A novel gene, Pog, is necessary for primordial germ cell proliferation in the mouse and underlies the germ cell deficient mutation, gcd

Alexander I. Agoulnik1,†, Baisong Lu1,†, Qichao Zhu1, Cavatina Truong1, Maria T. Ty1, Nelson Arango3, Kiran K. Chada4 and Colin E. Bishop1,2,*

1Department of Obstetrics and Gynecology and 2Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA, 3University of Texas, MD Anderson Cancer Center, Houston, TX, USA and 4University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA

Received July 18, 2002; Revised and Accepted September 13, 2002 DDBL/EMBL/GenBank accession nos. AF513619 and AF513620

Primordial germ cells (PGCs) are the precursor of the germ cells in adult gonads. They arise extra-gonadally and migrate through somatic tissues to the presumptive genital ridges, where they proliferate and differentiate into oogonia or spermatagonia cells. Abnormalities in this developmental process can cause embryonic depletion of germ cells leading to infertility in the adult. We report here that the mouse gcd (germ cell deficient) mutant phenotype, characterized by reduced numbers of PGCs and adult sterility, is due to reduced PGC proliferation rather than aberrant migration and is caused by the partial deletion of a single novel gene, Pog (proliferation of germ cells). Pog is critical for normal PGC proliferation, starting between 9.5 and 10.25 dpc when germ cells begin to migrate to the developing genital ridge. Deletion of Pog is also accompanied by reduced embryonic body weight and, on some genetic backgrounds, embryonic lethality. Thus, in addition to being necessary for PGC proliferation, Pog may have a wider significance in early embryonic development.

INTRODUCTION

Primordial germ cells (PGCs) are the founder cell population of the gametes. In the mouse they are distinguishable at 7.5 dpc (days post coitum) as a small pool of cells having high alkaline phosphatase expression (1–4). They emigrate from the hindgut epithelium at 9.5 dpc through the dorsal mesentery (10.5 dpc) and into the developing genital ridges by 11.5 dpc (5–7). There they continue to proliferate until 13.5 dpc when in the male they arrest at mitosis and in the female they enter meiosis. The germ cell deficient (gcd) mutation (8) is a recessive, transgenic insertional mutation in which there is a drastic reduction of PGCs in the developing genital ridge, resulting in male and female infertility in the adult. The male phenotype of a vacuolated testis, with very few functional tubules, is similar to the azoospermia phenotype seen in infertile human males. The female phenotype of rapid follicular depletion resembles the premature ovarian failure syndrome (POF) seen in infertile human males. The female phenotype of rapid follicular depletion resembles the premature ovarian failure syndrome (POF) seen in infertile human males. The female phenotype of rapid follicular depletion resembles the premature ovarian failure syndrome (POF) seen in infertile human males. Thus, determining the exact defect in the gcd mouse and its underlying genetic basis could have considerable impact on these surprisingly common clinical syndromes. We report here that the gcd phenotype is due to the deletion of a single novel gene, Pog (proliferation of germ cells) which encodes a protein with a plant homeodomain (PHD) motif at its C terminus (11). Pog is critical for normal PGC proliferation, starting between 9.5 and 10.25 dpc when germ cells begin to migrate into the developing genital ridge. It does not affect their normal migration pattern. Deletion of Pog is also accompanied by reduced embryonic body weight and, on some genetic backgrounds, embryonic lethality. Thus, in addition to being necessary for PGC proliferation, Pog may have a wider significance in early embryonic development.

RESULTS AND DISCUSSION

Total PGC numbers in 8.5 and 9.5 dpc gcd embryos were estimated after whole mount alkaline phosphatase (AP) staining. No difference in PGC number or migration pattern was seen between gcd/gcd and gcd/+ or +/+ littermates at these stages (Fig. 1A). 10.25–10.5 dpc embryos were then fully sectioned, stained for AP and the number and position of PGC's in each section recorded. It was found that the germ cell deficiency could be detected at 10.25 dpc with gcd/gcd homozygotes having approximately one third the number of

