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Methylparaben and butylparaben alter multipotent mesenchymal stem cell fates towards adipocyte lineage

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Abstract

Paraben esters and their salts are widely used as preservatives in cosmetics, personal care products, pharmaceuticals, and foods. We previously reported that parabens promoted adipocyte differentiation *in vitro* and increased adiposity but suppressed serum marker of bone formation *in vivo*. Here, we investigated the effects of parabens (methylparaben and butylparaben) on modulating cell fate of multipotent stem cell line C3H10T1/2. 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 but less pronounced effects. Moreover, butylparaben, but not methylparaben, was shown to activate peroxisome proliferator-activated receptor (PPAR) γ whereas neither of the paraben was shown to activate glucocorticoid receptor (GR) responsive reporter in C3H10T1/2 cells. The adipogenic effects of butylparaben were significantly attenuated by PPAR γ knockdown, but not by GR knockdown. In contrast, paraben's effects on osteoblast differentiation were affected by both knockdowns. Collectively, the results demonstrate opposing effects of parabens on adipogenic and osteoblastogenic/chondrogenic differentiation of multipotent stem cells. In light of the recent findings that parabens are detected in human placenta and milk, our studies provide rationales to study paraben exposure during early development of life in the future.

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DISCLOSURE

The authors declare no conflict of interest.

Keywords

Parabens; Endocrine disrupting chemicals; Adiposity; C3H10T1/2; Multipotent mesenchymal stem cell; Obesity

1. 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 are detected in human serum and urine [3, 4], milk [5, 6], placental tissues [7], seminal plasma [4], and breast tumor tissue samples [8]. The extent of this exposure is further reflected by the frequent detection of free and conjugated forms of parabens in urine samples in the general population [9–11]. Moreover, recent detection of parabens in human breast milk and placenta [7, 12] has raised the concerns about potential impact of perinatal exposure to parabens on early development in humans.

Many *in vitro* and animal studies have suggested that parabens are endocrine disrupting compounds (EDCs) with estrogenic and anti-androgenic properties. Emerging evidence also suggested that parabens may be metabolic disruptors possibly due to their abilities to activate nuclear receptors, peroxisome proliferator-activated receptor (PPAR) γ in particular [13–15], which is one of the properties shared by many known EDCs with metabolic disrupting capabilities [16, 17]. We reported [14] and later confirmed by others [15] that parabens promoted adipocyte differentiation of 3T3-L1 cells and primary culture derived from human adipose tissue. These effects may be mediated through activation of PPAR γ and/or glucocorticoid receptor (GR) [14, 15]. Moreover, we reported that post-weaning exposure to methyl- or butylparaben in the females of obesity-prone C57BL/6J mice under the chow diet-feeding induced changes in gene expression related to adipocyte differentiation and lipogenesis in the white adipose tissue (WAT) and the liver [18]. However, methylparaben exposed mice had significant increases of total white fat mass whereas butylparaben exposed mice did not show such increase. Remarkably, exposure to both parabens under the chow diet significantly decreased serum levels of procollagen type 1 N-terminal propeptide (PINP, a marker of bone formation) but had no effects on serum levels of C-terminal telopeptide of type I collagen (CTX-I, a marker of bone resorption), suggesting that post-weaning exposure to parabens may negatively affect both fat tissue and bone formation in mice.

It is believed that adipocytes are derived from common multipotent mesenchymal stem cells that also give rise to osteocytes and chondrocytes [18]. Some EDCs are able to sensitize multipotent stem cells to undergo adipogenesis, and to suppress their osteoblast differentiation [19]. In light of our studies demonstrating opposing effects of parabens on fat tissue and bone [20], we hypothesize that parabens may modulate the multipotent stem cell fate by promoting adipocyte differentiation but suppressing differentiation of osteocyte and/or chondrocyte of stem cells.

In this study, we investigate effects of methylparaben and butylparaben on stem cell differentiation into adipocytes, osteoblasts, or chondrocytes. Moreover, we explore the role of PPAR γ and GR in mediating the observed effects by parabens.

2. MATERIALS AND METHODS

2.1. Reagents

Methylisobutylxanthine (MIX), dexamethasone (DEX), insulin (Ins), 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). 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%).

2.2. 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, 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 a modified 3T3-L1 differentiation protocol. Briefly, on the day that the cells reached confluence (designated as day 0), cells were induced to differentiate with differentiation DMEM medium containing 10% FBS, 10 μ g/ml insulin, 1 μ M DEX, and 0.5 mM MIX 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 another 2 days before the cells were harvested for gene expression analysis or Oil red O (ORO) staining. Parabens or the vehicle control (0.1% DMSO in culture medium) were applied at the induction and at each change of the medium.

