type, dose, and exposure time of parabens. In murine fibroblasts, parabens enhanced the inflammatory response correlating with the increasing length of the linear alkyl side-chain (MP < PP < BP), where BP consistently generated the most significant response over time. In contrast, human fibroblasts' inflammatory response correlates with the decreasing length of the linear alkyl side-chain (BP < PP < MP). Methylparaben and PP provoke a higher fold and more sustainable inflammatory response than that of BP in the human BRF cells. Interestingly, MP is the type of paraben most

common in the environment and present in most abundance in the systemic fluids, making it the greatest risk in inducing inflammation in human breast fibroblasts (Greige-Gerges, Kaissi et al. 2013).

These results of increase mRNA expression of inflammatory markers are consistent with the activation of NF-κB reporter and signaling pathways by paraben treatment. However, we did not address the other signaling pathways that parabens are known to influence. Some of the variability in gene expression patterns observed could be a result of activating the intricate network of signaling pathways that lead to activation of NF-κB. An NF-κB signaling pathway is critical in mediating inflammatory response (da Cunha, Domingos et al. 2019). Two critical downstream events that lead to NF-κB activation are IκBα degradation and phosphorylation of p65 (Hayden and Ghosh 2004). The three markers identified to be upregulated by paraben exposure are all connected to the NF-κB signaling pathway (Li 2012, Kalluri 2016, Suman, Sharma et al. 2016, Tulotta and Ottewell 2018). Together, our results suggest that selected parabens at environmental achievable doses induced inflammatory gene expressions in both murine and human breast derived fibroblasts, which is accompanied by activation of NF-κB signaling pathways even though we do not conclusively show parabens directly activate NF-κB signaling. Future studies are warranted to confirm these results by conducting *in vitro* studies and *in vivo* studies using NF-κB knockdown or inhibitors to see if NF-κB is required in paraben-induced inflammatory effects .

Furthermore, we wanted to determine whether paraben stimulated inflammation in fibroblasts is sufficient to stimulate the proliferation of breast cancer cells, which is consistent with the impact of an inflamed stroma on carcinogenesis. Conditioned media was collected after treating the fibroblasts with parabens and washing the fibroblasts to remove any residual intact or hydrolyzed parabens, and used at a 1:1 ratio with the fresh media and was applied on autobioluminescent MCF7 cells. From both murine and human fibroblasts, paraben-stimulated conditioned media are sufficient to enhance the proliferation of MCF7 cells without direct exposure of parabens to the breast cancer cells.

At 1 µM, BP and PP, but not MP, significantly induced *Il1β* mRNA expression in 3T3-L1 fibroblasts; In contrast, MP and PP, not BP, significantly induced *IL1β* mRNA expression in human BRF cells. As one prominent cytokine in the inflamed stroma, IL1β has been shown to increase invasiveness and metastasis of breast tumors and correlates with worse prognosis (Brown 2014, Stender, Nwachukwu et al. 2017). Additionally, IL1β is also implicated in endocrine and chemotherapy resistance in ER+ breast cancers (Stender, Nwachukwu et al. 2017). Whether IL1β in the conditioned media promoted MCF proliferation remains to determined.

Upregulation of COX2 leads to the production of prostaglandins, such as PGE2 (Wang and Dubois 2006). PGE2 promotes tumor growth by binding to it receptors EP1-4 and activating signaling pathways that promote proliferation and inhibit apoptosis, such as Ras-MAPK pathway and EGFR/PI3K and Akt/ PPARδ pathway (Wang and Dubois 2006). At 1 µM, all tested paraben significantly induced *Cox2* mRNA expression in 3T3-L1 fibroblasts and BP and PP, but not MP significantly induced *COX2* mRNA expression in human BRF cells. It remains to be determined whether *Cox2*/PGE2 underlies the effects of the conditioned media collected from paraben-exposed fibroblasts.

Moreover, at 1 µM all tested parabens significantly induced *Cyp19a1* mRNA in 3T3-L1 fibroblasts and MP and BP, but PP, significantly induced *CYP19A1* mRNA expression in human BRF cells. It remains to be determined whether paraben significantly increases estrogen production from exposed fibroblasts thereby increasing ER+ MCF7 cell proliferation. Future studies are needed to determine the nature of the soluble factors that drive MCF7 cell proliferation from paraben exposed fibroblasts.

The insight of paraben-induced inflamed fibroblasts enhancing breast cancer proliferation is significant as women are exposed to these chemicals on a daily basis at low doses. As we only demonstrate the increase of proliferation using a functional assay, additional studies are needed to identify the critical soluble factors in the conditioned media from the

fibroblasts and the molecular mechanisms by which these soluble factors from paraben-exposed fibroblasts stimulate breast cancer cell proliferation.

In conclusion, we have shown that parabens, specifically MP, PP, and BP at environmental achievable doses, induce the upregulation of *IL1β, COX2, and MMP9* in both murine 3T3-L1 and human BRF cells *in vitro*. The inflammatory responses induced by parabens are accompanied by the activation of NF-κB signaling. Furthermore, we demonstrate that the secretions collected from paraben-exposed fibroblasts are sufficient to enhance breast cancer proliferation, suggesting that parabens at environmental achievable doses may promote inflammation in the stroma thereby promoting breast cancer progression.

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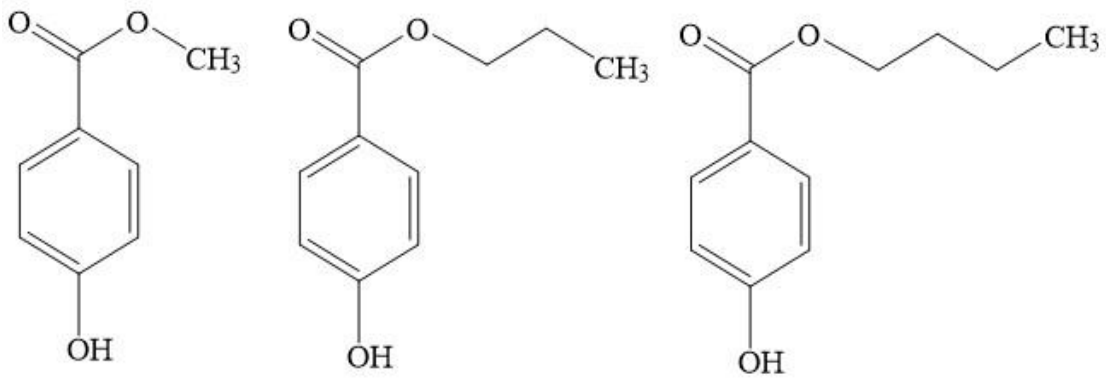
3.7. Appendix



Methylparaben **Propylparaben** **Butylparaben**

Figure 3.1. Paraben chemical structures.

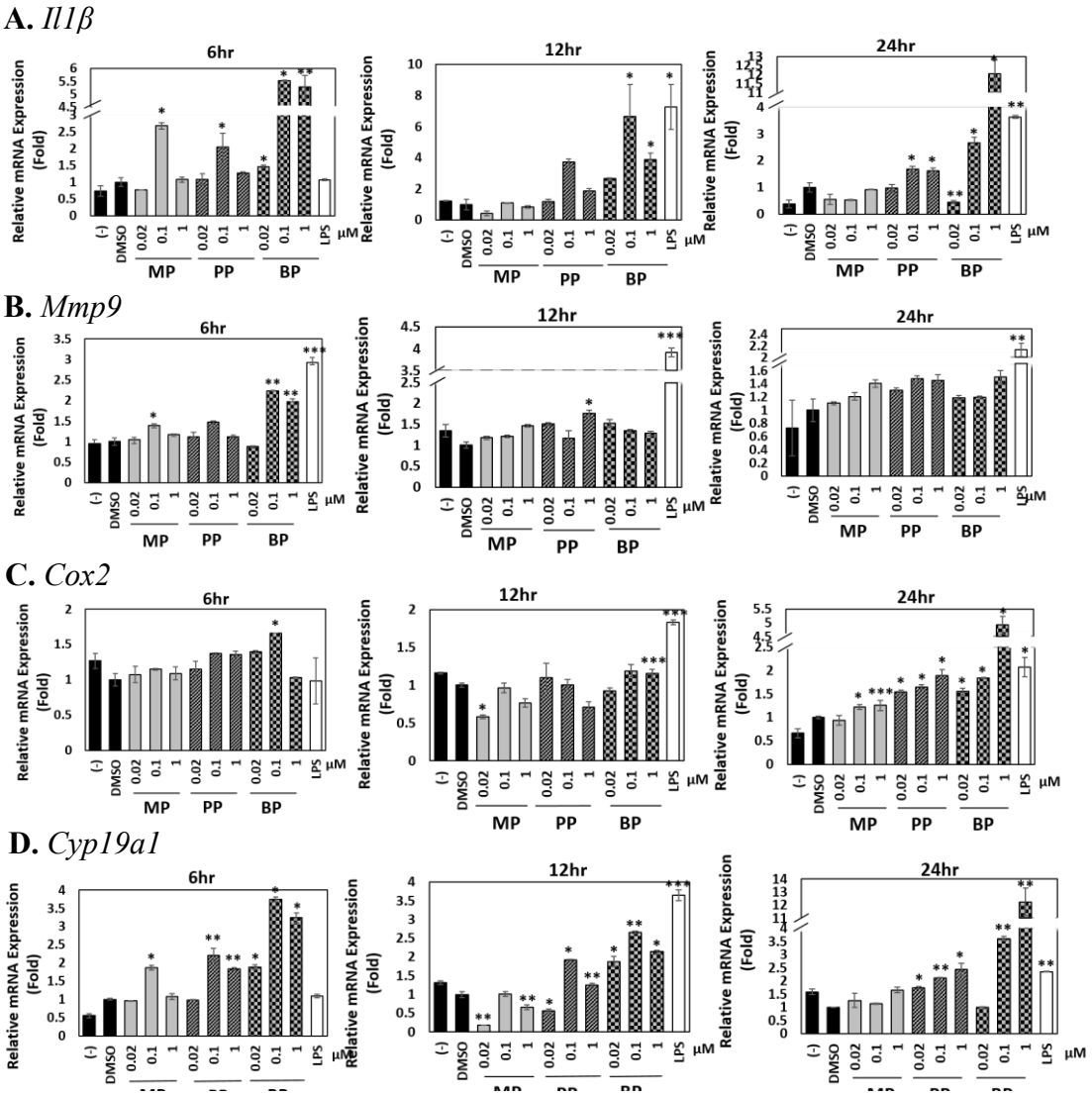


Figure 3.2. Parabens induce inflammatory gene expression in 3T3-L1 fibroblasts.

3T3-L1 fibroblasts were treated with selected parabens (0.02, 0.10, 1.0 μM) for the indicated time period. DMSO was the vehicle control and LPS was the positive control. Relative mRNA expression of inflammatory markers (**A.** *Il1β*, **B.** *Mmp9*, and **C.** *Cox2*) was analyzed. The relative mRNA expression was normalized to 36B4 and expressed as fold of that of the DMSO sample. Data are mean ± SE (n = 3). One-way ANOVA on Ranks was performed followed by multiple comparison tests with Student Newman-Keuls method to compare with the control. * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

