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Original Article

Prolonged development of normal and parthenogenetic postimplantation mouse embryos *in vitro*

LEONID I. PENKOV[1], EUGENE S. PLATONOV[1] and DENIS A.T. NEW[2]*

[1]*N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia and* [2]*The Physiological Laboratory, University of Cambridge, Cambridge, United Kingdom*

ABSTRACT **Parthenogenetic mammalian embryos show reduced placental development and do not develop beyond the 25-somite stage. But non-parthenogenetic embryos in culture, without a functional placenta, can develop to 40 somites or more. We have therefore examined the possibility that parthenogenetic embryos might also show prolonged development in culture. After parthenogenetic activation and diploidization, 23% of CBA and 56-58% of hybrid (CBAxC57BL/6) F1 mouse eggs developed in culture to blastocysts. When transferred to pseudopregnant recipients: 60% of the CBA blastocysts implanted and 26% of these developed to somite stage embryos; 71-72% of the hybrid blastocysts implanted and 11-17% of these developed to somite stage embryos. Improved development of postimplantation embryos explanted into culture at about the 15-20 somite stage was obtained by opening the visceral yolk sac (without exteriorizing the embryo). All the normal (non-parthenogenetic) embryos cultured in this way developed to more than 35 somites and many reached 45-55 somites. Under the same conditions, 11/17 diploid parthenogenetic CBA embryos developed in culture to more than 35 somites and 5 of these reached 45 somites; and 9/28 diploid parthenogenetic (CBAxC57BL/6) F1 embryos developed to 35 somites or more and 5 of these reached 45 somites. The size and protein content of the parthenogenetic embryos after culture was less than that of the normal embryos of equivalent stages. These results raise new possibilities for the analysis of parthenogenesis and genomic imprinting, including studies of the effects of adding to the culture medium specific growth factors and demethylating agents.**

KEY WORDS: *embryo culture, genomic imprinting, parthenogenesis*

Introduction

In mammals, parthenogenetic embryos usually die before or shortly after implantation. Few develop as far as the 25-somite stage (Kaufman *et al.*, 1977; Surani and Barton, 1983).

Death of parthenogenetic embryos results from the absence of the paternal genome. Because of genomic imprinting, there are differences in the expression of some homologous maternal and paternal alleles and both are necessary for normal development (DeChiara *et al.*, 1991; Ferguson-Smith *et al.*, 1993; Surani *et al.*, 1993).

One result of the lack of the paternal genome in parthenogenetic rodent embryos is poor development of the trophoblast, ectoplacental cone and placenta (Surani and Barton, 1983; Surani *et al.*, 1984), and this is probably the main reason for the failure of development of the embryo around the 25-somite stage. Barton *et al.* (1985) and Gardner *et al.* (1990) have shown that, if a composite embryo is made by combining a parthenogenetic inner cell mass with normal trophoblast (and endoderm), development may be prolonged to 40-44 somites (though the embryos die shortly after this stage – see Allen *et al.*, 1994 for further discussion).

When normal non-parthenogenetic rat or mouse embryos at head fold or early somite stages are explanted and grown in culture, there is usually reduced development of the trophoblast/ectoplacental cone. Nevertheless, such embryos may develop 40 somites or more (New, 1978, 1992; Hunter *et al.*, 1988). Since the trophoblast appears to be inessential to the embryo in these cultures, we thought it would be interesting to see whether cultured postimplantation parthenogenetic embryos, also lacking in trophoblast, would develop to a similar extent. If so, it could provide a useful new approach to the investigation of the mechanisms of genomic imprinting and parthenogenetic development. We therefore made the following experiments, using mouse eggs parthenogenetically activated, diploidized and transferred to the uterus of a foster mother by standard methods, then explanted at early somite stages and grown in culture. Parthenogenetic embryos were obtained from inbred CBA and from hybrid (CBAxC57BL/6) F1 mice. It was known from previous studies that, although the

***Address for reprints:** The Physiological Laboratory, University of Cambridge, Downing Street , Cambridge CB2 3EG, United Kingdom. FAX: 44.1223.333840.