Osteoblastogenic differentiation—C3H10T1/2 cells were differentiated into osteoblasts as previously described by Ducy et al [21]. Briefly, the cells were seeded at a density of $2 \times 10^4/\text{cm}^2$ in DMEM containing 10% FBS. After 24 h, the media was replaced with fresh medium supplemented with 200 ng/ml of human bone morphogenetic protein 7 (hBMP7) (R&D systems, Minneapolis, MI) when the cells reach confluence (designated as D0). 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 at each change of the medium.

Chondrogenic differentiation—C3H10T1/2 cells were differentiated into chondrocytes in a high-density micro-mass culture as previously described by Ahrens et al [22]. Briefly, C3H10T1/2 cells were trypsinized and re-suspended in Ham's F12K medium (CORNING, Tewksbury, MA) containing 10% FBS at a concentration of 10^7 cells/ml, and a 10 μ l-drop of

cell suspension was placed in the center of each well of 24-well cell culture plates. Cells were allowed to adhere for 1–2 h at 37°C under 5% CO₂, then 1 ml of the medium containing 100 ng/ml of hBMP2 (R&D systems, Minneapolis, MI) was added to the culture (designed as D0). Medium was replenished every two days until day 6. Parabens or the vehicle control (DMSO) were applied at the induction with hBMP2 and at each change of the medium.

2.3. 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). The primer sequences are summarized in Supplemental Table 1. Cycle conditions were 50°C 2 min, 95°C 15 min, then 40 cycles of 95°C for 15 s/60°C for 1 min.

2.4. ORO staining and quantification

Lipid accumulation in differentiated adipocytes were stained with oil red O (ORO) and quantified by ORO absorbance, as previously described [14].

2.5. Alcian blue staining and quantification

Chondrogenic differentiation was assessed as previously described by Ji et al [23]. Briefly, C3H10T1/2 cells undergone chondrogenic differentiation were rinsed twice with PBS, and fixed in 4% (w/v) paraformaldehyde for 15 min. Alcian blue 8-GX (1% (w/v)) in 0.1 N HCl (pH 1.0) was applied to stain cells overnight. For quantitative analysis, guanidine HCl (6 M) 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).

2.6. Reporter gene assays

C3H10T1/2 cells were seeded and transiently transfected with PPAR γ transactivation reporters or a GR responsive luciferase reporter (the mouse mammary tumor virus promoter driven luciferase reporter MMTV-Luc) and β -galactosidase (β -gal) control plasmid (for monitoring the transfection efficiency) with Lipofectamin 2000 according to the manufacturer's instructions (ThermoFisher Scientific). PPAR γ transactivation reporter system consists of a murine PPAR γ ligand binding domain (LBD) 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). The cells were treated as indicated in the figure legends for 18 h, cell lysates were then prepared and luciferase/ β -gal activity were measured using Glomax multidetection system (Promega, Madison, WI).

2.7. Lentiviral shRNA particle infection and generation of stably infected cell pools

C3H10T1/2 cells were plated at ~50% confluence in 6-well plates. The cells were then infected with MISSION shRNA lentiviral transduction particles for mouse PPAR γ , GR, or scrambled non-targeting controls according to manufacturer's instructions (Sigma-Aldrich). Stably infected cells were selected by puromycin (2 μ g/ml) for two weeks.

2.8. Statistical analysis

All data were presented as group mean $\pm$ SEM (n=3). Analysis of variance (ANOVA) was performed to compare the effects of methyl and butylparabens on various endpoints followed by Student–Newman–Keuls post hoc test. Statistical analysis was performed using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA). The level of significance was set at $p < 0.05$.

3. RESULTS

3.1. Differential effects of methyl- and butylparaben on adipogenic differentiation of multipotent stem cell C3H10T1/2

We investigated the effects of methyl- and butylparaben on modulating cell fates using a multipotent stem cell line C3H10T1/2, which have the potentials to be differentiated into adipocytes, osteocytes, or chondrocytes [24]. First, we examined the effects of parabens on adipogenic differentiation of C3H10T1/2 cells. C3H10T1/2 cells were subjected to adipogenic differentiation in the presence or absence of methylparaben, butylparaben, or the vehicle control (DMSO) for 8 days (Fig. 1). As early as 12 h upon treatment, butylparaben increased mRNA expression of adipocyte differentiation marker PPAR γ by 79% ($p < 0.01$) and CCAAT/enhancer binding protein alpha (C/EBP α) by 132% ($p < 0.01$) and decreased Runx2 mRNA level by 36% ($p < 0.01$). Methylparaben increased C/EBP α mRNA by 35% ($p < 0.05$) and decreased Runx2 mRNA by 32% ($p < 0.01$); however, it did not change PPAR γ mRNA at first 12 h of exposure (Fig. 1A **top**). At terminal differentiation (day 8), butylparaben increased mRNA level of PPAR γ by 105% ($p < 0.05$), C/EBP α by 174% ($p < 0.01$), and fatty acid binding protein 4 (FABP4) by 82% ($p < 0.01$) compared to the vehicle controls (Fig. 1A **bottom**). In contrast, methylparaben did not induce further increase on these markers compared to the controls at day 8. The findings of ORO-stained adipocyte morphology (Fig. 1B **right**) and quantification of lipid accumulation measured by ORO absorbance (Fig. 1B **left**) corroborated with the up-regulation of mRNA of adipocyte differentiation markers by butylparaben. Methylparaben, however, didn't induce noticeable enhancement of ORO absorbance compared to the controls at day 8 (Fig. 1B **right**).

