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Glutathione deficiency sensitizes cultured embryonic mouse ovaries to benzo[a]pyrene-induced germ cell apoptosis

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Abstract

Mice lacking the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in glutathione (GSH) synthesis, have decreased tissue GSH. We previously showed that *Gclm*−/− embryos have increased sensitivity to the prenatal *in vivo* ovarian toxicity of the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) compared with *Gclm*+/+ littermates. We also showed that BaP-induced germ cell death in cultured wild type embryonic ovaries is caspase-dependent. Here, we hypothesized that GSH deficiency increases sensitivity of cultured embryonic ovaries to BaP-induced germ cell death. 13.5 days post coitum (dpc) embryonic ovaries of all *Gclm* genotypes were fixed immediately or cultured for 24h in media supplemented with DMSO vehicle or 500 ng/ml BaP. The percentage of activated caspase-3 positive germ cells varied significantly among groups. Within each genotype, DMSO and BaP-treated groups had increased germ cell caspase-3 activation compared to uncultured. *Gclm*+/− ovaries had significantly increased caspase-3 activation with BaP treatment compared to DMSO, and caspase-3 activation increased non-significantly in *Gclm*−/− ovaries treated with BaP compared to DMSO. There was no statistically significant effect of BaP treatment on germ cell numbers at 24h, consistent with our prior observations in wild type ovaries, but *Gclm*−/− ovaries in both cultured groups had lower germ cell numbers than *Gclm*+/+ ovaries. There were no statistically significant BaP-treatment or genotype-related differences among groups in lipid peroxidation and germ cell proliferation. These data indicate that *Gclm* heterozygous or homozygous deletion sensitizes embryonic ovaries to BaP- and tissue culture-induced germ cell apoptosis.

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Conflict of Interest Statement

The authors declare that they have nothing to disclose.

Keywords

benzo[*a*]pyrene; glutathione; glutamate cysteine ligase modifier subunit; germ cells; apoptosis; embryonic ovary

Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (BaP), are ubiquitous environmental toxicants generated during incomplete combustion of organic materials, such as wood, fossil fuels, tobacco, and foods (ATSDR, 1995). PAHs generally require metabolism to reactive metabolites to exert toxicity (Xue and Warshawsky, 2005). Metabolism of BaP and other PAHs results in the formation of DNA adduct-forming, mutagenic metabolites (Xue and Warshawsky, 2005), and several of the metabolic pathways also generate reactive oxygen species (ROS) (Penning *et al.*, 1996; Wells *et al.*, 2009). ROS play a role in maintaining physiological functions as intracellular signaling molecules and in host defense systems, but excessive production of ROS can lead to the disruption of intracellular redox balance, a state of oxidative stress, damage to cell components, and ultimately cell death (Jones, 2006).

The tripeptide glutathione (GSH) is the most abundant intracellular nonprotein thiol, with intracellular concentrations in the millimolar range. GSH has numerous intracellular functions, not only in antioxidant defenses, but also in many metabolic processes (Shan *et al.*, 1990; Anderson and Luo, 1998). GSH can scavenge free radicals directly, participate in the reduction of hydrogen peroxide and lipid peroxides as a cofactor for glutathione peroxidases and peroxiredoxin, and detoxify electrophilic toxicants as a cofactor for glutathione transferases (Shan *et al.*, 1990; Anderson and Luo, 1998; Dalton *et al.*, 2004). Our work has shown that GSH is important in protecting mature ovarian follicles against apoptotic stimuli, including PAHs (Tsai-Turton and Luderer, 2006; Tsai-Turton *et al.*, 2007). 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 (Griffith and Mulcahy, 1999; Franklin *et al.*, 2009). GCLC is responsible for the catalytic activity of the enzyme, while binding of GCLM to GCLC decreases the Michaelis constant for the substrates glutamate and ATP and increases the inhibitory constant for GSH (Griffith and Mulcahy, 1999; Franklin *et al.*, 2009). *Gclc*−/− mice die during embryonic development (Dalton *et al.*, 2000), whereas *Gclm*−/− mice survive and reproduce, despite greatly decreased tissue levels of GSH (Yang *et al.*, 2002; McConnachie *et al.*, 2007).

Primordial germ cell (PGC) migration to the developing gonad, mitotic proliferation during migration and after arrival at the gonad, and the transition from mitosis to meiosis are critical prenatal developmental events that determine the size of the ovarian follicle reserve after birth. Disruption of any of these events can result in decreased ovarian reserve, resulting in premature ovarian senescence. PGCs in the mouse first emerge in the yolk sac at 7.25 days postcoitum (dpc), then migrate to the gonadal ridge (the future ovary), arriving at 10.5 dpc (McLaren, 2003; Pepling, 2006). During migration and after arriving in the ovary, PGCs actively proliferate until 13.5 dpc when they begin to enter meiosis (McLaren, 2003;

Pepling, 2006). Now called oocytes, they progress through prophase I of meiosis and arrest in the diplotene stage beginning at 17.5 dpc (Pepling, 2006). In humans, primordial germ cells arrive at the gonad around gestational week 3, begin to enter meiosis during week 10, and are enclosed in primordial follicles by the end of the second trimester. Establishment of this primordial follicle pool is vital for fertility and reproductive health in adulthood. The developing ovary is an important target for PAHs, including BaP. Maternal smoking, a primary route of human prenatal exposure to BaP, disturbs human ovarian development and endocrine signaling (Fowler *et al.*, 2014), decreases fecundity (Weinberg *et al.*, 1989) and is associated with earlier onset of menopause in daughters (Strohsnitter *et al.*, 2008). Our previous studies showed that exposure of pregnant mice to BaP from 6.5 to 15.5 dpc depletes germ cells, resulting in decreased ovarian reserve, decreased fertility, and epithelial ovarian tumors in the F1 female offspring (Lim *et al.*, 2013; Luderer *et al.*, 2017). Moreover, we showed that BaP treatment of cultured wild type embryonic day 13.5 ovaries depletes germ cells via induction of caspase-dependent, apoptotic death of these cells (Lim *et al.*, 2016).

