

**Exploring Novel Environmental Link to Obesity: Role of Parabens in
Adipogenesis *in vitro* and *in vivo***

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DEDICATION

I dedicate this work to my loved ones Chengxiang Hu, Yuanfang Long, Lan Hu, Ping Hu, Mason Hu and to Wen Ren. They have been supporting and encouraging me all the time.

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ABSTRACT

Parabens are a group of alkyl esters of p-hydroxybenzoic acid that include methylparaben, ethylparaben, propylparaben, butylparaben, and benzylparaben. Paraben esters and their salts are widely used as preservatives in cosmetics, toiletries, food, and pharmaceuticals. Humans are exposed to parabens through the use of such products from dermal contact, ingestion, and inhalation. The extent of the exposure is reflected by the frequent detection of these compounds in urine samples in the general population. Moreover, parabens have been detected in human serum, milk, placental tissues and breast tumor tissues. Parabens have been shown to preserve estrogenic/antiandrogenic effects, and can activate peroxisome proliferator-activated receptors. Therefore, they are considered endocrine disrupting chemicals (EDC). Here, the effects of parabens on adipogenesis in vitro and in vivo were investigated. We report that (1) parabens promote adipogenesis (or adipocyte differentiation) in murine 3T3-L1 cells through activation of nuclear receptor peroxisome proliferator-activated receptor γ and glucocorticoid receptor; (2) the adipogenic potency of parabens is increased with increasing length of the linear alkyl chain in the following potency ranking order: methyl- < ethyl- < propyl- < butylparaben; (3) parabens, butyl- and benzylparaben in particular, also promote adipose conversion of human adipose-derived multipotent stromal cells; (4) oral feeding of parabens in C57B6/J mice increase adiposity with altered metabolic biomarkers; and (5) exposure to parabens enhances adipocyte differentiation, but suppresses osteocyte and chondrocyte differentiation from a multipotent stem cell line. The results suggest that parabens may contribute to adipogenesis, through enhancing differentiation of predetermined preadipocytes, as well as through modulating the multipotent stem cells towards adipose lineage.

TABLE OF CONTENTS

INTRODUCTION	1
CHAPTER I LITERATURE REVIEW	3
1.1 Obesity and endocrine disrupting chemicals (EDCs)	4
1.1.1 Obesity and associated chronic diseases	4
1.1.2 Developmental origin of obesity	4
1.1.3 Environmental contribution to obesity: Endocrine disrupting chemicals	5
1.1.4 Mechanisms of metabolic disruption for EDCs	6
1.2 Models of adipogenesis	7
1.2.1 Cellular models of adipocyte differentiation	7
1.2.2 Transcriptional regulation of adipocyte differentiation	10
1.3 Parabens	15
1.3.1 Properties of paraben	15
1.3.2 Human exposure of parabens	15
1.3.3 Pharmacokinetics of paraben	16
1.3.4 Health concerns of paraben exposure	17
1.4 References	20
CHAPTER II EFFECTS OF PARABENS ON ADIPOCYTE DIFFERENTIATION	40
2.1 Abstract	42

2.2 Keywords	43
2.3 Introduction.....	44
2.4 Materials and methods	46
2.5 Results.....	51
2.6 Discussion	72
2.7 References	78
 CHAPTER III POST-WEANING EXPOSURE TO PARABENS INCREASE	
ADIPOSY AND PROMOTE DIFFERENTIATION OF MULTIPOTENT	
MESENCHYMAL STEM CELLS TOWARDS ADIPOSE LINEAGE.....	
	85
3.1 Abstract	87
3.2 Keywords	88
3.3 Introduction.....	89
3.4 Materials and methods	89
3.5 Results.....	94
3.6 Discussion	114
3.7 References	119
CONCLUSION AND FUTURE DIRECTIONS	126
VITA.....	129

LIST OF TABLES

Table 3.1 Ingredients of diets	93
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LIST OF FIGURES

Figure 2.1 Chemical structure of parabens, their common metabolite 4-hydroxybenzoic acid, and structurally related benzoic acid.	45
Figure 2.2 Parabens promote 3T3-L1 adipocyte differentiation.	52
Figure 2.3 The effect of exposure stage to paraben on 3T3-L1 adipocyte differentiation.	55
Figure 2.4 Parabens activate GR reporter and target gene without directly binding to, or modulating, the ligand binding of the receptor.	58
Figure 2.5 Parabens activate PPAR γ reporter and target genes.	62
Figure 2.6 The antagonists of GR and PPAR γ attenuate paraben-induced 3T3-L1 differentiation.	65
Figure 2.7 Paraben acts as a glucocorticoid-like compound to promote 3T3-L1 adipocyte differentiation.	66
Figure 2.8 Parabens promote adipocyte conversion of hADSC.	69
Figure 3.1 Effects of post-weaning exposure to parabens on body weight, body fat mass, adiposity and other metabolic biomarkers in mice fed with a chow diet with low phytoestrogens.	100
Figure 3.2 Effects of post-weaning exposure to parabens on gene expression in the WAT and liver in mice fed with the chow diet.	101
Figure 3.3 Effects of post-weaning exposure to parabens on body weight, body fat mass, adiposity and other metabolic biomarkers in mice fed with a high fat diet.	103
Figure 3.4 Effects of post-weaning exposure to parabens on gene expression in the WAT and liver in mice fed with the high fat diet.	107

Figure 3.5 Effects of parabens on adipogenic differentiation of multipotent stem cell	
C3H10T1/2.	108
Figure 3.6 Effects of parabens on osteogenic differentiation of multipotent stem cell	
C3H10T1/2.	111
Figure 3.7 Effects of parabens on chondrogenic differentiation of multipotent stem cell	
C3H10T1/2.	112

INTRODUCTION

Obesity is becoming a global epidemic that affects both adults and children. The 2012 report from National Health and Nutrition Examination Survey (NHANES) revealed that 35.7% of American adults are obesity (body mass index (BMI) > 30) and 68.8% are overweight (BMI >25) [1]. Obesity brings about mental problems and increases susceptibility to serious health conditions, such as hypertension [2], diabetes [3], and certain types of cancer [4]. Chronic low-grade inflammation in adipose tissue has been causally linked to insulin resistance, diabetes, and other metabolic complications, thus adipose tissue is now recognized as the target for obesity and associated metabolic syndromes.

Recent studies have suggested that obesity might result in part from prenatal and neonatal events when energy balance is interrupted. Early life exposure to endocrine disrupting chemicals (EDCs) is now considered to be contributing factors for the adverse environment that leads to childhood and adult obesity. EDCs are normally used in pesticides, plastic products, consumer products, industrial by-products and pollutants. Many EDCs, including bisphenol A (BPA), phthalates, organotins etc. have been related to increased risk of obesity in human [5-7].

Parabens are a group of alkyl esters of p-hydroxybenzoic acid that include methylparaben, ethylparaben, propylparaben, butylparaben, and benzylparaben. Parabens occur naturally in food, they are widely used as preservatives for their bactericidal and fungicidal properties in cosmetics, toiletries, pharmaceuticals and food [8]. Parabens are detected in human urine, milk, cord blood, and breast tissues [9-12]. Health concerns of paraben exposure have been raised since parabens have the ability to interact with estrogen receptor [13], androgen receptor [14], and peroxisome proliferator-activated receptor γ [15]. Studies have shown that paraben exposures are

associated with defects in sexual development [16-18], and might play roles in development of breast cancer and skin cancer [19,20,8].

Given the large-scale use of parabens and comprehensive daily exposure in humans, studies on adipogenic effects of parabens are limited. Butylparaben was shown to enhance adipocyte differentiation of 3T3-L1 cells, possibly through PPAR γ activation. Concentrations of paraben in urine samples during pregnancy were reported to be positively associated with weight growth between third ultrasound exam and birth, as well as with birth weight. Methylparaben in particular, was positively related to weight and abdominal circumference at 36 months [21]. These results suggest that exposure to parabens may contribute to obesity. This dissertation work was to systematically investigate the roles of parabens in adipogenesis, both *in vitro* and *in vivo*.

CHAPTER I
LITERATURE REVIEW

1.1 Obesity and endocrine disrupting chemicals (EDCs)

1.1.1 Obesity and associated chronic diseases

Obesity is becoming a global epidemic that affects both adults and children. It was reported in 2012 that 35.7% of American adults are obese (body mass index > 30) and 68.8% are overweight (BMI > 25) [1,2]. Obesity not only brings about mental pressures including social stigmatization and discrimination, but also increases the susceptibility to serious health conditions, such as hypertension [3], diabetes [4], and certain types of cancer [5].

Obesity is defined as excess of adipose tissue, which is generated from increases in adipocyte size and number [6]. It is increasingly recognized that obesity is associated with chronic low-grade inflammation in adipose tissue, which has been causally linked to insulin resistance, diabetes, and other metabolic complications. Chronic inflammation is characterized by proinflammatory cytokine and/or chemokine secretion, infiltration of immune cells, and generation of further proinflammatory response. Therefore, adipose tissue is now recognized as the key player in obesity and associated metabolic syndromes.

1.1.2 Developmental origin of obesity

Mounting evidence suggests that adult obesity might result in part from events taking place during pregnancy and postnatal development. Ravelli et al. has reported a higher incidence rate of obesity in 19-year-old males who had been exposed to the 1944-1945 Dutch famine during first half of gestation [7]. Another study of 50-year-old females who had been exposed to the famine in early gestation found that these women had higher BMI and waist circumference [8]. More recent studies showed that people with lower birth weight had higher body fat than high-birth-weight group [9,10], implying the association between low birth weight and increased obesity risk later in life.

Interestingly, higher birth weight was also found to be linked to higher adult BMI [11,12]. Therefore, it is postulated that adverse environment in utero or during infancy alters the programs of organ development and function, leading to energy imbalance and subsequently, obesity in adult life.

1.1.3 Environmental contribution to obesity: Endocrine disrupting chemicals

Early life exposure to endocrine disrupting chemicals (EDC) is now considered to be contributing factors for the adverse environment that leads to childhood and adult obesity. EDCs are chemicals that can disrupt hormonal regulation and endocrine homeostasis, resulting in health problems in animals and humans. EDCs are normally found in pesticides, plastic products, consumer products, and industrial by-products and pollutants.

Bisphenol A (BPA) is widely used as plasticizers for its cross-linking ability. Early life exposure to BPA has been associated with disruption of male and female reproductive system [13]. Animal studies found that perinatal exposure to BPA alters early adipogenesis and increases body weight [14,15], and BPA exposure prior to puberty was able to increase body weight as well [16]. Epidemiological study showed that urinary bisphenol A (BPA) concentration in a Chinese population was associated with adult obesity and insulin resistance [17].

Phthalates are mainly used as softeners in paint industry, but could also be found in personal-care products and medical devices. Phthalates have been shown to be antiandrogenic and can affect reproductive system development in male rodents [18], similar results were later confirmed in human studies [19]. Prenatal exposure to phthalates is found to be associated with the affected Orientation score and Alertness score of neonates [20], and is adversely associated with behavioral domains in children age 4 to 9 years that are diagnosed with conduct or attention deficit hyperactivity

disorders [21]. Certain phthalates metabolites in urine have been associated with obesity in children and adults [22,23].

Organotins are used to stabilize plastic and as fungicides, rodent repellents and in antifouling paints. Animal researches on tributyltin chloride (TBT), one of the well-known organotins, showed that it affected sexual development and reproductive system in both male and female rats [24]. In vitro studies found that organotin compounds stimulated differentiation of preadipocytes [25]. Mice studies further showed that in utero exposure to organotin compounds increased lipid accumulation in adipose tissue and liver, resulting in increased adipose mass in adults [26].

1.1.4 Mechanisms of metabolic disruption for EDCs

Hormones function mainly through their receptors. Molecular mechanisms have been proposed for the metabolic disrupting effects of EDCs : EDCs act through the hormone receptors.

Peroxisome proliferator-activated receptors γ (PPAR γ) is the master regulator for adipogenesis and plays important roles in lipid metabolism, and some EDCs are found to act as direct PPAR γ agonists. Organotins are readily measured in house dust and the presence was found in human liver and blood [27,28]. Studies showed that organotins are PPAR γ agonists [29,30], and that they activate RXR-PPAR heterodimers primarily through the interaction with RXR [31]. Phthalates are normally used as plasticizers and found in building materials, medicines and personal care products. Phthalates are found to activate PPAR γ and its target genes in adipocytes, leading to induction of adipocyte differentiation [32].

Glucocorticoid receptor (GR) and thyroid hormone receptor (TR) are two nuclear receptors that EDCs can work on. GR is ubiquitously expressed in the cytoplasm of

various cell types and plays important roles in metabolism, immune response, growth and development. TR regulates metabolism, growth and development. It has been reported that BPA and dicyclohexyl phthalate can promote adipogenesis in vitro through activation of GR [33]. Several EDCs have been shown to be capable of disrupting thyroid function, such as BPA [34], phthalates [35], polybrominated diphenyl esters (PBEDs) [36], and polychlorinated biphenyls (PCBs) [37].

Estrogen receptors (ERs) are activated by estrogen (17 β -estradiol), interruption of which could result in reproductive and developmental defects. ERs also play roles in many aspects of metabolism, such as glucose and fatty acid metabolism. The most well-known estrogenic EDC is BPA, known as a synthetic estrogen. As a persistent organic pollutant, polychlorinated biphenyls (PCBs) were shown to compete with natural ligand for binding to ER, suggesting the ability to interfere with ER signaling [38]. Hydroxylated PCB metabolites could inhibit the estrogen catabolism thus increase estradiol bioavailability, which might explain the estrogenic effects of PCBs [39]

1.2 Models of adipogenesis

Obesity is defined as excessive white adipose tissue (WAT), which is composed of a mixture of preadipocytes, adipocytes, stromal vascular tissue, lymph nodes, and tissue matrix. Obesity is a result of increased size and/or number of adipocytes. Both cellular models and animal models have been instrumental in understanding the development of obesity.

1.2.1 Cellular models of adipocyte differentiation

Cellular models of adipocyte differentiation have been developed and used to understand the molecular mechanisms by which precursor cells are converted into new functional adipocytes. The commonly used cellular models for adipogenesis are 3T3-L1 preadipocytes cell lines and C3H10T1/2 multipotent stem cells.

1.2.1.1 3T3-L1 differentiation

3T3 cells are originally established from primary embryonic fibroblast cells from mice [40]. 3T3 cells are subcultured every 3 days, at the rigid density of 3×10^5 cells/20 cm² dish, and 3T3-L1 is one of the cell lines that have been extensively used for studying in vitro cellular adipogenesis.

3T3-L1 cells have a fibroblast-like morphology before differentiation. When cells reach confluence, adipocyte differentiation is initiated by treatment of adipogenic cocktail. Adipocyte specific genes are induced and lipids are accumulated, and the cells adopt a round-shape, lipid-filing morphology at the end of differentiation.

Upon confluence, the cells are treated with differentiation DMEM containing 10% fetal bovine serum, 10 µg/ml insulin, 1 µM dexamethasone (Dex), and 0.5 mM 3-isobutyl-1-methylxanthine (MIX) (DMI) for 3 days. The cells are then grown in maintenance DMEM containing 10% FBS and 10 µg/ml insulin for an additional 2 days and then in DMEM containing 10% FBS until the end of the experiment. Typically, 99% of the cells are differentiated into mature adipocytes 8 days following the initiation of differentiation.

1.2.1.2 C3H10T1/2 differentiation

C3H10T1/2 is a multipotent stem cell line first established in Heidelberger laboratory at University of Wisconsin [41]. Derived from C3H mouse embryos, C3H10T1/2 cells possess the potential to differentiate into adipocytes, chondrocytes, osteocytes or myocytes.

C3H10T1/2 adipogenic differentiation

Differentiation of C3H10T1/2 into adipocyte lineage could use different protocols. Cheng et al. differentiated the cells in 0.1 µM Dex, 50 µM indomethacin and 5 µg/ml

insulin for 14 days [42], while Lee et al. adapted a protocol that is similar to 3T3-L1 protocol: 2 days in induction medium (1 μ M Dex, 0.5 mM MIX, 10 μ g/ml insulin), 6 days of maintenance medium (10 μ g/ml insulin) [43]. Bone Morphogenetic Protein 4 (BMP4) and BMP2 have been shown by several research groups to induce commitment of stem cells into adipose lineage and promote adipogenic differentiation of C3H10T1/2 cells [44-46]. Moreover, several cytoskeleton-associated proteins are found important in committing the stem cells to adipocyte lineage [45]. Adipocyte specific marker genes can be analyzed by the end of differentiation, including FABP4, PPAR γ , C/EBP α , adiponectin, fatty acid synthase (FASN).

C3H10T1/2 chondrogenic differentiation

Micromass technique modified by Ahrens et al. is used to induce chondrocyte differentiation of C3H10T1/2 cells [47]. Specifically, trypsinized C3H10T1/2 cells are suspended in Ham's F12 medium with 10% FBS at a concentration of 10^7 cells/ml, and 10 μ l drop of cell suspension are placed in the center of a well of 24-well tissue culture plate. Cells are allowed to adhere in incubator (37°C and 5% CO $_2$) for 2 to 3 h, then 1ml of medium containing BMP2 of 100 ng/ml are added to the culture. Medium are replenished every three days until the end of the experiment. Chondrocyte specific marker genes include link protein, type II collagen, cartilage oligomeric matrix protein (COMP), and the cartilage-like matrix can be stained by Alcian blue.

C3H10T1/2 osteogenic differentiation

Osteogenic differentiation of C3H101/2 are commonly induced by BMP2 [48], BMP7 [49], BMP9[50], or BMP4 [51]. Generally used osteoblast marker genes are osteocalcin (OC), alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BSP), and core binding factor alpha 1 (Cbfa1)/Runt-related transcription factor 2

(Runx2). Osteoblast differentiation of mesenchymal stem cells starts from cell proliferation to matrix maturation, during which state the ALP expression peaks and could be analyzed as a marker for successful differentiation. Then cells undergo matrix mineralization when OC, BSP, OPN are expressed. When mineralization is completed, calcium deposition is prominent and could be visualized by staining, such as Alizarin Red S staining.

1.2.2 Transcriptional regulation of adipocyte differentiation

Right after the induction of DMI, growth-arrested preadipocytes undergo several rounds of mitotic clonal expansion, which is a permissive state for subsequent differentiation [52]. The expression levels of *c-fos*, *c-jun*, *junB*, *c-myc* and CCAAT/enhancer binding protein β and δ (C/EBP β and δ) are increased [53]. C/EBP β and δ are believed to induce Krüppel-like transcription factor 15 (KLF15) [54], which acts in concert with the former two factors to mediate the expression of PPAR γ [55]. PPAR γ peaks at day 3 to day 4, then gradually decreases until day 7 [56,55]. C/EBP α was thought to be sufficient to initiate adipocyte differentiation, however it is not strictly required when ectopic PPAR γ expression is present [57]. C/EBP α becomes detectable after 2 days of DMI incubation under regulation of C/EBP β and δ [58]. PPAR γ and C/EBP α are key regulators of adipogenesis, they cross-regulate each other and transcriptionally induce expression of a set of adipocyte genes that encode proteins and enzymes involved in forming and maintain adipocyte phenotype.

PPAR γ

PPAR γ is a member of the nuclear hormone receptor family, it has restricted pattern of expression in white and brown adipose tissue at adult stage [59]. PPAR γ is the master regulator for adipocyte differentiation. Ectopic expression of PPAR γ enabled

nonadipogenic murine fibroblasts to initiate the adipogenic program and become fat cells capable of many functions of mature adipocytes [60] and specific PPAR γ knockdown in white adipose tissue induced growth retardation and lipodystrophy in mice [61].

PPAR γ is also required for maintenance of mature adipocyte state. Introduction of dominant-negative PPAR γ de-differentiated mature 3T3-L1 adipocytes and resulted in lipid loss as well as decreased expression of adipocyte markers [62]. Selective ablation of PPAR γ in mature adipocytes in mice led to death of the cells which were then replaced by PPAR γ -positive adipocytes, implying the important role of PPAR γ in mature adipocyte maintenance.

PPAR γ heterodimerizes with retinoid X receptor (RXR) and binds to PPAR response element (PPRE) in promoter regions of the target genes, initiating gene transcription. PPREs consists of direct repeat 1 (DR1), which was defined by a direct repeat of two core motifs (AGGTCA) spaced by one nucleotide [63] and is specific for PPAR-RXR heterodimer. Several cofactors have been identified to be in direct contacts with PPARs, including corepressors and coactivators. The nuclear receptor corepressor (N-CoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) are two well-characterized corepressors that repress PPAR γ transcription activity when ligand is not present; which could then be reversed by addition of PPAR γ ligand pioglitazone [64]. Coactivators enhance the transcriptional activation of PPAR-regulated genes. Some of the well-known coactivators are steroid receptor coactivator 1 (SRC-1), cAMP response element-binding protein (CREB) binding protein (CBP), PPAR γ coactivator 1 and 2 (PGC-1 and -2), PPAR-binding protein (PBP), and androgen receptor (AR) –associated protein 70 (ARA70).

PPAR γ expression could be positively regulated by many transcriptional factors, including C/EBP β and δ [65], KLF15 [55,66], Zfp423 [67] and sterol receptor element-binding protein 1c (SREBP-1c)/adipocyte determination and differentiation dependent factor 1 (ADD1) [68]. Some other transcriptional factors can negatively regulate PPAR γ activity, such as KLF2 [69], GATA-2 and GATA-3 [70]. Target genes of PPAR γ include adipokines adiponectin [71], leptin; genes related to lipogenesis and lipid secretion, such as stearoyl-CoA desaturase 1 (SCD1), FABP4 [72], FASN, lipoprotein lipase (LPL) [73], phosphoenolpyruvate carboxy kinase (PEPCK) [74], and many others.

GR

GR is a ubiquitously expressed nuclear receptor that plays roles in metabolism, immune response, growth and development. GR activation is necessary for adipocyte differentiation, as the synthetic glucocorticoid dexamethasone (Dex) is used in the standard adipogenic cocktail, even though excessive amount of glucocorticoids have been shown to have adverse effects such as excess visceral fat in patients with Cushing's syndrome [75]. As previously mentioned, PPAR γ activation is mediated downstream of C/EBP β and C/EBP δ , this process also needs the assistance of glucocorticoids [76]. GR activation by Dex was shown to prime the preadipocytes for the adipocyte fate, which can then be induced into differentiation by PPAR γ ligand MIX; but the temporal-reversed treatment failed to induce significant differentiation [77].

