

cated its ability to activate pro-gelatinase A is abolished (manuscript in preparation). □

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Disruption of *c-mos* causes parthenogenetic development of unfertilized mouse eggs

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THE *c-mos* proto-oncogene encodes a 37–39K cytoplasmic serine/threonine kinase¹ implicated in the meiotic maturation events during murine spermatogenesis² and oogenesis^{3–6}. In *Xenopus*, ectopic expression of pp39^{mos} can promote both the meiotic maturation of oocytes^{7–9} and also arrest the cleavage of blastomeres¹⁰. To elucidate the role of pp39^{mos} we have generated homozygous mutant mice by gene targeting in embryonic stem cells¹¹. These mice are viable and mutant males are fertile, demonstrating that pp39^{mos} is not essential for spermatogenesis. In contrast, mutant females, have a reduced fertility because of the failure of mature eggs to arrest during meiosis. *c-mos*^{-/-} oocytes undergo germinal vesicle breakdown and extrusion of both polar bodies followed in some cases by progression into cleavage. Mutant females also develop ovarian cysts. These results demonstrate that a major role for pp39^{mos} is to prevent the spontaneous parthenogenetic activation of unfertilized eggs.

The targeting vector, pMS1(neo)tk (Fig. 1a) contains a neomycin-resistance (*neo*^r) gene inserted into the middle of the single *c-mos* coding exon. This insertion introduces an amber stop codon into the *c-mos* reading frame to terminate translation upstream of sequences essential for kinase activity^{12,13}. pMS1(neo)tk was electroporated into CCE embryonic stem (ES) cells and clones resistant to both G418 and gancyclovir were screened for a targeting event. The expected genomic structure of the targeted *c-mos* allele was confirmed by Southern blotting using an external downstream genomic probe not present in the targeting construct. *Xba*I digestion, which does not cut within the *neo*^r gene, generated a fragment 1.8 kilobases (kb) larger than the wild-type fragment, a size increase consistent with insertion of the *neo*^r gene (Fig. 1b). *Xba*I–*Bam*HI double digestion (Fig. 1b) and *Pvu*II digestion (data not shown) confirmed that the insert within the *c-mos* coding exon was the *neo*^r gene.

Eight independently targeted clones were identified from 186 colonies and two were injected into C57Bl/6 blastocysts to give chimaeric animals. Seven out of 24 chimaeric males transmitted the ES cell genome to their progeny. Mice heterozygous for the disrupted *c-mos* allele (*mos*^{+/-}) were crossed to obtain mice homozygous for the mutation (*mos*^{-/-}) (Fig. 1b). Viable homozygous mutant mice were obtained at the expected frequency indicating that an intact *c-mos* gene is not required for normal embryonic development. Polymerase chain reaction (PCR) across the single *c-mos* coding exon using two different pairs of primers detected the presence of a wild-type exon only in mice with a wild-type allele and not in *mos*^{-/-} mice. Southern blot with a *mos* coding exon probe showed no intact wild-type *c-mos* exon in *mos*^{-/-} mice (Fig. 1b). Thus, the disrupted *c-mos* locus shows a genomic structure entirely consistent with the expected homologous replacement event.

Northern blot analysis of RNA extracted from *mos*^{+/+} ovaries and hybridized with *c-mos* exon fragments either upstream or downstream of the insert detected a clear band of messenger RNA of about 1.4 kb. There was no equivalent signal from equal amounts of RNA from *mos*^{-/-} ovaries suggesting that the insert may impair transcription or destabilize the transcript.

As pp39^{mos} has been implicated in both male and female gametogenesis we tested *mos*^{-/-} animals of both sexes for fertility. Homozygous mutant males sired offspring at comparable rates to control aged-matched litter mates (either *mos*^{+/+} or *mos*^{+/-}) indicating that an intact *c-mos* gene is not required for spermatogenesis. Histological examination of testes from null mutant animals also failed to identify gross differences from wild-type and showed the presence of mature sperm. In contrast, female mutant animals exhibited a reduced fecundity and gave rise to statistically significant small litters ($P < 0.005$). The average litter size from *mos*^{-/-} females was 2 ± 0.63 (7 breeding pairs, 12 offspring) whereas from a combined group of *mos*^{+/+} and *mos*^{+/-} females it was 7 ± 1.86 (12 pairs, 85 offspring). Such small litters were often cannibalized by the mother shortly after birth.

