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Use of triple tissue blastocyst reconstitution to study the development of diploid parthenogenetic primitive ectoderm in combination with fertilization-derived trophectoderm and primitive endoderm

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Summary

Diploid mouse conceptuses lacking a paternal genome can form morphologically normal but small fetuses of up to 25 somites, but they invariably fail to develop beyond mid-gestation. Such conceptuses differ from normal most notably in the poor development of extra-embryonic tissues which are largely of trophectodermal and primitive endodermal origin. However, it is not clear whether the demise of diploid parthenogenetic (P) or gynogenetic (G) conceptuses is attributable entirely to the defective development of these two tissues or whether differentiation of the primitive ectoderm, the precursor of the foetus, extra-embryonic mesoderm and amnion, is also impaired by the absence of a paternal genome. Therefore, a new blastocyst reconstitution technique was used which enabled primitive ectoderm from P blastocysts to be combined with primitive endoderm and trophectoderm from fertilization-derived (F) blastocysts. One third of the 'triple tissue' reconstituted blastocysts that implanted yielded foetuses. However, all foetuses recovered on the 11th or 12th day of gestation were small and, with one exception, either obviously retarded or arrested in development. The exception was a living 44 somite specimen which is the most advanced P foetus yet recorded. Foetuses were invariably degenerating in conceptuses recovered on the 13th day. In contrast, at least 16% of control reconstituted blastocysts with primitive ectoderm as well as primitive endoderm and trophectoderm of F origin developed normally on the 13th day of gestation or to term. Hence, the presence of a paternal genome seems to be essential for normal differentiation of all 3 primary tissues of the mouse blastocyst.

The P foetuses that developed from reconstituted blastocysts were so closely invested by their membranes that they often showed abnormal flexure of the posterior region of the body. Several also showed a deficiency of allantoic tissue. Therefore, the possibility that the defect in development of P primitive ectoderms resided in their extra-embryonic tissues was investigated by analysing a series of chimaeras produced by injecting them into intact F blastocysts. The foregoing anomalies were not discernible even when P cells made a large contribution to the extra-embryonic mesoderm or amnion plus umbilical cord. Furthermore, selection against P cells was no greater in extra-embryonic derivatives of the primitive ectoderm than in the foetus itself.

1. Introduction

Extensive cytogenetic studies have established that only very limited deviation from a normal karyotype is compatible with development to term in mammals (Epstein, 1986; Dyban & Baranov, 1987). Recently, it has also become clear that as well as being essentially euploid, the mouse zygote must possess both a paternally- and a maternally-inherited genome in order to complete its development. This unexpected finding emerged from experiments in which either the male or female pronucleus was removed from recently fertilized mouse eggs and replaced by another of the same or opposite parental origin (McGrath & Solter, 1983, 1984; Surani & Barton, 1983; Surani *et al.* 1984; Barton *et al.* 1984). Genetic studies in which translocations were used to produce chromosomally balanced zygotes from gametes with complementary duplications and deficiencies have demonstrated that such parental effects operate in regions of the mouse genome that are located on several different chromosomes (reviewed in Cattanach, 1986). However, none

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of the genes that are subject to such 'differential imprinting' have yet been identified. Nevertheless, certain transgenes in mice have been found to vary from generation to generation in DNA methylation and, in one case expression, according to whether they are inherited from the father or mother (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987).

Closer examination of the development of mouse zygotes with two paternal or two maternal genomes has revealed that both clearly deviate from normal before midgestation and, furthermore, exhibit essentially complementary phenotypes. Thus, while diploid parthenogenetic (P) or gynogenetic (G) conceptuses display a marked deficiency in growth of certain extra-embryonic membranes, in their androgenetic (A) counterparts it is the embryo itself that is either poorly developed or absent altogether (Surani, *et al.* 1984: Barton *et al.* 1984).

The possibility of an association between genomic imprinting and cell lineage is suggested by the patterns of development of A, P (or G) embryos. Thus, while A embryos show relatively normal differentiation of the trophectodermal and primitive endodermal lineages accompanied by defective development of the primitive ectoderm, P embryos exhibit the reverse situation (see Gardner, 1988*a* for an up-to-date fate map of the blastocyst). A simple explanation for these antipodal phenotypes would be that paternally-inherited copies of certain imprinted genes are necessary for normal development of the trophectodermal and primitive endodermal lineage, and maternally-inherited copies of other such genes for normal development of the primitive ectodermal lineage. Lineage specificity has already been established with respect to X-chromosome inactivation in female mouse embryos. While the paternal X is inactivated in both trophectoderm and primitive endoderm lineage, the process is evidently random in primitive ectoderm cells (reviewed in West, 1982). However, the proposition that parental imprinting of autosomal genes exhibits similar lineage specificity has yet to be tested critically.