GR is localized in cytoplasm when ligand is absent, by association with chaperone proteins which binds to the ligand binding domain (LBD) of GR [78]. Binding of GR to ligand disassociates chaperone protein, allowing GR subunits homodimerization and translocation into nucleus where it binds to glucocorticoid response element (GRE) in promoter region of target genes and regulates transcription [79]. GR can also crosstalk with other transcriptional factors to repress the target gene expression, such as Nuclear

Factor-kappa B (NF- κ B) and activator protein-1 (AP-1) [80]. GR also interacts with coactivators such as SRC-1, SRC-2 (also known as transcriptional mediators/intermediary factor 2 (TIF2)) and SRC-3 [81].

Even though Dex was commonly used as GR ligand to activate GR transcriptional activity, it decreased mRNA and protein level of GR by reducing transcription and shortening the half-life of GR protein, respectively [82]. Activation of GR can increase expression of hormone-sensitive lipase (HSL) and reduce expression of LPL, leading to enhanced lipolysis [83]. Preadipocytes factor 1(Pref-1) is a transmembrane protein that is abundant in preadipocytes but diminished during differentiation, it was found to be suppressed by Dex in 3T3L1 cells [84] and human mesenchymal stem cells (MSC) [85], which resulted in induced differentiation. Lipin 1 is required for adipocyte differentiation since it converts phosphatidate to diacylglycerol, the precursor for triglycerol. Lipin 1 gene expression was found to be up-regulated by the glucocorticoids and a GRE site was identified and characterized in lipin 1 promoter region [86].

Runx-related transcription factor 2 (Runx2)

Runx2 belongs to the runt-domain gene family, which can bind to DNA, protein and ATP [87]. Runx2 is highly expressed in mature osteocytes and has been shown to be critical for osteoblast differentiation. Runx2 deficient mice die after birth because of absence of osteoblasts, bone [88], or for a complete lack of ossification [89]. Forced expression of Runx2 in nonosteoblastic cells induced expression of principle osteoblast-specific genes [49].

Runx2 is also expressed in chondrocytes and increased along with the maturation of chondrocytes [90]. Runx2 deficient mice showed disturbed chondrocyte differentiation in the skeleton [91] and a lack of hypertrophic chondrocytes in anlagen of

the humerus [90], suggesting Runx2 could be a positive regulator for chondrocyte differentiation as well.

Runx2-deficient chondrocytes showed remarkably decreased expression of Pref-1 and underwent adipocyte differentiation [92], whereas induced expression of Runx2 by BMP2 in human marrow stromal precursor cells inhibited late stage adipocyte maturation [93]. These data suggest that Runx2 might be an inhibitory factor for adipocyte differentiation.

Since C3H10T1/2 has the potential to differentiate into osteocytes, chondrocytes or adipocytes, it is thus of rational sense to speculate that the differentiation into one lineage fate would reciprocally suppress the other. Indeed, an inverse relationship have been found between the amount of trabecular bone and the adipose tissue in bone marrow [94]. Studies in rat marrow stromal cells found that inhibited differentiation of adipocytes was concurrent with enhanced differentiation of osteogenic cells [95]. Overexpression of PPAR γ 2 stimulated FABP4 expression and fat accumulation in murine bone marrow-derived clonal cell lines, while suppressing Runx2 expression and osteoblast differentiation [96]. Activation of PPAR γ by ligand in mesenchymal cells also suppressed Runx2-mediated osteoblast differentiation [97]. Runx2 was identified as a downstream target of Dex, the downregulation of which was thought to be required for 3T3-L1 adipogenesis [98]. Lovastatin, a cholesterol-lowering drug, was reported to inhibit adipogenesis by suppressing PPAR γ 2 expression while in the meantime enhance osteoblast differentiation with increased expression of Runx2 [99]. These results suggest that Runx2 might play an important role in deciding the cell fate of multipotent stem cells in that it is necessary for osteocyte/chondrocyte differentiation, but suppressive for adipocyte differentiation.

1.3 Parabens

1.3.1 Properties of paraben

Parabens are a group of alkyl esters of p-hydroxybenzoic acid that include methylparaben, ethylparaben, propylparaben, butylparaben, and benzylparaben. Parabens are generally small colorless crystals or crystalline powders in pure form, and are normally stable in air, water and in acidic solutions. Parabens occur naturally in food, such as cloudberry, white wine, yellow passion fruit juice and the aerial part of *Stocksia brahuica* [100]. Paraben esters and their salts are widely used as preservatives for their bactericidal and fungicidal properties in cosmetics, toiletries, pharmaceuticals and food [101].

1.3.2 Human exposure of parabens

Human get exposed to parabens through food intake or via cosmetics and drugs. The combined consumption of parabens from all sources is estimated to be 76 mg/day: 1 mg/day from food, 50 mg/day from cosmetics and personal care products, and 25 mg/day from drugs [102].

Study of 100 urinary samples from a demographically diverse group with no known occupational exposure detected total methylparaben (free and conjugated forms) of 0.28 μM and 4 μM at 50th and 95th percentile, respectively [103]. Methyl- and propylparaben were detected in nearly all samples, while other parabens (ethyl-, butylparaben) were found in more than half of the samples [103].

Moreover, parabens have been detected in human milk, placental tissues, and breast tumor tissue. Defatted milk samples from cohort studies were used for evaluation of presence of parabens, and methyl-, ethyl-, propylparaben were detected [104]. Butylparaben were not detected, but it was postulated that butylparaben could possibly be present in the milk fat for its lipophilicity [104]. Finding of parabens in embryonic fluid,

placenta, fetal liver and fetal carcass after the pregnant rats were exposed to ethylparaben and butylparaben suggests the transfer of paraben from placenta to fetuses, and suggests that paraben exposure could start as early as embryonic period [105]. That higher concentrations of parabens were found in amniotic fluid than maternal plasma raised the concerns that concentration of paraben occurs in fetuses and the fetuses might be affected at a higher degree by parabens [105]. Towers and colleagues did a study on 50 patients and detected methylparaben in blood of 47 mothers (mean level 20.41 ng/L) and in 47 paired cord bloods (mean level 36.54 ng/L) [106]. Detection of intact parabens in human breast tumors were reported, raising the concerns that parabens in cosmetics might increase the incidence of breast cancer [107].

1.3.3 Pharmacokinetics of paraben

When orally exposed, almost all parabens are rapidly absorbed from the gastrointestinal tract in rats, rabbit, dogs, cats and humans [108-110]. Most absorbed parabens are hydrolyzed by esterase in different organs with *p*-hydroxybenzoic acid (PHBA) being the main metabolite; others excreted as glycine, glucuronic acid, and sulfuric acid conjugates of PHBA. Metabolized products occur rapidly in urine within 30 min after ingestion, and majority of the metabolites get cleared in 24 h [111,112]. Even after intravenous administration, paraben levels in serum quickly decline and remain low [111,112]. Esterases that hydrolyzes parabens are present in subcutaneous fat tissues, liver, kidney, and dermal skin. However, the hydrolysis of parabens could be impeded by esterases inhibitors that are present in some commonly-consumed food and drinks, such as grapefruit juice. Grapefruit juice has been shown to inhibit esterase activities due to the presence of many flavonoids [113,114], thus concurrent intake of parabens with inhibitory compounds for esterases could probably increase paraben escape from hydrolysis so that increased level of parabens are found in circulation.

Parabens are largely used in personal products and cosmetics and can be absorbed through skin. The dermal permeability of parabens is likely to be affected by the type of paraben, the partition coefficient, and other compounds present in the product [115,116]. Parabens can be hydrolyzed by cutaneous esterase and thus reduced amount of parabens can enter the systemic circulation [117,118].

1.3.4 Health concerns of paraben exposure

Despite the wide exposure of paraben to human, the health impacts of paraben have been investigated only recently [131,8]. Methyl-, ethyl-, propyl-, butyl-, benzylparaben all have been shown to possess estrogenic activity to some extent, and the activity increases with increasing length of the side chain [13,132]. Even though the ability of parabens for estrogen receptor (ER) binding is lower than some known estrogenic ligands and thus are called “weak estrogens”, parabens were able to function at the same magnitude as 17 β -estradiol in human breast cancer cells when the concentration is sufficient [132,13], suggesting that parabens can exert full agonist response as estrogen. Parabens were reported to be present in human breast tumors [118,12], and comparable concentrations of parabens were later found to be able to stimulate proliferation of human breast cancer cells [19], implying the possible involvement of parabens in development of breast cancer.

Parabens have also been shown to activate androgen receptor and possess antiandrogenic activity [14]. Animal study revealed alteration of spermatogenesis, testosterone secretion, and epididymal weight after repeated oral dosing of propyl- and butylparben in post-weaning rats and mice, which might be explained by its estrogenic activity and/or antiandrogenic activity that lowered androgen signaling in circulation [16-18]. Methyl- and ethylparaben didn't show anti-spermatogenic effects in juvenile rats though, levels of testosterone, luteinizing hormone (LH), follicle stimulating hormone

(FSH) was not changed either [133]. In humans, urinary parabens were found not to be associated with serum hormone levels and semen quality parameters, but positive association between butylparaben concentration and sperm DNA damage were noted [134].

Since estrogens have influence on skin composition and development, the question raises as to whether the estrogenic parabens can affect skin and related aging. It was reported that methylparaben decreased cell proliferation and caused changes in morphology [135]. Moreover, methylparaben potentiated ultraviolet-B (UVB) induced damages in keratinocytes as evidenced by decreased cell viability and enhanced lipid peroxidation [20]. Therefore, it has been hypothesized that higher rate of melanoma in younger people could possibly be correlated with greater use of paraben-containing personal products in this population [8].

Several studies have shown that parabens activate PPAR γ , one of the properties shared by many EDCs that are associated with metabolic disruption. It was found that butylparaben enhanced adipocyte differentiation of 3T3-L1 cells, along with upregulated expression and release of leptin, adiponectin and resistin [15]. Concentrations of parabens in urine during pregnancy were reported to be positively associated with weight growth between third ultrasound exam and birth, as well as with birth weight. Concentrations of methylparaben in particular, were positively related to weight and abdominal circumference at 36 months [21]. These results suggest that exposure to paraben may contribute to obesity.

In summary, parabens have been shown to interrupt with endocrine system and might contribute to obesity. However, there is no direct evidence that has confirmed the adipogenic effects of parabens *in vitro* or *in vivo*. Due to the widely use of parabens and general human exposure, it is necessary to systematically investigate the adipogenic

effects of parabens. The specific aims of the current study are: 1) to test whether parabens can induce adipocyte differentiation *in vitro* and what the adipogenic potency of various parabens are; 2) to study the molecular mechanisms underlying paraben's potential adipogenic effects; 3) to investigate the adipogenic effects of parabens *in vivo*; and 4) to explore the effects of parabens on modulating multipotent stem cell fate.

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CHAPTER II

EFFECTS OF PARABENS ON ADIPOCYTE DIFFERENTIATION

A version of this chapter was originally published by Pan Hu, Xin Chen, Rick J. Whitener, Eric T. Boder, Jeremy O. Jones, Aleksey Porollo, Jiangang Chen, and Ling Zhao under the title, “Effects of Parabens on Adipocyte Differentiation” *Toxicological sciences* (2013).

2.1 Abstract

Parabens are a group of alkyl esters of *p*-hydroxybenzoic acid that include methylparaben, ethylparaben, propylparaben, butylparaben, and benzylparaben. Paraben esters and their salts are widely used as preservatives in cosmetics, toiletries, food, and pharmaceuticals. Humans are exposed to parabens through the use of such products from dermal contact, ingestion, and inhalation. However, research on the effects of parabens on health is limited, and the effects of parabens on adipogenesis have not been systematically studied. Here, we report that (1) parabens promote adipogenesis (or adipocyte differentiation) in murine 3T3-L1 cells, as revealed by adipocyte morphology, lipid accumulation, and mRNA expression of adipocyte-specific markers; (2) the adipogenic potency of parabens is increased with increasing length of the linear alkyl chain in the following potency ranking order: methyl- < ethyl- < propyl- < butylparaben. The extension of the linear alkyl chain with an aromatic ring in benzylparaben further augments the adipogenic ability, whereas 4-hydroxybenzoic acid, the common metabolite of all parabens, and the structurally related benzoic acid (without the OH group) are inactive in promoting 3T3-L1 adipocyte differentiation; (3) parabens activate glucocorticoid receptor and/or peroxisome proliferator-activated receptor γ in 3T3-L1 preadipocytes; however, no direct binding to, or modulation of, the ligand binding domain of the glucocorticoid receptor by parabens was detected by glucocorticoid receptor competitor assays; and lastly, (4) parabens, butyl- and benzylparaben in particular, also promote adipose conversion of human adipose-derived multipotent stromal cells. Our results suggest that parabens may contribute to obesity epidemic, and the role of parabens in adipogenesis in vivo needs to be examined further.

2.2 Keywords

paraben; endocrine disrupting compound; adipocyte differentiation; glucocorticoid receptor; peroxisome proliferator–activated receptor.

2.3 Introduction

Parabens are a group of alkyl esters of *p*-hydroxybenzoic acid that include methylparaben, ethylparaben, propylparaben, butylparaben, and benzylparaben (Fig. 2.1). Paraben esters and their salts are widely used as preservatives in cosmetics, toiletries, food, and pharmaceuticals (Darbre and Harvey, 2008). A survey of 215 cosmetic products in 1995 found parabens in 99% of the leave-on cosmetic products and 77% of the rinse-off products (Rastogi et al., 1995). The systemic absorptions of parabens into the human body have been reported (Darbre et al., 2004; Ye et al., 2006). Intact parabens that escaped from metabolism by esterases from the intestine and skin were detected in human urine samples, with methylparaben and propylparaben being the most abundant followed by butylparaben. Total methylparaben (free and conjugated forms) levels were 43.9 (0.28 μ M) and 608 ng/ml (4 μ M) at 50th and 95th percentile of 100 urine samples collected from U.S. adult volunteers who had no known occupational exposure (Ye et al., 2006). The detection of parabens in human breast milk has also been reported, suggesting constant exposure of parabens early in life (Schlumpf et al., 2010).

Despite the wide use and human exposure, the health impacts of parabens have been examined only recently (Boberg et al., 2010; Darbre and Harvey, 2008). Both the in vitro (Chen et al., 2007; Terasaka et al., 2006; van Meeuwen et al., 2008) and in vivo rodent data (Oishi, 2001, 2002) have shown that parabens exert estrogenic/antiandrogenic activity, but with estrogenic potency of 3–6 orders of magnitude lower than that of 17 β -estradiol, although the studies with no effects of parabens have also been reported (Shaw and deCatanzaro, 2009). In addition, parabens could also act as a thyroid hormone receptor agonist/antagonist and as an

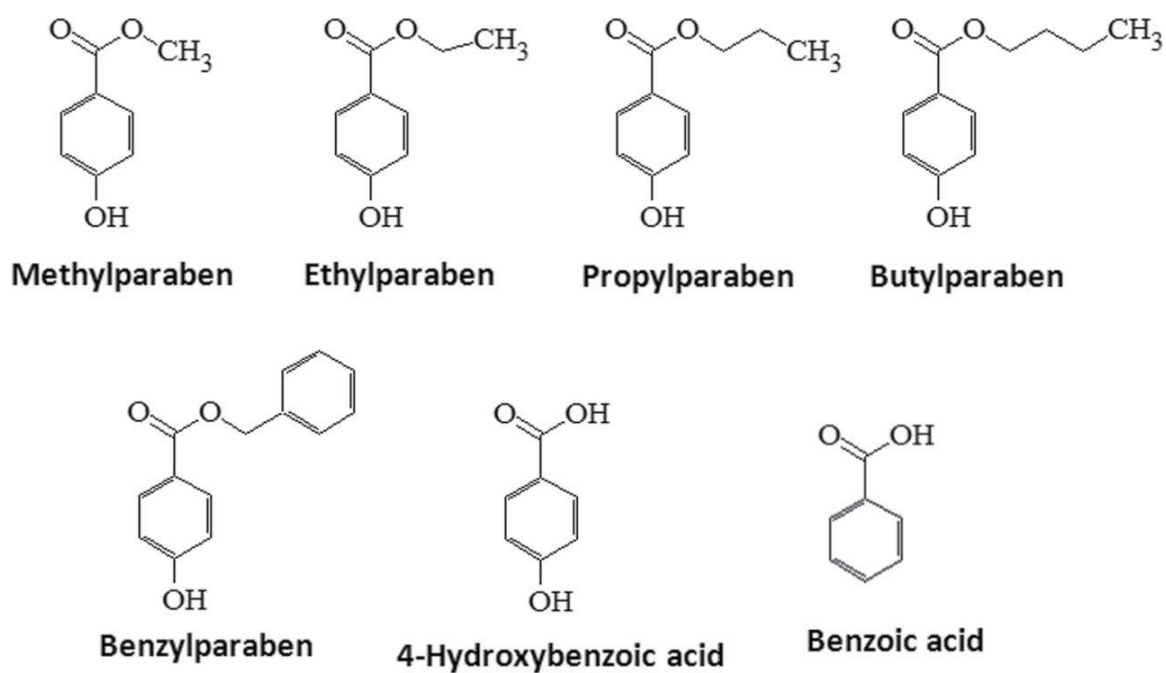


Figure 2.1 Chemical structure of parabens, their common metabolite 4-hydroxybenzoic acid, and structurally related benzoic acid.

agonist for peroxisome proliferator–activated receptor (PPAR) (Taxvig et al., 2012). Together, these data suggest that parabens are endocrine disrupting compounds (EDC).

Many environmental EDCs have been suggested to contribute to human obesity by interfering with lipid metabolism and/or adipogenesis, hence the term “obesogen” was coined (Grün and Blumberg, 2006). However, the effects of parabens on adipocyte differentiation (generation of new adipocytes or adipocyte hyperplasia), one of the contributing process to obesity, have not been systematically investigated. Murine 3T3-L1 is the most widely used in vitro cell model for adipocyte differentiation, by which fibroblast-like preadipocytes are converted into mature, spherical, and lipid-filled adipocytes through a multistage process (Green and Kehinde, 1975). Here we report the adipogenic effect of parabens on murine 3T3-L1 cells, as revealed by Oil Red O (ORO)–stained adipocyte morphology, lipid accumulation, and mRNA expression of specific adipocyte marker genes. The abilities of parabens in activating glucocorticoid receptor (GR) and peroxisome proliferator–activated receptor, the two established signaling pathways in adipocyte differentiation, are also investigated. Lastly, we examine the effects of parabens on adipose conversion of human adipose–derived multipotent stromal cells (hADSC).

2.4 Materials and methods

Reagents

Cortisone, methylisobutylxanthine (MIX), dexamethasone (DEX), insulin (Ins), peroxisome proliferator–activated receptor gamma (PPAR γ) agonist rosiglitazone, antagonists GW9662, bisphenol A diglycidyl ether (BADGE), and GR antagonist RU-486 were purchased from Sigma-Aldrich (St Louis, MO). Dimethyl sulfoxide; methyl-, ethyl-, butyl-, propyl-, and butylparaben; 4-hydroxybenzoic acid, and benzoic acid sodium salts

were all from Acros Organics (Thermo Fisher Scientific, Pittsburg, PA), and benzylparaben was from MP Biomedicals (Solon, OH).

Cell culture, induction of adipocyte differentiation, and paraben treatments.

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone) in 5% CO₂, 37°C environment until they reached confluence. To study the potentiating effects of parabens on differentiation induced by glucocorticoids, the standard differentiation protocol was modified using a weaker GR agonist cortisone, based on previous studies (Kim et al., 2007; Marcolongo et al., 2008; Park et al., 2011). Briefly, on the day of reaching confluence (designated as day 0), cells were treated with DMEM containing 10% fetal bovine serum (FBS, Atlas Biologicals), 0.5 mM MIX, 170 nM Ins, and 5 µM cortisone for 3 days (stage 1, day 0–3). The cells were then grown in maintenance DMEM containing 10% FBS and 170nM Ins for additional 2 days (stage 2, day 4–5) followed by growth in DMEM containing 10% FBS (stage 3, day 6–7) until day 7. hADSC were purchased from Zen-Bio (Research Triangle Park, NC) and were grown and differentiated according to the supplier's instructions. Briefly, the cells were seeded and grown in 60-mm tissue culture dishes in a preadipocyte medium until confluence. The differentiation was initiated with adipocyte differentiation medium for 7 days and maintained in adipocyte maintenance medium for additional 7 days. All media used for human primary cell culture were purchased from Zen-Bio.

Various parabens were added in the differentiation media of 3T3-L1 or hADSC.

Unless otherwise indicated, the parabens or the vehicle control (DMSO) were applied from the initiation and were reapplied at each change of medium for both cell types during the whole differentiation process. For the detection of early target genes, butylparaben or DMSO was added to the media with or without Dex or the differentiation

cocktails (Cortisone, MIX, and Ins; CMI) for indicated time. For the studies of the antagonists of GR or PPAR γ , the cells were pretreated with the antagonists of PPAR γ (GW9662 and BADGE) or GR (RU-486) or DMSO for 1h before the cells were cotreated with butylparaben or DMSO in the presence of the antagonist. The antagonist was reapplied together with butylparaben or the DMSO control at each change of the media.

ORO staining and quantification.

To quantify lipid accumulation, differentiated cells were fixed with 4% paraformaldehyde overnight and then rinsed with deionized water and stained with ORO solution (60% ORO in isopropanol) for 10 min. After staining, the plates were rinsed with deionized water and were scanned by a scanjet 3970 scanner (Hewlett-Packard Company, Palo Alto, CA). The images of the stained cells were taken by an integrated digital camera linked to a Micromaster inverted digital microscope (Thermo Fisher Scientific, Waltham, MA). To quantify the staining, the ORO was eluted with 100% isopropanol for 10min, and the OD absorbance at 500nm was measured in a Spectronic Genesys 5 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

RNA preparation and quantitative real-time PCR analysis.