Because ectopic expression of pp39^{mos} can induce meiotic maturation of *Xenopus* eggs it was possible that the small litter sizes were a consequence of the failure of some eggs to mature in response to hormonal signals. In mice, the final stages of egg maturation are initiated in response to a surge of luteinizing hormone (LH) in the bloodstream. After LH stimulation, oocytes undergo germinal vesicle breakdown (GVBD) and initiate meiotic division leading to first polar body extrusion. At this point, eggs are released from the ovary ready for fertilization. To study these initial events in *mos*^{-/-} animals, eggs were

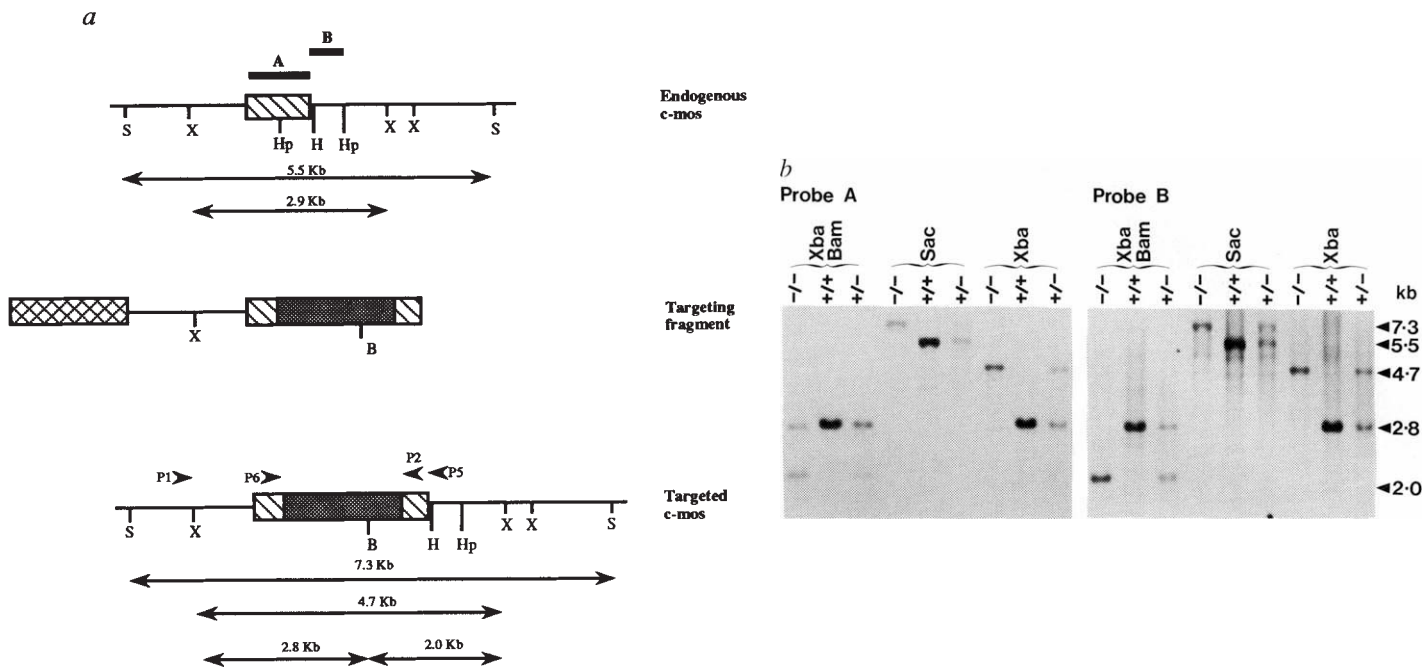


FIG. 1 Targeted disruption of the murine *c-mos* gene. *a*, Targeting vector and recombination at the *c-mos* locus. The *c-mos* 1-kb coding exon is shown hatched, the *neo*^r cassette stippled and the herpes simplex virus (HSV) thymidine kinase gene cross hatched. Bars marked A and B show the location of probes used to confirm targeting. PCR screening primers are shown by arrowheads. Expected restriction fragment sizes are indicated by arrows. B, *Bam*HI; H, *Hind*III; Hp, *Hpa*I; S, *Sac*I; X, *Xba*I. *b*, Southern blot analysis of the three genotype categories derived from heterozygote crosses.

