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Normal Gonadal Development in Mice Lacking GPBOX, a Homeobox Protein Expressed in Germ Cells at the Onset of Sexual Dimorphism

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***Gpbox* is a paired-like homeobox gene that colocalizes with two other members of the family, *PsxI* and *Pem*, on the proximal portion of the mouse X chromosome. *Gpbox* is expressed in the extraembryonic placenta and within the germ cells of the embryonic gonad. Beginning with the onset of sexual dimorphism (embryonic day [E]11.5 to 12.5), GPBOX transcripts accumulate faster in female than in male germ cells but disappear later in embryogenesis (E16) and have not been reported in adult tissues. To investigate the function of *Gpbox*, mouse cell lines lacking GPBOX were established using targeted mutagenesis in embryonic stem cells. Both homozygous *Gpbox* null female and hemizygous *Gpbox* null male mice were fertile and reproduced normally. Additionally, the development of male and female gonads in the null background was indistinguishable from that observed in normal littermates. The lack of an obvious phenotype raises the possibility that another member of this homeobox gene family provides the absent *Gpbox* function.**

Mouse gestation takes place over 19 days, and gender differences are normally observed in germ cells at embryonic day 13.5 (E13.5) when female (XX) cells begin to enter into the first meiotic prophase and male (XY) cells begin to mitotically arrest (1). Regardless of chromosomal sex, germ cells which populate testicular and ovarian primordia will undergo prenatal sexual differentiation toward spermatogenesis and oogenesis, respectively. The testicular environment prevents germ cells from entering into meiosis (a hallmark of oocyte development), but ectopically located germ cells, whether XX or XY, will enter into meiosis. Thus, it appears that germ cells will follow a constitutively female pathway unless diverted by the testicular environment (10, 19). However, the demise of ectopic or genetically altered female germ cells unable to establish primordial follicles with ovarian granulosa cells emphasizes the importance of somatic cell-germ cell interactions after birth (17, 23).

The expression patterns of relatively few genes have been investigated at the onset of oogenesis. Examples include *Zfx*, which encodes a zinc finger protein expressed in male and female germ cells (as well as elsewhere in the embryo). Although males and females remain fertile after the inactivation of the gene by targeted mutagenesis, there are decreased numbers of germ cells at E11.5 and female mice have a shortened reproductive life span (9). *Oct4* encodes a POU homeodomain transcription factor that is expressed in the blastocyst and appears to be essential for the pluripotentiality of the inner cell mass of the blastocyst (13). At E12.5 to 13.5, the OCT4 protein is present in both male and female germ cells but is later down regulated in oocytes. Mice lacking *Oct4* do not develop to the egg cylinder stage (12), and further analysis of its role in germ cells will require conditional mutants. Finally, *Figα*, an oocyte-specific basic helix-loop-helix transcription factor gene that is first expressed at E13, directs at least two oocyte-specific genetic pathways that result in the perinatal formation of primordial follicles and the expression of the zona pellucida genes (17).

To identify additional genes expressed early in germ cell development, the expression pattern of 3′-end-expressed sequence tags from sex-specific, E12 to 13 urogenital ridge RNA was determined. Transcripts of a gene, designated *Gpbox* (*germline-placenta-homeobox*), were detected in the placenta and germ cells but not elsewhere in the embryo or in adult tissues (20). *Gpbox* encodes a 227-amino-acid homeobox protein that is present in germ cells at the onset of sexual dimorphism. The single-copy gene is located on the X chromosome in close proximity to two other homeobox-encoding genes, *Pem* and *PsxI* (18, 20). The protein encoded by the latter gene is of the same length (6), has 87% identity in the homeodomain, and is expressed concurrently with *Gpbox* within germ cells during embryogenesis (20). Mice lacking PEM protein are fertile and have a normal phenotype (14). The consequence of the absence of either the PSXI or GPBOX function has not been reported.