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TABLE 1

DEVELOPMENT OF NORMAL (NON-PARTHENOGENETIC) EMBRYOS EXPLANTED AT DAY 9

Yolk sac	Number of embryos cultured	Number of embryos with 30 or more somites (36 h)	35 or more somites (48 h)	40 or more somites (72 h)	45 or more somites (72 h)	50 or more somites (96 h)
intact	22	22	10	2	—	—
Open (E)	20	20	20	9	2	1
Open (I)	28	28	28	25	12	7

Development of embryos cultured with the yolk sac intact, or with the yolk sac opened and the embryo exteriorized (Open E), or with the yolk sac opened and the embryo left inside (Open I).

percentage of activated CBA eggs that develop to the blastocyst stage is low, many of these blastocysts can develop *in vivo* to somite stages (Penkov and Platonov, 1992; Penkov *et al.*, 1992).

Results

Culture of normal (non-parthenogenetic) postimplantation embryos

The crown rump length of all the embryos at explantation was 1.6-1.8 mm. The results are summarized in Table 1.

In the first group, 22 embryos were cultured for 48-72 h with the visceral yolk sac intact. For the first 36 h, growth and development was similar to that *in vivo* up to 30-32 somites. 10 embryos developed to 35 somites or more but only 5 maintained a blood circulation in the yolk sac until this stage. Two of these embryos attained 40 and 42 somites respectively, but all were dead by 72 h.

In the second group (Open E), 20 embryos were grown under the same conditions, except that the yolk sac (and amnion) was opened and the embryo exteriorized at about the 30-somite stage. These embryos developed better than those with the yolk sac intact. All showed growth and development similar to that *in vivo* up to 35-38 somites, but in many the yolk sac blood circulation had failed by this stage. Only 9 survived to 72 h, with 40 or more somites.

In the third group (Open I), 28 embryos were grown under the same conditions, except that the yolk sac was opened at about the 30-somite stage (and later the amnion). The embryo was left inside the yolk sac. These embryos grew better than those of both the other two groups. Details are shown in Table 2. All the embryos showed growth and development similar to that *in vivo* up to 35-38 somites, and all still had a good yolk sac blood circulation at 48 h. After 48 h, growth was slower and the embryos were smaller than *in vivo*. Differentiation of the anterior part of the embryo continued without major anomalies but growth and differentiation of the hind part was reduced. 11 of the embryos showed an allantoic blood circulation up to 72 h, and all but two developed ocular pigmentation (Fig. 1). Six embryos developed 50 or more somites, and 5 of these still had a yolk sac blood circulation at 96 h. The blood circulation had ceased by 108 h, but one embryo developed to 56 somites.

Culture of diploid parthenogenetic embryos from the inbred strain CBA

After activation and diploidization, 23% of the eggs developed to blastocysts during 120 h in culture. 176 of the blastocysts were transferred to pseudopregnant CBA recipients; 105 (60%) of them implanted and 27 became somite stage embryos, 19 developing more than 14 somites (Table 3). 17 of these embryos were explanted into culture, with crown rump length 1.7-2.7 mm and a blood circulation in the yolk sac, but with only a very small ectoplacental cone. Details of their development in culture are shown in Tables 4 and 5. 11 of the embryos developed to 38 or more somites, and 3 of these finally reached 47 somites (Fig. 2). Up to about 35 somites, the embryos had normal morphology. After this, growth and development was retarded, particularly in the hind part of the embryo, including the hind limb buds. 9 of the embryos developed ocular pigmentation, but none had a yolk sac blood circulation at 48 h, and none developed an allantoic circulation. The embryos were smaller, and had a lower protein content, than the cultured normal embryos of equivalent stages.