METHODS. To generate the targeting plasmid, a 2.8 kb *Sac*I-*Hind*III *c-mos* genomic fragment from pMS-1²⁰ was cloned into the corresponding sites of pSP64 to give pSP(MS-1). A PGKneo cassette²¹ from pKJ-1

was released by *Eco*RI-*Hind*III digestion and blunt ligated into the unique *Hpa*I site within the *c-mos* coding region of pSP(MS-1) to generate pMS1(neo). A 2.0 kb blunt-ended *tk*-fragment driven by a modified HSV promoter obtained from pMC1neo was subcloned into the *Sac*I site of pMS1(neo) to generate the targeting vector pMS1(neo)tk. ES cells were cultured and electroporated with 5 µg linearized targeting construct as described previously²². G418^r/ganc^r clones were screened for a targeting event using a 500 bp *Bgl*II-*Hpa*I fragment (probe B) immediately downstream of the *c-mos* coding sequence. Lack of an intact *c-mos* coding exon was confirmed using a 900 bp *Ava*I-*Hind*III coding fragment (probe A).

isolated from Graffian follicles and matured *in vitro*¹⁴. Both mutant and wild-type eggs initiated GVBD and extruded the 1st polar body (Fig. 2) indicating that wild-type pp39^{mos} is not essential for normal oocyte maturation. This is in contrast to one of the functions of *c-mos* in *Xenopus* where ectopic expression promotes egg GVBD. As expected, eggs from wild-type animals arrested after formation of the first polar body (Fig. 2). However, *mos*^{-/-} eggs failed to arrest at this stage and proceeded to extrude the second polar body, consistent with parthenogenetic activation.

During maturation, *Xenopus* eggs accumulate pp39^{mos} which is rapidly degraded by the calcium-dependent cystein protease

calpain after fertilization¹⁵. Microinjection of synthetic *mos* RNA arrests the mitotic division of *Xenopus* blastomeres¹⁰, suggesting that one possible function of pp39^{mos} is as a component of the cytostatic factor (CSF), which holds unfertilized eggs at meiotic metaphase II. Degradation of pp39^{mos} after fertilization would allow completion of meiosis. But degradation of pp39^{mos} may not be involved in the release from metaphase arrest because the kinetics of pp39^{mos} destruction are not consistent with this^{16,17}. Also, microinjection of antisense *c-mos* oligonucleotides into murine oocytes results in chromosome decondensation and reformation of a nucleus after meiosis I, suggesting that pp39^{mos} is required for meiosis II (ref. 6). To resolve the biological func-

FIG. 2 Parthenogenetic activation of *mos*^{-/-} eggs. Animals were injected intraperitoneally with 5 IU of PMS followed 48 h later by 5 IU of hCG. Representative photomicrographs of eggs at the times indicated (in h) after hCG administration (time zero). Polar bodies are indicated by arrowheads, pronuclei by arrows. Eggs isolated from Graffian follicles; 0 h, showing germinal vesicle; 12 h, germinal vesicle breakdown has occurred and first polar body extrusion; 24 h, second polar body extrusion; 29 h pronucleus formation; Eggs isolated from oviducts; 48 h, cleavage to two cells each with a pronucleus; 72 h, 4 cell parthenogenone.

METHODS. Eggs were isolated from Graffian follicles and cultured as described in ref. 14. Egg isolation from oviducts was performed as outlined in ref. 23.

