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Reprint of "*In utero* exposure to benzo[a]pyrene increases adiposity and causes hepatic steatosis in female mice, and glutathione deficiency is protective"☆

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HIGHLIGHTS

- Metabolic effects of prenatal exposure to the pollutant benzo[a]pyrene were examined.
- Benzo[a]pyrene-exposed female offspring had increased adipose tissue and body weights and hepatic lipid.
- Glutathione-deficient offspring were resistant to these effects.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene (BaP), are ubiquitous environmental pollutants found in tobacco smoke, air pollution, and grilled foods. Reactive metabolites and reactive oxygen species generated during PAH metabolism are detoxified by reactions involving glutathione (GSH). Early life exposures to tobacco smoke and air pollution have been linked to increased risk of obesity and metabolic syndrome. We investigated the independent and interactive effects of prenatal exposure to BaP and GSH deficiency due to deletion of the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in GSH synthesis, on adiposity and hepatic steatosis in adult female F1 offspring. We mated $Gclm^{+/-}$ dams with $Gclm^{+/-}$ males and treated the pregnant dams with 0, 2, or 10 mg/kg/day BaP in sesame oil by oral gavage daily from gestational day 7 through 16. We analyzed metabolic endpoints in female $Gclm^{-/-}$ and $Gclm^{+/+}$ littermate F1 offspring. Prenatal BaP exposure significantly increased visceral adipose tissue weight, weight gain between 3 weeks and 7.5 months of age, hepatic lipid content measured by oil red O staining, and hepatic fatty acid beta-oxidation gene expression in $Gclm^{+/+}$, but not in $Gclm^{-/-}$, female offspring. Hepatic expression of lipid biosynthesis and antioxidant genes were decreased and increased, respectively, in $Gclm^{-/-}$ mice. Our results suggest that reported effects of pre- and peri-natal air pollution and tobacco smoke exposure on obesity may be mediated in part by PAHs. GSH deficiency is protective against the metabolic effects of prenatal BaP exposure.

1. Introduction

In recent years, it has become increasingly clear that various aspects of the intrauterine environment, such as exposure to environmental pollutants, influence the developmental origins of obesity and other risk factors for cardiovascular disease (Janesick and Blumberg, 2011a,b; La Merrill and Birnbaum, 2011).

Maternal smoking during pregnancy is associated with increased risk of obesity, diabetes, and hypertension in offspring (Ino, 2010; Morley et al., 1995; Oken et al., 2005; Power and Jefferis, 2002). Children of mothers who smoked during pregnancy were more likely to display hallmarks of metabolic syndrome, including higher body mass index (BMI), higher LDL and lower HDL concentrations, higher triglycerides, and higher systolic and diastolic blood

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pressure at eight years of age (Huang et al., 2007). Maternal exposure to second-hand tobacco smoke during pregnancy has been associated with increased BMI in offspring at 2 and 3 years of age (Braun et al., 2011). Gestational treatment with nicotine decreased pancreatic islet size and number and caused weight gain, adipocyte hypertrophy, glucose intolerance, and insulin resistance in male rats (Somm et al., 2008). However, tobacco smoke is a complex mixture, and the effects of other components of tobacco smoke such as PAHs have not been studied for their ability to prenatally program obesity. Tobacco smoke contains numerous PAHs, such as benzo[a]pyrene (BaP). The total carcinogenic PAH content of one cigarette has been estimated at 25–250 ng (Lodovici et al., 2004; Shopland et al., 2001). PAH exposure also occurs with exposure to sidestream tobacco smoke. Sidestream or second-hand tobacco smoke contains 10-fold higher concentrations of PAHs than mainstream smoke, or about 2.3–3.9 μg total PAHs and 0.5–1.2 μg carcinogenic PAHs per cigarette (Lodovici et al., 2004).

Particulate matter (PM) air pollution, especially the fine particulate fraction ($PM_{2.5}$), is rich in PAHs, and PAHs in PM are thought to mediate many adverse effects of PM (Lewtas, 2007). Concentrations of PAHs in ambient urban air are 10-fold higher than in rural air. Total PAH intake from ambient air has been estimated at 0.2 μg/day (range 0.02–3 μg/day) (ATSDR, 1995; Menzie et al., 1992). Long-term exposures to $PM_{2.5}$ were associated with increased cardiovascular mortality in a large study of participants from many US cities (Pope et al., 2004). Another multi-city study found that long term $PM_{2.5}$ exposure was associated with increased incidence of nonfatal cardiovascular events and of deaths from cardiovascular diseases in postmenopausal women (Miller et al., 2007). Several recent studies found increased risk of insulin resistance and type II diabetes with exposure to traffic-related air pollution (Krämer et al., 2010; Pruett et al., 2011) and $PM_{2.5}$ (Pearson et al., 2010; Xu et al., 2011) and increased risk of hypertension with exposure to $PM_{2.5}$ (Fuks et al., 2011). Exposure to traffic-related air pollution during childhood was associated with increased attained BMI (Jerrett et al., 2010). Early life exposure of mice to $PM_{2.5}$ increased adiposity, and caused insulin resistance and vascular dysfunction (Xu et al., 2010).

The other major source of PAH exposure in non-smokers is through the diet. Studies in the US and Europe have estimated that average daily intake of PAH from food is 1–17 μg/day, with the higher intakes associated with frequent consumption of grilled or smoked foods (ATSDR, 1995; Menzie et al., 1992).

PAHs require metabolism by microsomal cytochrome P450 enzymes and epoxide hydrolase to dihydrodiols, such as BaP-7,8-*trans*-dihydrodiol to exert toxicity (Kleiner et al., 2004; Shimada and Fujii-Kuriyama, 2004). This dihydrodiol can undergo further oxidation by cytochrome P450s to 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene (BPDE) or can be metabolized by aldo-keto reductases to BaP-7,8-dione (Xue and Warshawsky, 2005). BPDE forms bulky DNA adducts in the nucleus and mitochondria and is mutagenic (Allen and Coombs, 1980; Denissenko et al., 1996; Mass et al., 1993). BaP-7,8-dione is an arylhydrocarbon receptor (AHR) ligand, enabling it to be shuttled to the nucleus, where it undergoes redox cycling, generating reactive oxygen species (ROS) and oxidative DNA lesions, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) (Park et al., 2009). Glutathione transferase-mediated conjugation with glutathione (GSH) is a major Phase II biotransformation/detoxification pathway for BPDE and BaP-7,8-dione metabolites of BaP (Jernström et al., 1996; Romert et al., 1989; Xue and Warshawsky, 2005). As a major cellular antioxidant, GSH also detoxifies ROS that are produced as a result of BaP metabolism.