MATERIALS AND METHODS

Isolation of GPBOX genomic DNA and construction of the targeting vector. Bacteriophage (1.8×10^6) of a lambda 129/Sv mouse genomic library (Stratagene, La Jolla, Calif.) were screened by plaque hybridization (16) using ^{32}P-labeled mouse *Gpbox* cDNA (20). Six positive phage clones were isolated, and the identities of four were confirmed using oligonuculeotide primers specific to *Gpbox* introns (20). The 24-kbp insert of one was subcloned into Bluescript KS, its identity was confirmed by nucleic acid sequencing, and it was used to construct a targeting vector in pPNT (21). A 2.3-kbp *Bam*HI fragment from the 5′ flank region of *Gpbox* was cloned between the phosphoglycerate kinase (PGK)-Neo^r

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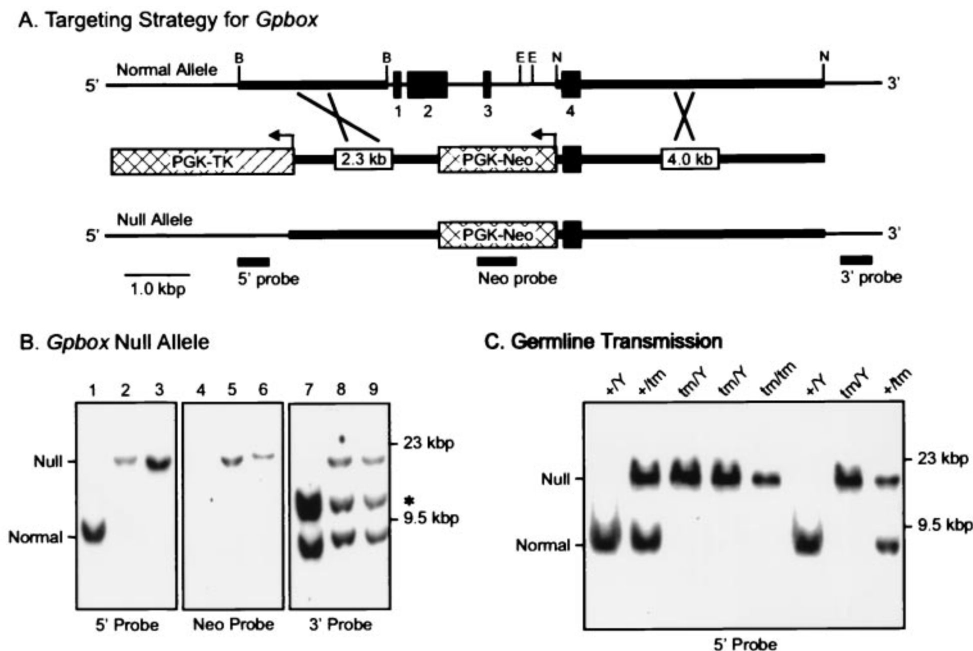

FIG. 1. Generation of mouse cell lines lacking GPBOX. (A) The top diagram is a schematic representation of the normal *Gpbox* allele with four exons; the middle diagram shows the targeting construct with PGK-Neo^r and PGK-thymidine kinase (PGK-TK) as positive and negative selectable markers, respectively, which was designed to delete the 2.2 kbp of *Gpbox* that included the putative translational start site and the first three exons. Homologous regions included 2.3 kbp in the promoter and 4.0 kbp that encompassed exon 4 and the region 3′ to the gene. The bottom diagram shows the *Gpbox* allele mutated by homologous recombination. The boxes with Arabic numbers below represent exons, thick horizontal lines indicate the extent of the homologous DNA, and the positions of the 5′ probe, the 3′ probe, and the Neo^r probe are indicated under the null allele. Restriction enzyme cut sites are indicated by E (*Eco*RI), B (*Bam*HI), and N (*Nhe*I). Arrows indicate the direction of transcription of the two selectable markers. (B) Genotyping of ES cells by Southern blot analysis of purified DNA hybridized with ^{32}P-labeled 5′ (left), Neo^r (middle), and 3′ (right) probes. After digestion with *Eco*RI, the normal and mutant alleles detected with the 5′ probe had restriction enzyme fragments of 7.8 and 20 kbp, respectively, and the normal and mutant alleles detected with either the 3′ or the Neo^r probe had restriction enzyme fragments of 10 and 20 kbp, respectively. Lanes 2, 5, and 8 and 3, 6, and 9 show DNAs from two targeted RI ES cell lines, and lanes 1, 4, and 7 show DNAs from normal RI cells. The ES cell line has an XY genotype, and the single-copy *Gpbox* is located on the X chromosome. *, comigrating *PsxI* and *Gpbox* fragments. (C) Genotyping by Southern blot analysis of DNA purified from tails of F_2 mice generated from a $Gpbox^{+/tm} \times Gpbox^{tm/Y}$ cross after restriction digestion with *Eco*RI and hybridization with the ^{32}P-labeled 5′ probe. Heterozygous (+/tm) and homozygous (tm/tm) female and normal (+/Y) and hemizygous (tm/Y) male *Gpbox* null genotypes were present in the expected Mendelian ratios of a single-copy mutant gene.