At indicated times, total RNA was prepared from preadipocytes or adipocytes using TRIzol (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Total RNA abundance was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. mRNA expression of various adipocyte marker genes and loading control 36B4 or 18S were measured quantitatively using Absolute Blue QPCR SYBR Green ROX mix (Thermo Fisher Scientific, Waltham, MA) or by gene-specific TaqMan gene expression assays (Applied Biosystems,

Carlsbad, CA) (primer sequences and TaqMan probe IDs are provided in the Supplemental materials) and were run in a 96-well format using an ABI 7900HT Real-Time PCR System. Cycle conditions were 50°C 2 min, 95°C 15 min, and then 40 cycles of 95°C for 15 s/60°C for 1 min. Relative gene expression was calculated using $2(-\Delta\Delta Ct)$ method (Dawson et al., 2012).

Small RNA interference.

Silencer Select predesigned and validated siRNA targeting mouse GR or PPAR γ were purchased from Applied Biosystems (Carlsbad, CA). 3T3-L1 preadipocytes were seeded onto six-well plates and transfected with siRNA oligos targeting GR and PPAR γ or with nontargeting negative control oligos using DeliverX plus system (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol. We have achieved more than 75% knockdown efficiency with siGR and siPPAR γ (data not shown).

Transfection and reporter gene assays.

A GR-responsive luciferase reporter construct (the mouse mammary tumor virus promoter-driven luciferase reporter MMTV-Luc) was a gift from Dr Vickie Wilson, U.S. EPA (Wilson et al., 2002). Murine PPAR γ ligand binding domain coupled to the Gal4 DNA binding domain (DBD) (mPPAR γ -Gal4) and a reporter construct containing an upstream activating sequence (UAS)-linked luciferase, 4xUAS-TK-luc (TK: thymidine kinase), were gifts from Dr Susanne Mandrup (University of Southern Denmark, Denmark) (Taxvig et al., 2012). 3T3-L1 preadipocytes stably transfected with MMTV-Luc were generated by selecting the stably expressing individual clones with G418 resistance. For transient transfection, 3T3-L1 preadipocytes were seeded the day before and transiently transfected with mPPAR γ -Gal4, 4xUAS-TK-Luc, and β -galactosidase (β -gal) control plasmid (for monitoring the transfection efficiency) with Fugene HD transfection reagent (Promega, Madison, WI) according to the manufacturer's protocol.

To further define the activation of parabens on GR, COS-7 cells, which have no or little endogenous GR expression, were seeded and transfected with GR or the empty vector together with MMTV-Luc and β -gal control plasmid. The cells were treated as indicated in the figure legends for 18 h, and cell lysates were prepared and the luciferase and β -gal activities were measured using Glomax multidetection system (Promega, Madison, WI).

Glucocorticoid receptor competitor assay.

The competitive binding of parabens for GR was evaluated using the PolarScreen GR competitor assay according to the manufacturer's instructions (Life Technologies, Grand Island, NY) using a multimode microplate reader Synergy 2 (BioTek Instruments, Winooski, VT) (485-nm excitation and 535-nm emission). Briefly, full-length human GR is added to a fluorescent glucocorticoid ligand, Fluormone GS1, in the presence of test compounds in a 96-well plate. When a test compound competes with Fluormone GS1, the GR/Fluormone GS1 complex will not form and Fluormone GS1 will tumble rapidly, resulting in a low polarization value. The decrease in polarization value is used to determine the relative affinity of the test compound(s) for GR.

Statistical analysis.

All data were presented as means $\pm$ SE in the figures. Each experiment was repeated at least three times. Within an experiment, measurements were performed in triplicates. Data were log transformed when appropriate for statistical analysis purposes. Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc.). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls Method or Holm-Sidak method to compare with controls to determine the differences between the treatment groups or time points. The level of significance was set at $p < 0.05$.

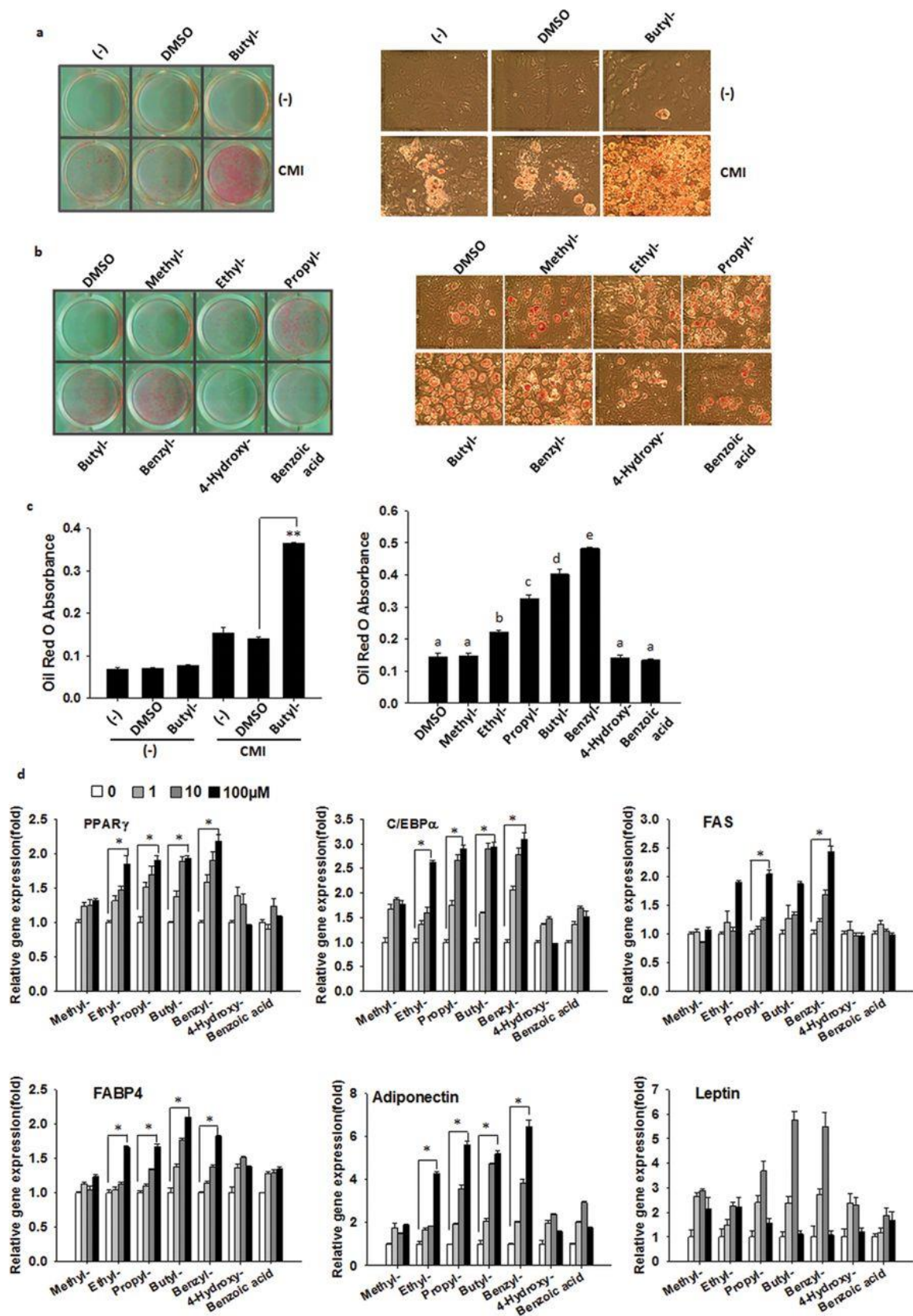
2.5 Results

Parabens Dose-Dependently Promote Murine 3T3-L1 Adipocyte Differentiation

We first examined the effects of parabens on 3T3-L1 adipocyte differentiation, one of the most commonly used in vitro adipogenesis models. The standard 3T3-L1 differentiation cocktail involves using the synthetic glucocorticoid DEX, MIX, and Ins. To better explore the potentiating effects of parabens on differentiation induced by glucocorticoids, we have chosen to use cortisone, a weaker glucocorticoid as previously reported (Kim et al., 2007; Marcolongo et al., 2008; Park et al., 2011). The concentrations of parabens were chosen based on the reported human exposure (Ye et al., 2006) and previous in vitro studies (Byford et al., 2002; Darbre et al., 2002). Confluent 3T3-L1 preadipocytes were induced to differentiate with butylparaben or DMSO, in the presence or absence of the differentiation cocktail that is composed of CMI. Butylparaben, in the presence of CMI, enhanced the 3T3-L1 adipocyte differentiation compared with DMSO, as revealed by the ORO-stained lipid accumulation (Fig. 2.2a, left panel), adipocyte morphologies (Fig. 2.2a, right panel), and ORO absorbance (Fig. 2.2c, left panel). We further compared the potency of various selected parabens and their common metabolite in enhancing the differentiation in the presence of CMI. Parabens enhanced 3T3-L1 adipocyte differentiation with the potency that increases as the length of the linear alkyl chain increases in the following ranking order: methyl- < ethyl- < propyl- < butylparaben. The extension of the linear alkyl chain with an aromatic ring in benzylparaben further augmented the adipogenic ability, as revealed by ORO-stained lipid accumulation and adipocyte morphologies (Fig. 2.2b) and ORO absorbance (Fig. 2.2c, right panel). However, the common metabolite 4-hydroxybenzoic acid or the structurally related benzoic acid (Fig. 2.1) did not have the similar effects (Figs. 2.2b and 2.2c, right panel). The effects of various parabens were further confirmed

Figure 2.2 Parabens promote 3T3-L1 adipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate with butylparaben or DMSO (a) or with various selected parabens, the common metabolite 4-hydroxybenzoic acid or the structurally related benzoic acid (b), in the presence or absence of the differentiation cocktail (cortisone 5 μ M, MIX 0.5 mM, and insulin 170 nM—CMI) during the 7-day differentiation process. Oil Red O (ORO) staining of lipid accumulation (left panel) and adipocyte morphology (right panel) and quantification of ORO absorbance (c) at D7 are shown. (d) 3T3-L1 preadipocytes were differentiated in the presence or absence of increasing doses of selected parabens (1, 10, 100 μ M) during the differentiation process. Relative mRNA expression of adipocyte markers was analyzed. The relative mRNA expression was normalized to 36B4 and expressed as fold of that of the 0 μ M sample (set at 1) for each paraben. Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method for structure-function relationship (c, right panel) or dose response (d) or Holm-Sidak method (c, left panel) to compare with the respective control. Different letters indicate significant difference ($p < 0.05$) (c, right panel). The bar indicates the dose-dependent responses (d). *, **, $p < 0.05$, and $p < 0.01$, respectively. Scale bar = 127 μ m.



by the dose-dependent induction of mRNA expression of adipocyte marker genes, which include the master transcriptional factors, peroxisome proliferator-activated receptor (PPAR) γ , and CCAAT-enhancer-binding protein (C/EBP) α , and the genes involved in lipid metabolism FABP4 and FAS (Fig. 2.2d). Parabens also dose-dependently induced adipocyte-specific adipokine adiponectin mRNA and leptin mRNA (up to 10 μ M for propyl-, butyl-, benzylparaben) (Fig. 2.2d). No cytotoxicity of parabens at concentrations $\leq$ 100 μ M was detected by MTT assays in 3T3-L1 cells (data not shown).

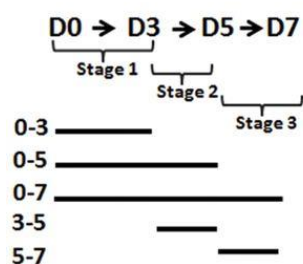
Effects of Exposure Stage to Paraben on 3T3-L1 Adipocyte Differentiation

Standard 3T3-L1 adipocyte differentiation protocol involves stimulating postconfluent cells with differentiation cocktails for 3 days (stage 1, day 0–3) and maintaining them in maintenance media for 2 days (stage 2, day 3–5), followed by basal media for the remainder 2 days until day 7 (stage 3, day 5–7), during which growth-arrested confluent preadipocytes reenter the cell cycle for two rounds of division, known as mitotic clonal expansion (stage 1), followed by postmitotic intermediate stage (stage 2) and the terminal stage (stage 3) (Gregoire et al., 1998). To characterize the effects of parabens on differentiation process, we attempted to pinpoint the critical stage(s) during which parabens promote differentiation. We focused on butylparaben due to both its high adipogenic potency (Fig. 2.2b) and wide use. Confluent 3T3-L1 preadipocytes were induced to differentiate with the differentiation cocktail CMI in the presence of butylparaben or DMSO at indicated time shown in Fig. 2.3a. The exposure of butylparaben in both stages 1 and 2 (days 0–5) induced the strongest adipogenic effects compared with that in either stage 1 (day 0–3) or stage 2 (day 3–5) alone, as revealed by ORO staining (Figs. 2.3b and 2.3c) and mRNA expression of adipocyte-specific markers (Fig. 2.3d). No further enhancement of differentiation was observed in 0–7 group when the cells were exposed to butylparaben for the whole 7-day differentiation

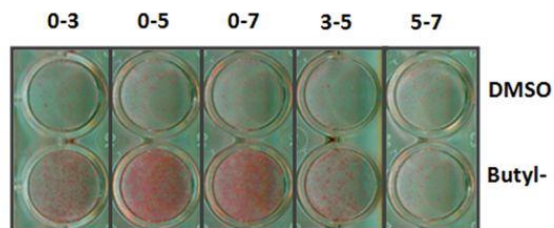
Figure 2.3 The effect of exposure stage to paraben on 3T3-L1 adipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate with the differentiation cocktail CMI in the presence of butylparaben (100 μ M) or DMSO at indicated time shown in (a). Oil Red O (ORO) staining of lipid accumulation (b), quantification of ORO absorbance (c), and relative mRNA expression of adipocyte markers at D7 (d) are shown. The relative gene expression was normalized to 36B4 and expressed as fold of DMSO 0–3 samples (set at 1). Data are the mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method to compare among different treatment groups. Different letters indicate significant difference ($p < 0.05$).

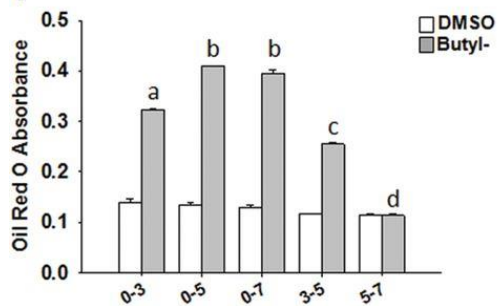
a Differentiation Stages



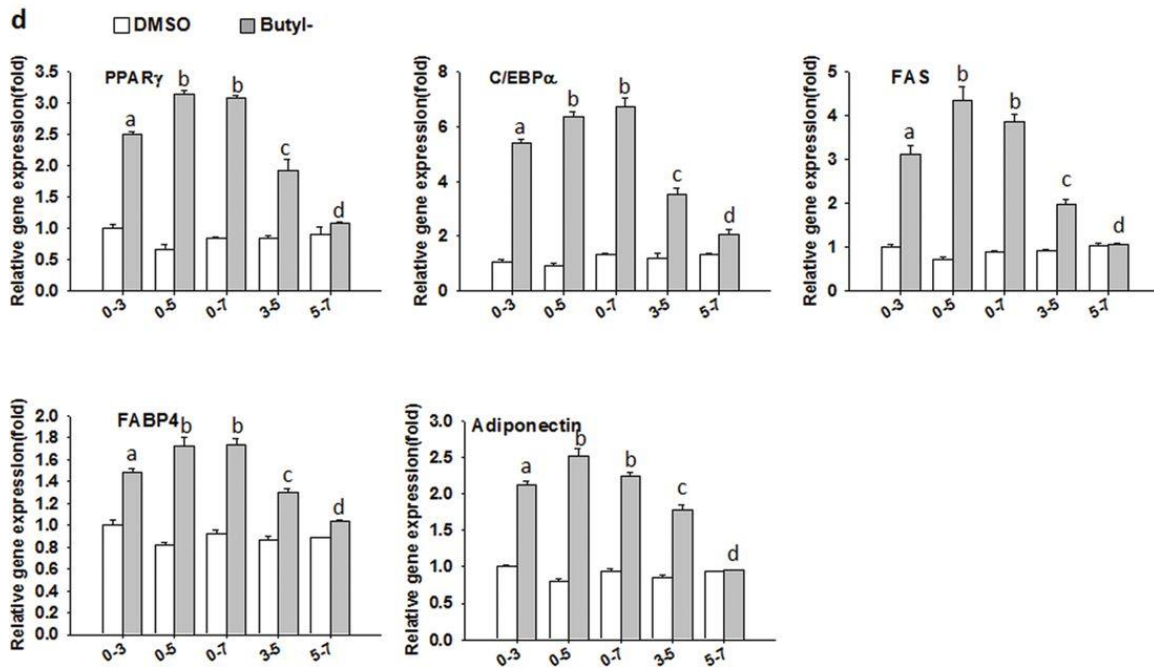
b



c



d



process. The presence of butylparaben in stage 1 was more effective than the presence in stage 2 in promoting differentiation. The exposure of butylparaben in stage 3 (day 5–7) had minimal effects, compared with the DMSO group. These results suggest that butylparaben may modulate the early events of the differentiation process to promote differentiation.

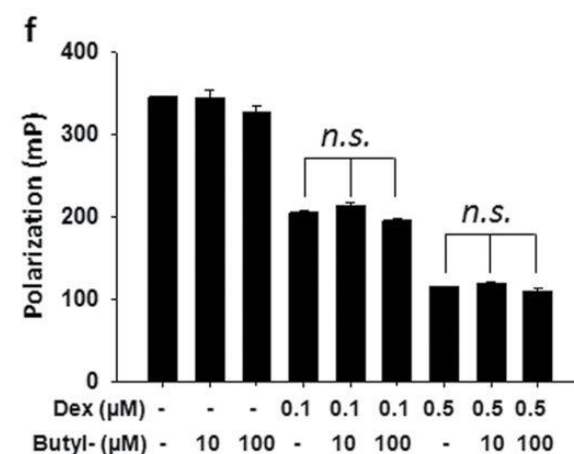
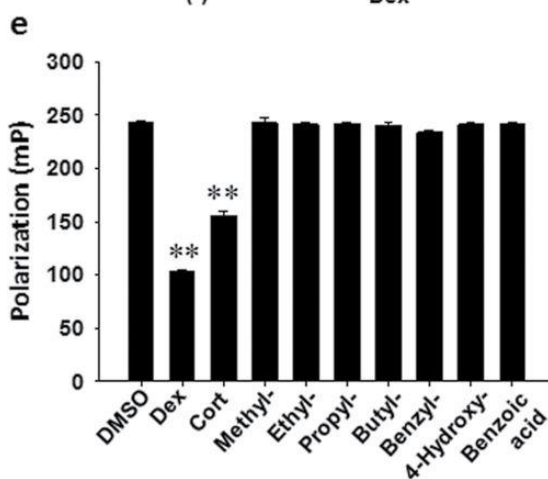
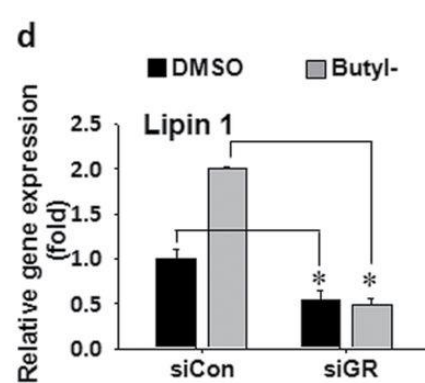
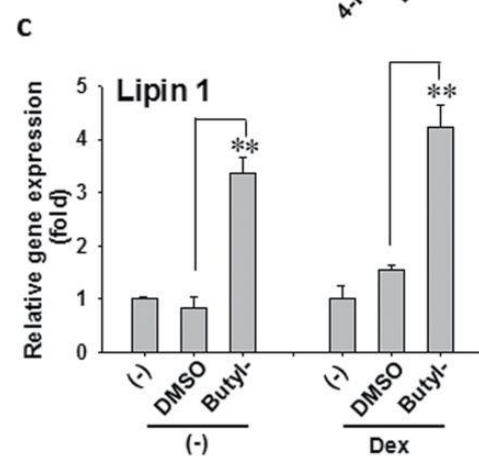
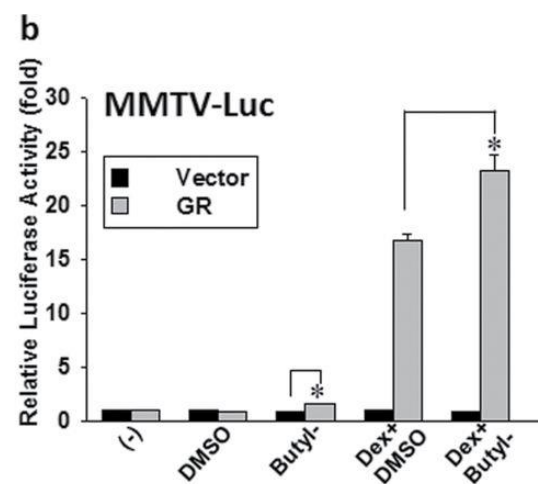
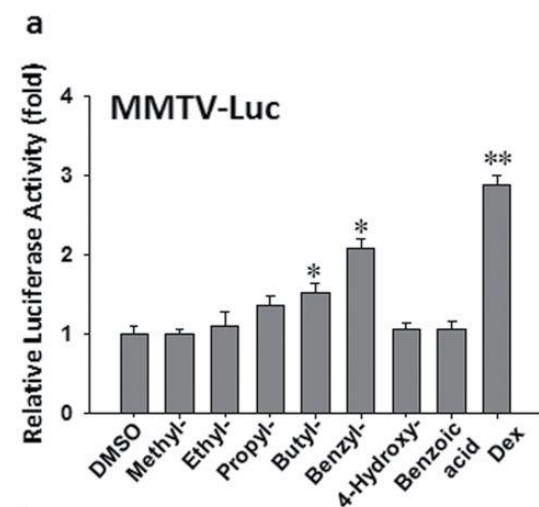
Parabens Activate GR in 3T3-L1 Preadipocytes

Many EDCs have been shown to induce adipocyte differentiation in vitro through activation of GR (Sargis et al., 2010). Therefore, we tested whether parabens have glucocorticoid-like activity using a GR-responsive luciferase reporter (MMTV-Luc). 3T3-L1 preadipocytes stably transfected with MMTV-Luc were treated with selected parabens for 18h. Parabens activated MMTV promoter mediated–luciferase activities, and the potency increased as the length of the linear alkyl chain increased in the following ranking order: methyl- < ethyl- < propyl- < butylparaben in 3T3-L1 preadipocytes (Fig. 2.4a). The extension of the linear alkyl chain with an aromatic ring in benzylparaben further increased the ability to activate the reporter. To better define the interaction of paraben with GR, COS-7 cells, known to have no or little endogenous GR expression (Charmandari et al., 2005; Danielsen et al., 1989; Xu et al., 1996), were transfected with full length of GR or the empty vector, together with MMTV-Luc and the control plasmid β -gal. Transfection of full-length GR, but not the empty vector, conferred the effects of butylparaben alone and the synergistic effects on glucocorticoid ligand Dex-induced GR activation (Fig. 2.4b), demonstrating the activation of GR by the paraben.