We also performed dose-response studies of methyl- and butylparaben on adipogenic differentiation of C3H10T1/2 cells. Butylparaben (0.1, 1, 10, and 100 μ M) dose-dependently enhanced adipogenic differentiation as revealed by increased mRNA expression of PPAR γ , C/EBP α , and FABP4 (Supplemental Fig. 1). In contrast, we did not detect dose dependent response of methylparaben using standard differentiation protocol (Supplemental Fig. 1).

3.2. Differential effects of methyl- and butylparaben on osteoblastogenic differentiation of C3H10T1/2

Next, we examined the effects of parabens on osteogenic differentiation of C3H10T1/2 cells. C3H10T1/2 cells were subjected to osteoblastogenic differentiation in the presence or absence of methylparaben, butylparaben or the DMSO for 12 days (Fig. 2). Upon initial 12 h treatment, methylparaben and butylparaben suppressed Runx 2 mRNA expression by 21% ($p<0.05$) and 32% ($p<0.01$), respectively (Fig. 2A **left**). At terminal differentiation on day 12, methylparaben suppressed mRNA expression of osteoblastogenic marker alkaline phosphatase (ALP) by 35% ($p<0.01$), osteocalcin (OCA) by 25% ($p<0.05$), and osteopontin (OPN) by 18% ($p<0.05$). Butylparaben suppressed mRNA expression of ALP by 41% ($p<0.01$), OCA by 81% ($p<0.01$), and OPN by 24% ($p<0.01$) (Fig. 2A **right**). Microscopic examination at day 12 revealed uniform osteoblasts with cytoplasmic storage materials in the controls. Methylparaben treated cells were less uniform and with less cytoplasmic storage materials whereas butylparaben treated cells were flat, not uniform, and almost with no cytoplasmic storage materials, indicating a strong suppression of differentiation by butylparaben and modest suppression by methylparaben compared with the differentiated control cells (Fig. 2B).

We performed dose-response studies of methyl- and butylparaben on osteoblastogenic differentiation of C3H10T1/2 cells. When compared at day 12, there were no significant effects of methylparaben or butylparaben on mRNA of ALP, OCA, and OPN when used at doses less than 100 μ M (Supplemental Fig. 2).

3.3. Differential effects of methyl- and butylparabens on chondrogenic differentiation of C3H10T1/2

Further, we examined the effects of parabens on chondrogenic differentiation of C3H10T1/2 cells. C3H10T1/2 cells were subjected to chondrogenic differentiation in the presence or absence of methylparaben, butylparaben or DMSO for 6 days (Fig. 3). After 12 h treatment, methylparaben suppressed mRNA expression of chondrogenic marker Runx2 by 42% ($p<0.01$), Col II by 59% ($p<0.01$), and Col X by 42% ($p<0.01$). Similarly, butylparaben suppressed mRNA expression of Runx2 by 30% ($p<0.01$), Col II by 61% ($p<0.01$), and Col X by 51% ($p<0.01$) (Fig. 3A **top**). At terminal differentiation on day 6, butylparaben suppressed mRNA expression of Agg to the basal level ($\sim 100\%$, $p<0.01$), Col II by 96% ($p<0.01$), and Col X by 43% ($p<0.01$) compared with the vehicle control (Fig. 3A **bottom**). In contrast, methylparaben did not induce any noticeable effects on these markers. The effects were confirmed by Alcian blue staining at termination (Fig. 3B **left**), which indicates that Alcian blue staining was much less in the micromass treated by butylparaben. The quantifications of the staining absorbance is shown in Fig. 3B **right**. Microscopic examination revealed (i) a large portion of undifferentiated cells in the periphery of the butylparaben-treated micromass; and (ii) a much smaller portion of undifferentiated cells in the periphery of methylparaben-treated micromass compared with the vehicle control (Fig. 3C).