Our prior work showed that GSH-deficient *Gclm−/−* female mice have increased sensitivity to destruction of germ cells by *in vivo* transplacental exposure to BaP compared with *Gclm +/+* littermates (Lim *et al.*, 2013), indicating that GSH is protective against the prenatal ovarian toxicity of BaP. Here, we tested the hypotheses that *Gclm* deficient embryonic ovaries are more sensitive to induction of oxidative damage and germ cell apoptosis in response to the stress of culture and BaP treatment than *Gclm+/+* ovaries.

Material and Methods

Animals

Gclm−/− mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a β-galactosidase/neomycin phosphotransferase fusion gene and were backcrossed onto a C57BL/6J genetic background (Giordano *et al.*, 2006; McConnachie *et al.*, 2007). Mice for these experiments were bred in our colony and housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility, with free access to deionized water and laboratory chow (Harlan Teklad 2919) on a 14:10h light-dark cycle. Temperature was maintained at 69–75°F. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were approved by the Institutional Animal Care and Use Committee at the University of California Irvine. 10-week old *Gclm+/−* females were mated with *Gclm+/+* or *Gclm−/−* males on the evening of proestrus, determined by vaginal cytology, and the morning after overnight mating was considered 0.5 days postcoitum (dpc) if a vaginal plug was found. Pregnant mice were sacrificed by CO_2 euthanasia on 13.5 dpc, and the embryos were quickly removed from the uterus. Embryos were dissected using a stereomicroscope, and the sex was determined by the morphology of the gonads. DNA was extracted from embryo tails for *Gclm* genotyping as previously described (Giordano *et al.*, 2006).

Embryonic ovary culture and BaP treatment

Ovaries from 13.5 dpc embryos were dissected with mesonephros intact and either fixed immediately (0h) or cultured as described previously (Lim *et al.*, 2016) for 24h in media with 0.005% dimethyl sulfoxide (DMSO) alone or with 500 ng/ml BaP (Supelco, Bellefonte, PA, USA) in Dulbecco's modified Eagle's medium/Ham F12 (1:1) (Gibco, Grand Island, NY, USA) supplemented with 0.1% bovine serum albumin, 100 μg/ml streptomycin and 100 IU/ml penicillin G. The two ovaries from the same embryo were always assigned to different treatment groups. Ovaries from embryos of 4 to 8 different pregnant dams were used or each of the 9 experimental groups (3 *Gclm* genotypes times 3 experimental conditions). We chose the concentration and duration of culture based on our prior study in which we observed statistically significant caspase-9 and -3 activation at 24h and decreased germ cell numbers at 48h in wild type embryonic ovaries exposed to 1000 ng/ml, but not 500 ng/ml, BaP (Lim *et al.*, 2016). We chose a concentration that did not induce apoptosis in wild type ovaries so that we would be able to detect increased sensitivity of the *Gclm* deficient ovaries. A fresh aliquot of stock solution (20 mg/ml) was used to make the BaP treatment media for each experimental run. Ovaries were placed on 0.4 μm Millicell-CM Biopore membranes (Millipore, Billerica, MA, USA) floating on 400 μL culture medium in tissue culture dishes and cultured at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide. At the end of the culture, ovaries were fixed in Bouin's solution overnight at 4°C and embedded in OCT before being stored at −80°C. The embedded ovaries were sectioned at 5 μm for immunostaining.

Germ cell counting

Complete serial sections were cut for every ovary and mounted so that there were four sets of slides with four sections per slide, which were separated by 3 intervening sections. One complete set of slides, containing every 4th section through the entire ovary was immunostained with TRA98 antibody for germ cell counts, and the other sets were used for immunostaining with other antibodies as described below. We performed immunofluorescence assays for germ cell-specific antigen (TRA98) using a rat anti-TRA98 monoclonal IgG antibody (1:200; Abcam #82527, Cambridge, MA, USA) as previously described (Lim *et al.*, 2016). After incubation with Alexa 488 goat anti-rabbit IgG, the germ cells were identified by their green fluorescence and distinctive morphology with large size and spherical shape. We counted germ cells in every fourth section and multiplied the sum of the values obtained for the observed sections of one ovary by 4 to obtain a total count of germ cells per ovary. All counts were carried out blind to treatment using ImageJ software for counting (National Institutes of Health, Bethesda, MD, USA) on images captured using a Retiga 2000R digital camera with an Olympus BX60 microscope equipped with fluorescence filters.