To confirm the activation of GR by parabens, we further examined the effects of butylparaben on mRNA expression of the known target gene of GR, lipin 1 (Zhang et al., 2008), in 3T3-L1 cells. Lipin 1 was initially identified as a phosphatidic acid phosphatase-

Figure 2.4 Parabens activate GR reporter and target gene without directly binding to, or modulating, the ligand binding of the receptor.

(a) 3T3-L1 preadipocytes stably transfected with MMTV-Luc were seeded and treated with various parabens (100 μ M) for 18h. (b) COS-7 cells were seeded and transfected with the empty vector or the full length of GR, with MMTV-Luc reporter and β -gal control plasmid for 24h before the cells were treated with butylparaben (100 μ M) or DMSO in the presence or absence of Dex (1 μ M) for 18h. The reporter gene assays were performed. Luciferase activities were normalized with the β -gal activities. (c) Confluent 3T3-L1 preadipocytes were treated with butylparaben (100 μ M) or DMSO in the presence or absence of Dex (1 μ M) for 4h. (d) 3T3-L1 cells were transfected with siRNA targeting GR (siGR) or nontargeting control (siCON) for 24h. The cells were then treated with butylparaben or DMSO in the presence of Dex for 4h. The relative gene expression was normalized to 36B4 and expressed as fold of the respective control (set at 1). (e, f) Human GR/fluormone complex was incubated with various parabens (100 μ M), Dex (1 μ M), or cortisone (5 μ M) (e) or with butylparaben (10, 100 μ M) in combination with Dex (0.1 and 0.5 μ M) (f). Polarization was measured. Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method for structure-function relationship (a) or to compare among different groups (b) or Holm-Sidak method (c–f) to compare with the respective control. *, **, p < 0.05, and p < 0.01, respectively; N.S., not significant.



1 enzyme, which catalyzes the conversion of phosphatidate to diacylglycerol, the immediate substrate for the synthesis of triacylglycerol and other phospholipids (Han et al., 2006). The upregulation of lipin 1 mRNA by Dex (Zhang et al., 2008) is an early event during adipocyte differentiation. Stimulation by butylparaben in the presence or absence of Dex for 4h induced lipin 1 mRNA (Fig. 2.4c). Consistently, the upregulation of lipin 1 mRNA by butylparaben was attenuated by transfection of siRNA targeting GR, but not the negative control (Fig. 2.4d).

EDCs have been shown to modulate GR activity by competing the ligand binding to the receptor (Gumy et al., 2008; Johansson et al., 1998, 2005). To determine whether parabens can bind to the ligand binding domain of GR, we performed the competitive binding assays of parabens with the full-length human GR using PolarScreen GR competitor assays in which the complex of human GR and a tracer glucocorticoid (Fluormone GS1) was incubated with various parabens. No competitive binding of parabens to GR was detected (Fig. 2.4e), as revealed by no decreases in polarization value. In contrast, the positive control Dex (1 μ M) and Cort (5 μ M) showed competitive binding to the GR, as revealed by the decrease in polarization (Fig. 2.4e). To determine whether parabens may be an allosteric modulator of the ligand binding of GR, we performed the competitive binding assays with increasing doses of butylparaben in the presence of low concentrations of Dex (0.1 and 0.5 μ M). As shown in Fig. 2.4f, butylparaben did not appreciably enhance the binding of Dex to the GR at the tested concentrations, as revealed by no further decrease in the polarization values of Dex. Similar results were observed with methylparaben and benzylparaben (data not shown). These results suggest that parabens may not compete for, or allosterically modulate, the ligand binding of GR.

Parabens Activate PPAR γ in 3T3-L1 Preadipocytes

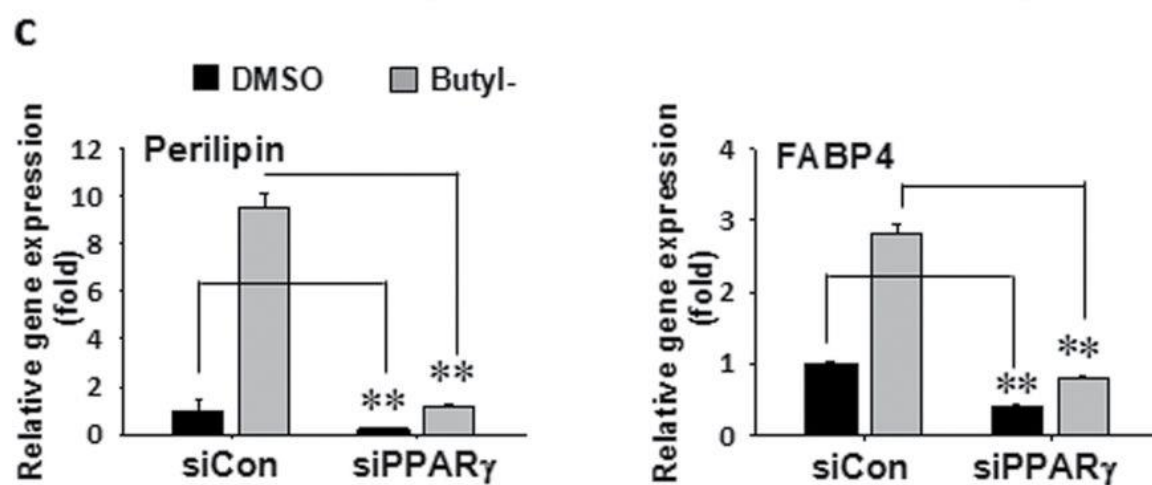
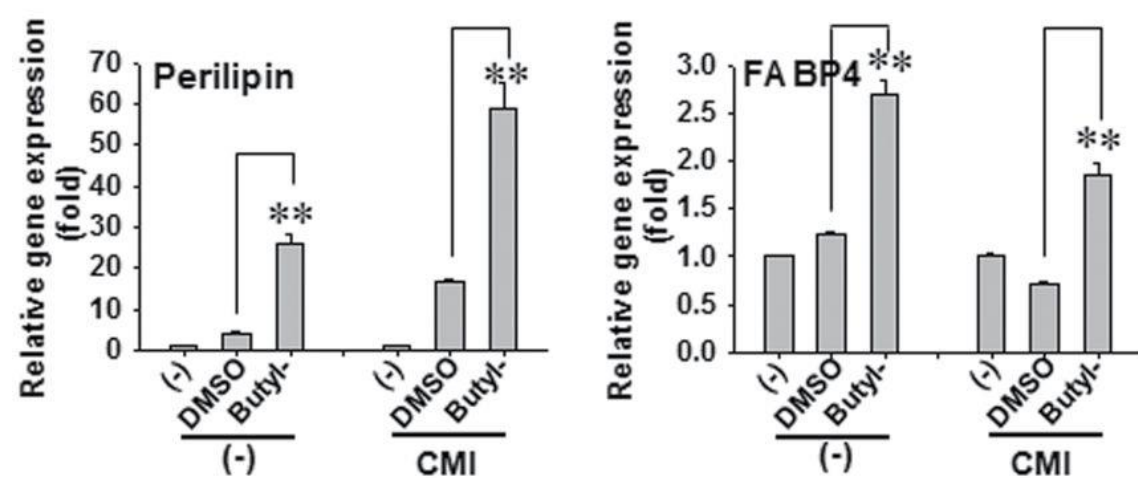
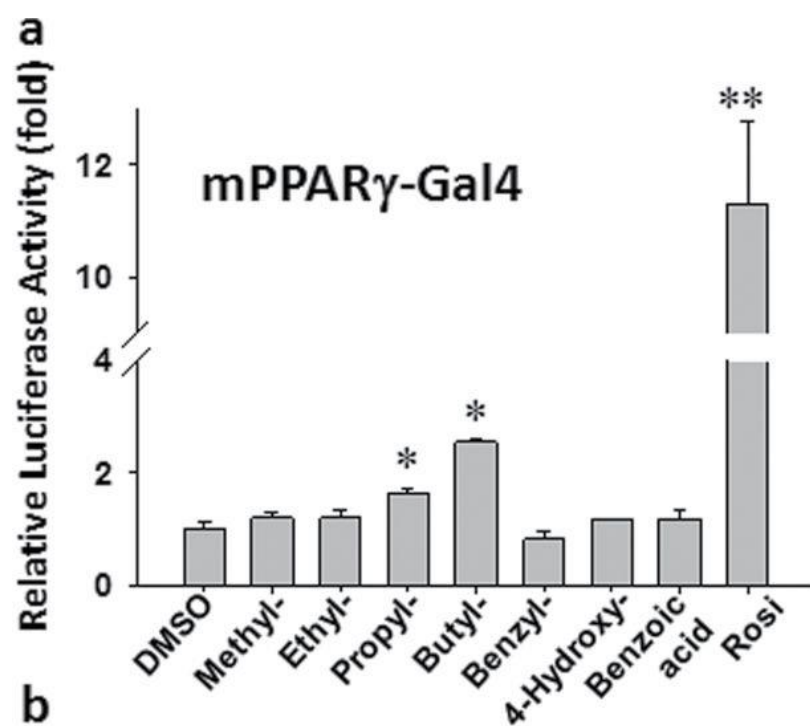
Paraben also has been recently reported to act as a PPAR γ agonist (Taxvig et al., 2012). We tested the effects of parabens on PPAR γ transactivation in 3T3-L1 preadipocytes using the PPAR γ ligand binding domain coupled with the DBD of Gal4 and a reporter containing a UAS-linked luciferase, 4xUAS-TK-luc. As shown in Fig. 2.5a, parabens transactivated PPAR γ , and the potency increased as the length of the linear alkyl chain increased in the following ranking order: methyl- < ethyl- < propyl- < butylparaben. However, benzylparaben, which has the most potent adipogenic effects, did not significantly activate PPAR γ , which may suggest differential mechanisms underlying the adipogenic effects of various parabens.

To confirm the activation of paraben on PPAR γ , we further examined the effects of butylparaben on mRNA expression of the known target genes of PPAR γ . We have focused on PPAR γ target gene perilipin (Arimura et al., 2004) and FABP4 (Frohnert et al., 1999; Martin et al., 2000) in 3T3-L1 cells. Perilipin belongs to a family of proteins found on the surface of lipid droplets in adipocytes and has been suggested to act as regulator of lipolysis (Greenberg et al., 1993; Souza et al., 1998). FABP4 is an adipocyte-specific fatty acid binding protein that controls the transport of fatty acids in adipocytes (Martin et al., 2000). Stimulation of butylparaben in the presence or absence of the differentiation cocktail (CMI) for 24h induced perilipin and FABP4 mRNA, compared with the DMSO group (Fig. 2.5b). Consistently, the upregulation of the mRNA expression of perilipin and FABP4 by butylparaben was attenuated by transfection of siRNA targeting PPAR γ , but not the negative control (Fig. 2.5c). These results further support the fact that the effects of parabens are mediated through PPAR γ .

Figure 2.5 Parabens activate PPAR γ reporter and target genes.

(a) 3T3-L1 preadipocytes were transiently transfected with mPPAR γ -Gal4, 4xUAS-TK-luc, and β -gal for 24h and treated with various parabens (100 μ M) for 18h. The reporter gene assays were performed. Luciferase activities were normalized with β -gal activities.

(b) Confluent 3T3-L1 cells were treated with butylparaben (100 μ M) or DMSO in the presence or absence of the differentiation cocktail CMI for 24h. (c) 3T3-L1 cells were transfected with siRNA targeting PPAR γ (siPPAR γ) or nontargeting control (siCON) for 24h. The cells were treated with butylparaben or DMSO in the presence of CMI for further 24h. The relative gene expression was normalized to 36B4 and expressed as fold of the respective control (set at 1). Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method for structure-function relationship (a) or Holm-Sidak method (b, c) to compare with the respective control. *, **, p < 0.05 and p < 0.01, respectively.



Antagonizing GR or PPAR γ Suppresses the Adipogenic Effects of Parabens

To further confirm whether the adipogenic effects of parabens are through activation of GR or PPAR γ , the effects of PPAR γ and GR antagonists were investigated. 3T3-L1 cells were pretreated with the antagonist of GR (RU-486), PPAR γ (GW9662 and BADGE), or the vehicle control DMSO for 1 h before the cells were cotreated with butylparaben or DMSO and the antagonist in the presence of the differentiation cocktail (CMI). The antagonist was reapplied together with butylparaben at each change of the media during the differentiation process. PPAR γ antagonists GW9662 and BADGE and GR antagonist RU-486 all significantly suppressed butylparaben's effects on adipocyte differentiation, as revealed by attenuated ORO-stained lipid accumulation (Fig. 2.6a) and mRNA expression of specific marker genes (Fig. 2.6b).

We next asked the question whether paraben can replace the glucocorticoid in the standard differentiation cocktail (Dex + MIX + Ins). Confluent 3T3-L1 preadipocytes were induced to differentiate with butylparaben or DMSO in the presence of medium only, MIX + Ins (without Dex), or the standard differentiation cocktail (Dex + MIX + Ins). In the presence of MIX and Ins (without Dex), butylparaben was sufficient to stimulate 3T3-L1 adipocyte differentiation to a level that is comparable to that induced by the standard differentiation cocktail (Dex + MIX + Ins), as revealed by ORO-stained lipid accumulation (Figs. 2.7a and 2.7b) and mRNA expression of adipocyte-specific markers, compared with the respective control (Fig. 2.7c). As expected, butylparaben significantly enhanced the differentiation induced by the standard differentiation cocktail (Dex + MIX + Ins), demonstrating the synergistic effects of paraben on adipogenesis (Figs. 2.7a–c). Butylparaben alone in the absence of MIX and Ins was not sufficient to enhance differentiation (Figs. 2.7a–c).

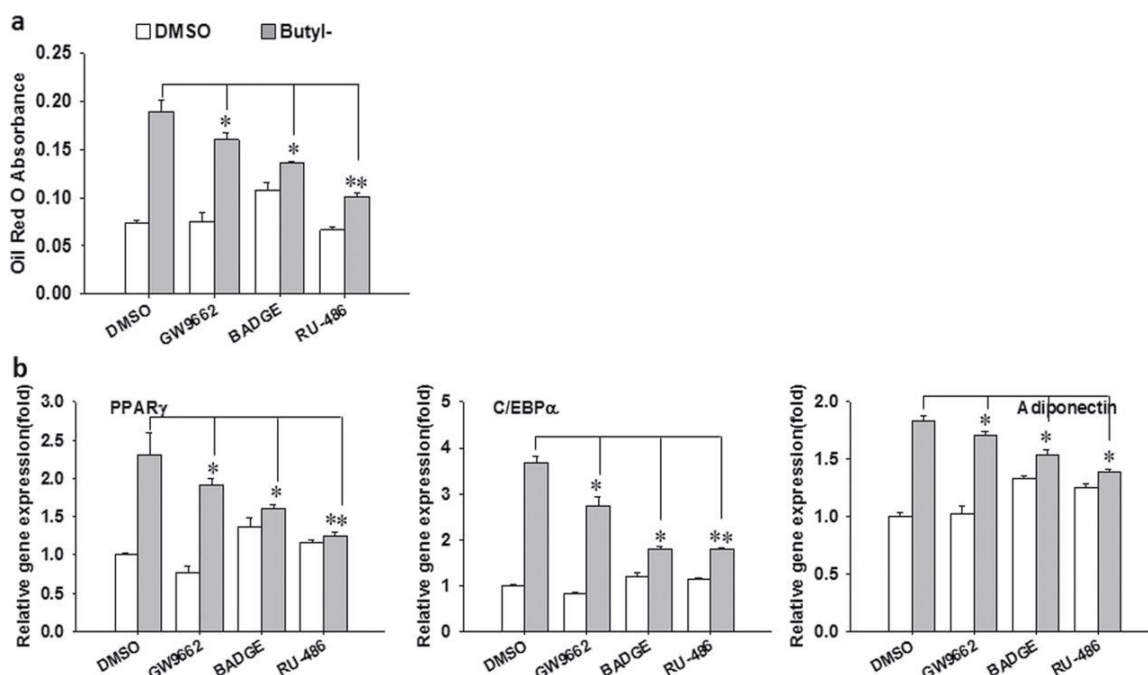
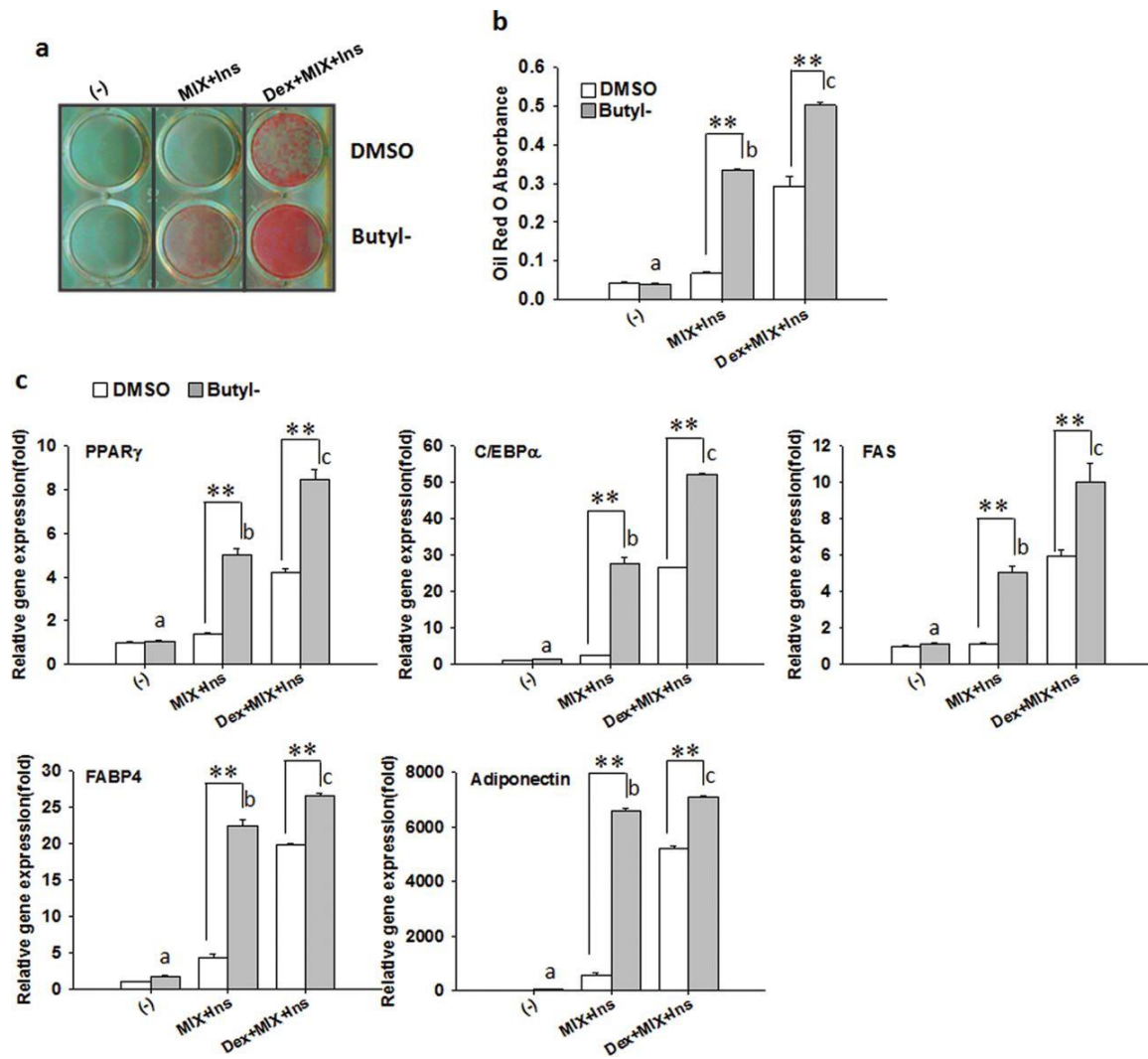


Figure 2.6 The antagonists of GR and PPAR γ attenuate paraben-induced 3T3-L1 differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate with butylparaben (100 μ M) or DMSO in the presence of the differentiation cocktail (CMI), with or without the GR antagonist RU-486 (10 μ M), the PPAR γ antagonist GW9662 (20 μ M), or BADGE (50 μ M) during the process. Quantification of Oil Red O absorbance (a) and relative mRNA expression of adipocyte markers (b) are shown. The relative gene expression was normalized to 36B4 and expressed as fold of the DMSO-treated samples (set at 1). Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Holm-Sidak method to compare the antagonist with the respective control. *, **, p < 0.05 and p < 0.01, respectively.

Figure 2.7 Paraben acts as a glucocorticoid-like compound to promote 3T3-L1 adipocyte differentiation.

Confluent 3T3-L1 preadipocytes were induced to differentiate with butylparaben or DMSO in the presence of the medium only, MIX + Ins (without Dex), or the standard differentiation cocktail (Dex + MIX + Ins). Oil Red O (ORO) staining of lipid accumulation (a), quantification of ORO absorbance (b), and relative mRNA expression of adipocyte markers (c) are shown. The relative gene expression was normalized to 36B4 and expressed as fold of the respective control (set at 1). Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by multiple comparison tests with Student-Newman-Keuls method. Different letters indicate significant difference ($p < 0.05$) among the butylparaben-treated groups. **, $p < 0.01$ versus the respective control.