GSH is synthesized in two ATP-dependent reactions. The first, rate-limiting reaction is catalyzed by GCL, a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit (Franklin et al., 2009; Griffith, 1999). Mice that lack *Gclc* die during embryonic development (Dalton et al., 2000, 2004; Shi et al., 2000). Mice that lack *Gclm* survive and reproduce, but have low GSH concentrations (Giordano et al., 2006; Yang et al., 2002). *Gclm* null mice have increased sensitivity to acetaminophen and domoic acid toxicity (Giordano et al., 2006, 2007; McConnachie et al., 2007). Our previous studies showed that $Gclm^{-/-}$ mice are more sensitive to the gonadal toxicity of gestational exposure to BaP than wild type littermates (Lim et al., 2013; Nakamura et al., 2012) and that female $Gclm^{-/-}$ mice are subfertile (Nakamura et al., 2011). In contrast, *Gclm* null mice are protected against diet-induced steatohepatitis, showing upregulation of hepatic antioxidant genes and downregulation of triglyceride synthesis and fatty acid β-oxidation (Haque et al., 2010; Kendig et al., 2011). Nonalcoholic hepatic steatosis, also called nonalcoholic fatty liver disease, is an independent risk factor for Type 2 diabetes and is prevalent in individuals with metabolic syndrome (Sung and Kim, 2011). *GCLC* polymorphisms are associated with increased risk of progression of nonalcoholic hepatic steatosis to nonalcoholic steatohepatitis in humans (Oliveira et al., 2010).

In our studies designed to test the modifying effects of GSH deficiency on the ovarian and testicular toxicity of prenatal BaP exposure (Lim et al., 2013; Nakamura et al., 2012), we observed increased weight gain in the BaP-exposed female offspring. We therefore investigated the effects of prenatal BaP exposure and *Gclm* genotype on adiposity and hepatic steatosis in these offspring.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

2.2. Animals

Gclm null mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a beta-galactosidase/neomycin phospho-transferase fusion minigene (Giordano et al., 2006; McConnachie et al., 2007). The mice were backcrossed 8 times onto a C57BL/6 J genetic background (B6.129-$Gclm^{tm1Tjka}$; hereafter referred to as $Gclm^{-/-}$). Mice for these experiments were generated at the University of California Irvine (UC Irvine) by mating $Gclm^{+/-}$ males with $Gclm^{+/-}$ females. Offspring were genotyped by PCR using primers for both the native *Gclm* sequence and the *β-Geo* sequence on DNA extracted from tail or toe snips as previously described (Giordano et al., 2006). All mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility, with free access to deionized water and soy-free laboratory chow (Harlan 2019, 23% of calories from fat), on a 14:10 h light–dark cycle. Temperature was maintained at 21–23 °C. The experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996) and were approved by the Institutional Animal Care and Use Committee at UC Irvine.

2.3. Monitoring of estrous cycles

Estrous cycle stage in individually housed adult female mice was evaluated every morning by microscopic examination of fresh vaginal lavage fluid obtained in 0.9% sodium chloride (Cooper et al., 1993).

2.4. Experimental protocol

$Gclm^{+/-}$ female mice were mated with $Gclm^{+/-}$ or $Gclm^{-/-}$ male mice on the afternoon of proestrus based on vaginal cytology. Females were checked for vaginal plugs the following morning. The day of vaginal plug detection in the female was designated gestational day (GD) 1. Dams were treated by oral gavage with 10 mg/kg benzo[a]pyrene in sesame oil daily from GD7 to GD16 (Block 1) or 2 mg/kg/day from GD7 to GD16 (Block 2). Control animals were gavaged with the same volume (2 ml/kg) of sesame oil alone in both blocks. Dams were randomly assigned to treatment group using a random number table. The dosing regimen in Block 1 was based on a previous study in CD-1 mice, which showed that offspring treated with this dose had reduced fertility compared to controls but were not completely infertile (MacKenzie and Angevine, 1981). The lower dose was used in the second block because of an apparently, but not statistically significantly, increased intrauterine mortality of $Gclm^{-/-}$ female fetuses in Block 1, which resulted in only one litter of eight BaP-treated litters having any $Gclm^{-/-}$ female offspring (Lim et al., 2013). Dams

were allowed to deliver and care for their litters. Litters were weaned on post-natal day (PND) 21. Only the female offspring were used for the present study.

Gclm$^{-/-}$ and *Gclm*$^{+/+}$ female offspring were bred with wild type males for 20 weeks starting at 8 weeks of age (Lim et al., 2013). After the last litter was delivered, estrous cycles were monitored for at least 14 days and mice were killed by carbon dioxide asphyxiation on the next morning of estrus or on day 15 if they were not cycling. Therefore, female mice were 7.5–8 months old at the time of euthanasia. Body weights and weights of livers and kidneys, and combined ovarian, mesenteric, and perirenal (visceral) adipose depot weights were obtained at the time of euthanasia. Pieces of livers were fixed in 10% neutral buffered formalin overnight, then stored in 70% ethanol in both studies. For Block 2 only, pieces of liver were also snap frozen for oil red O staining and for RNA extraction.

2.5. Quantitative real-time reverse transcriptase PCR (qRTPCR)

Total RNA was extracted from liver using the Qiagen RNeasy Kit (Qiagen, Valenica, CA) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed to cDNA using Transcriptor Reverse Transcriptase (Roche, Nutley, NJ) following the manufacturer's protocol. Aliquots of cDNA from 62.5 ng starting RNA were subjected to PCR in duplicate using gene-specific forward (F) and reverse (R) primers (purchased from Invitrogen) and the FastStart SYBR Green QPCR Master Mix (Roche, Nutley, NJ) in 20 μl reaction volumes. The PCR amplification of all transcripts was performed on the DNA Opticon Thermal Cycler (MJ Research, Watertown, MA) using the following program: (1) initial incubation at 95 °C for 10 min to activate FastStart Taq DNA polymerase; (2) each cycle (total 40 cycles) at 95 °C for 10 s, followed by incubation at an average annealing temperature of forward and reverse primers for 30 s according to the primers used (Supplemental Table S1), and final elongation at 72 °C for 10 s. The quality and identity of each PCR product was determined by melting curve analysis. Expression of each target gene relative to expression of the mouse *36B4* gene (housekeeping gene) was calculated by the method of Pfaffl (Pfaffl, 2001), which takes account of differences in PCR efficiency between the target gene and the housekeeping gene. Standard curves derived from serial dilutions of mouse liver RNA were used to determine the efficiencies of the PCR reactions. Forward (F) and reverse (R) primer sequences (5′–3′) are shown in Supplemental Table S1. Primer sequences were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank/) or were designed using PerlPrimer (version 1.1.14; copyright 2003–2006, O. Marshall).

