### Title
Glutathione-Deficient Mice Have Increased Sensitivity to Transplacental Benzo[a]pyrene-Induced Premature Ovarian Failure and Ovarian Tumorigenesis




### Journal
Cancer Research, 73(2)

### ISSN
0008-5472



### Authors
Lim, Jinhwan
Lawson, Gregory W
Nakamura, Brooke N
et al.



### Publication Date
2013-01-15

### DOI
10.1158/0008-5472.can-12-3636

Published in final edited form as:
*Cancer Res*. 2013 January 15; 73(2): 908–917. doi:10.1158/0008-5472.CAN-12-3636.

# Glutathione-deficient mice have increased sensitivity to transplacental benzo[a]pyrene-induced premature ovarian failure and ovarian tumorigenesis

**Jinhwan Lim**[1], **Gregory W. Lawson**[2], **Brooke N. Nakamura**[1], **Laura Ortiz**[1], **Jin A. Hur**[1], **Terrance J. Kavanagh**[3], and **Ulrike Luderer**[1,3,4]

[1]Department of Medicine, University of California Irvine

[2]Department of Laboratory Animal Medicine, University of California Los Angeles

[3]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA

[4]Department of Developmental and Cell Biology, University of California Irvine

## Abstract

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are ubiquitous environmental pollutants found in tobacco smoke, air pollution and grilled foods. Prenatal exposure to BaP causes premature reproductive senescence in mice, and other PAHs are transplacental ovarian carcinogens. Glutathione (GSH) is critical for detoxification of the reactive metabolites of PAHs. Therefore, we hypothesized that mice that are genetically deficient in GSH synthesis, due to deletion of the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in GSH synthesis, have increased destruction of oogonia, premature ovarian failure, and ovarian tumorigenesis after transplacental BaP exposure compared to *Gclm+/+* females. *Gclm+/−* female and male mice were mated, and dams were treated with 0, 2, or 10 mg/kg/day BaP in sesame oil by gavage from gestational days 7–16. Compared to oil-treated F1 females of the same genotype, *Gclm−/−* prenatally BaP-treated females had significantly greater decrements in offspring production than *Gclm+/+* BaP-treated females. Similarly, we observed significant BaP dose × *Gclm* genotype interactions on ovarian follicle counts and ovarian tumor multiplicity at 7.5 months of age, with *Gclm−/−* females having greater decrements in follicle numbers and more ovarian tumors in response to prenatal BaP exposure than *Gclm+/+* females. The ovarian tumors were positive for the epithelial marker cytokeratin. Our results demonstrate that prenatal exposure of females to BaP causes premature ovarian failure and ovarian tumorigenesis and that embryonic GSH deficiency due to deletion of *Gclm* increases sensitivity to these transplacental ovarian effects of BaP.

### Keywords

polycyclic aromatic hydrocarbons; glutathione; ovarian cancer; glutamate cysteine ligase; ovarian follicle; benzo[a]pyrene

[3]**Corresponding Author:** Dr. Ulrike Luderer, Center for Occupational and Environmental Health, 100 Theory Drive, Suite 100, Irvine, CA 92617, uluderer@uci.edu, Tel: 949-824-8641.

**Conflicts of Interest:** The authors disclose no potential conflicts of interest.

## INTRODUCTION

Sixty percent of women diagnosed with ovarian cancer will die of the disease; it is the leading cause of death from gynecological cancers. Because ovarian cancer tends to be asymptomatic until it has reached an advanced stage, treatment is often ineffective. Understanding the causes of ovarian cancer is critical for the development of preventive strategies and methods for early diagnosis, yet the underlying cause of most ovarian cancers remains elusive. Ninety percent of malignant ovarian cancers in women are histologically classified as epithelial, while about 7% are classified as stromal (1). Epithelial ovarian cancers are thought to originate from the ovarian surface epithelium, and stromal ovarian cancers are thought to originate from granulosa cells, theca cells, other stromal cells, or their counterparts in the testicular sex cords (Sertoli cells and Leydig cells) (1).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants found in air pollution, cooked foods, and tobacco smoke. Human biomonitoring data demonstrate that essentially all Americans are exposed to PAHs (2). It has been known for decades that PAHs like benzo[a]pyrene (BaP) are toxic to the ovary, destroying ovarian follicles and causing ovarian tumors in rodents. The PAHs 7,12-dimethylbenzanthracene (DMBA), 3-methylcholanthrene, and BaP all destroyed follicles in pre- and peri-pubertal mice after both single high doses (3, 4) and after repeated low doses (5). The significant PAH content of tobacco smoke is thought to play a role in the known ovarian toxicity of smoking. Women who smoke have decreased per menstrual cycle probability of pregnancy compared to women who do not smoke (6, 7), and the onset of menopause occurs several years earlier in women who smoke (8). Treatment of adult mice with various PAHs by several routes induced ovarian tumors (9–11), and women who smoke have increased risk of epithelial ovarian cancer (12, 13).

There is increasing evidence that environmental exposures that occur during embryonic and fetal development modify the risk for developing diseases, such as cancer, in adulthood. *In utero* exposure to PAHs causes ovarian toxicity to the female offspring. Treatment of pregnant mice with BaP dose-dependently decreased fertility and resulted in atrophic ovaries with few follicles in female offspring (14). Transplacental ovarian carcinogenesis has been demonstrated for the PAHs DMBA (15) and dibenz[*def,p*]chrysene (16), but to the best of our knowledge has not been reported for BaP. BaP, like dibenz[*def,p*]chrysene, is relevant to humans because environmental exposure to these two PAHs is ubiquitous, whereas DMBA is not present in the environment.

In order to exert their toxicity, PAHs generally require metabolic activation to toxic metabolites by the Phase I metabolizing enzymes, cytochromes P450 (CYPs) and microsomal epoxide hydrolase (17). Of the major CYPs involved in PAH metabolism, *Cyp1b1* is expressed in mouse embryos from embryonic day (E) 11 to E17, while *Cyp1a1* is expressed on E7, but not later, and *Cyp1a2* is not expressed (18, 19). To our knowledge, no information is available on fetal ovary expression of any of these CYPs in mice. Prostaglandin-endoperoxide synthases are also key enzymes involved in bioactivation of PAHs in the developing embryo (20, 21). Prostaglandin-endoperoxide synthases oxidize PAHs to free radical intermediates, which can initiate reactive oxygen species (ROS) generation (21). Phase II metabolism or detoxification of toxic BaP metabolites occurs via glutathione-*S*-transferase-catalyzed conjugation with glutathione (GSH) (22, 23). During mouse development, embryos express GSH synthetic enzymes and synthesize GSH beginning at the blastocyst stage (24). Expression of *Gsta4* is present in the whole embryo on E7.5 and E8.5 and in the ovary on E14.5, and *Gstp1, Gstp2, Gstm1,* and *Gstm2* are expressed throughout the embryo on E14.5 (25).

GSH is the most abundant intracellular nonprotein thiol and one of the most important intracellular antioxidants. It is present in cells at millimolar concentrations. GSH has numerous intracellular functions including reduction of hydrogen peroxide and lipid peroxides as a cofactor for GSH peroxidases, detoxification of electrophilic toxicants as a cofactor for glutathione-*S*-transferases, regulation of protein function, and regulation of nucleotide metabolism. GSH is synthesized in two ATP-dependent reactions (26). The first, rate-limiting reaction is catalyzed by GCL, a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit. Mice that lack *Gclc* die during embryonic development (27, 28). Mice that lack *Gclm* survive and reproduce, but have greatly reduced tissue levels of GSH (29, 30). We recently reported that female *Gclm−/−* mice have compromised fertility due to early preimplantation embryo mortality (31).

In view of the importance of GSH in maintaining cellular redox status and in detoxifying reactive metabolites of BaP, we hypothesized that *Gclm* null mice have greater sensitivity to the ovarian toxicity of prenatal exposure to BaP. We further hypothesized that BaP is a transplacental ovarian carcinogen and that female *Gclm* null mice are more susceptible than wild type littermates to ovarian tumorigenesis resulting from *in utero* BaP exposure.

## MATERIALS AND METHODS

### Materials

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

### Animals

*Gclm* null mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a beta-galactosidase/neomycin phospho-transferase fusion minigene (30, 32). The mice were backcrossed 8 times onto a C57BL/6J 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). 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 (32). The 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, on a 14:10h 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* and were approved by the Institutional Animal Care and Use Committee at UC Irvine.

