Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8787–8791, October 1991
Developmental Biology

Germ-cell deficient (*gcd*), an insertional mutation manifested as infertility in transgenic mice

(primordial germ cells/genetics/premature ovarian failure)

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Communicated by Aaron J. Shatkin, July 11, 1991 (received for review March 6, 1991)

ABSTRACT A genetic analysis is necessary to gain a greater understanding of the complex developmental processes in mammals. Toward this end, an insertional transgenic mouse mutant has been isolated that results in abnormal germ-cell development. This recessive mutation manifests as infertility in both males and females and is specific for the reproductive organs, since all other tissues examined were histologically normal. A developmental analysis of the gonadal tissues demonstrated that the germ cells were specifically depleted as early as day 11.5 of embryonic development, while the various somatic cells were apparently unaffected. Therefore, the mutated locus must play a critical role in the migration/proliferation of primordial germ cells to the genital ridges of developing embryos. In addition, females homozygous for the mutation could potentially be a valuable animal model of a human syndrome, premature ovarian failure. This mutation has been named germ-cell deficient, *gcd*.

Mammalian germ-cell development is well understood at the morphological level (reviewed in ref. 1). Primordial germ cells (PGCs) in the mouse embryo are first recognized histochemically by their high content of alkaline phosphatase at ≈7 days postcoitum (dpc) in the extraembryonic mesoderm (2), and by 8 dpc they have migrated to the caudal end of the primitive streak, allantoic bud, and yolk sac splanchnopleure (3). The PGCs then proliferate mitotically during their migration through the hindgut, dorsal mesentery, and mesenteric root and across the coelomic angles, reaching the gonadal ridges by 11.5–12.5 dpc (3). Finally, at 12.5–13.5 dpc, the presumptive testis differentiates from the presumptive ovary by the formation of testicular cords, which contain the developing germ cells and primitive Sertoli cells.

A genetic analysis is necessary to further advance our understanding of germ-cell development. The spontaneous mutations that have been identified which affect germ cells include the *W* (white) (3), *Sl* (steel) (4), *an* (Hertwig's anemia) (5, 6), and *at* (atrichosis) (7) mouse loci. Unfortunately, these mutations are pleiotropic, eliciting defects not only in germ-cell development but also in hematopoiesis (*an*, *W*, and *Sl*) and hair density (*at*). This therefore restricts their utility in the analysis of germ-cell development.

Transgenic mouse technology (8) has allowed the identification of genes or loci that affect the mature germ cells in two different ways. First, overexpression of foreign genes in the testis can impair spermatogenesis (9, 10). Second, the insertion of foreign DNA will occasionally disrupt an active genomic locus, causing a mutation. Previously reported insertional mutants have all resulted in male infertility, secondary to germ-cell formation, either due to a chromosomal translocation (11) or due to a mutation in a specific gene necessary for normal spermatogenesis (12). Interestingly, one mutation has been reported where hemizygous males do not transmit the transgene to their progeny (13). In contrast to the previously described mutations, the insertional mutation described herein causes a deficiency of primordial germ cells that leads to infertility without apparent pleiotropism.

MATERIALS AND METHODS

Transgenic Mice. Eight transgenic founder mice were generated by microinjection of an 8.0-kilobase (kb) *Xba* I–*Xba* I DNA fragment containing the goat fetal β-globin gene (Fig. 1*a*) (14). The transgenic mouse lines were established by mating the founders to (CBA/J × C57BL/6J)F_1 mice and the progeny were analyzed for inheritance of the transgene by Southern blot analysis (15). Hemizygous mice within each line were mated to produce progeny homozygous for the integration site and genotype determined by quantitative dot blot analysis (Fig. 1*b*) (15). The radiolabeled probe (16) for both Southern and dot blot analysis was the microinjected 8-kb goat fetal β-globin DNA fragment.

Breeding Analysis. Matings were confirmed by the presence of copulatory vaginal plugs, and the time of their appearance was designated as 0.5 dpc. Superovulation of female mice was induced by 5 international units of pregnant mare serum gonadotropin (Gestyl, Diosynth, Holland) i.p., followed 46–48 hr later by 5 international units of human chorionic gonadotropin (Sigma) i.p. Fresh ejaculates were flushed from the uterus of superovulated females with an equal volume of medium (17) 20 min after matings evidenced by copulatory vaginal plugs.