2.6. Hepatic histology

Formalin-fixed pieces of liver were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Sections were evaluated blind to genotype and treatment for steatosis (0 = <5% of cells with steatosis; 1 = 5–33% of cells; 2 = >33–66% of cells; 3 = >66% of cells), ballooning (0 = absent; 1 = present), lobular inflammation (0 = no foci; 1 = <2 foci/200× field; 2 = ≥2 foci), and central vein or periportal inflammation (0 = none/minimal; 1 = greater than minimal), and a nonalcoholic fatty liver disease score was calculated as the sum of these subscores for each mouse (Kleiner et al., 2005).

2.7. Hepatic oil red O staining

Snap frozen liver samples were embedded in optimal cutting temperature embedding compound and sectioned at 10 μm using a cryostat. They were then fixed in 4% paraformaldehyde in PBS, sequentially washed with PBS, deionized water, and 60% isopropanol, then stained with oil red O (4 g/L in 60% isopropanol), washed with 60% isopropanol, and counterstained with hematoxylin. Sections were scored for oil red O staining blind to genotype and treatment as follows: no or minimal staining, some staining, or abundant staining.

2.8. Statistical analyses

Because Blocks 1 and 2 were conducted about two years apart, the effect of block on various endpoints (body weight, body weight gain, visceral adipose tissue weight, kidney weight, liver weight) was examined for *Gclm*$^{+/+}$ females exposed prenatally to 0 mg/kg BaP. As there were no significant effects of block on any of these endpoints, the data were combined for our primary analyses. We also analyzed the data from Block 2 separately and present these results in Supplemental Table S2. Because *Gclm* heterozygous female mice were mated with heterozygous males and then treated with oil or BaP, there were both *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ offspring in most litters. In developmental toxicology studies, it is very important to control for litter effects. Taking the litter average for a given endpoint is not a statistically powerful way to adjust for litter effects. Moreover, in our case, we would have had to take separate litter averages for *Gclm* wild type and homozygous null offspring, and therefore would not have been able to adjust for possible litter effects between these genotypes. Use of a generalized estimating equation (GEE, a form of generalized linear model) approach is considered to be a preferred way to adjust for litter effects because it enables data from multiple animals per litter to be used while adjusting for correlations within litters (Ryan, 1992). It is also used to adjust for repeated measures within individuals (Ehrlich et al., 2012). Therefore, for those endpoints for which data from more than one animal per litter were available, the effects of genotype and BaP dose were analyzed using GEE models. BaP dose, *Gclm* genotype, and BaP × genotype interaction were modeled as fixed effects, and litter number was modeled as a subject effect to adjust for litter effects. Because variances were not assumed to be homogeneous between *Gclm*$^{-/-}$ and *Gclm*$^{+/+}$ offspring from the same litter, an unstructured working correlation matrix was used. The estimated marginal means and standard errors of the mean (SEM) from the GEE models are presented in the tables and figures.

For the quantitative real time RTPCR endpoints, data from only one animal per litter were analyzed; therefore General Linear Models were used with the same fixed effects. For the latter analyses, if the overall *P*-values for genotype or treatment were <0.05, then intergroup comparisons were made using Fisher's least significant difference (LSD) test. To assess the effect of BaP dose or genotype on an ordinal endpoint, such as the extent of hepatic oil red O staining, Kendall's tau test was used. Statistical analyses were performed using SPSS Statistics Version 20 for MacIntosh.

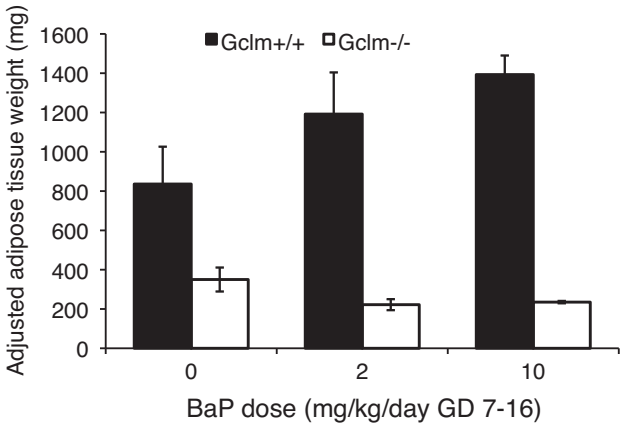

Fig. 1. Prenatal exposure to BaP increased visceral adipose tissue weights in *Gclm*$^{+/+}$ females, but not in *Gclm*$^{-/-}$ females. Pregnant *Gclm*$^{+/-}$ females, which had been mated with *Gclm*$^{+/-}$ males, were treated with 0, 2, or 10 mg/kg/day BaP in sesame oil by oral gavage from GD 7–16. The graphs show the estimated marginal mean ± SEM of the combined periovarian, mesenteric, and perirenal (visceral) adipose tissue depot weights in F1 female offspring at 7.5 months of age. The effects of *Gclm* genotype and the BaP dose × genotype interaction were statistically significant ($P < 0.001$). Data are from 6–13 offspring from 4–10 litters/group, except *Gclm*$^{-/-}$ 10 mg/kg BaP, data are from 2 offspring from 1 litter.

3. Results

3.1. Effects of prenatal BaP exposure and Gclm genotype on adipose tissue, kidney, liver, and body weights

Detailed information about the effects of prenatal BaP treatment on litter size, litter mortality, and offspring genotype distributions were previously published (Lim et al., 2013). Briefly, there were no statistically significant effects of BaP treatment on any of these parameters.