### 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.

### Experimental protocols

*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 BaP in sesame oil daily from GD7 to GD16 (Experiment 1) or 2 mg/kg/day from GD7 to GD16 (Experiment 2). Control animals were gavaged with the same volume of sesame oil alone in both experiments. The dosing regimen in Experiment 1 was based on a previous study in mice, which showed that offspring of dams treated with this dose had reduced fertility compared to controls but were not completely infertile (14). We used the lower dose in the second study because of an

apparently increased intrauterine mortality of *Gclm−/−* female fetuses in Experiment 1. Of 8 litters born to dams treated with 10 mg/kg BaP, only 2 *Gclm−/−* females (both from the same litter) were born, compared to 7 *Gclm+/+* females from 5 litters. Dams were allowed to deliver and care for their litters. Litters were weaned on post-natal day (PND) 21, and dams were euthanized with $CO_2$ on the day of weaning. Maternal ovaries were processed for histomorphometry.

Fertility of *Gclm−/−* and *Gclm+/+* female offspring was tested in a continuous breeding assay (see below). At the end of the breeding assay, female mice were removed from their mates. After the last litter was delivered, estrous cycles were monitored for at least 14 days and mice were killed by $CO_2$ asphyxiation on the next morning of estrus or on day 15 if they were not cycling. Skin, mammary glands, livers, kidneys, lungs, uteri, and ovaries were carefully examined for gross evidence of tumors at necropsy. In Experiment 1, both ovaries were fixed in Bouin's fixative, processed for histology, serially sectioned at 5 μm, stained with hematoxylin and eosin, and used for histomorphometry, including diagnosis of ovarian tumors. In Experiment 2, both ovaries were examined for ovarian tumors. One ovary was processed as for Experiment 1 and used for histomorphometry, and the other was fixed in 4% paraformaldehyde, cryopreserved in 15% sucrose in PBS, embedded in OCT, and every fourth section was used for hematoxylin and eosin staining. *Gclm+/−* female offspring were sacrificed by $CO_2$ euthanasia at 35 days of age, and ovaries were processed as for Experiment 1 for ovarian histomorphometry.

### Female fertility assessment

Beginning at two months of age control or BaP-treated *Gclm+/+* or *Gclm−/−* female mice were mated to wild type, 10 week old C57BL/6J male mice (Jackson Labs) for a continuous breeding assay. Females were checked daily for pups. On the day of birth, live and dead pups were counted and sexed, live pups were weighed, and pups were humanely euthanized.

### Ovarian histomorphometry

Complete serial histological sections from each animal were evaluated by two of the investigators (U.L. and J.L.) without knowledge of genotype or treatment using an Olympus BX60 microscope equipped with Plan Fluor 10x, 20x, and 40x objectives, Plan Achromat 4x objective (Olympus America, Melville, NY), and a Retiga 2000R cooled CCD mono digital camera (QImaging, Surrey, BC, Canada). Ovarian follicles were classified as primordial (single layer of flattened granulosa cells), primary (single layer of cuboidal or mixed cuboidal/flattened granulosa cells), secondary (greater than one layer of granulosa cells), antral (possessing an antral cavity or several fluid filled vesicles in the case of early antral follicles) and were further classified as healthy or atretic. Atretic secondary and antral follicles were identified by the presence of 3 or more pyknotic granulosa cells per largest cross-section and separation of the oocyte from the granulosa cells (33). Atretic primordial and primary follicles were identified by eosinophilic oocytes (34). Primordial, primary, and secondary follicles were counted in every $5^{th}$ serial section. The counts were multiplied times 5 to obtain estimates of the total number of follicles per ovary (35). Antral follicles were followed through every serial section, taking care to only count each of these structures once. Each section of the ovaries from 7.5 month old mice was also evaluated for the presence of ovarian tumors or cysts by a board-certified veterinary pathologist (G.W.L.) blind to genotype and treatment. Perpendicular tumor diameters were measured in the largest tumor cross-section. The average tumor diameter was calculated for each animal. Several tumors contained very large cysts. For these cystic tumors, the largest diameters with and without the cyst were measured, and average diameters were calculated with and without the cyst. Sensitivity analyses compared the effect of genotype on average tumor diameter when cysts were included versus not included in the measurements.

### Immunostaining

One slide was selected from each ovarian tumor for destaining with 1% hydrochloric acid and 1.5% ammonium hydroxide in 70% ethanol and subsequent immunostaining with an antibody directed against mouse pancytokeratin (Sigma C1801, Clone PCK-25), a marker for epithelial ovarian tumors (36). Immunostaining was done using the MOM Kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Briefly, slides were subjected to antigen retrieval in 10 mM sodium citrate with 0.05% Tween-20 at 95°C, blocked with mouse MOM IgG blocking reagent, washed, blocked with avidin and biotin blocking reagents, washed, incubated with 1:50 dilution of primary antibody in MOM diluent, washed, incubated with 3% hydrogen peroxide in PBS to block endogenous peroxidases, incubated with biotinylated secondary antibody, washed, incubated with ABC reagent, washed, incubated with diaminobenzidine substrate in peroxide buffer, and counterstained with hematoxylin. Negative control slides (without primary antibody, primary antibody replaced with mouse Ig, without secondary antibody) and an untreated ovary control were run simultaneously with the experimental slides. All negative control slides showed no non-specific staining. The untreated ovary showed staining only in the ovarian surface epithelium.

### Statistical Analyses

Because Experiments 1 and 2 were conducted two years apart, the effect of experiment on various endpoints was examined for *Gclm+/+* females exposed prenatally to 0 mg/kg BaP. As there were no significant effects of experiment on any of the endpoints, the data were combined for subsequent analyses. The effects of genotype and BaP dose on continuous outcome variables were analyzed using Generalized Estimating Equations, a form of Generalized Linear Models, with BaP dose, *Gclm* genotype, and BaP times genotype interaction modeled as fixed effects. In order to adjust for litter effects, litter numbers were entered into the model as a subject effect. An unstructured working correlation matrix was used for the litter effects, except in the rare cases when the model did not converge, then an exchangeable working correlation matrix was used (assumes homogeneous correlation among littermates). When the outcome variable was a fraction (e.g. fraction of cornified or leukocytic vaginal cytology), the data were first arcsine square root transformed prior to entering into the models; for these analyses only, the unadjusted means and SEMs were used for presentation of the data. Statistical analyses were performed using SPSS 20.0 for MacIntosh.

## RESULTS

### Effects of gestational treatment with BaP on F0 dams

Data on pregnancy outcomes are shown in Table 1. The percentage of F0 females with vaginal plugs that delivered litters did not differ by BaP dose. Litter size did not differ by BaP dose. Full-litter mortality occurred for five out of thirteen 10 mg/kg BaP-treated dams, four out of nineteen 2 mg/kg BaP-treated dams, and six out of twenty-six control dams; the effect of BaP dose was not statistically significant. Although fewer *Gclm−/−* female offspring were born to 10 mg/kg BaP-treated females than to 2 mg/kg BaP-treated or control females, the effect of BaP dose on offspring genotype distributions was not statistically significant (Table 1).

Ovarian follicle numbers in the dams were not affected by the 10 mg/kg BaP dose (1354±242 healthy follicles per ovary in 0 mg/kg/day BaP-treated dams and 1363±320 in 10 mg/kg/day BaP-treated dams).

## Gestational BaP treatment decreased the fertility and disrupted estrous cycling of F1 female offspring

The results of the continuous breeding studies of *Gclm−/−* and *Gclm+/+* female offspring are shown in Figure 1. Two mice had prolapsed uteri and had to be euthanized prior to the end of the 20 week breeding study in Experiment 1. Both were wild type, one in the control group and one in the 10 mg/kg/day BaP-treated group. Among the remaining mice, *in utero* treatment with 10 mg/kg/day BaP profoundly decreased fertility of female mice of both genotypes, whereas treatment with 2 mg/kg/day BaP decreased fertility to a much greater extent in *Gclm−/−* females than in *Gclm+/+* females. Two of two 10 mg/kg/day BaP-treated *Gclm−/−* mice and four of six *Gclm+/+* 10 mg/kg/day BaP-treated mice delivered no litters and showed no signs of pregnancy during the study period. The other two surviving *Gclm+/+* 10 mg/kg/day BaP-treated mice had 1 and 3 litters, and the BaP-treated mouse with uterine prolapse had delivered the second litter, when she was euthanized at week 11 of the study. The effects of genotype ($p<0.001$) and BaP dose ($p<0.001$) on the number of litters produced in 20 weeks were statistically significant (Figure 1A). In addition, to having fewer litters, BaP-treated females produced fewer offspring in 20 weeks ($p<0.001$, effect of BaP dose); this effect of prenatal BaP treatment was more pronounced in the *Gclm−/−* females than the *Gclm+/+* females ($p<0.001$, BaP dose × genotype interaction; Figure 1B). *Gclm−/−* females produced significantly fewer pups compared to *Gclm+/+* littermates ($p<0.001$, effect of genotype), as we have previously reported (31).

