Development of Gynogenetic Eggs in the Mouse: Implications for Parthenogenetic Embryos

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Abstract. *Mouse eggs with different genetic constitutions were prepared by micromanipulation of fertilized diploids and triploids. The diploid gynogenones, activated by the male gamete which was then removed, developed at best to about the 25-somite stage as did the genetically similar diploid parthenogenones stimulated to develop in the complete absence of the male gamete. The failure of development to term in both cases may be due to homozygosity and does not appear to be due to a lack of extragenetic contribution from spermatozoa.*

Embryos that have been stimulated to develop in the complete absence of the male gamete are termed parthenogenetic. Embryos that develop as a result of activation by the male gamete, but contain only the maternal chromosomes because the male gamete degenerates before fusing with the egg nucleus, are termed gynogenetic. The development of gynogenetic or parthenogenetic embryos to term could have application in animal breeding both because they would be female, with similarities to the maternal genotype (*1*), and because they would be useful in detecting recessive genes. However, parthenogenetic mouse embryos do not develop to term because of homozygosity and expression of recessive genes or because of a lack of extragenetic contribution from spermatozoa (*2*). Diploid parthenogenones develop at best to the 25-somite stage (*3*) although in combination with fertilized embryos they can develop as chimeras to adulthood (*4, 5*) with viable germ cells (*6*). However, it has been reported that fertilized enucleated eggs with nuclei transplanted from parthenogenetic embryos have developed into viable young (*7*). Mice have also been obtained from diploidized androgenetic (containing only paternal chromosomes) and gynogenetic embryos (*8*), although in the human the androgenetic embryos give rise to hydatidiform moles (*9*). Nevertheless, cytoplasmic and nongenetic factors contributed by spermatozoa have been suggested to be crucial for development (*7, 8*). By contrast, in some studies it has not been possible to obtain development of homozygous embryos beyond the immediate implantation period (*1, 10–12*). We therefore examined the development of eggs of various genetic constitution prepared by micromanipulation from fertilized diploid and dygynic triploid eggs (*13, 14*). We demonstrate that even dygynic embryos, which presumably contain cytoplasmic and nongenetic factors contributed by spermatozoa, develop to about the same level as parthenogenones. This suggests that failure to reach term in both cases is probably due to homozygosity and not to the lack of extragenetic contribution from spermatozoa.

Fertilized eggs were obtained from superovulated females from four strains of mice (see Tables 1 and 2). Fertilized eggs or triploids obtained by the suppression of the second polar body extrusion (*13, 14*) were used to prepare embryos with varying genetic constitution by micromanipulation (Fig. 1). All the manipulated and control eggs were cultured overnight in Brinster's medium (BMOC-3) containing bovine serum albumin (BSA) (*15*) at 37°C in humidified 5 percent CO_2 in air. The hemizygous haploid eggs were cultured in the presence of cytochalasin B (5 μg/ml) to suppress the first cleavage division and restore the diploid state (*8, 11, 16*).

The next morning the eggs cleaved to the two-cell stage, excepting those in medium containing cytochalasin B which were arrested at the one-cell stage but in which two distinct nuclei were usually detected. These eggs were washed extensively in BMOC-3 containing BSA and cultured for a further 1 to 2 hours. Experimental embryos were then transferred to the right oviducts of day-1 pseudopregnant MF1 females (obtained by mating with vasectomized males of known sterility; day 1 = day of plug) and normal two-cell fertilized eggs were

Table 1. The development of manipulated MF1 triploid and diploid eggs. The recipient embryos in all but the restored diploid group C were examined between days 9 and 11 of pregnancy. This exceeded the expected time of deterioration of most embryos but still enabled us to assess the stage reached and carry out GPI typing (*28*). In group A retarded embryos were detected, 18 of them with 15 to 26 somites and a beating heart, but on the whole they were deteriorating. There was also a failure to "turn" properly and one "squirrel" embryo with the tail fused to the head was found; some embryos had enlarged head regions. The remaining 12 embryos were at the presomite stage to about six somites; most small embryos had prominent amnion and abnormal allantois. However, in all cases the yolk sac and trophoblast were much more substantial than in group B, where all the embryos were in small yolk sacs and had meager trophoblasts. Embryos on day 10 ranged from eight-somite to early headfold stages; a day-11 embryo with 25 somites and deteriorating tissues is shown in Fig. 2. The animals in group C were examined on day 19 of pregnancy when normal live young were obtained by autopsy. In group E, only one small egg cylinder was detected, while in group F no embryonic derivatives were found. It is likely that in both groups E and F very few embryos reached the blastocyst stage and even these were probably deficient in inner cell mass. N.E., not examined.

