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Lack of Maternal Glutamate Cysteine Ligase Modifier Subunit (*Gclm*) Decreases Oocyte Glutathione Concentrations and Disrupts Preimplantation Development in Mice

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Glutathione (GSH) is the most abundant intracellular thiol and an important regulator of cellular redox status. Mice that lack the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in GSH synthesis, have decreased GSH synthesis. Nicotinamide nucleotide transhydrogenase, an inner mitochondrial membrane protein, catalyzes the interconversion of reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate; reduced nicotinamide adenine dinucleotide phosphate is required for reduction of GSH disulfide. Previous work supports roles for GSH in preimplantation development. We hypothesized that *Gclm*−/− mice have increased preimplantation embryonic mortality and that this effect is enhanced by absence of a functioning *Nnt* gene. *Gclm*−/− females produced significantly fewer pups per litter than *Gclm*+/+ littermates. Numbers of oocytes ovulated in a natural estrous cycle or upon superovulation did not differ by genotype. Fewer uterine implantation sites were observed in the *Gclm*−/− females. Prepubertal *Gclm*−/− and *Gclm*+/+ females were superovulated, then mated overnight with a *Gclm*+/+ male. At 0.5 d postcoitum, *Gclm*−/− females had significantly lower percentages of zygotes with two pronuclei and higher percentages of zygotes with one pronucleus than *Gclm*+/+ or *Gclm*+/− females. At 3.5 d postcoitum, a significantly lower percentage of blastocyst stage embryos was recovered from uteri of *Gclm*−/− females than *Gclm*+/+ females. Embryonic development to the blastocyst stage, but not the two-cell stage, was significantly decreased after *in vitro* fertilization of oocytes from *Gclm*−/− females compared with *Gclm*+/+ females. The *Nnt* mutation did not enhance the effects of *Gclm* genotype on female fertility. These results demonstrate critical roles for maternal GSH in supporting normal preimplantation development. **(*Endocrinology* 152: 2806–2815, 2011)**

Reactive oxygen species (ROS) result from the sequential addition of electrons to molecular oxygen, forming superoxide anion radical, hydrogen peroxide, and hydroxyl radical. Superoxide anion radical can react with nitric oxide, forming the reactive nitrogen species peroxynitrite. ROS/reactive nitrogen species can function as intracellular signaling molecules, but because some of them are highly reactive, they can damage important cellular components by reacting with lipids, proteins, and nucleic acids. ROS are generated as byproducts of normal cellular processes, such as oxidative phosphorylation in all tissues and steroidogenesis in the gonads and adrenal glands (1). To prevent the adverse effects of high levels of ROS, the ovary and oviduct possess antioxidant defenses, including vitamins A, C, and E, the antioxidant tripeptide glutathione (GSH), and ROS scavenging enzymes, such as

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Abbreviations: BSO, Buthionine sulfoximine; CG, chorionic gonadotropin; dpc, day postcoitum; FHM, flushing and holding medium; GCL, glutamate cysteine ligase; GCLC, GCL catalytic subunit; GCLM, GCL modifier subunit; GD, gestational day; GSH, glutathione; GSSG, GSH disulfide; hCG, human CG; NADPH, reduced nicotinamide adenine dinucleotide phosphate; *Nnt*, nicotinamide nucleotide transhydrogenase; ROS, reactive oxygen species.

superoxide dismutases, GSH peroxidases, catalase, and GSH-S-transferases (2–8).

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 GSH-S-transferases, regulation of protein function, and regulation of nucleotide metabolism (9). In the process of reducing peroxides, GSH is oxidized to the disulfide, GSH disulfide (GSSG). GSSG is reduced back to GSH via the action of the enzyme GSH reductase, which requires reduced nicotinamide adenine dinucleotide phosphate (NADPH). C57BL/6J mice have been reported to carry a mutation in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene (10, 11), which results in the complete absence of mature NNT protein. NNT is an inner mitochondrial membrane protein that catalyzes the interconversion of reduced nicotinamide adenine dinucleotide and NADPH. Under conditions of oxidative stress, the generation of NADPH by NNT is favored. In the nematode *Caenorhabdis elegans*, *Nnt* deletion mutants had significantly greater inhibition of growth in response to oxidative stress than nematodes with wild-type *Nnt* (12).

GSH is synthesized in two ATP-dependent reactions. The first, rate-limiting reaction is catalyzed by glutamate cysteine ligase (GCL), a heterodimer composed of a catalytic subunit [GCL catalytic subunit (GCLC)] and a modifier subunit [GCL modifier subunit (GCLM)] (13, 14). GCLC possesses all the catalytic activity, whereas binding of GCLM decreases the Michaelis constant for the enzyme substrates glutamate and ATP and increases the inhibitory constant for GSH (13, 14).

Ovulated oocytes possess among the highest GSH concentrations of any cell type, on the order of 10 mM (15, 16). Previous studies suggest that oocyte GSH is important for sperm nucleus decondensation and male pronucleus formation after fertilization (16, 17) and for formation of the meiotic spindle during meiosis II (18). The preimplantation embryo has limited ability to up-regulate GSH synthesis before the blastocyst stage and relies on GSH from the oocyte (19–21).

Mice that lack *Gclc* die during embryonic development (9, 22, 23). Mice that lack *Gclm* survive and reproduce but have greatly reduced tissue levels of GSH (9, 24–26). Although *Gclm*−/− mice are able to reproduce, detailed studies of the effects of knocking out *Gclm* on female fertility have not been conducted. We hypothesized that *Gclm*−/− females have low GSH concentrations in oocytes, resulting in abnormal development of preimplantation embryos and decreased fertility and that the effect of *Gclm* genotype is enhanced by the absence of a functioning *Nnt* gene.

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 β-galactosidase/neomycin phosphotransferase fusion minigene (26). The mice have been backcrossed eight times onto a C57BL/6J genetic background (B6.129-*Gclm*^{tm1Tjka}; hereafter referred to as *Gclm*−/−). Mice for these experiments were generated by mating *Gclm*+/− males with *Gclm*+/− females. Genotyping was carried out using DNA extracted from tail or toe snips as described (24, 26). Our production of over 650 offspring from heterozygous crosses has revealed a slight deviation from the expected Mendelian distribution, with 20% *Gclm*−/−, 53% *Gclm*+/−, and 27% *Gclm*+/+ offspring produced. We have not observed any sex differences in the genotype distributions. The mice were housed in American Association for the Accreditation of Laboratory Animal Care-accredited facilities, with free access to deionized water and irradiated, soy-free laboratory chow (Teklad 2919; Harlan Laboratories, Inc., Indianapolis, IN), on a 14-h light, 10-h dark cycle, with lights on at 0630 h. Temperature was maintained at 20.6–23.9 C. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (27) and were approved by the Institutional Animal Care and Use Committee at the University of California Irvine.

Female fertility assessment

Beginning at 2 months of age, *Gclm*+/+ or *Gclm*−/− female mice were mated to wild-type, 8-wk-old C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME) for a continuous breeding assay (28). 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 then humanely euthanized by decapitation after anesthetizing on ice.