TABLE 2

DEVELOPMENT OF 28 NORMAL EMBRYOS EXPLANTED AT DAY 9 AND CULTURED WITH OPENED YOLK SAC

Embryo No.	Crown-rump length (mm) at 72 h	Yolk sac circulation 48 h	72 h	96 h	Fore limb (mm)	Hind limb (mm)	Final somite number	Ocular pigment	Embryo protein (μg)
1	5.5	+	+		1.0	0.6	45	+	
2	4.5	+			0.8	0.5	42	+	496
3	4.8	+			0.6	0.5	41	+	500
4	5.9	+	+	+	1.2	1.1	53	++	464
5	3.8	+			0.6	0.4	40	+	
6	5.3	+			0.5	0.4	42	+	408
7	4.1	+			0.7	0.4	40	+	304
8	4.6	+	+		0.8	0.7	47	++	
9	5.0	+			0.6	0.4	40	+	388
10	6.0	+	+		1.0	0.6	47	++	576
11	5.3	+	+	+	1.2	1.1	51	++	
12	3.3	+			0.5	0.2	37	-	448
13	4.8	+			0.5	0.3	39	-	440
14	4.3	+			0.5	0.4	41	+	
15	4.9	+	+		0.7	0.5	47	++	
16	5.3	+			0.7	0.5	46	+	608
17	4.7	+	+		0.9	0.6	50	++	560
18	4.1	+			1.0	0.6	42	+	
19	*	+			0.9	0.5	42	+	
20	4.4	+			0.7	0.3	40	+	364
21	5.0	+	+		1.1	0.7	47	+	552
22	6.5	+	+	+	1.3	1.2	56	+++	
23	3.0	+			0.7	0.2	38	+	408
24	3.6	+			0.6	0.3	40	+	412
25	5.8	+	+	+	1.2	1.1	55	+++	
26	5.0	+			0.6	0.5	44	+	
27	6.0	+	+	+	1.0	0.7	52	++	
28	4.8	+			0.6	0.5	40	+	

(Embryos of bottom row of Table 1). *This embryo was removed from culture at 48h, with crown-rump length 4.4 mm.