Estrous cycle data collected after completion of the breeding study are shown in Table 2. Two of 13 *Gclm+/+* and 2 of 12 *Gclm−/−* oil-treated female offspring were not cycling at 7.5 months of age. None of the 10 mg/kg/day BaP-treated offspring displayed estrous cycles ($p<0.05$, effect of BaP on presence or absence of cycles). Most of the 10 mg/kg/day BaP-treated mice had persistently cornified vaginal cytology, whereas most of the irregularly cycling control and 2 mg/kg/d BaP mice had predominantly leukocytic vaginal cytology ($p<0.005$, effect of BaP on percentage of cornified and percentage of leukocytic vaginal smears). Predominantly cornified vaginal cytology, termed “persistent vaginal estrus,” is uncommon in normal aged, reproductively senescent mice, while predominantly leukocytic vaginal cytology is commonly observed in aged mice. Persistent cornified vaginal histology has been reported following neonatal treatment with various estrogenic chemicals. The number of transitions between cornified and leukocytic cytology, consistent with an estrus to metestrus transition, in 14 days declined significantly with BaP dose ($p<0.001$, effect of BaP). There were no effects of BaP dose or genotype on estrous cycle length in the mice that were cycling. *Gclm−/−* mice had fewer leukocytic vaginal smears than *Gclm+/+* females ($p<0.001$, effect of genotype).

## Gestational BaP treatment decreased ovarian follicle numbers in offspring

To assess the effect of gestational treatment with BaP on prepubertal ovarian follicle numbers, ovarian histomorphometry was performed on a subset of the *Gclm* heterozygous F1 female offspring from Experiments 1 and 2 at 35 days of age. Exposure to BaP from GD 7–16 dose-dependently decreased the total number of healthy ovarian follicles from 2250±348 in 0 mg/kg BaP exposed, to 1036±338 in 2 mg/kg BaP-exposed, and 34±16 in 10 mg/kg BaP-exposed ($p<0.001$, effect of BaP dose). The effect of BaP dose was statistically significant for numbers of healthy primordial follicles, primary follicles, secondary follicles, and antral follicles ($p=0.001$, $p=0.007$, $p<0.001$, $p=0.041$), as well as for numbers of atretic secondary and antral follicles ($p=0.005$; $p=0.031$; Table 3). Very few atretic primordial or primary follicles were observed in any BaP dose group, and there were no apparent effects of BaP dose on these outcomes (data not shown).

Ovarian follicle counts were also performed on both ovaries per mouse in the breeding study from Experiment 1 and one ovary per mouse from the breeding study in Experiment 2, both at about 7.5 months of age (Figure 2). Prenatal BaP exposure dose-dependently decreased the total number of healthy follicles, as well as the numbers of healthy primordial, primary, secondary, and antral follicles ($p<0.001$, effects of BaP dose). Consistent with the lack of litters in the 10 mg/kg/d BaP-treated mice after week 12 of the breeding study, there were no antral or secondary follicles in any of the 10 mg/kg/day BaP-exposed ovaries. Deletion of *Gclm* was associated with greater sensitivity to follicle depletion due to prenatal BaP exposure. The interaction between BaP dose and *Gclm* genotype was statistically significant for total follicle count ($p<0.001$; Figure 2A), primordial follicle count ($p<0.001$; Figure 2B), primary follicle count ($p=0.025$; Figure 2C) and secondary follicle count ($p=0.032$, Figure 2D). In addition, deletion of *Gclm* was independently associated with fewer primordial, primary, and secondary follicles and total healthy follicles ($p<0.001$, $p=0.007$, $p=0.032$, $p<0.001$, effect of genotype respectively).

## Gestational BaP treatment caused epithelial ovarian tumors in offspring at 7.5 months of age

Serial sections of both ovaries were evaluated for ovarian tumors. The results are shown in Table 4, Figure 3, and Supplemental Figure S1. Two of two 10 mg/kg/day BaP-treated *Gclm −/−* female offspring had bilateral ovarian tumors that replaced the entire ovarian parenchyma and were invading the periovarian fat. Three of six 10 mg/kg/day BaP-treated *Gclm+/+* offspring had bilateral ovarian tumors, two of six had unilateral ovarian tumors, and one of six had no ovarian tumors; four of the eight tumors were invading the periovarian fat (Supplemental Figure S1B). Four of six 10 mg/kg/day BaP-treated *Gclm+/+* offspring had very large, complex, fluid-filled cysts within the ovaries; two of these were in tumors (Supplemental Figure S1C and Figure 3C). One of nine 2 mg/kg/day BaP-treated *Gclm−/−* females had a unilateral ovarian tumor (Supplemental Figure S1F and Figure 3D). None of the ovaries from 2 mg/kg/day BaP-treated *Gclm+/+* females or from 0 mg/kg/day BaP-treated females of either genotype had any ovarian tumors. The adjusted average number of ovarian tumors per mouse was 0±0 for both 0 mg/kg/day BaP treated groups and the *Gclm+/+* 2 mg/kg/day BaP-treated group, 0.11±0.09 in the *Gclm−/−* 2 mg/kg/day BaP-treated group, 1.3±0.2 in the *Gclm+/+* 10 mg/kg/day BaP-treated group, and 2.0±0 in the *Gclm−/−* 10 mg/kg/day BaP-treated group ($p<0.001$, effects of BaP dose, genotype, and dose × genotype interaction). In addition to a larger percentage of the tumors showing invasion of the periovarian fat in the *Gclm−/−* females, their tumors also tended to be larger. The average largest tumor diameter, excluding cystic structures in two tumors, was 1894±203 μm in the 10 mg/kg/day BaP-treated *Gclm−/−* females and 1239±252 μm in the 10 mg/kg/day BaP-treated *Gclm+/+* females ($p=0.02$ by t-test). When cysts were included in the diameters, the tumors were still larger in the *Gclm−/−* females (1894±203 μm versus 1396±444 μm in the *Gclm+/+* females), but the difference was no longer statistically significant ($p=0.20$).

All of the ovarian tumors were initially classified as stromal tumors based on their histological appearance (Supplemental Figure S1). Although invasion of the periovarian fat was noted for several tumors, the relative paucity of mitotic figures and lack of metastases was consistent with benign tumors. Immunostaining for the epithelial marker cytokeratin showed that all but two of the tumors strongly expressed cytokeratin in the cells lining the abundant tubule-like structures in these tumors (Figure 3A–C). One of these cytokeratin-negative tumors, in a *Gclm+/+* 10 mg/kg/day BaP-exposed female, contained a large cyst, and only the cells lining the cyst and the ovarian surface epithelium displayed cytokeratin immunostaining (not shown). The other cytokeratin-negative tumor was the only tumor found in a 2 mg/kg/day BaP-exposed female (Supplemental Figure S1F and Figure 3D);

only the ovarian surface epithelium and a small epithelial inclusion showed cytokeratin immunostaining. Cytokeratin is expressed only in the ovarian surface epithelium in normal ovaries (36). In view of this pattern of cytokeratin immunostaining, we concluded that the majority of the tumors induced by prenatal BaP exposure were benign epithelial tumors, most likely tubular adenomas. The two cytokeratin negative tumors were diagnosed as granulosa theca cell tumors based on their histological appearance.

We did not observe gross tumors in any organ besides ovaries at necropsy. Histological examination of the livers and kidneys of the 10 mg/kg/day BaP-exposed mice also did not reveal any tumors.

## DISCUSSION

Our results demonstrate for the first time that the ubiquitous PAH BaP is a transplacental ovarian tumorigen at doses that do not produce tumors in other organs. The majority of the ovarian tumors induced by prenatal exposure to BaP were positive for the epithelial marker cytokeratin. Our study confirms earlier findings that prenatal oral exposure to the PAH BaP causes premature reproductive senescence in F1 female mice (14), and our data show that this is due to premature depletion of ovarian follicles. Importantly, our data demonstrate that GSH deficiency due to deletion of *Gclm* sensitizes female embryos to the ovotoxicity and the transplacental ovarian tumorigenicity of BaP.