The capacity of diploid blastomeres lacking either a maternal or paternal genome to participate in development when combined with normal fertilization-derived (F) blastomeres possessing both parental genomes has been investigated in several laboratories. Androgenetic (A), parthenogenetic (P) or gynogenetic (G) cells seem to be allocated normally to trophectoderm and ICM in these circumstances (Clarke *et al.* 1988; Thomson & Solter, 1989). However, very marked selection against them occurs as early as gastrulation. This is evident both from a dramatic decline in the incidence of chimaeras by this stage and the consistent failure to detect A, P or G cells in particular tissues in the minority of conceptuses in which chimaerism persists (Clarke *et al.* 1988; Thomson & Solter, 1988; Surani *et al.* 1987, 1988). Thus, in most cases, A cells appear to be almost entirely confined to trophectoderm derivatives and P or G cells to primitive ectoderm derivatives. Hence cells lacking one or other parental genome give enduring chimaerism only in the lineage whose development is most normal in conceptuses that are composed wholly of those cells (Surani *et al.* 1987, 1988). Because selection against such cells continues during later development even in this lineage (Fundele *et al.* 1989, 1990; Nagy *et al.* 1987, 1989; Paldi *et al.* 1989; Surani *et al.* 1987, 1988), it has been inferred that their capacity to differentiate all lineages is impaired. However, consistent extreme selection against one genotype, notably BALB/c, has also been documented repeatedly in aggregation experiments in which both participating embryos were of F origin (Mullen & Whitten, 1971; Nagy *et al.* 1987; Paldi *et al.* 1989). Therefore caution is warranted in drawing conclusions about the developmental potential of early embryonic cells from their performance in a competitive situation.

Barton *et al.* (1985) avoided this complication by studying the development of P or G ICMs in reconstituted blastocysts whose trophectoderm was of F origin. They found that while development of such conceptuses was superior to that of those composed entirely of cells lacking a paternal genome, it still did not proceed beyond mid-gestation. However, since the primitive endoderm and primitive ectoderm both originate from the ICM (Gardner, 1985*a*), failure to obtain better foetal development in these experiments could have been due to deficiencies in extra-embryonic endoderm cells carrying two maternal genomes (Nagy *et al.* 1987; Surani *et al.* 1987, 1988) rather than to any compromizing effect of such a genetic constitution on cells of the primitive ectodermal lineage.

Refinements in microsurgical technique have recently enabled the reconstitution of viable blastocysts whose trophectoderm, primitive endoderm and primitive ectoderm are derived from different embryos (Gardner, 1988*b*). The principal aim of the present investigation was to exploit this 'triple tissue' reconstitution technique to examine the development of P primitive ectoderm in blastocysts whose other two tissues were of F origin. Additional experiments were undertaken in which the fate of P primitive ectoderm tissue injected into entire F blastocysts was studied. Earlier work failed to reveal any differences in developmental potential between diploid P and G embryos (Barton *et al.* 1985; Thomson & Solter, 1988). Therefore, because experimentally-induced parthenogenesis is much simpler to undertake on a large scale, it was used throughout this study to provide blastocysts composed of diploid cells lacking a paternal genome.

2. Materials and Methods

Donors of F blastocysts and pseudopregnant recipients were spontaneously ovulating females from stocks derived from the PO (Pathology, Oxford) random-

bred albino strain. The former were invariably homozygous for the a allozyme of glucosephosphate isomerase (= *Gpi-1 a/Gpi-1a*) and the latter usually homozygous for the b allozyme (*Gpi-1b/Gpi-1b*). They were housed in rooms kept at 23 °C which were illuminated daily either between 07.00–19.00 h (standard lighting) or between 13.00–01.00 h (altered lighting) and checked for oestrus by external inspection (Champlin *et al.* 1973) before being placed with fertile or vasectomized males throughout the dark period. Females used to provide 5th day blastocysts as a source of primitive endoderm tissue were kept in standard lighting conditions and were invariably mated with males of the same stock. Similarly, females for use as pseudopregnant recipients were kept in standard lighting. However, either altered or standard lighting was used for donors of 4th day blastocysts. These females were mated with either inbred C57BL/6J (*Gpi-1b/Gpi-1b*) or *Gpi-1a/Gpi-1a* PO males when used to provide trophectoderm donor blastocysts and with *Gpi-1a/Gpi-1a* PO males to provide host blastocysts in tissue transplantation experiments. In all cases females were checked for vaginal plugs the morning after pairing, and those that were positive recorded as being in the first day of pregnancy or pseudopregnancy.

