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Abstract

Background: In mice, germ cells are specified through signalling between layers of cells comprising the primitive embryo. The function of Dppa3 (also known as Pgc7 or stella), a gene expressed in primordial germ cells at the time of their emergence in gastrulating embryos, is unknown, but a recent study has claimed that it plays a central role in germ cell specification.

Results: To test Dppa3's role in germ cell development, we disrupted the gene in mouse embryonic stem cells and generated mutant animals. We were able to obtain viable and fertile Dppa3-deficient animals of both sexes. Examination of embryonic and adult germ cells and gonads in Dppa3-deficient animals did not reveal any defects. However, most embryos derived from Dppa3-deficient oocytes failed to develop normally beyond the four-cell stage.

Conclusion: We found that Dppa3 is an important maternal factor in the cleavage stages of mouse embryogenesis. However, it is not required for germ cell specification.

Background

Among the many specialized cell types present in adult mammals, the first to be programmed or specified during embryogenesis are germ cells, which give rise to eggs and sperm. Which molecules direct this programming of germ cells? In many other animals, including flies and worms, material known as "germ plasm" is laid down in the egg before fertilization, and its subsequent passage to a subset of embryonic cells dictates their fate as germ cells [1,2]. In mammalian embryos, germ cells are specified in a very different manner, through signalling between layers of cells comprising the primitive embryo [3,4].

Recently, Saitou, Barton and Surani proposed a molecular pathway by which these intercellular signals are translated into germ cell fate in mice [5,6]. Central to this proposed program of germ cell specification is stella / PGC7 / Dppa3, a gene expressed in primordial germ cells and their descendants, including oocytes [5,7,8]. Here we will use the name Dppa3, as approved by the Mouse Genome Informatics Database, when referring to this gene. Saitou and colleagues' model of Dppa3's role in germ cell specification was based on the timing and site of the gene's expression, not on functional analysis. Nonetheless, the model makes clear predictions as to the phenotype of mice lacking Dppa3 function: such embryos should not form germ cells. We tested this prediction and sought to clarify the gene's importance by generating Dppa3-deficient mice and examining their germline development.
Results and Discussion

We disrupted the Dppa3 gene in cultured embryonic stem (ES) cells and thereby generated Dppa3-deficient mice. Specifically, we replaced the entire open reading frame of Dppa3 in mouse V6.5 ES cells [9] with a hygromycin-thymidine kinase selection cassette flanked by loxP sites (Figure 1A). The selection cassette was subsequently removed via transient expression of Cre recombinase in targeted ES cells. The resulting heterozygous Dppa3tm1WHT/+ ES cells were used to generate chimeric mice, which transmitted the mutation to offspring. Intercrosses between Dppa3tm1WHT/+ heterozygous animals yielded Dppa3tm1WHT/Dppa3tm1WHT homozygotes as well as Dppa3tm1WHT/+ heterozygotes and +/- offspring, demonstrating that zygotic function of Dppa3 is not essential for viability (Figure 1B). This allowed us to characterize germ cell development in animals lacking Dppa3.

Dppa3 is not required for germ cell specification

Our findings do not support the proposed centrality of Dppa3 in germ cell programming. First, the gonads of Dppa3tm1WHT/Dppa3tm1WHT embryos contained germ cells, identified by expression of alkaline phosphatase, in numbers comparable to those of Dppa3tm1WHT/+ and +/- embryos (Figure 2A). Second, the ovaries of Dppa3tm1WHT/Dppa3tm1WHT adult females expressed Oct4, a marker of oocytes [10,11], despite the absence of Dppa3 expression (Figure 2B). Third, histological examination of the gonads of Dppa3tm1WHT/Dppa3tm1WHT adults revealed no morphological defects; spermatogenesis in males and ovarian follicle development in females appeared to be normal (Figure 2C,2D). Finally, we obtained fertile Dppa3tm1WHT/Dppa3tm1WHT mice of both sexes (though litters from Dppa3tm1WHT/Dppa3tm1WHT females were small, as described below). Each of these findings demonstrates that the Dppa3 gene is not required for germ cell specification.

Moreover, this function is not readily ascribed to a gene closely related to Dppa3. We electronically searched the sequenced mouse genome for Dppa3 homologues. We identified several processed (intron-less) pseudogenes of
Dppa3, but no functional, full-length homologue. As judged by RT-PCR analysis, the Dppa3 pseudogenes are not expressed in embryonic or adult tissues (data not shown).

**Dppa3 is a potent maternal factor**

We found that Dppa3 plays an important role in early embryonic development as a maternal factor. While Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup> males were fully fertile, Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup> females had small litters. This was true regardless of whether such females were crossed with Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup>, Dppa3<sup>tm1WHT</sup>/+ or wild type males (3.5 ± 1.5, 3.1 ± 2.1, or 3.0 ± 0.9 viable pups/litter, respectively). By contrast, Dppa3<sup>tm1WHT</sup>/+ females of the same (mixed) genetic background had large litters when mated to Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup> or Dppa3<sup>tm1WHT</sup>/+ males (9.4 ± 3.5 or 10.1 ± 3.2 viable pups/litter, respectively).