Adult Tissue Histology. Organs were weighed immediately upon dissection and then fixed in neutral buffered formalin or Bouin's fixative. They were dehydrated in a graded ethanol series to 95% ethanol and embedded in JB-4 Plus plastic resin (Polysciences). Sections of 1.5–2.0 μm were stained with epoxy tissue stain (Electron Microscopy Sciences) and differentiated in 0.1% acetic acid solution.

Fetal Gonads. At 13.5 dpc, fetuses were bisected into cranial and caudal halves and the former was used as a source of DNA for genotyping. The gonads with attached mesonephros were dissected from the caudal half of the fetus. Gonadal sex was determined by the presence or absence of testicular cords. The gonads were fixed in periodate/lysine/paraformaldehyde fixative (10 mM $NaIO_4$/37.5 mM sodium phosphate buffer/2% paraformaldehyde, pH 6.2) for 3 hr at

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Abbreviations: dpc, days postcoitum; PGC, primordial germ cell.

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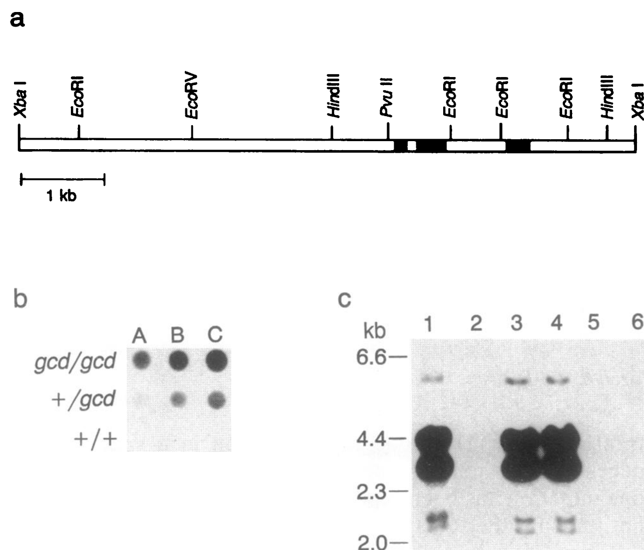

FIG. 1. (*a*) Restriction map of the goat fetal β-globin gene that was microinjected to generate the *gcd* mice. The black boxes represent the exons. (*b*) Quantitative dot blot of 11.5-dpc embryonic DNA hybridized with the above gene. The amount of DNA loaded in dots A, B, and C, respectively, was as follows: for *gcd/gcd*, 1.9, 3.8, and 7.6 μg; for *gcd*/+, 2.7, 5.4, and 10.8 μg; for +/+, 4.7, 9.4, and 18.8 μg. (*c*) Southern blot of *Hin*dIII-digested DNA from the *gcd* founder (lane 1) and four progeny (lanes 3–6) hybridized to the above gene.

5°C and transferred into 0.1 M phosphate buffer/5% sucrose, pH 7.4, at 5°C until dehydration and embedding.

Gonads were processed in plastic and stained either for alkaline phosphatase as described by Fallon (18) or by conventional methods (see above). Tissue blocks were stored at 5°C in the dark until sectioned. Sections were cut at 10 μm, floated onto water droplets on albumin-coated microscope slides, and fixed onto slides by air drying overnight in the dark at room temperature. Slides were then stored in the dark at 5°C and stained for alkaline phosphatase in a flat humid chamber at 37°C for 2–4 hr with 0.2-μm-filtered alkaline phosphatase reagents prepared at 37°C (Sigma). Alkaline phosphatase activity was identified by either red or blue staining, according to the base dye in the staining kit chosen.