and PGK-thymidine kinase cassettes. A 4.0-kbp *Nhe*I fragment was cloned into the pSE380 Superlinker vector (Invitrogen), excised with *Sal*I and *Xho*I, and cloned into the *Xho*I site of the pPNT vector upstream of PGK-Neo^r.

The plasmid was linearized by digestion with *Not*I and electroporated into RI embryonic stem (ES) cells (11) which had been cultured on neomycin-resistant fibroblasts. After double-drug selection with G418 (Gibco) and ganciclovir (Roche Discovery), individual colonies were analyzed by Southern analysis of genomic DNA using ^{32}P-labeled 5′ (0.7-kbp *Pst*I fragment), 3′ (0.5-kbp *Xho*I-*Not*I fragment), and Neo^r fragment (0.6-kbp *Pst*I fragment isolated from PGK-Neo^r) probes. After digestion with *Eco*RI, the 5′ and 3′ probes detected 7.8- and 10-kbp fragments, respectively, from the normal allele and an ~20-kbp fragment from the null allele. Targeted ES cells were microinjected into C57BL/6 blastocysts to produce chimeric mice which were mated with CF-1 mice and bred to produce homozygous null females and hemizygous null males (15). Comparisons of null mutant litter sizes to normal litter sizes were performed by unpaired *t* test using InStat version 3.02 for Windows 95 (GraphPad Software, San Diego, Calif.). All experiments using mice were conducted under protocols approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases-National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

RNase protection assay and RT-PCR. Total RNA was isolated from urogenital ridges or extraembryonic placentas using RNazol B (Cinna/Biotex Laboratories). The RNase protection assay and the reverse transcription (RT)-PCR were performed as previously described (20). The GPBOX probe (nucleotides [nt] 619 to 829 of the 880-nt transcript) for the RNase protection assay includes the region encoded by exon 3 and portions of exons 2 and 4. The 5′ primer for RT-PCR spans the junction between exons 2 and 3, and the 3′ primer is in exon 4 (nt 619 to 638 and nt 816 to 835 of the transcript, respectively).

Histology. Mullerian structures and gonads were dissected, placed in phosphate-buffered saline, and photographed immediately under a dissecting microscope. Gonads isolated from mice were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight, rinsed in the same buffer without fixative, and transferred to 70% ethanol. Tissues were dehydrated and embedded in methacrylate, and 2-μm-thick sections were cut from them (American Histolabs). Mounted sections were stained with periodic acid-Schiff reagent and hematoxylin prior to photomicroscopy.