Immunohistochemistry

We previously validated the antibodies against the mitotic marker Ki67, lipid peroxidation marker 4-hydroxynonenal (4-HNE) and apoptosis marker cleaved caspase-3 in mouse embryonic and adult ovaries (Lim *et al.*, 2015; Lim *et al.*, 2016). Slides were thawed and heated for 15 min at 95°C in a 10mM citrate buffer (pH 6.0) for antigen retrieval. The

primary antibodies, rabbit anti-cleaved caspase-3 Asp 175 (1:100; Cell Signaling #9664, Beverly, MA, USA), rabbit anti-4-HNE (1:500; Alpha diagnostic #HNE11-S, San Antonio, TX, USA), and rabbit anti-Ki67 (1:500; Abcam #15580), were detected using biotinylated goat anti-rabbit secondary antibodies in 5% normal goat serum and avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized using 3,3′-diaminobenzidine (DAB) substrate. Slides were counterstained with hematoxylin. Germ cells that stained positively and negatively for cleaved caspase-3 and Ki67 were counted as previously described (Lim *et al.*, 2016) and quantities expressed as fractions were used for statistical analyses. Briefly, hematoxylin-stained PGCs were easily distinguished from somatic cells in the embryonic ovary by morphology alone. Pre-meiotic and early meiotic PGCs at 13.5–14.5 dpc were clearly identified by their large and spherical nuclei with patches of condensed chromatin at the periphery of the nuclei (pre-meiotic) or by their thread-like chromosomes in nuclei (leptotene stage) (Baltus *et al.*, 2006; Lim *et al.*, 2016). Moreover, during this developmental stage, most PGCs are surrounded by somatic cells in a germ cell cyst. Thus, only cleaved caspase-3 or Ki-67 positive PGCs within the cyst were counted for quantification.

Because 4-HNE immunostaining was observed throughout the embryonic ovaries, we determined the intensity of DAB staining in the ovary sections. Images stained with DAB and hematoxylin were white-balanced prior to image capture. We used the Fiji version of Image J (http://fiji.sc/Fiji) to run color deconvolution on the images and the result of color deconvolution led to the production of DAB and hematoxylin images. Because chromogen stains have lower intensity values with increasing staining, we subtracted the mean intensity of DAB staining in an ovary section from 250 (the maximum intensity observed in white, unstained areas) (Nguyen *et al.*, 2013). This “reciprocal intensity” is positively correlated with increasing darkness of DAB chromogen. Negative controls (primary antibody replaced with nonimmune IgG, primary antibody without secondary antibody, or secondary antibody without primary antibody) were included in every experiment.

Statistical analysis

All values are presented as mean ± SEM in figures. For germ cell counts and oxidative lipid damage, we used two-way ANOVA to examine the main effects of experimental group and *Gclm* genotype. In order to better understand how *Gclm* genotype and experimental group interacted, we then carried out one-way ANOVA using Fisher’s LSD test for intergroup comparisons. Apoptosis and germ cell proliferation were expressed as fractions of positive germ cells and were subjected to arcsine square root transformation (Pasternack and Shore, 1982) prior to two-way ANOVA analysis. We also analyzed these data using the nonparametric Kruskal-Wallis test. We conducted post-hoc testing for intergroup comparisons using the Mann Whitney U test if the results of both of these tests agreed with one another. Statistical analyses were performed using SPSS 24.0 for Mac OS X (IBM Software).

Results

Gclm deletion increases BaP-induced germ cell apoptosis

The active form of caspase-3, the executioner caspase, is generated by cleavage of the pro-enzyme by both the intrinsic and extrinsic apoptotic pathways. We localized activated caspase-3 protein in ovarian sections by immunohistochemistry (Figure 1). Immunoreactivity for caspase-3 was observed primarily in the nuclei of germ cells or, with more intense staining, in the apoptotic bodies (Figure 1B–G). Few apoptotic, activated caspase-3-positive germ cells were detected in uncultured (0h) 13.5 dpc ovaries (< 0.5% of total germ cells) of all genotypes. About 1–2% of germ cells in DMSO ovaries and about 2.5–4.5% germ cells in BaP-treated ovaries across the genotypes were activated caspase-3 positive after culture for 24h (Figure 1A). The effects of treatment group and genotype were statistically significant ($F=34.62$, $df=2$, $P<0.00001$ for experimental group and $F=4.10$, $df=2$, $P=0.022$ for genotype, two-way ANOVA after arcsine transformation on the fraction data). Similarly, there were highly statistically significant differences in germ cell caspase 3 activation among groups by non-parametric Kruskal-Wallis test (Chi-square = 41.69, $P<0.00001$). Within each genotype, DMSO and BaP-treated had increased caspase-3 activation compared to uncultured (0h) ($P\leq0.019$, Mann Whitney $U\leq4.0$). BaP treatment significantly increased the percentage of activated caspase-3-positive germ cells compared to DMSO in *Gclm*+/− ovaries ($P=0.002$, Mann-Whitney $U=1.00$). Non-significantly higher percentages of activated caspase-3-positive germ cells were observed in *Gclm*−/− BaP-treated ovaries compared to *Gclm*−/− DMSO ovaries ($P=0.093$, Mann-Whitney $U=16.00$) and in *Gclm*+/+ BaP-treated ovaries compared to *Gclm*+/+ DMSO ovaries ($P=0.115$, Mann Whitney $U=17.00$).