3.4. Butylparaben, activates PPAR γ , but not a GR responsive reporter, in C3H10T1/2 cells

We have previously reported that parabens, butylparaben in particular, activated PPAR γ and GR using the PPAR γ transactivation reporters and MMTV-Luc (a GR responsive reporter), respectively [14]. Moreover, the adipogenic effects of butylparaben was attenuated by the knockdown of PPAR γ or GR [14]. Therefore, we further determined whether methylparaben or butylparaben activates PPAR γ or GR in C3H10T1/2 cells using the same reporter assays (Fig. 4A). Rosiglitazone (Rosi), a synthetic ligand for PPAR γ , and dexamethasone, a synthetic ligand for GR, were included as positive controls. We found that butylparaben, but not methylparaben, activated PPAR γ in C3H10T1/2 cells ($p < 0.01$) (Fig. 4A **left**). In contrast, we did not observe activation of MMTV-Luc by either paraben in C3H10T1/2 cells (Fig. 4A, **right**). We further examined parabens with various length in linear alkyl side chain. Activation of PPAR γ by paraben in C3H10T1/2 cells increased with increasing length of the alkyl side chain, similar to what was reported in 3T3-L1 cells (Supplemental Fig. 1A). However, we did not observe activation of MMTV-Luc by any of the tested parabens in C3H10T1/2 cells (Supplemental Fig. 1B).

To further determine whether PPAR γ or GR is required for the observed effects of parabens in C3H10T1/2 cells, we generated C3H10T1/2 cells with stable knockdown (KD) of PPAR γ , GR, or non-targeting controls by infecting the cells with lentiviral particles encoding shRNA targeting either PPAR γ , GR, or a non-targeting scrambled (SCR) shRNA followed by puromycin selection (Fig. 4B). As determined by western analysis, the knockdown efficiency for PPAR γ and GR was 98.7% and 98.3%, respectively (Fig. 4B **right**). We further demonstrated that butylparaben, but not methylparaben, activated PPAR γ in the scrambled controls to a similar degree as shown in the non-infected cells (Fig. 4A). As expected, PPAR γ activation by butylparaben or Rosi was significantly attenuated in PPAR γ KD cells, but not in GR KD cells (Fig. 4C **left**). In contrast, both methylparaben and butylparaben did not activate GR in the scramble controls. GR activation by the positive control Dex was significantly attenuated in GR KD cells, but not in PPAR γ KD cells (Fig. 4C **right**).

3.5. Adipogenic effects of butylparaben are attenuated by PPAR γ knockdown, but not by GR knockdown in C3H10T1/2 cells

To determine whether PPAR γ or GR is required for the paraben's adipogenic effects in C3H10T1/2 cells, we compared adipogenic effects of parabens among the PPAR γ KD, GR KD, and the control SCR cells (Fig. 5). Increased mRNA expression of adipogenic marker C/EBP α , FABP4, and PLIN by butylparaben was significantly attenuated in PPAR γ KD cells, but not in GR KD cells, compared to the SCR controls ($p < 0.05$) (Fig. 5A). Consistently, increased lipid accumulation by butylparaben as assessed by ORO absorbance was also significantly attenuated in PPAR γ KD cells, not in GR KD cells, compared to the SCR controls (Fig. 5B). Interestingly, GR knockdown potentiated the upregulation of FABP4 mRNA by both butylparaben and methylparaben compared to the SCR controls (Fig. 5A).

3.6. Suppression of osteoblastogenic differentiation by parabens is affected by knockdown of either PPAR γ or GR in C3H10T1/2 cells

To determine whether PPAR γ or GR is required for paraben's suppression on osteoblastogenesis, we compared the effects of parabens on osteoblastogenic differentiation among the PPAR γ KD, GR KD, and SCR cells (Fig. 6). Suppression of ALP mRNA by butylparaben was attenuated in both PPAR γ KD ($p<0.05$) and GR KD cells ($p<0.05$). Similarly, suppression of ALP by methylparaben also appeared to be attenuated in PPAR γ KD or GR KD cells, but neither reached statistical significance. Suppression of OCA mRNA by methylparaben was attenuated in PPAR γ KD and GR KD cells; however, suppression of OCA mRNA by butylparaben was not affected in either PPAR γ KD or GR KD cells. Further, suppression of OPN mRNA by butylparaben was attenuated in GR KD ($p<0.05$) cells but was potentiated in PPAR γ KD cells ($p<0.05$). In contrast, suppression of OPN mRNA by methylparaben was not affected in either PPAR γ KD or GR KD cells (Fig. 6A).

We noticed that butylparaben treatment was associated with lipid droplet formation in osteoblastogenically differentiated C3H10T1/2 cells; therefore, we further examined adipogenic marker expression in the osteoblastogenic differentiation of the stable knockdown cells and the controls. We confirmed that butylparaben increased mRNA expression of adipogenic marker C/EBP α , FABP4, and PLIN in the SCR controls, all of which were almost completely attenuated in PPAR γ KD cells ($p<0.05$) (Fig. 6B). In contrast, butylparaben's effects on C/EBP α mRNA were not attenuated and its effects on mRNA of FABP4 and PLN were only partially attenuated ($p<0.05$) in GR KD cells compared with the SCR controls (Fig. 6B). Methylparaben did not significantly affect adipogenic gene expression in the osteoblastogenic differentiation compared with the SCR controls (Fig. 6B).