RESULTS

Establishment of GPBOX-deficient mice. The targeting vector was designed to remove the first three exons of *Gpbox*, which included the translational start site and much of the homeodomain (Fig. 1A). After linearization, the targeting plasmid was electroporated into R1 ES cells and potentially targeted cells were identified by positive-negative selection using G418 and ganciclovir. *Gpbox* is a single-copy gene located on the X chromosome, and the XY genotype of the ES cells ensures a single allele. The gene was highly homologous to *PsxI* over 12.5 kbp, including 2 kbp 5′ to the translational start site

TABLE 1. Litter size of *Gpbox* null mice

Male	Litter size[a] produced in crosses with females with the following genotype:		
	Normal (*Gpbox*$^{+/+}$)	Heterozygous (*Gpbox*$^{+/tm}$)	Homozygous (*Gpbox*$^{tm/tm}$)
Normal (*Gpbox*$^{+/Y}$)	8.8 ± 2.2 (5)	10.2 ± 0.6 (24)	6.5 ± 0.6 (21)
Hemizygous (*Gpbox*$^{tm/Y}$)	7.2 ± 0.8 (19)	8.7 ± 0.8 (24)	7.5 ± 1.5 (13)

[a] Average litter size ± standard error of the mean (total number of litters of at least five mating pairs).

and extending 8 kbp 3′ to the last exon. Therefore, to accurately discriminate between the two genes, a 0.7-kb region, specific to and 5′ to the coding region of *Gpbox*, was used as a probe to screen genomic DNA from ES cells (Fig. 1B). The normal (Fig. 1B, lane 1) and targeted (Fig. 1B, lanes 2 and 3) *Gpbox* alleles were detected as single 7.8 and 20-kbp bands, respectively, after digestion with *Eco*RI. The presence of the null allele was confirmed using a Neo^r probe (Fig. 1B, lanes 5 and 6).

Because of cross-hybridization with related homeodomain genes, the pattern obtained with the 3′ probe (0.5 kbp) was more complex. Cross-hybridizing *PsxI* and *Gpbox EcoRI* fragments (10 kbp) comigrated in normal ES cells (Fig. 1B, lane 7). As expected, in the targeted ES cell lines, the *Gpbox* null allele was present as a 20-kbp fragment (Fig. 1B, lanes 8 and 9). The persistence of the 10-kbp fragment in the targeted lines represented the nontargeted *PsxI* gene and was consistent with the continued expression of *PsxI* in the *Gpbox* null mice (see below). The origin of the 8-kbp band detected with the 3′ probe is not predicted by known restriction sites of either *Gpbox* or *PsxI* and most likely reflects cross-hybridization with *Pem* (or with another closely homologous gene).

Two independently targeted ES cell lines were injected into C57BL/6 host blastocysts, and the resulting chimeric male mice were bred to CF-1 females. Mice in which the null mutation was passed through the germ line were bred to obtain hemizygous null males (tm/Y), heterozygous null females (+/tm), and homozygous null females (tm/tm) (Fig. 1C). Mice of all genotypes had normal fertility and produced litters, the average size of which was statistically indistinguishable from that of normal mice when litter sizes were compared using an unpaired t test ($P > 0.1$) (Table 1). The ratios of genotypes obtained from mating null and normal mice with one another followed the predicted Mendelian frequencies (data not shown).

Normal gonadogenesis in the absence of *Gpbox* expression. Using an RNase protection assay (Fig. 2A), GPBOX transcripts were not detected at E12.5 either in the placentas or the urogenital ridges of the *Gpbox* null mice. The lower band in the urogenital ridge was observed intermittently and may represent an aberrant expression of exon 4, only 151 nt of which could be detected by the probe. In both tissues, PSXI mRNA was detected and did not appear to differ significantly from that of normal mice. Thus, despite the high degree of similarity of the *Gpbox* and *PsxI* genes, the targeting strategy was specific to the *Gpbox* locus. To confirm the absence of GPBOX tran-