Transplacental exposure to the PAHs DMBA and dibenzo[*def,p*]chrysene has been reported to cause sex-cord stromal ovarian tumors (15, 16), and our current results demonstrate that BaP is also a transplacental ovarian carcinogen. Importantly, immunostaining showed that most of the BaP-induced ovarian tumors were strongly positive for cytokeratin, an epithelial cell marker, indicating that they were of epithelial and not stromal origin (36). Adult exposure to PAHs, including BaP, by several routes has long been known to induce ovarian tumors in mice (9–11). Although the doses used in those studies were high compared to the present study, the incidences of females with ovarian tumors tended to be lower than we observed in the 10 mg/kg/day BaP treated group. Taken together, these results suggest that the period of ovarian development is a sensitive window for ovarian carcinogenesis. Our data also demonstrate for the first time that embryonic GSH deficiency increases the sensitivity to transplacental ovarian tumorigenesis by BaP. Our findings that transplacental PAH exposure causes epithelial ovarian cancers in mice and that GSH deficient embryos have increased sensitivity to this effect have relevance to humans. Mainstream and sidestream tobacco smoke contain dozens of carcinogenic PAHs, including BaP, and women who smoke have increased risk of epithelial ovarian cancer (12, 13). Polymorphisms in *GCLM* and *GCLC*which affect GSH synthesis, exist in humans (37–40) and may modulate sensitivity to transplacental ovarian toxicity and tumorigenesis of PAHs.

Decreased ability to detoxify reactive metabolites of BaP and/or ROS produced as a result of BaP metabolism likely causes the increased sensitivity of the *Gclm*−/− embryonic ovary to BaP. GSH conjugation is an important Phase II detoxification mechanism for the diol epoxide metabolites (22, 23), which are thought to be the ovotoxic metabolites in peripubertal mice (41). GSH conjugation is also important in Phase II metabolism of arene oxide and quinone metabolites of PAHs. CYPs 1A1, 1A2, and 1B1 are the major P450 enzymes involved in the metabolic activation of BaP *in vitro*although *in vivo* metabolism of BaP by CYP1A1 appears to be more important in detoxification (17, 42). Expression of most CYPs remains low until after birth. Exceptions include transient constitutive expression of *Cyp1a1* on GD 7 in the mouse, induction of *Cyp1a1* expression in the liver and lung as early as GD 12.5 by exposure to PAHs in the mouse, and constitutive expression of *Cyp1b1* in the mouse embryo from GD 11 onward and in the human fetal thymus and

kidney (18–20, 43). Previous work has shown that embryos constitutively express prostaglandin-endoperoxide synthase 1 and *2*that prostaglandin-endoperoxide synthases can bioactivate BaP to free radical intermediates that initiate ROS formation, that gestational treatment with BaP increases embryonic oxidative protein and DNA damage, and that antioxidants are protective against teratogenesis caused by BaP (21, 44, 45). Although these previous studies did not examine developmental toxicity to the reproductive system, our findings of increased sensitivity of GSH-deficient female embryos to transplacental ovarian toxicity of BaP in the present study and our observation of increased sensitivity of GSH-deficient male embryos to transplacental testicular toxicity of BaP (46) provide further support for a role for oxidative stress in the developmental toxicity of BaP.

The mechanism by which transplacental exposure to BaP and other PAHs causes ovarian tumors is uncertain. It is well known that metabolism of PAHs leads to the formation of mutagenic metabolites, including diol epoxides and ROS (17). Although we are not aware of any published studies describing PAH-induced ovarian mutations, BaP treatment caused testicular DNA adducts and germline mutations in male mice (47), and BaP treatment resulted in the formation of ovarian DNA adducts in adult mice and rats (48, 49). Different histopathological types of ovarian tumors have been reported in *Cyp1b1* null mice (cystadenomas) versus wild type mice (granulosa cell tumors) treated with DMBA (48). The authors postulated that this could be due to the absence of *Cyp1a1* expression in stromal cells, versus its presence in ovarian surface epithelial cells where it could compensate for the lack of *Cyp1b1*. It is also possible that different PAH metabolites have different mechanisms of ovarian tumorigenesis. Oocyte depletion has been proposed to play a role in ovarian tumorigenesis by PAHs (10, 11). While most of the tumors that we observed occurred in ovaries that were devoid of oocytes and follicles, the tumor that occurred in the 2 mg/kg/day BaP-treated *Gclm*−/− female occurred in an ovary that still contained hundreds of healthy follicles. This supports the contention that oocyte depletion and the resultant high levels of gonadotropin hormone secretion may promote ovarian tumor growth, but may not be required for ovarian tumor initiation.

The $ED_{50}$ for primordial follicle destruction by 15 daily i.p. doses of BaP in peripubertal mice was 3 mg/kg/day (5). In contrast, we observed no effect on follicle numbers in the F0 dams and an $ED_{50}$ of about 2 mg/kg/day in the *Gclm* wild type and heterozygous F1 offspring with 10 days of oral dosing to the pregnant dam in the present study. Although comparisons with earlier studies of BaP on ovarian follicle destruction are complicated by their use of i.p. dosing (3–5), taken together the results suggest that the period of *in utero* development is the most sensitive to the ovotoxicity of BaP, followed by the peripubertal period, with adults being least sensitive. In a previous study, oral gavage of CD-1 dams with 10 mg/kg/day BaP during the same dosing window that we used in the present study caused about a 50% decrease in pup production by female offspring (14). In contrast, we observed a 96% reduction in pup production in the wild type 10 mg/kg/day BaP-treated F1 females. This is consistent with earlier reports that C57BL/6 mice are more sensitive to the peripubertal ovarian toxicity of BaP than other strains (3, 4, 41). An alternate explanation is that the *Gclm* heterozygosity of the mothers may have contributed to the increased ovarian toxicity of the transplacental exposure compared to the earlier study. In the future, this could be tested by transferring embryos from heterozygous dams mated with heterozygous males to pseudopregnant wild type recipients, then treating the recipient dams with BaP. Treatment with the PAH DMBA destroyed small follicles in a dose- and time-dependent manner in human ovarian explants that were implanted subcutaneously in mice (50), demonstrating that human ovarian follicles are susceptible to PAH-induced destruction.

Our findings show that the PAH BaP is a transplacental ovarian tumorigen, which causes mainly epithelial ovarian tumors in mice. Further, deficiency of GSH due to deletion of

*Gclm* increases sensitivity to premature ovarian failure and ovarian tumorigenesis induced by *in utero* exposure to BaP. These findings have broader implications because human exposure to BaP and other PAHs is ubiquitous and known human polymorphisms in *GCLC* and *GCLM* may define a sensitive subpopulation of women at greater risk for ovarian toxicity and tumorigenesis from early life exposures to PAHs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Dr. Christine McLaren of the UC Irvine Chao Family Comprehensive Cancer Center Statistical Core for advice regarding statistical analyses.

GRANT SUPPORT

This work was supported by the National Institutes of Health (R01ES020454, and R21AG032087 to U.L.; P30CA062203, the University of California Irvine (UC Irvine) Chao Family Comprehensive Cancer Center; R01ES010849 and P50ES015915 to T.J.K.; P30ES007033, the University of Washington (UW) Center for Ecogenetics and Environmental Health); the UC Irvine Office of Research; and the UC Irvine Center for Occupational and Environmental Health.