4. DISCUSSION

The multipotent stem cell line C3H10T1/2, which has the potential to be differentiated into adipocytes, chondrocytes, osteocytes or myocytes [24], is a widely used model to study the modulations of stem cell fate under nutritional and environmental influence [25–29]. Consistent with the effects of parabens on 3T3-L1 adipocyte differentiation [14], butylparaben markedly promoted adipogenic differentiation (Fig. 1) but suppressed osteoblastogenic (Fig. 2) and chondrogenic differentiation (Fig. 3) of C3H10T1/2 cells at both early (i.e., 12 h) and later time points (i.e., day 12 or day 6). Although methylparaben suppressed osteoblastogenic differentiation at both time points (Fig. 2), its suppression on chondrogenic (Fig. 3) and adipogenic differentiation (Fig. 1) was only detected at early time point and not detected at the end of the differentiation. Moreover, we demonstrated that while adipogenic effects by butylparaben on adipogenic differentiation (Fig. 5) and osteoblastogenic differentiation (Fig. 6B) of C3H10T1/2 cells may be mainly mediated by PPAR γ , the effects on osteoblastogenic differentiation by both methyl- and butylparaben may be mediated by both PPAR γ and GR (Fig. 6). To our knowledge, this is the first report demonstrating the opposing effects of parabens on adipogenic and osteoblastogenic or chondrogenic differentiation using a multipotent stem cell line. The results suggest that parabens may modulate stem cell fate by favoring the differentiation of adipocytes at the

expense of osteoblasts or chondrocytes since these cell types are known to share the same stem cell population [30–32].

We have focused on methylparaben and butylparaben in the current studies because these two parabens not only represent parabens with either a short or long linear alkyl side chain, respectively, but also they are among the most commonly used parabens [33]. Adipogenic potency of parabens on 3T3-L1 adipocyte differentiation increases with the length of linear alkyl side chain with methylparaben being the weakest and butylparaben being the strongest, which correlated with their abilities to activate PPAR γ and GR [14]. We found a similar trend of PPAR γ activation by parabens in C3H10T1/2 cells (Supplemental Fig. 1A). In contrast to what was reported in 3T3-L1 cells, none of the tested parabens was shown to activate MMTV-Luc, a GR responsive reporter in C3H10T1/2 cells (Supplemental Fig. 1B). However, it was reported that propylparaben and butylparaben at the concentration of 1 μ M induced 1.60 and 1.57 fold activation of a stably transfected MMTV-Luc reporter over the control in MDA-kb2 cells, which express high levels of functional endogenous GR [34]; therefore, it is possible that the lack of GR activation by paraben in C3H10T1/2 cells may be due to the low endogenous GR expression levels in these cells.

We previously reported critical roles of nuclear transcription factor PPAR γ and GR in mediating parabens' adipogenic effects in 3T3-L1 adipocyte differentiation [14]. In this report we explored their roles in mediating stem cell differentiation in the presence of parabens. Butylparaben activated PPAR γ (Fig. 4) while knockdown of PPAR γ almost completely attenuated butylparaben's adipogenic effects as demonstrated by mRNA of adipocyte markers (C/EBP α , FABP4, and PLIN) (Fig. 5) and lipid accumulation as assessed by ORO absorbance (Fig. 5). These data suggest that butylparaben's adipogenic effects on C3H10T1/2 stem cells may be primarily mediated through PPAR γ . In contrast, the suppressive effects of methylparaben or butylparaben on osteoblastogenic differentiation were significantly affected by the knockdown of either PPAR γ or GR depending on the specific osteoblast marker examined (Fig. 6). These data suggest that osteoblastogenic effects of parabens in C3H10T1/2 stem cells may be mediated through both PPAR γ and GR.

The roles of PPAR γ in EDCs' opposing effects of adipogenic versus osteogenic differentiation of stem cells have been suggested. Phthalates and organotin compounds have been reported to activate PPAR γ and are adipogenic both *in vitro* and/or *in vivo*; therefore, they are recognized as environmental obesogens [35]. Interestingly, they also suppressed osteogenesis in mouse primary culture of bone marrow-derived stem cells and there was a strong correlation between their abilities to induce adipogenesis and the abilities to suppress osteogenesis [36]. Consistently, adipose tissue derived stromal stem cells (ADSCs) from mice exposed to tributyltin (TBT) in utero showed increased adipogenic capacity and reduced osteogenic capacity [19]. It is worth noting that activation of PPAR γ by rosiglitazone or over expression of PPAR γ was reported to promote adipogenesis but suppress osteogenesis both *in vitro* and *in vivo* [37, 38]. On the other hand, decreasing PPAR γ expression or treatment with PPAR γ antagonist led to reduced adipogenesis and increased bone mass [39, 40]. Together, our results added to the increasing evidence that some EDCs may elicit opposing effects on adipogenesis and osteogenesis through modulation of PPAR γ .

