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Research article

Dppa3 / *Pgc7* / *stella* is a maternal factor and is not required for germ cell specification in mice

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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.

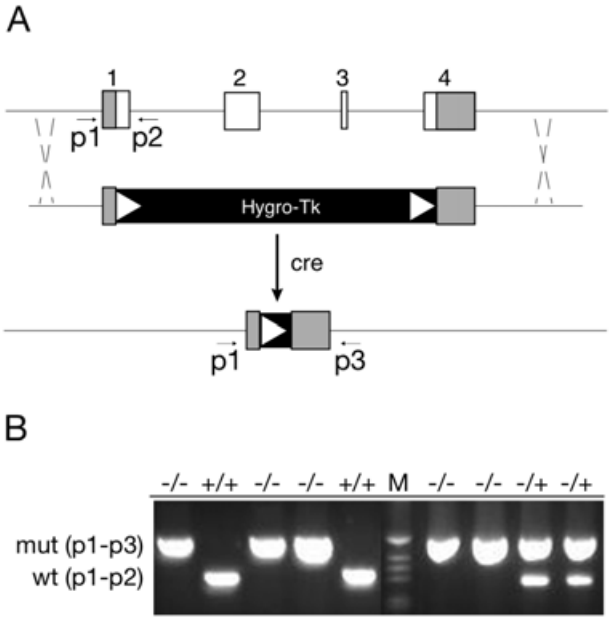

Figure 1
Generation of *Dppa3*-deficient animals. A, Schematic representation of genomic ablation of *Dppa3*. The gene's four exons are shown; non-coding regions of the first and last exons are shaded gray. The hygromycin-thymidine kinase (Hygro-TK) cassette replaces the entire open reading frame of the gene. *Cre*-mediated excision of the selection cassette leaves only the non-coding portions of the gene, together with a single *loxP* site (white triangle). Also shown are the locations of genotyping primers p1, p2 and p3 in wild-type and mutated *Dppa3* alleles. **B**, PCR genotyping of the offspring of an intercross between *Dppa3*^{tm1WHT}/+ animals. Inferred genotypes are shown above the gel image. The wild type allele yields a PCR product of 304 bp with primers p1 and p2. The mutant allele (*Dppa3*^{tm1WHT}) yields a PCR product of 492 bp with primers p1 and p3. M, DNA molecular weight marker.

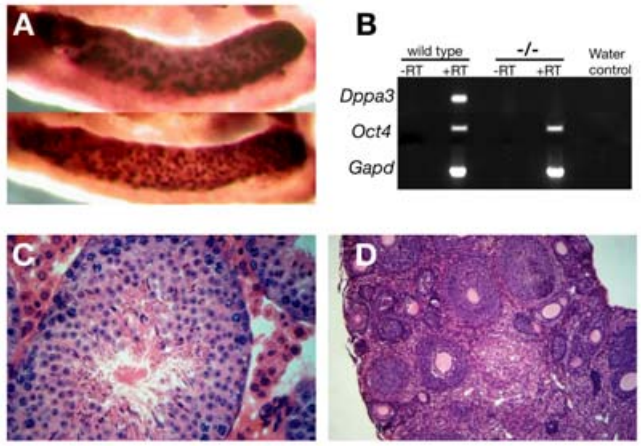

Figure 2
Normal germ cell development in the absence of *Dppa3*. A, Gonads from E12.5 embryos (above: wild type; below: *Dppa3*^{tm1WHT}/*Dppa3*^{tm1WHT}) stained for alkaline phosphatase to reveal primordial germ cells. **B**, RT-PCR analysis of gene expression in wild-type and *Dppa3*^{tm1WHT}/*Dppa3*^{tm1WHT} adult ovaries. **C,D**, *Dppa3*^{tm1WHT}/*Dppa3*^{tm1WHT} testis (**C**) and ovary (**D**) are histologically normal.

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 *Dppa3*^{tm1WHT}/+ ES cells were used to generate chimeric mice, which transmitted the mutation to offspring. Intercrosses between *Dppa3*^{tm1WHT}/+ heterozygous animals yielded *Dppa3*^{tm1WHT}/*Dppa3*^{tm1WHT} homozygotes as well as *Dppa3*^{tm1WHT}/+ 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 Dppa3^{tm1WHT}/Dppa3^{tm1WHT} embryos contained germ cells, identified by expression of alkaline phosphatase, in numbers comparable to those of Dppa3^{tm1WHT}/ + and +/+ embryos (Figure 2A). Second, the ovaries of Dppa3^{tm1WHT}/Dppa3^{tm1WHT} 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 Dppa3^{tm1WHT}/ Dppa3^{tm1WHT} 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 Dppa3^{tm1WHT}/Dppa3^{tm1WHT} mice of both sexes (though litters from Dppa3^{tm1WHT}/Dppa3^{tm1WHT} 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 $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ males were fully fertile, $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ females had small litters. This was true regardless of whether such females were crossed with $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$, $Dppa3^{tm1WHT}/+$ or wild type males (3.5 ± 1.5, 3.1 ± 2.1, or 3.0 ± 0.9 viable pups/litter, respectively). By contrast, $Dppa3^{tm1WHT}/+$ females of the same (mixed) genetic background had large litters when mated to $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ or $Dppa3^{tm1WHT}/+$ males (9.4 ± 3.5 or 10.1 ± 3.2 viable pups/litter, respectively).