Estrous cycling and ovarian histology

Estrous cycling in individually housed, 7-month-old female mice after completion of the fertility study was evaluated every morning for a minimum of 14 d by vaginal cytology (29, 30). Mice were euthanized by CO_2 inhalation on estrus, and ovaries were collected for enumeration of ovulated oocytes and corpora lutea by evaluation of hematoxylin and eosin-stained complete serial sections of the entire ovary by an observer blind to genotype.

Timed breeding studies

For assessment of implantation and of postimplantation mortality, 2-month-old *Gclm*−/− or *Gclm*+/+ females were mated with wild-type male proven breeders on the late afternoon of proestrus, as determined by vaginal cytology. The next morning,

females were checked for vaginal plugs. The day of vaginal plug was designated gestational day (GD) 1. Females were killed by CO_2 euthanasia on the morning of GD 18. Live and dead fetuses were counted, live fetuses were weighed, and obvious resorption sites were noted. All uteri were then stained for 4.5 h in 10% ammonium sulfide, and a final count of resorption sites was made (31). Females were weighed before mating and on the day of euthanasia, and gestational weight gain was calculated.

Superovulation and collection of preimplantation embryos

Twenty-seven-day-old *Gclm*−/−, *Gclm*+/−, and *Gclm*+/+ females were injected with 5 IU equine chorionic gonadotropin (CG) ip at 1400 h, then injected with 5 IU human CG (hCG) ip 46 h later at 1200 h. Ovulated oocytes were collected from the ampullae of oviducts at 22–24 h after hCG injection.

For collection of embryos, *Gclm*−/−, *Gclm*+/−, and *Gclm*+/+ mice were mated with a wild-type male in the late afternoon after hCG injection. The next morning, females were checked for vaginal plugs. The morning of vaginal plug detection was designated 0.5 d postcoitum (dpc). Zygotes were collected from the ampullae of oviducts at 0.5 dpc, and blastocysts were collected by flushing the uterine horns at 3.5 dpc.

Oocytes and zygotes were collected into flushing and holding medium (FHM) (Specialty Media, Phillipsburg, NJ) containing hyaluronidase (0.3 mg/ml) to release cumulus cells for a maximum of 5 min, then transferred to FHM. Blastocysts were collected into FHM. All embryos were washed by transfer through three drops of FHM under mineral oil. The numbers of fertilized and nonfertilized oocytes and embryos were counted at ×400 using a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany) equipped with differential interference contrast optics. At the 0.5-dpc time point, oocytes were evaluated for the presence of sperm and/or one or two pronuclei; fragmentation was also noted. For the 3.5-dpc time point, conceptuses were scored for stage of development (unfertilized oocyte, blastocyst, morula, two- to eight-cell embryo, fragmented or degenerating embryo). All evaluations were performed by an observer blinded to genotype.

GSH assays

After treatment with hyaluronidase and initial wash in FHM, oocytes were washed through four drops of collection media [Hanks' balanced salt solution without calcium or magnesium (Invitrogen, Carlsbad, CA), with 5 mg/ml HEPES, and 1 mg/ml BSA] under mineral oil. Oocytes were collected in a small volume of collection medium into tared microcentrifuge tubes. One quarter volume (based on weight of the sample) of 5% sulfosalicylic acid was added, the oocytes were disrupted by homogenization with a handheld mortar and pestle Kontes dounce homogenizer (Kimble-Chase, Vineland, NJ) on ice, incubated on ice for 15 min, centrifuged at 15800 × *g* at 4 C, and the supernatant was stored frozen at −80 C. Total and oxidized GSH were measured in pooled supernatants from 150 to 231 oocytes per sample (oocytes from two to five mice) using a modification of an enzymatic recycling assay developed by Griffith (6, 32, 33). All samples were assayed in triplicate. Standards of known concentrations of GSSG were prepared in four parts buffer with 1 part 5% sulfosalicylic acid. Total GSH was measured by the addition of reaction mixture containing 0.26 mM NADPH, 0.74 mM 5,5′-dithiobis (2′-dinitrobenzoic acid), and 0.62 mM GSH reductase in sodium phosphate EDTA buffer. The reduction of 5,5′-dithiobis (2′-dinitrobenzoic acid) to thiobis(2-nitrobenzoic acid) was monitored every 10 sec using a microplate spectrophotometer (VersaMax; Molecular Devices, Sunnyvale, CA). GSSG assay was carried using the same procedure, except that reduced GSH was first removed by conjugation with 2-vinylpyridine, followed by chloroform extraction.

Modulation of the reproductive phenotype of *Gclm*−/− females by mutation in the *Nnt* gene

C57BL/6J mice have been reported to carry a mutation in the *Nnt* gene (10, 11), which results in the complete absence of mature NNT protein. NNT is one of several enzymes that can provide NADPH, which is required for the regeneration of GSH by GSSG reductase. Therefore, the decreased fertility and preimplantation embryonic death observed in *Gclm*−/− females on a C57BL/6J background could be partially due to the presence of the *Nnt* mutation. To test this, we generated two new lines of mice, which both carry the knockout *Gclm* allele, one in the presence of the mutant *Nnt* and one in the presence of wild-type *Nnt*. These lines were generated by crossing a *Gclm*−/− male with a C57BL/6JEiJ female mouse. The latter subline separated from the parental C57BL/6J in 1976. It has been maintained as a separately inbreeding subline ever since, and it is known not to carry the *Nnt* mutation (http://jaxmice.jax.org/strain/000924.html). The *Gclm*+/−;*NntWT/MUT* and *Gclm*+/−;*NntWT/MUT* offspring of this first cross were mated to generate the *Gclm*+/−;*NntMUT* and *Gclm*+/−;*NntWT* founders of the two new sublines, which we designate B6.Cg-*Gclm*^{tm1Tjka} *Nnt*$^{C57BL/6J}$/Lud (abbreviated *Gclm/NntMUT*) and B6.Cg-*Gclm*^{tm1Tjka} *Nnt*$^{+}$/Lud (abbreviated *Gclm/NntWT*).

Gclm−/−/*NntWT*, *Gclm*−/−/*NntMUT*, *Gclm*+/+/*NntWT*, and *Gclm*+/+/*NntMUT* female mice (n = 6–8 per group) were individually bred with wild-type C57BL/6J males for 16 wk beginning at 3 months of age. Females were checked daily for litters. Male and female offspring were counted, weighed, and euthanized on the day of birth.

Effect of *Gclm* genotype on *in vitro* fertilization and embryonic development

Gclm−/−/*NntWT*, *Gclm*−/−/*NntMUT*, *Gclm*+/+/*NntWT*, and *Gclm*+/+/*NntMUT* female mice were superovulated as described above, and oocytes were collected from the ampullae of the oviducts at 13 h after hCG injection. Oocytes from each mouse were placed in K-RVFE-50 *in vitro* fertilization medium (Cook Medical, Inc., Bloomington, IN) and were mixed with fresh epididymal sperm from a *Gclm*+/+ C57BL/6J male. After incubation at 37 C in 5% CO_2, 5% O_2, and 90% N_2 for 4–6 h, the oocytes were washed through four drops of K-RVFE-50 media and incubated for another 20 h. The zygotes were then transferred to potassium simplex optimized medium + ½ amino acids media (Millipore, Bedford, MA) and cultured for four more days. Embryos were scored daily for stage of development by observers blind to genotype.