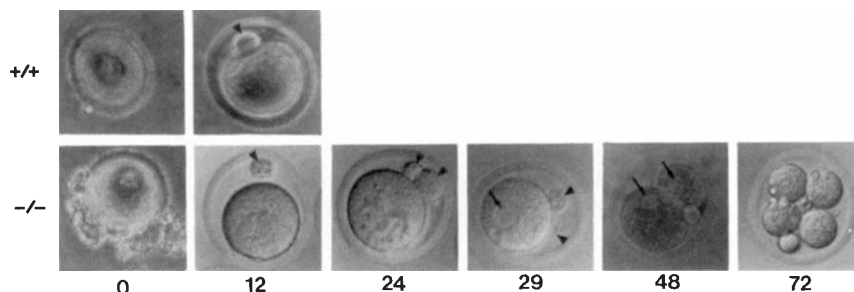


TABLE 1 Incidence of parthenogenetic activation in unfertilized eggs

Time post hCG	Genotype	Total no. of eggs			
		Class 0	Class 1	Class 2	Class 3
18 h	+/+	10	20	0	0
	-/-	0	6	33	0
32 h	+/+	0	80	0	2
	+/-	0	115	0	0
	-/-	0	0	42	129

Four-week-old mice were superovulated by PMS and hCG administration and eggs flushed from oviducts at the times indicated into M16 medium. The eggs were scored immediately into the following classes: class 0, germinal vesicle breakdown complete, no polar body; 1, eggs with a single polar body; 2, eggs with two polar bodies; 3, eggs showing cytoplasmic fragmentation.

tion of pp39^{mos} we superovulated 4-week-old virgin females and scored for the presence of eggs that had failed to arrest.

Eggs isolated from mutant animals 18 h after human chorionic gonadotropin (hCG) administration predominantly had two polar bodies and no pronuclei (Table 1). Hoechst staining indicated that both polar bodies contained DNA. Eggs isolated from normal litter mates only had a single polar body. When mutant eggs with two polar bodies were maintained in culture, about 40% developed a pronucleus (Fig. 2), indicative of true parthenogenetic development. About 30% of these cleaved to two

cells, each containing a nucleus (Fig. 2), and occasionally to four cells. Development was not observed past the four-cell stage, consistent with the limited developmental potential of haploid parthenogenones. Wild-type eggs never formed second polar bodies. Mutant eggs that failed to cleave often underwent cytoplasmic fragmentation which was rarely observed in wild-type eggs. This was most noticeable in eggs isolated 32-h after the hCG injection (Table 1). We only observed parthenogenetically activated eggs in homozygous mutant animals, demonstrating that pp39^{mos} is involved in maintaining metaphase II arrest. We suggest that the small numbers of normal offspring that arise from *mos*^{-/-} mothers may be derived from eggs that were fertilized shortly after maturation, and before parthenogenetic activation renders them incapable of being fertilized.

In the LT/Sv strain of mice, 10% of oocytes undergo spontaneous parthenogenetic activation¹⁸. These mice develop teratomas containing a variety of different cell types. Importantly, we found ovarian cysts in five mutant animals but never in age-matched *mos*^{+/+} (19 animals) or *mos*^{+/-} (10 animals) controls. The incidence of the cysts was high (4/7) in animals older than six months but we found a small (<2 mm diameter) cyst in an animal as young as one month. The cysts were typically serous, bilateral, multilocular in older mice and in some cases (2/4) infiltrated with blood. Two types of cyst were observed. The more common (3/5) simple smaller cysts were composed of predominantly epithelial tissue (Fig. 3a) but the larger cysts (2/5), which appeared to develop at a site internal to the ovary, consisted of several tissue types typical of a teratoma. These included simple columnar epithelia, pigmented epithelia syncytial cells with large nuclei very similar in appearance to trophoblast giant cells (Fig. 3b) and folliculated tissue delineated by a basement membrane (Fig. 3d) containing eosinophilic colloid (Fig. 3c, d). This latter tissue, at least superficially, has a thyroid-like appearance but this remains to be confirmed immunohistologically. Within areas of this tissue we also observed hyperplasia of secretory epithelia forming papilliform processes and showing active resorption of colloid material (Fig. 3e, f).