In utero BaP-treated *Gclm*$^{+/+}$ females in both experiments had increased visceral adipose depot weights (combined ovarian, perirenal, and mesenteric adipose tissue depots) compared to oil-treated *Gclm*$^{+/+}$ females (Fig. 1). The mean adipose tissue weights in the control, oil-treated *Gclm*$^{+/+}$ female mice were also increased compared to their null littermates, but *Gclm*$^{-/-}$ females exposed to BaP *in utero* did not have increased adipose tissue weights compared to oil-treated *Gclm*$^{-/-}$ females (Fig. 1). The effect of genotype ($P < 0.001$) and the interaction effect between genotype and BaP treatment ($P < 0.001$) on adipose tissue depot weights were statistically significant, with prenatal BaP exposure increasing adipose tissue weights in the *Gclm*$^{+/+}$ females, but not in the *Gclm*$^{-/-}$ females. Similar and statistically significant effects of genotype and BaP × genotype interaction on visceral adipose tissue weights were observed when the data from Block 2 (0 and 2 mg/kg/day BaP) were analyzed alone (Supplemental Table S2).

The BaP-treated *Gclm*$^{+/+}$ females weighed about 3 g more on average at 7.5 months of age and gained about 2 g more during

Table 1
Effects of prenatal BaP exposure and *Gclm* genotype on organ and body weights in female mice.

	Estimated marginal means ± SEM					
	$Gclm^{+/+}$			$Gclm^{-/-}$		
BaP dose (mg/kg/day GD7-16)	0	2	10	0	2	10
Body weight (g)[a]	25.6 ± 0.9	29.0 ± 0.7	28.4 ± 0.8	23.0 ± 0.6	22.5 ± 0.4	19.0 ± 0.5
Body weight gain (g)[b]	6.5 ± 0.6	6.5 ± 1.3	8.7 ± 0.5	5.7 ± 0.4	3.6 ± 0.7	0.3 ± 0.3
Adipose tissue weight (mg)[c]	836 ± 190	1192 ± 212	1393 ± 97	350 ± 61	222 ± 28	235 ± 6
Liver weight (g)[d]	1.45 ± 0.06	1.50 ± 0.04	1.40 ± 0.04	1.30 ± 0.02	1.25 ± 0.05	1.17 ± 0.01
Adjusted liver weight (mg/g BW)[e]	52.4 ± 1.4	52.8 ± 1.3	49.1 ± 1.1	58.2 ± 0.8	53.9 ± 1.5	57.0 ± 0.3
Kidney weight (paired, mg)[f]	374 ± 10	408 ± 11	375 ± 5	385 ± 8	382 ± 18	287 ± 0.4
Adjusted kidney weight (mg/g BW)[g]	14.2 ± 0.3	15.2 ± 0.6	13.3 ± 0.3	15.7 ± 0.4	16.3 ± 0.4	14.3 ± 0.2

6–13 offspring, from 4–10 litters/group, except $Gclm^{-/-}$ 10 mg/kg BaP, 2 offspring from 1 litter.
[a] $P = 0.046$, effect of BaP dose; $P < 0.001$, effect of genotype; $P < 0.001$, dose × genotype interaction.
[b] $P = 0.031$, effect of BaP dose; $P < 0.001$, effect of genotype; $P < 0.001$, dose × genotype interaction.
[c] $P = 0.202$, effect of BaP dose; $P < 0.001$, effect of genotype; $P < 0.001$, dose × genotype interaction.
[d] $P = 0.063$, effect of BaP dose; $P < 0.001$, effect of genotype; $P = 0.346$, dose × genotype interaction.
[e] $P = 0.193$, effect of BaP dose; $P < 0.001$, effect of genotype; $P = 0.002$, dose × genotype interaction.
[f] $P < 0.001$, effect of BaP dose; $P < 0.001$, effect of genotype; $P < 0.001$, dose × genotype interaction.
[g] $P < 0.001$, effect of BaP dose; $P < 0.001$, effect of genotype; $P = 0.109$, dose × genotype interaction.

the course of the study than oil-treated $Gclm^{+/+}$ females (Table 1). In contrast, $Gclm^{-/-}$ females exposed prenatally to 10 mg/kg/day BaP weighed less and gained less weight than 0 or 2 mg/kg/day BaP exposed $Gclm^{-/-}$ females (Table 1). The effects of prenatal BaP treatment ($P = 0.046$ for body weight and $P = 0.031$ for weight gain) and the BaP dose times genotype interaction ($P < 0.001$ for both body weight and weight gain) were statistically significant. There were also statistically significant effects of genotype on female offspring body weight and body weight gain between 2 and 7.5 months of age, with $Gclm^{+/+}$ females weighing significantly more and gaining more weight than $Gclm^{-/-}$ females ($P < 0.001$; Table 1). Similar and statistically significant effects of genotype and BaP × genotype interaction on body weight were observed when the data from Block 2 were analyzed alone (Supplemental Table S2). For body weight gain in Block 2 alone, only the effect of genotype was statistically significant (Supplemental Table S2).

There was a statistically significant effect of genotype ($P < 0.001$) on liver weight unadjusted for body weight, with lower liver weights in $Gclm^{-/-}$ and BaP-treated mice (Table 1). When liver weight was adjusted for body weight, the interaction between BaP dose and genotype also became statistically significant ($P = 0.002$; Table 1). There were statistically significant effects of genotype, BaP dose, and genotype times dose interaction on kidney weight unadjusted for body weight (all $P < 0.001$) and of genotype and BaP dose on kidney weight adjusted for body weight (both $P < 0.001$), with lower kidney weights in $Gclm^{-/-}$ mice overall and in BaP-treated $Gclm^{-/-}$ mice, but increased kidney weights in the $Gclm^{+/+}$ mice prenatally exposed to 2 mg/kg/day BaP (Table 1). When the data from Block 2 were analyzed alone, similar and statistically significant effects of genotype and BaP × genotype interaction were observed on unadjusted liver and adjusted kidney weights; only the BaP × genotype interaction was statistically significant for unadjusted kidney weights, and there were no statistically significant effects on adjusted liver weights (Supplemental Table S2).