Statistical analyses

For continuous variables, Levene's test was used to test for homogeneity of variances. The effects of *Gclm* genotype on various endpoints were analyzed by independent samples *t* test for equal or unequal variances or by ANOVA, as appropriate. Quantities expressed as fractions (fraction of embryos at a par-

ticular developmental stage) were subjected to arcsine square root transformation before analysis (34). Alternatively, if variances were not homogeneous, nonparametric Kruskal-Wallis or Mann-Whitney tests were used. Data are presented as mean ± SEM in figures and tables. Statistical analyses were performed using SPSS 17.0 for Macintosh (SPSS, Inc., Chicago, IL).

Results

Decreased oocyte GSH concentrations in *Gclm*−/− females

Concentrations of total GSH were measured in pools of ovulated oocytes collected from superovulated *Gclm*−/−, *Gclm*+/−, and *Gclm*+/+ mice (150–231 oocytes/pool; n = 2–3 pools/genotype). Total GSH concentrations in oocytes ovulated from *Gclm*−/− mice were below the limit of detection of the assay, indicating that *Gclm*−/− oocytes had GSH concentrations that were less than 20% those of oocytes ovulated from *Gclm*+/+ (1.03 ± 0.11 pmol/oocyte) and *Gclm*+/− (1.04 ± 0.25 pmol/oocyte) mice. GSH concentrations in oocytes from *Gclm*+/− mice did not differ significantly from those of *Gclm*+/+ mice. GSSG was undetectable in all samples.

Gclm−/− females have decreased fertility

To assess the effects of low GSH concentrations on fertility, we conducted a continuous breeding study, in which *Gclm*−/− and *Gclm*+/+ females were bred with wild-type males for 20 wk from 2 to 7 months of age. Cumulative litter and pup production of *Gclm*−/− and *Gclm*+/+ females are shown in Fig. 1. *Gclm*−/− and *Gclm*+/+ females produced equal numbers of litters in 20 wk (5.3 ± 0.3 *vs.* 5.4 ± 0.4, respectively). However, the *Gclm*−/− females produced significantly fewer offspring in 20 wk (19.2 ± 2.6 *vs.* 45.4 ± 4.1; t = −5.63, *df* = 12, $P < 0.001$ by *t* test).

Smaller litter size of Gclm−/− females is not due to lower ovulation rate

Ovulated oocytes in the oviduct, fresh corpora lutea, and older corpora lutea were enumerated in serial sections of both ovaries collected on estrous morning. As expected, the numbers of ovulated oocytes and stage 1 (freshly ovulated) (35) corpora lutea were highly correlated, with a correlation coefficient of 0.97, and did not differ by *Gclm* genotype (t = −0.30, *df* = 12, $P = 0.77$; t = −1.09, *df* = 12, $P = 0.30$) (Table 1). The number of stage 2 corpora lutea, which is indicative of numbers of ovulations in previous estrous cycles, also did not differ by genotype (t = −1.26, *df* = 12, $P = 0.23$) (Table 1). The numbers of oocytes ovulated after a superovulation protocol in prepubertal *Gclm*+/+, *Gclm*+/−, and *Gclm*−/− mice also did not differ by genotype (F = 0.49, *df* = 34, $P = 0.62$) (Table 1).

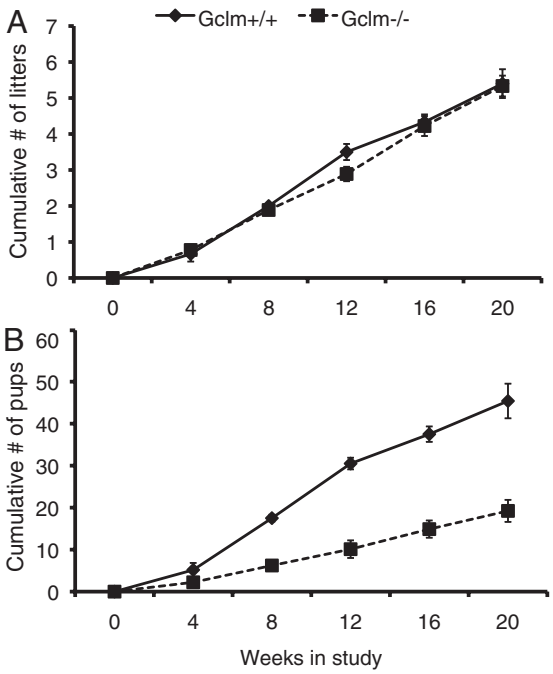

FIG. 1. *Gclm*−/− female mice have decreased fertility compared with *Gclm*+/+ mice. *Gclm*−/− and *Gclm*+/+ mice were mated with wild-type males for 20 wk beginning at 8 wk of age. A, Cumulative mean ± SEM number of litters produced in 20 wk. B, Cumulative mean ± SEM number of pups produced in 20 wk. The effect of genotype on total pup production was statistically significant ($P <$ 0.001 by *t* test). n = 5–9 per genotype.

Smaller litter size in *Gclm*−/− females is not due to postimplantation embryo death

The number of live fetuses per litter on GD 18 was statistically significantly lower in timed-mated *Gclm*−/− females than in *Gclm*+/+ females (t = −3.69, *df* = 18, $P = 0.002$) (Table 2). The mean maternal gestational weight gain was 7.7 ± 1.3 g in the *Gclm*−/− females compared with 14.1 ± 1.2 g in the *Gclm*+/+ females. The difference in weight gain was not statistically significant when adjusted for number of fetuses (F= 0.48, *df* = 20, $P = 0.497$). The numbers of dead fetuses and resorption

TABLE 1. Effect of lack of maternal *Gclm* on ovulation

	Gclm+/+ (mean±SEM)	*Gclm*−/− (mean±SEM)
Oocytes in oviducts on estrus[a]	8.8 ± 0.7	8.3 ± 1.1
Class 1 corpora lutea on estrus[a]	9.4 ± 0.5	8.0 ± 0.9
Class 2 corpora lutea on estrus[a]	9.8 ± 1.7	12.0 ± 0.9

	Gclm+/+	*Gclm*+/−	*Gclm*−/−
Oocytes in oviduct after superovulation[b]	44.6 ± 8.0	45.0 ± 5.6	52.5 ± 5.9

[a] *Gclm*+/+, *n* = 5; *Gclm*−/−, *n* = 9.
[b] *Gclm*+/+, *n* = 10; *Gclm*+/−, *n* = 13; *Gclm*−/−, *n* = 12.

TABLE 2. Effect of lack of maternal *Gclm* on postimplantation survival

	Gclm+/+ (mean±SEM)	*Gclm*−/− (mean±SEM)
Live fetuses	6.8 ± 0.9	2.4 ± 0.8[a]
Dead fetuses	0	0.2
Resorption sites	0.9 ± 0.4	1.7 ± 0.4
Total implantations	7.9 ± 0.8	4.3 ± 0.7[a]

n = 8–10 per group.
[a] $P < 0.005$ by t test.

sites did not differ by genotype (t = 1.5, $df = 9$, $P = 0.17$ and t = −1.38, $df = 15$, $P = 0.19$, respectively). The total number of implants (live plus dead fetuses plus resorptions) was significantly lower in *Gclm*−/− females than in *Gclm*+/+ females (t = −3.35, $df = 15$, $P = 0.004$). The mean fetal weight per litter did not differ significantly by genotype (data not shown).