We attribute the small litters from Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup> mothers to abnormalities that manifest early in embryogenesis, during the cleavage stages of pre-implantation development. While nearly all embryos derived from Dppa3-deficient oocytes developed to the 2-cell or 4-cell stage (Figure 3A,3B,3C,3D), subsequent development was severely compromised in most such embryos (Figure 3E,3F). Some embryos derived from Dppa3-deficient oocytes failed to reach the 8-cell stage and instead showed evidence of compaction at the 4-cell stage. Other embryos derived from Dppa3-deficient oocytes cleaved to form 8 to 16 blastomeres, but failed to compact (Figure 3E,3F). These observations suggest that maternally supplied Dppa3 function is important in the cleavage stages of pre-implantation development.

Might maternal Dppa3 induce zygotic expression of Oct4/Pou5f1, which encodes a transcription factor that is crucial to pre-implantation development [10,12]? To test this possibility, we crossed Dppa3<sup>tm1WHT</sup>/Dppa3<sup>tm1WHT</sup> females with Dppa3<sup>+</sup>+, Tg(Oct4<sup>−/−</sup>ΔPE-GFP)<sup>10WHT</sup>/Tg(Oct4<sup>−/−</sup>ΔPE-GFP)<sup>10WHT</sup> males, the latter transmitting an Oct4-GFP transgene, with the Oct4 promoter driving expression of GFP. We retrieved the resulting embryos at the 2-cell stage and cultured them in vitro for 72 hours to monitor expression of the Oct4-GFP transgene. All such embryos were observed to express the fluorescent marker, regardless of the degree to which the embryos developed or failed to develop during the culture period (Figure 3G,3H). Thus, the poor development of many embryos derived from Dppa3-deficient oocytes cannot be attributed to the absence of zygotic expression of Oct4. Further analysis of the maternal-effect phenotype of Dppa3 should illuminate the molecular and biological context and consequences of the gene's activity.
Conclusions
We conclude that Dppa3 is not required for germ cell specification in mice. The identity of the mammalian gene or genes that program germ cells remains an open question. Dppa3 appears to function as a maternal factor, with an important role early in embryogenesis, during cleavage.

Methods
Generation of Dppa3-deficient animals
The Dppa3 targeting construct contained 1.3-kb and 3-kb segments of mouse genomic DNA, the former located 5’ of Dppa3’s translation initiation site and the latter located 3’ of the termination codon (Figure 1). At the center of the construct was a 3-kb hygromycin-thymidine kinase selection cassette (Hygro-TK) flanked by two loxP direct repeats. V6.5 (C57BL/6 × 129/Sv)F1 ES cells [9] were transfected by electroporation, and recombined clones were selected in the presence of hygromycin (Invitrogen). Correctly targeted clones were identified by long-distance PCR. The Hygro-TK cassette was removed via transient transfection of ES cells with a Cre-expressing plasmid in the presence of ganciclovir (Sigma). The final genomic structure of the resulting clones was verified by Southern analysis. Two independently targeted ES cell clones were microinjected into Balb/c blastocysts to generate chimeras. Animals used in this study were of a mixed C57BL/6 × 129/Sv genetic background.

RNA isolation and RT-PCR
Total RNAs were isolated from mouse tissues, and expression of Dppa3, Oct4, and Gapd was assayed by RT-PCR, all as described previously[8].

Alkaline phosphatase staining of primordial germ cells
Gonads were dissected from wild type and Dppa3tm1WHT / Dppa3tm1WHT embryos on day 12.5 of gestation and stained for alkaline phosphatase as described previously [13].

Histology
Dissected adult testes and ovaries were fixed overnight in Bouin’s solution, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Generation of Oct4-GFP transgenic animals
Mice bearing an Oct4-GFP transgene were generated by microinjection of a 14-kb Oct4.A PE-GFP linear DNA fragment into C57BL/6 × SJL Fl2 hybrid mouse eggs. This construct essentially reproduces the previously described GOF18A PE-lacZ construct [14] but contains a gene for enhanced green fluorescent protein (EGFP, Clontech) in place of lacZ at the ATG of Oct4. Mice bearing transgene Tg(Oct4Sfi1 ΔPE-GFP)10WHT accurately reproduced the previously reported Oct4 expression pattern [14] and were bred to generate Tg(Oct4Sfi1 ΔPE-GFP)10WHT / Tg(Oct4Sfi1 ΔPE-GFP)10WHT homozygous animals.

Isolation, culture and analysis of cleavage stage embryos
2-cell embryos were flushed from oviducts at E1.5 and cultured for up to 72 hours in microdrops of KSOM (Speciality Media) under light mineral oil (Squibb) with 5% CO2 in air. E3.5 embryos were flushed from uteri.

Authors’ contributions
AB conducted molecular biological, ES cell culture and embryological studies, and co-wrote the manuscript. MG carried out blastocyst injections. ML assisted in mouse and embryological studies. DP coordinated the study and co-wrote the manuscript. All authors read and approved the final manuscript.

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