*To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, Baylor College of Medicine, 6550 Fannin Street (#880), Houston, TX 77030, USA. Tel: +1 7137988221; Fax: +1 7137985074; Email: bishop@bcm.tmc.edu
†The authors wish it to be known that, in their opinion, these two authors should be considered as joint First Authors.
Figure 1. Analysis of PGC development in gcd embryos. (A) Migration of the primordial germ cells (in yellow) in hindgut mesentery (in red) at 9.5 dpc embryo (a). Equal number of primordial germ cells (arrows) in 9.5 dpc gcd/gcd homozygote (b) and wild type embryo (c). (B) gcd/gcd embryos have normal PGC migration, impaired PGC proliferation and lower body weight. (a) Section of 10.25 dpc gcd/+ embryo. PGCs (arrowed) were seen in the dorsal mesentery and developing genital ridge. (b) Section of 10.25 dpc gcd/gcd embryo. Less PGCs were present compared with a. (c) Section of 10.5 dpc +/- embryo. PGCs were mainly seen in the developing genital ridge. (d) Section of 10.5 dpc gcd/gcd embryo, less PGCs were present compared with c. (e) Section of 11.5 dpc +/- developing genital ridge showing PGCs in the genital ridge. (f) Section of 11.5 dpc gcd/gcd genital ridge. PGCs are present but in lower numbers. ao, Dorsal aorta; dm, dorsal mesentery; gr, genital ridge; nt, neurotube; pc, peritoneal cavity. (C) Total PGC counts: 10.5 dpc gcd/gcd embryos (n = 5) had approximately one third PGC numbers of that of 10.5 dpc gcd/+ embryos (n = 4). (D) gcd/gcd embryos (top row in each panel) are visibly smaller than gcd/+ and +/- embryos (bottom row in each panel) at 10.5 dpc (top panel) and 11.5 dpc (lower panel). (E) gcd/gcd embryos have a lower body weight (~30% lower) at 10.5 dpc and 11.5 dpc compared to gcd/+ and +/- embryos.
PCGs found in their normal gcd/+ or +/- littermates (Fig. 1A–C). Homozygous gcd/gcd PCGs were only found in the developing genital ridge or in the dorsal mesentery near the genital ridge (5,7). No PCGs were found in any other tissues indicating that germ cell migration was following the normal pathway. Further, PCGs from gcd/gcd and +/- 11.5 dpc embryos were found to have colonized the genital ridge indicating that there was no temporal change in the migration pattern (Fig. 1B e and f).

In this analysis, gcd/gcd homozygotes were generated by intercrossing (C57BL/6xFVB)F1 gcd/+ heterozygotes and all genotypes were obtained in the expected Mendelian ratio. In contrast, on a predominantly C57BL/6 genetic background (N8), it was found that although gcd/gcd mice could be obtained in normal numbers up to 9.5 dpc they were virtually absent by 10.5 dpc. It was also noted that on the permissive C57BL6xFVB background, although normal numbers of embryos were found to have colonized the genital ridge (Fig. 1B e and f).

Taken together, these data suggest that the germ cell deficiency is due to a defect in normal PGC proliferation, rather than migration, beginning between 9.5–10.25 dpc, just as the first PCGs begin to enter into the genital ridge. They also show that the development of the embryo proper is compromised by the gcd mutation at this point.

In order to identify and clone the gene responsible for the gcd phenotype, sequences flanking both ends of the transgenic insertion (3.3.1 and 3.3.19) were obtained from a gcd/gcd genomic phage library. A single 200 kb BAC clone (RPC1253D2) spanning both breakpoints was subsequently identified and its gene content estimated by shotgun sample sequencing (Fig. 2A). Altogether 360 subclones were sequenced, five of which matched EST records in the database corresponding to two distinct genes, Vrk2 (mouse ortholog of the human vaccinia virus related kinase 2, VRK2) a serine/threonine kinase (12,13) and a novel gene provisionally termed Pog. The insertion of the goat β-globin transgene caused an ~150 kb deletion of mouse genomic DNA removing exons 2–11 of Vrk2 and exons 4–14 of Pog (Fig. 2A). Vrk2 and Pog are transcribed in opposite orientations, their last exons, (exon 11 of Vrk2 and exon 14 of Pog) overlap and are transcribed off complementary DNA strands. Northern analysis and RT–PCR indicate that both genes have a widespread expression pattern, including 10.5 dpc embryos and adult gonads (Fig. 2B, ref. 13).