Smaller litter size in *Gclm*−/− females is due to embryonic death before the blastocyst stage

Because *Gclm*−/− females had similar ovulation rates as *Gclm*+/+ females, but fewer implantations, we reasoned that either oocytes of *Gclm*−/− females had lower fertilization rates and/or that conceptuses of *Gclm*−/− females died before implantation. To test this, we superovulated immature females of both genotypes, mated them with wild-type males overnight, and collected oocytes and conceptuses at 0.5 and 3.5 dpc.

Figure 2A shows the effect of *Gclm* genotype on zygote development at 0.5 dpc. The effect of genotype was statistically significant for the percentage of zygotes with one pronucleus ($\chi^2 = 11.1$, $df = 2$. $P = 0.004$ by Kruskal-Wallis test), the percentage of zygotes with two pronuclei ($\chi^2 = 11.2$, $df = 2$, $P = 0.004$), and the percentage of oocytes with sperm but no pronuclei ($\chi^2 = 6.4$, $df = 2$, $P = 0.041$). Intergroup comparisons showed that a significantly smaller percentage of zygotes of *Gclm*−/− dams than of *Gclm*+/+ or *Gclm*+/− dams had two pronuclei (Z = −2.74, $P = 0.006$ and Z = −2.89, $P = 0.004$, respectively, by Mann-Whitney test), and a significantly larger percentage of zygotes of *Gclm*−/− dams had only one pronucleus (Z = −2.74, $P = 0.006$ and Z = −2.89, $P = 0.004$, respectively). The percentage of eggs with a sperm that had penetrated the zona pellucida, but no pronuclei, was higher in *Gclm*−/− females than in *Gclm*+/− females (Z = −2.56, $P = 0.010$ by Mann-Whitney test). In most oocytes with one pronucleus in *Gclm*−/− females, the pronucleus was located near the second polar body, and there was an intact sperm visible in the egg. The latter observations are consistent with lack of sperm nucleus decondensation resulting in lack of male pronucleus formation. Intergroup comparisons showed no statistically significant differences between *Gclm*+/− and *Gclm*+/+ females.

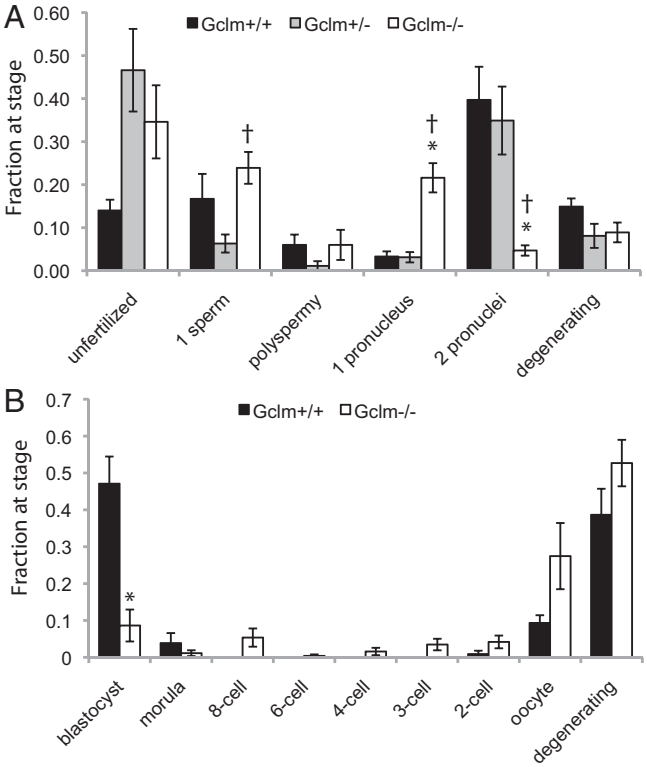

FIG. 2. *Gclm*−/− female mice have decreased zygote development and decreased percentages of embryos that reach the blastocyst stage. Superovulated, prepubertal mice of the indicated genotypes were mated with wild-type males, and conceptuses were collected from the oviducts at 0.5 dpc or from the uterus at 3.5 dpc as described in *Materials and Methods*. A, Mean ± SEM fraction of embryos at 0.5 dpc at the indicated stages of development (n = 5–6 per genotype). The effect of genotype was statistically significant by Kruskal-Wallis test for fraction of embryos with one pronucleus ($P = 0.004$), fraction with two pronuclei ($P = 0.004$), and oocytes with sperm in the cytoplasm ($P = 0.041$). B, Mean ± SEM fraction of embryos at 3.5 dpc at the indicated stages of development (n = 6–8 per genotype). *, $P < 0.05$ *vs.* *Gclm*+/+ by Mann-Whitney test; †, $P < 0.05$ *vs.* *Gclm*+/− by Mann-Whitney test.

Figure 2B shows the percentages of embryos that attained various developmental stages at 3.5 dpc by maternal genotype. Compared with *Gclm*+/+ dams, a significantly smaller percentage of embryos of *Gclm*−/− dams reached the blastocyst stage (Z = −2.74, $P = 0.006$ by Mann-Whitney test), and larger percentages of embryos of *Gclm*−/− dams tended to be unfertilized (Z = −1.75, $P = 0.081$) or to be at the three-cell (Z = −1.94, $P = 0.052$) and eight-cell (Z = −1.94, $P = 0.052$) stages at 3.5 dpc. The percentage of degenerating embryos was nonsignificantly increased in *Gclm*−/− dams. Maternal genotype did not affect the total number of conceptuses recovered from the uteri at 3.5 dpc (19.9 ± 3.3 in *Gclm*−/− *vs.* 14.8 ± 1.7 in *Gclm*+/+).

Nnt mutation does not worsen fertility deficit of *Gclm*−/− females

The presence of a mutant *Nnt* gene, which results in absence of mature NNT protein (10, 11), significantly

modulated the cumulative number of litters (F = 5.72, *df* = 28, *P* = 0.025, effect of *Nnt* genotype) but did not significantly modulate the cumulative number of offspring (F = 0.993, *df* = 28, *P* = 0.329, effect of *Nnt* genotype) produced by *Gclm*−/− female mice during a 16-wk breeding study from 3 to 7 months of age (Fig. 3, A and B). The percentage of pups born dead during the breeding study also appeared to be modulated by *Nnt* genotype (χ^2 = 14.97, *df* = 3, *P* = 0.002, effect of group by Kruskal-Wallis test) (Fig. 3C). Contrary to our hypothesis that the *Nnt* mutation would exacerbate the effects of lack of *Gclm*, the *Nnt* mutation appeared to partially rescue the effects of lack of *Gclm* on cumulative number of litters produced and percent of pups born dead. Interestingly, the effect of the lack of *Gclm* on female fertility appeared to be more severe on the mixed C57BL/6J x C57BL/6JEiJ background than on the pure C57BL/6J background in that *Gclm*−/− females of the mixed background not only produced cumulatively fewer offspring (F = 176.5, *df* =28, *P* < 0.001, effect of *Gclm* genotype) but also produced fewer litters (F = 11.9, *df* = 28, *P* = 0.002) than *Gclm*+/+ littermates, regardless of *Nnt* genotype (compare Fig. 3, A and B, with Fig. 1, A and B).