Donors of eggs for parthenogenetic activation were 4–6 week-old non-albino (C57BL/6J X CBA/Ca)F1 (= F1) *Gpi-1b/Gpi-1b* females bred from stocks supplied commercially (Bantin & Kingman, U.K.). They were superovulated by intra-peritoneal injection of 7·5 i.u. pregnant mare's serum gonadotrophin followed 42–48 h later by 7·5 i.u. human chorionic gonadotrophin (HCG) (both from Intervet Ltd, Cambridge). Some of these females were mated with F_1 males to provide control F1 × F1 fertilized eggs.

Young mature F1 females weighing approximately 18–20 g were mated with vasectomized males for use as recipients of P and F primitive ectoderm donor embryos. These embryos were transplanted to the oviducts at the 2-cell stage or, in a small minority of cases, at the 4–8 cell or late morulae/early blastocyst stage. All F1 animals were housed in rooms maintained at 23 °C which were illuminated between 06.00 and 20.00 h.

(i) *Production of diploid parthenogenetic embryos*

Eggs were recovered from the F1 females between 16·5–17·5 h after HCG injection and their cumulus cells dispersed as described previously (Fundele *et al.* 1989). They were activated by exposure to 7% ethanol in T6 medium supplemented with bovine serum albumin (BSA) for 4·5 min. at room temperature (Kaufman, 1982; Cuthbertson, 1983), rinsed 6 times in T6 + BSA, and then cultured for 3·5–4·0 h at 37·8 °C in medium containing 5 μg/ml cytochalasin B, as described by Fundele *et al.* (1989). Following culture, the eggs were rinsed 9 times in T6 + BSA and incubated for a further 2 h before being scored for pronuclei. Those with 2 pronuclei were cultured overnight or, occasionally, to day 3 (4–8 cell stage) or day 4 (late morula/early blastocyst) prior to transfer to the oviducts of F1 females on the first day of pseudopregnancy. F1 × F1 control fertilized eggs were recovered *ca.* 20 h after HCG injection and cultured to day 3 before being transferred similarly. All F1 recipients were transported from Cambridge to Oxford by rail on the 4th day after mating.

(ii) '*Triple tissues reconstitution*' *of blastocysts*

Throughout this investigation blastocysts were recovered from the uterus, and both stored and manipulated *in vitro* in PB1 medium (Whittingham & Wales, 1969) which was modified as described elsewhere (Cockroft & Gardner, 1987). Furthermore, blastocyst dissection and injection were carried out at *ca.* 10 °C. Both primitive ectoderm and primitive endoderm donor blastocysts were recovered between approximately 11.30 and 15.30 h on the 5th day of gestation. Because implantation is usually well advanced by this juncture, the excised uterine horns had to be inflated and then flushed repeatedly with medium to obtain a satisfactory yield of blastocysts. Blastocysts recovered from different donors were pooled and those with small ICMs or lysed polar trophectoderm cells discarded. Usually, a substantially higher proportion of P than F blastocysts had to be rejected. The remainder were placed individually in hanging drops of medium in manipulation chambers (Puliv, Leitz, West Germany) where they were torn open murally with two siliconized (Repelcote, Hopkin & Williams, U.K.), sharp-tipped solid glass needles held in Leitz micromanipulators. Once opened, the blastocysts were incubated in calcium-magnesium-free Tyrode's saline containing 0·5% (w/v) trypsin and 2·5% (w/v) pancreatin (both enzyme preparations from Difco, U.S.A.) for 18 min. at *ca.* 4 °C. Following a rinse in PB1, they were returned to the manipulation chamber for isolation of the primitive endoderm and primitive ectoderm which was done as described previously (Gardner, 1985*b*). Isolated tissues resulting from dissections that appeared to give clean separation were pooled according to type and stored at *ca.* 10 °C until they could be recombined with partners of a different genotype or injected directly into 4th day host blastocysts. Recombination of primitive ectoderm with endoderm was achieved by placing pairs of the tissues in individual microdrops of alpha medium (Stanners *et al.* 1971) covered with paraffin oil in 60 mm bacteriological dishes (Sterilin, U.K.). The dishes were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air for up to 4 h during the first part of which they were removed briefly from the incubator at *ca.* 20 min. intervals so that any pairs that had not adhered could be pushed together again.