Effects of Gclm genotype, culture, and BaP treatment on germ cell number

The effect of treatment on germ cell numbers was not statistically significant ($F=2.79$, $df=2$, $P=0.070$ two-way ANOVA; Figure 2), while the effect of *Gclm* genotype on germ cell number per ovary was statistically significant ($F=6.50$, $df=2$, $P=0.003$, two-way ANOVA). One-way ANOVA similarly showed statistically significant differences among experimental groups ($F=2.61$, $df=8$, $P=0.018$), and we therefore sought to better understand how *Gclm* genotype and BaP treatment interacted by analyzing intergroup differences. Within the BaP-treated groups, intergroup comparisons revealed that germ cell numbers were lower in *Gclm*−/− ovaries compared to *Gclm*+/+ ($P=0.033$), but not *Gclm*+/− ovaries. Within the DMSO groups, intergroup comparisons revealed that germ cell numbers were lower in *Gclm*−/− ovaries compared to *Gclm*+/+ ($P=0.004$), but not *Gclm*+/− ovaries. Within all three *Gclm* genotypes, intergroup comparisons revealed no statistically significant differences in germ cell numbers among uncultured, DMSO, and BaP-treated groups, similar to our prior observations in wild type cultured ovaries at 24h of culture (Lim *et al.*, 2016).

Gclm deletion and BaP treatment did not increase 4-HNE immunostaining in mouse embryonic ovaries

To assess the involvement of oxidative stress in germ cell death by BaP in 13.5 dpc ovaries, we measured the levels of oxidative lipid damage by immunohistochemistry, using an antibody specific for 4-HNE (Figure 3). Color deconvolution was used to remove

hematoxylin staining from images prior to determining the intensity of 4-HNE immunostaining (Figure 3H–J). There was a statistically significant effect of treatment group (F=4.417, df=2, P=0.0170, 2-way ANOVA), with DMSO groups having lower immunostaining than uncultured (0h) or BaP groups. The effect of *Gclm* genotype on 4-HNE immunostaining intensity was not statistically significant (F=1.03, df=2, P=0.367). There were no statistically significant differences among experimental groups in 4-HNE immunostaining intensity by non-parametric test (Chi-square=11.33, df=8, P=0.184, Kruskal Wallis).

Gclm deletion and BaP treatment do not affect germ cell proliferation in cultured mouse embryonic ovaries

We assessed the effects of *Gclm* deletion and BaP treatment on germ cell proliferation in the ovaries by immunostaining for the mitosis-specific protein, Ki-67 (Scholzen and Gerdes, 2000) (Figure 4). Ki-67 staining identified mitotic germ cells only, whereas meiotic germ cells were identified by their morphology as described in Methods. About half of the germ cells of all genotypes in uncultured (0h) 13.5 dpc ovaries were Ki-67 positive. As expected because of the transition from mitosis to meiosis, the percentage of Ki-67-positive germ cells in both DMSO and BaP-treated ovaries dropped to less than 20% after 24h of culture, while the percentage of meiotic germ cells increased commensurately. The effect of treatment group was statistically significant F=2133.42, df=2, P<0.001, two way ANOVA), while the effect of genotype was not (F=0.33, df=2, P=0.72). There were statistically significant differences among experimental groups by non-parametric test as well (Chi-square=31.46, df=8, P=0.0001, Kruskal-Wallis test). These effects were driven the above-mentioned large drop in Ki-67 positive cells after 24h of culture. Within each genotype, intergroup comparisons between cultured DMSO controls (11–17%) and cultured BaP-treated (13–15%) were not statistically significant (P>0.347 by Mann Whitney U test).

Discussion

Our findings support that cultured embryonic *Gclm+/−* and *Gclm−/−* ovaries are more sensitive to induction of germ cell apoptosis by BaP than *Gclm+/+* ovaries. Moreover, *Gclm −/−* ovaries have increased germ cell apoptosis in response to the stress of culture compared to *Gclm+/−* and *Gclm+/+* ovaries even in the absence of BaP exposure. We found no effects of *Gclm* deficiency or BaP treatment on germ cell mitosis, germ cell meiotic entry and progression, or ovarian oxidative lipid damage.

Maternal cigarette smoking during pregnancy is associated with decreased fertility and earlier onset of menopause in daughters exposed in utero (Weinberg *et al.*, 1989; Strohsnitter *et al.*, 2008). BaP exposure in mainstream smoke ranges from 20 to 40 ng cigarette (Shopland *et al.*, 2001; Lodovici *et al.*, 2004), and the concentration of BaP in ovarian follicular fluid of women who smoke has been measured at 1.32±0.68 ng/ml (Neal *et al.*, 2008). The concentration of BaP used in this study is therefore high compared to levels measured in women. However, humans are simultaneously exposed to many PAHs from multiple sources, and total exposure to PAHs in highly exposed humans (smokers who are

exposed to polluted urban air and consume grilled foods) is in the neighborhood of 0.5 μg/kg/day (Menzie *et al.*, 1992; ATSDR, 1995; Shopland *et al.*, 2001; Lodovici *et al.*, 2004).