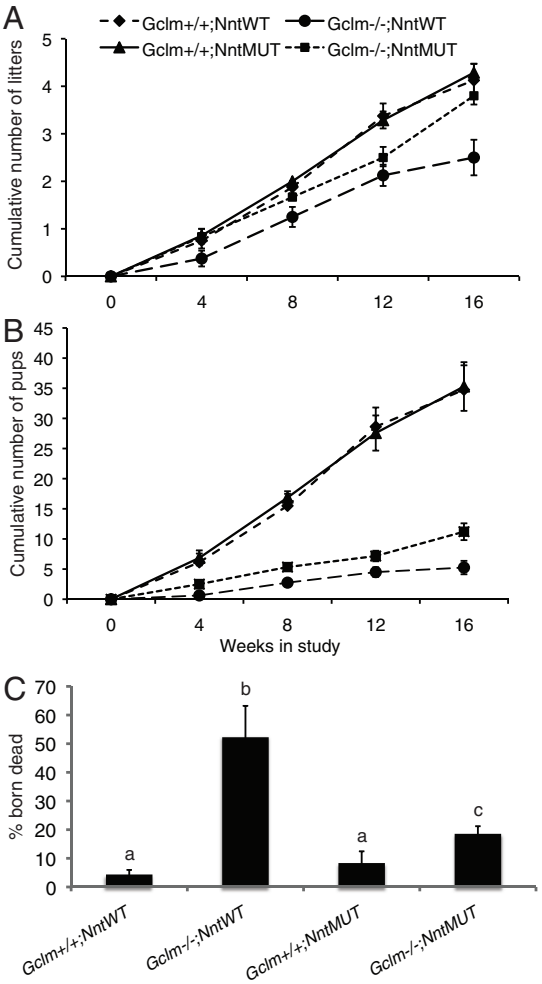

FIG. 3. The presence of a mutation in the *Nnt* gene modulates the effects of deletion of *Gclm* on female fertility. *Gclm*−/−/*NntWT*, *Gclm*+/+/*NntWT*, *Gclm*−/−/*NntMUT*, and *Gclm*+/+/*NntMUT* females were mated with wild-type males from 3 months of age for 16 wk (n = 6–8 per group). A, Mean ± SEM cumulative number of litters produced during the breeding study by genotype. B, Mean ± SEM cumulative number of offspring produced during the breeding study by genotype. The effect of *Gclm* genotype was statistically significant for cumulative litters and offspring (*P* = 0.002 and *P* < 0.001, respectively, by two-way ANOVA). The effect of *Nnt* genotype was statistically significant for cumulative number of litters (*P* = 0.025) but not for cumulative number of offspring (*P* = 0.329). The interaction between the genotypes was not statistically significant in either case (*P* = 0.074 and *P* = 0.414, respectively). C, Mean ± SEM percent of pups born dead to mothers of the indicated genotypes. The effect of genotype was significant overall (*P* = 0.002 by Kruskal-Wallis test). *Bars with different letters* differed significantly from one another by Mann-Whitney test, *P* < 0.04.

Effects of female *Gclm* knockout and *Nnt* mutation on *in vitro* fertilization and embryo development in culture

Oocytes of *Gclm*−/−/*NntWT*, *Gclm*−/−/*NntMUT*, *Gclm*+/+/*NntWT*, and *Gclm*+/+/*NntMUT* females fertilized *in vitro* with sperm from wild-type males progressed to the two-cell stage by 30 h after gamete mixing at similar rates (Table 3). However, by 78 h after gamete mixing, significantly lower percentages of embryos derived from oocytes of *Gclm*−/− females had progressed to the six-cell through morula stages (F = 9.28, *df* = 14, *P* = 0.011, effect of *Gclm* genotype) (Table 3). By 102 h after gamete mixing, significantly lower percentages of embryos derived from oocytes of *Gclm*−/− females had progressed at least to the six-cell stage (F = 14.93, *df* = 14, *P* = 0.003, effect of *Gclm* genotype) or had reached the blastocyst stage (F = 9.28, *df* = 14, *P* = 0.011, effect of *Gclm* genotype) (Table 3). *Nnt* genotype did not significantly affect embryo development in culture (F < 1.5) (Table 3).

Discussion

This study definitively demonstrates a critical role for maternal GSH in preimplantation embryonic development. Specifically, lack of maternal *Gclm* resulted in dramatically decreased GSH concentrations in ovulated oocytes and in decreased rates of formation of the second pronucleus at 0.5 dpc, decreased development of embryos to the blastocyst stage at 3.5 dpc, decreased uterine implantation rates, and decreased litter sizes. Interestingly, oocytes from *Gclm*−/− dams that were fertilized *in vitro* developed to the two-cell stage by 30 h after gamete mixing at similar rates as oocytes from *Gclm*+/+ dams but failed to progress to the blastocyst

TABLE 3. Effects of female *Gclm* knockout and *Nnt* mutation on *in vitro* fertilization and embryo development in culture

Genotype of oocyte donor	% two to three cell at 30 h (±SEM)	% six-cell to morula at 78 h (±SEM)[a]	% six-cell to blastocyst at 102 h (±SEM)[b]	% blastocysts at 102 h (±SEM)[a]	N
Gclm+/+;*NntMUT*	55.7 ± 14.6	18.9 ± 6.1	29.1 ± 7.5	15.7 ± 7.6	4
Gclm−/−;*NntMUT*	34.1 ± 19.4	3.3 ± 3.3	4.2 ± 3.0	0	3
Gclm+/+;*NntWT*	63.9 ± 10.9	27.8 ± 13.4	34.0 ± 12.4	22.0 ± 18.4	3
Gclm−/−;*NntWT*	58.1 ± 10.9	7.1 ± 2.7	9.0 ± 3.7	0.4 ± 0.4	4

[a] $P = 0.011$, effect of *Gclm* genotype by two-way ANOVA on arcsine square root transformed data.

[b] $P = 0.003$, effect of *Gclm* genotype by two-way ANOVA on arcsine square root transformed data.

stage. Taken together, these results suggest that lack of *Gclm* and resulting GSH deficiency exerts adverse effects at several stages of early development.