3.2. Hepatic steatosis develops in $Gclm^{+/+}$ female mice exposed to BaP prenatally, but not in $Gclm^{-/-}$ female mice exposed to BaP prenatally

Hepatic lipid content, as detected by oil red O staining, was significantly increased in $Gclm^{+/+}$ females treated with 2 mg/kg/day BaP *in utero* compared to control oil-treated $Gclm^{+/+}$ females ($P = 0.037$) and 2 mg/kg/day BaP-treated $Gclm^{-/-}$ females ($P < 0.001$; Fig. 2A, B and Table 2). Oil red O staining was not performed in Block 1. Hepatic histology in the prenatally BaP-exposed $Gclm^{+/+}$ mice showed features of non-alcoholic fatty liver disease, with steatosis, mild lobular inflammatory infiltrates, and periportal and central vein inflammatory infiltrates (Kleiner et al., 2005) (Fig. 2E, G, I, J). Livers of $Gclm^{-/-}$ mice consistently showed less of these features, regardless of prenatal BaP exposure (Fig. 2F, H). Control $Gclm^{+/+}$ mice exposed (Fig. 2C) prenatally to oil vehicle had levels of hepatic steatosis and inflammation intermediate between $Gclm^{-/-}$ mice (Fig. 2D) and BaP-exposed $Gclm^{+/+}$ mice (Fig. 2E, G). Consistent with these observations, there were statistically significant effects of genotype ($P < 0.001$) and BaP dose ($P = 0.018$) on nonalcoholic fatty liver disease scores (Table 3). No ballooning, a feature of steatohepatitis, was noted in any of the livers. The nonalcoholic fatty liver disease scores we obtained in the control $Gclm^{-/-}$ and $Gclm^{+/+}$ mice in the present study are very similar to those reported for control $Gclm^{-/-}$ and $Gclm^{+/+}$ mice in another recent study (Haque et al., 2010).

Table 2
Effects of prenatal BaP exposure and *Gclm* genotype on hepatic oil red O staining for lipids.

	No staining	Some staining	Abundant staining
	N (% within BaP dose and genotype group)		
$Gclm^{+/+}$[a]			
0 mg/kg/day BaP	1 (20)	3 (60)	1 (20)
2 mg/kg/day BaP[b]	0 (0)	2 (33)	4 (67)
$Gclm^{-/-}$			
0 mg/kg/day BaP	0 (0)	3 (100)	0 (0)
2 mg/kg/day BaP[b]	1 (20)	4 (80)	0 (0)

[a] $P = 0.037$, effect of BaP dose within $Gclm^{+/+}$ genotype.
[b] $P < 0.001$, effect of genotype within 2 mg/kg/day BaP-exposed groups.

Table 3
Effects of prenatal BaP exposure and *Gclm* genotype on hepatic nonalcoholic fatty liver disease (NAFLD) scores (estimated marginal means ± SEM).

	NAFLD score	*N*
$Gclm^{+/+}$		
0 mg/kg/day BaP	2.6 ± 0.3	5
2 mg/kg/day BaP	2.7 ± 0.4	6
10 mg/kg/day BaP	2.8 ± 0.2	6
$Gclm^{-/-}$		
0 mg/kg/day BaP	1.4 ± 0.3	5
2 mg/kg/day BaP	2.0 ± 0.2	5
10 mg/kg/day BaP	1.0 ± 0.0	2

$P < 0.001$, effect of genotype; $P = 0.018$, effect of BaP dose; $P = 0.128$, genotype × BaP dose.