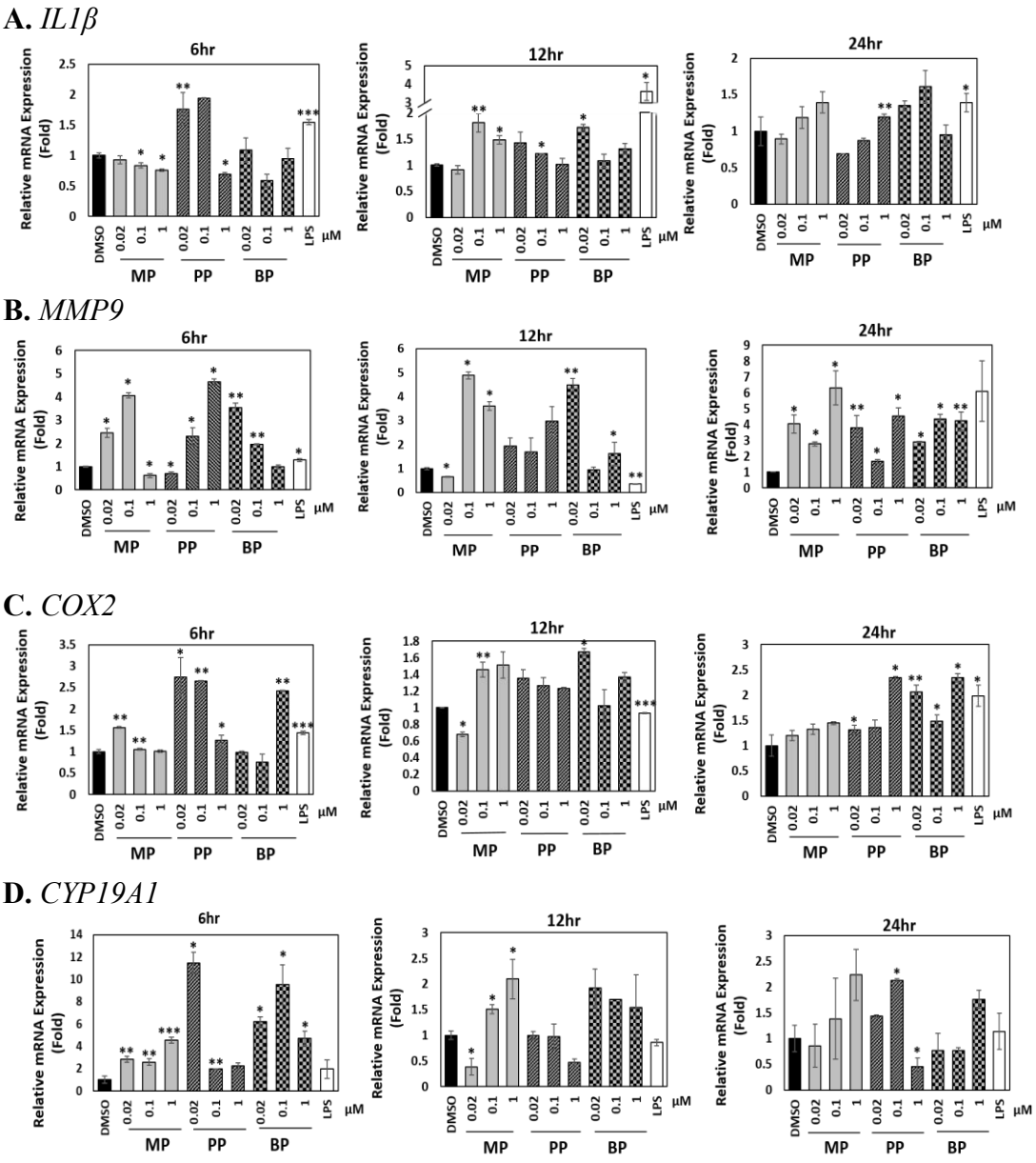


Figure 3.3. Parabens induce inflammatory gene expression in human BRF fibroblasts.

BRF fibroblasts were treated with selected parabens (0.02, 0.10, 1.0 μM) for the indicated time period. DMSO was the vehicle control and LPS was the positive control. Relative mRNA expression of inflammatory markers (**A.** *Il1β,* **B.** *MMP-9*, and **C.** *Cox2*) was analyzed. The relative mRNA expression was normalized to 36B4 and expressed as fold of that of the DMSO sample. Data are mean ± SE (n = 3). One-way ANOVA on Ranks was performed followed by multiple comparison tests with Student Newman-Keuls method to compare with the control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

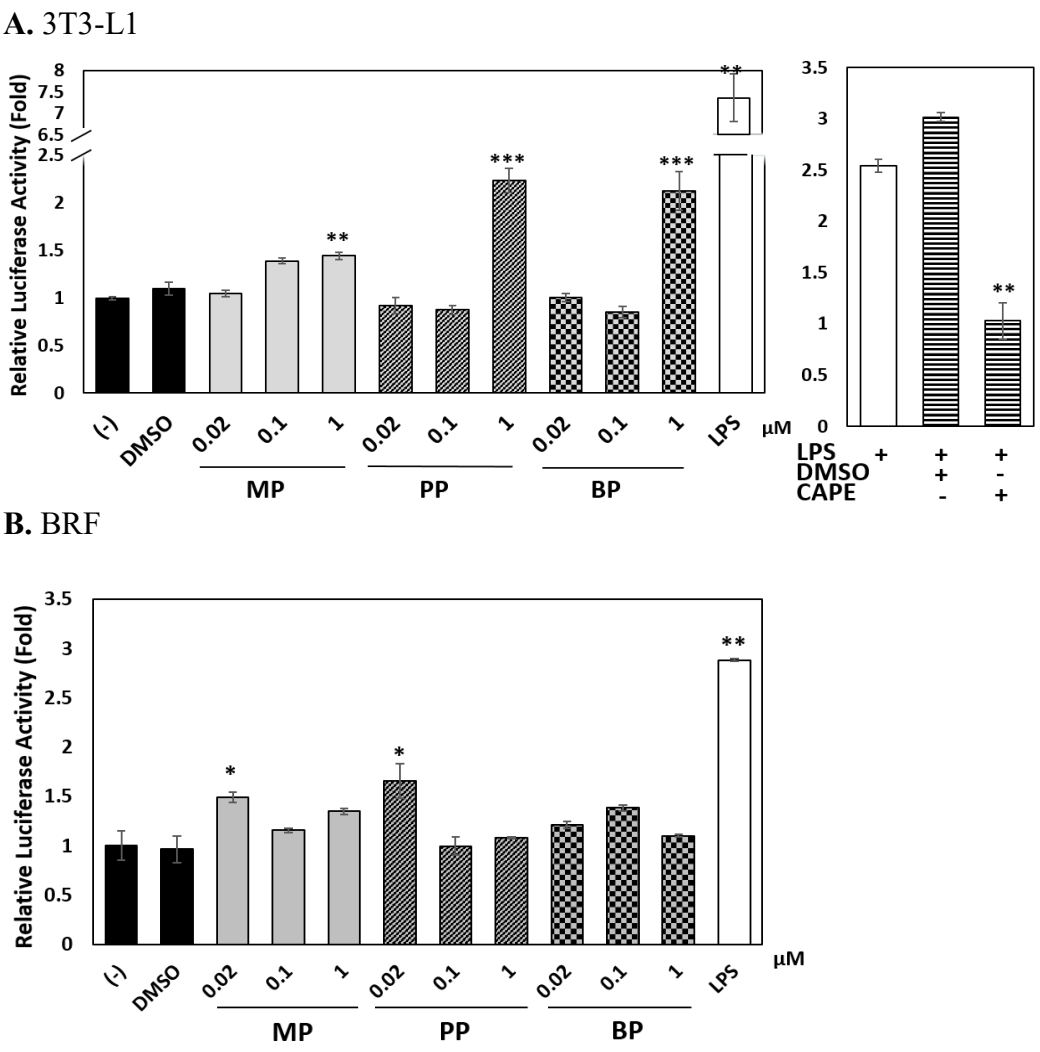


Figure 3.4. Parabens activate NFκB reporter in both murine and human fibroblasts.

A. 3T3-L1 and **B.** BRF fibroblasts were infected with adenovirus containing NFκB-Luc and β-galactosidase (β-gal) for 15 hours and stimulated with 0.02, 0.1, or 1 μM MP, PP, or BP along with appropriate controls for 12 hours. Reporter gene assays were conducted and relative luciferase activity was normalized by β-gal for each sample and expressed as fold. Data are means ± SE (n=3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

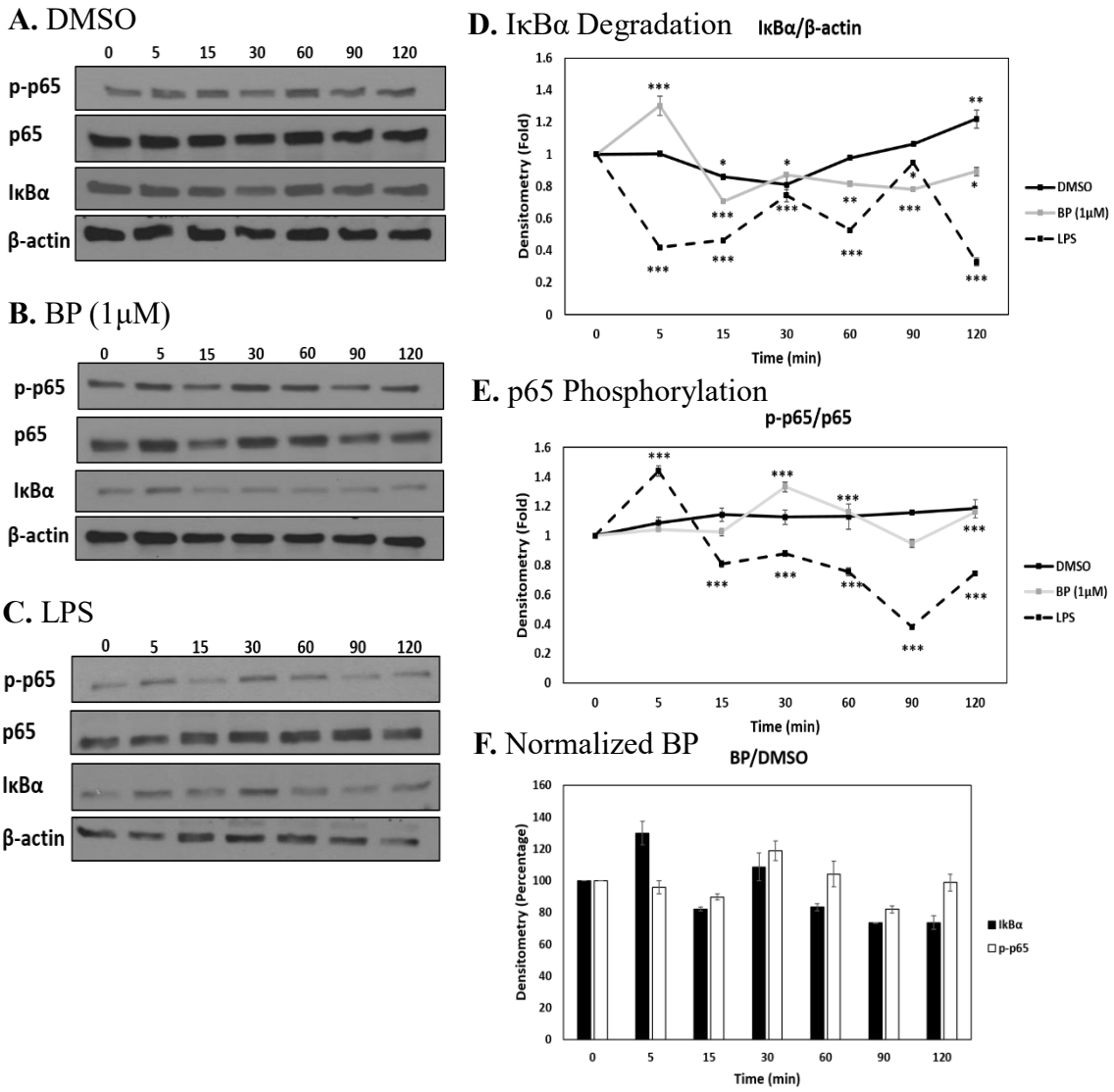


Figure 3.5. Paraben activates NFκB signaling in murine fibroblasts.

Fibroblasts were serum starved and treated with vehicle control **A.** DMSO, **B**. BP (1μM), or **C**. LPS (5 ng/mL) for indicated times, and whole cell lysate was prepared and analyzed by Western analysis with p-p65, p65, IκBα, and β-actin antibodies. Relative phosphorylation of p65 was normalized by p65 for each sample and expressed as fold. Relative expression of IκBα was normalized by β-actin for each sample and expressed as fold. **D.** IκBα degradation and **E.** phosphorylation of p65 comparison between each treatment with their own time 0. **F.** BP induced expression of IκBα and p-p65 normalized with DMSO. Data are means $\pm$ SE (n=3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