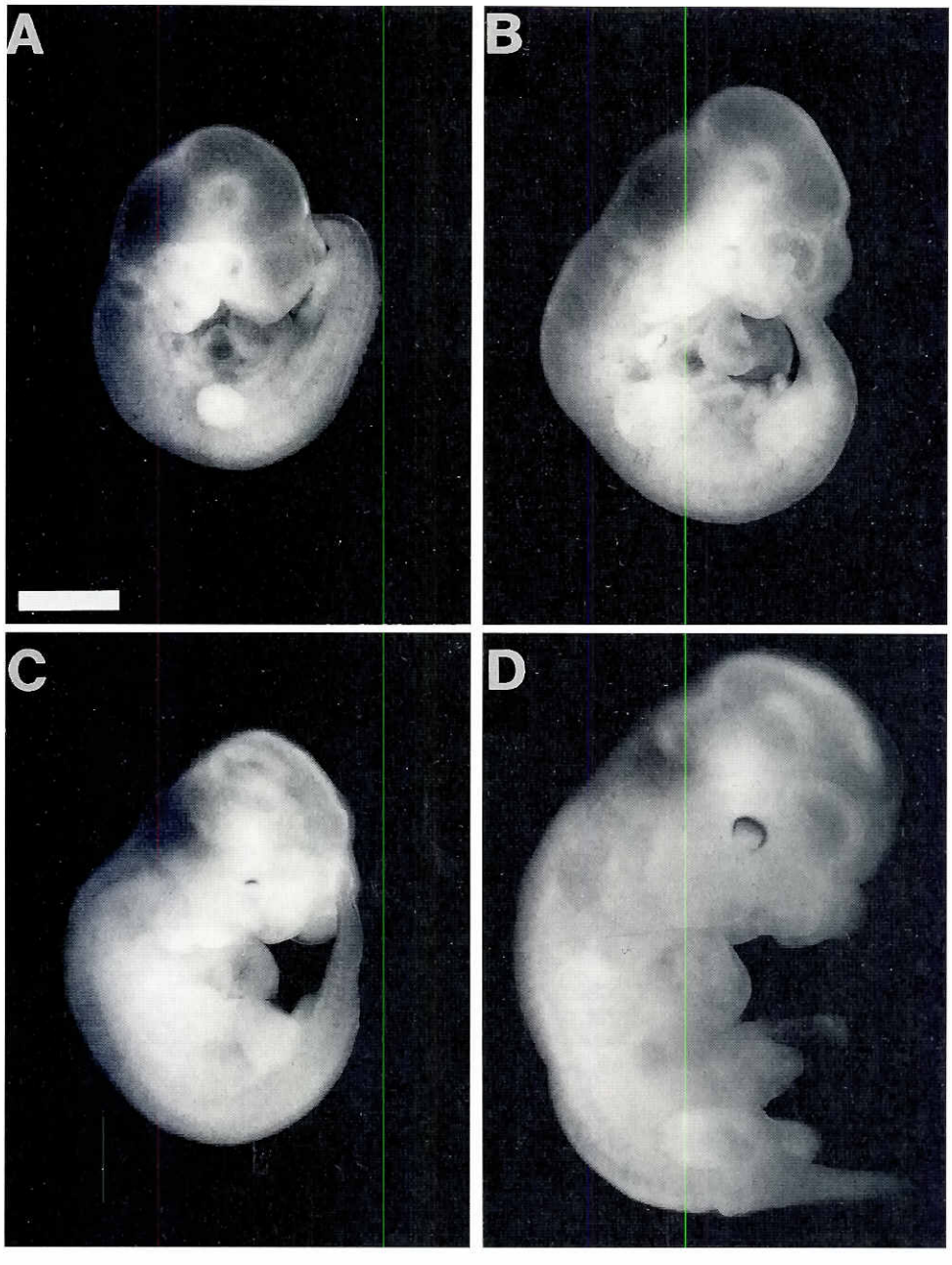

Fig. 1. Normal (non-parthenogenetic) embryos after various periods in culture: (A) *24 h, embryo with 33 somites;* **(B)** *48 h, 42 somites (Table 2, No. 19);* **(C)** *72 h, 47 somites (Table 2, No. 8);* **(D)** *96 h, 56 somites (Table 2, No. 22).* **(B,C and D)** *show ocular pigment. All embryos were photographed before fixation and are shown at the same magnification: bar, 1 mm. [Embryo C was slightly larger than B after culture (Table 2) but shunk during 24 h storage in saline before photography].*

Culture of diploid parthenogenetic embryos from the (CBAxC57BL/6) F1 hybrid

After activation and diploidization, 56-58% of the eggs developed to blastocyst stages after 96 h in culture. 119 of the blastocysts were transferred to CBA recipients: 86 (72%) implanted and 9 became somite stage embryos, 5 developing more than 14 somites. 852 blastocysts were transferred to (CBAxC57BL/6) F1 recipients: 602 (71%) implanted and 105 became somite stage embryos, 29 developing more than 14 somites (Table 3). 5 of the first group and 23 of the second group were chosen for culturing, and the development in culture of these 28 embryos is shown in Tables 4 and 6. The crown rump length of the embryos at explantation ranged from 1.5 to 2.8 mm; all had only a very small ectoplacental cone, and only 22 had a yolk sac blood circulation, in some poorly developed. 18 of the embryos developed 30 or more somites, but many had major anomalies – microcephaly, failure of closure of the posterior neural tube, and failure of chorio-allantoic fusion. Only 9 embryos developed 35 somites or more (without anomalies), 7 of them showing ocular pigmentation. Five continued to develop to 45 somites (Fig. 2). None of the embryos had a yolk sac blood circulation at 48 h, and none developed an allantoic circulation. The embryos were smaller and had a lower protein content than the cultured normal embryos of equivalent stages.

TABLE 3

DEVELOPMENT OF PARTHENOGENETIC EMBRYOS, FIRST *IN VITRO*, THEN AFTER TRANSFER TO PSEUDOPREGNANT RECIPIENTS

Strain of eggs	Number of eggs	Number of blastocysts (% of eggs)	Number of blastocysts transferred	Strain of recipient	Number of implantations (% of blastocysts)	Number of somite embryos (% of implants)	Number of embryos with >14 somites
CBA	827	191 (23%)	176	CBA	105 (60%)	27 (26%)	19
F, (CBA x C57BL/6)	217	122 (56%)	119	CBA	86 (72%)	9 (11%)	5
F, (CBA x C57BL/6)	1537	888 (58%)	852	F, (CBA x C57BL/6)	602 (71%)	105 (17%)	29

Discussion

It is known that rat embryos explanted at head-fold or early somite stages can develop in culture to 40-45 somites (New *et al.*, 1976; New, 1992), and if explanted later (at 38-43 somites) may reach 54-55 somites (New and Coppola, 1970; Cockroft, 1976). But as far as we are aware, the maximum development previously reported for mouse embryos in culture is 30-32 somites (Sadler and New, 1981; Hunter *et al.*, 1988). Before culturing parthenogenetic mouse embryos, we improved the culture method for normal (non-parthenogenetic) mouse embryos.