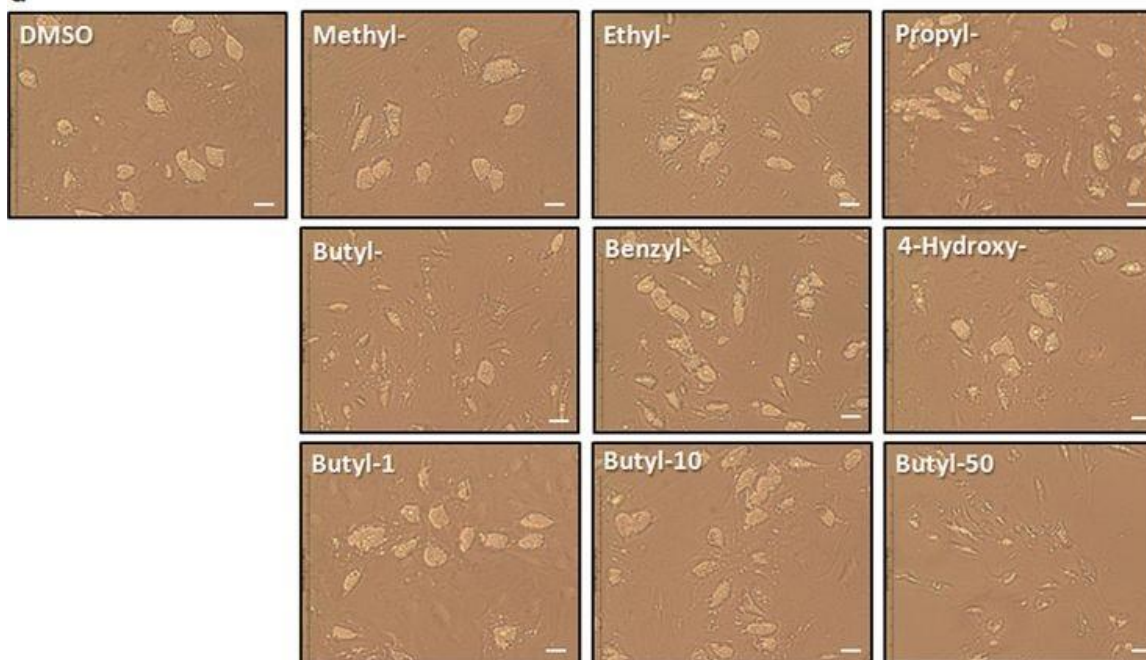
Effects of Parabens on Adipose Conversion of hADSC

To further explore the adipogenic effects of parabens in human cells, we evaluated the effects of parabens on adipose conversion of hADSC. As butyl- and benzylparaben caused cell toxicity when used at 100 μ M (MTT assays, data not shown) in hADSC cells, we differentiated hADSC with various parabens at 50 μ M or DMSO in the presence of the differentiation media, which include Dex, MIX, Ins, and PPAR γ agonist, for 7 days. The cells were then maintained in the adipocyte maintenance media with parabens or DMSO for additional 7 days until the cells were fully differentiated into adipocytes. Among the tested parabens, butyl- and benzylparaben promoted lipid accumulation as early as day 3 and continued throughout the differentiation process, as revealed by the lipids-containing cell morphologies (Fig. 2.8a) and upregulation of adipocyte-specific lipid binding protein FABP4 mRNA (Fig. 2.8b). On day 14, benzylparaben showed the most potent adipogenic effects, as revealed by upregulation of mRNA expression of adipocyte marker genes (FABP4, FAS, and adiponectin), in addition to the lipid-filled adipocyte morphology (Fig. 2.8b). Moreover, all parabens tested significantly suppressed leptin mRNA on day 14. At 50 μ M, butylparaben did not induce mRNA expression of adipocyte marker genes except for FABP4 in hADSC cells. Further dose-response analysis of butylparaben (1, 10, 50 μ M) showed that butylparaben at 1 μ M had the strongest adipogenic effects, as revealed by the lipid-filled adipocyte morphology (Fig. 2.8a) and upregulation of mRNA expression of adipocyte marker genes (PPAR γ , C/EBP α , FABP4, FAS, and adiponectin) (Fig. 2.8c), whereas other parabens showed no significant effects at either 1 or 10 μ M concentrations (data not shown).

Figure 2.8 Parabens promote adipocyte conversion of hADSC.

hADSC cells were differentiated with various parabens (50 μ M) (a, b) or increasing doses of butylparaben (1, 10, 50 μ M) (a, c) in the presence the differentiation media. Human adipocyte morphologies (a) and mRNA expression of adipocyte-specific markers (b and c) are shown. The relative gene expression was normalized to 18S and expressed as fold of the DMSO-treated samples (set at 1). Data are mean $\pm$ SE (n = 3). One-way ANOVA was performed followed by the tests with Holm-Sidak method to compare various parabens or doses with the DMSO group. *, **, p < 0.05, and p < 0.01 versus the DMSO control, respectively. Scale bar = 127 μ m.

a



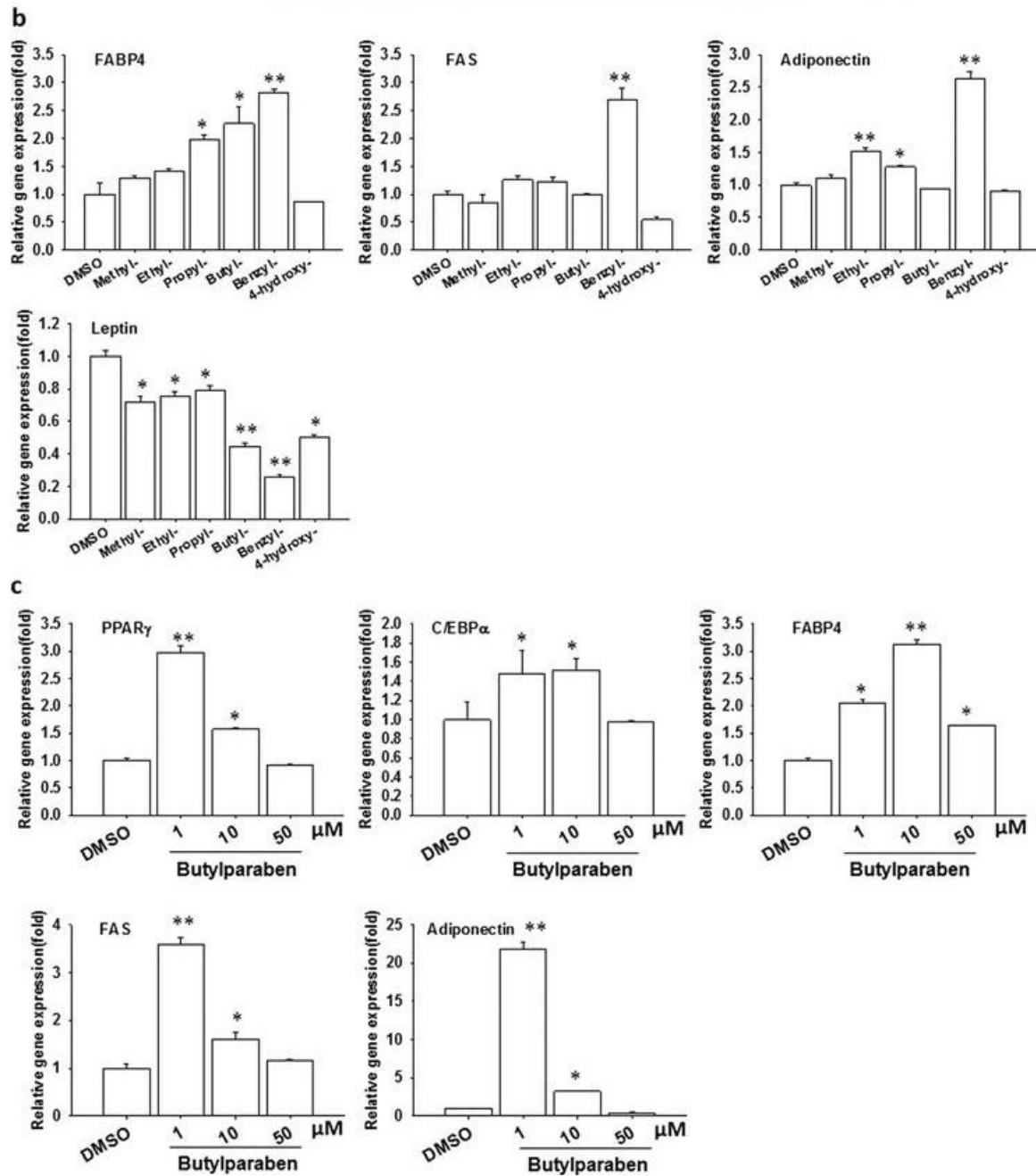


Figure 2.8 Continued

2.6 Discussion

As preservatives, parabens have been widely used in personal care products, pharmaceuticals, and food and beverage processing. The most common parabens used in cosmetic products are methylparaben, propylparaben, and butylparaben; and more than one paraben is often used in a single product (US FDA, 2007). In Europe, up to 0.4% for one ester or a maximal of 0.8% for a mixture of paraben esters is allowed in finished products (EU Cosmetics Directive 76/768/EEC). In the United States, the Cosmetic Ingredient Review (CIR) reviewed the safety of parabens in 1984 and concluded that they were safe to use in cosmetic products at levels up to 25%. Although the CIR began to reopen the safety assessments of parabens in 2003 and 2005, there have been no changes to the original conclusion that parabens are safe as used in cosmetics (U.S. FDA, 2007). As a result, there continues to be a constant exposure of parabens from a wide variety of sources (Darbre and Harvey, 2008).

Despite the wide use and constant daily human exposure, the health impact of parabens has just begun to be examined (Boberg et al., 2010; Darbre and Harvey, 2008). Recent studies have suggested that parabens are EDCs with estrogenic/antiandrogenic activities (Chen et al., 2007; Oishi, 2001, 2002; Shaw and deCatanzaro, 2009; van Meeuwen et al., 2008). Here, we report, for the first time, the adipogenic effects of parabens in both murine 3T3-L1 and hADSC, the two most commonly used in vitro adipogenesis models. Our studies show that parabens, butylparaben and benzylparaben in particular, but not the common metabolite 4-hydroxybenzoic acid or structurally related benzoic acid, promote adipogenesis in vitro in both systems. Moreover, we show that parabens activate GR (without directly binding to or modulating the ligand binding of GR) and/or PPAR γ , thereby promoting adipogenesis.

Several recent studies have demonstrated the potentials of parabens entering into human body in intact and unmetabolized forms. Traditionally, it was believed that parabens are rapidly absorbed from gastrointestinal tract or intact skin followed by ester linkage hydroxylation and glucuronidation or sulfation before being excreted from the urine, which partially contributes to their low toxicity and broad “inertness” (Darbre, 2004). However, Ye et al. have reported the detection of free, unconjugated parabens in majority of urine samples (99% and 96%, for methyl and propylparaben; 58%, 69%, and 39% for ethyl, butyl, and benzylparaben, respectively) collected from humans with no known occupational exposures (Ye et al., 2006). Intact parabens were also detected in normal human placenta and breast tumors (Darbre et al., 2004). Moreover, Janjua et al. have reported the detection of butylparaben in serum of human volunteers who were exposed for 1 week to a cosmetic formulation containing butylparaben and phthalates (Janjua et al., 2007). In fact, the insufficiency of skin esterase to hydrolyze all paraben esters to completion was revealed by the rapid absorption of parabens through the skin even with a single dose of body care product into the human body, and permeation of parabens through human skin increases with repeated doses ex vivo (El Hussein et al., 2007).

Human exposure to parabens appears to be influenced by many other factors (Bando et al., 1997; Soni et al., 2005). The common food components such as flavonoids in grapefruit juice and grape seed extract are esterase inhibitors and have been shown to effectively increase the bioavailability of ester pro drugs into the circulation without being extensively metabolized (Li et al., 2007). Moreover, the presence of penetration enhancers or various surfactants found in personal care products can alter the penetration of parabens and consequently their absorption (Esposito et al., 2003; Komatsu et al., 1986). Furthermore, coexposure to various ester

pesticides and ester pharmaceuticals can also enhance the bioavailability of parabens in the body of the individual as they compete with parabens for esterase (Li et al., 2007). Lastly, interindividual differences in dermal metabolic capacities among humans do exist (Calafat et al., 2010; Jewell et al., 2007). Overall, with wide interindividual variability in exposure levels, these studies support the physiological relevance of our results with intact parabens.

We have attempted to address the molecular mechanisms by which parabens elicit the adipogenic effects. Our results show that the adipogenic potential of paraben increases as the length of linear alkyl chain increases, and the extension of the linear alkyl chain with an aromatic ring in benzylparaben further augments the adipogenic ability. The results are consistent with the abilities of parabens to activate GR-responsive reporter (Fig. 2.4a). GR plays a critical role in adipocyte differentiation. GR signaling is important for inducing the expression of C/EBPs, which, in turn, increases PPAR γ expression, the master regulator of adipogenesis (Farmer, 2006; Gregoire et al., 1998). The activation of GR by parabens has been further supported by the fact that transfection of COS-7 cells (with no or low endogenous GR expression) with full-length GR expression plasmid confers the GR activation by butylparaben with or without Dex (Fig. 2.4b). Moreover, butylparaben induces mRNA expression of the known GR target gene lipin 1 (Zhang et al., 2008) both in the presence and absence of Dex, and the knockdown of GR by siRNA attenuates the upregulation of lipin 1 mRNA by butylparaben (Figs. 2.4c and 2.4d). Furthermore, the GR antagonist RU-486 attenuates the adipogenic effects of butylparaben (Fig. 2.6). Overall, these results suggest that the effects of butylparaben are mediated through the GR signaling pathway.

EDCs have been shown to modulate GR activity by competing with the ligand binding to the receptor. Lund and associates reported that tolylfluanid and

methysulfonyl-PCBs competed with glucocorticoid for binding to GR (Johansson et al., 2005). However, GR competitor assays showed that there was no competitive binding of GR by the parabens, unlike DEX or cortisone (Fig. 2.4e). To this end, some EDCs have also been shown to alter the ligand binding affinity through allosteric effects on the receptor. Dibutyltin, one of the organotins used as stabilizer in the production of polyvinyl chloride plastics, can inhibit GR activation through insertion at an allosteric site near the steroid-binding pocket (Gumy et al., 2008). It is possible that parabens may enhance the binding of glucocorticoids through modulation of the ligand-binding domain of GR. However, further GR competitor assays with the paraben and Dex added together did not support this possibility, suggesting that parabens may not modulate the ligand binding of GR (Fig. 2.4f). On the other hand, some EDCs have been shown to modulate glucocorticoid activation by modulating the glucocorticoid metabolizing enzymes: 11 β -hydroxysteroid dehydrogenase-1 and-2, which catalyze the conversion between cortisone and cortisol (Draper and Stewart, 2005). Although our preliminary results show the induction of 11 β -hydroxysteroid dehydrogenase-1 by parabens as the differentiation proceeds, the effects of parabens on 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD1) mRNA expression do not seem to fully explain the results of parabens on GR activation and adipogenesis as there is no clear trend of induction of 11 β -HSD1 mRNA with increasing length of the linear alkyl chain of parabens (data not shown). Moreover, even with charcoal-stripped serum with minimal endogenous glucocorticoids, parabens still activate GR in GR-responsive reporter assays (data not shown), and butylparaben can substitute DEX to induce marked differentiation when it is combined with MIX and Ins (Fig. 2.7), suggesting that 11 β -HSD1 mRNA upregulation may not be the main mechanism underlying the adipogenic effects of parabens. Further studies are needed to elucidate the mode of action of parabens on GR, thereby promoting adipogenesis.

Consistent with the recent report by Taxvig et al. (2012), our results show that parabens transactivate PPAR γ (Fig. 2.5a). Consistently, we show that butylparaben induces the expression of target genes (e.g., perilipin and FABP4), and the upregulation of the target genes by butylparaben is attenuated by siRNA targeting PPAR γ (Figs. 2.5b and 2.5c). Moreover, PPAR γ antagonists (GW9662 and BADGE) suppress the adipogenic effects of butylparaben in 3T3-L1 cells (Fig. 2.6). Interestingly, benzylparaben, which shows the potent adipogenic effects, does not activate PPAR γ in the transactivation assay. Why benzylparaben does not transactivate PPAR γ , yet still has a strong adipogenic effect, is currently unknown. However, it has been reported that some EDCs induce 3T3-L1 adipocyte differentiation in a PPAR γ -independent manner (Chamorro-Garcia et al., 2012). We have observed that benzylparaben induces the greatest activation of GR as assessed by MMTV promoter–linked luciferase (Fig. 2.4a). Therefore, it is possible that the adipogenic effects of parabens may be mediated through multiple nuclear receptors.

Taken together, our results suggest that parabens may activate multiple nuclear receptors, thereby promoting adipogenesis. Future studies are necessary to reveal parabens' effects on other endogenous adipogenic signals.

Information generated from preadipose cell lines and primary preadipocytes suggests that the committed preadipocytes have to go through growth arrest and mitotic clonal expansion, leading to the clonal amplification of committed cells in early stage, to intermediate stage and then to terminal differentiation, when the cells take on morphology and functions of mature adipocytes (Gregoire et al., 1998). The fact that parabens show more potent adipogenic effects when treated at early stage (stage 1, day 0–3) suggests that parabens may affect these early events necessary for differentiation.

Further studies are needed to elucidate the mechanisms of parabens' action during early stage of adipocyte differentiation.

In addition to the adipogenic effects, parabens are also shown to modulate mRNA expression of adipokines, adiponectin and leptin during differentiation. Parabens upregulate adiponectin mRNA expression in both 3T3-L1 adipocytes and the primary adipocytes derived from human adipose multipotent stromal cells, consistent with the potentiating effects of parabens on differentiation. In contrast, parabens suppress leptin mRNA in human primary adipocytes (Fig. 2.8b) but differentially increase leptin mRNA in 3T3-L1 adipocytes (Fig. 2.2d). However, the reasons for parabens inducing differential effects on leptin mRNA in the two cell models are currently unknown. Leptin plays important roles in regulating food intake and metabolic and endocrine functions (Stofkova, 2009). It has been reported that rats exposed to butylparaben at 100-mg/kg body weight in utero from gestation day (GD) 11–20 had low serum leptin levels at GD 21, compared with the controls, suggesting a role of butylparaben in the developmental programming of the metabolic system (Boberg et al., 2008).

In summary, parabens, butylparaben and benzylparaben in particular, promote adipogenesis in vitro in both murine 3T3-L1 cells as well as hADSC. The adipogenic effects of parabens are mediated through GR and/or PPAR γ . Future studies are required to delineate the molecular mechanisms by which parabens act on adipogenesis in vitro and to define the parabens' action in vivo.

2.7 References

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CHAPTER III

POST-WEANING EXPOSURE TO PARABENS INCREASE ADIPOSITY AND PROMOTE DIFFERENTIATION OF MULTIPOTENT MESENCHYMAL STEM CELLS TOWARDS ADIPOSE LINEAGE

A version of this chapter will be submitted by Pan Hu, Rebekah Kennedy, Xin Chen, Jiangang Chen, and Ling Zhao under the title, “Post-weaning Exposure to Parabens Increase Adiposity and Promote Differentiation of Multipotent Mesenchymal Stem Cells Towards Adipose Lineage”.

3.1 Abstract

Paraben esters and their salts are widely used as preservatives in cosmetics, personal care products, pharmaceuticals, and foods. Human exposure to parabens has been confirmed. It has been reported that parabens promote adipogenesis *in vitro*. In this study, we investigated the effects of post-weaning exposure to parabens (methylparaben and butylparaben) on metabolic biomarkers in female obesity prone C57B6/J mice fed with a chow diet with low phytoestrogens or a high fat diet (45% kcal from fat). We also examined the effects of parabens on modulating cell fate of a multipotent stem cell line C3H10T1/2. Methylparaben exposure by daily oral gavage (100 mg/kg/day) increased body weight, body fat mass, adiposity, and serum adiponectin and leptin levels in mice fed with the chow diet, but not in mice fed with the high fat diet. Butylparaben exposure only increased fasting glucose levels in mice fed with the chow diet, but had no effects in mice fed with the high fat diet. Moreover, both parabens modulated adipogenic, osteogenic, and chondrogenic differentiation of C3H10T1/2 cells *in vitro*. Butylparaben markedly promoted adipogenic differentiation, but suppressed osteogenic and chondrogenic differentiation whereas methylparaben showed similar effects but to a much less extent. Collectively, these results suggest that parabens, methylparaben in particular, increase adiposity under the chow diet, possibly through modulation of multipotent stem cell fate towards adipose lineage at the expense of osteoblast and chondrocyte lineage. Our studies have provided rationales to study paraben exposure at a range of doses and during the perinatal period, a presumably more sensitive window of development, in the future.

3.2 Keywords

Paraben, Endocrine disrupting chemical, Adiposity, C3H10T1/2, Multipotent mesenchymal stem cell, Obesity.

3.3 Introduction

Paraben esters and their salts are widely used as preservatives in cosmetics, personal care products [1], pharmaceuticals, and foods [2]. Systemic human exposure to parabens has been confirmed; parabens have been detected in human serum [3,4], milk [5], placental tissues [6], seminal plasma [4], and breast tumors [7]. The extent of this exposure is reflected by the frequent detection of free and conjugated forms of parabens in urine samples in the general population [8-10].

The detection of parabens in human breast milk and placenta [6,5] has raised the concerns about impact of perinatal exposure to parabens on human health. It has been reported that when pregnant rats were exposed to ethylparaben and butylparaben, the concentrations of both free and conjugated parabens were higher in the embryonic fluid, fetal carcass and liver than in the maternal plasma, indicating that parabens can cross the placenta, cumulating in the fetuses [11].

The health implications of paraben exposure have mostly been from *in vitro* and animal studies. Using human reporter cell lines, parabens, including butyl-, propyl-, and ethylparaben, can activate estrogen receptors alpha and beta (ER α and ER β) [12]. At concentrations detected in the human breast tumors, parabens enhanced colony growth of a human breast cancer cell line, suggesting a role of parabens in the development of breast tumor [13]. Exposure to parabens have been shown to be uterotrophic in immature or ovariectomized female mice and rats [14] and lead to decreased sperm counts and testosterone levels in male mice and rats [15-17], suggesting that parabens are estrogenic/antiandrogenic *in vivo*, although the interpretation of results are still under debate [18].