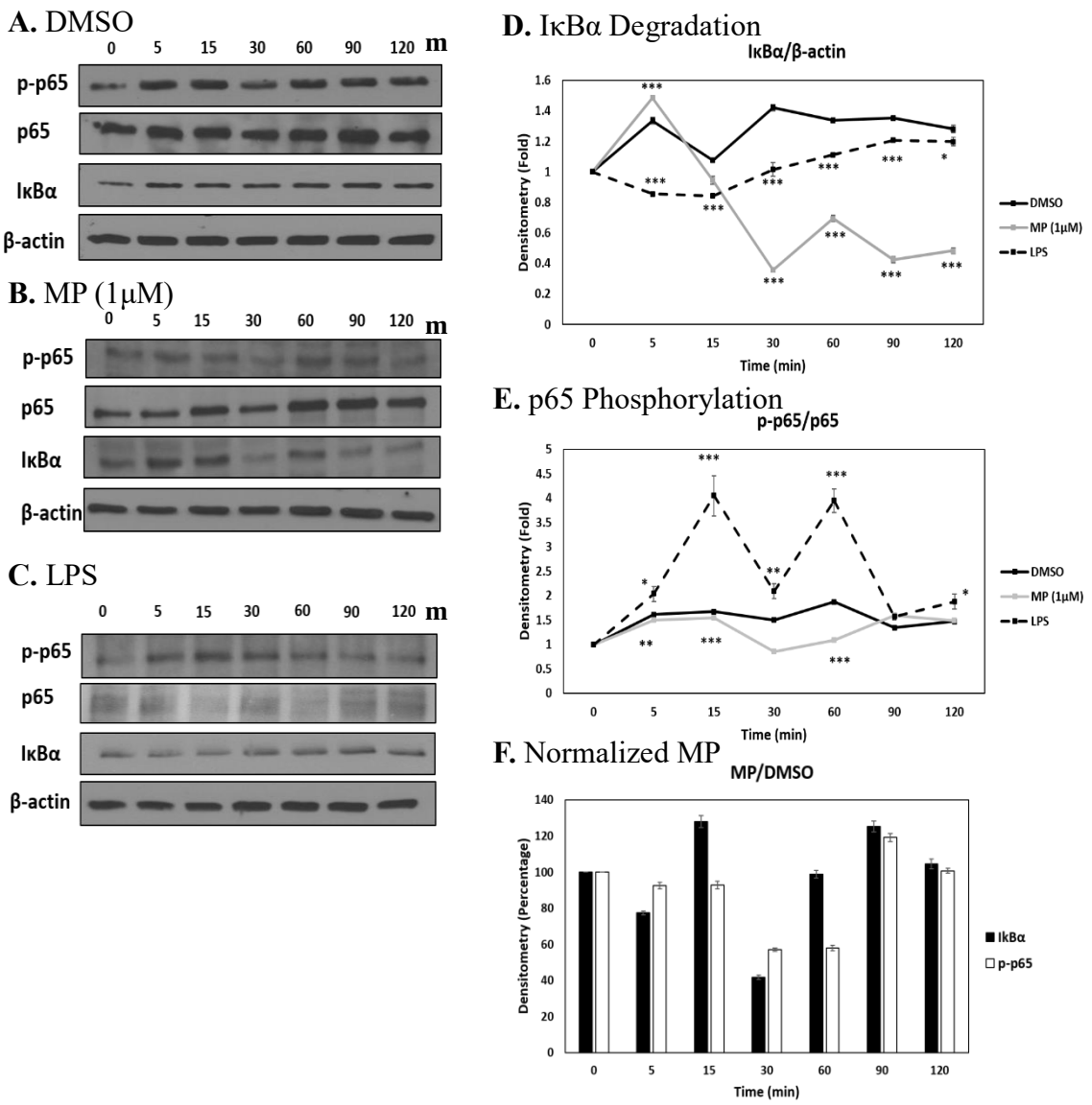


Figure 3.6. Paraben activates NFκB signaling in human BRF fibroblasts.

Fibroblasts were serum starved and treated with **A.** vehicle control (DMSO), **B.** MP (1μM), and **C.** LPS (5 ng/mL) for indicated times, and whole cell lysate was prepared and analyzed by Western with p-p65, p65, IκBα, and β-actin antibodies. Relative phosphorylation of p65 was normalized by p65 for each sample and expressed as fold. Relative expression of IκBα was normalized by β-actin for each sample and expressed as fold. **D.** IκBα degradation and **E.** phosphorylation of p65 comparison between each treatment with their own time 0. **F.** MP induced expression of IκBα and p-p65 normalized with DMSO. Data are means $\pm$ SE (n=3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

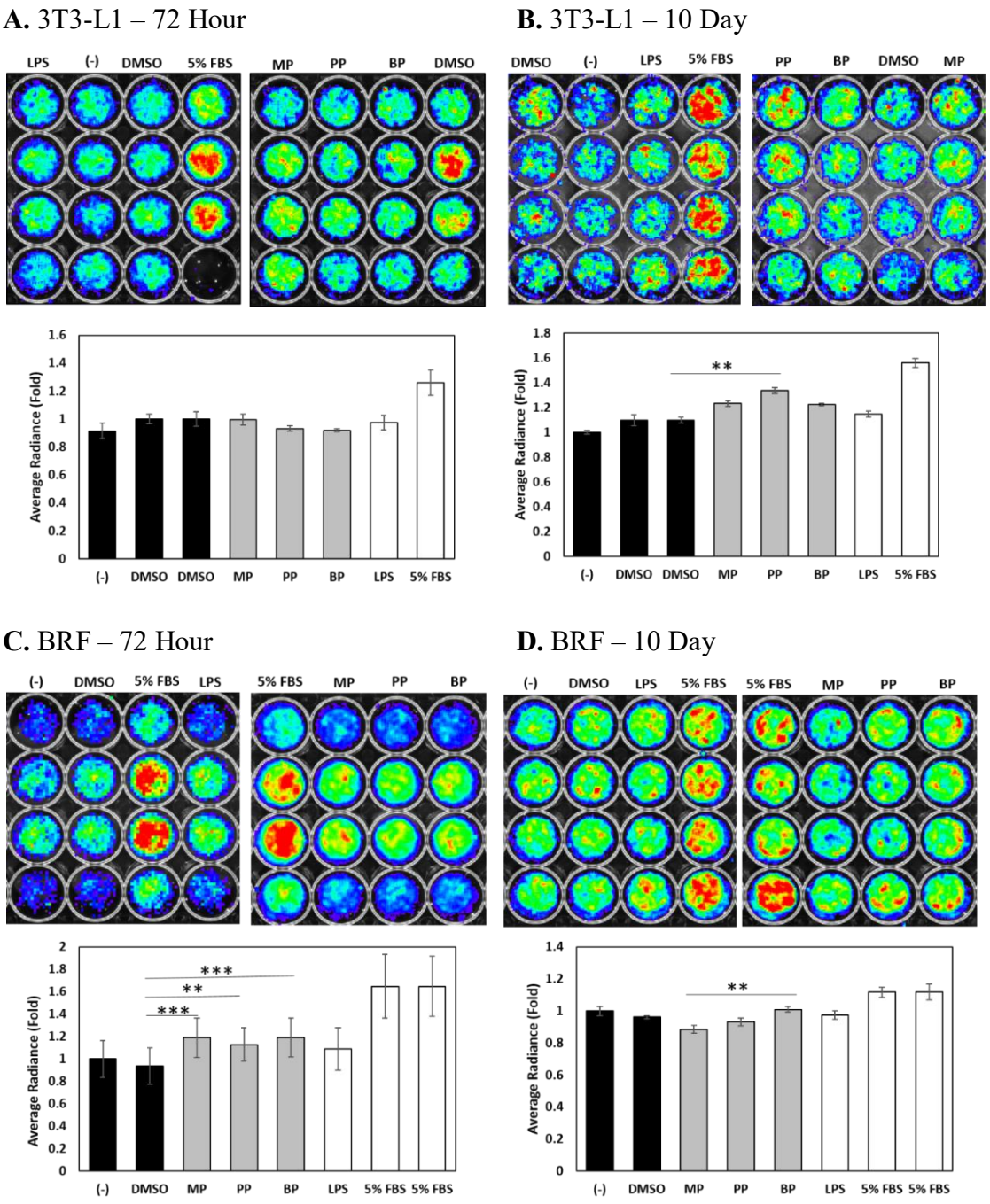


Figure 3.7. The conditioned media from paraben-treated fibroblasts promotes breast cancer cell proliferation.

Fibroblasts were serum starved and treated with the negative (-), the vehicle control (DMSO), MP (1μM), PP (1μM), BP (1μM), or LPS (5 ng/mL) for 72 hr (**A.** 3T3-L1 and **C.** BRF) or 10 days (**B.** 3T3-L1 and **D.** BRF). CM was collected and incubated with autobioluminescent MCF7 cell at a 1:1 ratio with fresh media. Average luminescence was measured after 7 days and normalized to the vehicle control and expressed as fold. Data are means ± SE (n=6). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 3.1. Primer sequences for qRT-PCR using SYBR green

Gene	Type	Sequence
36b4	Murine	FOR – GCTTCGTGTTCACCAAGGAGGA REV – GTCCTAGACCAGTGTTCTGAGC
36B4 (RPLP0)	Human	FOR – TGGTCATCCAGCAGGTGTTCGA REV - ACAGACACTGGCAACATTGCGG
Mmp9	Murine	FOR – GCTGACTACGATAAGGACGGCA REV – TAGTGGTGCAGGCAGAGTAGGA
MMP9	Human	FOR – GCCACTACTGTGCCTTTGAGTC REV – CCCTCAGAGAATCGCCAGTACT
Il1β	Murine	FOR – TGGACCTTCCAGGATGAGGACA REV – GTTCATCTCGGAGCCTGTAGTG
IL1β	Human	FOR – CCACAGACCTTCCAGGAGAATG REV – GTGCAGTTCAGTGATCGTACAGG
Cox2	Murine	FOR – GCGACATACTCAAGCAGGAGCA REV – AGTGGTAACCGCTCAGGTGTTG
COX2	Human	FOR – CGGTGAAACTCTGGCTAGACAG REV – GCAAACCGTAGATGCTCAGGGA
Cyp19a1	Murine	FOR – AAGCTCTGACGGGCCCTGGT REV – ACGTAGCCCGAGGTGTCGGT
CYP19A1	Human	FOR – CACATCCTCAATACCAGGTCC REV – CAGAGATCCAGACTCGCATG

CHAPTER 4 :
PARABENS INDUCE INFLAMMATION IN STROMAL ADIPOCYTES LEADING TO BREAST CANCER CELL PROLIFERATION

4.1. Abstract

Background: Breast stromal inflammation is being identified as a key contributor to breast carcinogenesis and breast cancer progression. Understanding the role and contribution of each stromal cell type in breast carcinogenesis can lead to novel and effective therapeutic or preventative strategies. Parabens are classified as a group of 4-hydroxybenzoic acid esters, found ubiquitously in pharmaceuticals, food, personal care products, and industry working as anti-microbial agents. Studies have shown that parabens are estrogenic endocrine disruptive chemicals in experimental animals and can accumulate in a higher amount in the breast cancer samples. Therefore, we hypothesize that parabens may promote breast carcinogenesis by negatively modulating breast stromal adipocytes nearby breast epithelial cells.

Methods: 3T3-L1 murine and human BRF adipocytes differentiated from respective precursor cells were treated with the three most common parabens, methyl-, propyl-, and butylparaben (MP, PP, and BP) at environmentally relevant doses (0.02, 0.1, and 1 μM) for 6, 12, and 24 h. Gene expression was evaluated by RT-PCR. The effects of parabens on NF-κB activation were studied by NF-κB reporter gene assays in differentiated 3T3-L1 CARΔ1 cells, which are engineered to express adenovirus receptor to enable adenoviral DNA delivery, and by western analysis of p-p65 and IκBα degradation, two critical events for NF-κB signaling pathways. Lastly, paraben-treated adipocyte conditioned media was studied for their abilities to promote the proliferation of autobioluminescent MCF7 breast cancer cells.

Results: A single exposure of BP increases *Il1β, Cox2,* and *Mmp-9* at 6, 12 and 24 h in murine adipocytes. A single exposure of MP and BP upregulates *MMP-9* in human adipocytes. Exposure of MP, PP, and BP downregulates *IL1β* and *COX2* in human adipocytes. The NF-κB pathway is activated by parabens shown by the induction of the NF-κB reporter gene and by increased phosphorylation of p65 and/or degradation of IκBα in murine adipocytes. In contrast, parabens only induce a modest response in human BRF adipocytes. Consistently, the conditioned media from paraben-treated murine adipocytes,

but not from human BRF adipocytes, promotes the proliferation of breast cancer MCF7 cells.

Conclusion: Parabens may play important roles in breast cancer progression by inducing inflammation and aromatase expression in the breast stromal adipocytes.

4.2. Introduction

Breast cancer continues to be the second most common cause of cancer-related death among women of various races, despite the improving survival rate due to advances in screening and treatment (ACS 2018). Hereditability accounts for less than 10% of breast cancer incidences, where the remaining 90% are attributed to modifiable risk factors (Darbre 2006, Darbre and Harvey 2014). A few modifiable environmental risk factors are obesity, xenoestrogen exposure, alcohol consumption, hormone therapy, and oral contraceptives (ACS 2018). The tumor microenvironment or stroma is the collection of various cell types, adipocytes, fibroblasts, macrophage, neutrophils, and endothelial cells, surrounding tumorigenic epithelial cells that provide a niche conducive to tumor growth. The stroma has been found to play a critical role in angiogenesis, proliferation, invasion, and metastasis and is greatly affected by these modifiable risk factors (Hanahan 2011).