These data demonstrate genetically that one function for pp39^{mos} is to arrest developing mammalian eggs during meiosis before fertilization and that it is essential for mammalian cyto-static activity. Eggs lacking a functional pp39^{mos} undergo spontaneous parthenogenetic activation, as defined by completion of the second meiotic division, extrusion of the second polar body and pronucleus formation without fertilization. The mutant mice also develop ovarian cysts, some of which consist of several tissue types including possible thyroid tissue. In humans, about 10% of all benign cystic teratomas of the ovary contain thyroid tissue¹⁹ and it will be of interest to investigate the status of the *c-mos* locus in these tumours and to analyse the *mos*^{-/-} mice further to see if they provide an animal model for this type of human ovarian pathology. □

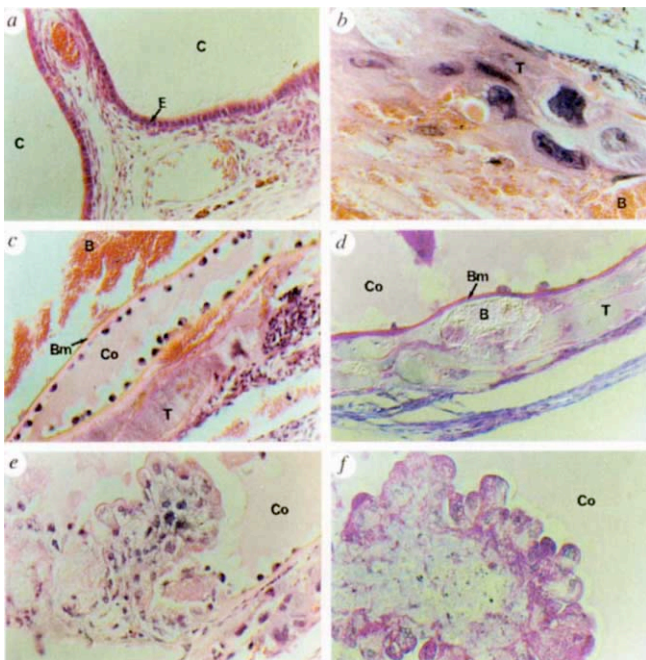


FIG. 3 Histology of ovarian cysts a, Columnar epithelium (E) lining the wall of a simple cyst (C) ($\times 200$, haematoxylin and eosin (H and E) stain); b, trophoblast-like giant cells (T) in a blood (B) filled cyst ($\times 200$, H and E stain); c, thyroid-like tissue illustrating close association with trophoblast-like cells ($\times 200$, H and E stain; Co, colloid); d, thyroid-like tissue stained with periodic acid-schiff (PAS) to illustrate basement membrane (Bm) layer surrounding the follicle ($\times 200$, PAS, H and E stain); e, hyperplastic secretory epithelia infiltrating colloid material ($\times 200$, H and E stain); f, secretory epithelia stained with PAS to show glycoproteins and illustrate high level of secretory activity ($\times 400$, PAS, H and E).

METHODS. Cysts were fixed in 4% phosphate buffered formalin, embedded in paraffin wax and 10- μ m sections cut and stained.

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Parthenogenetic activation of oocytes in *c-mos*-deficient mice

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In *Xenopus* the *c-mos* proto-oncogene product (Mos) is essential for the initiation of oocyte maturation¹, for the progression from meiosis I to meiosis II^{2,3} and for the second meiotic metaphase arrest, acting as an essential component of the cytostatic factor CSF^{4,5}. Its function in mouse oocytes is unclear^{6–9}, however, as is the biological significance of *c-mos* mRNA expression in testes^{1,10} and several somatic tissues^{1,10,11}. We have generated *c-mos*-deficient mice by gene targeting in embryonic stem cells. These mice grew at the same rate as their wild-type counterparts and reproduction was normal in the males, but the fertility of the females was very low. The *c-mos*-deficient female mice developed ovarian teratomas at a high frequency. Oocytes from these females matured to the second meiotic metaphase both *in vivo* and *in vitro*, but were activated without fertilization. The results indicate that in mice Mos plays a role in the second meiotic metaphase arrest, but does not seem to be essential for the initiation of oocyte maturation, spermatogenesis or somatic cell cycle.