Mouse Pog encodes a protein of 375 amino acids and contains a plant homeodomain (PHD) zinc-finger motif (C4HC3 consensus sequence) at the C-terminus (11) (Fig. 2C). A blast search of GenBank revealed a human cDNA sequence with 77% nucleotide identity to mouse Pog (Access. No. AK001197). Analysis of the human genomic sequence showed identical intron–exon organization and orientation of mouse/human Pog/POG and Vrk2/VRK2 genes. The POG protein is well conserved between mouse and human, showing an overall 79% amino acid identity. Drosophila melanogaster and Arabidopsis thaliana each has a protein sequence with weak similarity to mouse and human POG (Fig. 2C). The similarity is not confined to the PHD domain although it is more prominent in this region.

To test whether deletion of Vrk2 was responsible for the gcd phenotype, BAC rescue was performed. BAC 253D2, which contains the complete mouse Vrk2 coding sequence and upstream promoter/control elements, was microinjected into fertilized FVB/N eggs. Expression of the resulting transgene in the adult gonads was confirmed by RT–PCR using a polymorphism in the 3’ UTR of Vrk2 to distinguish endogenous (FVB/N) from transgenic (C57BL/6) expression. The Vrk2 transgene was introduced into the homozygous gcd/gcd mouse background using a standard mating scheme and the histology of adult (4–6 week) testis and ovariies was examined. The phenotype of the transgenic Vrk2, gcd/gcd males and females was indistinguishable from that of the non-transgenic gcd/gcd littermates (data not show). This lack of complementation suggested that Vrk2 does not underlie the gcd phenotype and implied a role for Pog.

We examined the biological role of Pog using an insertional gene targeting approach developed by Zheng et al. (14,15). As shown in Figure 3, homologous recombination leads to double stranded gap repair, duplication of Pog exons 9–13 and the insertion of the vector backbone DNA between the duplicated segments. The C-terminus of POG, which contains the putative PHD domain, was thus disrupted.

Pog⁺/Pog⁻ targeted males (where Pog⁻ is a targeted allele) were crossed to gcd/+ females to produce compound heterozygotes, or intercrossed to Pog⁺/Pog⁻ females to produce Pog⁻/Pog⁻ null mice. Transcription of Pog was checked in the gonads and spleen of the resulting homozygous Pog⁺/Pog⁻ targeted mice using RT–PCR and primers specific for the 3’ and the 3’ ends of the gene. No transcription could be detected, suggesting that disruption of the 3’ end of the transcript has led to mRNA instability and degradation. As expected, the expression of mouse Vrk2 mRNA was not affected and could be detected in Pog⁻/Pog⁻ gonad RNA (data not shown).

A histological examination of 4 week adult gcd/gcd, gcd/ Pog⁻ and Pog⁻/Pog⁻ gonads (Fig. 4) showed that they had virtually identical sterility phenotypes with abnormal testis and ovariys, characteristic of the original gcd/gcd mutation (8). Testes were one third normal size with over 90% of the tubules containing vacuolated Sertoli cells but no developing germ cells (Fig. 4A–D). It is interesting to note that in both gcd/gcd and Pog⁻/Pog⁻ adult males there is some degree of re-population of the seminiferous tubules by germ cells (Lu and Bishop, unpublished data). In the affected female, the ovary was extremely small and the uterine horns were pale and thin. Very few developing follicles were seen compared to Pog⁺/ Pog⁻ or Pog⁺/Pog⁻ controls (Fig. 4E–G). In addition, germ cell deficiency was confirmed in 10.5 dpc Pog⁻/gcd embryonic gonads (Fig. 5). Significantly, the latter embryos contain an intact copy of the Vrk2 gene, but nevertheless manifest the mutant germ cell deletion phenotype. No histological abnormalities could be detected in the Pog⁻/Pog⁻ lung, heart, spleen, kidney or liver (data not shown). Similar to the gcd/gcd crosses, Pog⁻/Pog⁻ and gcd/Pog⁻ embryonic lethality was seen on pure 129/Sv and (129/SvxB6)F1 backgrounds. In these experiments Pog⁻/Pog⁻ and gcd/Pog⁻ mice were generated (in normal numbers) on a mixed (129/SvxFVB)F1 and (129/ svxFVB)xB6 background, respectively.