In our recent study using the same 13.5 dpc ovary culture model, 1000 ng/ml BaP caused a significant increase in activated caspase-3 positive germ cells after 24h and significant decrease in germ cell numbers after 48h of culture in wild type ovaries, while 500 ng/ml did not cause significant changes in either endpoint (Lim *et al.*, 2016), and the findings in *Gclm +/+* ovaries in the present study recapitulate those results. We also previously observed greater sensitivity of *Gclm−/−* female embryos to reduced ovarian follicle reserve after *in vivo* exposure to BaP from 6.5 through 15.5 dpc (Lim *et al.*, 2013). Therefore, we hypothesized that cultured *Gclm* heterozygous and homozygous null ovaries would be more sensitive to BaP-induced germ cell apoptosis than *Gclm+/+* ovaries, due to decreased ability to detoxify reactive metabolites, including ROS. We observed that there were no *Gclm* genotype-related differences in percentages of activated caspase-3 positive germ cells or in the number of germ cells per ovary in uncultured embryonic ovaries (Figure 1), consistent with our published work showing that *Gclm−/−* neonatal mice have similar numbers of ovarian germ cells/follicles as their *Gclm+/+* littermates (Lim *et al.*, 2015). Percentages of activated caspase-3-positive germ cells increased with BaP treatment in all three genotypes, but the relative increase was statistically significant only in *Gclm+/−* (Figure 1). We showed in our prior publication (Lim *et al.*, 2016) that treatment with a pan caspase inhibitor completely prevented germ cell depletion by 1000 ng/mL BaP in wild type ovaries, demonstrating the requirement for caspase activation for germ cell depletion by BaP in cultured 13.5 dpc ovaries. Caspase 3 activation has been demonstrated to occur very rapidly, on the order of 5 minutes once initiated and is followed rapidly by downstream events such as DNA cleavage and death of the cell (Tyas *et al.*, 2000). However, this process is not expected to occur simultaneously in all the germ cells. Therefore, it is expected that at any one time point only a fraction of those germ cells which will undergo caspase 3 activation and die are activated caspase 3 positive.

Our results are consistent with decreased ability of *Gclm−/−* or *Gclm+/−* ovaries to detoxify reactive metabolites of BaP and/or ROS generated during BaP metabolism compared to *Gclm+/+* ovaries. Biotransformation of BaP by cytochrome P450s, epoxide hydrolase, and aldo ketoductases generates BaP diol epoxides, which form DNA adducts, as well as BaP quinones with the concomitant production of ROS that directly attack DNA (Penning *et al.*, 1996; Xue and Warshawsky, 2005). In addition, prostaglandin-endoperoxide synthase 2 (PTGS2) is an important enzyme involved in bioactivation of PAHs in the developing embryo (Wells *et al.*, 2009). PTGS2 metabolism of BaP generates reactive metabolites and ROS that can induce oxidative damage and teratogenesis (Winn and Wells, 1997; Parman and Wells, 2002). Due to relatively low expression of cytochrome P450s involved in BaP metabolism in developing embryos (Miller *et al.*, 1996), PTGS2-mediated metabolism may be the predominant metabolic pathway for BaP in the embryo.

GSH is a critical intracellular antioxidant that prevents or delays oxidative cell damage resulting from excessive ROS. Although the preimplantation embryo has limited ability to upregulate GSH synthesis prior to the blastocyst stage and relies on GSH from the oocyte (Gardiner and Reed, 1995; Gardiner *et al.*, 1998; Stover *et al.*, 2000; Salmen *et al.*, 2005),

Gclm and *Gclc* mRNA are highly expressed in post-implantation embryos during mouse development, particularly in the liver and CNS (Diaz *et al.*, 2002). Therefore, we hypothesized that *Gclm−/−* ovaries may display increased germ cell oxidative damage and apoptosis upon being exposed to the stress of standard culture conditions with supraphysiological oxygen levels even in the absence of BaP exposure. Tissue oxygen tensions *in vivo* are in the range of 1–10 mm Hg, while culture in 95% room air exposes cells to 150 mm Hg oxygen (Halliwell, 2003). Consistent with our hypothesis, culturing alone significantly increased germ cell caspase-3 activation in *Gclm−/−* ovaries compared to *Gclm+/−* ovaries and nonsignificantly compared to *Gclm+/+* ovaries, while there were no differences among *Gclm* genotypes in uncultured, 13.5 dpc ovaries (Figure 1). We previously reported that immunostaining for 4-HNE, a marker of oxidative lipid damage, was increased in in granulosa cells and theca cells of secondary follicles of prepubertal and young adult *Gclm−/−* mice compared to *Gclm+/+* mice (Lim *et al.*, 2015). 4-HNE is a product of lipid hydroperoxide decomposition by ROS-mediated chain reactions in polyunsaturated fatty acids and lipoproteins, causing structural changes and reduced activity of membrane-bound enzymes (Dean *et al.*, 1993; Roberts *et al.*, 2009). Whereas oxidative damage in other tissues and organs has been extensively studied, information on prenatal oxidative damage in germ cells remains limited. To examine the involvement of oxidative lipid damage in germ cell death by BaP, we evaluated the intensity of 4-HNE immunostaining in 13.5 dpc ovaries. To our knowledge, this is the first work to examine oxidative lipid damage in the embryonic ovary. We observed that there were no apparent effects of *Gclm* genotype or BaP treatment on 4-HNE immunostaining. This may suggest that 24h of exposure to BaP at this developmental stage does not induce oxidative stress, perhaps because excess ROS in the ovaries are controlled by repair responses and antioxidant defense systems. However, we cannot rule out the possibility that lipid peroxidation may not be a good indicator of oxidative stress in 13.5 dpc ovaries. Future studies should measure the effects of *Gclm*-deficiency and BaP treatment on PTGS2 expression and ROS production, as well as markers of oxidative DNA and protein damage, in embryonic ovaries to clarify the role of oxidative stress in mediating BaP-induced germ cell apoptosis in the developing ovary.