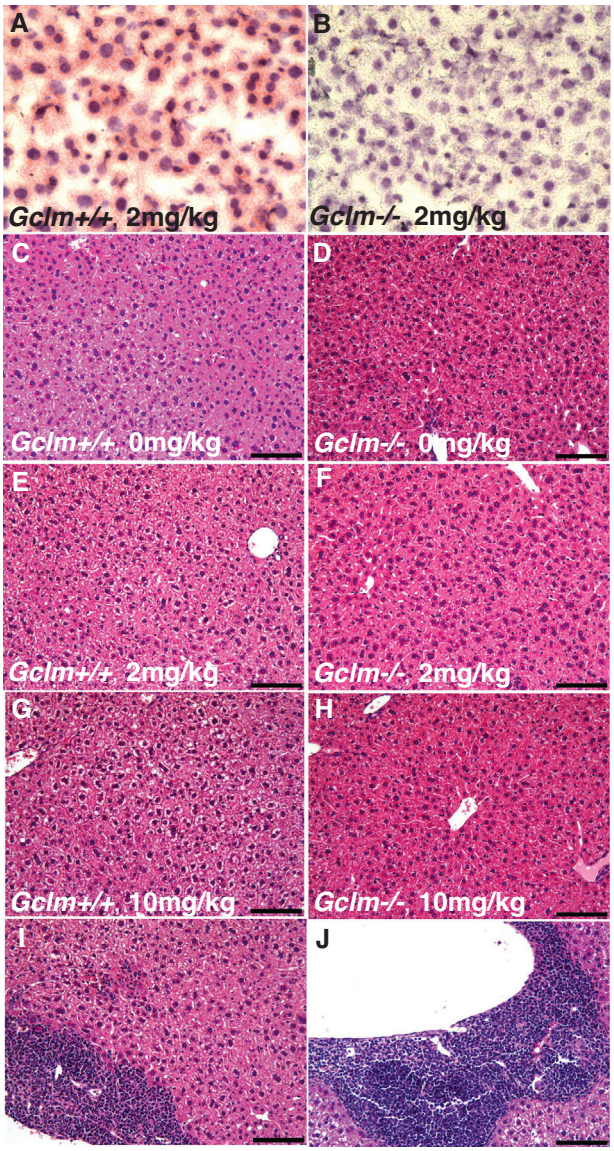

Fig. 2. Prenatal exposure to BaP increased hepatic lipid accumulation and histological evidence of hepatic steatosis in *Gclm*$^{+/+}$ females, but not in *Gclm*$^{-/-}$ females. Hepatic lipid deposition was evaluated by oil red O staining (A, B), and liver histology was evaluated in hematoxylin and eosin stained sections (C–I), in subsets of the F1 offspring. (A) Abundant lipid (red staining) was noted in liver of 2 mg/kg/day BaP-exposed *Gclm*$^{+/+}$ female. Original magnification, 400×. (B) Minimal lipid was noted in liver of 2 mg/kg/day BaP-exposed Gclm$^{-/-}$ female; 0 mg/kg BaP-exposed Gclm$^{-/-}$ females had similarly low levels of hepatic oil red O staining. Original magnification, 400×. (C) No steatosis in liver of 0 mg/kg/day BaP-exposed *Gclm*$^{+/+}$ female. (D) No steatosis in liver of 0 mg/kg/day BaP-exposed *Gclm*$^{-/-}$ female. (E) Mild steatosis in liver of 2 mg/kg/day BaP-exposed *Gclm*$^{+/+}$ female. (F) Minimal steatosis in liver of 2 mg/kg/day BaP-exposed *Gclm*$^{-/-}$ female. (G) Hepatocyte steatosis (white spaces in many hepatocytes) in liver of 10 mg/kg BaP-exposed *Gclm*$^{+/+}$ female. (H) Minimal steatosis in liver of 10 mg/kg/day BaP-exposed *Gclm*$^{-/-}$ female. (I) Inflammatory infiltrate adjacent to central vein in the liver of the same mouse as in E. (J) Inflammatory infiltrate adjacent to central vein in the liver of the same mouse as in G. Inflammatory infiltrates as in I and J were never observed in *Gclm*$^{-/-}$ females. Scale bars, 100 μm.

3.3. Effects of prenatal BaP exposure and Gclm genotype on hepatic gene expression

To begin to investigate the mechanisms by which prenatal BaP exposure and *Gclm* genotype affect hepatic steatosis, we analyzed hepatic expression of genes related to lipid metabolism, adipogenesis, and oxidative stress response in liver tissue from mice prenatally exposed to 0 or 2 mg/kg/day BaP in Block 2. Three major

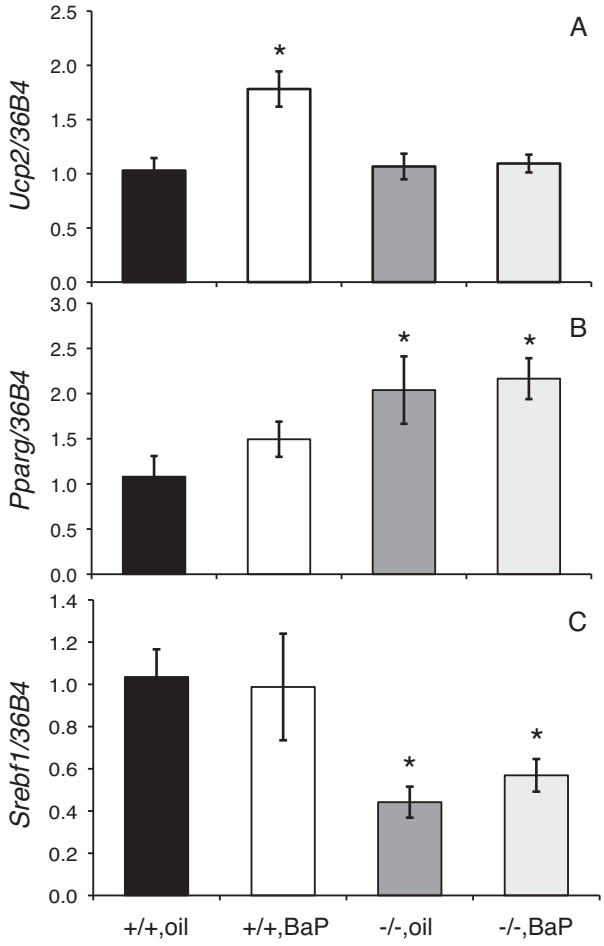

Fig. 3. Effects of prenatal exposure to BaP and *Gclm* genotype on hepatic gene expression. RNA was extracted from liver of one mouse per genotype per litter from Block 2 (exposed prenatally to 0 or 2 mg/kg/day BaP as described in Fig. 1), and qRTPCR was used to measure expression of genes related to lipid metabolism, adipogenesis, fatty acid beta-oxidation, and oxidative stress response, as described in Section 2. (A) Uncoupling protein 2 (*Ucp2*), which uncouples mitochondrial ATP production from substrate oxidation, showed increased expression in *Gclm*$^{+/+}$ BaP-treated females compared to *Gclm*$^{+/+}$ oil-treated controls and compared to both groups of *Gclm*$^{-/-}$ females ($P < 0.03$, effects of BaP dose, genotype, and dose × genotype). (B) *Pparg*, a major regulator of adipocyte differentiation, showed nonsignificantly increased expression in prenatally BaP exposed *Gclm*$^{+/+}$ females and significantly increased expression in both *Gclm*$^{-/-}$ groups ($P = 0.005$, effect of genotype; $P = 0.293$, effect of BaP dose; $P = 0.571$ dose × genotype). (C) Sterol regulatory element binding transcription factor 1 (*Srebf1*), a major regulator of lipogenesis, showed significantly decreased expression in both *Gclm*$^{-/-}$ groups ($P = 0.011$, effect of genotype; $P = 0.819$, effect of BaP dose; $P = 0.619$ dose × genotype. $^*P < 0.05$ *versus* 0 mg/kg/day BaP exposed *Gclm*$^{+/+}$ group by LSD test. $N = 5$/group, except $N = 3$, *Gclm*$^{-/-}$, 0 mg/kg BaP group.

patterns of hepatic gene expression were observed. The first pattern, exemplified by the expression of uncoupling protein 2 (*Ucp2*), a protein that uncouples mitochondrial ATP production from oxidation of substrates, showed increased expression in *Gclm*$^{+/+}$ BaP-treated females compared to *Gclm*$^{+/+}$ oil-treated controls and compared to both groups of *Gclm*$^{-/-}$ females ($P < 0.03$, effects of BaP dose, genotype, and dose × genotype; Fig. 3A and Table 4). A similar pattern was also observed for Acyl-CoA oxidase (*Acox1*), the rate limiting enzyme in microsomal fatty acid β-oxidation; Carnitine palmitoyltransferase-1a (*Cpt1a*), a rate-limiting enzyme in mitochondrial fatty acid β-oxidation; peroxisome proliferator activated receptor-α (*Ppara*), a transcriptional regulator of *Cpt1a* and *Ucp2*; and phosphoenolpyruvate carboxykinase (*Pck2*, also known as *Pepck*), a target of peroxisome proliferator activated receptor-γ

Table 4
Effects of prenatal BaP exposure and *Gclm* genotype on hepatic gene expression.