## REFERENCES

1. Chen VW, Ruiz B, Killeen JL, Cote TR, Wu XC, Correa CN. Pathology and Classification of Ovarian Tumors. Cancer. 2003; 97:2631–2642. [PubMed: 12733128]
2. Li Z, Sandau CD, Romanoff LC, Caudill SP, Sjodin A, Needham LL, et al. Concentration and Profile of 22 Urinary Polycyclic Aromatic Hydrocarbon Metabolites in the US Population. Environ Res. 2008; 107:320–331. [PubMed: 18313659]
3. Mattison DR. Difference in Sensitivity of Rat and Mouse Primordial Oocytes to Destruction by Polycyclic Aromatic Hydrocarbons. Chem Biol Interact. 1979; 28:133–137. [PubMed: 115601]
4. Mattison DR, Thorgeirsson SS. Ovarian Aryl Hydrocarbon Hydroxylase Activity and Primordial Oocyte Toxicity of Polycyclic Aromatic Hydrocarbons in Mice. Cancer Res. 1979; 39:3471–3475. [PubMed: 113091]
5. Borman SM, Christian PJ, Sipes IG, Hoyer PB. Ovotoxicity in Female Fischer Rats and B6 Mice Induced by Low-Dose Exposure to Three Polycyclic Aromatic Hydrocarbons: Comparison through Calculation of an Ovotoxic Index. Toxicol Appl Pharmacol. 2000; 167:191–198. [PubMed: 10986010]
6. Baird DD, Wilcox AJ. Cigarette Smoking Associated with Delayed Conception. JAMA. 1985; 253:2979–2983. [PubMed: 3999259]
7. Jensen TK, Henriksen TB, Hjollund NH, Scheike T, Kolstad H, Giwercman A, et al. Adult and Prenatal Exposures to Tobacco Smoke as Risk Indicators of Fertility among 430 Danish Couples. Am J Epidemiol. 1998; 148:992–997. [PubMed: 9829871]
8. Harlow BL, Signorello LB. Factors Associated with Early Menopause. Maturitas. 2000; 35:3–9. [PubMed: 10802394]
9. Biancifiori C, Bonser GM, Caschera F. Ovarian and Mammary Tumours in Intact C3Hb Virgin Mice Following a Limited Dose of Four Carcinogenic Chemicals. Br J Cancer. 1961; 15:270–283. [PubMed: 21772453]
10. Krarup T. 9L10-Dimethyl-1;2-Benzanthracene Induced Ovarian Tumours in Mice. Acta Pathol Microbiol Scand. 1967; 70:241–248. [PubMed: 6050377]
11. Taguchi O, Michael SD, Nishizuka Y. Rapid Induction of Ovarian Granulosa Cell Tumors by 7,2-Dimethylbenz(a)anthracene in Neonatally Estrogenized Mice. Cancer Res. 1988; 48:425–429. [PubMed: 3121173]
12. Gates MA, Rosner BA, Hecht JL, Tworoger SS. Risk Factors for Epithelial Ovarian Cancer by Histologic Subtype. Am J Epidemiol. 2010; 171:45–53. [PubMed: 19910378]

13. Terry PD, Miller AB, Jones JG, Rohan TE. Cigarette Smoking and the Risk of Invasive Epithelial Ovarian Cancer in a Prospective Cohort Study. Eur J Cancer. 2003; 39:1157–1164. [PubMed: 12736118]

14. MacKenzie KM, Angevine DM. Infertility in Mice Exposed in Utero to Benzo(a)pyrene. Biol Reprod. 1981; 24:183–191. [PubMed: 7470542]

15. Goerttler K, Loehrke H, Hesse B, Milz A, Schweizer J. Diaplacental Initiation of NMRI Mice with 7,12-dimethylbenz[a]anthracene during Gestation Days 6–20 and Postnatal Treatment of the F1-generation with the Phorbol Ester 12-O-tetradecanoylphorbol-13-acetate: Tumor Incidence in Organs Other than Skin. Carcinogenesis. 1981; 2:1087–1094. [PubMed: 6797749]

16. Shorey LE, Castro DJ, Baird WM, Siddens LK, Löhr CV, Matzke MM, et al. Transplacental Carcinogenesis with Dibenz[*def,p*]chrysene (DBC): Timing of Maternal Exposures Determines Target Tissue Response in Offspring. Cancer Lett. 2012; 317:49–55. [PubMed: 22085489]

17. Xue W, Warshawsky D. Metabolic Activation of Polycyclic Aromatic Hydrocarbon and Heterocyclic Aromatic Hydrocarbons and DNA Damage: A Review. Toxicol Appl Pharmacol. 2005; 206:73–93. [PubMed: 15963346]

18. Choudhary D, Jansson I, Schenkman JB, Sarfarazi M, Stoilov I. Comparative Expression Profiling of 40 Mouse Cytochrome P450 Genes in Embryonic and Adult Tissues. Arch Biochem Biophys. 2003; 414:91–100. [PubMed: 12745259]

19. Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Expression Pattern of Mouse and Human CYP Orthologs (Families 1–4) during Development and in Different Adult Tissues. Arch Biochem Biophys. 2005; 436:50–61. [PubMed: 15752708]

20. Rich KJ, Boobis AR. Expression and Inducibility of P450 Enzymes during Liver Ontogeny. Microsc Res Tech. 1997; 39:424–435. [PubMed: 9408909]

21. Wells PG, McCallum GP, Chen CS, Henderson JT, Lee CJJ, Perstin J, et al. Oxidative Stress in Developmental Origins of Disease: Teratogenesis, Neurodevelopmental Deficits, and Cancer. Toxicol Sci. 2009; 108:4–18. [PubMed: 19126598]

22. Jernström B, Funk M, Frank H, Mannervik B, Seidel A. Glutathione-S-Transferase A1-1-Catalysed Conjugation of Bay and Fjord Region Diol Epoxides of Polycyclic Aromatic Hydrocarbons with Glutathione. Carcinogenesis. 1996; 17:1491–1498. [PubMed: 8706254]

23. Romert L, Dock L, Jenssen D, Jernström B. Effects of Glutathione Transferase Activity on Benzo[a]pyrene 7,8-dihydrodiol Metabolism and Mutagenesis Studied in a Mammalian Cell Co-cultivation Assay. Cancer Res. 1989; 10:1701–1707.

24. Stover SK, Gushansky GA, Salmen JJ, Gardiner CS. Regulation of γ-Glutamate Cysteine Ligase Expression by Oxidative Stress in the Mouse Preimplantation Embryo. Toxicol Appl Pharmacol. 2000; 168:153–159. [PubMed: 11032771]

25. Richardson, L.; Venkataraman, S.; Stevenson, P.; Yang, Y.; Burton, N.; Rao, J., et al. [cited 09/19/12] EMAGE mouse embryo spatial gene expression database. 2009. Available from: http://www.emouseatlas.org/emage/

26. Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, Kavanagh TJ. Structure, Function, and Post-Translational Regulation of the Catalytic and Modifier Subunits of Glutamate Cysteine Ligase. Mol Aspects Med. 2009; 30:86–98. [PubMed: 18812186]

27. Dalton TP, Dieter MZ, Yang Y, Shertzer HG, Nebert DW. Knockout of the Mouse Glutamate Cysteine Ligase Catalytic Subunit (Gclc) Gene: Embryonic Lethal When Homozygous, and Proposed Model for Moderate Glutathione Deficiency When Heterozygous. Biochem Biophys Res Commun. 2000; 279:324–329. [PubMed: 11118286]

28. Shi Z-Z, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, et al. Glutathione Synthesis is Essential for Mouse Development but not for Cell Growth in Culture. Proc Natl Acad Sci. 2000; 97:5101–5106. [PubMed: 10805773]

29. Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP. Initial Characterization of the Glutamate Cysteine Ligase Modifier Subunit *Gclm (−/−)* Knockout Mouse: Novel Model System for Severely Compromised Oxidative Stress Response. J Biol Chem. 2002; 277:49446–49452. [PubMed: 12384496]

30. McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, et al. Glutamate Cysteine Ligase Modifier Subunit Deficiency and Gender as Determinants of Acetaminophen-Induced Hepatotoxicity in Mice. Toxicol Sci. 2007; 99:628–6236. [PubMed: 17584759]

31. Nakamura BN, Fielder TJ, Hoang YD, Lim J, McConnachie LA, Kavanagh TJ, et al. Lack of Maternal Glutamate Cysteine Ligase Modifier Subunit (*Gclm*) Decreases Oocyte Glutathione Concentrations and Disrupts Preimplantation Development in Mice. Endocrinology. 2011; 152:2806–2815. [PubMed: 21558310]

32. Giordano G, White CC, McConnachie LA, Fernandez C, Kavanagh TJ, Costa LG. Neurotoxicity of Domoic Acid in Cerebellar Granule Neurons in a Genetic Model of Glutathione Deficiency. Mol Pharmacol. 2006; 70:2116–2126. [PubMed: 17000861]

33. Hirshfield AN. Size-Frequency Analysis of Atresia in Cycling Rats. Biol Reprod. 1988; 38:1181–1188. [PubMed: 3408785]

34. Desmeules P, Devine PJ. Characterizing the Ovotoxicity of Cyclophosphamide Metabolites on Cultured Mouse Ovaries. Toxicol Sci. 2006; 90:500–509. [PubMed: 16381661]