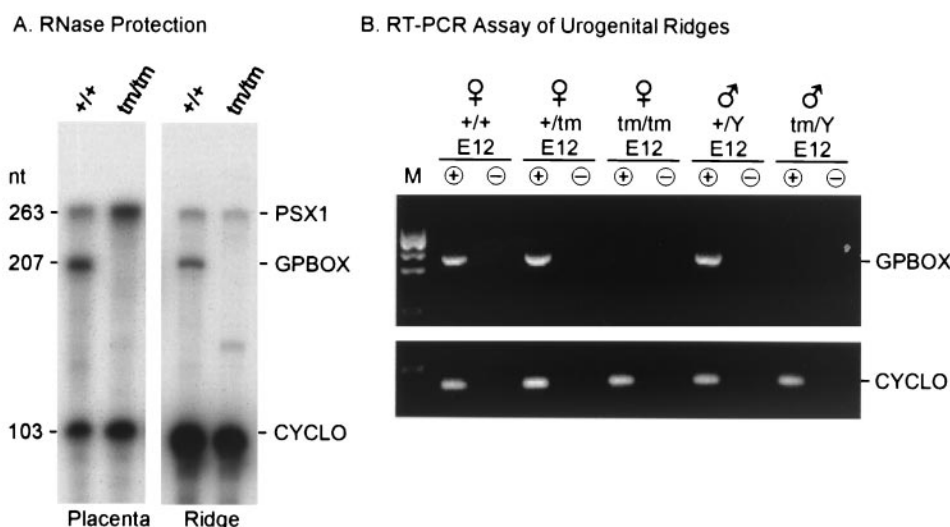

FIG. 2. Expression of *Gpbox* in null mice. (A) RNase protection analysis was performed using total RNA (3 to 10 μg) isolated from E12.5 normal and *Gpbox* null placentas (left panel) and urogenital ridges (right panel). The RNA samples were hybridized to ^{32}P-labeled GPBOX and PSX1 antisense riboprobes, and a CYCLOPHILIN (CYCLO) probe served as a load control. The protected PSXI (263-nt), GPBOX (207-nt), and CYCLOPHILIN (103-nt) fragments were detected by autoradiography after RNase A or T_1 digestion. (B) RT-PCR analysis was performed using total RNA (1 μg) isolated from E12 normal mice and mice with *Gpbox* null urogenital ridges in the presence (+) or absence (−) of reverse transcriptase. GPBOX transcripts (217-bp PCR product) were detected in normal male (+/Y) and female (+/+) and heterozygous female (+/tm) urogenital ridges but not in homozygous null female (tm/tm) or hemizygous null male (tm/Y) urogenital ridges. CYCLOPHILIN transcripts (93-bp PCR product, positive control) were present in both normal and mutant ridges. Lane M, *Hae*III digest of ϕ/174, used for molecular weight markers.