Trophectoderm donor blastocysts were flushed

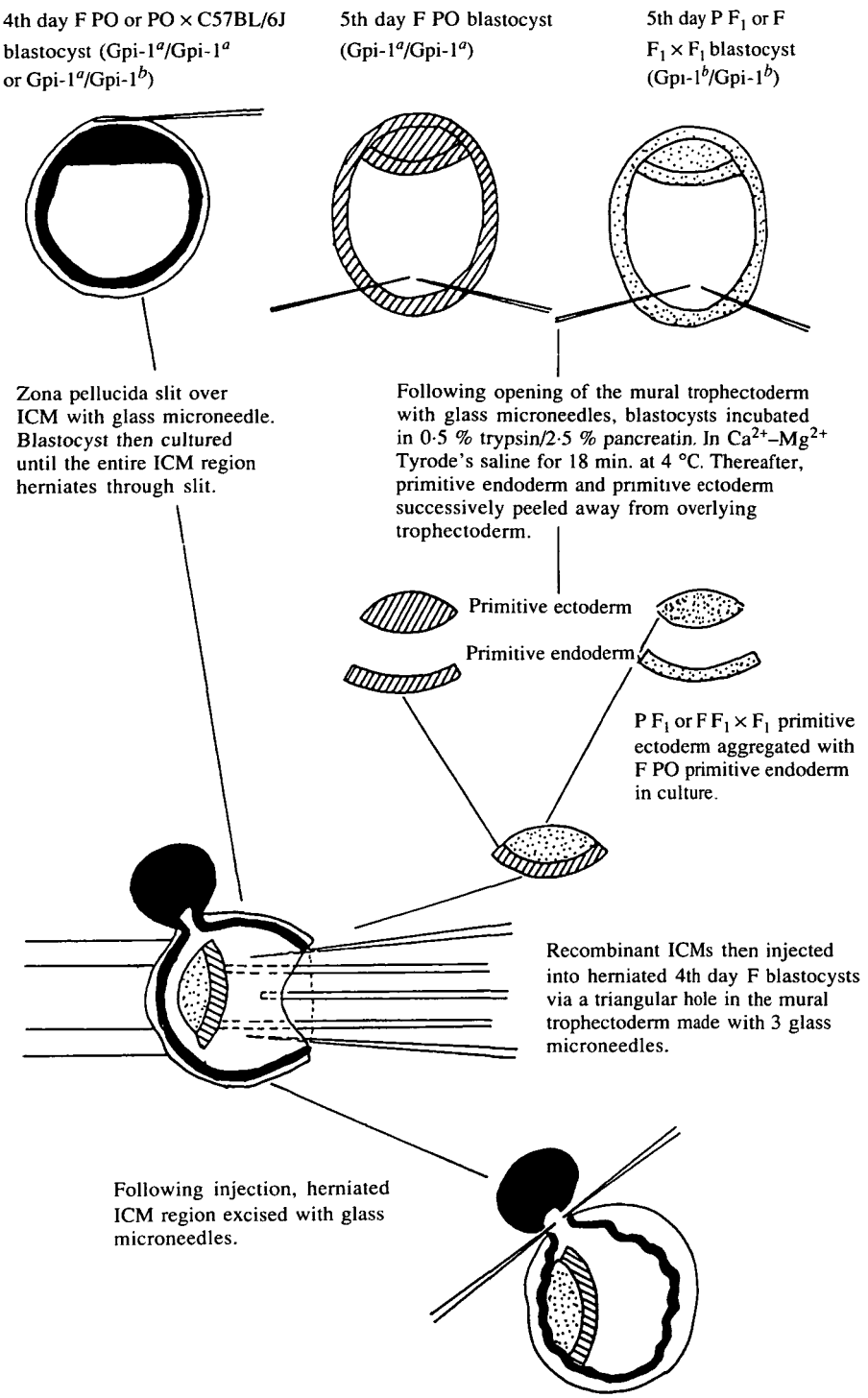

Fig. 1. Diagram illustrating the principal stages in the 'triple tissue' reconstitution of blastocysts.

from the uteri of females on altered or standard lighting between 09.00 and 11.00 h on the 4th day of pregnancy. A slit approximately $\frac{1}{5}$ of its diameter in length was made in the zona pellucida over the centre of the ICM (Tsunoda *et al.* 1986; Nichols & Gardner, 1989), following which the blastocysts were incubated for up to 5 h in alpha medium to encourage herniation of the embryonic pole. Blastocysts from females kept under standard lighting conditions were used in all later experiments because herniation was found to be satisfactory only when the zona was slit before it had begun to thin appreciably. Those whose entire ICM region was outside the zona and connected to the remainder of the trophectoderm by a narrow waist following incubation were used in the reconstitution operation. This entailed injecting each blastocyst with a recombinant ICM and excising the herniated host ICM region immediately thereafter. The injection was done as described earlier (Gardner *et al.* 1973; Gardner, 1978), except that trophectoderm donor blastocysts were immobilized by applying the holding pipette to one side of the hernia. In addition, the

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injection pipette was usually made wide enough to allow donor ICMs to be drawn inside it without compressing them unduly. Excision of host ICMs following injection was done with a pair of needles mounted on a second Leitz micromanipulator assembly, as described by Papaioannou (1982). The entire procedure for 'triple tissue reconstitution' of blastocysts is summarized diagrammatically in Fig. 1. Both reconstituted blastocysts and intact blastocysts injected with parthenogenetic primitive ectoderm were cultured post-operatively for between $\frac{1}{2}$ and 2 h before being transferred to the uteri of recipient females late on the 3rd day of pseudopregnancy. Such transfers were usually unilateral with, in many cases, unoperated PO blastocysts being placed in the contralateral horn.