Fetuses at 11.5 dpc. Since different fetuses from the same dam can vary in their stage of development, 11.5-dpc fetuses on dissection were classified by morphology of their hind limb buds (17). Only stage 4 or stage 5 fetuses, with the hind limb bud posterior and anterior reentrant (stage 4) or symmetric and with a circular outline (stage 5) were analyzed. These fetuses were bisected into cranial and caudal halves and the former was used as a source of DNA for genotyping. The entire caudal half of the fetus was fixed in periodate/lysine/paraformaldehyde fixative for 3 hr at 5°C, stored in 0.1 M phosphate buffer/5% sucrose at 5°C, and embedded in JB-4 Plus plastic resin. Transverse 8-μm sections were air-dried onto albumin-coated slides and stained for alkaline phosphatase activity as described above. Cross sections of gonadal ridges were analyzed for cross-sectional area (mm^2) and concentration of PGCs (no./mm^2) with the aid of a morphometric analysis system (SMI, Atlanta, GA). This system used a television camera, mounted on a microscope, that fed into a monitor for simultaneous visualization of the microscopic image and the data input from a digitizing pad. Setup parameters used were count, area, and density (counts per unit area).

RESULTS

Seven out of eight transgenic mouse lines harboring the 8-kb goat fetal β-globin gene produced mice that were homozygous for the insertion site and were phenotypically normal. One founder, GF49, transmitted the transgene to its progeny (Fig. 1*c*), but when they were intercrossed, the resulting homozygotes were infertile. Microinjection of DNA into the pronucleus of fertilized mouse eggs can occasionally cause translocations (11) that may lead to an infertile phenotype (19). Therefore, a karyotypic analysis of homozygous mice from line GF49 was performed and revealed no detectable abnormalities, within the sensitivity of cytogenetic analysis (Muriel Davisson and Ellen Akeson, personal communication).

The genotypes of progeny obtained from segregation analysis (negative, hemizygous, and homozygous for the transgene) were 25%, 57%, and 18% of males (n = 100) and 21%, 64%, and 15% of females (n = 144), respectively. These observed frequencies were not significantly different from the expected frequencies ($P < 0.005$ for both males and females). All of the above mice that were homozygous for the insertion exhibited abnormally small gonads whereas hemizygous (n = 63) and wild-type (n = 20) littermates were unaffected, thus demonstrating that the mutation segregates with the transgene.

Infertility was not caused by aberrant sexual behavior, since copulation plugs were observed for both homozygous males and females when mated with their wild-type counterparts. Thirteen matings of GF49 homozygous adult males to wild-type females produced only one pregnancy of two grossly normal embryos (dissected 10.5 dpc). In addition, seven matings of GF49 homozygous females resulted in no offspring.

The initial characterization of the infertile phenotype involved the investigation of the mature eggs and sperm released from the homozygous female and male mice. Sperm counts from fresh ejaculates contained 10% the normal concentration, while epididymal sperm counts were 10–20% of normal (Table 1). However, the spermatozoa appeared normal in motility and morphology (unpublished data). Most importantly, the released sperm were proven to be functional: eight homozygous males were mated with (CBA/J × C57BL/6J)F_1 superovulated females and produced two litters from two different males, containing 23 viable offspring. The genotypes of the sires were confirmed as homozygous since every pup in each litter was shown to be positive for the transgene. Ten 8- to 12-week-old homozygous females were superovulated with exogenous gonadotropins, mated with (CBA/J × C57BL/6J)F_1 males and the oviducts were dissected the following day. No eggs were recovered from homozygous females, whereas 13 normal females averaged 23 eggs each. Eggs have been recovered from younger homozygous females (3–6 weeks of age) superovulated by exogenous gonadotropins; however, the average number of eggs ovulated (5 ± 4, n = 11) by these females was significantly less as compared with their hemizygous and negative littermates (27 ± 6, n = 24; $P \leq 0.001$). In addition, when 18 fertilized eggs from the superovulated homozygous females were transferred to normal foster mothers, four viable offspring, all positive for the transgene, were produced. When eggs from wild-type females were transferred

Table 1. Ejaculatory and epididymal sperm concentrations

	Sperm concentration*	
Genotype	Ejaculate	Epididymis
+/+	7.3 ± 2.2	7.9 ± 3.6
+/*gcd*	6.8 ± 2.3	8.3 ± 3.4
gcd/gcd	0.6 ± 0.1	1.3 ± 1.3

*Mean sperm concentration is expressed as no. of sperm per ml × 10^{-4} (ejaculate; n = 2, 2, and 12 for +/+, +/*gcd*, and *gcd/gcd*) or × 10^{-6} (epididymis; n = 3, 4, and 7 for +/+, +/*gcd*, and *gcd/gcd*).