Several studies [19-21] have shown that parabens also activate nuclear receptors, PPAR γ in particular, which is one of the properties shared by many known

EDCs with metabolic disruption capabilities [22,23]. Indeed, we have reported [20] and later confirmed by others [21] that parabens promote adipocyte differentiation of 3T3-L1 cells and primary culture derived from human adipose tissue and that the effects may be mediated through activation of PPAR γ and/or glucocorticoid receptor (GR). Recently, a study of 520 mother-son pairs from French EDEN cohort reported that concentrations of measured parabens (methyl-, ethyl-, propyl-, and butylparaben) in the mother's urine during pregnancy were positively associated with weight growth between the third ultrasound exam and birth, and with birth weight. The concentrations of methylparaben were also positively related to weight and abdominal circumference at 36 months [24]. These results suggest that early life exposure to parabens could potentially contribute to weight gain.

Adipocytes are derived from common multipotent mesenchymal stem cells that also give rise to osteocytes and chondrocytes [25]. Some EDCs are able to sensitize multipotent stem cells to undergo adipogenesis, but suppress their osteoblast differentiation [26]. The adipogenic effects of these EDCs were blocked by PPAR γ antagonist [26].

In this study, we investigated effects of post-weaning exposure to parabens on metabolic biomarkers in mice fed with either a chow or a high fat diet. We have chosen methylparaben and butylparaben, since not only they are commonly used, but also they have the shortest and longest linear alkyl side chain, showing the weakest and strongest adipogenic activities *in vitro* [20]. We further investigated the effects of methylparaben and butylparaben on modulating cell fate using a multipotent stromal stem cell line C3H10T1/2. We report here that post-weaning exposure to methylparaben increased body weight, body fat mass, and adiposity under a chow diet with low phytoestrogens. However, the effects were diminished under a high fat diet. Butylparaben exposure

showed only marginal effects. Moreover, both parabens modulated multipotent stem cell fate *in vitro*. Butylparaben markedly promoted adipogenic differentiation, but suppressed osteogenic and chondrogenic differentiation of C3H10T1/2 cells whereas methylparaben showed similar effects, but to a much less extent.

3.4 Materials and methods

Reagents

Methylisobutylxanthine (MIX), dexamethasone (DEX), insulin (Ins), peroxisome proliferator-activated receptor gamma (PPAR γ) agonist rosiglitazone were purchased from Sigma-Aldrich (St. Louis, MO). Methyl-, butylparaben ($\geq 99\%$) and dimethyl sulfoxide (DMSO) were purchased from Acros Organics (Thermo Fisher Scientific, Pittsburg, PA). For animal treatment, parabens were dissolved in absolute alcohol at a concentration of 1.0 g/ml followed by dilution with corn oil to reach the final concentration of 10 mg/ml. The mice were then given 10 μ l/g of body weight. For cell culture, parabens were dissolved in DMSO at a stock concentration of 100 mM. The working concentration of parabens was 100 μ M, which was obtained by diluting the stock with the culture medium (final concentration of DMSO was 0.1%).

Animal husbandry and paraben treatment

Female C57BL/6J mice (~3 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice were acclimated in our animal facility with 12 h light/dark cycle for one week. Following acclimation, mice were lined up based on body weight and were randomly assigned to the vehicle control (corn oil), methylparaben, or butylparaben group (n=8 per group) so that each group had a similar average body weights. Mice were orally gavaged daily for 12 weeks with either corn oil, methylparaben, or butylparaben at 100 mg/kg/day, a level that is 10 times of the current recommended acceptable daily intake (ADI) for the sum of methylparaben, ethylparaben

and their sodium salts by European Food Safety Authority (EFSA). During treatment, the mice were given ad libitum access to water and either a chow diet with minimal natural phytoestrogens (2020X) or a high-fat diet (45% kcal from fat, TD.120059) (Harlan laboratories, Madison, WI). Mice were terminated at 16 weeks of age. At termination, whole blood was collected by cardiac puncture and mice were euthanized by CO₂. Various fat pads and the liver were isolated, weighed, and stored for histological and gene expression analysis. The animal procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Knoxville.

Plasma and Serum measurements

Plasma insulin was measured using Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL). Serum leptin and adiponectin were measured using Mouse Leptin ELISA Kit (Crystal Chem, Downers Grove, IL) and Mouse Adiponectin Quantikine ELISA Kit (R&D systems, Minneapolis, MN), respectively. Serum triglyceride was measured using kits from Wako (Wako Chemical USA, Richmond, VA).

Glucose Tolerance and Insulin Tolerance Tests (GTT and ITT)

The mice were given the GTT at 14 weeks and ITT at 15 weeks of age. For GTT, the mice were orally given a dextrose solution at 2 g/kg after an overnight fast. For ITT, the mice were intraperitoneally (ip) injected with insulin (Humulin® R) (Lilly, Indianapolis, IN) at 0.75 U/kg after a 5 h fast. The blood glucose was measured at 0, 15, 30, 60, 90, 120 min post the treatment with a handheld glucometer (LIFESCAN, Milpitas, CA).

C3H10T1/2 cell culture and differentiation

Multipotent stem cell line C3H10T1/2 (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO) in 5% CO₂, 37°C environment until confluence. Cells were differentiated into adipocytes, osteocytes,

Table 3.1 Ingredients of diets

Diet	2020X		TD. 120059	
Formula	Ingredient	g/kg	Ingredient	g/kg
	Crude Protein	191	Casein	195.0
	Fat (acid hydrolysis)	6.5	L-Cystine	3.0
	Carbohydrate		Sucrose	340.0
	(available)	470	Corn Starch	56.86
	Crude Fiber	27	Maltodextrin	60.0
	Neutral Detergent	123	Anhydrous Milkfat	210.0
	Fiber	51	Corn Oil	20.0
	Ash		Cellulose	50.0
			Mineral Mix, AIN-93G-MX (94046)	43.0
			Vitamin Mix, AIN-93-VX (94047)	19.0
			Choline Bitartrate	3.0
			TBHQ, antioxidant	0.04
			Green Food Color	0.1
<i>Selected Nutrition Information</i>				
	% by weight	% kcal from	% by weight	% kcal from
Protein	26.3	24	17.3	14.8
Carbohydrate	65.9	60	47.6	40.6
Fat	7.8	16	23.2	44.6

or chondrocytes in the presence or absence of methylparaben or butylparaben (100 μ M) as follows:

Adipogenic differentiation C3H10T1/2 cells were induced to differentiate using the standard 3T3-L1 differentiation protocol [27]. Briefly, on the day that the cells reached confluence (designated as day 0 or D0), cells were induced to differentiate with differentiation DMEM medium containing 10% FBS, 10 μ g/ml insulin, 1 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for 4 days. Cells were then switched to the maintenance DMEM containing 10% FBS and 10 μ g/ml insulin for an additional 2 days followed by DMEM medium with 10% FBS for additional 2 days before the cells were harvested for analysis or oil red O staining. Parabens or the vehicle control (0.1% DMSO in culture medium) were applied at the induction and were reapplied at each change of the medium.

Osteogenic differentiation C3H10T1/2 cells were differentiated into osteocytes as described [28]. Briefly, the cells were plated at a density of $2 \times 10^4/\text{cm}^2$, medium was replaced by fresh medium supplemented with 200 ng/ml of hBMP7 (R&D systems, Minneapolis, MI) 24 h later. Fresh medium with hBMP7 was replenished every two days until day 12. Parabens or the vehicle control (DMSO) were applied at the induction with hBMP7, and was reapplied at each change of the medium.

Chondrogenic differentiation C3H10T1/2 cells were differentiated into chondrocytes in a high-density micro-mass culture as described [29]. Briefly, C3H10T1/2 cells were trypsinized and resuspended in Ham's F12K medium (CORNING, Tewksbury, MA) containing 10% FBS at a concentration of 10^7 cells/ml, and 10 μ l drop of cell suspension were placed in the center of a well of 24-well tissue culture plates. Cells are allowed to adhere for 1-2 h at 37°C under 5% CO₂, then 1 ml of medium containing 100 ng/ml of hBMP2 (R&D systems, Minneapolis, MI) was added to the culture. Medium was

replenished every two days until day 6. Parabens or the vehicle control (DMSO) were applied at the induction with hBMP2, and was reapplied at each change of the medium.

RNA preparation and quantitative real-time PCR analysis

At indicated times, total RNA was prepared from C3H10T1/2 cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instruction. Total RNA abundance was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. mRNA expression of various genes, and the loading control 36B4 were measured by ABI 7300HT quantitatively using gene-specific primers and SYBR master mix (Thermo Fisher Scientific). Cycle conditions were 50°C 2min, 95°C 15 min, then 40 cycles of 95°C for 15 s/60°C for 1 min.

Oil red O (ORO) staining and quantification

Lipid accumulation in differentiated adipocytes were stained with oil red O (ORO) and quantified by ORO absorbance, as previously described [20].

Alkaline Phosphatase (ALP) staining

C3H10T1/2 cells that have undergone osteocyte differentiation were washed with PBS and fixed with enough neutral buffered formalin (10%). After 60 seconds, the fixatives were removed and the cells were washed with washing buffer (PBS containing 0.05% Tween 20). The cells were then covered by enough BCIP/NBT substrate solution (Thermo Fisher Scientific, Rockford, IL) and incubated at room temperature in the dark for 5-10 min. The staining progression was checked every 2-3 min. When appropriate staining was achieved, the substrate solution was removed and the cells were washed and stored under PBS.

Alcian blue staining and quantification

Chondrogenic differentiation was assessed as described [30]. Briefly, C3H10T1/2 cells that have undergone chondrogenic differentiation were rinsed twice with PBS, and fixed in 4% (w/v) paraformaldehyde for 15 min. 1% (w/v) Alcian blue 8-GX (Sigma) in 0.1 N HCl (pH 1.0) was applied to stain cells overnight. For quantitative analysis, 6 M guanidine HCl were used to recover the Alcian blue staining overnight at room temperature. Absorbance at 650 nm in the extracted dye was read in Glomax multidetection system (Promega, Madison, WI).

Statistical analysis

All data were presented as means $\pm$ SEM of each group (animal study) or triplicates (in vitro study). Statistical analysis was performed using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA) with unpaired student's t test. The level of significance was set at $p < 0.05$.

3.5 Results

Effects of post-weaning exposure to parabens on metabolic biomarkers in mice fed with a chow diet with low phytoestrogens.

It is known that biological activities of EDCs in animal models are associated with many factors, such as strain and sex of animals examined, type of diet used and route of exposure [31-33]. In our studies, female obesity prone C57B6/J mice were orally gavaged for 12 weeks with the vehicle control (corn oil), methylparaben, or butylparaben at 100 mg/kg/day from weaning (4 weeks of age). We first investigated the effects of exposure under a chow diet with low phytoestrogens. Methylparaben-exposed mice were a little heavier than the control group and butylparaben-exposed mice, starting from one week of exposure. This trend continued until the end of the study at 16 weeks old. At termination, methylparaben-exposed mice were modestly but significantly heavier

(20.2 ± 0.2 g) than the controls (19.4 ± 0.3 g) ($p < 0.05$); whereas there were no significant differences in body weight between butylparaben-exposed mice (19.0 ± 0.3 g) and the controls (Fig. 3.1A). Further analysis revealed that methylparaben-exposed mice had a significant increase in total body fat mass by weight (0.82 ± 0.09 g) ($p < 0.05$) and adiposity, as defined by adiposity index, i.e., percentage fat by weight (4.20 ± 0.46 %), ($p < 0.05$) compared with those of the controls (0.51 ± 0.05 g; 2.70 ± 0.25 %); no differences were observed between butylparaben-exposed mice and the controls in either body fat mass (0.54 ± 0.03 g) or adiposity (2.97 ± 0.15 %) (Fig. 3.1A).

Fasting glucose levels were not changed by methylparaben exposure (110 ± 3 mg/dL), but were significantly elevated in butylparaben-exposed mice (141 ± 7 mg/dL) ($p < 0.05$), compared with the controls (115 ± 6 mg/dL) (Fig. 3.1B). No significant differences were observed in plasma insulin, or serum triglyceride levels among the groups. In contrast, mice exposed to methylparaben had elevated adiponectin (17.04 ± 0.2 μ g/ml) ($p < 0.05$) and leptin concentration (5.50 ± 0.50 ng/ml) ($p < 0.01$) compared with the controls (15.88 ± 0.44 μ g/ml for adiponectin; 2.74 ± 0.51 ng/ml for leptin). No differences were observed between butylparaben-exposed mice (15.8 ± 0.44 μ g/ml for adiponectin; 3.96 ± 0.55 ng/ml for leptin) and the controls (Fig. 3.1B).

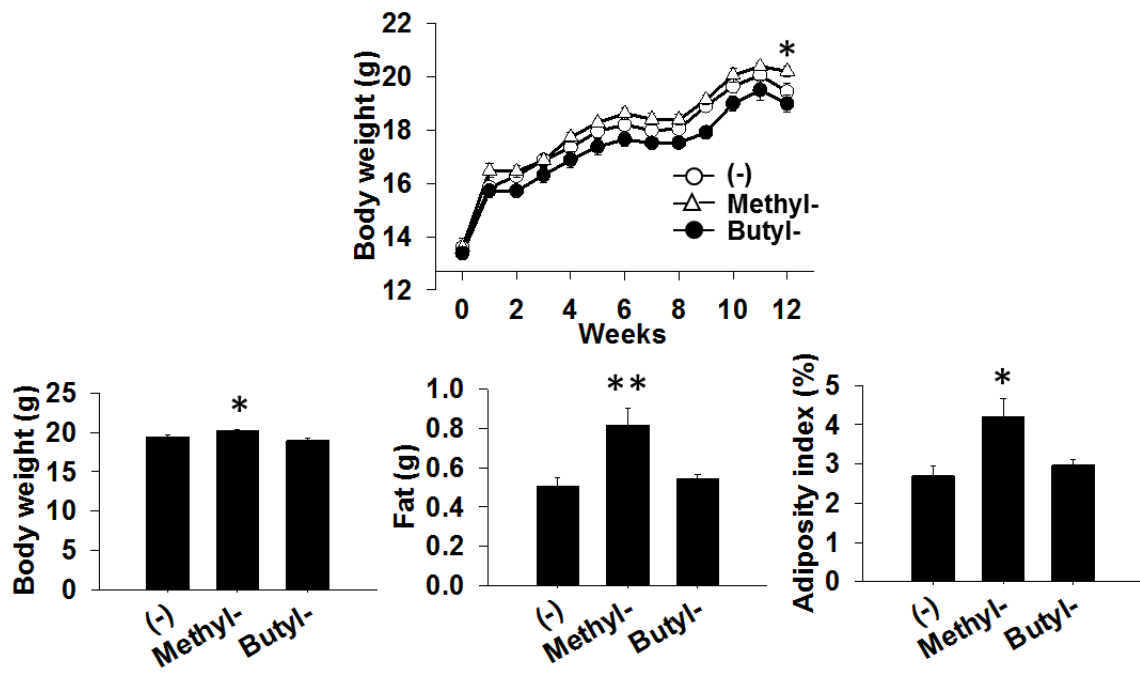
To further evaluate the glucose and insulin sensitivities, we performed glucose (GTT) and insulin tolerance tests (ITT). There were no significant differences among groups when area under the curves (AUC) were calculated and compared (Fig. 3.1C and 3.1D).

We further analyzed gene expression in white adipose tissue (WAT) and liver. Methylparaben-exposed mice had significantly increased mRNA expression of lipogenic marker FAS, SCD, and lipin 1 and leptin mRNA, but decreased adiponectin mRNA in the WAT (Fig. 3.2A). Butylparaben-exposed mice, on the other hand, had markedly

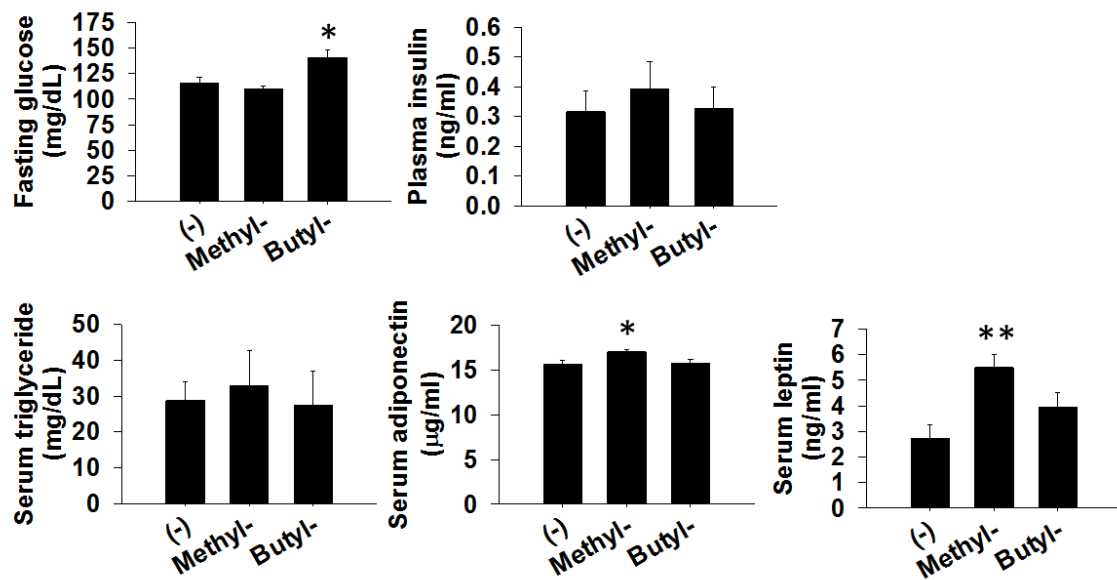
Figure 3.1 Effects of post-weaning exposure to parabens on body weight, body fat mass, adiposity and other metabolic biomarkers in mice fed with a chow diet with low phytoestrogens.

Female C57B6/J mice were orally gavaged with the vehicle control, methyl- or butylparaben from weaning (4 weeks of age) for 12 weeks. Body weights were measured every week and at termination, whole blood was collected and white fat pads were isolated and weighed. (A) Body weights by week, body fat mass, adiposity index (body fat percentage) are shown. (B) After overnight fasting at termination, blood glucose, plasma insulin, serum triglyceride, adiponectin and leptin are shown. (C) Glucose tolerance tests and calculations of area under the curve (AUC). (D) Insulin tolerance tests and calculations of AUC. Data are mean $\pm$ SEM (n=8). *, **, $p < 0.05$ and $p < 0.01$ versus the controls, respectively.

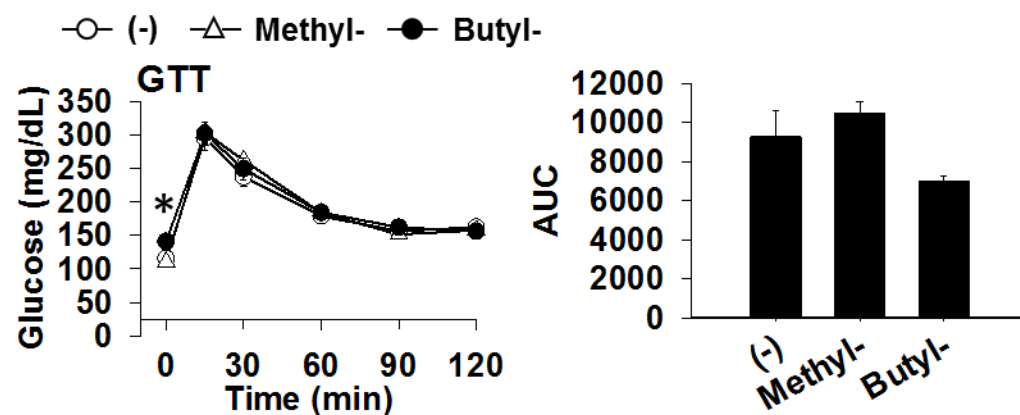
A Chow Diet



B



C



D

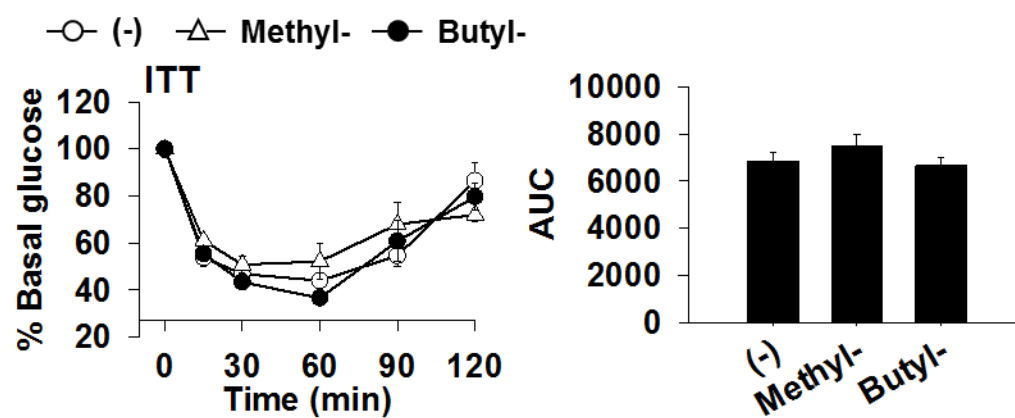


Figure 3.1 Continued

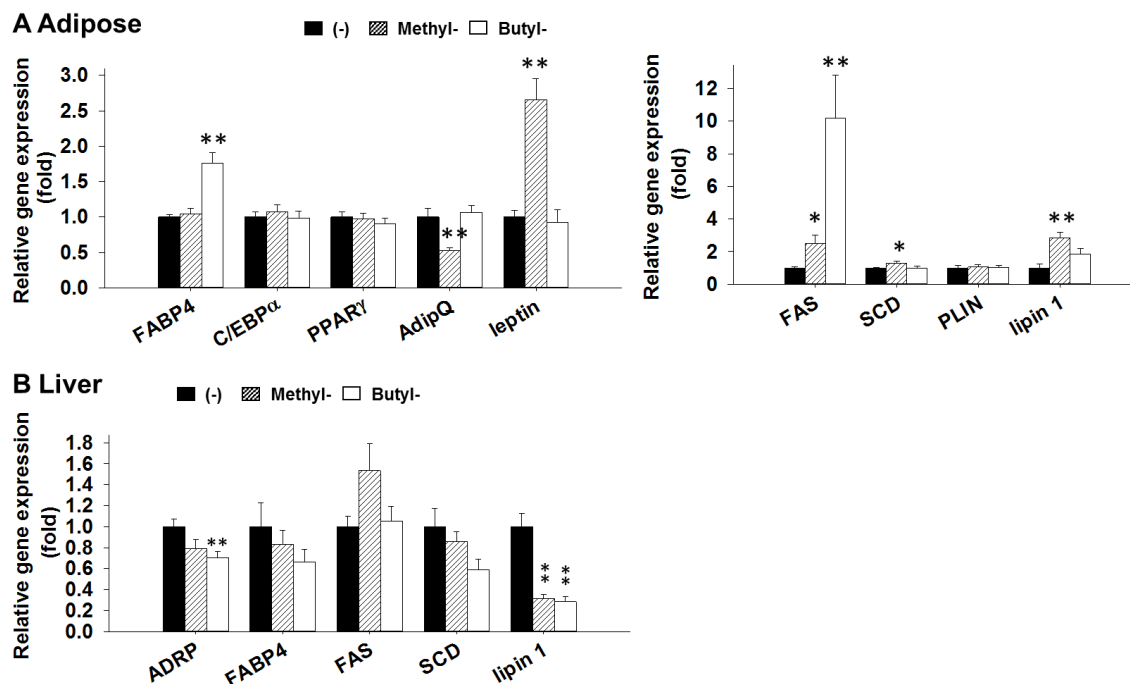


Figure 3.2 Effects of post-weaning exposure to parabens on gene expression in the WAT and liver in mice fed with the chow diet.