(iii) *Appraisal of the development of reconstituted and injected blastocysts*

Some of the recipients of control reconstituted blastocysts composed entirely of F tissues were allowed to go to term. All others were killed on the 11th, 12th or 13th day post-coitum (pc). This period of gestation was chosen because it extends from the end-point of normal development recorded for P blastocysts or their ICMs in previous studies to approximately two days beyond this limit (Kaufman, 1983; Barton *et al.* 1985). Dissections were done with watchmaker's forceps and the decidual swellings viewed in a Wild M8 stereo-zoom microscope while they were opened and their contents inspected. When present, foetuses were examined morphologically with particular attention being paid to their overall form, condition of the vascular system, number of somites, and the presence or absence of ocular pigmentation. In some cases they were photographed and crown-rump length was also measured, either directly or from camera lucida drawings which included an appropriate scale bar. Finally, conceptuses were either dissected into fractions for GPI analysis or fixed in 10% formalin-saline for wax-embedding and serial sectioning at 6–7 microns. In the dissections the foetus, amnion plus umbilical cord, visceral yolk sac mesoderm, and visceral yolk sac endoderm were isolated as separate fractions where practicable since these are most instructive for GPI analysis. This is because, according to previous experience with blastocyst reconstitution, contamination of primitive ectoderm with primitive endoderm cells or vice versa or of trophectoderm with ICM cells is more likely than contamination of ICM tissues with trophectoderm cells (Gardner, 1988*b* and unpublished observations). The placenta was not used because it contains cells derived from all 3 lineages of the blastocyst (Gardner 1988*a*), as well as those of maternal origin. Furthermore, substantial maternal contamination of both mural trophoblast and, more surprisingly, parietal endoderm (K. Becker, R. L. Gardner & J. Green, unpublished observations) occurs around mid-gestation that can mask weak chimaerism in these tissues. Allozymes of GPI were resolved electrophoretically on Titan III cellulose acetate plates (Helena Laboratories, U.S.A.) by the method of Eicher & Washburn (1978). Following staining, plates carrying samples of conceptuses that had developed from reconstituted blastocysts were scored visually in order to determine whether the tissues used in the reconstitution operation had been dissected cleanly. A scanning densitometer (Gelman DCD16) was used for quantitating chimaerism in fractions of conceptuses obtained in experiments in which P primitive ectoderms were injected into F blastocysts. Serially-sectioned conceptuses were stained with haemalum and eosin prior to examination.

Recipients that were left to carry their litters to term were allowed to deliver naturally and their offspring examined for coat chimaerism at 2–3 weeks postpartum.

3. Results

(i) *Effects of* in vitro *manipulation and in vivo transportation on development*

It was essential to ensure that the viability of early embryos which had been manipulated and cultured *in vitro* prior to transfer to the oviduct was not seriously impaired by subsequently transporting recipients from Cambridge to Oxford by rail late in their preimplantation phase of pregnancy. Hence 4 F1 females each carrying 12 *in vitro* manipulated F1 × F1 F preimplantation embryos which had been cultured for 42 h before transfer to their oviducts on the first day of pseudopregnancy were dispatched to Oxford on the 4th day. All 4 recipients were pregnant when killed on the 15th or 16th day post-coitum. Thirty nine of the 48 embryos had implanted and 30 of the implantation sites contained living foetuses, giving a satisfactory rate of normal development of 63%.

(ii) *Triple tissue blastocyst reconstitutions*

Despite the fairly high rate of normal development of F1 × F1 F embryos that were left in vivo, the morphology of those recovered as implanting blastocysts on the 5th day was often so poor that only a small minority were considered suitable for microsurgery. This was also true of diploid P F1 embryos, and seemed to be due to an unusual fragility of both categories of blastocyst which was evident particularly during their dissection. Since it was not encountered in blastocysts that had developed entirely in vivo, this fragility was most probably a consequence of the initial period of culture of all P and F F1 embryos. Hence, in both cases, those used to provide primitive ectoderms for blastocyst reconstitution or injection were a carefully selected sub-group of the total recovered.