We attribute the small litters from $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ 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 $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ females with *Dppa3* +/+, $Tg^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}/Tg^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}$ 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.

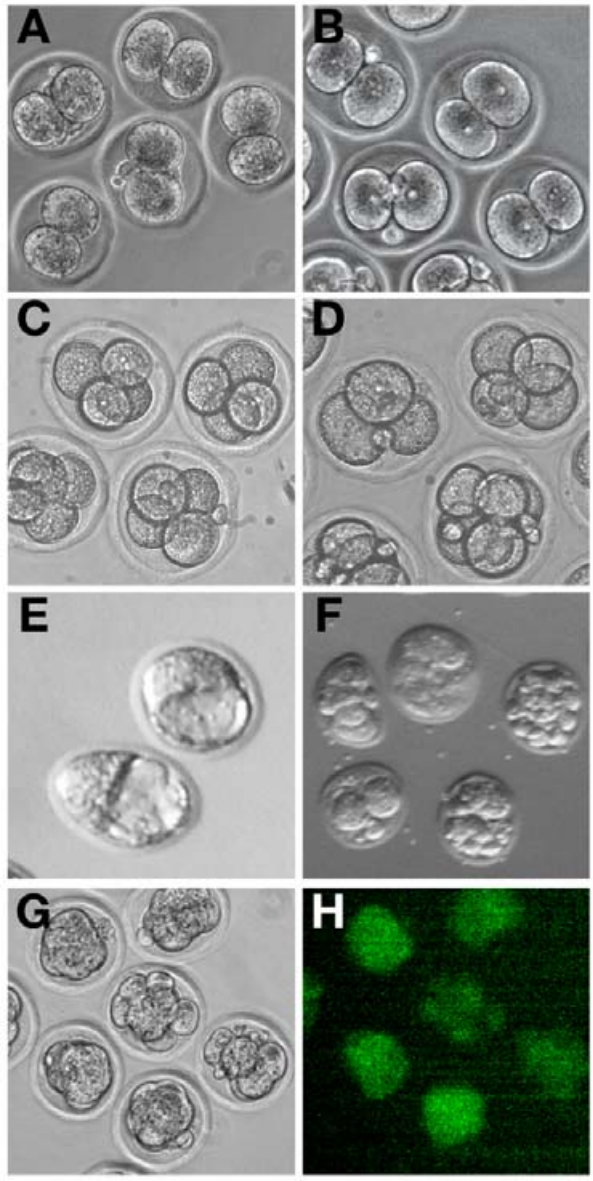

Figure 3
Abnormal pre-implantation development of embryos derived from *Dppa3*–deficient oocytes. A,C, Cultured 2-cell (**A**) and 4-cell (**C**) control embryos derived from wild-type matings. **B,D**, Cultured 2-cell (**B**) and 4-cell (**D**) embryos produced by crossing $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ females with wild-type males. **E,F**, E3.5 control embryos derived from wild-type matings have progressed to the blastocyst stage (**E**). By contrast, most E3.5 embryos produced by crossing $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ females with wild-type males have not progressed to the blastocyst stage and instead cleave abnormally and degenerate (**F**). **G,H**, Many embryos produced by crossing $Dppa3^{tm1WHT}/Dppa3^{tm1WHT}$ females with *Dppa3* +/+, $Tg^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}/Tg^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}$ males fail to develop normally beyond the 4-cell stage (**G**) but nonetheless express the Oct4-GFP marker (**H**).

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 genomic 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.

Primers for PCR genotyping were as follows: p1 (5' TAG CCT GGG GGT AGA CTC GGC TGT AT 3'); p2 (5' AAC GAG AAG AGA AGG GAG GGC TTC 3'); and p3 (5' TCA CAT AAA TCT GGA TCG TTG TGC ATC 3'). The wild type allele gives rise to a PCR product of 304 bp with primers p1 and p2. The mutant allele (*Dppa3*^{tm1WHT}) gives rise to a PCR product of 492 bp with primers p1 and p3.

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 *Dppa3*^{tm1WHT} / *Dppa3*^{tm1WHT} 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 *Oct4Δ PE-GFP* linear DNA fragment into C57Bl/6 × SJL F2 hybrid mouse eggs. This construct essentially reproduces the previously described *GOF18Δ 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*$^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}$ accurately reproduced the previously reported *Oct4* expression pattern [14] and were bred to generate *Tg*$^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}$ /*Tg*$^{(Pou5f1\ \Delta PE\text{-}GFP)10WHT}$ 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 (Specialty Media) under light mineral oil (Squibb) with 5% CO_2 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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