Obesity, defined by the body mass index (BMI) ≥ 30 kg/m^2 and characterized by excess of adipose tissue accumulation, is a critical risk factor linked to breast cancer development, recurrence, and decreased survivability (Chan, Vieira et al. 2014, Gathirua-Mwangi, Zollinger et al. 2015, Playdon, Bracken et al. 2015). Adipose tissue functions as an endocrine organ secreting soluble factors such as cytokines, adipokines, hormones, etc., and an obese individual can experience hypertrophy of adipocytes where the volume can significantly multiply (Divella, De Luca et al. 2016). As adipocytes reach the limit of fat containment, the integrity of the cell membrane becomes comprised and can induce hypoxia and necrosis ultimately leading to inflammation (Divella, De Luca et al. 2016). Adipocytes can also rupture from excess lipid accumulation, attracting macrophage to remove debris further exacerbating inflammation via NF-κB activation (Divella, De Luca

et al. 2016, Iyengar, Gucalp et al. 2016). Inflammation within the obese stroma signals the release of cytokines, growth factors, and other proteins, thereby promoting changes conducive to cancer proliferation, such as mitogenesis, angiogenesis, and ECM remodeling (Radisky and Radisky 2007, Iyengar, Gucalp et al. 2016).

As the predominant cell type in breast tissue is the adipocyte, increased adiposity in the stroma leads to enhanced estrogen production surrounding breast epithelial cells and raised serum estradiol in obese individuals (Howe, Subbaramaiah et al. 2013, Hoy, Balaban et al. 2017). Moreover, inflammatory cytokines, PGE2, a COX2 product, and adipokines can influence the production of estrogen biosynthesis through the induction of aromatase (Brown 2014, Iyengar, Gucalp et al. 2016). Aromatase is a cytochrome P450 enzyme encoded by the *CYP19A1* gene that functions as the rate-limiting step in the conversion of androgens to estrogen (Gerard and Brown 2018). Aromatase overexpression and activity has been identified in the breast tissue of obese individuals and have a positive association with the presence of crown-like structures (CLS), which develop when adipocytes become necrotic (Howe, Subbaramaiah et al. 2013, Brown 2014, Gerard and Brown 2018). Taken together, this suggests a critical role of stromal inflammation contributes to the obesity-inflammation-estrogen axis (Gerard and Brown 2018).

It is recognized that environmental exposures particularly EDCs with estrogenic activities pose as risk factors for breast cancer development (Cui, Balshaw et al. 2016). EDCs are industrial chemicals utilized to enhance product sustainability and extend shelf-life but have been identified to dysregulate endocrine systems, causing adverse health outcomes (Cui, Balshaw et al. 2016). Parabens are alkyl esters of *p*-hydroxybenzoic acid incorporated into personal care products, pharmaceuticals, food packaging, and paper products for their anti-microbial and anti-fungal properties. The most common parabens used in the commercial products and therefore often being detected in human biological samples are methyl-, propyl-, and butylparaben (MP, PP, and BP) (Giulivo, Lopez de Alda et al. 2016).

Parabens have been classified as weak EDCs, however, previous studies have identified at low-dose exposures their ability to bind hormone receptors, enhance estrogenicity, and promote progression breast cancer (Darbre and Harvey 2014). Moreover, total parabens have been detected within normal breast tissue at an average concentration of 85 ng/g of tissue with MP and PP in the highest concentrations at 16.6 and 16.8 ng/g tissue respectively, indicating the presence of parabens around breast epithelial cells (Barr, Metaxas et al. 2012). Furthermore, parabens have been detected in higher amounts in breast cancer samples than their normal breast tissue controls, ranging from 17.3 ng/g tissue to 115 ng/g tissue (Darbre, Aljarrah et al. 2004). Furthermore, parabens have been shown to be capable to promote breast epithelial cell growth, however, it is not clear whether parabens also negatively affect breast stroma (Darbre and Harvey 2008).

The objective of this study was to determine the effects of common parabens (MP, PP, and BP) at environmentally achievable doses (0.02, 0.1, and 1 μM) on mRNA expression of inflammatory genes and *Cyp19A1* in the stromal adipocytes from murine and human origins and the ability of exposed adipocytes to enhance breast cancer cell proliferation. We demonstrate that parabens induce the upregulation of inflammatory genes (*Il1β* and *Cox2*) and ECM remodeling gene *Mmp9* and induce activation of the NF-κB pathway. We also show paraben-exposed adipocytes conditioned media induces substantial MCF7 proliferation.

4.3. Materials and Methods

4.3.1 Reagents

Methylisobutylxanthine (MIX), dexamethasone (DEX) and insulin (Ins) were purchased from Sigma Aldrich (St Louis, MO). Dimethyl sulfoxide (DMSO); methyl-, propyl-, and butylparaben were from Acros Organics (>99%, Thermo Fisher Scientific, Pittsburg, PA). Ultra-pure lipopolysaccharide (LPS) was from List Biological Laboratories (Campbell, CA). Phospho-NF-κB p65 (Ser536) (no. 3033) and NF-κB -p65 (D14E12) XP (no. 8242) were purchased from Cell Signaling Technology. IκBα (C-21): sc-371 was purchased from

Santa Cruz Biotechnology. Anti-β-actin (AC-15) (no. A1978) was from Sigma-Aldrich. Neomycin (G-418) was purchased from Research Products International (Mount Prospect, IL)

4.3.2. Cell Culture

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagles medium (DMEM) containing 10% calf serum (HyClone). Murine 3T3-L1 expressing coxsackievirus and adenovirus receptor (CARΔ1) cells were donated by Dr. Orlicky (Orlicky DJ 2001) and were grown in DMEM containing 10% fetal bovine serum (FBS) (Atlanta Biological, Flowery Branch, GA). Human subcutaneous breast fat preadipocytes (BRF) (Zenbio, NC) were grown in Preadipocyte Medium (PM-1) according to the manufacturer's instructions (Zenbio, NC). Human Autobioluminescent MCF7 cell line was a gift from Dr. Ripp (490 BioTech, Inc., Knoxville, TN) (Xu, Ripp et al. 2014) and was grown in DMEM containing 10% FBS with G-418. All were grown in 5% CO_2, 37^{o}C conditions.

4.3.3. Immortalization of Primary BRF Cells

Immortalization of primary BRF cells was completed by infecting the BRF cells with lentivirus encoding the SV40LT antigen. SV40LT antigen was cloned into a lentiviral vector carrying the neomycin phosphotransferase gene (Alwin Prem Anand, Gowri Sankar et al. 2012). Lentivirus particles were generated by co-transfecting SV40LT vector with packaging (psPAX2) and envelope (pMD2.G) vectors (Addgene; Boston, MA) in HEK293Ta cells (GeneCopoeia, Inc; Rockville, MD) using FuGENE® HD transfection reagent (Promega Corporation; Madison, WI) following manufacturer's guidelines. Once developed, the lentivirus particles were used to infect the BRF cells. Stable cell lines were selected using G418 antibiotic (300 µg/ml) for two weeks.

4.3.4. Adipocyte Differentiation

3T3-L1 fibroblasts were grown to confluency, and on the day of confluency cells were treated with DMEM containing 10% FBS, 0.5mM MIX, 10µg/mL Ins, and 1µM of DEX for 3 days (day 0-3). The cells were then grown in DMEM containing 10% FBS and 10µg/mL Ins for 2 days (day 4-5). Lastly, the cells are maintained in their differentiated state with DMEM containing 10% FBS (day 6-7). BRF fibroblasts were grown to confluency, on the day of confluency cells were treated with Adipocyte Differentiation Media (DM-2) (Zenbio, NC) for 7 days. After 7 days, DM-2 was removed and Adipocyte Maintenance Media (AM-1) (Zenbio, NC) was added for an additional 7 days.

4.3.5. Oil red O Staining and Quantification

Lipid accumulation after differentiation of adipocytes was stained with oil red O (ORO) as previously described (Hu, Chen et al. 2013).

4.3.6. Paraben Treatment

To detect the inflammatory effects of low-dose paraben on 3T3-L1 or BRF fibroblasts, a single exposure of 0.02, 0.10, or 1.0 µM methyl-, propyl-, or butylparaben was administered in low-serum conditions, 0.25% calf serum (HyClone) for 3T3-L1 cells and 1% charcoal-stripped fetal bovine serum (CS-FBS) (HyClone) for BRF cells. DMSO and LPS were used as vehicle control and positive control, respectively.

4.3.7. RNA and Quantitative Real-Time PCR

Total RNA was prepared from adipocytes using TRIzol (Invitrogen Coorporation, Carlsbad, CA) following the manufacturer's protocol. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was utilized to measure total RNA. Reverse transcription was completed with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) following the manufacturer's protocol. mRNA expression of adipocyte inflammation markers and housekeeping gene 36B4 (Table S1) were analyzed quantitatively using PowerUp SYBR Green Master Mix (Thermo Fisher

Scientific) in a 96-well PCR plates with an ABI 7900HT Real-Time PCR System with cycle conditions of 50°C 2 min, 95 °C 2 min, and 40 cycles of 95°C for 15s/60°C for 1 min. The relative gene expression was calculated utilizing $2^{(-\Delta\Delta Ct)}$ method and normalized with 36B4.

4.3.8. Reporter Gene Assays

3T3-L1 CARΔ1 were seeded in a 24-well plate format and infected with adenovirus encoding NF-κB-Luc reporter gene and adenovirus encoding β-galactosidase (β-gal) control plasmid for 15 h, as described previously (Purohit, Hu et al. 2013). After infection, the cells were differentiated following the 3T3-L1 protocol. The cells were probed with various parabens for 6 and 12 h. The cell lysate was prepared utilizing Reporter Lysis 5X Buffer (Promega). Reporter luciferase activity was analyzed using GloMax Luminometer (Promega, Madison, WI) and normalized by β-gal activities.

4.3.9. Western Blot Analysis

Total cell lysates of adipocytes were prepared, centrifuged, and protein concentrations measured by Pierce BCA protein assay kit (Thermo Fisher Scientific). 20-40 μg of total cell lysate were run through a polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk or 5% bovine serum albumin (BSA). The membrane was immunoblotted with primary antibodies at 4 °C overnight followed by secondary antibody conjugated with horseradish peroxidase. The signal was detected utilizing SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), HyBlot CL autoradiography film (Denville Scientific), and a Konica SRX-101A film processor. Quantification by densitometry was performed by the ImageJ software.

4.3.10. Conditioned Media Collection

3T3-L1 and BRF fibroblasts were seeded into 60 mm dishes and probed to differentiate following their respective protocols. Each plate of cells was treated with MP, PP, BP (each at 1 μM) or controls (DMSO or LPS) in 5% CS-FBS. After 72 h or 10 days, the treatment was removed, and the cells were washed twice with phenol-red free (PRF) media (HyClone). To collect the conditioned media (CM), cells were cultured for 48 h in PRF media with 5% CS-FBS, penicillin, and streptomycin. After 48 h, the media was collected and centrifuged at 600 rpm for 5 minutes. The supernatant was transferred into a fresh tube and stored at 4ºC before use.

4.3.11. Bioluminescent imaging

Autobioluminescent MCF7 cells were plated at 30% confluency in duplicate wells of 24-well opaque, clear bottom plates (Labnet/Corning Inc., Cary, NC). After attachment overnight, MCF7 cells were washed with PRF media and treated with CM: 5% CS-FBS PRF media in a 1:1 ratio. Treatment persisted for 7 days with changing CM at day 4. On day 7, plates were imaged utilizing a CCD-based IVIS Lumina imaging system (PerkinElmer). Autobioluminescent readings were acquired 3 times at 10 min intervals according to the manufacturer's instructions.

4.3.12. Statistical Analysis

All data are presented as means $\pm$ SE. Measurements were performed in triplicates unless otherwise indicated. Statistical analysis was performed using SigmaPlot 14.0 (Systat Software). One-way ANOVA on ranks or one-way ANOVA with repeated measures followed by multiple comparison tests (Student-Newman-Keuls method) were used to determine the differences among treatment groups or time points. Student's *t*-tests were conducted to evaluate LPS compared to the control. The level of significance was set at $P < 0.05$. The effects of treatment and plate location on fold change of average radiance were analyzed using two-way ANOVA analysis for each time point in 3T3-L1 and human BRF adipocytes.Post hoc multiple comparisons were performed with Tukey's adjustment.

Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M4 for Windows 64x (SAS institute Inc., Cary, NC).

4.4. Results

4.4.1. Parabens Induce mRNA Expression of inflammatory genes and CYP19a1 in Murine 3T3-L1 Adipocytes and Human BRF Adipocytes

3T3-L1 fibroblasts were first differentiated following the 7-day differentiation protocol (Figure 4.1.A.) and then exposed to MP, PP, or BP or their vehicle control at 0.02, 0.1, or 1 µM for 6, 12, or 24 h. LPS was included as a positive control. Typically, there is ~100% and ~50% differentiation of 3T3-L1 and BRF cells, respectively (Figure 4.1.A-B). The inflammatory response to parabens differs depending on the length of the side chain, dose, and time of exposure. Propylparaben and BP significantly upregulated inflammatory *Il1β* mRNA expression in 3T3-L1 adipocytes. Compared to the vehicle control (DMSO), PP increased maximally ~ 7 fold of *Il1β* mRNA expression at 1 µM at 12 h ($p<0.001$), but was not sustained at 24 h ($p<0.001$) of exposure. BP at all tested doses significantly increased *Il1β* mRNA expression at 6, 12, and 24 h of exposure. At 1 µM BP increased the maximal ~ 17 fold of *Il1β* mRNA expression at 6 h ($p<0.01$) and ~ 3 fold ($p<0.05$) and ~ 4.5 fold ($p<0.001$) at 12 and 24 h, respectively (Figure 4.2.A). In contrast, MP did not influence *Il1β* mRNA expression at 6 or 12 h but downregulated it at 24 h (Figure 4.2.A). A maximal 2 fold *Il1β* mRNA level was induced by LPS at 24h of exposure (Figure 4.2.A).