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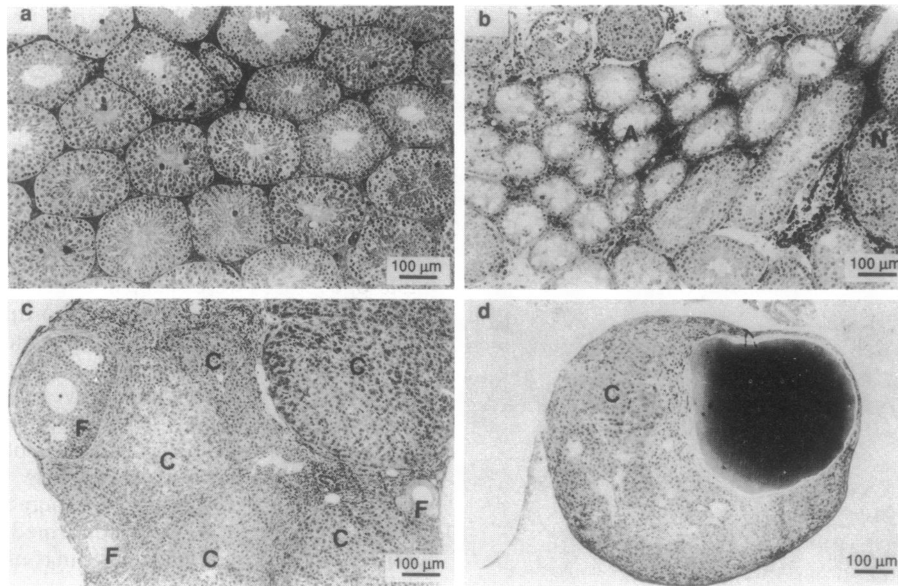

FIG. 2. Adult gonads. Histological sections of male (*a* and *b*) and female (*c* and *d*) gonads are shown, comparing the gonads of +/+ (*a* and *c*) and *gcd/gcd* mice (*b* and *d*). The homozygous *gcd/gcd* testis (b) contained groups of normal (N) and abnormal (A) seminiferous tubules. The abnormal seminiferous tubules contained only large, vacuolated Sertoli cells. The homozygous *gcd/gcd* ovary (*d*) is much smaller than the normal ovary (*c*) and is relatively inactive, containing no developing follicles (F) and only a few, old corpora lutea (C). Also, a well-delineated hemorrhagic cyst is present in the upper-right quadrant of the homozygous ovary (*d*). The number of animals histologically analyzed is as follows: +/+, 8 males and 3 females; +/*gcd*, 8 males and 3 females; *gcd/gcd*, 14 males and 6 females.

into pseudopregnant homozygous females, no pregnancies were produced (unpublished data).

To define the mutation more precisely, gross and histopathological analyses of tissues from homozygous, hemizygous, and negative (with respect to the transgene) adult mice were performed. Tissues from homozygous mice that were normal both in wet weight and in histology as compared to hemizygous and negative mice included skin, blood, bone marrow, spleen, lymph nodes, thymus, brain, liver, kidneys, lungs, heart, adrenals, thyroid, pancreas, stomach, and intestine. The homozygous male accessory sex glands—comprising the seminal vesicles, coagulating glands, prostate, bulbourethral glands, and preputial glands—were also normal in wet weight and histological appearance. However, testicular and epididymal masses were one-third normal (unpublished data). Histological examination of the homozygous testis revealed that 30–50% of the seminiferous tubules were normal with sperm in all stages of development and a full complement of accessory cells. The remaining tubules were devoid of all but Sertoli cells appearing as syncytial