The roles of GR activation on osteogenesis have also been reported. Low glucocorticoids (GC) concentrations promote mesenchymal stem cell commitment and osteoblastogenic differentiation [41, 42]; while high concentrations and long term treatments inhibit osteoblasts maturation and terminal differentiation [43, 44]. Even though we could not detect GR activation using MMTV-Luc reporter assays, knockdown of GR clearly attenuated the suppression of ALP mRNA, potentiated the suppression of OPN mRNA, and had no effects on suppression on OCA mRNA, by butylparaben. In addition to their reported activation of PPAR γ and suppression on osteogenesis discussed above, phthalates (diethyl hexyl phthalate and diphenyl isophthalate) have been shown to activate a stably transfected MMTV-Luc reporter in MDA-kb2 cells (which express high levels of functional endogenous GR) [34, 45] whereas tributyltin did not activate MMTV-Luc in 3T3-L1 cells when used at 1 μ M concentration [46]; however, the role of GR in their suppression on osteogenesis have not been reported. Therefore, more studies are needed to clarify the role of GR in EDC's effects on osteogenesis.

How can parabens induce adipogenic differentiation but suppress osteogenic differentiation downstream of activation of PPAR γ and/or GR? We demonstrated that both methyl- and butylparabens down-regulated Runx2 during differentiation of adipocytes, osteoblasts and chondrocytes of C3H10T1/2. Runx2 mRNA was suppressed by both parabens as early as 12 h upon the treatment during osteoblastogenic and chondrogenic differentiation (Fig. 2 and 3). Runx2, which belongs to the runt-domain gene family, is highly expressed in osteoblasts and chondrocytes, and its up-regulation is required for differentiation of osteoblasts [21, 47, 48] and chondrocytes [49, 50]. Moreover, Runx2 was shown to be an inhibitor for adipocyte differentiation as knockdown of Runx2 expression promoted whereas overexpression of Runx2 inhibited adipocyte differentiation [51]. Consistently, we found that Runx2 mRNA was also suppressed at 12 h upon the treatment in adipogenic differentiation of C3H10T1/2 cells (Fig. 1). Moreover, Runx2 has been identified as a downstream target of GR signaling [51]. Therefore, it is possible that parabens could modulate stem cell fate through downregulating Runx2 expression, which could be downstream events of GR and/or PPAR γ activation. Future studies are necessary to further explore the role of Runx2 and delineate the pathways by which parabens modulate stem cell fates.

Opposing effects of paraben on adipogenic versus osteogenic differentiation of stem cells reported herein provides some explanation for the reported effects of post-weaning exposure to parabens *in vivo* [20]. Post-weaning exposure to methylparaben, but not butylparaben, significantly increased total white fat mass whereas both paraben exposure decreased serum P1NP levels [20]. It is known that WAT starts to develop shortly after birth and continues throughout life in rodents [52]. In contrast, endochondral bone development starts from early to midgestation in rodents [53]. Even though linear growth may stop after puberty, bone remodeling and repair continue throughout life [54]. Decrease of P1NP levels found in both methyl- and butylparaben exposed mice [20] are consistent with the suppressive effects of both parabens on osteoblastogenic differentiation of stem cells. However, discrepancy between the *in vitro* and *in vivo* adipogenic effects by parabens was noted. Post-weaning exposure to methylparaben significantly increased total white fat mass, which is in contrast to the modest adipogenic effects of methylparaben on C3H10T1/2 cells [20]. Although we did not detect PPAR γ activation by methylparaben in C3H10T1/2 cells, methylparaben has

been reported to dose-dependently activate a stably transfected PPARE-Luc, a luciferase reporter driven by a promoter containing peroxisome proliferator hormone response elements, in PPAR γ -CALUX cells. This PPAR γ -CALUX cells are human osteoblast cells with a stable transfection of human PPAR γ cDNA [15]. Moreover, methylparaben was shown to dose-dependently promote lipid accumulation in 3T3-L1 differentiation induced by insulin alone [15]. Therefore, the fact that we could not detect PPAR γ activation by methylparaben via transient transfection of PPAR γ activation reporters in C3H10T1/2 cells may be due to low transfection efficiency of C3H10T1/2 cells. Moreover, higher basal adipogenic differentiation in the C3H10T1/2 cells induced by the standard program, which includes treatment with DEX, MIX, and insulin (see materials and methods), may have prevented us from detecting the differences between the methylparaben-treated cells and the controls at the end of the differentiation.