In addition to *Il1β*, we also examined *Mmp9* mRNA expression, a key MMP in matrix remodeling in breast cancer growth. Even though LPS significantly upregulated ~ 3.5 fold of *Mmp9* mRNA expression at 6 h ($p<0.001$), reaching ~ 8 fold at 12 h ($p<0.001$), and ~ 3.5 fold at 24 h exposure, there was not much effects by parabens except that PP increased 3 fold at 12 h at 1 µM ($p<0.001$) and BP increased 2.5 fold at 6 h at 1 µM ($p<0.001$) and 2.5 fold at 12 h at 0.02 µM ($p<0.01$) compared to DMSO (Figure 4.2.B). We examined the effects of parabens on *Cox2* mRNA expression in adipocytes as COX2 upregulation is greatly associated with progression of many cancers, including breast cancer (Denkert, Winzer et al. 2004, Sano, Kogashiwa et al. 2018). Propylparaben and BP significantly

upregulated *Cox2* mRNA expression at 6, 12, and 24 h ($p<0.05$). Propylparaben induced ~ 2.5 fold of *Cox2* mRNA expression at 6 h at 1 µM, which has remained at 12 h but subsided at 24 h. All tested doses of BP induced significant Cox2 mRNA at 6 h and 24 h, with a maximal 4.5 fold at 1 µM ($p<0.001$) compared to the control. In contrast, LPS only increased 2 fold of *Cox2* mRNA expression at 6 h ($p<0.05$) (Figure 4.2.C).

Lastly, we analyzed mRNA expression of *Cyp19a1*, the enzyme responsible for the conversion of androstenedione and estrone to estrogen (Gerard and Brown 2018). Parabens tested, PP and BP in particular, significantly upregulated *Cyp19a1* mRNA expression. PP at all tested concentrations significantly upregulated *Cyp19a1* mRNA expression at both 6 and 12 h ($p<0.001$). PP at 1µM induced ~ 4 fold of *Cyp19a1* mRNA expression at 6 h, which was increased to ~ 6 fold at 12 h and subsided to the basal level at 24 h. BP at all tested concentrations significantly upregulated *Cyp19a1* mRNA expression at 6, 12, and 24 h. Butylparaben at 1 µM induced a maximal 10 fold of *Cyp1a* mRNA expression at 6 h ($p<0.001$), which was decreased to 3.5 fold at 12 and 24 h ($p<0.05$) compared to the vehicle control. In contrast, LPS only increased ~ 2 fold of *Cyp19a1* mRNA expression at 24 h exposure ($p<0.001$) (Figure 4.2.D).

Similar to those of murine adipocytes, all three parabens also induced differential responses in the human BRF adipocytes, depending on the length of the side chain, dose, and time of exposure (Figure 4.3.). Butylparaben induced maximal ~ 3 fold of *IL1β* mRNA expression at 12 h at 0.1 µM but downregulated it at 6 and 24 h at all tested in human concentrations in the BRF adipocytes (Figure 4.3.A). Methylparaben and PP both downregulated *IL1β* mRNA expression at 6 h while MP and PP had minimal effect at 12 and 24 h (Figure 4.3.A). In contrast, LPS induced a maximal 2.5 fold of *IL1β* mRNA expression at 12 h and had minimal effects at 6 and 24 h (Figure 4.3.A).

In contrast, all three parabens induced more robust responses of MMP9 in human BRF cells. Compared to the controls, BP induced ~ 2 fold of *MMP9* mRNA expression at 6 h and ~ 3.5 fold at 24 h at 1 µM. MP induced ~ 4 fold of *MMP9* mRNA at 0.1 µM ($p<0.01$)

and ~ 4.5 fold at 1 μM ($p<0.05$) only at 24 h ($p<0.05$) (Figure 4.3.B). PP had no significant induction of *MMP9* mRNA expression. LPS induced ~ 2.5 fold of *MMP9* mRNA expression at 6 h in human BRF cells (Figure 4.3.B). All three parabens downregulate *COX2* mRNA expression at 6 and 12 h with no significant changes at 24 h (Figure 4.3.C).

Lastly, *CYP19A1* mRNA expression was significantly downregulated by all three tested parabens at 6 h with no significant response at 12 or 24 h except BP at 0.02 μM at 12 h of exposure, which increased *CYP1A1* to ~ 1.3 fold compared to the vehicle control ($p<0.05$). Propylparaben also significantly upregulates *CYP19A1* mRNA expression by 1.5 fold at 0.02 μM at 24 h ($p<0.01$). Similarly, LPS does not induced an increase of *CYP19A1* mRNA expression at 6, 12, or 24 h in human BRF adipocytes (Figure 4.3.D).

4.4.2. Parabens Regulate NF-κB Signaling in Murine and Human Derived Adipocytes

Next, we investigated the effects of paraben on the NF-κB signaling pathway, which is critical signaling that drives inflammatory gene expression. First, we investigated NF-κB activation using NF-κB-responsive luciferase reporter assays. We validated the infection efficiency of adenovirus-mediated infection in differentiated 3T3-L1 CARΔ1 adipocytes using adenoviral GFP (Figure 4.4.A). Then 3T3-L1 CARΔ1 fibroblasts were infected with adenovirus encoding an NF-κB-Luc receptor and then differentiated. At 6 h, MP activated NF-κB in 3T3-L1 CARΔ1 adipocytes only at 0.02 μM ($p<0.05$). LPS induced ~ 3 fold of activation at 6 h, which was attenuated by an NF-κB inhibitor CAPE (Figure 4.4.B). Similar to the gene expression results, PP induced a modest but significant NF-κB activation in 3T3-L1 adipocytes only at 1 μM and at 12 h ($p<0.05$). (Figure 4.3.C). Butylparaben induced ~ 1.5 fold of activation only at 0.02 μM and at 12 h ($p<0.05$). In contrast, LPS induced 4 fold of NF-κB activation in 3T3-L1 adipocytes at 12 h.

We further confirmed paraben-induced NF-κB activation in 3T3-L1 cells by analyzing the phosphorylation of p65 and degradation of IκBα, two key events leading to NF-κB activation (Figure 4.5.A). Quantification of densitometry showed that compared to the respective vehicle control (DMSO), BP at 1 μM induced the degradation of IκBα beginning

at 30 min ($p<0.001$) (Figure 4.5.D) and phosphorylation of p65 also at 30 min ($p<0.05$) (Figure 4.5.E). LPS also showed significant degradation of IκBα and phosphorylation of p65 beginning at 5 min ($p<0.001$). When MP NF-κB activation was normalized by DMSO, IκBα degradation had a minimal response, however p65 phosphorylation was significantly enhanced demonstrating the bimodal activation at 5 and 30 minutes (Figure 4.5.F). NF-κB activation by BRF cells was not tested as gene expression analysis showed a decrease in inflammatory mRNA markers.

4.4.3. Conditioned Media from Parabens-Treated Adipocytes Regulates Breast Cancer Cell Proliferation

To explore the effects of paraben exposure on stromal induction and cancer progression, we evaluated the effects of the CM collected from paraben-treated adipocytes on the proliferation of human breast cancer cells MCF7. We evaluated CM collected after short-term (72 h) and long-term (10 days) exposure of parabens on both 3T3-L1 and BRF adipocytes. The CM was then used to induce the proliferation of autobioluminescent MCF7 cells. At 72 h, PP induced greater proliferation of MCF7 cells compared to those of other parabens, but it did not reach statistical significance. Moreover, paraben stimulated proliferation is not significantly greater than the vehicle control (DMSO) (Figure 4.6.A). In contrast, the CM collected from BP, but not MP or PP-treated 3T3-L1 adipocytes for 10 days induced significant proliferation of autobioluminescent MCF7 cells compared to the vehicle control ($p<0.05$) (Figure 4.6.B). Interestingly, the CM from both MP and BP exposed human BRF adipocytes appeared to suppress the proliferation of MCF7 cells at 72 h ($p<0.001$), whereas the CM from PP exposed BRF adipocytes had no effects compared to the vehicle control at 72 h (Figure 4.6.C). Lastly, MCF7 cell proliferation is diminished upon treatment with the CM from MP, PP, and BP exposed adipocytes for 10 days ($p<0.001$), confirming downregulated gene expression results (Figure 4.6.D).

4.5. Discussion and Conclusion

To our knowledge, our study is the first to demonstrate the effect of parabens at environmental achievable doses influence mRNA expression of inflammatory genes and aromatase, *CYP19A1*, in murine and human adipocytes differentiated from human breast stromal cells. Using conditioned media, we determined the effect of secretion from low-dose parabens exposed adipocytes on breast cancer cell growth. Our results demonstrate that typical exposure to parabens within the environment induce a pro-inflammatory response and upregulate aromatase *Cyp19a1* in murine adipocytes. In contrast, parabens were found to be protective against inflammation by downregulation of pro-inflammatory genes and *CYP19A1* in human BRF adipocytes. Consistently, the conditioned media from paraben-exposed murine adipocytes, but not from human BRF adipocytes, promotes breast cancer cell proliferation.

Parabens are preservatives utilized in industry including personal care products, pharmaceuticals, food, etc. causing a daily ubiquitous presence in the environment (Darbre and Harvey 2008, Darbre and Harvey 2014). These chemicals found in creams and lotions are absorbed dermally while absorption through the gastrointestinal (GI) tract is another avenue, particularly for food and pharmaceutical products. Esterases in the skin and in the GI tract hydrolyze parabens quickly, however intact parabens are detected in the blood, breast milk, placenta, and urine (Sandanger, Huber et al. 2011, Zhao, Liu et al. 2014, Kolatorova 2017, Shen, Liang et al. 2017). Within 5 h unconjugated parabens are present at low levels in human serum, at 12 h hydrolyzed metabolites are no longer present in serum, and by 24 h conjugated parabens are the all that remain (Shin, Shin et al. 2019). All paraben and metabolites are excreted by 72 h after a single exposure (Shin, Shin et al. 2019).

Parabens influence a variety of endocrine-related diseases including obesity as obesogens and reproductive diseases in both men and women (Hu, Chen et al. 2013, Kirchhof and de Gannes 2013, Hu, Kennedy et al. 2016, Guerra, Sanabria et al. 2017). Some of these diseases are related to the estrogenic nature of parabens, however, they do influence other

endocrine pathways. Parabens have been identified to promote breast cancer cell proliferation, invasion, and metastasis possible through modulation of estrogen receptor, human epidermal growth factor receptor, androgen receptor, G protein-coupled estrogen receptor 1. (Darbre and Harvey 2014, Lillo, Nichols et al. 2017).

A breast cancer tumor requires the cooperation of the surrounding stromal cells to create a niche that provides the perfect environment for growth and proliferation (Hanahan 2011). The stroma is a complex network of fibroblasts, adipocytes, immune cells, endothelial cells, macrophages, etc. all communicating with each other and adjacent epithelial cells (Bussard, Mutkus et al. 2016). Stromal adipocytes are the most abundant in the obese breast and function as the estrogen source for postmenopausal women. Adipocytes are endocrine organs, capable of secreting adipokines, pro-inflammatory cytokines, chemokines, estrogen, and other factors, which then influence surround breast epithelial cells through cross-talk. Adipocytes can be trained to support and enable tumor growth and therapy resistance through local inflammation (Majidinia and Yousefi 2017). In addition to the ability to store energy in the form of lipids, adipocytes release glycerol and fatty acids into the stroma, which are necessary for the synthesis of epithelial cell membranes and metabolic components (Hoy, Balaban et al. 2017). Adipocytes found in a cancerous microenvironment have increased expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines, including interleukin (IL1β) and cyclooxygenase (COX2) (Bussard, Mutkus et al. 2016, Divella, De Luca et al. 2016, Iyengar, Gucalp et al. 2016). Our findings add to the established literature by demonstrating parabens' abilities to induce inflammation and ECM remodeling gene in murine adipocytes, creating a conducive environment promoting breast cancer cell growth.