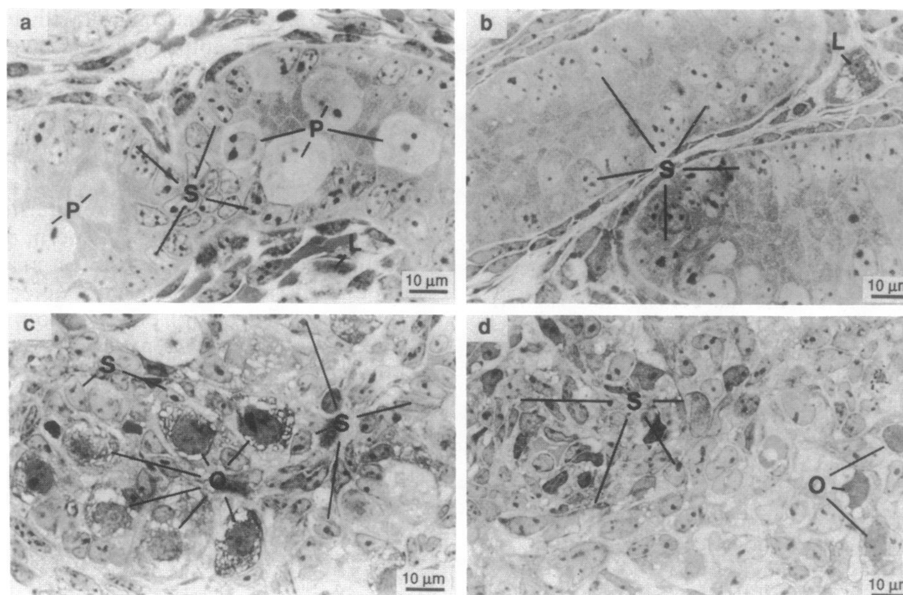

FIG. 3. Gonads of newborn mice. Histological sections of male (*a* and *b*) and female (*c* and *d*) newborn gonads are shown, comparing the gonads of +/+ (*a* and *c*) and *gcd/gcd* (*b* and *d*) newborn mice. The sex cords (seminiferous tubules) of a normal testis (*a*) contained large prospermatogonia (P) along with smaller supporting cells (S) situated peripherally along the basement membrane. In contrast, homozygous *gcd/gcd* testis (*b*) contained both normal and abnormal seminiferous tubules, with the abnormal tubules containing supporting cells only. Similar to the homozygous newborn testis, the homozygous newborn ovary (*d*) contained mostly supporting cells (S) and fewer oogonia (O) than normal (c). The number of newborns analyzed is as follows: +/+, 2 males and 8 females; +/*gcd*, 7 males and 18 females; *gcd/gcd*, 4 males and 8 females.

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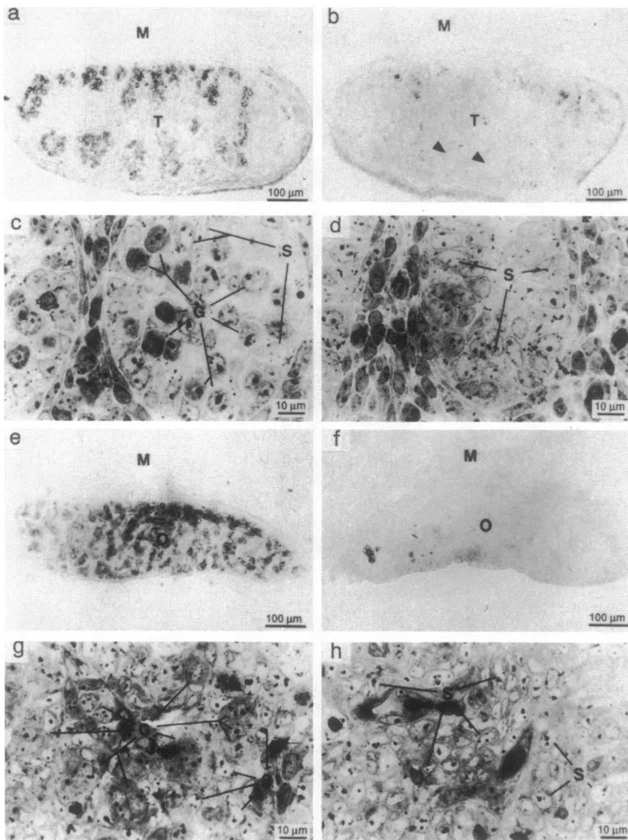