These data demonstrate that it is the deletion of the Pog gene alone which is responsible for the germ cell deficiency,
reduced embryonic body weight and, on some genetic backgrounds, embryonic lethality. Deletion of \( \text{Vrk2} \) in the original \( \text{gcd} \) mice does not appear to contribute to the phenotype as gonadal dysfunction appears identical between \( \text{Pog}^+/\text{C}0 \) and \( \text{gcd}^+/\text{Pog}^+/\text{C}0 \) mice even though the latter express \( \text{Vrk2} \). Any somatic function of \( \text{Vrk2} \) can presumably be replaced by redundancy within the protein kinase family (12). Formal proof that \( \text{Vrk2} \) does not play a role in the phenotype could be evaluated in future knockout experiments. The experimental data obtained here show that \( \text{Pog} \) is not necessary for correct germ cell migration, but is critical for proliferation of PGCs beginning between 9.5–10.25 dpc. It is at this point that embryos show lower body weight (or embryonic lethality occurs) suggesting that \( \text{Pog} \) also affects the development of the somatic tissues.

Only a few genes are known to specifically affect PGC proliferation in vivo. TGF beta signaling molecules, in particular, BMP4 and BMP8b (16) and their downstream signal mediators SMAD1 and SMAD5 (17,18), all affect PGC development to various degrees, but there is no evidence that \( \text{POG} \) is part of this pathway. Decreased PGC migration and reduced proliferation are also associated with the \( W \) (white

---

**Figure 2.** Structure of the \( \text{gcd} \) locus. (A) A region of ~150 kb on chromosome 11 was deleted and replaced with 10 tandem repeats of a goat \( \beta \)-globin transgene. \( \lambda \) phage clone 3.19 and 3.1 encode sequences spanning the two integration sites. The structure of \( \text{Vrk2} \) (open boxes for exons) and \( \text{Pog} \) (filled boxes for exons) are shown. Note the 3' UTR of both genes overlap and are transcribed from opposite DNA strands. (B) Expression analysis of the \( \text{Pog} \) and \( \text{Vrk2} \) genes by RT–PCR. RNA was isolated from: 1, brain; 2, kidney; 3, testis; 4, ovary; 5, lung; 6, heart; 7, 10.5 dpc embryo. Lane 8 is a mouse genomic DNA; lane 9 is negative (water) control. (C) Alignment of the PHD domain (C4HC3) of mouse and human \( \text{POG} \), with the POG homologous protein from \( \text{Drosophila melanogaster} \) and \( \text{Arabidopsis thaliana} \). Conserved cysteine and histidine residues are shaded.
spotting) and Sl (Steel) mutants which involve the c-Kit receptor kinase gene and its ligand Scf (stem cell factor) respectively (19). Unlike the W and Sl mutations Pog/C0 mice do not show defects in hematopoiesis or melanocyte development. The gcd phenotype is quite similar to that of Tiar/C0 mice (20). Both have reduced numbers of PGCs as early as 11.5 dpc and both show partial embryonic lethality.

TIAR is an RNA binding protein involved in apoptosis (21, 22). Our preliminary data using a TUNEL assay indicates that apoptosis is not involved in the gcd phenotype indicating that POG acts through a different mechanism.

As yet, is not yet known how the Pog gene exerts its effect on PGC proliferation. An analysis of the protein structure suggests that it is an intracellular protein encoding a PHD domain at the C-terminus. This motif is believed to be important in the regulation of gene expression, through interaction with other proteins and subsequent modulation of chromatin structure.

In summary, we have identified a novel gene, Pog, the deletion of which is responsible for the phenotypic lack of germ cells seen in the gcd mouse mutant. Pog is critical for normal PGC proliferation beginning at the time of their entry into the developing genital ridge, at 9.5–10.25 dpc. Beginning at the same time point, Pog null mice also show reduced embryonic body weight or lethality depending on genetic background suggesting that it may also play a wider role in embryogenesis. Identification of the pathway through which the POG protein acts during PGC proliferation should provide a better understanding of the biology of early germ cell development.