It has been reported that almost all germ cells were positive for the proliferation marker Ki-67 in 12.5 dpc mouse ovaries (Atchison *et al.*, 2003). Our and others prior work demonstrated that about half of the germ cells are Ki-67 positive in 13.5 dpc mouse ovaries, which is consistent with the onset of entry into meiosis on 13.5 dpc (Menke *et al.*, 2003; Baltus *et al.*, 2006; Lim *et al.*, 2016). In the current study, we similarly observed parallel patterns of proliferation and meiotic progression of germ cells during the period of 24h culture in 13.5 dpc ovaries of all genotypes and BaP treatment groups. Around 50% of germ cells were Ki67-positive in all genotypes in uncultured 13.5 dpc ovaries, and the percentages of Ki-67-positive germ cells after 24h culture declined to about 14% in both DMSO controls and BaP-treated ovaries, while the percentages of meiotic germ cells increased commensurately (Figure 4), indicating that *Gclm* deficiency and BaP treatment have no effects on meiotic entry and progression of germ cells in 13.5 dpc mouse ovaries.

The results of the present study demonstrate that cultured *Gclm+/−* and *Gclm−/−* embryonic ovaries are more sensitive to induction of germ cell apoptosis by BaP exposure than wild

type ovaries. Moreover, our results support that *Gclm−/−* embryonic ovaries are more sensitive to the stress of culture than *Gclm+/−* or *Gclm+/+* ovaries. Future studies should aim to disentangle the relative contributions of the antioxidant and Phase II biotransformation roles of GSH in preventing germ cell death caused by exposure to BaP, as well as investigating the potential protective role of upregulation of other antioxidant defenses in *Gclm−/−* ovaries compared to *Gclm+/−* ovaries.

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Research Highlights

1. *Gclm* is a subunit of the rate-limiting enzyme in glutathione synthesis.
2. Embryonic *Gclm+/+, Gclm+/−* and *Gclm−/−* ovaries were exposed to benzo[a]pyrene (BaP) or vehicle.
3. *Gclm+/−* and *Gclm−/−* ovaries were more sensitive to BaP-induced germ cell apoptosis than *Gclm+/+* ovaries.

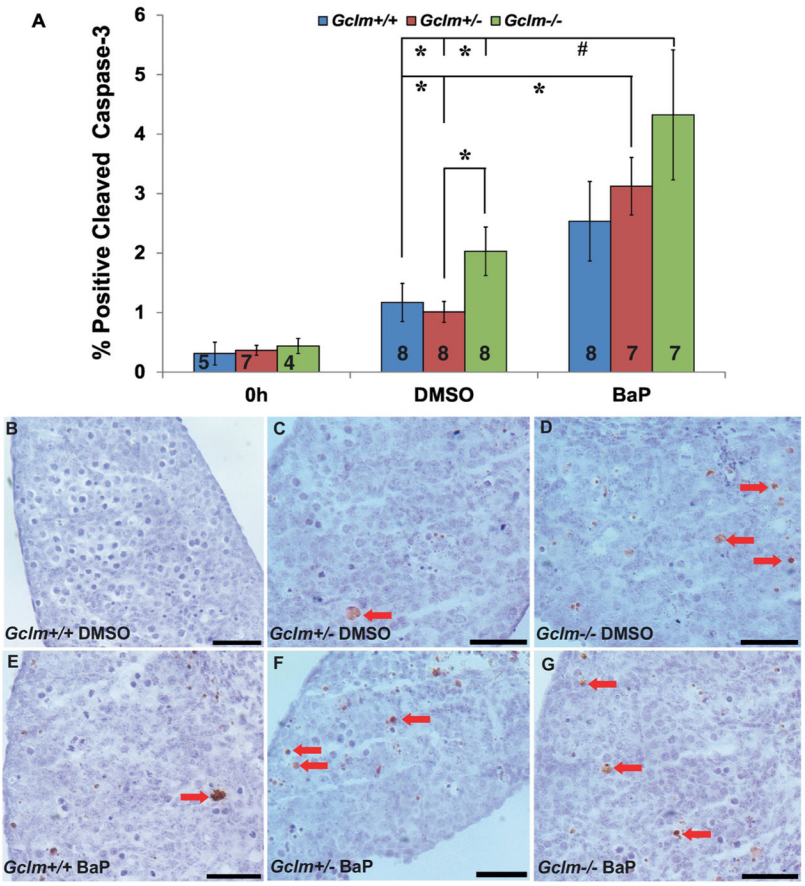

Figure 1. BaP and *Gclm* deletion interact to induce germ cell apoptosis

13.5 dpc ovaries from *Gclm+/+, Gclm+/− and Gclm−/−* embryos were fixed immediately (uncultured, 0h) or cultured for 24h with 0.005% DMSO or 500ng/ml BaP. Germ cell apoptosis was detected by cleaved caspase-3 immunostaining. (A) The graph shows means ± SEM percentage of activated caspase-3 positive germ cells per ovary. The effect of experimental group was statistically significant (P<0.00001, Kruskal Wallis test). Statistical intergroup comparisons showed no *Gclm* genotype-related differences in 0h controls, while DMSO and BaP-treatment significantly increased the percentage of positive cells compared to 0h within every genotype. Comparisons between DMSO and BaP-treated groups are indicated on the graph: * P<0.05; # P=0.093, Mann-Whitney test. N per group is indicated on each bar. (B–G) Representative immunostaining images show activated caspase-3 positive germ cells (brown staining) in embryonic ovaries of the indicated *Gclm* genotypes cultured with DMSO vehicle or BaP. Red arrows point to examples of activated caspase-3 positive germ cells. Ovaries were counterstained with hematoxylin. Scale bars, 50 μm.