FIG. 4. Gonads of 13.5-dpc fetuses. Histological sections of male (*a–d*) and female (*e–h*) fetal gonads are shown, comparing the gonads of +/+ fetuses (*a, c, e,* and *g*) with the abnormal gonads of *gcd/gcd* fetuses (*b, d, f,* and *h*). Gonads were stained with epoxy tissue stain (*c, d, g,* and *h*) or for alkaline phosphatase activity (*a, b, e,* and *f*). Many testicular cords of the 13.5-dpc *gcd/gcd* testis (T) were deficient in alkaline phosphatase-positive cells (germ cells) (*b*, arrowheads point to deficient testicular cords). The deficiency of germ cells was confirmed by conventional staining (*d*) where many primordial tubules contained supporting cells (S) but lacked germ cells (G). The analogous situation existed in fetal ovaries (O): *gcd/gcd* ovaries lacked germ cells (G), identified both by alkaline phosphatase activity and by conventional staining, but had a normal complement of supporting cells (S). M, mesonephros. The number of 13.5-dpc fetuses analyzed is as follows: +/+, 7 males and 5 females; +/*gcd*, 20 males and 19 females; *gcd/gcd*, 2 males and 15 females.

masses of vacuolated cells (Fig. 2). Interstitial Leydig cells appeared to be present in normal concentration and appearance.

Similarly, all homozygous female tissues examined were normal except for the ovaries, uterus, cervix, and vagina, which were reduced in mass to 10–20% of wild type (unpublished data). The entire homozygous female reproductive tract was pale, thin, and "threadlike" with a castrate appearance. Gross examination showed that the ovaries had no developing follicles and few corpora lutea while, frequently, solitary cysts were present on the ovaries, some of which were hemorrhagic (Fig. 2). Histological analysis of 2.5- to 5-month-old homozygous ovaries revealed a significant absence of developing follicles and only a few corpora lutea. In the homozygous uterus, the thickness of the muscularis externa was markedly reduced and the epithelium appeared to be low in profile and "inactive" (unpublished data).

Since the infertile phenotype seemed to be caused by a deficiency of mature germ cells rather than a behavioral defect, an analysis of germ-cell development was undertaken. When the gonads from newborn mice were dissected, their morphology correlated with the pattern seen in adults (Fig. 3). In the homozygous newborn testis, the majority of seminiferous tubules lacked prospermatogonia but had the normal complement of supporting (Sertoli) cells. Homozygous newborn ovaries were deficient in oogonia but also had the normal complement of supporting cells. These results suggested that the deficiency of germ cells occurred prenatally, and therefore the developmental analysis was continued.

At 13.5 dpc, germ cells are organized within testicular cords in the presumptive testis while they are randomly distributed throughout the presumptive ovary. Gonads of 13.5-dpc fetuses were sexed and then fixed for histochemical analysis. PGCs between 7 and 13.5 dpc can be histochemically identified by their high endogenous alkaline phosphatase activity (2, 20). Therefore, histological sections of 13.5-dpc fetal gonads were stained, revealing a stark deficiency of positively stained cells in homozygotes of both sexes (Fig. 4). Since it was possible that the lack of positive cells might reflect a deficiency of the enzyme alkaline phosphatase without a real decrease in PGC number, the deficiency of PGCs in homozygotes was confirmed by identifying PGCs strictly on a morphological basis in routinely stained sections (Fig. 4).

Fetuses at 11.5 dpc were selected next for analysis because by that stage the majority of PGCs have completed their migration from their extragonadal origins into the gonadal ridges. PGC concentrations (number per unit cross-sectional area) in gonadal ridges at 11.5 dpc were determined on histological sections stained for alkaline phosphatase activity (Table 2). A reduction in PGC concentration in the gonadal ridges was observed in homozygous fetuses when compared with their negative or hemizygous littermates. There was no significant difference in mean cross-sectional area of the gonadal ridges between genotypes. Also, there were no discernible differences among genotypes in the number or location of the occasionally observed extragonadal PGCs (unpublished data).

DISCUSSION

The experimental results demonstrate that a transgenic mouse harboring 10–15 copies of a goat fetal β-globin gene gave rise to an insertional mutant with an infertile phenotype. The mutation was autosomal recessive with no phenotypic differences observed between wild-type and hemizygous littermates. It affected both sexes and led to a vastly reduced number of mature germ cells in the homozygous adults. However, the remaining germ cells were functional in that viable offspring could be derived from homozygous animals. Therefore, this suggests that the infertility is directly due to a lack of germ cells and not to sperm or egg dysfunction.