Several genetically modified mouse models of decreased antioxidant capacity have been developed, but few of these have been systematically evaluated for effects on reproductive function. Mice that lack the transcription factor *Nrf2* have decreased antioxidant capacity due to decreased basal and inducible transcription of numerous antioxidant defense genes, including the genes of GSH synthesis (36, 37). *Nrf2*−/− female mice have been reported to have significantly smaller litters than wild-type C57BL/6 mice at 6–20 wk of age, but the cause of smaller litter sizes was not investigated (38). Female mice null for *Sod1* are also subfertile, but the cause remains unclear. One group reported that ovaries of *Sod1*−/− mice had few large antral follicles and corpora lutea, suggesting a defect in the later stages of follicle maturation (39). Another group reported normal ovulation and conception, but increased embryonic lethality in *Sod1*−/− females (40). Although *Sod2*−/− mice die within several weeks of birth, transplantation of *Sod2*−/− ovaries to wild-type hosts resulted in normal follicular development, ovulation, and viable offspring, suggesting that *Sod2* is not required for normal ovarian function (39). Normal fertility has been reported in mice that lack *Gpx1* (41) or catalase (42) or that bear an inactivating mutation in GSH reductase (43–45), whereas disruption of *Gpx4* (46–48) or *Gclc* (22, 23) results in embryonic lethality. In contrast, γ-glutamyl transpeptidase 1 (*Ggt1*) null mice display a shortened lifespan, stunted growth, and a severe female reproductive phenotype, with complete infertility, lack of ovarian large antral follicles and corpora lutea, and absent ovulatory response to exogenous gonadotropins (49–51). These mice have decreased ovarian cysteine concentrations, but normal ovarian GSH concentrations, compared with wild-type controls, and the female reproductive phenotype is completely rescued by cysteine replacement (49). Detailed reproductive studies of another *Gclm* null strain that was generated using a different targeting vector and on a different genetic background have not been published (25), but the females of that strain are reportedly also subfertile (Chen, Y., personal communication). Thus, the limited data to date do not suggest that knockout of different antioxidant genes results in similar female reproductive phenotypes but do suggest that GSH and cysteine are important for female fertility.

We did not observe a significant difference in oocyte GSH concentrations between *Gclm*+/− and *Gclm*+/+ females in the present study. Intermediate concentrations of GSH in *Gclm*+/− mice, which are higher than the levels in *Gclm*−/− and lower than levels in *Gclm*+/+, have been reported for some, but not all, tissues and cells tested (25, 26, 52). The slight differences in preimplantation embryo development between *Gclm*+/− and *Gclm*+/+ females observed in the present study (Fig. 2A) may be caused by differences in GSH concentrations in other cell types besides the oocytes.

Brief depletion of GSH associated with an oxidant stimulus (diamide or iodoacetamide) in ovulated hamster oocytes before *in vitro* fertilization disrupted normal hamster oocyte meiotic spindle formation leading to the formation of abnormal female pronuclei (18). However, no spindle abnormalities were reported when bovine oocytes were depleted of GSH using buthionine sulfoximine (BSO), a specific inhibitor of GCL (17). These results suggest that meiotic spindle formation may be sensitive to oxidative stress but not to GSH depletion *per se*. In our *Gclm*−/− model, oocyte GSH concentrations are chronically low, but we observed apparently normal formation of the female pronucleus. Biochemical depletion of GSH with BSO during ooctye maturation *in vitro* (16, 17) or *in vivo* (15) prevented sperm head decondensation and formation of the male pronucleus after *in vitro* fertilization. This was believed to be due to the requirement for reduction of protamine disulfide bonds in the sperm nucleus for sperm nuclear reactivation to occur (16, 53). Our finding that very few zygotes of *Gclm*−/− dams had two pronuclei at 0.5 dpc provide further evidence that high concentrations of GSH in the oocyte are required for normal formation of the male pronucleus. Our *in vitro* fertilization data suggest that the low GSH concentrations in oocytes of *Gclm*−/− females may be sufficient to permit eventual decondensation of the sperm nucleus, with sim-

ilar rates of cleavage stage embryos observed 30 h after gamete mixing for both *Gclm*−/− and *Gclm*+/+ females. However, the embryos derived from oocytes of *Gclm*−/− females failed to develop to the blastocyst stage at 102 h after gamete mixing. The latter results are consistent with a continued requirement for GSH from the oocyte for development beyond the cleavage stage to the blastocyst stage.

Before the blastocyst stage, preimplantation embryos do not express *Gclc* and are not able to up-regulate GSH synthesis in response to treatment with agents that deplete GSH by conjugation, such as diethyl maleate (19, 20). In contrast, two-cell embryos are able to respond to stimuli that induce oxidative stress by up-regulating *Gclc* expression and GSH synthesis (19). *In vivo* treatment with BSO beginning before initiation of a superovulation protocol significantly diminished concentrations of GSH in oocytes and increased the percentage of degenerating embryos retrieved at 1.5 dpc (21). These results are consistent with our observation of decreased *in vitro* development of embryos of *Gclm*−/− females to the blastocyst stage compared with embryos of *Gclm*+/+ females despite similar rates of development to the cleavage stage. In contrast, BSO injections beginning at 0.5 dpc significantly decreased GSH concentrations in oviductal and uterine secretions, but did not decrease zygote GSH concentrations or affect embryonic development at 1.5 or 2.5 dpc (21). Taken together with the results of the present study, these findings support the conclusion that GSH present in the oocyte is critical for normal preimplantation development, whereas GSH in reproductive tract secretions appears to play a lesser, if any, role. Consistent with a role for oocyte GSH in early preimplantation development, zygotes from mice overexpressing GSH synthetase showed better *in vitro* development than zygotes from wild-type mice (54).

Genetic background is known to modulate the effects of gene deletions. For example, the effects of *Dax* knockout on gonadal differentiation are much more pronounced on a C57BL/6J background than on a 129/SvlmJ background (55). The effects of lack of *Sod2* also vary with background strain, with embryonic lethality occurring on a C57BL/6J background compared with survival to postnatal d 8 on a DBA/2J background (11). Huang *et al.* (11) showed that this differential sensitivity to *Sod2* deletion was associated with a mutation in the *Nnt* gene in the C57BL/6J strain. NNT is a nuclear-encoded, inner mitochondrial membrane protein that catalyzes the interconversion of reduced nicotinamide adenine dinucleotide and NADPH, with the generation of NADPH predominating. NADPH is required for the reduction of GSSG by GSSG reductase. The *Nnt* mutation has also been reported to account for the glucose intolerance observed in C57BL/6J mice (10). In the present study, we found that the presence of a mutant *Nnt* gene modulated the effect of *Gclm* deletion on female fertility but not in the hypothesized direction. The females that were homozygous null for *Gclm* and harbored the *Nnt* mutation (*Gclm*−/−;*NntMUT*) produced significantly more litters and lower percentages of pups that were born dead and nonsignificantly more live offspring than *Gclm*−/−;*NntWT* females. This suggests a beneficial effect of the *Nnt* mutation in the setting of low GSH synthesis, which is difficult to explain. One possibility is that the presence of the *Nnt* mutation leads to up-regulation of other antioxidant genes, partially compensating for the deficiency of *Gclm*. An alternative explanation is that C57BL/6JEiJ mice may differ from C57BL/6J mice in loci besides *Nnt* that compensate for the compromised GSH status in *Gclm*−/− mice. These possibilities should be investigated in future studies.