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Table 1. *Summary of development of experimental and control reconstituted blastocysts and unoperated ( ) blastocysts transferred to contralateral horns*

	Type of reconstitution	Stage of analysis	Blastocysts		No. of decidua	Contents of decidua					Loose decidua masses
			Total No. transferred	No. transferred to females that became pregnant		Normal foetuses or young	Markedly retarded or arrested foetuses	ICM derivatives without foetus	Trophoblast only	No identifiable embryonic tissues	
A	Control reconstitutions with $F_1 \times F_1$ F ectoderms	13th day pc	21(9)	21(9)	12*(9)	2**(9)	0(0)	0(0)	0(0)	10(0)	1*(0)
B	Control reconstitutions with $F_1 \times F_1$ F ectoderms	Postnatal	24(21)	24(21)	—	8(15)	—	—	—	—	—
	Total controls		45(30)	45(30)	—	10(24)	0(0)	0(0)	0(0)	10(0)	1*(0)
C	Exptl. reconstitution with F_1 P ectoderms	11th day pc	11(8)	11(8)	10(4)	0(4)	3(0)	2(0)	3(0)	2(0)	0(0)
D	Exptl. reconstitution with F_1 P ectoderms	12th day pc	43(29)	33(18)	28*(15)	0(13)	9(0)	4(0)	2(0)	11(2)	2*(0)
E	Exptl. reconstitution with large F_1 P ectoderms	12th day	20(10)	18(6)	9(6)	1***(5)	1(1)	0(0)	1(0)	6(0)	0(0)
F	Exptl. reconstitution with large F_1 P ectoderms	13th day	7(6)	7(6)	7(0)	0(0)	6(0)	1(0)	0(0)	0(0)	0(0)
	Total experimentals	—	81(53)	69(38)	54*(35)	1(22)	19(0)	7(0)	6(0)	19(3)	2*(0)

* Minimum because loose decidual masses difficult to count.
** 1 small for age but not markedly retarded.
*** Forty-four somite conceptus with vigorous heart beat plus visceral yolk sac circulation (= conceptus no. 13 in Tables 2 and 3).

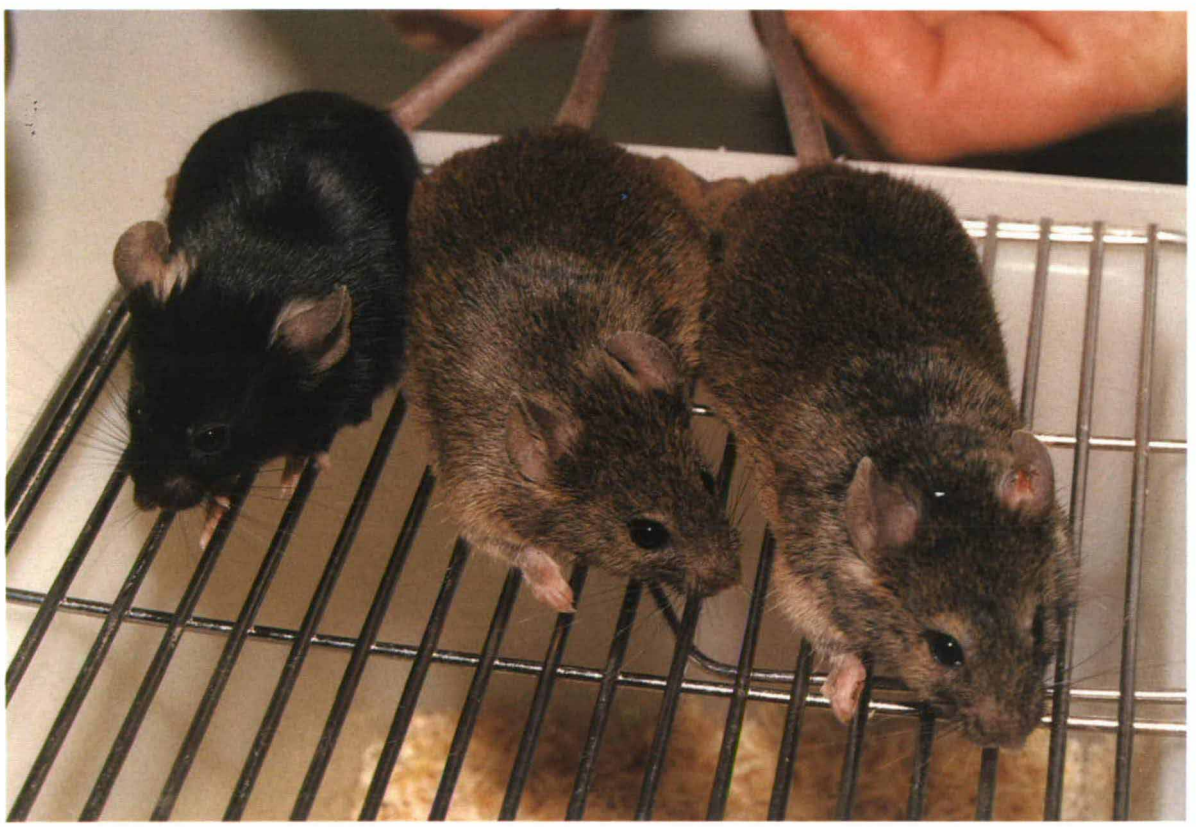

FIGURE 2. A non-agouti female and two agouti male mice derived from blastocysts that were reconstituted with primitive ectoderm from F1xF1 blastocysts (pigmented) and primitive endoderm and trophectoderm from PO blastocysts (albino).