Group*	Number of eggs survived/ number operated	Cleaved	Number transferred		Number implanted (%)		GPI typing†
			Total	To recipients that became pregnant	Total	With embryos	
A Triploid			118	78	44 (56)	30 (38) Retarded	ab
B Dygynic	112/131	101	97	77	34 (44)	7 (9) Retarded	aa
C Restored diploid from triploid	69/82	69	62	44	24 (55)	16 (36) Normal‡	ab
D Fertilized			134	97	79 (81)	75 (77) Normal	ab
E Hemizygous diploid gynogenetic	151/186		140	95	15 (16)	1 (1) Very retarded	N.E.
F Hemizygous diploid androgenetic	90/120		89	62	4 (6)	0	

*The letters and descriptions correspond to the eggs illustrated in Fig. 1. †MF1 albino female mice are homozygous for the isozyme of glucose phosphate isomerase (GPI) and are referred to as Gpi-1^a. These Gpi-1^a females were mated with (C57BL × CBA)F_1 males which are also homozygous for GPI and are nonalbino, and are referred to as Gpi-1^b. ‡Live young delivered on day 19 of pregnancy by autopsy. All other embryos were examined between days 9 and 11 of pregnancy.

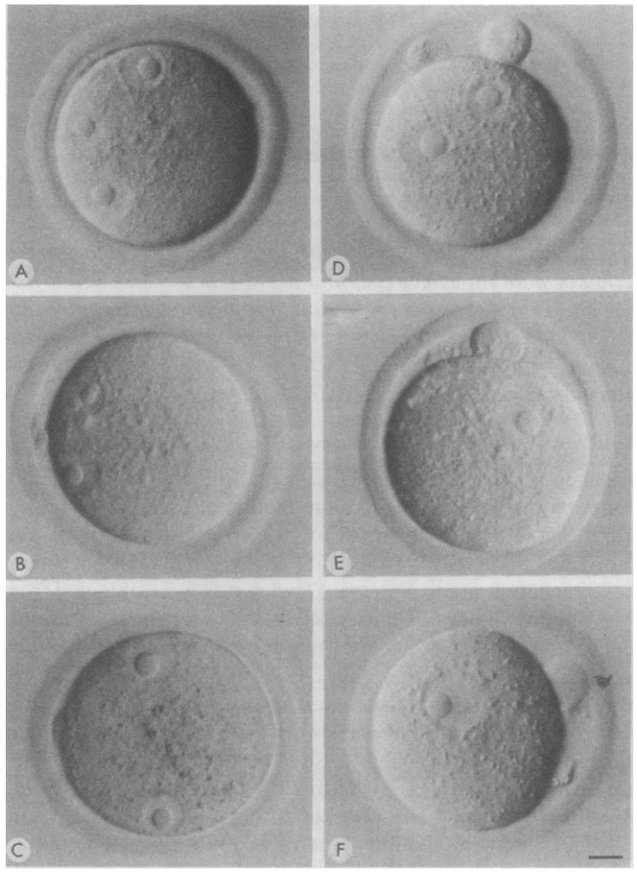
transferred to the left oviducts as controls.

The results in the MF1 strain (ARC colony) (Table 1 and Fig. 2) indicate that the genetic constitution of eggs had considerable impact on the development of embryos. When the hemizygous diploidized gynogenetic embryos were transferred, 16 percent implanted and only one very retarded embryo was obtained on day 9 of pregnancy. The hemizygous diploid androgenetic embryos fared worst of all, probably because the half of them with a Y chromosome could not cleave more than twice (*17*). However,

Table 2. Development of manipulated eggs. BALB/c females were mated with the F_1 males; the other two strains of females were mated with MF1 males. N.E., not examined.

Strain of mice	Group	Number of eggs survived/ number operated	Cleaved	Number transferred		Number implanted*		GPI typing
				Total	To recipients that became pregnant	Total	With embryos	
(C57BL × CBA)F_1	Hemizygous diploid gynogenetic	25/26		25	20	0	0	
	Dygynic	59/61	54	54	36	14	10†	bb
C57BL/6	Hemizygous diploid gynogenetic	141/176		121	92	6	0	
	Dygynic	100/125	37	31	16	7	1‡	N.E.
BALB/c	Hemizygous diploid gynogenetic	96/128		67	57	3	0	
	Dygynic	67/82	8	8	7	1	0	

*On day 13 of pregnancy. †Retarded embryos. ‡Small abnormal mass of embryonic tissue.