The results of the present study may be relevant to female reproductive aging. Declines in oocyte quality, such as aneuploidy, decreased mitochondrial fraction, increased dilated smooth endoplasmic reticulum and Golgi fractions, and increased mitochondrial membrane rupture (56–58), are observed with aging in experimental animals and women. Diminished oocyte quality, in turn, is thought to play a role in the decline in litter size (59) and increased preimplantation and postimplantation embryo loss (60, 61) observed in aging mice and the decreased success rates of assisted reproduction in older women (57, 62). Expression of *Gclm* (56) and concentrations of GSH (63) were reportedly decreased in oocytes from aged mice compared with young adult mice. Our results suggest that the age-related declines in oocyte quality may be caused in part by the decreased capacity of aged oocytes to synthesize GSH. In addition, polymorphisms in *Gclm* and *Gclc* (64–67), which affect GSH synthesis, exist in humans, and our results predict that they may affect oocyte quality and fertility in women.

In conclusion, maternal GSH is essential for normal zygote development and normal fertility in female mice. *Gclm*−/− females have low oocyte GSH concentrations and decreased male pronucleus formation after fertilization, resulting in fewer implantations and smaller litter sizes. This decreased oocyte quality as a result of GSH deficiency may be relevant to female reproductive aging and to fertility in women with polymorphisms in *Gclc* and *Gclm*.

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References

1. **Hanukoglu I** 2006 Antioxidant protective mechanisms against reactive oxygen species (ROS) generated by mitochondrial P450 systemes in steroidogenic cells. Drug Metab Rev 38:171–196
2. **Sato EF, Kobuchi H, Edashige K, Takahashi M, Yoshioka T, Utsumi K, Inoue M** 1992 Dynamic aspects of ovarian superoxide dismutase isozymes during the ovulatory process in the rat. FEBS Lett 303: 121–125
3. **Hsieh CH, Tsai SP, Yeh HI, Sheu TC, Tam MF** 1997 Mass spectrometric analysis of rat ovary and testis cytosolic glutathione S-transferases (GSTs): identification of a novel class-α GST, rGSTA6, in rat testis. Biochem J 323:503–510
4. **Aten RF, Duarte KM, Behrman HR** 1992 Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin $F_{2\alpha}$. Biol Reprod 46:401–407
5. **Gardiner CS, Salmen JJ, Brandt CJ, Stover SK** 1998 Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biol Reprod 59:431–436
6. **Luderer U, Kavanagh TJ, White CC, Faustman EM** 2001 Gonadotropin regulation of glutathione synthesis in the rat ovary. Reprod Toxicol 15:495–504
7. **Tilly JL, Tilly KI** 1995 Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology 136:242–252
8. **Mattison DR, Shiromizu K, Pendergrass JA, Thorgeirsson SS** 1983 Ontogeny of ovarian glutathione and sensitivity to primordial oocyte destruction by cyclophosphamide. Pediatr Pharmacol 3:49–55
9. **Dalton TP, Chen Y, Schneider SN, Nebert DW, Shertzer HG** 2004 Genetically altered mice to evaluate glutathione homeostasis in health and disease. Free Radic Biol Med 37:1511–1526
10. **Freeman HC, Hugill A, Dear NT, Ashcroft FM, Cox RD** 2006 Deletion of nicotinamide nucleotide transhydrogenase. A new quantitative trait locus accounting for glucose intolerance in C57BL/6J mice. Diabetes 55:2153–2156
11. **Huang TT, Naeemuddin M, Elchuri S, Yamaguchi M, Kozy HM, Carlson EJ, Epstein CJ** 2006 Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. Hum Mol Genet 15:1187–1194
12. **Arkblad EL, Tuck S, Pestov NB, Dmitriev RI, Kostina MB, Stenvall J, Tranberg M, Rydström J** 2005 A *Caenorhabdis elegans* mutant lacking functional nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative stress. Free Radic Biol Med 38:1518–1525
13. **Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, Kavanagh TJ** 2009 Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. Mol Aspects Med 30:86–98
14. **Griffith OW, Mulcahy RT** 1999 The enzymes of glutathione synthesis: γ-glutamylcysteine synthetase. Adv Enzymol Rel Areas Mol Biol 73:209–267
15. **Calvin HI, Grosshans K, Blake EJ** 1986 Estimation and manipulation of glutathione levels in prepuberal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. Gamete Res 14:265–275
16. **Perreault SD, Barbee RR, Slott VL** 1988 Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. Dev Biol 125:181–186
17. **Sutovsky P, Schatten G** 1997 Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. Biol Reprod 56:1503–1512
18. **Zuelke KA, Jones DP, Perreault SD** 1997 Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. Biol Reprod 57:1413–1419
19. **Stover SK, Gushansky GA, Salmen JJ, Gardiner CS** 2000 Regulation of γ-glutamate cysteine ligase expression by oxidative stress in the mouse preimplantation embryo. Toxicol Appl Pharmacol 168:153–159
20. **Gardiner CS, Reed DJ** 1995 Synthesis of glutathione in the preimplantation mouse embryo. Arch Biochem Biophys 318:30–36
21. **Salmen JJ, Skufca F, Matt A, Gushansky G, Mason A, Gardiner CS** 2005 Role of glutathione in reproductive tract secretions on mouse preimplantation embryo development. Biol Reprod 73:308–314
22. **Dalton TP, Dieter MZ, Yang Y, Shertzer HG, Nebert DW** 2000 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 279:324–329
23. **Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW** 2000 Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci USA 97:5101–5106
24. **Giordano G, White CC, McConnachie LA, Fernandez C, Kavanagh TJ, Costa LG** 2006 Neurotoxicity of domoic acid in cerebellar granule neurons in a genetic model of glutathione deficiency. Mol Pharmacol 70:2116–2126
25. **Yang Y, Dieter MZ, Chen Y, Shertzer HG, Nebert DW, Dalton TP** 2002 Initial characterization of the glutamate cysteine ligase modifier subunit *Gclm* (−/−) knockout mouse: novel model system for severely compromised oxidative stress response. J Biol Chem 277: 49446–49452
26. **McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, Chatterton-Kirchmeier S, White CC, Pierce RH, Kavanagh TJ** 2007 Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. Toxicol Sci 99:628–636
27. **National Research Council** 1996 Guide for the care and use of laboratory animals. Washington, DC: National Research Council, National Academy of Sciences
28. **Chapin RE, Sloane RA** 1997 Reproductive assessment by continuous breeding: evolving study design and summaries of ninety studies. Environ Health Perspect 105(Suppl 1):199–205
29. **Cooper RL, Goldman JM, Vandenbergh JG** 1993 Monitoring of the estrous cycle in the laboratory rodent by vaginal lavage. In: Heindel JJ, Chapin RE, eds. Female reproductive toxicology. San Diego: Academic Press, Inc.; 45–55