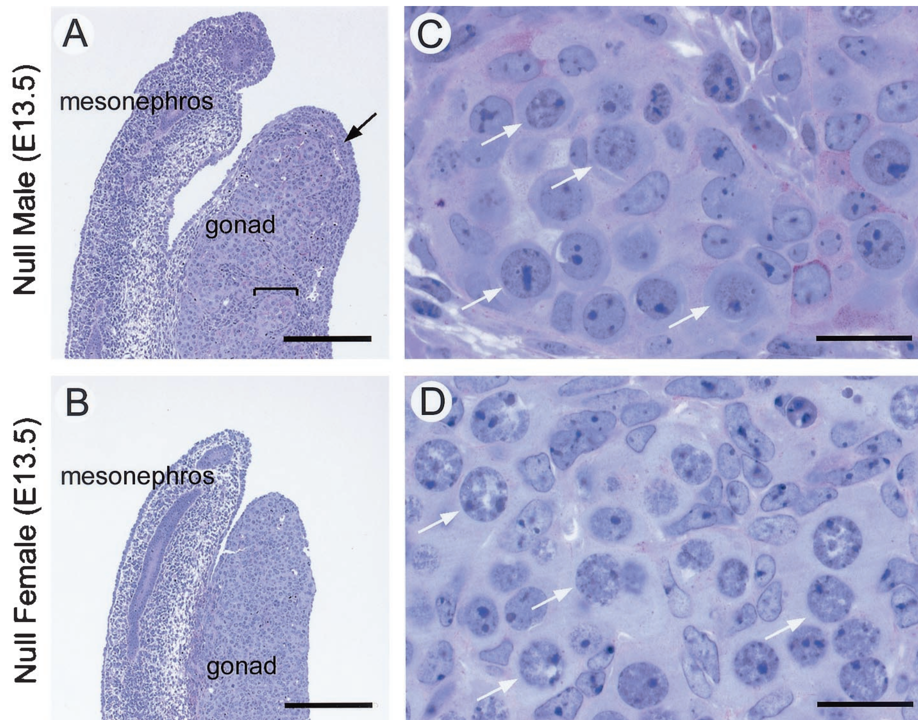

FIG. 3. Histology of developing gonads. Gonads dissected from *Gpbox* null male (tm/Y) and female (tm/tm) mice at E13.5 were fixed, embedded in plastic, and stained with periodic acid-Schiffs' reagent and hematoxylin. Photomicrographs were obtained with 5× (A and B) and 63× (C and D) lens objectives. Mesonephroi and gonads are labeled (A and B). The dark arrow indicates the tunica albuginea at the surface of the testes (not present in the ovary), and the bracket marks a testicular cord (A). Representative germ cells in the male (C) and female (D) gonads are indicated with light arrows. Scale bars, 1.0 mm (A and B) and 0.1 mm (C and D).

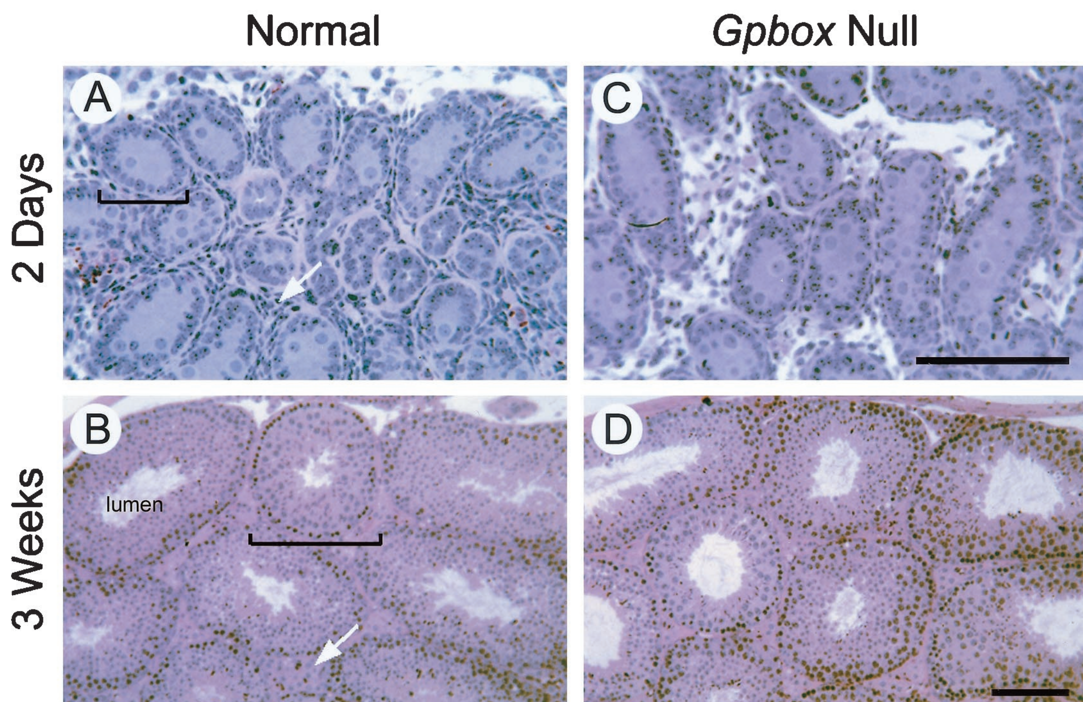

FIG. 4. Testicular histology. Gonads were dissected from normal (A and B) and hemizygous (tm/Y) *Gpbox* null (C and D) males at 2 days (A and C) and at 3 weeks (B and D) after birth. Brackets indicate testicular cords in which germ cells (large cells with prominent nuclei) were separated from somatic Sertoli cells at the periphery. The resumption of germ cell mitosis and the proliferation of Sertoli cells after the morphology of the cords, which by 3 weeks after birth develop lumens (B) in their centers that contain mature but immotile spermatozoa. The interstitium (light arrows) contained Leydig and peritubular myoid cells surrounding seminiferous tubules. Scale bars, 0.2 mm.