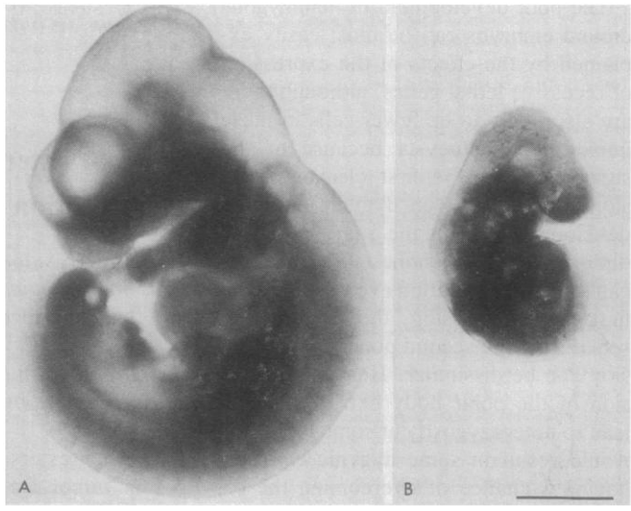


Fig. 1 (left). Fertilized eggs were isolated at approximately 16 to 17 hours after ovulation, and the cumulus cells were removed with hyaluronidase (300 IU/ml) in phosphate-buffered medium containing BSA (4 mg/ml) (*25*). We added cytochalasin B (5 μg/ml) to the BMOC-3 and BSA to suppress the second polar body extrusion and cause formation of triploids (*13*) (10 to 60 percent of fertilized eggs) during culture at 37°C in humidified 5 percent CO_2 in air. The eggs were examined periodically over 6 to 7 hours for pronuclei and the triploid eggs resulting from the suppression of the second polar body were separated from normal diploid fertilized eggs. One of the pronuclei was microsurgically removed from the eggs on a Leitz micromanipulator under a Zeiss microscope fitted with Nomarski optics. The procedure was carried out in medium PB1 with BSA and cytochalasin B (5 μg/ml) alone (*7*, *16*, *26*) or with Colcemid (0.1 μg/ml) as well (*27*). The pronuclei were pinched off from eggs as small karyoplast fragments essentially as described recently (*27*). The male pronucleus was normally distinguished by its large size and position among the pronuclei and relative to the second polar body when present. Eggs in which the identity of the pronuclei was ambiguous were rejected, and any resulting embryos were checked by GPI typing. (A) Triploid egg with two female pronuclei and one male pronucleus. Note the male pronucleus at the top end of the egg and the absence of a polar body but some remains of the first polar body. (B) Dygynic egg after the removal of the male pronucleus from a triploid egg. (C) Egg restored to a normal genetic constitution after removal of one of the two female pronuclei from a triploid egg retaining one male and one female pronucleus. (D) Normal fertilized egg. Compare with the triploid egg and note that the second polar body has been extruded. (E) Hemizygous haploid gynogenetic egg after the removal of the male pronucleus. (F) Hemizygous haploid androgenetic egg after the removal of the female pronucleus. The eggs in (E) and (F) were diploidized before transfer to recipients as described in the text. Scale bar, 10 μm. Fig. 2 (right). (A) Control MF1 embryo on day 11 of pregnancy with approximately 35 somites. (B) The dygynic MF1 embryo from the contralateral horn of the same female with about 25 somites. The heart was beating but the rest of the embryo was deteriorating. A second dygynic embryo with ten somites and beating heart but otherwise grossly disorganized was also found in the same female. Scale bar, 1 mm.

the dygynic embryos developed better since 44 percent implanted; seven embryos, albeit retarded, were found. These were confirmed as dygynic embryos homozygous for glucose phosphate isomerase (GPI-aa). Significantly, in triploid eggs where the genetic constitution was restored to normal after the removal of one of the two female pronuclei, development continued to term. Over 50 percent of such embryos implanted and 16 (36 percent, with dark eyes and GPI-ab) developed to term. The unoperated triploid embryos also implanted (38 percent) and 30 embryos with a variety of abnormalities were obtained on day 11 of pregnancy, similar to a previous study (*18*). Development of hemizygous diploid gynogenetic and dygynic embryos from inbred C57BL/6 and BALB/c (from Olac, Bicester, England) and (C57BL × CBA)F_1 mice was also examined (Table 2). Although a number of them implanted, only 11 sites contained dygynic embryonic derivatives.