When we cultured normal mouse embryos with the visceral yolk sac intact, growth and development of the embryos for the first 36 h was similar to that reported by other workers (Sadler, 1979; Hunter *et al.*, 1988) and only 10/22 of the embryos continued to develop beyond 34 somites. However, when we partially opened the yolk sac and exteriorized the embryo, as in the procedure of Cockroft (1973) for rat embryos, or used a modification of this procedure in which we opened the yolk sac but left the embryo inside, development was much improved. Martin and Swanson (1993) obtained good development of mouse embryos cultured by the Cockroft procedure. In our experiments the embryos developing inside the opened yolk sac gave the best results, but direct comparison of our results with those of Martin and Swanson is invalid because our embryos were younger at the beginning of culture and our culture period was much longer. All the embryos cultured by our method still had a yolk sac blood circulation after 48 h and many developed normally to 35-38 somites (Table 2). The most advanced embryos reached 50-56 somites after 96 h in culture (Fig. 1).

In the parthenogenetic embryos (both CBA and hybrid), formation of blastocysts, implantation rate and development to somite stages was similar to that found in our previous studies (Penkov and Platonov, 1992; Penkov *et al.*, 1992), although the substrain CBA/CaBkl used here was different from the substrain CBA/CaLac used previously. (The results with the hybrid embryos at the preimplantation stages were similar to those obtained by other workers, e.g. Cuthbertson, 1983 and Robertson, 1987). There were considerable differences between the CBA and the hybrid embryos, and between the hybrid embryos transferred to CBA recipients and the hybrid embryos transferred to hybrid recipients. The hybrid eggs yielded much higher percentages of blastocysts and implantations, but a larger proportion of the implanted CBA embryos reached somite stages (Table 3). In comparison with normal (non-parthenogenetic) embryos, the number of somites in the parthenogenetic embryos at explantation showed a much wider range, and the parthenogenetic embryos both at explantation and during subsequent development were smaller and with a lower protein content than the normal embryos at equivalent stages of differentiation.

TABLE 4

DEVELOPMENT IN CULTURE OF PARTHENOGENETIC EMBRYOS (FROM TABLE 3) EXPLANTED AT DAY 10.

Strain	Number of embryos cultured	Number of embryos with			
		30 or more somites (24 h)	35 or more somites (36 h)	40 or more somites (48 h)	45 or more somites (48 h)
CBA	17	14	11	9*	5
F, (CBAX C57BL/6)	28	18	9*	5	5

*2 (live) embryos removed and fixed

Many of the parthenogenetic embryos developed to about 35 somites, and a few to 45 somites or more, with normal morphology. This amount of development has been obtained previously only *in vivo* with composite embryos made from parthenogenetic inner cell mass and normal trophoblast/endoderm (Barton *et al.*, 1985; Gardner *et al.*, 1990), and more recently, with wholly parthenogenetic embryos after treatment with 5-azacytidine (Penkov *et al.*, 1996). Now that such prolonged development of parthenogenetic embryos can be achieved in culture, new approaches are possible for the analysis of parthenogenesis and genomic imprinting, including studies of the effects of adding to the culture medium specific growth factors and demethylating agents.

Materials and Methods

Vasectomy, pseudopregnancy and pregnancy

All mice were 2-3 months old. Males and females mated for normal pregnancies were (CBAxC57BL6) F1 hybrids. Vasectomized males were from an outbred strain (TO). Vasectomy was by a standard