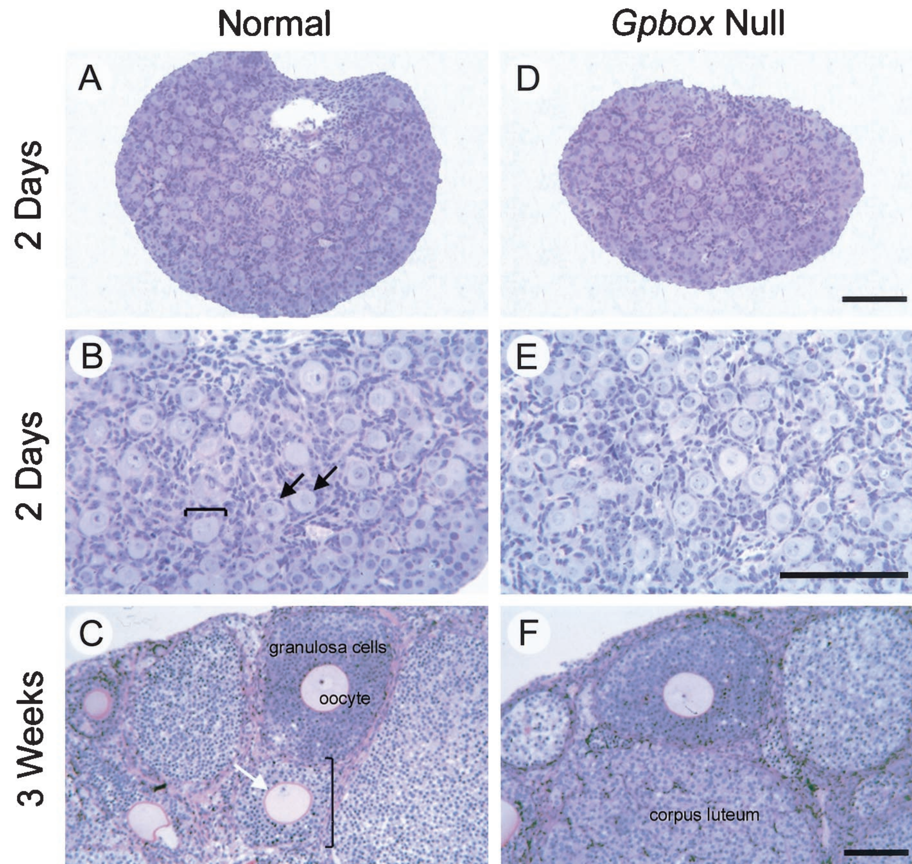

FIG. 5. Ovarian histology. Gonads were dissected from normal (A to C) and homozygous (tm/tm) *Gpbox* null (D to F) females at 2 days (A, B, D, and E) and at 3 weeks (C and F) after birth. Primordial follicles (bracket) with resting oocytes (dark arrows) were present in normal and null mice 2 days after birth (B). By 3 weeks after birth (C and F), the ovaries of both normal and null mice contained growing follicles (bracket) in which oocytes were surrounded by a zona pellucida matrix and layers of proliferating granulosa cells. Occasional corpora lutea (F) were observed in both normal and *Gpbox* null ovaries. Scale bars, 0.2 mm.

scripts in the null mice, total RNA from E12 urogenital ridges was analyzed by RT-PCR, using the absence of reverse transcriptase as a negative control. Using primers in the second and fourth exons, GPBOX transcripts were not detected in hemizygous null males or homozygous null females but were present in heterozygous and normal mice (Fig. 2B).