	Gclm$^{+/+}$		*Gclm*$^{-/-}$	
	BaP dose (mg/kg/day GD 7–16)			
	0	2	0	2
	Mean ± SEM expression relative to 36B4 expression			
Fatty acid beta-oxidation genes				
Ucp2[a]	1.03 ± 0.12	1.78 ± 0.16[d]	1.07 ± 0.12	1.09 ± 0.08
Ppara	1.15 ± 0.35	1.43 ± 0.26	1.22 ± 0.22	0.86 ± 0.12
Cpt1a[b]	1.07 ± 0.19	1.37 ± 0.10	0.98 ± 0.11	0.90 ± 0.04
Acox1	1.04 ± 0.14	1.25 ± 0.11	1.02 ± 0.12	1.00 ± 0.10
Antioxidant genes				
Gclc[b]	1.04 ± 0.15	1.78 ± 0.27[d]	2.38 ± 0.50[d]	2.33 ± 0.45[d]
Gclm	1.04 ± 0.14	1.41 ± 0.13	ND	ND
Gstm1[c]	1.02 ± 0.11	1.14 ± 0.17	3.52 ± 0.91[d]	3.51 ± 0.37[d]
Gstm2[c]	1.06 ± 0.18	1.15 ± 0.36	2.53 ± 0.75[d]	2.09 ± 0.32[d]
Gstp1[c]	1.01 ± 0.08	1.03 ± 0.07	2.97 ± 0.47[d]	3.33 ± 0.40[d]
Gsta4	1.00 ± 0.06	1.03 ± 0.11	1.05 ± 0.18	1.35 ± 0.17
Gpx1	1.05 ± 0.15	1.48 ± 0.16	1.13 ± 0.53	1.22 ± 0.20
Gsr	1.05 ± 0.16	1.49 ± 0.27	1.17 ± 0.18	1.40 ± 0.14
Sod1	1.13 ± 0.27	1.27 ± 0.08	1.28 ± 0.39	1.80 ± 0.32
Sod2[c]	1.05 ± 0.16	1.59 ± 0.16[d]	2.09 ± 0.31[d]	1.45 ± 0.09[d]
Prdx3	1.03 ± 0.13	0.92 ± 0.08	0.95 ± 0.04	1.01 ± 0.22
Txn[c]	1.01 ± 0.08	1.15 ± 0.09	1.54 ± 0.28[d]	1.45 ± 0.08[d]
Txn2	1.02 ± 0.11	1.16 ± 0.05	1.11 ± 0.01	1.03 ± 0.15
Txnrd1	1.06 ± 0.18	1.38 ± 0.17	1.33 ± 0.16	1.11 ± 0.10
Glrx	1.00 ± 0.03	1.12 ± 0.09	1.04 ± 0.13	0.87 ± 0.05
Cat	1.06 ± 0.18	1.46 ± 0.21	1.52 ± 0.26	1.24 ± 0.13
Lipogenesis and adipogenesis genes				
Pparg[c]	1.08 ± 0.23	1.49 ± 0.20	2.04 ± 0.37[d]	2.17 ± 0.23[d]
Fabp4[c]	1.00 ± 0.06	1.15 ± 0.22	0.65 ± 0.19	0.80 ± 0.11
Srebf1[c]	1.03 ± 0.13	0.99 ± 0.25	0.44 ± 0.07[d]	0.57 ± 0.08[d]
Fasn[c]	1.02 ± 0.09	0.68 ± 0.20	0.30 ± 0.09[d]	0.40 ± 0.08[d]
Pck2	1.11 ± 0.24	1.59 ± 0.24	1.47 ± 0.03	1.07 ± 0.09

$N = 5$/group, except *Gclm*$^{-/-}$, 0 mg/kg BaP, $N = 3$.
ND = not detected.
[a] $P < 0.05$, effects of BaP dose, genotype, dose × genotype interaction.
[b] $P = 0.05$, effect of genotype. Effects of BaP dose and dose × genotype interaction not significant.
[c] $P < 0.05$, effect of genotype. Effects of BaP dose and dose × genotype interaction not significant.
[d] $P < 0.05$, significantly different from *Gclm*$^{+/+}$, 0 mg/kg/day BaP by LSD test.

(*Pparg*) involved in repression of hepatic gluconeogenesis, although the differences were not statistically significant (Table 4).

The second pattern of expression was observed for hepatic *Pparg*, which was increased by 1.5-fold in *Gclm*$^{+/+}$ BaP-treated females and significantly increased by >2-fold in both *Gclm*$^{-/-}$ groups compared to *Gclm*$^{+/+}$ oil-treated controls (Fig. 3B and Table 4). Several antioxidant genes displayed a similar pattern of significant 1.5-fold upregulation (*Gclc*, *Sod2)* or no change (glutathione-*S*-transferases, *Gstm1*, *Gstm2*, *Gstp1*, and Thioredoxin, *Txn*) in hepatic expression in *Gclm*$^{+/+}$ BaP-treated females and two-fold or greater upregulation in both *Gclm*$^{-/-}$ groups compared to *Gclm*$^{+/+}$ oil controls.

The third pattern of hepatic gene expression, with significant downregulation in both *Gclm*$^{-/-}$ groups and no effect of prenatal BaP exposure in *Gclm*$^{+/+}$ females, was noted for the lipogenesis genes *Srebf1* (sterol regulatory element binding transcription factor 1, also known as *Srebp-1c*), *Fasn* (fatty acid synthase), and *Fabp4* (Fatty acid binding protein 4; Fig. 3C and Table 4). *Srebf1* is a known regulator of *Fasn* and *Fabp4* transcription (Latasa et al., 2003; Nakachi et al., 2008).

4. Discussion

Our data demonstrate that prenatal exposure to BaP *via* a route of exposure relevant to humans increases body weight gain and visceral adipose tissue depot weights and causes hepatic steatosis in wild type female mice, but not in GSH-deficient, *Gclm*$^{-/-}$ female mice. Importantly, these effects were noted in mice maintained on a breeding diet (23% of calories from fat), not on a high fat diet (variably defined as 30–60% of calories from fat (Buettner et al., 2006)). The increased hepatic steatosis in BaP-exposed wild type mice is associated with increased expression of *Ucp2*, a major regulator of hepatic fatty acid β-oxidation, and of *Pparg* a major regulator of adipogenesis. The apparent resistance to hepatic lipid accumulation in the *Gclm*$^{-/-}$ mice may be caused by pronounced hepatic down-regulation of lipogenesis genes and upregulation of other antioxidant genes.