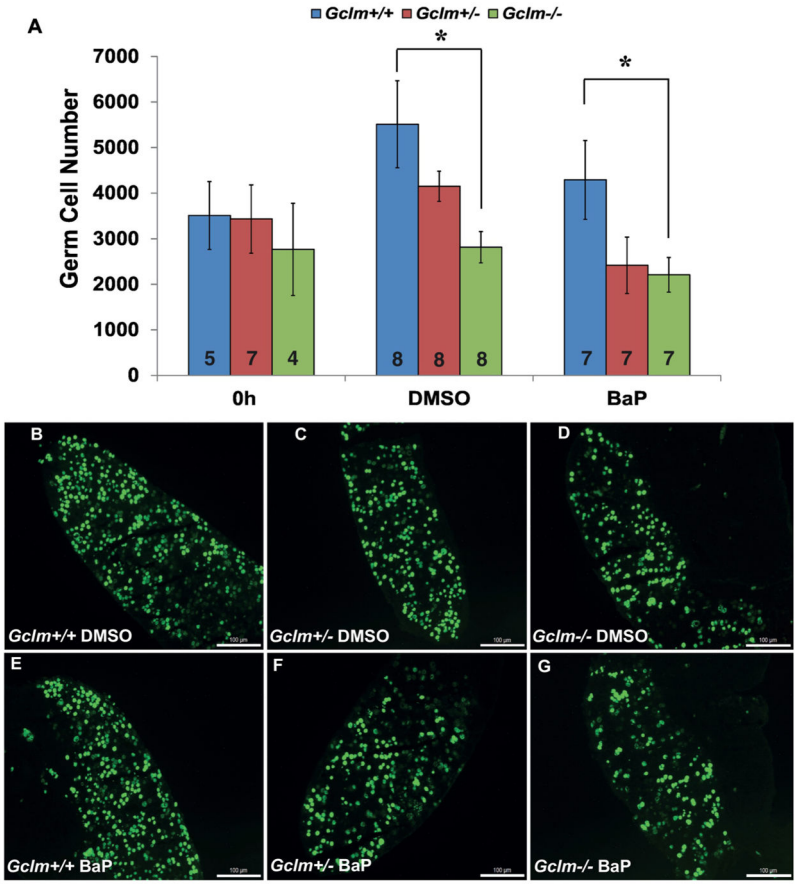

Figure 2. Effects of BaP and *Gclm* deficiency on germ cell (GC) numbers

Germ cells were detected by germ cell specific antibody (TRA98) immunofluorescence staining in uncultured (0h) ovaries or ovaries cultured for 24h as described for Figure 1. (A) The graph shows means ± SEM number of germ cells per ovary. The effect of experimental group was statistically significant (P=0.018, one-way ANOVA). There were no statistically significant intergroup differences between 0h, DMSO and BaP-treated groups of the same genotype. Statistically significant differences between genotypes within treatment groups are indicated on the graph (*P<0.05, LSD test). N per group is indicated on each bar. (B–G) Representative immunofluorescence images identify germ cells by TRA98 immunopositivity (spherical and strong green fluorescence) in embryonic ovaries of the indicated *Gclm* genotypes cultured with DMSO vehicle or BaP. Scale bars, 100 μm.

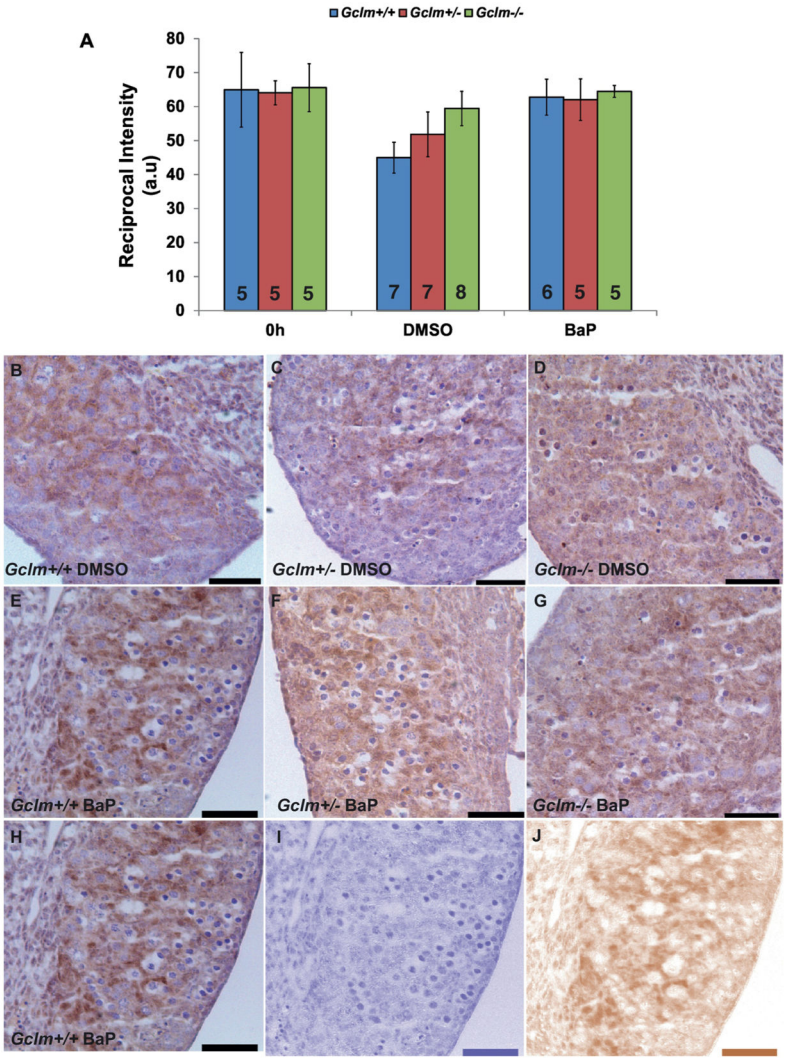

Figure 3. *Gclm* deletion and BaP treatment do not affect 4-HNE immunostaining in cultured 13.5 dpc ovaries