35. Canning J, Takai Y, Tilly JL. Evidence for Genetic Modifiers of Ovarian Follicular Endowment and Development from Studies of Five Inbred Mouse Strains. Endocrinology. 2002; 144:9–12. [PubMed: 12488324]

36. Yang W-L, Cai KQ, Smedberg JL, Smith ER, Klein-Szanto A, Hamilton TC, et al. A Reduction of Cyclooxygenase 2 Gene Dosage Counters the Ovarian Morphological Aging and Tumor Phenotype in Wv Mice. Am J Pathol. 2007; 170:1325–1336. [PubMed: 17392171]

37. Le TM, Willis AS, Barr FE, Cunningham GR, Canter JA, Owens SE, et al. An Ethnic-Specific Polymorphism in the Catalytic Subunit of Glutamate-Cysteine Ligase Impairs the Production of Glutathione Intermediates *in Vitro*. Mol Genet Metab. 2010; 101:55–61. [PubMed: 20655259]

38. Nakamura S-I, Kugiyama K, Sugiyama S, Miyamoto S, Koide S-I, Fukushima H, et al. Polymorphism in the 5'-Flanking Region of the Human Glutamate-Cysteine Ligase Modifier Subunit Gene Is Associated with Myocardial Infarction. Circulation. 2002; 105:2968–2973. [PubMed: 12081989]

39. Nichenametla SN, Ellison I, Calcagnotto A, Lazarus P, Muscat JE, Richie JPJ. Functional Significance of the CAG Trinucleotide-Repeat Polymorphism in the Gene for the Catalytic Subunit of γ-glutamylcysteine Ligase. Free Radic Biol Med. 2008; 45:645–650. [PubMed: 18549827]

40. Walsh AC, Feulner JA, Reilly A. Evidence for Functionally Significant Polymorphism of Human Glutamate Cysteine Ligase Catalytic Subunit: Association with Glutathione Levels and Drug Resistance in the National Cancer Institute Tumor Cell Line Panel. Toxicol Sci. 2001; 61:218–223. [PubMed: 11353130]

41. Takizawa K, Yagi H, Jerina DM, Mattison DR. Murine Strain Differences in Ovotoxicity following Intraovarian Injection with Benzo(*a*)pyrene, (+)-(7*R*,8*S*)-oxide, (−)-(7*R*,8*S*)-Dihydrodiol, or (+)-(7*R*,8*S*)-Diol-(9*S*,10*R*)-epoxide-2. Cancer Res. 1984; 44:2571–2576. [PubMed: 6327019]

42. Uno S, Dalton TP, Dragin N, Curran CP, Derkenne S, Miller ML, et al. Oral Benzo[a]pyrene in Cyp1 Knockout Mouse Lines: CYP1A1 Important in Detoxication, CYP1B1 Metabolism Required for Immune Damage Independent of Total-Body Burden and Clearance Rate. Mol Pharmacol. 2006; 69:1103–1114. [PubMed: 16377763]

43. Dey A, Westphal H, Nebert DW. Cell-Specific Induction of Mouse Cyp1a1 RNA during Development. Proc Natl Acad Sci U S A. 1989; 86:7446–7450. [PubMed: 2477842]

44. Winn LM, Wells PG. Evidence for Embryonic Prostaglandin H Synthase Catalyzed Bioactivation and Reactive Oxygen Species-Mediated Oxidation of Cellular Macromolecules in Pheytoin and Benzo[a]pyrene Teratogenesis. Free Radic Biol Med. 1997; 22:607–621. [PubMed: 9013124]

45. Parman T, Wells PG. Embryonic Prostaglandin H Synthase-2 (PHS-2) Expression and Benzo[*a*]pyrene Teratogenicity in PHS-2 Knockout Mice. FASEB J. 2002; 16:1001–1009. [PubMed: 12087061]

46. Nakamura BN, Mohar I, Lawson GW, Hoang YD, Cortés MM, Ortiz L, et al. Increased Sensitivity to Testicular Toxicity of Transplacental Benzo[a]pyrene Exposure in Male Glutamate Cysteine

Ligase Modifier Subunit *Gclm*−/−) Knockout Mice. Toxicol Sci. 2012; 126:227–241. [PubMed: 22253057]

47. Verhofstad N, van Oostrom CTM, Zwart E, Mass LM, van Benthem J, van Schooten F-J, et al. Evaluation of Benzo(*a*)pyrene-Induced Gene Mutations in Male Germ Cells. Toxicol Sci. 2011; 119:218–223. [PubMed: 20961952]
48. Buters J, Quintanilla-Martinez L, Schober W, Soballa VJ, Hintermair J, Wolff T, et al. CYP1B1 Determines Susceptibility to Low Doses of 7,12-Dimethylbenz[a]anthracene-Induced Ovarian Cancers in Mice: Correlation of CYP1B1-Mediated DNA Adducts with Carcinogenicity. Carcinogenesis. 2003; 24:327–334. [PubMed: 12584184]
49. Ramesh A, Archibong AE, Niaz MS. Ovarian Susceptibility to Benzo[a]Pyrene: Tissue Burden of Metabolites and DNA Adducts in F-344 Rats. J Toxicol Environ Health, Part A. 2010; 73:1611–1625. [PubMed: 20967675]
50. Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu H-Y, et al. Aromatic Hydrocarbon Receptor-Driven Bax Gene Expression is Required for Premature Ovarian Failure Caused by Biohazardous Environmental Chemicals. Nat Genet. 2001; 28:355–360. [PubMed: 11455387]