Although the defect was manifest in adult mice, it became clear that the mutation caused a defect during PGC development. A deficiency of PGCs at both 11.5 and 13.5 dpc in homozygous fetuses was apparent. At 11.5 dpc, PGC concentration in homozygotes was 25–40% of that in wild-type or hemizygous (+/*gcd*) mice, with no significant difference in gonadal size.

Table 2. Gonadal ridge area and PGC concentration at 11.5 dpc

Genotype	Cross-sectional area, $mm^2 \times 10^{-3}$	PGCs, no. per mm^2
+/+	34.5 ± 11.2	1249 ± 436
+/*gcd*	24.4 ± 18.1	1839 ± 464
gcd/gcd	20.3 ± 16.1	485 ± 205

Number of 11.5-dpc fetuses used was 2, 4, and 5 for +/+, +/*gcd*, and *gcd/gcd*, respectively. The cross-sectional areas of the 11.5-dpc genital ridge were not significantly different between genotypes. Homozygous PGC concentrations were significantly lower than negative or hemizygous concentrations by t test ($P < 0.05$).

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Adult homozygous males compensate for the initial deficiency of germ cells because sperm are produced from a stem-cell population (spermatogonia). Thus, their testes had some normal seminiferous tubules containing Sertoli cells and sperm in all stages of development. However, most seminiferous tubules were abnormal, containing only syncytial masses of vacuolated Sertoli cells. These morphological alterations of Sertoli cells are common in germ-cell-depleted seminiferous epithelium (21, 22). Therefore, the oligospermia observed in homozygous males was probably a result of the overall reduction in functional seminiferous epithelium. However, adult females are unable to form a stem-cell population and thus *gcd/gcd* females are more severely affected than are *gcd/gcd* males. Most oocytes present at birth undergo atresia and only a small proportion reach maturity and ovulation (23). Therefore, the mutant females have an initial limited follicular stock, which presumably is quickly eliminated by atresia. It is not surprising that adult homozygous females cannot maintain a pregnancy to term, since there is a hormonal imbalance (see below) probably due to the reduced amount of ovulation. Since the infertility of line GF49 is due to a lack of germ cells, this mutation is designated germ-cell deficient (*gcd*).

Oocyte depletion has been hypothesized to be the primary cause of reproductive senescence in female mammals (24), with complete sterility occurring by ≈400 days of age in mice (23). In *gcd/gcd* mice, the ovaries exhibit histological changes typical of old age (23), including reduced ovarian mass, a lack of follicular development, and the appearance of cysts at ≈2 months. Additionally, *gcd/gcd* females have elevated levels of follicle-stimulating hormone (unpublished data). This is reminiscent of the phenotype of the human disease premature ovarian failure, in which females as young as 11 years can exhibit premature menopause associated with elevated gonadotropins (25). This disease is known to have a genetic component and one of the proposed etiologies is a dearth of germ cells reaching the genital ridge during embryonic development (26).

The *gcd* mutation is unique in that it causes infertility in both sexes due to only a specific disruption of germ-cell development. This is in contrast to the phenotype of the other known spontaneous or insertional mutants that exhibit infertility of only one sex or are associated with other physiological defects. Complementation analysis has demonstrated that *gcd* is not allelic with *an, at, W*, or *Sl* (unpublished data). Interestingly, another spontaneous mutant, *ter* (teratoma; ref. 27) is classified as germ-cell deficient for both sexes; however, only males are sterile.

Further studies are needed to determine whether the germ-cell deficiency arises due to migration or proliferation of the germ cells. Finally, because the mutant cosegregates with the transgene, it should be possible to clone out the disrupted locus to ultimately define the role of the *gcd* gene product in germ-cell development.

We are grateful to Richard Nowakowski for use of the morphometric analysis system and to Muriel T. Davisson and Ellen C. Akeson for karyotypic analysis. This work was supported by a National Institutes of Health grant (GM38731), a Basil O'Connor Starter Scholarship (Colonel Sanders Memorial Fund) (K.C.), a New Jersey Cancer Commission research grant (687-006), a Center for Advanced Biotechnology and Medicine and New Jersey Commission on Science and Technology grant (89-240350-1), a Cooley's Anemia Foundation fellowship (T.C.P.) and a March of Dimes predoctoral graduate research training fellowship (18-89-36) (S.S.P.).

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