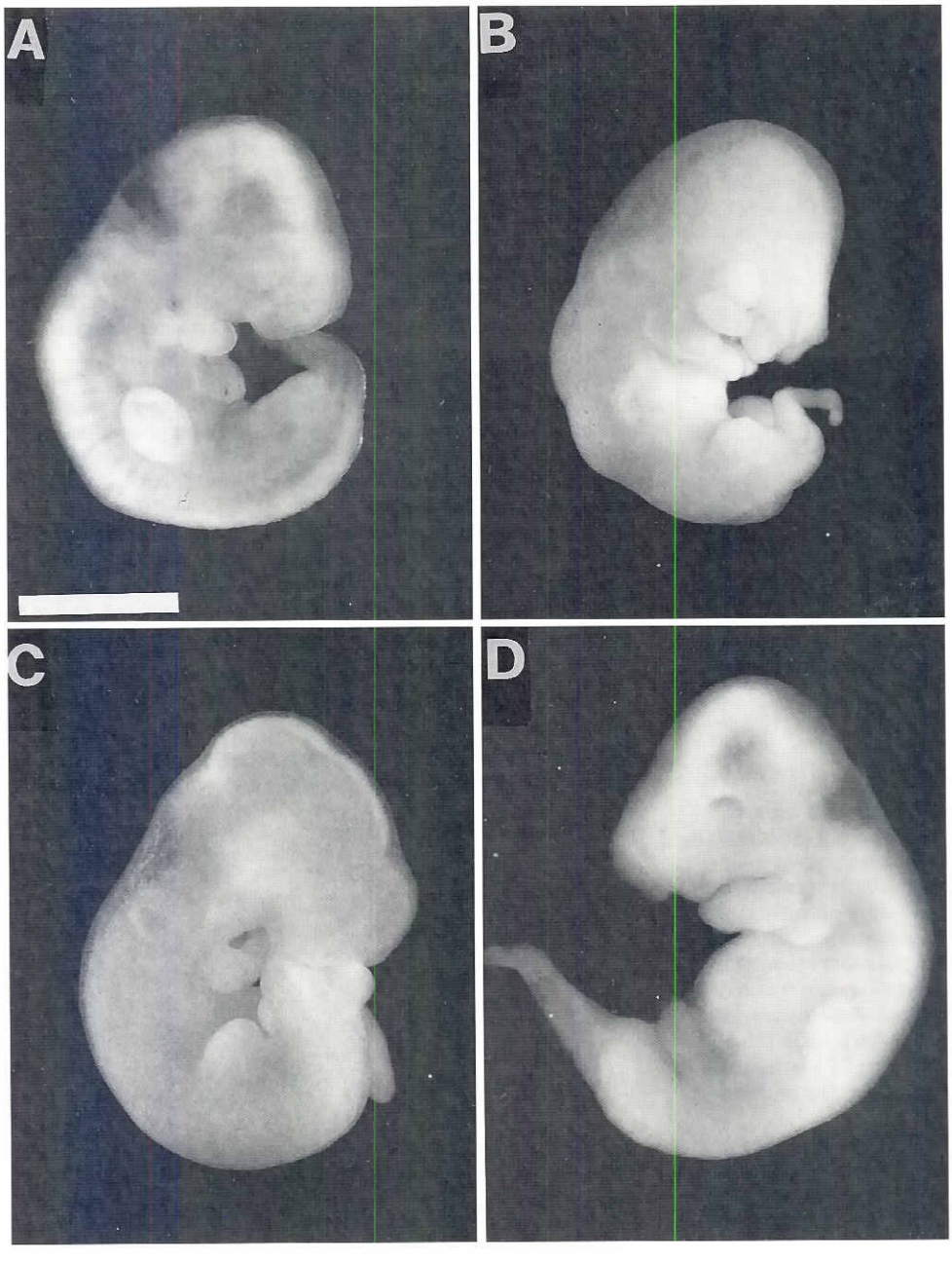

Fig. 2. Parthenogenetic embryos after various periods in culture: (A) *48 h, embryo with 42 somites (Table 5, No. 14);* **(B)** *64 h, 47 somites (Table 5, No. 7);* **(C)** *48 h, 38 somites (Table 6, No. 12);* **(D)** *64 h, 45 somites, showing ocular pigment (Table 6, No. 28). All embryos were photographed after fixation and are shown at the same magnification: bar, 1 mm.*

method (Hogan *et al.*, 1986). Females for pseudopregnancy were CBA (substrain CaBkl) or (CBAxC57BL6) F1 hybrids. Both pregnancy and pseudopregnancy (following mating with a vasectomized male) were timed from the appearance of a vaginal plug; the day of discovery of the plug was designated day 1 of pregnancy or pseudopregnancy.

Diploid parthenogenetic embryos

Embryos for parthenogenetic development were obtained from CBA (substrain CaBkl) or from (CBAxC57BL/6) F1 mice. Females, 4-6 weeks old, were superovulated by intraperitoneal injection of 8-10 IU of a follicle stimulating hormone (Folligon, Intervet Ltd., Cambridge, UK), followed after 42-48 h by injection of 5-6 IU of human chorionic gonadotrophin (Chorulon, Intervet Ltd., Cambridge, UK). After a further 20 h, the mice were killed by cervical dislocation, the eggs extracted from the oviducts in Dulbecco's phosphate buffer solution (Sigma), and the cumulus cells removed in 100 IU/ml hyaluronidase (Sigma). Eggs were parthenogenetically activated by immersion for 5 min in 7% ethanol (Kaufman, 1982; Cuthbertson, 1983) in modified Whitten's medium at 27°C (Whitten, 1971; Abramczuk *et al.*, 1977) and were diploidized by culture at 37°C for 6 h in modified Whitten's medium containing 5 μg/ml cytochalasin B (Sigma). After a further 2-4 h culture (without cytochalasin B), the eggs were scored for pronuclei and any haploid or fragmented eggs were removed. Finally, the eggs were cultured for 96 or 120 h to the blastocyst stage in sealed tubes containing 0.5% Ultroser-G (Gibco BRL) in modified Whitten's medium and 5% CO_2, 5% O_2, 90% N_2, and transferred to the uterus of recipient females on day 3 of pseudopregnancy (Penkov *et al.*, 1996).