In this study, we have aspired to address the effects of common, household parabens at environmentally achievable doses on stromal adipocytes and the subsequent effect of exposed adipocytes on breast cancer cell proliferation. Our results demonstrate that parabens at low concentrations upregulate mRNA for *Cox2, Il1β,* and *Cyp19a1* in murine adipocytes and upregulate *MMP9* in human adipocytes in type, dose, and exposure time-

dependent manner. In murine adipocytes, parabens elicited an inflammatory response with the increasing length of the alkyl side-chain (MP < PP < BP), where BP most often induced the most significant response over time.

The increased inflammatory mRNA expression in murine adipocytes is confirmed by the activation of NF-κB reporter and the signaling events of phosphorylation of p65 and IκBα degradation. Additionally, the timing of activation of NF-κB reporter mimics the gene expression in that MP induces a response in a shorter period of time compared to that of PP or BP. Although we demonstrate parallel effects of NF-κB activation with inflammatory gene *Il-1β* and *Cox-2* by parabens, whether inflammatory effects induced by parabens in adipocytes are mediated by NF-κB pathway need to be confirmed in the future. We did not identified that parabens directly activate NF-κB pathway, but they likely activate other signaling pathways that network with NF-κB leading to activation. More studies are necessary to elucidate the exact signaling mechanisms activated in stromal adipocytes upon paraben treatment, especially considering parabens ability to bind and activate multiple hormone and protein receptors.

In contrast, parabens induced a modest response of inflammatory genes and *CYP19A1* in human adipocytes, demonstrating a species-specific response to parabens in the stromal adipocytes. A single exposure of MP and BP only provokes a higher and sustainable *MMP9* mRNA expression in the human BRF adipocytes, suggesting that these human adipocytes may be more influential in ECM remodeling than inflammation. Interestingly, the downregulation of inflammatory gene expression upon paraben treatment suggests a protective effect in human adipocytes.

Furthermore, we aimed to determine the effect of paraben-stimulated adipocytes can stimulate the proliferation of breast cancer cells. Conditioned media was acquired after treating adipocytes with parabens and washing to remove any residual intact or hydrolyzed parabens. The CM was used in a 1:1 ratio with fresh media to support the growth of autobioluminescent MCF7 cells. The CM collected 72 h from the paraben-exposed murine

adipocytes did not stimulate the proliferation of MCF7 cells, but the CM collected 10 days from BP-exposed adipocytes was sufficient to enhance MCF cell proliferation. The finding of secretion from inflamed adipocytes inducing proliferation of breast cancer cells agrees with the understood effects of inflammation in the stroma on breast cancer (Howe, Subbaramaiah et al. 2013, Gerard and Brown 2018). Interestingly, the CM collected from paraben-exposed human adipocytes showed decreased proliferation of MCF7 cells, which is consistent with the suppression of *IL1β, COX2* and *CYP19A1* mRNA expression by parabens, suggesting that parabens could be protective in these human adipocytes.

Previous studies have indicated that body fatness is protective against breast cancer in premenopausal women and deleterious in postmenopausal women (Amadou, Ferrari et al. 2013, Nichols, Schoemaker et al. 2017). Therefore, a future study comparing the effects of CM collected from both pre- and postmenopausal stromal adipocytes with known BMI on breast cancer cell proliferation could elucidate these results. Additional studies to solidify results such as identifying the soluble factors within the CM from the adipocytes and the molecular mechanisms activated from paraben-exposed adipocytes are also necessary.

In conclusion, we have shown that parabens, common preservatives in household products, at environmentally achievable doses promote mRNA expression of pro-inflammatory genes and *Cyp19a1* in 3T3-L1 adipocytes. The inflammatory response of upregulated *Il1β* and *Cox2* expression induced by parabens are consistent with NF-κB activation. In contrast, parabens only induce a modest response in human BRF adipocytes. Further studies are needed to study the effects of long term with repeated exposure of parabens in adipocytes and to confirm the stromal response to parabens *in vivo* to further define the roles of parabens in breast cancer development.

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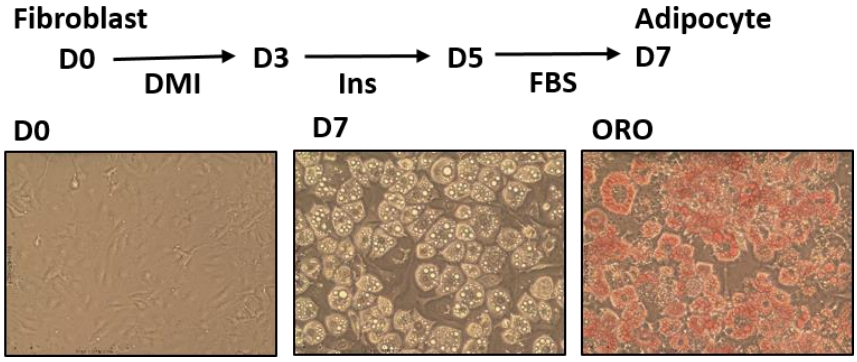
4.7. Appendix

A. 3T3-L1



B. BRF

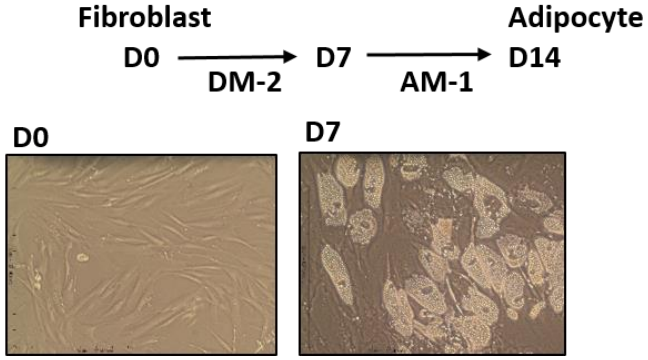


Figure 4.1. Adipocyte differentiation of 3T3-L1 and the human BRF cells.

The cell images demonstrating 3T3-L1 (A) and BRF fibroblasts (B) differentiation into adipocytes and lipid accumulation (stained by ORO lipophilic stain) following standard protocols.

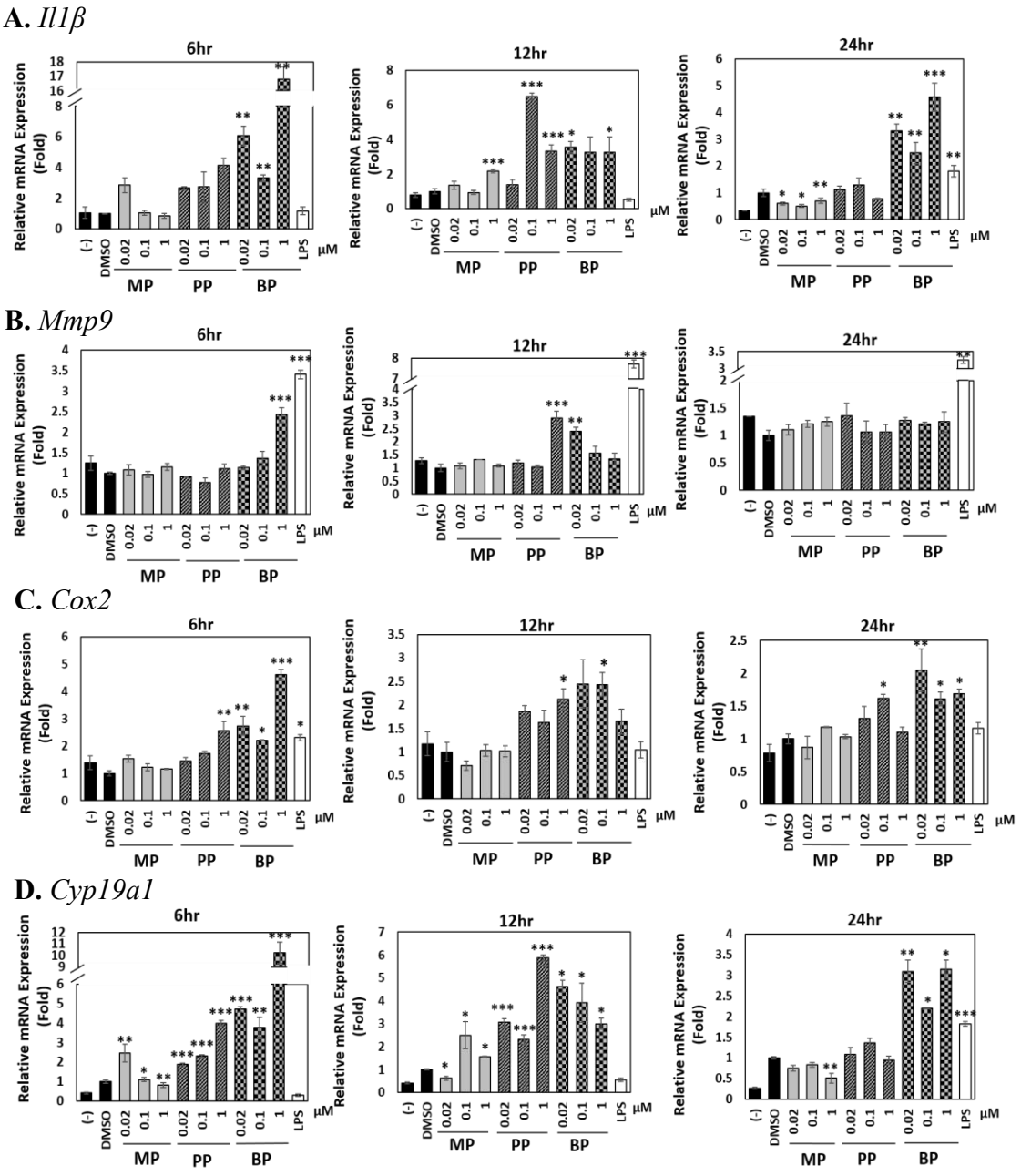


Figure 4.2. Parabens induce mRNA expression of inflammatory genes and *Cyp19a1* in 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated with selected parabens (0.02, 0.10, 1.0μM) for the indicated time period. DMSO was the vehicle control and LPS was the positive control. Relative mRNA expression of inflammatory markers (**A.** *Il1β*, **B.** *Mmp9*, **C.** *Cox2*, and **D.** *Cyp19a1*) was analyzed. The relative mRNA expression was normalized to 36B4 and expressed as fold of that of the DMSO sample. Data are mean ± SE (n = 3). One-way ANOVA on Ranks was performed followed by multiple comparison tests with Student Newman-Keuls method to compare with the control. * , $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$.

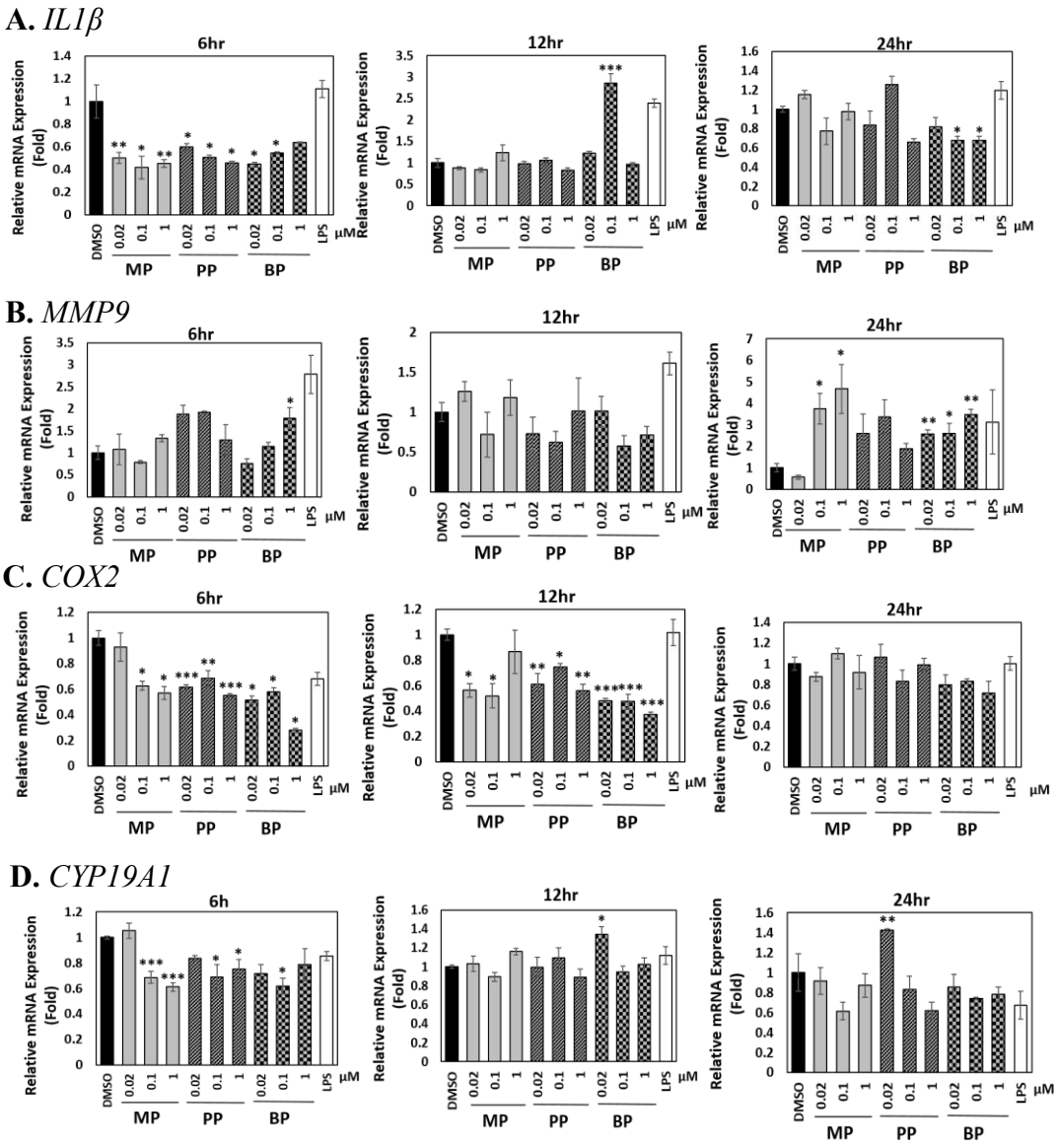


Figure 4.3. Parabens induce mRNA expression of inflammatory genes and *CYP19A1* in human BRF adipocytes.