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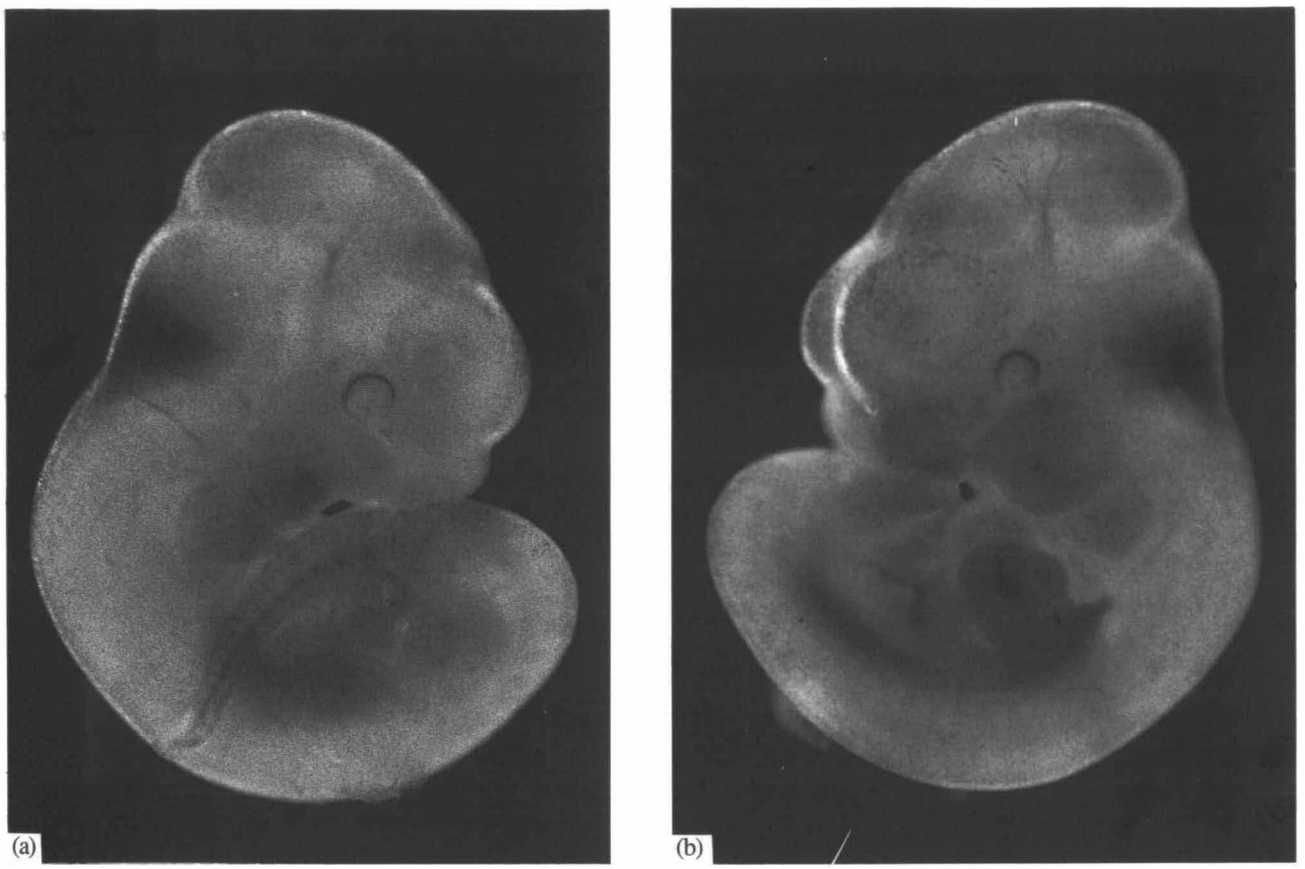

Fig. 3. Right (a) and left (b) views of the most advanced parthenogenetic foetus obtained from a 'triple tissue' reconstituted blastocyst. Note the normal morphology and uniformly pigmented eyes of this 44 somite foetus which was living when recovered on the 12th day of gestation.