TABLE 5

DEVELOPMENT IN CULTURE OF 17 PARTHENOGENETIC CBA EMBRYOS AFTER EXPLANTATION AT DAY 10

Embryo No.	Duration of culture (h)	Crown rump length (mm) 0h	48h	64h	Yolk sac circulation 0h	24h	Fore limb (mm)	Hind limb (mm)	Final somite number	Ocular pigment	Embryo protein (μg)
1	24	1.7			+	-	0.2	-	27	-	
2	48	1.8	2.8		+	+	0.4	0.2	40	+	
3	48	1.8	2.0		+	+	0.2	-	25	-	
4	48	2.2	2.8		+	+	0.5	0.2	42	+	
5	64	1.8	2.5	2.0	+	+	0.5	0.2	47	+	
6	64	2.3	3.0	3.5	+	+	0.7	0.6	47	+	
7	64	2.5	3.2	2.5	+	+	0.4	0.3	47	+	
8	64	1.5	2.4	2.3	+	+	0.3	0.1	33	-	
9	64	1.8	2.8	2.2	+	+	0.3	0.1	38	+	
10	64	2.1	2.5	2.6	+	+	0.2	0.1	31	-	71
11	64	2.5	2.7	2.7	+	+	0.5	0.3	45	+	116
12	48	2.4	3.0		+	+	0.5	0.2	40	-	75
13	48	2.0	2.4		+	+	0.4	0.1	38	-	97
14	48	2.7	2.9		+	+	0.5	0.3	42	+	
15	64	2.5	2.7	2.7	+	+	0.5	0.3	45	+	92
16	24	2.5			+	-	0.3	0.1	30	-	81
17	24	1.8			+	-	0.3	-	25	-	45

(Embryos of top row of Table 4).

Culture of postimplantation embryos

Normal embryos were explanted late on day 9 of pregnancy, parthenogenetic embryos late on day 10 from the beginning of pseudopregnancy. The embryos were explanted with their embryonic membranes; Reichert's membrane was torn open but the other membranes were left intact. Only completely turned embryos were cultured. The semi-transparency of the yolk sac allowed accurate determination of the crown-rump length of each embryo at explantation but not of the number of somites. Initial somite numbers of the cultured embryos were therefore estimated by comparison with other similar embryos removed from the yolk sac. Normal embryos at this stage had about 14-16 somites (35-46 μg protein); parthenogenetic embryos ranged from 5 to 25 somites but only those assessed as having 14-25 somites (20-47 μg protein) were cultured.

The culture method was based on established techniques (Sadler and New, 1981; Hunter *et al.*, 1988). The embryos were incubated at 37°C in cylindrical bottles of 30 ml capacity, rotating at 30 rpm, with a continuous gas flow (New and Cockroft, 1979). Each bottle contained 4 ml of culture medium, consisting of three parts Dulbecco's Modified Eagle's Medium (DMEM) and one part heat-inactivated immediately-centrifuged rat serum (Steele and New, 1974), with 0.06 mg/ml penicillin and 0.1 mg/ml streptomycin. The medium was renewed every 24 h. For the first 24 h, two embryos were placed in each bottle and the gas mixture was 40% O_2, 5% CO_2, 55% N_2; for the remainder of the culture period, one embryo was placed in each bottle and the gas mixture was 95% O_2, 5% CO_2.

Normal (non-parthenogenetic) embryos were cultured in three groups, each litter of embryos being divided among the three groups:

Group 1 The yolk sac was left intact throughout the culture period.

Group 2 When the embryos had developed to about the 30-somite stage (30-36 h of culture), a slit was cut in the yolk sac in a region free of large blood vessels, the embryo pulled though it (i.e. exteriorised), and the amnion removed, as in the method of Cockroft (1973).