MATERIALS AND METHODS

Alkaline phosphatase staining of PGC’s

The morning vaginal plugs were found was taken as 0.5 dpc. Embryos were dissected from the decidua and DNA extracted from the yolk sac or the embryo proper was used for genotyping by PCR. Embryos were fixed in 4% paraformaldehyde for 2 h at 4°C. For whole mount staining, the embryos were processed as described (17) and stained with Fast Red (Sigma) (23). For staining sections, the embryos were fixed, dehydrated, embedded in Paraplast X-tra (Fisher) at 54°C and mounted on silanated slides (Sigma). Sections were dewaxed in Histo-Clear (National Diagnostics, GA) rehydrated and stained with Fast Red.

Gene targeting

A 5.3 kb plasmid was isolated from a mouse genomic DNA library (14) using the Pog cDNA as probe. It cut with Hpal and religated to generate a 300 bp ‘gapped’ clone which was used for electroporation of AB1 ES cells. ES cells were selected with 0.35 mg/ml G418 and PCR was used to screen for targeted clones. The primers used for amplifying the 3 kb fragment in the screening were P3527, 5'-AGAAGAGCGAATGACATTTGTT-3' (from the targeting vector) and gap1 5'-ATGCTGCTGCTTCTGTA-3' (from the deleted 300 bp fragment). The primers used for amplifying the 2 kb fragment were Bzp1, 5'-CGGAAAGCAGCCCATATAC-3' (from the
targeting vector), and gap2, 5'-TCAGAAGTGAGCAGCAGCAT-3'. (from the deleted 300 bp fragment). Pog+/Pog- ES clones were then injected into C57BL/6 blastocysts, transferred to pseudopregnant females and resulting germ line chimeras identified by crossing to C57BL/6 females.

**Maintenance of gcd mice and Pog knockout mice**

gcd mice, originally obtained on a mixed (C57BL/6xCBA/H) F1 background, were subsequently backcrossed to C57BL/6 for 8 generations in our facilities. Homozygous gcd/gcd mice were obtained by intercrossing heterozygous C57BL/6 gcd+/ mice and heterozygous gcd/+ (FVB/NxC57BL/6)F1's. Pog+/Pog- targeted mice were maintained on a 129/Sv background. Pog-/Pog- mice were obtained by intercrossing 129/sv Pog+/Pog- or 129/SvxFVB/N)F1 heterozygotes.

**Genotyping**

Mouse ear or tail pieces were digested in 50 µl of digestion buffer (10 mmol/l Tris-HCl, pH 9.0, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1% Triton X-100, 0.45% IGEPAL, 0.8 mg/ml proteinase K) at 55°C for 4 h to overnight. It was heated at 94°C for 10 minutes, centrifuged at 10 000 g for 5 min and the supernatant used for PCR. Primers for amplification of goat β-globin DNA were gcd/intF, 5'-CCTGTGGAAACCACACC TTG-3' and gcd/intR 5'-TGTTGCTTGTTTGTAGCTG-3'. Primers for amplification of the deleted region in gcd: gcdF1 5'-CTTTGGAAGACTGACTCTGCCTAACC-3' and gcdR1 5'-GTTGCTTGGAAGGACACAGCGATAG-3'. Primers for amplification of targeted allele in Pog knockout mice: Type1 5'-CCACAAACAACGTGTTATGCGTC-3' and Bzp1 as describe in ‘gene targeting’ section.
Acknowledgements

We would like to thank Helen Martinez for excellent technical assistance and Dr Jan Rohozinski for critical reading of the manuscript. We thank Dr Peter J. Donovan for his comments and suggestions. This research was supported by NIH grant P01 HD36289 to C.E.B.

References


Histology

Tissue samples were fixed in Bouin’s Solution (Sigma) and processed for routine histology. 8μl sections were cut and stained with haematoxylin and eosin.

Figure 5. Reduction of the primordial germ cells in Pog knockout mutants at 10.5 dpc embryos. Alkaline-stained primordial germ cells (arrows) in wildtype (A) and Pog'gcd (B) embryos. ao, Dorsal aorta; pc, peritoneal cavity.