Increased visceral, as compared to subcutaneous, adipose tissue is strongly related to increased risks of insulin resistance, diabetes mellitus, dyslipidemia, hypertension, and atherosclerotic heart disease (Tran and Kahn, 2010). Although the daily dose of PAHs in highly exposed humans (about 0.002 mg/kg/day from tobacco smoke, food, and air pollution) is about three orders of magnitude lower than the 2 mg/kg/day dose of BaP in the present study (Lodovici et al., 2004; Shopland et al., 2001; ATSDR, 1995; Menzie et al., 1992), the cumulative dose to a highly exposed woman during all of pregnancy (about 0.55 mg/kg PAHs) is only 36-fold lower than our cumulative dose during gestation of 20 mg/kg in the 2 mg/kg/day dose group. Therefore, our observations that prenatal exposure to BaP increased visceral adipose tissue weight and body weight gain in female mice (Section 3.1) suggest that BaP and other PAHs found in tobacco smoke and PM air pollution may be responsible, at least in part, for the reported associations between prenatal tobacco smoke exposure and increased BMI and obesity (Huang et al., 2007; Oken et al., 2005; Power and Jefferis, 2002) and between childhood PM exposure and increased BMI (Jerrett et al., 2010). In contrast to our findings with prenatal exposure in mice, neonatal treatment of rat pups with 2 mg/kg/day BaP by oral gavage on PND 5–11 was reported to decrease adult body weight (Chen et al., 2012), but the diet fed the rats was not specified and effects on adipose tissue and liver were not examined in that study.

Some BaP metabolites are arylhydrocarbon receptor ligands (Park et al., 2009). Arylhydrocarbon receptor constitutively represses fatty acid synthesis genes in mouse liver and in human hepatoma cells and hepatocytes (Tanos et al., 2012). In addition, activation of arylhydrocarbon receptor by the agonist β-naphthoflavone downregulates expression of fatty acid synthesis genes in mouse liver and primary human hepatocytes (Tanos et al., 2012). This suggests that future studies should investigate whether the effects of prenatal BaP exposure on hepatic steatosis are mediated by down-regulation of arylhydrocarbon receptor expression.

The significantly increased hepatic expression of *Ucp2* in prenatally BaP-exposed *Gclm*$^{+/+}$ mice (Section 3.2) is consistent with increased hepatic fatty acid β-oxidation, as has been reported in patients with non-alcoholic steatosis and steatohepatitis (Koek et al., 2011; Pessayre and Fromenty, 2005). Increased hepatic fatty acid β-oxidation is initially adaptive in that it increases the activity of the mitochondrial electron transport chain, thereby limiting ROS production; however, because it decreases the proton gradient across the inner mitochondrial membrane and uncouples ATP production from electron transport, it leads to ATP depletion (Koek et al., 2011). The latter can lead to hepatocyte necrosis (Koek et al., 2011). Increased hepatic expression of *Ucp2* has also been reported in mice with hepatic steatosis induced by a methionine and choline deficient diet (Haque et al., 2010). UCP2 is also known to attenuate the pancreatic insulin response to glucose and to increase insulin resistance in white adipose tissue, thus exacerbating the diabetic phenotype (Azzu et al., 2010; Chan and Harper, 2006). Our finding of increased *Ucp2* expression thus supports a link between prenatal exposure to BaP and other PAHs and hepatic steatosis and metabolic syndrome.

The transcription factor *Pparg* is a major regulator of adipogenesis (Lowe et al., 2011). Upregulation of hepatic *Pparg* is thought to play a role in the pathogenesis of diet-induced fatty liver (Morán-Salvador et al., 2011). The trend toward increased *Pparg* expression in wild type females exposed to BaP *in utero* is consistent with their increased hepatic oil red O staining (Sections 3.2 and 3.3). Interestingly and consistent with two recent reports that *Gclm*$^{-/-}$ mice are resistant to the induction of nonalcoholic hepatic steatosis by both a high fat diet (Kendig et al., 2011) and by a methionine and choline deficient diet (Haque et al., 2010), we observed minimal hepatic lipid despite upregulation of hepatic *Pparg* expression in female *Gclm*$^{-/-}$ livers. Our results add prenatal exposure to BaP to the list of stimuli of hepatic steatosis to which the *Gclm* null mice are resistant. These groups also reported that *Gclm*$^{-/-}$ mice have decreased hepatic expression of lipid biosynthesis genes, and the authors concluded that the down-regulation of these other genes is an important factor in their resistance to hepatic steatosis (Haque et al., 2010; Kendig et al., 2011). In the present study, we similarly observed significantly decreased hepatic expression of *Srebf1* and its target *Fasn*, as well as of the *Pparg*- and *Srebf1*-regulated gene *Fabp4*, in *Gclm*$^{-/-}$ mice. An association between a polymorphism in the promoter region of the *GCLC* gene, which is thought to decrease the GSH synthesis response to oxidative stress, and increased risk of progression of nonalcoholic hepatic steatosis to steatohepatitis was recently reported (Oliveira et al., 2010). Oxidative stress is thought to play an important role in the progression of hepatic steatosis to steatohepatitis (Koek et al., 2011; Pessayre and Fromenty, 2005), and the significantly increased hepatic expression of numerous antioxidant genes in *Gclm* null mice (Section 3.3) may contribute to their resistance to hepatic steatosis. Taken together, the above findings suggest that investigations of associations between polymorphisms in the *GCLC* and the *GCLM* genes (Le et al., 2010; Nakamura et al., 2002, 2003; Oliveira et al., 2010; Walsh et al., 2001; Willis et al., 2003) and obesity and hepatic steatosis may prove fruitful.

In conclusion, prenatal exposure to the PAH BaP increased visceral adiposity and caused hepatic steatosis in wild type female mice. Mice deficient in GSH due to deletion of *Gclm* were resistant to these effects of transplacental exposure to BaP; this resistance was associated with hepatic downregulation of several genes involved in lipid biosynthesis and hepatic upregulation of antioxidant genes. PAHs in tobacco smoke and particulate matter air pollution may play a role in the reported associations between early life exposure to these pollutants and propensity to develop obesity and metabolic syndrome. Polymorphisms in *GCLC* and *GCLM* should be examined for possible roles in human obesity and hepatic steatosis.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2013.11.017.

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