BRF adipocytes were treated with selected parabens (0.02, 0.10, 1.0μM) for the indicated time period. DMSO was the vehicle control and LPS was the positive control. Relative mRNA expression of inflammatory markers (**A.** *IL1β*, **B.** *MMP9*, **C.** *COX2*, and **D.** *CYP19A1*) was analyzed. The relative mRNA expression was normalized to 36B4 and expressed as fold of that of the DMSO sample. Data are mean $\pm$ SE (n = 3). One-way ANOVA on Ranks was performed followed by multiple comparison tests with Student Newman-Keuls method to compare with the control. * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

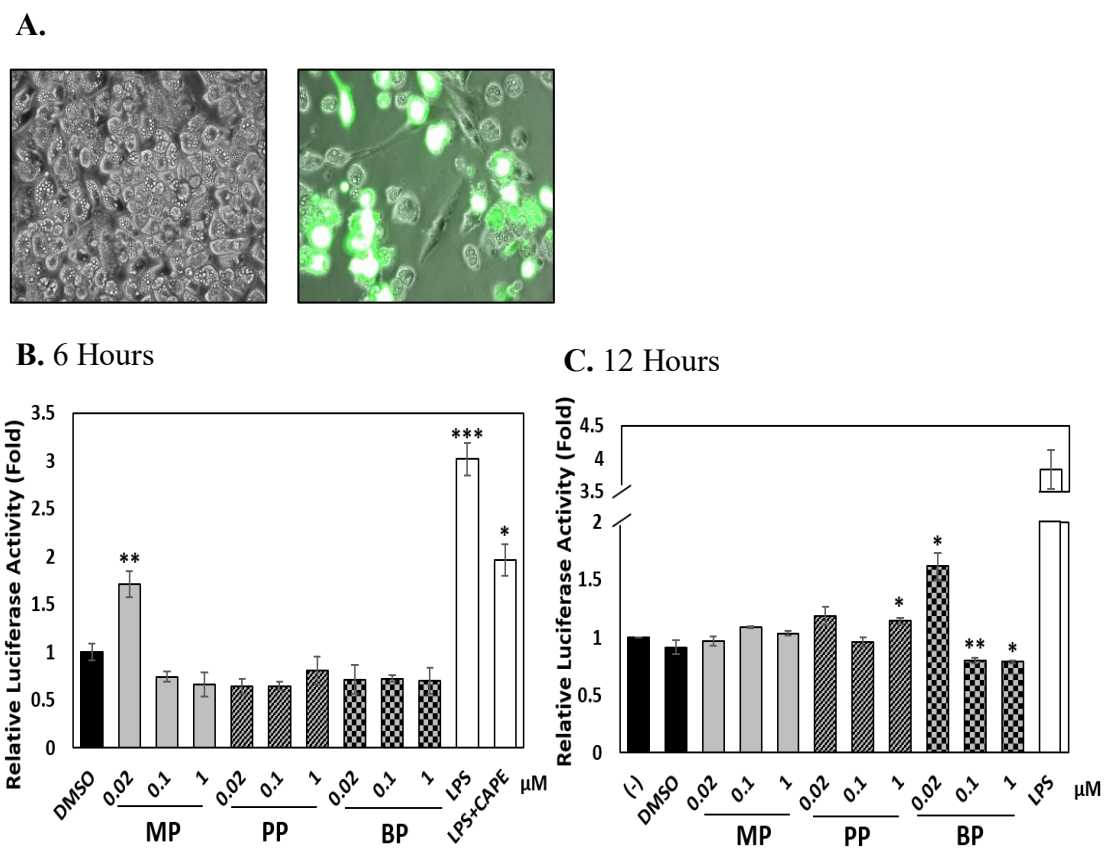


Figure 4.4. Parabens activate NFκB reporter in murine adipocytes.

3T3-L1 CARΔ1 fibroblasts were infected with adenovirus containing NFκB-Luc and β-galactosidase (β-gal) for 15 h and then differentiated following 3T3-L1 differentiation protocol. **A.** GFP luminescence verifying infection of adenovirus into 3T3-L1 CARΔ1 adipocytes. Adipocytes were stimulated with 0.02, 0.1, or 1 μM MP, PP, or BP along with DMSO and 5 ng/mL LPS as controls for **B.** 6 h and **C.** 12 h. Reporter gene assays were conducted and relative luciferase activity was normalized by β-gal for each sample and expressed as fold. Data are means ± SE (n=3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

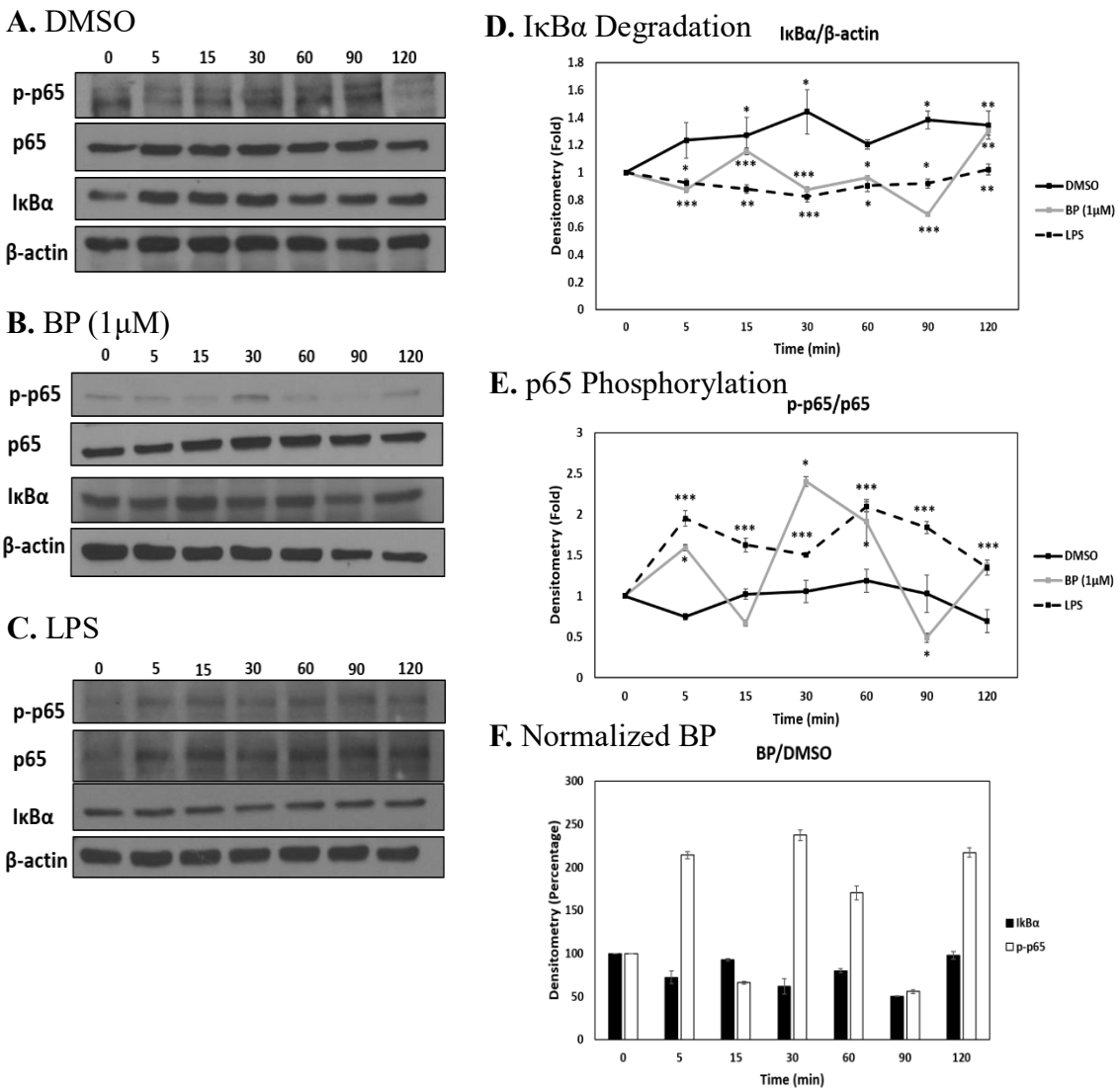


Figure 4.5. Paraben activates NFκB signaling in murine adipocytes.

Adipocytes were serum starved and treated with vehicle control **A.** DMSO, **B.** BP (1μM), or **C.** LPS (5 ng/mL) for indicated times, and whole cell lysate was prepared and analyzed by Western analysis with p-p65, p65, IκBα, and β-actin antibodies. Relative phosphorylation of p65 was normalized by p65 for each sample and expressed as fold. Relative expression of IκBα was normalized by β-actin for each sample and expressed as fold. **D.** IκBα degradation and **E.** phosphorylation of p65 comparison between each treatment with their own time 0. **F.** BP induced expression of IκBα and p-p65 normalized with DMSO. Data are means ± SE (n=3). * $P < 0.05$, ** $P < 0.01$, and

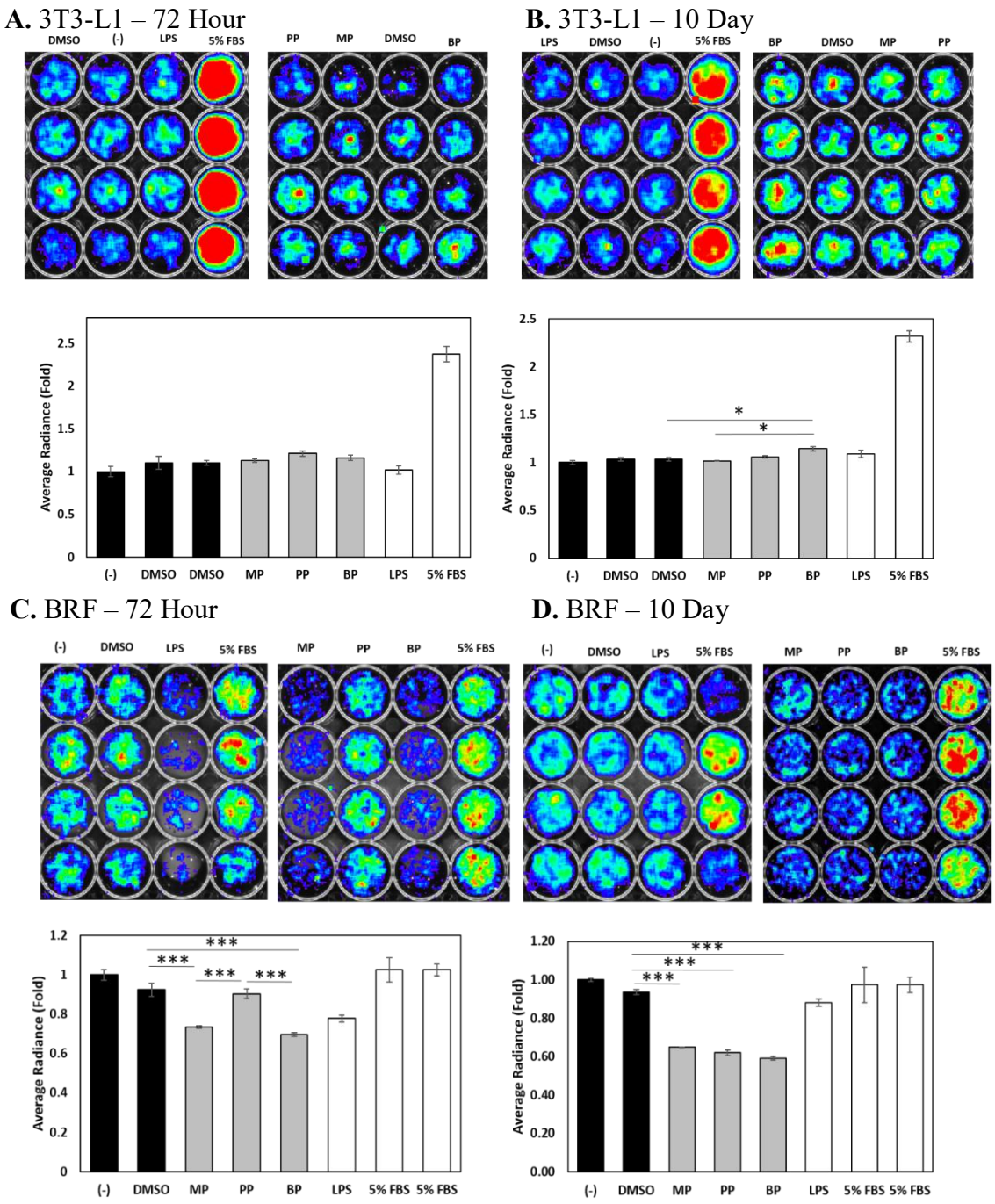


Figure 4.6. The conditioned media from paraben-treated adipocytes promotes breast cancer cell proliferation.