Group 3 When the embryos had developed to about the 30-somite stage (30-36 h of culture), a hole was torn in the yolk sac in a region free of large blood vessels, as shown in Figure 3. The embryo was left inside. Sometimes the hole resealed during further culture and when this happened it was torn open again. When the embryo reached 35-40 somites, the amnion was also torn open.

All the parthenogenetic embryos were treated as the normal embryos of Group 3, i.e. a hole was made in the yolk sac at about the 30-somite stage but the embryo was not exteriorised. (Because the stage of development of these embryos at explantation was more variable than that of the normal embryos, the duration in culture before the yolk sac was

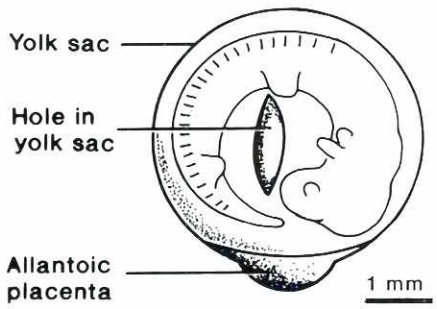

Fig. 3. Diagram showing the position and size of the hole made in the yolk sac of the cultured embryos at about the 30-somite stage.

TABLE 6

DEVELOPMENT IN CULTURE OF 28 PARTHENOGENETIC (CBA X C57BL/6) F1 EMBRYOS AFTER EXPLANTATION AT DAY 10

Embryo No.	Duration of culture (h)	Crown rump length (mm) 0h	48h	64h	Yolk sac circulation 0h	24h	Fore limb (mm)	Hind limb (mm)	Final somite number	Ocular pigment	Embryo protein (μg)
1	48	1.7	2.4		-	-	0.2	-	27	-	
2	48	2.0	3.0		+	+	0.3	0.1	33	-	
3	48	1.5	2.2		-	-	0.1	-	22	-	
4	48	1.5	2.0		-	-	-	-	20	-	
5	64	2.3	3.0	2.9	+	+	0.7	0.3	45	+	
6	48	2.0	2.3		+	+	0.3	-	30	-	
7	48	2.2	2.4		+	+	0.3	0.1	34	-	
8	48	2.2	3.2		+	+	0.6	0.5	45	+	
9	48	1.5			-	-	-	-	17	-	
10	48	1.5	2.2		-	-	-	-	20	-	
11	48	2.0	3.0		+	+	0.4	0.2	35	+	
12	48	2.4	3.3		+	+	0.6	0.2	38	+	
13	48	2.0	2.7		+	-	0.3	0.1	32	-	
14	48	1.5	2.0		+	-	0.2	-	27	-	
15	48	1.5	1.8		+	-	0.2	-	25	-	
16	48	1.8	2.0		+	+	0.3	0.1	33	-	
17	48	1.7	1.8		-	-	0.2	-	26	-	
18	48	1.7	2.0		+	-	0.3	-	30	-	
19	48	1.5	2.0		+	+	0.3	-	32	-	
20	48	2.2	2.2		+	+	0.5	0.3	35	-	212
21	48	1.4	1.8		+	+	0.3	-	25	-	43
22	48	1.5	1.7		+	+	0.3	-	22	-	31
23	64	1.8	2.4	2.4	+	+	0.4	0.3	45	+	124
24	48	1.9	2.0		+	+	0.4	0.1	32	-	63
25	64	2.2	2.4	2.2	+	+	0.6	0.2	36	-	116
26	64	2.8	3.3	3.1	+	+	0.6	0.5	45	++	
27	48	2.3	2.9		+	+	0.5	0.1	34	-	136
28	64	2.8	3.4	3.2	+	+	0.7	0.6	45	++	

(Embryos of bottom row of Table 4).

opened was also more variable. The embryos explanted at 14-20 somites had the yolk sacs opened after 30-36 h, those explanted at 21-25 somites had the yolk sacs opened after 18-24 h).

Assessment of somites, embryo protein and ocular pigmentation

In determining the number of somites in an embryo, all the somites were counted if the embryo was without hind limb buds; if hind limb buds were present, the somite immediately posterior to the bud was considered to be number 31 and the count continued posteriorly.

Protein content of the embryo (freed from the embryonic membranes) was determined by the method of Lowry *et al.* (1951).

Ocular pigmentation, as shown in Tables 2,5 and 6, was recorded in three grades: +, less than a semi-circle of pigment; ++, a thick semicircle of pigment; +++, a half-moon of pigment.

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