(A) The WAT and (B) liver samples were isolated after termination, and were analyzed for relative gene expression. Expression was normalized to 36B4 and expressed as fold of the controls. Data are mean $\pm$ SEM (n=8). *, **, p<0.05, and p<0.01 versus the controls, respectively.

increased mRNA expression of FABP4 and FAS in the WAT (Fig. 3.2A). We also examined lipogenic genes in the liver. Both methyl- and butylparaben-exposed mice had significantly decreased lipin 1 mRNA expression. Butylparaben-exposed mice also had decreased ADRP mRNA (Fig. 3.2B).

Effects of post-weaning exposure to paraben on metabolic biomarkers in mice fed with a high fat diet.

We further investigated the effects of post-weaning exposure to parabens in mice fed with a high fat diet. At termination, there were no significant differences in body weight among the groups (23.9 ± 1.2 g for the control; 23.2 ± 0.7 g for the methyl- group; and 24.5 ± 0.7 g for the butyl- group) (Fig. 3.3A). No significant differences in the total body fat mass (1.89 ± 0.31 g for the controls; 1.58 ± 0.27 g for the methyl- group; and 1.94 ± 0.22 g for the butyl- group) and adiposity index (7.66 ± 0.92 % for the control; 6.60 ± 0.96 % for the methyl- group; and 7.86 ± 0.74 % for the butyl- group) were noted (Fig. 3.3A). Except for the plasma insulin levels, which were decreased in both methyl- (0.27 ± 0.06 ng/ml) ($p < 0.01$) and butylparaben-exposed mice (0.21 ± 0.05 ng/ml) ($p < 0.01$) compared to the controls (0.39 ± 0.07 ng/ml), there were no significant differences in fasting glucose, serum triglyceride, adiponectin, and leptin levels among the groups (Fig. 3.3B).

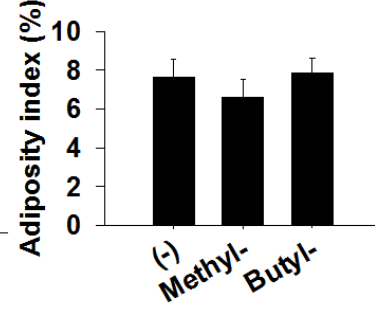
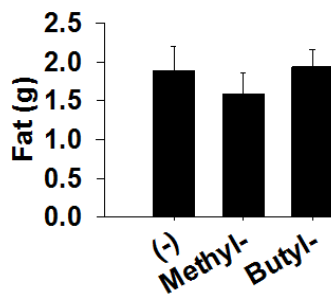
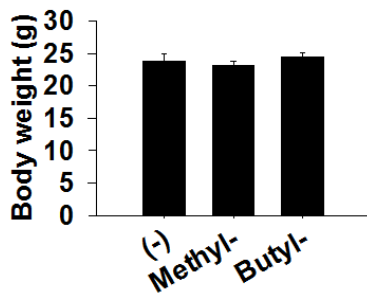
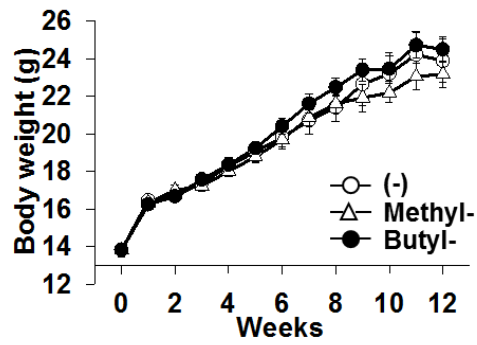
We also investigated the glucose and insulin sensitivities using GTT and ITT. We did not observe any significant differences in glucose tolerance tests among groups. However, at 15 min and 30 min, both paraben-exposed groups were more resistant to insulin compared to the controls ($p < 0.05$), although the overall insulin sensitivities, as indicated by the AUC, were not significantly different among the groups (Fig. 3.3C and 3.3D).

Gene expression analysis revealed that butylparaben-exposed mice had

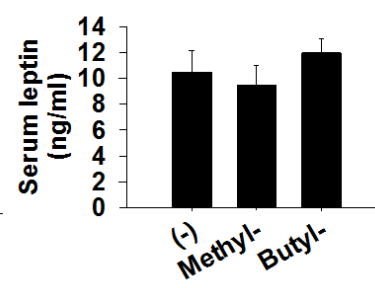
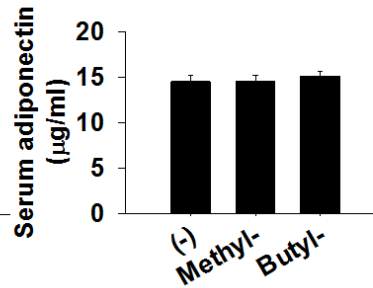
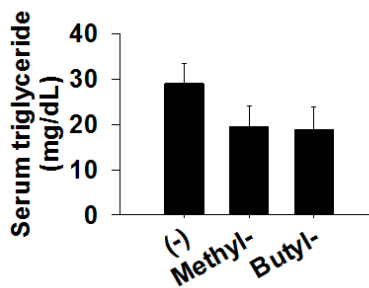
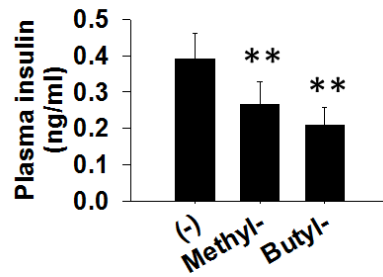
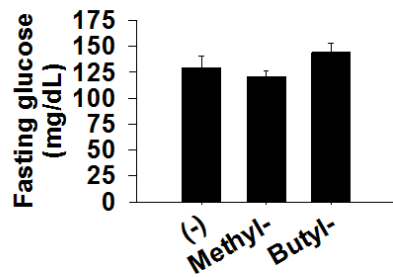
Figure 3.3 Effects of post-weaning exposure to parabens on body weight, body fat mass, adiposity and other metabolic biomarkers in mice fed with a high fat diet.

Female C57B6/J mice were orally gavaged with the vehicle control, methyl- or butylparaben from weaning (4 weeks of age) weaning (4 weeks of age) for 12 weeks. Body weights were measured every week and at termination, whole blood was collected and white fat pads were isolated and weighed. (A) Body weights by week, body fat mass, adiposity index (body fat percentage) are shown. (B) After overnight fasting at termination, blood glucose, plasma insulin, serum triglyceride, adiponectin and leptin are shown. (C) Glucose tolerance tests and calculations of area under the curve (AUC). (D) Insulin tolerance tests and calculations of AUC. Data are mean $\pm$ SEM (n=8). #, *, $p < 0.05$ for methyl- and butylparaben relative to the controls at the same time points, respectively.

A High Fat Diet

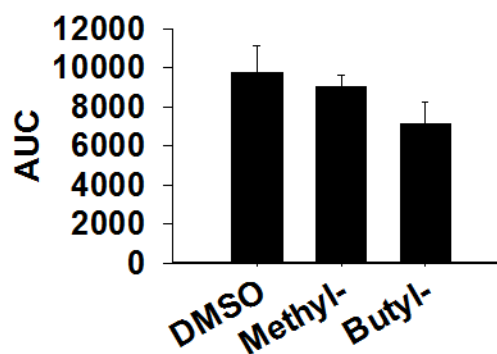
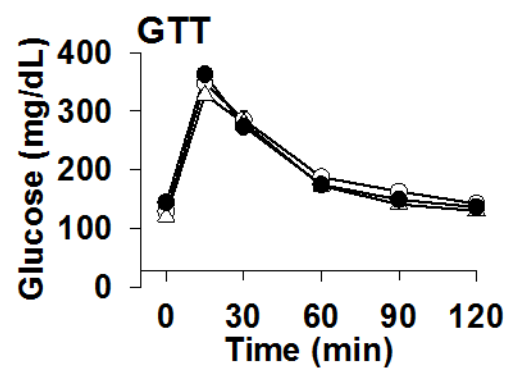


B



C

○ (-) △ Methyl- ● Butyl-



D

○ (-) △ Methyl- ● Butyl-

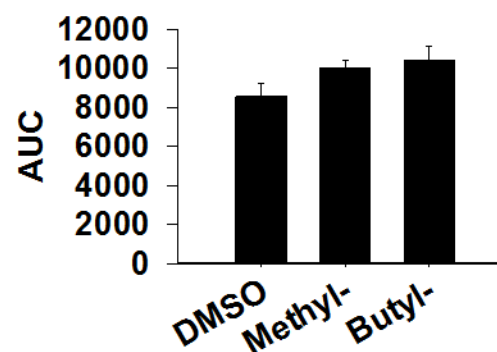
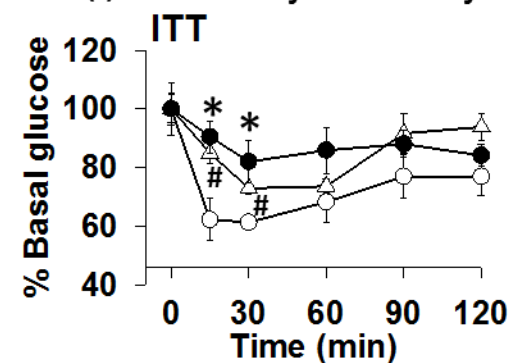


Figure 3.3 Continued

decreased adiponectin and increased lipin 1 mRNA expression in WAT (Fig. 3.4A). On the other hand, methylparaben-exposed mice had decreased perilipin (PLIN) mRNA expression (Fig. 3.4A). In the liver, mRNA expression of lipogenic FAS and lipin 1 were increased in butylparaben-exposed mice whereas methylparaben-exposed mice didn't show any differences in mRNA of the genes examined (Fig. 3.4B).

Effects of parabens on adipogenic differentiation of multipotent stem cell

C3H10T1/2.

The findings that post-weaning exposure to parabens, methylparaben in particular, increased adiposity promoted us to examine the modulation of stem cell fate by parabens. We examined the effects of methyl- and butylparaben on modulation of cell fates using a multipotent stem cell line C3H10T1/2, which has the potentials to be differentiated into adipocytes, osteocytes, or chondrocytes [34]. First, we examined the effects of parabens on adipogenic differentiation of C3H10T1/2 cells. After 12 h treatment, both methyl- and butylparaben significantly suppressed Runx2 mRNA expression by 35% ($p<0.05$) and 40% ($p<0.05$), respectively (Fig. 3.5A). Runx2 is a transcription factor, whose expression is down-regulated in adipogenic differentiation, but up-regulated in both osteogenic and chondrogenic differentiation of C3H10T1/2 cells. Butylparaben significantly induced PPAR γ and C/EBP α mRNA expression by 70% ($p<0.01$) and 130% ($p<0.01$), respectively (Fig. 3.5A). Methylparaben only increased C/EBP α mRNA expression by 30% ($p<0.01$) (Fig. 3.5A). We further investigated whether the transcriptional modulation by parabens at early stage lead to differences in terminal differentiation. On day 8, butylparaben treatment increased mRNA expression of adipocyte marker gene PPAR γ by 100% ($p<0.05$) C/EBP α by 150% ($p<0.01$), FABP4 by 75% ($p<0.01$) compared to the DMSO controls (Fig. 3.5A), demonstrating an enhanced adipogenic differentiation, which was consistent with ORO stained cell morphology and

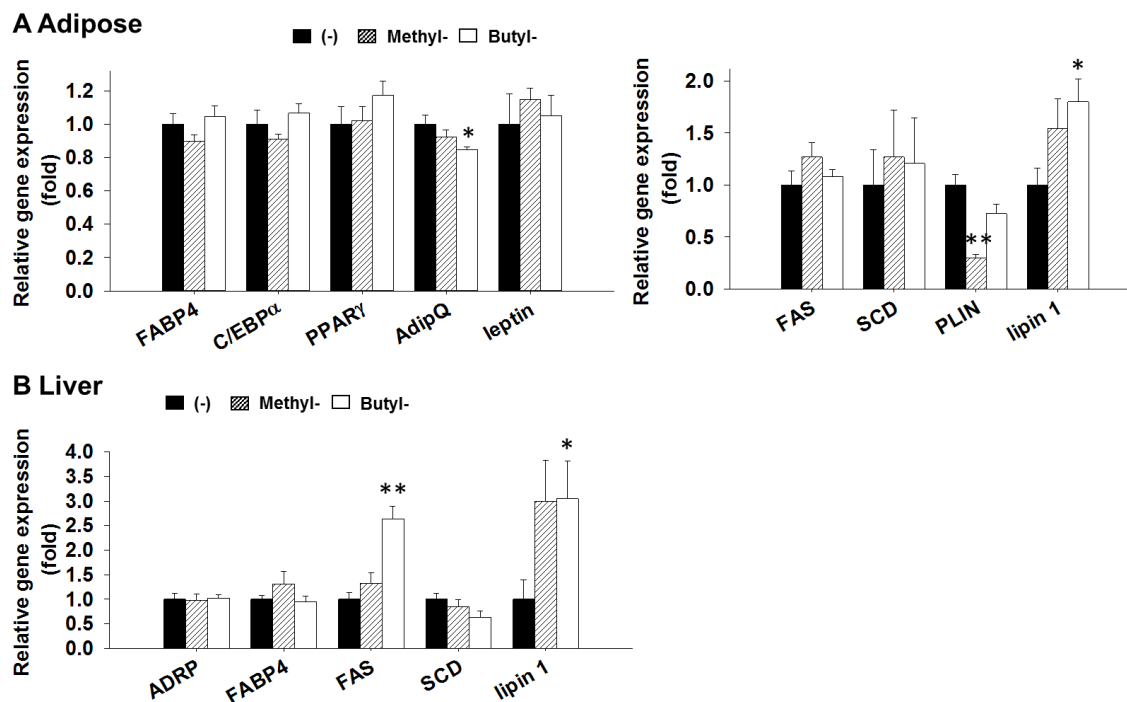


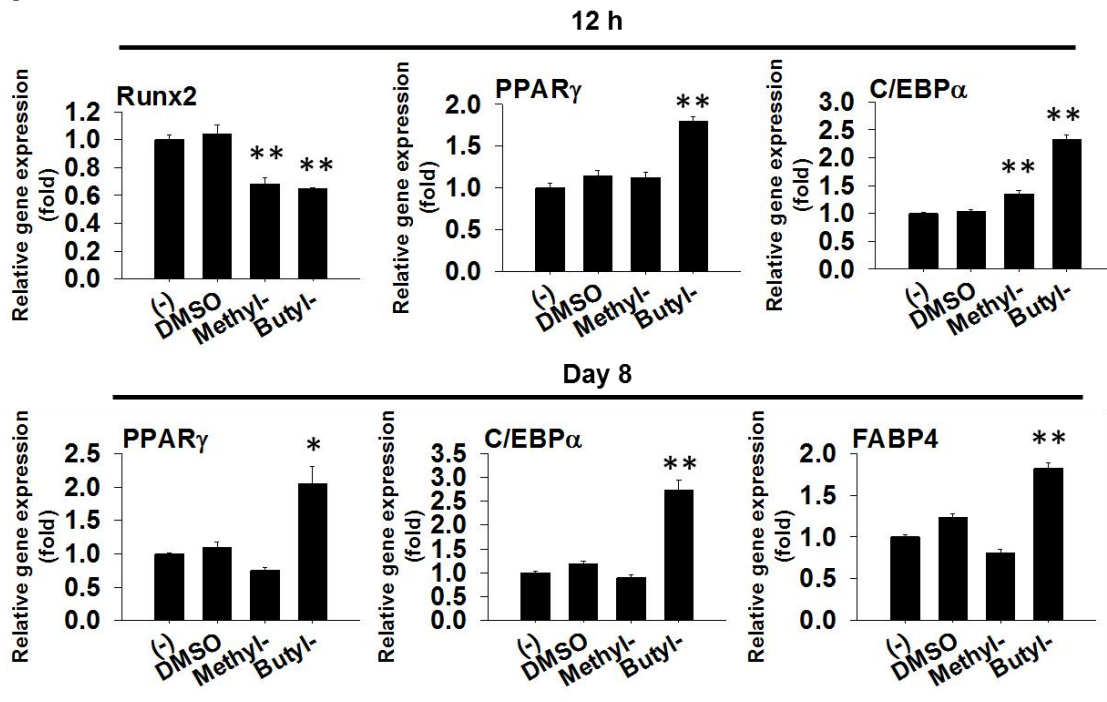
Figure 3.4 Effects of post-weaning exposure to parabens on gene expression in the WAT and liver in mice fed with the high fat diet.

(A) The WAT and (B) liver samples were isolated after termination, and were analyzed for relative gene expression. Expression was normalized to 36B4 and expressed as fold of the controls. Data are mean $\pm$ SEM (n=8). *, **, $p < 0.05$, and $p < 0.01$ versus the controls, respectively.

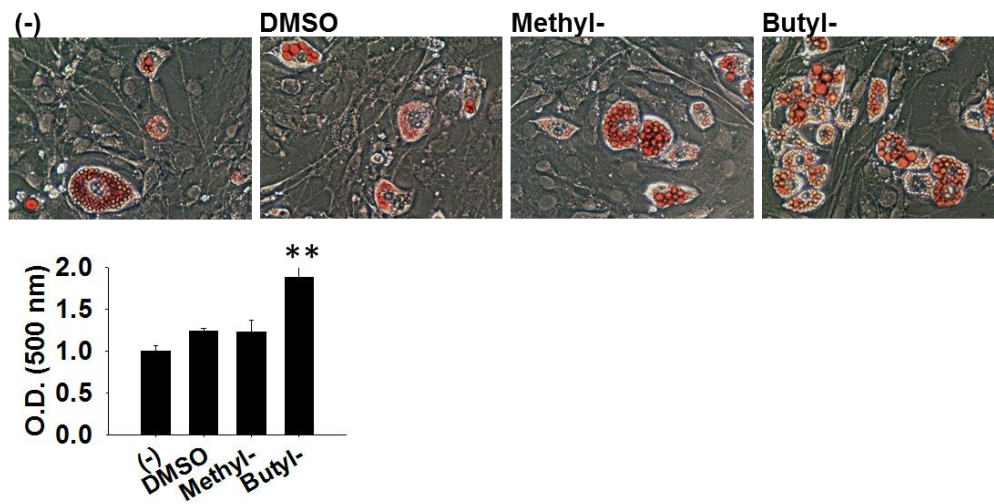
Figure 3.5 Effects of parabens on adipogenic differentiation of multipotent stem cell C3H10T1/2.

C3H10T1/2 cells were induced for adipogenic differentiation in the presence or absence of methyl-, butylparaben or the vehicle control DMSO. (A) mRNA expression of Runx2, PPAR γ , and C/EBP α were analyzed at 12 h after the initiation of differentiation. mRNA expression of PPAR γ , C/EBP α , and FABP4 were analyzed on day 8 after the initiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) Oil red O (ORO) staining of cell morphology at day 8 and quantifications of ORO absorbance were shown. Data are mean $\pm$ SEM (n=3). *, **, p<0.05 and p<0.01 versus the vehicle control DMSO, respectively.

A



B



quantifications of ORO absorbance (Fig. 3.5B). Methylparaben, however, didn't show noticeable enhancement on terminal adipogenic differentiation of C3H10T1/2 cells (Fig. 3.5A and 3.5B).

Effects of parabens on osteogenic differentiation of C3H10T1/2 cells.

Next, we examined the effects of parabens on osteogenic differentiation of C3H10T1/2 cells. After 12 h treatment, methyl- and butylparaben significantly suppressed Runx2 mRNA expression by 20% ($p<0.05$), and 30% ($p<0.01$), respectively (Fig. 3.6A). Consistently, both methyl- and butylparaben suppressed mRNA expression of osteogenic marker gene alkaline phosphatase (ALP) by 40% ($p<0.01$) at day 12. Methyl- and butylparaben suppressed mRNA of osteocalcin (OCA) by 25% ($p<0.05$) and 80% ($p<0.01$), respectively, and suppressed osteopontin (OPN) by 20% ($p<0.05$) and 30% ($p<0.01$), respectively (Fig. 3.6A). ALP staining in differentiating cells revealed marked suppression of staining by butylparaben and modest suppression by methylparaben (Fig. 3.6B). Microscopic images at day 12 revealed a strong suppression of differentiation by butylparaben and modest suppression by methylparaben compared with the well differentiated controls (Fig. 3.6C).

Effects of parabens on chondrogenic differentiation of C3H10T1/2 cells.