Why did butylparaben induce strong adipogenic effects in C3H10T1/2 cells, but not increase total white fat mass in the post-weaning mice? It is believed that parabens are subject to hydrolysis by non-specific esterase in the body, mostly in the gastrointestinal tract and the liver. In addition, the efficiency and pattern of hydrolysis depend on the length of alkyl side chain and the location of the hydrolysis [55, 56]. Butylparaben was found to be more efficiently hydrolyzed than methylparaben by microsomes prepared from rat liver and small intestine as well as by rat plasma [56]. Therefore, it is conceivable that higher hydrolysis of butylparaben in the exposed mice may have contributed to less pronounced effects of butylparaben on total white fat mass *in vivo*. Less efficient hydrolysis of methylparaben may have led to unmetabolized methylparaben accumulation in the plasma or tissue compartment, resulting in more pronounced *in vivo* effects. Future investigations are needed to confirm paraben's opposing effects on adipogenic and osteogenic effects *in vivo*.

One limitation of our current studies is that we have focused on one dose of paraben (100 μ M) for most of our studies. Although this dose of paraben falls in the range of doses reported for paraben studies in the current literature [14, 57–59], our knowledge about human systemic exposure to parabens remains to be limited. It has been suggested that the levels of paraben detected in human subjects vary depending on the age, gender, and ethnicity, possibly reflecting frequency of exposure, the level of parabens contained in the products, and the percentage of skin areas covered with paraben containing cosmetic products [11, 60]. Women have higher exposure to parabens compared to men probably due to the higher use frequency of paraben-containing cosmetic products [11, 60]. In addition, paraben exposure was reportedly higher in children with atopic dermatitis, possibly due to increased use of specific emollients for treatment and/or impaired skin barrier [61]. Future human studies are warranted to assess paraben exposure in various populations and the health impact of parabens exposure in the risk groups. Moreover, systematic examination of dose-response of methylparaben and butylparaben on adipogenic and osteoblastogenic differentiation of the stem cells are needed in the future. When examined at Day 8, the end of the adipogenic differentiation of C3H10T1/2 cells, butylparaben, not methylparaben, appeared to dose-dependently increase adipocyte marker gene expression (Supplemental Fig. 1). However, methylparaben significantly increased C/EBP α and suppressed Runx2 mRNA expression as early as 12 h upon treatment (Fig. 1A). On the other hand, based on gene expression analysis at Day 12 of the osteoblastogenic differentiation of C3H10T1/2

cells, there were no linear dose response by either methylparaben or butylparaben (Supplemental Figure 2). Therefore, it may be necessary to analyze more time points, examine various marker genes and endpoints, or even decrease the strength of the differentiation stimulation (e.g., in case of adipogenic differentiation) to assess dose-response of parabens, including methylparaben, on adipogenic and osteoblastogenic differentiation of the stem cells.

In conclusion, we have demonstrated that butylparaben markedly promoted adipogenic differentiation but suppressed osteogenic and chondrogenic differentiation of C3H10T1/2 cells whereas methylparaben showed similar but less pronounced effects. Mechanistically, we have demonstrated the role of PPAR γ and GR in mediating the modulation of stem cell differentiation by parabens. In light of the recent findings that parabens are detected in human placenta and milk, our studies provide rationales to study paraben exposure during early development of life in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ALP	Alkaline Phosphatase
Agg	Aggrecan
Col II	Collagen II
Col X	Collagen X
C/EBPα	CCAAT/enhancer binding protein alpha
EDCs	endocrine disrupting chemicals
FABP4	Fatty acid binding protein 4
FAS	Fatty acid Synthase
GR	glucocorticoid receptor
OCA	Osteocalcin
OPN	Osteopontin
PLIN	perilipin
PPARγ	peroxisome proliferator-activated receptor gamma

Runx2	Runt-related transcription factor 2
SCD	Stearoyl-CoA desaturase

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Highlights

- Butylparaben modulates mesenchymal stem cell fate towards adipocytes
- Methylparaben has similar effects but to a less extent
- The effects of parabens are mediated through PPAR γ and/or GR
- Paraben exposure during early life development warrants further investigation

Figure 1.

Effects of methyl- and butylparaben on adipogenic differentiation of multipotent stem cell C3H10T1/2. C3H10T1/2 cells were induced for adipogenic differentiation in the presence or absence of methyl- (100 μ M), butylparaben (100 μ M) or the vehicle control DMSO. (A) mRNA expression of PPAR γ , C/EBP α , Runx2, or FABP4 were analyzed 12 h (Top panels) and 8 days (Low panels) after initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) ORO staining of cell morphology at day 8 (Left panels) and quantifications of ORO absorbance (Right panel) were shown. Data are mean $\pm$ SEM (n=3). *, **, p<0.05 and p<0.01 versus the vehicle control DMSO, respectively.