The poor development of hemizygous diploid embryos can be most easily explained by the effects of the expression of recessive lethal genes, although there are also likely to be fewer cells in these implanting blastocysts because of the suppression of the first cleavage division. However, even dygynic embryos developed to about the same extent as diploid parthenogenones (*3, 12*). Because of recombination events during the first meiotic division, the two haploid sets during the second polar body extrusion can be dissimilar. Hence, suppression of the polar body extrusion could lead to heterozygosity at some loci. This would result in some dygynic embryos having a chance of overcoming the effects of recessive lethal genes and might partly account for their relatively better development. In outbred amphibians a few viable dygynic adults have been obtained (*19–21*). In mammals there is a great excess of genes and chance combination leading to viable dygynic genotype is perhaps far less. However, in some tissues, such as trophectoderm, there is preferential inactivation of the paternal X chromosome (*22, 23*). Hence, errors in X-inactivation after implantation may occur which could provide an explanation for the developmental failure of both gynogenones and parthenogenones, since they lack a paternal X chromosome, although X-inactivation does occur in extraembryonic membranes of parthenogenones (*24*). We have consistently noticed very meager development of trophoblasts in parthenogenones and gynogenones.

The role of the extragenetic contribution from spermatozoa in development still needs explanation, since a few eggs with nuclei from parthenogenetic embryos of strain LT/Sv transplanted to enucleated fertilized eggs develop to term (*7*). This result suggests that some of the dygynic embryos from the four strains of mice used in the present study should also develop to term, but thus far they have not. Therefore, the role of cytoplasmic factors in the development of gynogenones and parthenogenones remains to be determined.

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Phorbol Ester Receptors: Autoradiographic Identification in the Developing Rat

Abstract. *Autoradiography with ^{3}H-labeled phorbol dibutyrate was used for the light microscopic detection of phorbol ester receptors in rat fetuses. In 15- and 18-day fetuses, as well as in adult rats, receptors were found to be concentrated in the central nervous system. The localization of receptors in the ventral marginal zone of the fetal neural tube, the lens of the eye, and other sites suggests a role for phorbol ester receptors in cellular process extension and cell-cell interaction.*

Phorbol esters are among the most potent tumor-promoting agents known. Their effects are mediated by specific cell membrane receptors that can be labeled with [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) (*1*). These receptors are distributed ubiquitously in animal tissues but their highest concentrations are in the brain (*2*). Solubilized phorbol ester receptors copurify with protein kinase C, a calcium- and phospholipid-dependent enzyme (*3*) that is stimulated by phorbol esters (*4*). Phorbol ester receptors may be relevant to normal cell growth and differentiation, since phorbol esters induce a number of enzymes—including epidermal transglutaminase and ornithine decarboxylase (*5*)—which participate in rapid tissue growth (*6*). We now report selective localizations of phorbol ester receptors in rat fetuses. Our findings suggest that phorbol ester receptors have a role in ontogenetic process extension rather than in cell division.

We used light microscopic autoradiography (*7*) to determine the locus of [^{3}H]PDBu (New England Nuclear) binding to slide-mounted, unfixed tissue sections (8 to 10 μm) derived from 15- and 18-day rat fetuses and adult rat brain. Sprague-Dawley rats were obtained from Charles River. We assigned gestational age by using the appearance of the vaginal plug as day 0 of gestation. Unfixed tissue was used to avoid altering the properties of the [^{3}H]PDBu binding sites. Slide-mounted tissue binds [^{3}H]PDBu with an affinity of 10 n*M*, and 4β-phorbol 12-myristate 13-acetate inhibits binding at 50 to 100 n*M*. Equilibrium is attained within 30 minutes. These properties are consistent with the [^{3}H]PDBu sites labeled in studies with tissue homogenates (*2*). Slide-mounted sections were incubated with 2 n*M* [^{3}H]PDBu in 100 m*M* NaCl and 50 m*M* tris-HCl, *p*H 7.7, for 1 hour at room temperature. Separate sections were incubated with 2 n*M* [^{3}H]PDBu and 1 μ*M* 4β-phorbol 12-myristate 13-acetate (Sig-