30. **McClintock MK** 1981 Social control of the ovarian cycle and the function of estrous synchrony. Am Zool 21:243–256
31. **Narotsky MG, Brownie CF, Kavlock RJ** 1997 Critical period of carbon tetrachloride-induced pregnancy loss in Fischer-344 rats, with insights into the detection of resorption sites by ammonium sulfide staining. Teratology 56:252–261
32. **Griffith OW** 1980 Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207–212
33. **Tsai-Turton M, Luderer U** 2005 Gonadotropin regulation of glutamate cysteine ligase catalytic and modifier subunit expression in the rat ovary is subunit and follicle stage-specific. Am J Physiol 289:E391–E402
34. **Pasternack BS, Shore RE** 1982 Analysis of dichotomous response data from toxicological experiments involving stable laboratory mouse populations. Biometrics 38:1057–1067
35. **Erickson GF, Nakatani A, Ling N, Shimasaki S** 1993 Insulin-like growth factor binding protein-3 gene expression is restricted to involuting corpora lutea in rat ovaries. Endocrinology 133:1147–1157
36. **Chan JY, Kwong M** 2000 Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochim Biophys Acta 1517:19–26
37. **Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW** 2001 Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in *nrf2* transcription factor-deficient mice. Proc Natl Acad Sci USA 98:3410–3415
38. **Hu X, Roberts JR, Apopa PL, Kan YW, Ma Q** 2006 Accelerated ovarian failure induced by 4-vinyl cyclohexene diepoxide in Nrf2 null mice. Mol Cell Biol 26:940–954
39. **Matzuk MM, Dionne L, Guo Q, Kumar TR, Lebovitz RM** 1998 Ovarian function in superoxide dismutase 1 and 2 knockout mice. Endocrinology 139:4008–4011
40. **Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ** 1998 Reduced fertility in female mice lacking copper-zinc superoxide dismutase. J Biol Chem 273:7765–7769
41. **Ho YS, Magnenat JL, Bronson RT, Cao J, Gargano M, Sugawara M, Funk CD** 1997 Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. J Biol Chem 272:16644–16651
42. **Ho YS, Xiong Y, Ma W, Spector A, Ho DS** 2004 Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. J Biol Chem 279:32804–32812
43. **Pretsch W** 1999 Glutathione reductase activity deficiency in homozygoug $Gr1^{a1Neu}$ mice does not cause haemolytic anaemia. Genet Res 73:1–5
44. **Rogers LK, Bates CM, Welty SE, Smith CV** 2006 Diquat induces renal proximal tubule injury in glutathione reductase-deficient mice. Toxicol Appl Pharmacol 217:289–298
45. **Rogers LK, Tamura T, Rogers BJ, Welty SE, Hansen TN, Smith CV** 2004 Analyses of glutathione rductase hypomorphic mice indicate a genetic knockout. Toxicol Sci 82:367–373
46. **Yant LJ, Ran Q, Rao L, Van Remmen H, Shibatani T, Belter JG, Motta L, Richardson A, Prolla TA** 2003 The Selenoprotein GPX4 Is Essential for mouse development and protects from radiation and oxidative damage insults. Free Radic Biol Med 34:496–502
47. **Liang H, Yoo SE, Na R, Walter CA, Richardson A, Ran Q** 2009 Short form glutathione peroxidase 4 is the essential isoform required for survival and somatic mitochondrial functions. J Biol Chem 284:30836–30844
48. **Garry MR, Kavanagh TJ, Faustman EM, Sidhu JS, Liao R, Ware C, Vliet PA, Deeb SS** 2008 Sensitivity of mouse lung fibroblasts heterozygous for GPx4 to oxidative stress. Free Radic Biol Med 44:1075–1087
49. **Kumar TR, Wiseman AL, Kala G, Kala SV, Matzuk MM, Lieberman MW** 2000 Reproductive defects in γ-glutamyl transpeptidase deficient mice. Endocrinology 141:4270–4277
50. **Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chévez-Barrios P, Wang Y, Habib GM, Goodman JC, Huang SL, Lebovitz RM, Matzuk MM** 1996 Growth retardation and cysteine deficiency in γ-glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci USA 93:7923–7926
51. **Will Y, Fishcher KA, Horton RA, Kaetzel RS, Brown MK, Hedstrom O, Lieberman MW, Reed DJ** 2000 γ-Glutamyltranspeptidase-deficient knockout mice as a model to study the relationship between glutathione status, mitochondrial function, and cellular function. Hepatology 32:740–749
52. **Giordano G, White CC, Mohar I, Kavanagh TJ, Cotsta LG** 2007 Glutathione levels modulate domoic acid-induced apoptosis in mouse cerebellar granule cells. Toxicol Sci 100:433–444
53. **Perreault SD, Wolff RA, Zirkin BR** 1984 The role of disulfide bond reduction during mammalian sperm nuclear decondensation *in vivo*. Dev Biol 101:160–167
54. **Rzucidlo SJ, Brackett BG** 2000 Developmental patterns of zygotes from transgenic female mice with elevated tissue glutathione. J Exp Zool 286:173–180
55. **Park SY, Lee EJ, Emge D, Jahn CL, Jameson JL** 2008 A Phenotypic spectrum of sexual development in *Dax1* (*Nr0b1*)-deficient mice: consequence of the C57BL/6J strain on sex determination. Biol Reprod 79:1038–1045
56. **Pan H, Ma P, Zhu W, Schultz RM** 2008 Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. Dev Biol 316:397–407
57. **Ottolenghi C, Uda M, Hamatani T, Crisponi L, Garcia JE, Ko M, Pilia G, Sforza C, Schlessinger D, Forabosco A** 2004 Aging of oocyte, ovary, and human reproduction. Ann NY Acad Sci 1034:117–131
58. **Vogt E, Kirsch-Volders M, Parry J, Eichenlaub-Ritter U** 2008 Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mutat Res 651:14–29
59. **Flurkey K, Brandvain Y, Klebanov S, Austad SN, Miller RA, Yuan R, Harrison DE** 2007 PohnB6F1: a cross of wild and domestic mice that is a new model of extended female reproductive life span. J Gerontol A Biol Sci Med Sci 62A:1187–1198
60. **Lopes FL, Fortier AL, Darricarrère N, Chan D, Arnold DR, Trasler JM** 2009 Reproductive and epigenetic outcomes associated with aging mouse oocytes. Hum Mol Genet 18:2032–2044
61. **Holinka CF, Tseng YC, Finch CE** 1979 Reproductive aging in C57BL/6J mice: plasma progesterone, viable embryos and resorption frequency throughout pregnancy. Biol Reprod 20:1201–1211
62. **Broekmans FJ, Knauff EAH, te Velde ER, Macklon NS, Fauser BC** 2007 Female reproductive ageing: current knowledge and future trends. Trends Endocrinol Metab 18:58–65
63. **Tarín JJ** 1996 Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. Mol Hum Reprod 2:717–724
64. **Nakamura S-I, Kugiyama K, Sugiyama S, Miyamoto S, Koide S-I, Fukushima H, Honda O, Yoshimura M, Ogawa H** 2002 Polymorphism in the 5′-flanking region of the human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. Circulation 105:2968–2973
65. **Nakamura S-I, Sugiyama S, Fujioka D, Kawabata K-I, Ogawa H, Kugiyama K** 2003 Polymorphism in glutamate cysteine ligase modifier subunit gene is associated with impairment of nitric oxide-mediated coronary vasomoter dilation. Circulation 108:1425–1427
66. **Walsh AC, Feulner JA, Reilly A** 2001 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 61:218–223
67. **Walsh AC, Li W, Rosen DR, Lawrence DA** 1996 Genetic mapping of GLCLC, the human gene encoding the catalytic subunit of γ-glutamyl-cysteine synthetase, to chromosome band 6p12 and characterization of a polymorphic trinucleotide repeat within its 5′ untranslated region. Cytogen Cell Gen 75:14–16