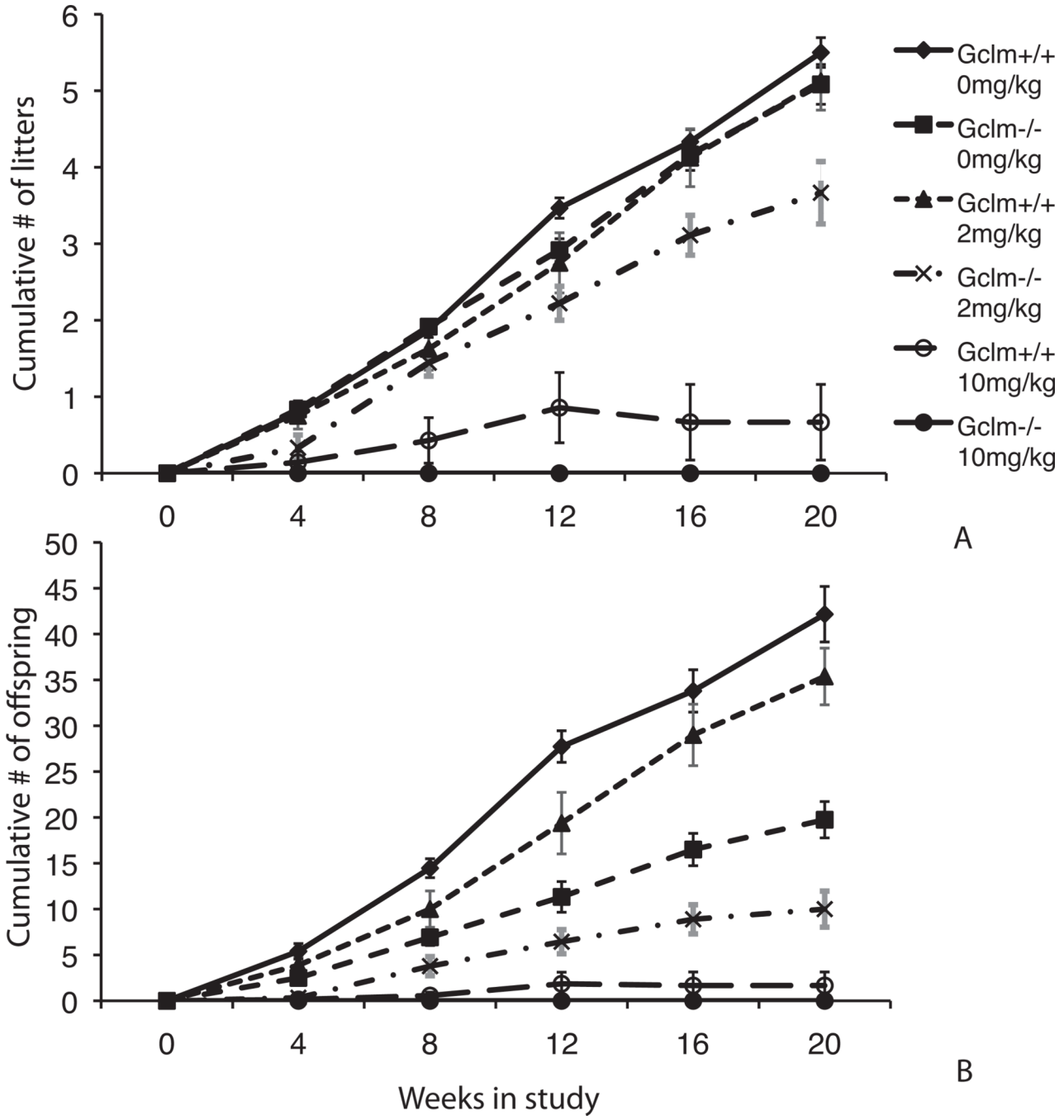


**Figure 1.**

*Gclm−/−* F1 females produce fewer offspring following transplacental exposure to BaP than *Gclm+/+* littermates. F0 females were dosed by oral gavage with 0, 2, or 10 mg/kg/day BaP from GD7–16. Each *Gclm−/−* and *Gclm+/+* F1 female offspring was continuously bred with a wild type male for 20 weeks beginning at 8 weeks of age. Cumulative numbers of litters (A) and offspring (B) are shown as means ± SEM. A) The effects of BaP dose ($p<0.001$) and *Gclm* genotype ($p<0.001$) were statistically significant; the dose × genotype interaction was not ($p=0.738$). B) The effects of BaP dose ($p<0.001$), *Gclm* genotype ($p<0.001$), and dose × genotype interaction ($p<0.001$) were statistically significant.

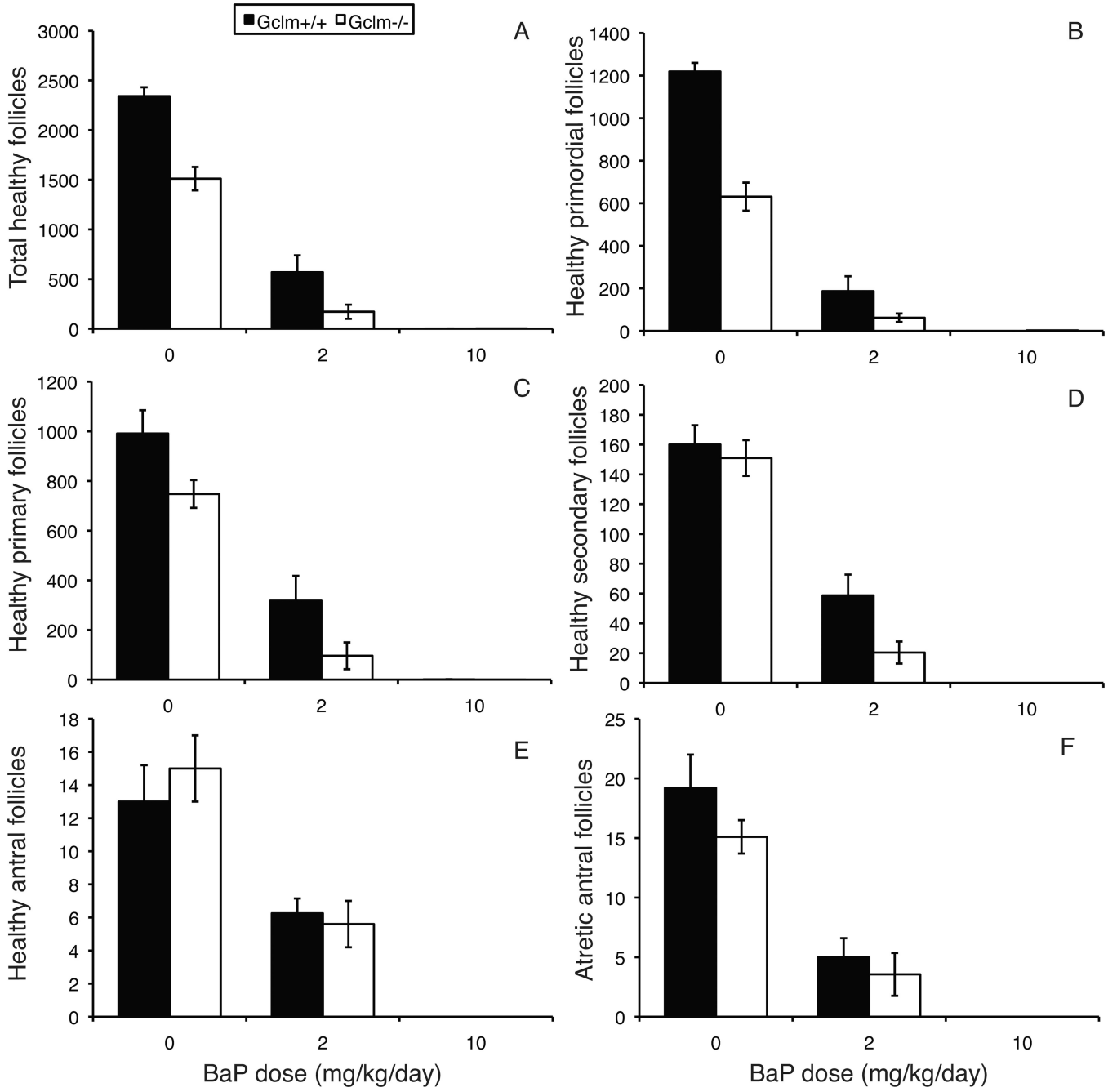


**Figure 2.**

*Gclm−/−* F1 females have greater ovarian follicle depletion in response to transplacental exposure to BaP than *Gclm+/+* littermates. Ovaries were harvested after the end of the breeding study described in Figure 1. Healthy follicles were counted in serial sections of ovaries as described in Materials and Methods. The graphs show the estimated marginal means ± SEM number of follicles in both ovaries from models adjusted for litter effects. A) Total number of healthy follicles of all stages combined. The effects of BaP dose ($p<0.001$), *Gclm* genotype ($p<0.001$), and dose × genotype interaction ($p=0.002$) were statistically significant. B) Healthy primordial follicles. The effects of BaP dose ($p<0.001$), *Gclm* genotype ($p<0.001$), and dose × genotype interaction ($p<0.001$) were statistically significant.

C) Healthy primary follicles. The effects of BaP dose ($p<0.001$), *Gclm* genotype ($p=0.007$), and the dose × genotype interaction ($p=0.025$) were statistically significant. D) Healthy secondary follicles. The effects of BaP dose ($p<0.001$), *Gclm* genotype ($p=0.032$) and the dose × genotype interaction were statistically significant ($p=0.032$). E) Healthy antral follicles. Only the effect of BaP dose was statistically significant ($P<0.001$). F) Atretic antral follicles. Only the effect of BaP dose was statistically significant ($P<0.001$). N=6–13, from 4–10 litters/group, except *Gclm*−/− 10 mg/kg BaP, N=2 from 1 litter.