Lastly, we examined the effects of parabens on chondrogenic differentiation of C3H10T1/2 cells. After 12 h treatment, methyl- and butylparaben suppressed Runx2 mRNA expression of by 40% ($p<0.01$) and 30% ($p<0.01$), respectively. Consistently, both suppressed collagen type II (Col II) by 40% ($p<0.01$), and suppressed collagen type X (Col X) by 40% ($p<0.01$) and 50% ($p<0.01$), respectively (Fig. 3.7A). Aggrecan (Agg) mRNA expression was not changed by either paraben at this time point (data not shown). At day 6, butylparaben abolished Agg mRNA expression by almost 100% ($p<0.01$), Col II by 95% ($p<0.01$), and Col X by 40 % ($p<0.01$) (Fig. 3.7A) compared with

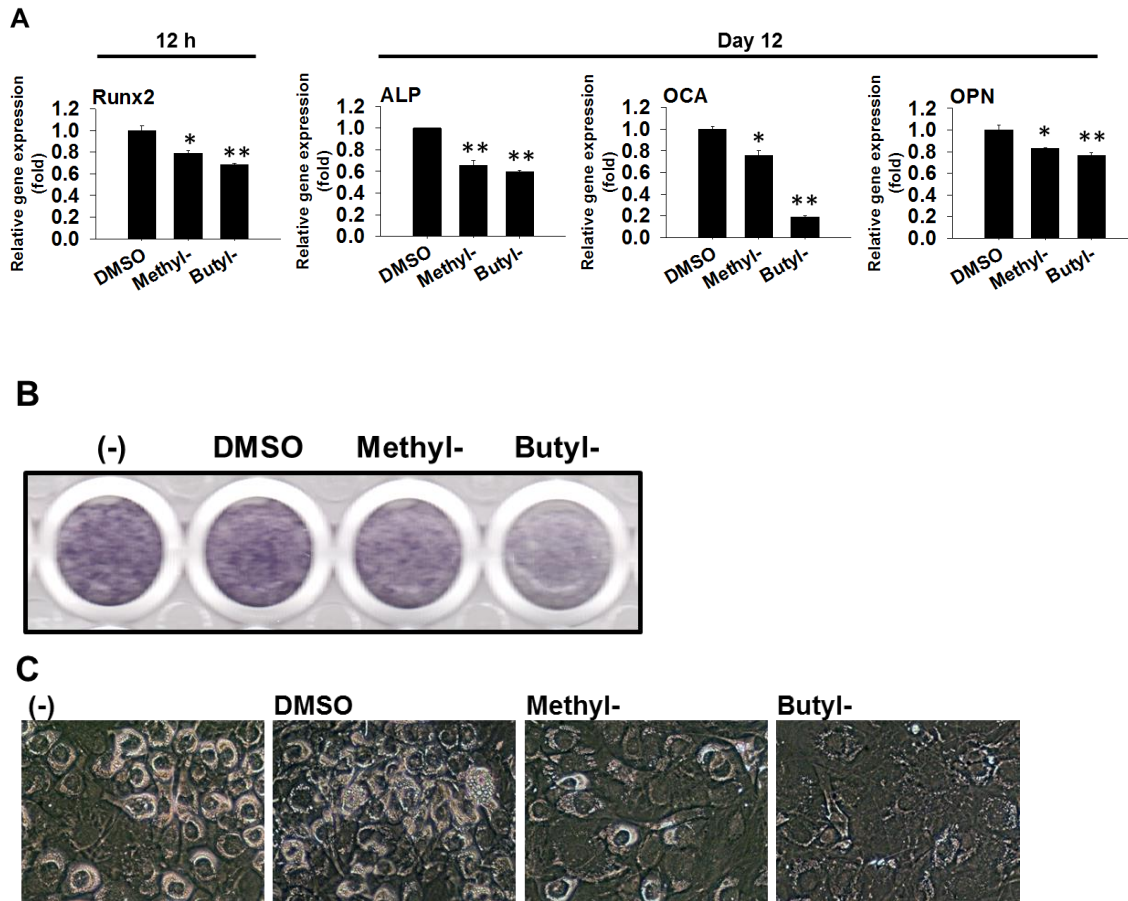
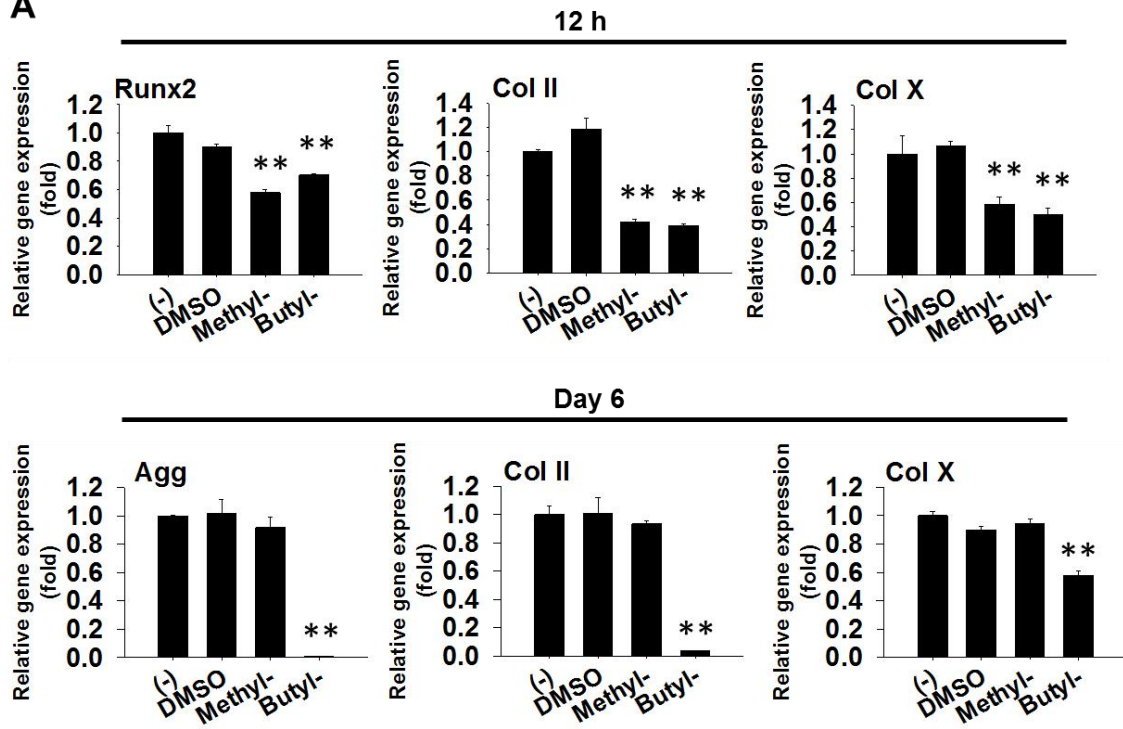
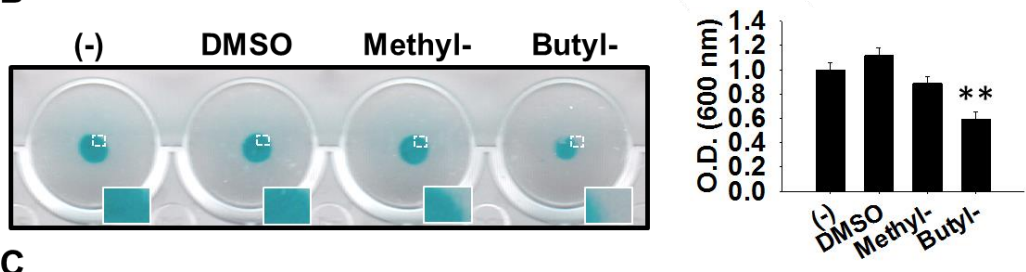
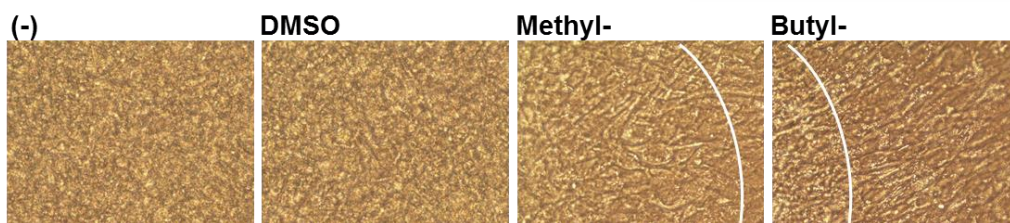


Figure 3.6 Effects of parabens on osteogenic differentiation of multipotent stem cell C3H10T1/2.

C3H10T1/2 cells were induced for osteogenic differentiation in the presence or absence of methyl-, butylparaben, or the vehicle control DMSO. (A) mRNA of Runx2 was analyzed at 12 h and mRNA expression of ALP, OCA and OPN were analyzed on day 12 after the initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). Data are mean $\pm$ SEM (n=3). *,**, $p < 0.05$ and $p < 0.01$ versus the vehicle control DMSO, respectively. (B) ALP staining of the differentiating cells were performed on day 2. (C) Microscopic images of the cells at day 12 were shown.

Figure 3.7 Effects of parabens on chondrogenic differentiation of multipotent stem cell C3H10T1/2.

Micromass of C3H10T1/2 cells were seeded and induced for chondrogenic differentiation in the presence or absence of methyl-, butylparaben, or the vehicle control DMSO. (A) mRNA expression of Runx2, Agg, Col II and Col X were analyzed at 12 h and day 6 after initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) Alcian blue staining and quantifications of the absorbance were performed on day 6. Data are mean $\pm$ SEM (n=3). **, p<0.01 versus the vehicle control DMSO. (C) Microscopic images of the micromass at day 6 were shown.

A**B****C**

the DMSO control. Methylparaben did not show any noticeable effects on mRNA of these marker genes. The effects were confirmed by Alcian blue staining of the differentiated cells and by the quantifications of the absorbance of the staining (Fig. 3.7B). Microscopic images revealed a large portion of undifferentiated cells in the peripheral of the micromass by butylparaben and a much smaller portion of undifferentiated cells in the peripheral by methylparaben compared with the DMSO controls (Fig. 3.7C).

3.6 Discussion

Parabens are widely used as preservatives in personal care products, cosmetics, toiletries, pharmaceuticals and foods. Based on the industry estimates of the daily use of cosmetics and assumption that parabens are used at the highest permissible concentration (0.8%), it was estimated that daily dose of total parabens from cosmetics is 142.08 mg and 3.024 mg for adults and infants, respectively. The paraben dose for an adult weighing 60 kg is then equaled to 2.368 mg/kg body weight/day. Widespread production and use of parabens have resulted in wide detections of parabens in environmental samples, such as surface water, soils, sediments and sludge, air and dust, and even in fish meat, which could presumably get into human body through oral ingestion and inhalation (reviewed in Bledzka et al 2014). To this end, it has been reported that almost all common foodstuffs in China contained at least one of the parabens analyzed, although the concentrations of parabens reported were generally low in those foods [2]. Our exposure of parabens was set at 100 mg/kg/day, which is 10 times of the current recommended acceptable daily intake (ADI) for the sum of methylparaben, ethylparaben and their sodium salts by European Food Safety Authority (EFSA). This dose seems to be relevant and consistent with others reported in *in vivo* rodent studies [11,15-17]. Due to the non-monotonic dose-response relationship

reported for some known EDCs, such as bisphenol A (BPA), studies that use a range of doses of parabens are needed in the future.

Studies on the effects of parabens have mostly been focused on reproductive systems [15-17]. To the best of our knowledge, our study is the first to investigate the adipogenesis, associated metabolic consequences of paraben exposure, and their interactions with diet *in vivo*. Notably, methylparaben had more apparent effects than butylparaben in increasing body fat mass, adiposity, and serum leptin in mice fed with the chow diet, compared with the controls. This is in contrast to the fact that methylparaben was much less effective than butylparaben in promoting adipogenesis *in vitro* [20]. Following oral administration, parabens are known to be subjected to hydrolysis by widely distributed, non-specific esterase in the gastrointestinal tract and liver. It has been reported that efficiency and pattern of hydrolysis of parabens vary considerably depending on the alkyl chain length and tissue [35,36]. The hydrolytic activity of human carboxylesterase 2 (hCE-2), the predominant form found in human small intestine, increases with the increasing length of the alkyl chain of paraben esters [36]. Similarly, butylparaben seemed to be more effectively hydrolyzed by the microsomes from the rat liver and the small intestine, compared to parabens with shorter alkyl side chain [35]. Therefore, our findings that more apparent effects were observed in methylparaben-exposed mice may be due to more parental methylparaben available in the exposed mice. Notably, even though butylparaben had no significant effects on body weight, adiposity, and serum adipokines, it significantly increased fasting glucose levels (Fig. 3.1B) and mRNA expression of some adipogenic markers, such as FABP4 and FAS in the WAT in mice fed with the chow diet (Fig. 3.2A), suggesting that some tissues, such as the liver, insulin-secreting pancreatic exocrine cells (the former two organs are important for regulating fasting glucose levels), and stromal stem cells in the WAT,

capable of becoming mature fat cells, may be more sensitive to a presumably low concentration of butylparaben.

Interactions of diet with EDC exposure have been reported. While perinatal exposure to BPA increased body weight and impaired glucose tolerance in adult offspring rats fed with normal diet, high fat feeding (28.5% kcal from fat) further accelerated and exacerbated the detrimental effects [31]. In another study, male adult rats exposed to BPA displayed impairment of glucose homeostasis under regular chow diet, and the disruption was aggravated by high fat diet (41.3% kcal from fat) [37]. However, several endpoints, such as serum insulin level and HOMA-IR at 35 weeks, showed more pronounced differences by BPA exposure under the chow diet than under a high fat diet [37]. In our study we used a high fat diet with 45% kcal from fat with lipid profiles mimicking a western diet. The high fat feeding did not synergize with the effects of paraben exposure. In contrast, many effects observed in methylparaben-exposed mice under the chow diet were attenuated by the high fat diet. Future studies with diets with a lower range of kcal from fat may provide additional information on the interaction of paraben exposure with diet.

There is a general consensus that perinatal periods (i.e., during gestation and lactation) are sensitive window of development, most susceptible to nutritional and environmental insults and the influences occur during early development can impact health later in life. This is often referred as “developmental origins of health and disease” (DoHAD) paradigm. Studies of exposure of pregnant dams to ethyl- or butylparaben from gestational day 7 to 21 revealed that parabens can cross the placenta, and concentrate in the fetuses, resulting in higher concentrations of free and glucuronidated and sulfated parabens in the embryonic fluid, fetal carcass and fetal liver than in the maternal plasma [11]. We have used a post-weaning exposure model to investigate the

adipogenic effects of paraben exposure *in vivo* in the current studies. Our findings from the post-weaning exposure have provided additional rationales to study the effects of perinatal exposure of parabens. Therefore, further studies on the effects of perinatal exposure to parabens on the development of obesity and metabolic dysfunction in later life are warranted.

Multipotent stem cell C3H10T1/2, which possess the potential to be differentiated into adipocytes, chondrocytes, osteocytes or myocytes [34], has been widely used as a model to study the alteration of stem cell fate. Consistent with potent effects on 3T3-L1 adipocyte differentiation [20], butylparaben markedly enhanced adipogenic differentiation of C3H10T1/2 cells. Methylparaben suppressed Runx2, but up-regulated C/EBP α , not PPAR γ at the early time point, but it did not enhance adipogenic differentiation at day 8. In contrast, butylparaben markedly suppressed differentiation of osteoblasts and chondrocytes from C3H10T1/2 whereas methylparaben suppressed osteogenic differentiation to a much lesser degree and had minimal effects on chondrogenic differentiation (Fig. 3.6 and 3.7). Our results suggest that increased adiposity with modestly increased gross body weight by post-weaning exposure to parabens, methylparaben in particular, may be attributed to the modulation of multipotent stem cell fate by parabens.

The mechanisms by which parabens modulate stem cell fates are not clear at this point. It has been reported that tributyltin (TBT), an EDC mostly found in seafood and food crops from the use of TBT as an antifouling agents on boats and fungicides, sensitized adipose-derived multipotent stem cells (MSC) to differentiate into adipocytes while suppressing their osteogenic differentiation [26]. TBT was shown to be an agonist of PPAR γ , a known master transcription factor controlling adipocyte differentiation. It is thought that the effects of TBT were mediated through PPAR γ . Overexpression of

PPAR γ 2 stimulated adipocyte marker FABP4 expression and fat accumulation in murine bone marrow-derived stem cells, while suppressing Runx2 expression and osteogenic differentiation [38]. Activation of PPAR γ by its ligand in mesenchymal cells inhibited the expression of osteocalcin, an osteoblast specific protein, both by suppressing the expression and the transactivation ability of Runx2 [39]. Transcription factor Runx2, which belongs to the runt-domain gene family, is highly expressed in osteoblasts and chondrocyte and its up-regulation is required for differentiation of osteoblasts [40,41,28] and chondrocytes [42,43]. In contrast, Runx2 has been shown to be an inhibitor for adipocyte differentiation and its down-regulation promotes adipocyte differentiation [44]. Moreover, Runx2 has been identified as a downstream target of glucocorticoid receptor (GR) signaling as Runx2 mRNA was suppressed by the synthetic glucocorticoid dexamethasone, a key component of adipogenic cocktail, during adipocyte differentiation [44]. Since parabens have been reported to activate both GR and PPAR γ , it is possible that parabens could modulate stem cell fate through down-regulating Runx2 expression downstream of GR and/or PPAR γ activation. Consistently, we have found that both parabens down-regulated Runx2 during differentiation of adipocytes, osteoblasts and chondrocytes of C3H10T1/2 (Fig. 3.5, 3.6 and 3.7). Future studies are necessary to confirm the role of Runx2 in the modulation of stem cell fates by parabens.

In conclusion, we show that parabens, methylparaben in particular, increase adiposity in mice fed with the chow diet, possibly through modulation of multipotent mesenchymal stem cell fate towards adipose lineage at the expense of osteoblast and chondrocyte lineage. Our studies have provided additional rationales to study paraben exposure at a range of doses and during the perinatal period, a presumably more sensitive window of development, in the future.

3.7 References

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CONCLUSION AND FUTURE DIRECTIONS

Conclusion

In this dissertation, we have shown that parabens promote adipocyte differentiation in murine 3T3-L1 cells, which might be mediated through activation of nuclear receptor PPAR γ and/or GR. The adipogenic potency of parabens is increased with increasing length of the linear alkyl chain in following potency ranking order: methyl- < ethyl- < propyl- < butylparaben. Benzylparaben has an aromatic ring and preserves strong adipogenic potency; the common metabolite of all parabens 4-hydroxybenzoic acid, and the structurally related benzoic acid are inactive in promoting 3T3-L1 adipocyte differentiation. We find that parabens, butyl- and benzylparaben in particular, can promote adipose conversion of human adipose-derived multipotent stromal cells. We demonstrate that exposure to methyl- and butylparaben in female C57B6/J mice increased adiposity and altered metabolic markers. Both parabens showed effects on modulating stem cell fates, and butylparaben is more potent in promoting adipocyte differentiation but suppressing differentiation of osteocytes and chondrocytes from C3H10T1/2 cells. We conclude that parabens exposure could increase adiposity through enhancing differentiation of predetermined preadipocytes, as well as through modulating the multipotent stem cells towards adipose lineage at the expense of osteocyte and chondrocyte lineage.

Future Directions

Obesity might be resulted from events taking place early in life, such as during gestation and postnatal development when individuals are exposed to various EDCs including parabens. In an attempt to explore the possible effects of paraben exposure on adipogenesis we have performed studies both *in vitro* and *in vivo*.

We have shown that parabens enhance adipocyte differentiation in murine cell line 3T3-L1, which is possibly mediated through activation of GR. EDCs have been shown to modulate GR activity by competing with the ligand binding to the receptor, but the GR competitor assays in our study didn't show signs of competitive binding of GR by parabens, nor did it show that. Parabens modulate ligand binding of GR. Some EDCs have been shown to modulate GR activation by working on glucocorticoid metabolizing enzymes (such as 11 β -HSD1 and 2), but our preliminary study didn't provide sufficient evidence to support this hypothesis. Future studies on the mechanisms by which parabens modulate GR activation are needed to elucidate the roles of parabens in promoting adipogenesis.

Butylparaben showed much stronger adipogenic potency than methylparaben when used in 3T3-L1 cells. For the animal study, we have chosen methyl- and butylparaben since these two parabens are commonly used and they bracket the length of the linear alkyl side chain. However, we found more significant homeostasis disruption caused by methylparaben in mice, and butylparaben only had marginal effects. Since parabens are subject to hydrolysis by esterase after oral administration, the abilities of parabens to escape the hydrolysis might be the key to explain the discrepancies between in vitro vs in vivo we observed. Future studies on the hydrolysis efficiency of different esterases on specific parabens are needed to further validate the results of our in vivo studies.

Runx2 belongs to runt-domain gene family and is highly expressed in mature osteoblasts and chondrocytes. Up-regulation of Runx2 is required for differentiation of osteoblasts and chondrocytes, while its down-regulation promotes adipogenic differentiation. We have shown that both methyl- and butylparaben suppressed Runx2 expression at the early stage in adipogenic, osteogenic, and chondrogenic differentiation

of C3H10T1/2 cells. In-depth mechanistic study on how parabens work on Runx2 expression and its transcriptional activity would provide us with more insight into the mechanisms by which parabens induce adiposity in vivo.

VITA

Pan Hu was born to Chengxiang Hu and Yuanfang Long on June 19th 1985 in Wuhan, China. After he finished elementary school and middle school on Wulongquan Street in Jiangxia District, he attended Jiangxia No.1 High School and received his high school diploma in 2004. He obtained the Bachelor of Science degree from School of Life Sciences at Wuhan University in 2008. In November of 2009 he joined Dr. Liwen Jiang's lab as research assistant at Chinese University of Hong Kong. He enrolled in the doctoral program in Nutritional Sciences at University of Tennessee at Knoxville in fall 2010, where he studied the roles of endocrine disrupting chemical parabens in adipogenesis under supervision of Dr. Ling Zhao. During his graduate study in Department of Nutrition, he worked as graduate teaching assistant for Nutrition 100 instructed by Lee Murphy, and obtained a Master of Science degree in Statistics. He defended his doctoral dissertation on November 26th 2014. He married Wen Ren in Wuhan on July 30th 2010. Their son, Mason Hu, was born in Parkwest Medical Center on September 22nd 2014. His sisters Lan Hu and Ping Hu live in China.