Figure 2.

Effects of methyl- and butylparaben on osteoblastogenic differentiation of multipotent stem cell C3H10T1/2. C3H10T1/2 cells were induced for osteoblastogenic differentiation in the presence or absence of methyl- (100 μ M), butylparaben (100 μ M), or the vehicle control DMSO. (A) Runx 2 mRNA was analyzed 12 h (Left panel) and mRNA of ALP, OCA, and OPN was analyzed 12 days (Right panels) after the initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) Microscopic images of the cells at day 12 were shown. Data are mean $\pm$ SEM (n=3). *, **, p<0.05 and p<0.01 versus the vehicle control DMSO, respectively.

Figure 3.

Effects of methyl- and butylparaben 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- (100 μ M), butylparaben (100 μ M), or the vehicle control DMSO. (A) mRNA expression of Runx2, Agg, Col II and Col X were analyzed at 12 h (Top panels) and 6 days (Low panels) after initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) Alcian blue staining (Left panels) and quantifications of the absorbance (Right panel) were

performed on day 6. (C) Microscopic images of the micromass at day 6 were shown. Data are mean $\pm$ SEM (n=3). **, p<0.01 versus the vehicle control DMSO.

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Figure 4.

Butylparaben activates PPAR γ , but not a GR responsive reporter, in C3H10T1/2 cells. (A) C3H10T1/2 cells were seeded and transiently transfected with PPAR γ transactivation reporters (Left panel) or MMTV-Luc (Right panel) and β -gal control plasmid for 24 h and treated with or without methyl- (100 μ M), butylparaben (100 μ M), DMSO, Rosi (1 μ M) or Dex (1 μ M) as indicated for 18 h before the cells were lysed and reporter activities were measured. Relative luciferase activities are luciferase activities normalized by β -gal activities and are expressed as fold of the negative control (-) (set at 1). (B) C3H10T1/2 cells were stably infected with lentiviral shRNA particles targeting against GR, PPAR γ , or a scrambled control (SCR). Knockdown (KD) efficiency was determined by western analysis

(Left panel) and quantified by densitometry (Right panels). (C) C3H10T1/2 cells with stable knockdown of PPAR γ (PPAR γ KD), GR (GR KD), or the scrambled controls (SCR) were seeded and transiently transfected with PPAR γ transactivation reporters (Left panels) or MMTV-Luc (Right panels) and β -gal control plasmid for 24 h and treated with or without methyl- (100 μ M), butylparaben (100 μ M), DMSO, Rosi (1 μ M) or Dex (1 μ M) for 18 h before the cells were lysed and reporter activities were measured. Relative luciferase activities are luciferase activities normalized by β -gal activities and are expressed as fold of the negative control (–) (set at 1). Data are mean $\pm$ SEM (n=3). *, ***, p<0.05 and p<0.01 versus DMSO, respectively.

Figure 5.

Adipogenic effects of butylparaben are attenuated by PPAR γ knockdown, but not by GR knockdown in C3H10T1/2 cells. C3H10T1/2 cells with stable knockdown of PPAR γ (PPAR γ KD), GR (GR KD), or the scrambled controls (SCR) were seeded at the same density and induced for adipogenic differentiation for 8 days after reaching confluence in the presence or absence of methyl- (100 μ M), butylparaben (100 μ M) or the vehicle control DMSO. (A) mRNA expression of C/EBP α , FABP4, and PLIN were analyzed on day 8 after initiation of differentiation. Expression was normalized to 36B4 and expressed as fold of the controls (set at 1). (B) Quantifications of ORO absorbance at day 8 are shown. Data are mean $\pm$ SEM (n=3). *, **, p<0.05 and p<0.01 versus the SCR controls (A) or versus the vehicle control DMSO (B), respectively.

Figure 6.

Suppression of osteoblastogenic differentiation by parabens was affected by knockdown of either PPAR γ or GR in C3H10T1/2 cells. C3H10T1/2 cells with stable knockdown of PPAR γ (PPAR γ KD), GR (GR KD), or the scrambled controls (SCR) were seeded at the same density ($\sim 5.0 \times 10^4$) per well of 12-well plates and induced for osteoblastogenic differentiation for 12 days after reaching confluence. C3H10T1/2 cells were induced for osteoblastogenic differentiation in the presence or absence of methyl- (100 μ M), butylparaben (100 μ M), or the vehicle control DMSO. (A) mRNA expression of osteoblastogenic marker ALP, OCA and OPN were analyzed on day 12 after the initiation of differentiation. (B) mRNA expression of adipogenic and lipid associated marker C/EBP α , FABP4, and PLIN were analyzed on day 12 after the initiation of osteoblastogenic 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 SCR controls, respectively.