The *c-mos*-deficient mice were generated by homologous recombination in TT2 embryonic stem (ES) cells^{12,13} (for details, see Fig. 1 legend). The disruption of the *c-mos* gene in these mice was confirmed both from its genomic structure by Southern blot analysis (Fig. 1b) and by RNase protection assay of messenger RNA from the testis and ovary (Fig. 1c), where *c-mos* mRNA is most highly expressed in adult mice¹⁰.

Both *c-mos*-deficient male and female mice were apparently normal. No histological abnormality was found in brain, heart, lung, kidney, mammary gland, epididymis or placenta, in all of which low but significant levels of *c-mos* mRNA expression have been reported^{10,11}. Testes of homozygous mutant mice were also

TABLE 1 Breeding of *c-mos*-deficient females

Number of pregnancies	Number of mice		Average number of offspring	
	+/-	-/-	+/-	-/-
0	0	10	—	—
1	0	11	—	1.7
2	5	4	6.5	1.8
3	17	0	7.2	—
4	3	0	7.1	—

Twenty-five heterozygous (+/-) or homozygous (-/-) *c-mos*-deficient females were consecutively mated with fertile normal males from 2–6 months of age. Four non-pregnant (-/-) females developed massive ovarian teratomas at 4–6 months of age. These are included in zero frequency of pregnancy. Wild-type mice were not used for breeding, but the results for heterozygous mice were normal.

histologically normal (data not shown), although strong expression of *c-mos* mRNA¹⁰ and its protein¹⁴ occur in this organ. Eleven test-mated males all fathered offspring normally, with an average litter size of 6.5. Thus Mos appears to be non-essential in somatic cell growth and male germ-cell differentiation. In contrast, fertility of *c-mos*-deficient females was very low (Table 1). Ten out of 25 females examined were infertile, and even in fertile females, the frequency of pregnancy during the mating period and the number of offspring were also markedly low compared with heterozygous females. Ovarian histology of *c-mos*-deficient females showed normally differentiated germ cells and somatic cells, but significant numbers of oocytes appeared to be parthenogenetically activated (see below: Fig. 2e, f).

To determine the stage of oocyte maturation at which Mos participates, immature oocytes were collected and cultured *in vitro*¹⁵. As shown in Fig. 3a, the oocytes from *c-mos*-deficient female mice underwent germinal vesicle breakdown (GVBD) over a similar timescale to those from heterozygous and wild-type mice. These results contradict reports in which GVBD of mouse oocytes was blocked by the electrotransferred antibody against Mos⁶, although these earlier results were dependent on the antibodies used^{6,7}.

After GVBD, oocytes from both wild-type and *c-mos*-deficient female mice progressed through the first meiotic metaphase, emitted the first polar body, reached the second meiotic metaphase in 20 hours after liberation from ovarian follicles, and remained at this stage for at least 8 hours; frequency of oocytes with polar body was 77 and 74% among 236 wild-type and 289 *c-mos*-deficient oocytes, respectively. Neither chromosome decondensation nor DNA synthesis occurred throughout the maturation process (results not shown). However, the first polar body emission in *c-mos*-deficient oocytes was reproducibly delayed compared with that in wild-type oocytes (not shown), although the emission occurred asynchronously. There have been two observations relating to the role of Mos in mouse oocyte maturation. (1) *c-mos*-specific antisense oligonucleotides injected into oocytes prevent extrusion of the first polar body⁸; as this result was obtained in relatively short-term culture (<15 h), it could correspond to a delay in the first polar body emission that we have noted. (2) *c-mos*-specific antisense oligonucleotides failed to inhibit polar body emission⁹, which is consistent with our result but the oocytes injected with the nucleotides did not appear to enter the second meiotic metaphase (see below).

When oocytes were observed at 36–41 h after liberation from ovarian follicles, almost all wild-type oocytes remained arrested at the second meiotic metaphase, but as many as 66% of *c-mos*-deficient oocytes were released from the arrest, as evidenced by their nuclear formation (Fig. 2a, b). Most of the released oocytes had one or two nuclei, and some had divided into two or more cells. DNA synthesis was detected in nuclei of all these released oocytes, indicating their parthenogenetic activation (Fig. 2c, d). These results are apparently consistent with those from oocytes

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