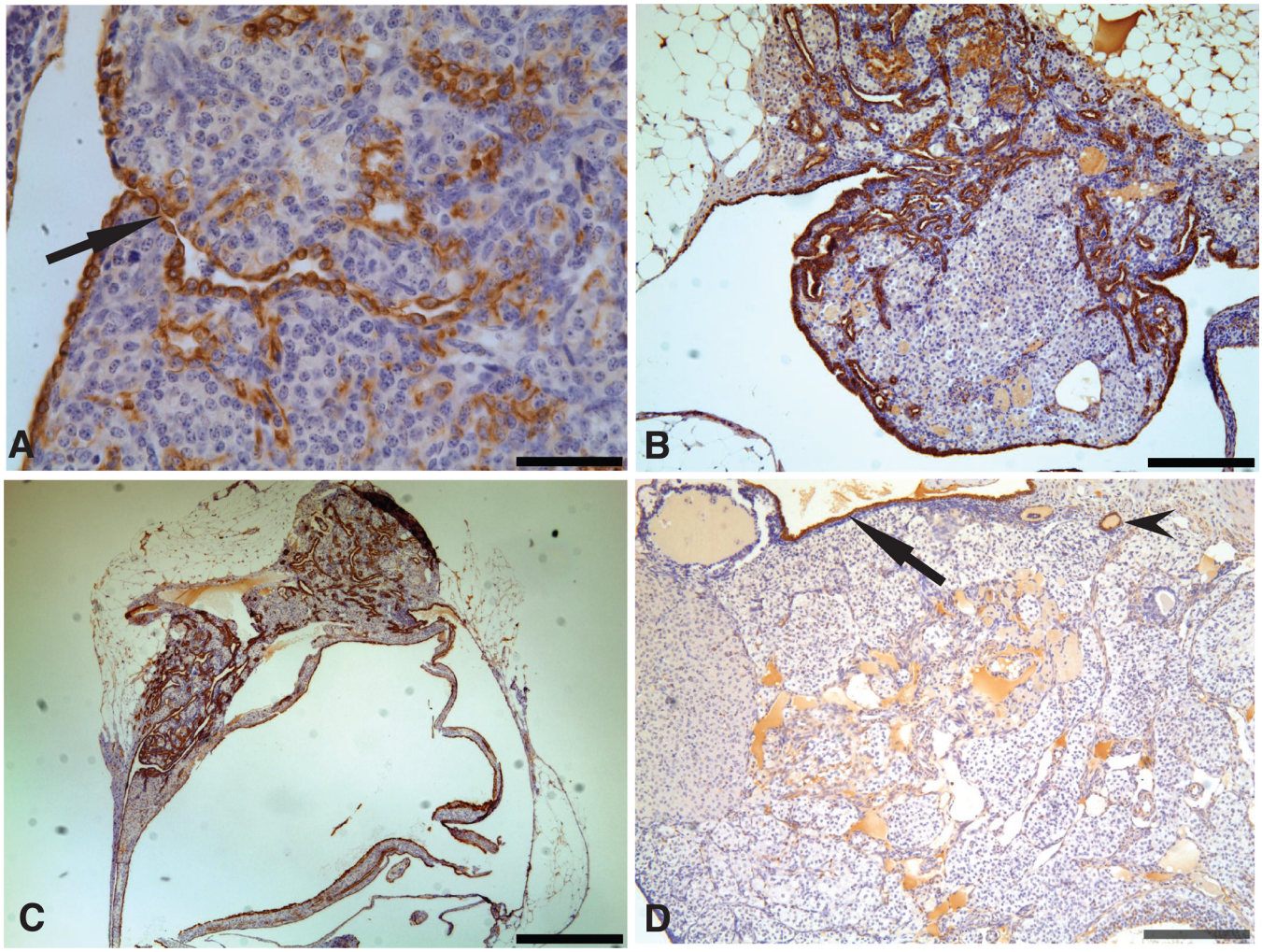


**Figure 3.**

Prenatal BaP exposure causes epithelial ovarian tumors in F1 offspring. F0 females were treated with BaP or vehicle as described in Figure 1. Ovarian tumor sections were immunostained with a pancytokeratin antibody (brown) and counterstained with hematoxylin as described in Materials and Methods. A) Ovarian tumor from a *Gclm−/−* female prenatally exposed to 10 mg/kg/day BaP. Linings of numerous small tubular structures stain positively for cytokeratin, as does deep invagination that is contiguous with the ovarian surface epithelium (arrow). Scale bar 50 μm. B) Ovarian tumor from a *Gclm+/+* female prenatally exposed to 10 mg/kg/day BaP. Cells lining tubular structures as well as ovarian surface epithelium again stain positive for cytokeratin. Note also the invasion of the tumor into the periovarian fat at top. Scale bar 500 μm. C) Cystic ovarian tumor from a *Gclm+/+* female prenatally exposed to 10 mg/kg/day BaP. Cells lining the large cysts, as well as those lining the smaller tubular structures at top stain positively for cytokeratin. Scale bar 500 μm. D) Ovary of the same *Gclm−/−* 2 mg/kg/day BaP treated F1 female as in Supplemental Figure S1F showing strong cytokeratin immunostaining in ovarian surface epithelial cells (arrow) and small epithelial inclusion cyst (arrowhead), but no staining in adjacent granulosa theca cell tumor. Scale bar 200 μm.

**Table 1**

Effect of BaP treatment from GD 7–16 on birth outcomes of F0 dams

| BaP dose (mg/kg/day) | % plugged delivered litter | litter size* | % pups dead PND0 | dead litters/total litters PND0 (%) | Female offspring genotypes (% of total females) | | | Male offspring genotypes (% of total males) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | KO | Het | WT | KO | Het | WT |
| 0 | 71 | 6.5±0.4 | 29±8 | 6/26 (23) | 12 (18) | 36 (54) | 19 (28) | 20 (30) | 25 (38) | 21 (32) |
| 2 | 82 | 5.7±0.5 | 33±9 | 4/19 (21) | 9 (25) | 19 (53) | 8 (22) | 6 (15) | 19 (46) | 16 (39) |
| 10 | 83 | 6.1±0.8 | 39±14 | 5/13 (38) | 2 (8) | 16 (64) | 7 (28) | 7 (19) | 21 (57) | 9 (24) |

* includes pups found dead on day of birth

**Table 2**

Effect of prenatal BaP on estrous cycling in F1 females

| | # not cycling (N of group)[a] | Mean ± SEM cycle length (days)[*] | Mean ± SEM % days with leukocytic cytology[**], [b] | Mean ± SEM % days with cornified cytology[**], [c] | Number of leukocytic to cornified transitions in 14 days[*],[d] |
|---|---|---|---|---|---|
| *Gclm+/+* | | | | | |
| 0 mg/kg/d BaP | 2 (13) | 5.6±0.5 | 53.1±3.8 | 22.2±2.3 | 2.5±0.2 |
| 2 mg/kg/d BaP | 3 (8) | 4.9±0.1 | 50.0±3.8 | 21.6±4.8 | 2.3±0.3 |
| 10 mg/kg/d BaP | 6 (6) | NA | 33.2±15.0 | 58.3±17.9 | 0.5±0.1 |
| *Gclm−/−* | | | | | |
| 0 mg/kg/d BaP | 2 (12) | 5.3±0.5 | 53.8±2.6 | 21.6.±3.0 | 2.3±0.3 |
| 2 mg/kg/d BaP | 2 (9) | 5.3±0.2 | 46.7±6.6 | 33.6±7.6 | 2.2±0.2 |
| 10 mg/kg/d BaP | 2 (2) | NA | 5.6±0.0 | 94.2±0.2 | 1.0±0.0 |

[*] Estimated marginal means and SEMs from models adjusted for litter effects

[**] Unadjusted means and SEMs

[a] p<0.05, effect of BaP dose for both genotypes by Likelihood ratio chi-square probability

[b] p=0.004, effect of BaP dose; p=0.905, effect of genotype; p=0.222, genotype × dose

[c] p<0.001, effect of BaP dose; p<0.001, effect of genotype; p=0.052, genotype × dose

[d] p<0.001, effect of BaP dose; p=0.626, effect of genotype; p=0.083, genotype × dose

NA: Not applicable, animals not cycling

**Table 3**

Effect of prenatal BaP on ovarian follicle counts in 35 day old *Gclm+/−* F1 offspring

| | Healthy primordial follicles* | Healthy primary follicles* | Healthy secondary follicles* | Atretic secondary follicles* | Healthy antral follicles** | Atretic antral follicles** |
|---|---|---|---|---|---|---|
| BaP dose (mg/kg/d) | | | | | | |
| 0 | 1721±279 | 404±75 | 116±11 | 28.6±5.5 | 9.1±2.6 | 15.7±2.6 |
| 2 | 636±252 | 303±71 | 83±17 | 18.0±3.4 | 14.2±2.5 | 19.4±1.5 |
| 10 | 10±7 | 13±5 | 9±6 | 1.3±1.3 | 2.5±2.5 | 7.5±3.2 |

N=7, 5, 4 per 0, 2, 10 mg/kg/d dose group, respectively, each from separate litters

* P<0.01,

** P<0.05, effect of BaP dose.

**Table 4**

Effects of prenatal BaP exposure on ovarian tumorigenesis in F1 offspring

| | % without tumors (N) | % unilateral tumors (N) | % bilateral tumors (N) |
|---|---|---|---|
| *Gclm+/+* | | | |
| 0 mg/kg BaP | 100 (13) | 0 | 0 |
| 2 mg/kg BaP | 100 (8) | 0 | 0 |
| 10 mg/kg BaP | 17 (1) | 33 (2) | 50 (3) |
| *Gclm−/−* | | | |
| 0 mg/kg BaP | 100 (12) | 0 | 0 |
| 2 mg/kg BaP | 89 (8) | 11 (1) | 0 |
| 10 mg/kg BaP | 0 | 0 | 100 (2) |

P<0.001, effects of BaP dose, genotype, and dose × genotype interaction on tumor multiplicity per mouse.