Urogenital ridges (gonad and mesonephros) were isolated from embryos after timed pregnancies, and gestational age was determined morphologically. At E13.5, both hemizygous null males and homozygous null females had normal histology (Fig. 3). At this period of development, the mesonephroi and the gonad are linked. The male gonad, with the the tunica albuginea at its periphery, normally contains testicular cords in which clusters of large, prominent germ cells are surrounded by somatic cells (Fig. 3A and C). In the female, no cords are visible (Fig. 3B and D) and the appearance of the gonad under low magnification is similar to that observed earlier in the development of either sex (e.g., E11).

Male gonads were also examined 2 days postpartum and at 3 weeks of age (Fig. 4). Normal and *Gpbox* null testes were roughly the same size and weight and had normal histology. Testicular cords in which germ cells (large cells with prominent nuclei) were separated by somatic Sertoli cells at the periphery were prominent 2 days after birth. The resumption of germ cell mitosis (~7 days after birth) and the proliferation of Sertoli cells dramatically altered the morphology of the cords. By 3 weeks of age, lumens in the cell centers contained mature but immotile spermatazoa. The interstitium contained Leydig and peritubular myoid cells surrounding the seminiferous tubules. Similarly, female gonads were examined 2 days postpartum and at 3 weeks of age. Mullerian structures (oviducts, uteri, and upper vagina) in normal and *Gpbox* null mice were roughly the same size (data not shown), and ovaries from normal and mutant mice had normal histology (Fig. 5). Primordial follicles and resting oocytes were prominent at 2 days after birth. By 3 weeks of age, the ovaries were filled with growing follicles containing central germ cells encased in a zona pellucida and surrounded with layers of granulosa cells. Occasional corpora lutea were observed in both normal and homozygous *Gpbox* null ovaries, which presumably reflect sporadic oocyte loss in advanced follicles or episodic prepubertal ovulation.

DISCUSSION

Homeobox genes encode transcription factors that regulate organogenesis and axis formation during development. These

factors contain a 60-amino-acid DNA-binding motif termed a homeodomain that forms a helix-helix-turn-helix tertiary structure and is conserved among metazoans. There are distinct subclasses (4), and the structure of GPBOX most closely resembles that of a paired-like homeobox protein. Although the sequence of the GPBOX homeodomain has diverged significantly from those of the other members of the paired-like family, the positions of two introns interrupting its homeodomain are maintained (20). The observation that the mice lacking GPBOX protein had no obvious gonadal dysfunction and reproduced normally suggests that another homeobox protein provides a comparable function during development.

The colocalization of three homeodomain genes, *Gpbox, PsxI*, and *Pem*, in the proximal portion of the X chromosome (2, 7, 20) suggests that they arose from gene duplication. Both the GPBOX and PSXI proteins are composed of 227 amino acids (82% identity), and although PEM is less well conserved (210 amino acids, 31% identity), all three genes are expressed in the developing gonads as well as in the placenta (5–8, 20, 22). *Pem* is expressed in migrating germ cells at E8.5, and transcripts persist in males and females until E14 and are present in somatic cells of the gonad postnatally. The observation that *Pem* null male and female mice have intact gonads, normal reproduction, and no discernable abnormalities (14) suggests possible compensation by other homeodomain proteins or the presence of sufficient residual PEM protein for biological activity (3).

However, *PsxI* seems a more likely candidate than *Pem* to replace *Gpbox* function. *Gpbox* and *PsxI* have similar developmental patterns of expression, and as noted above, they encode proteins that are highly homologous (5, 20). The disruption of *Gpbox* expression in homozygous null females and hemizygous null male mice does not affect the expression of *PsxI*, which is also expressed in the placenta and in germ cells within the urogenital ridge. While it would be of interest to observe the phenotype of mice lacking both GPBOX and PSXI, the close proximity of the two genes on the X chromosome precludes crossing mice with differing individual null mutations. A better strategy may be to genetically engineer ES cell lines with disruption of the two loci and use them to establish de novo mouse lines for analysis of the double *Gpbox*/*PsxI* null phenotype.

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