Adipocytes were serum starved and treated with the negative (-), vehicle control (DMSO), MP (1μM), PP (1μM), BP (1μM), or LPS (5 ng/mL) for 72 h (**A.** 3T3-L1 and **C.** BRF) and 10 days (**B.** 3T3-L1 and **D.** BRF). CM was collected and incubated with autobioluminescent MCF7 cell at a 1:1 ratio with fresh media. Average radiance was measured after 7 days and normalized to the vehicle control and expressed as fold. Data are means ± SE (n=6). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

CHAPTER 5 :
CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Conclusions

This dissertation contains two primary experiments detailed in Chapters 3 and 4. Chapter 3 details the inflammatory effect of MP, PP, and BP at environmentally achievable doses on fibroblasts. Low-dose (i.e., 0.2–1 μM) paraben exposure led to the upregulation of inflammatory mRNA expression including *Il1β* and *Cox2* in both murine 3T3-L1 and human BRF fibroblasts. *MMP9* mRNA expression was also upregulated by parabens in murine cells but more significantly in human fibroblasts. These results demonstrate the ability for a single exposure of parabens to fibroblasts to influences inflammatory and matrix remodeling gene expression patterns. Additionally, parabens increased the mRNA expression of *Cyp19A1* or aromatase implicating parabens' ability to increase estrogen production. Activation of the NF-κB signaling pathway was confirmed by reporter gene and western blot analysis, consistent with an inflammatory gene response. The wavering trends of IκBα degradation and p65 phosphorylation could be due to paraben's ability to activate a variety of different signaling pathways that ultimately result in influencing NF-κB. The CM of the paraben treated fibroblasts enhanced proliferation of MCF7 breast epithelial cells compared to their vehicle control. Therefore, parabens at low-doses are able to stimulate inflammation in stromal fibroblasts, which in turn are able to promote the proliferation of breast cancer cells.

Chapter 4 addresses the inflammatory effect of MP, PP, and BP on stromal adipocytes. Paraben exposures led to an upregulation of inflammatory *Il1β, Mmp9,* and *Cox2* in murine adipocytes, but surprisingly decreased *Il1β* and *Cox2* mRNA expression in the human adipocytes. Moreover, both 3T3-L1 and BRF adipocytes induced the upregulation of *Cyp19a1* or aromatase upon paraben treatment. Parabens activated the NF-κB pathway as indicated by the reporter gene and western blot analysis in 3T3-L1 adipocytes. Similar to the fibroblasts, more detailed analysis of the upstream activation of NF-κB could aid in understanding the variance in inflammatory gene expression and NF-κB patterns. Consistently, long-term (10 days) exposure of parabens to differentiated 3T3-L1 adipocytes led to CM that enhanced MCF7 cell proliferation. However, paraben exposed human adipocytes suppressed MCF7 cell proliferation compared to vehicle control

(DMSO) similar to gene expression, demonstrating a species-specific response of adipocytes to paraben exposure.

In summary, both murine and human stromal fibroblasts and adipocytes showed response when exposed to environmentally relevant doses of paraben. Murine fibroblasts and adipocytes activate inflammatory signaling pathways and upregulating cytokines that are closely associated with breast cancer progression. Murine fibroblasts and adipocytes have consistent upregulation of inflammatory mRNA expression as well as NF-κB activation and MCF7 growth promotion. Even though human fibroblasts follow suit with murine cells, human adipocytes appear to have an alternate effect leading to protection against inflammation.

5.2. Significance of the Study

Breast cancer is a complex disease that affects women globally. As improvements are being made with treatment causing a decline in death rates, women are still being diagnosed with invasive breast cancer at rates similar to previous decades. With the stroma play a critical role in breast cancer, understanding the contribution from each cell type is necessary to identify potential prevention or treatment strategies. In breast cancer microenvironment, fibroblasts and adipocytes are the two most common stromal cells.

The study of the negative effects of environmental chemical exposure on breast cancer via a stromal inflammation can give new insight into addressing breast cancer from a different perspective. As parabens, a commonly used anti-microbial found in personal care products, food, pharmaceuticals, and more, have the ability to induce inflammation in stromal cells, the local inflammation surround breast epithelial cells is enhanced. Inflammation functions as a promoter to cancer progression in that it stimulates the opening of DNA strands, increasing vulnerability to mutations. As human are exposed to fresh parabens multiple times a day, unconjugated parabens found in serum stimulate inflammation in stromal cells continuously leading to an inflamed microenvironment. If there are abnormal epithelial cells already present in the stroma (e.g., DCIS), this paraben-induced inflammation can

lead to the promotion of breast cancer. Therefore, this study indicates that parabens at daily human exposure levels can induce stromal inflammation, which is then to enhance breast cancer progression.

When addressing obesity and breast cancer, aromatase is often thought of to be the link bringing the two together. Aromatase functions in the conversion of estrogens to estradiol, and greater levels of estradiol contacting ER+ breast cancers can obviously stimulate proliferation. Parabens are shown to increase the expression of aromatase in fibroblasts and adipocytes. Assuming the increase in aromatase leads to increased estrogen secretion into the conditioned media, both fibroblasts and adipocytes can be influence the amount of estrogen present in the microenvironment ultimately leading to breast cancer progression in ER+ breast cancers.

Therefore, this study demonstrates how parabens have the potential to enhance breast cancer proliferation solely from parabens' effect on stromal cells.

5.3. Limitations and Future Directions

As an exploratory study to identify the effects of parabens on stromal cells, there are several limitations to this study. A single exposure to parabens was used in both fibroblasts and adipocytes, however, individuals are exposed to parabens multiple times per day for years. Therefore, longer term exposures could provide additional insights into parabens genomic effects such as altered epigenetics.

Moreover, the contents of the CM were not analyzed and doing so via mass spectrometry would provide more information on which cytokines from paraben stimulation were translated into protein and secreted triggering breast cancer cell proliferation. Additionally, identifying the signaling pathway within the breast cancer epithelial cells that were enhanced upon treatment with CM could also solidify the findings. Therefore, conducting a more mechanistic signaling study with the MCF7 breast cancer cells would be a logical next step.

The human stromal cells utilized in this model had a difficult time differentiating after being infected with the lentivirus to make them immortal. With additional funding, a better technique would be to analyze primary stromal cells without infection. Primary stromal cells differentiated into adipocytes would be a better translatable model. Acquiring breast stromal cells from both premenopausal and postmenopausal women of both normal and obese BMI would enhance the study as well. Comparing the effects of BMI and menopause could show differences in the imposition of parabens and the effect of stromal inflammation of breast cancer cells.

Since only *in vitro* studies were performed, future studies are needed to confirm the effects of parabens in breast stromal cells and breast cancer progression *in vivo* via xenograft of paraben treated stromal cells mixed with tumorigenic MCF7 cells in immunodeficient mice.

To enhance this study, the utilization of breast epithelial cells representing all possible cancer types would provide significantly more information on the influence of inflamed stroma. For instance, imposing conditioned media upon non-cancerous breast epithelial cells (MCF10A), ER+/PR-/HER2- cells (MCF7), ER+/PR+/HER2- cells (T-47D), ER-/PR-/HER2+ cells (SkBr3), and triple negative breast cancer cells (MDA-MB-231). The utilization of all these cancer cell types could expand our understanding of how paraben-induced stromal inflammation on breast cancer development and progression and could lead to novel strategies for breast cancer treatment and prevention.

VITA

Emily Nicole Hager was born to Sherry and Dave Hardgrave in Knoxville, TN, completing grade school in Knox County. She then attended East Tennessee State University (ETSU) where she received her Bachelors of Science degree in Biology/Biochemistry with honors as *Summa Cum Laude* with a 3.9 GPA. During this time, Emily received many awards and honors including Dean's List, Honors in Discipline Biology, and Biology Department Faculty Award. While pursuing her B.S., Emily worked for one year in the Writing Center and for four years in a variety of laboratories researching polycystic kidney disease and neuroscience. While at ETSU, Emily has authored and co-authored the following oral and poster presentations:

- *Search for intelligence in honey bee queen behavior. North Carolina Honey Bee Research Consortium, 5th Annual Meeting, University of North Carolina at Greensboro, 17 April, 2010. (abstract)*
- *Lack of rhythmicity in honey bee queen behavior. North Carolina Honey Bee Research Consortium, 5th Annual Meeting, University of North Carolina at Greensboro, 17 April, 2010 (abstract)*
- *Temporal behavior patterns of the honey bee (Apis mellifera). Poster presentation for the Appalachian Student Research Forum, 2009*

Emily has also been an author on publication as an undergraduate at ETSU

- *Absence of consistent diel rhythmicity in mated honey bee queen behavior. Journal of Insect Physiology. 2010*

After graduating from ETSU, Emily commissioned into the United States Navy as a Naval Officer with jobs in anti-submarine warfare. After serving four years, Emily returned to Knoxville with the family to tend to her mother diagnosed and succumbed to metastatic breast cancer.

Emily began pursuing her Doctor of Philosophy degree in Cellular and Molecular Nutrition at the University of Tennessee at Knoxville (UTK). Whilst working toward her doctoral research on breast cancer, Emily has received many scholarships and worked as a graduate

teaching assistant (GTA) and graduate research assistant (GRA). In the time that Emily has been working towards her doctorate in Nutritional Science, she has lost her grandmother, her previous husband, and birthed two children. Throughout these trying times, Emily has continued to maintain a 3.9 GPA and completed all necessary experiments for her dissertation in Dr. Ling Zhao's laboratory, which defended her dissertation on November 1st, 2019. The experimental skills honed during this research include cell culture, genetic manipulation, cellular immortalization, cell functionality assays, rodent handling, surgical experience, bioluminescent imaging, etc. Computer programs mastered include Microsoft Word, Excel, PowerPoint, Sigma Plot, EndNote, SAS, SPSS, Canvas, MacMillan, and ImageJ.

As a GRA, Emily managed the equipment and facilities of five labs. She learned and perfected communication skills with peers and external service companies. She coordinated repairs on a variety of experimental instruments critical for every study performed in the Cellular and Molecular Nutrition Department. Emily also functioned as the Bio-Safety contact for the Nutrition Department. Emily was also required to order and stock ethanol, liquid Nitrogen, Carbon Dioxide, and other tools necessary for daily laboratory operations.

As a GTA for Nutrition 100, Emily helped teach undergraduates introductory nutrition and metabolism in a class setting. Emily was critical in the implementation of new programs utilized for this class including iClicker and MacMillan.

While at UTK, Emily has authored the following poster presentations and publications:

- *Methylparaben and butylparaben alter multipotent mesenchymal stem cell fates toward adipocyte lineage. Toxicology and Applied Pharmacology. 2017*

Alongside academia, Emily has put countless hours into developing and implementing programs within Knoxville and the state of Tennessee in Veteran suicide prevention as the chairwoman of the Department of Veteran Affairs Knoxville Regional Mental Health Council, member of the Tennessee Veteran Suicide Prevention Task Force, and

chairwoman of Vet to Vet Tennessee Woman Veterans Committee. Emily organized and hosted two Women Veterans Summits in Knoxville in October 2018 and 2019. Emily is currently producing a theatrical play for suicide prevention geared toward the two highest-risk populations, youth and Veterans, and she is developing a program for suicide prevention to be taught in Monroe County schools. Emily is currently a certified QPR-Suicide Prevention Gatekeeper Instructor. Lastly, Emily is also collaborating with UTK Law School in hosting expungement clinics throughout Eastern Tennessee.