Oxidative lipid damage was assessed by 4-HNE immunostaining in uncultured (0h) ovaries or ovaries cultured for 24h as described for Figure 1. (A) The graph shows means ± SEM reciprocal intensity of brown immunostaining after color deconvolution to remove hematoxylin staining. There were no statistically significant differences among groups in oxidative lipid peroxidation (P=0.218, Kruskal-Wallis). N=5–8/group. (B–G) Representative immunostaining images show 4-HNE immunopositivity (brown staining) in embryonic ovaries of the indicated *Gclm* genotypes cultured with DMSO vehicle or BaP. Slides were counterstained with hematoxylin (purple). (H–J) Representative images showing before (H) and after (I: hematoxylin and J: DAB) color deconvolution. Scale bars, 50 μm.

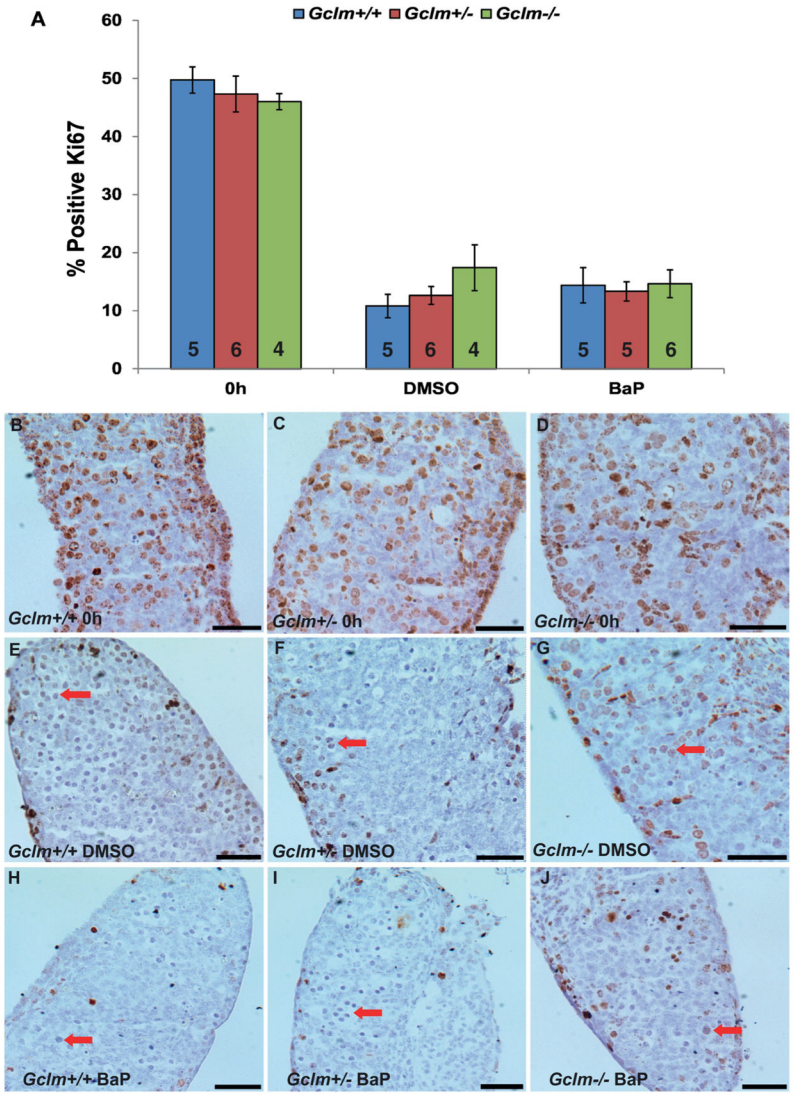

Figure 4. *Gclm* deletion and BaP treatment have no effect on germ cell proliferation in mouse embryonic ovaries

Germ cell proliferation was assessed by Ki-67 immunostaining in uncultured (0h) ovaries or ovaries cultured for 24h as described for Figure 1. (A) The graph shows means ± SEM number of Ki-67-positive germ cells per ovary. There were statistically significant differences among groups (P=0.0001, Kruskal-Wallis test). Within each genotype, intergroup comparisons between 0h and DMSO or 0h and BaP were statistically significant (P<0.021 for DMSO and P<0.034 for BaP, Mann Whitney), while cultured DMSO controls and cultured BaP-treated did not differ (P>0.462 by Mann Whitney). N=4–6/group. (B–J) Representative immunostaining images show Ki-67-positive cells (brown staining) in embryonic ovaries of the indicated *Gclm* genotypes uncultured (0h) or cultured with DMSO vehicle or BaP. Red arrows point to examples of meiotic germ cells typical of the leptotene stage. Sections were counterstained with hematoxylin. Scale bars, 100 μm.