A summary of the development of control and experimental reconstituted blastocysts is presented in Table 1. A total of 10 pigmented foetuses and young were obtained in a series of 45 control reconstitutions using trophectoderm and primitive endoderm from PO F blastocysts and primitive ectoderm from F1 × F1 F blastocysts (Table 1, rows A & B). Both control 13th day foetuses were living at the time of recovery although one was rather small. Like their membrane fractions, these foetuses were composed entirely of cells of the expected genotype by GPI analysis. One of the 8 young obtained in the control reconstitutions disappeared without trace shortly after birth so that its genotypic composition could not be ascertained. It was presumably composed largely, if not exclusively, of F1 × F1 cells since it had deeply and uniformly pigmented eyes. Two of the other 7 control young had uniformly pigmented eyes when dissected within 48 h of birth and also exhibited only the B allozyme of GPI in brain, thymus, heart, liver, intestine and kidneys, as well as in the residual carcass. Three of the remaining five that were left to develop postnatally also appeared to be composed entirely of cells of F1 × F1 origin judging by their uniformly agouti or non-agouti coats (Fig. 2). However, the other two were unequivocal chimaeras with a minor albino contribution. It is not possible to identify the source of this contamination because trophectoderm and primitive endoderm donor blastocysts were of the same genotype in these control reconstitutions. Nevertheless, normal development of F1 × F1 F primitive ectoderm occurred in at least 16% (7/45) of reconstituted blastocysts in which contamination of the tissue was not discernible.

In an initial series of experimental reconstitutions all primitive ectoderms isolated satisfactorily from P blastocysts were recombined with primitive endoderms and trophectoderms from F blastocysts and the recipient females autopsied on the 11th or 12th day of gestation (Table 1 *C*, *D*). Then, in order to determine whether the very variable state of development of conceptuses in the first series was due to damage to the P ectoderms during their isolation, a further series was undertaken in which only larger P ectoderms or aggregated pairs of smaller ones were used (Table 1 *E*). The recovery of a single normal-looking but small living foetus on the 12th day in this series (Fig. 3) prompted a further one in which conceptuses developing from blastocysts reconstituted with large P ectoderms were recovered one day later in gestation (Table 1 *F*).

The overall rate of implantation of blastocysts reconstituted from P primitive ectoderm, F primitive endoderm and F trophectoderm was 78% compared with 92% for unoperated PO blastocysts transferred

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Table 2. *State of development and condition at recovery of foetuses derived from blastocysts reconstituted with P primitive ectoderm*

Foetus no.	Recipient (series in Table 1)	State of development			Condition on recovery			Mode of analysis
		Crown rump length (mm)	Somite no.	Ocular pigmentation	Heart beat visceral yolk sac circulation	Arrested	Degenerate	
1	11th (C)	nr	7	–	–	+	·	GPI
2*	11th (C)	nr	14	–	+	·	·	GPI
3*	11th (C)	nr	14	–	+	·	·	GPI
4*	12th (D)	nr	30	–	–	+	·	GPI
5	12th (D)	nr	Presomite	–	–	+	·	GPI
6	12th (D)	nr	43	+	–	+	·	GPI
7*	12th (D)	nr	34	–	–	·	+	GPI
8	12th (D)	nr	43	+	–	+	·	GPI
9	12th (D)	nr	31	–	+	·	·	GPI
10	12th (D)	nr	34	–	–	·	+	GPI
11	12th (D)	nr	28	–	–	·	+	Histology
12	12th (D)	nr	39	–	–	+	·	GPI
13	12th (E)	5·0	44	+	+	·	·	GPI
14	12th (E)	nr	12	–	–	·	+	GPI
15	13th (F)	4·7	nr	+	–	·	+	Histology
16	13th (F)	4·4	nr	+	–	·	+	GPI
17	13th (F)	4·6	nr	+	–	·	+	GPI
18	13th (F)	5·5	nr	+	–	·	+	Histology
19	13th (F)	3·9	30	–	–	+	·	GPI
20	13th (F)	2·4	23**	–	–	+	·	GPI

* Deficient in allantoic tissue.
** Minimum number because damaged during dissection.
nr = not recorded.

to contralateral horns of the majority of recipients (Table 1). However, the proportion of decidua induced by reconstituted blastocysts that contained foetuses or definitive embryos was markedly lower than recorded for controlateral unoperated controls (37% vs. 63%). Interestingly, it was significantly higher in recipients in which control conceptuses were developing in the opposite uterine horn (17/29) than in those which they were not (3/25) (χ^2 1 d.f. = 12·51; $P < 0{\cdot}001$). Invariably, decidua induced by blastocysts reconstituted with P ectoderm that contained foetuses were conspicuously smaller than normal. Twelfth day specimens, for example, were of a size appropriate to the 11th day of gestation.

Details of foetuses derived from all 20 blastocysts reconstituted with P ectoderm that exhibited organized differentiation are provided in Table 2. As shown in this table they varied widely in stage of development, ranging from incipient somitogenesis to 44 somites. A heartbeat and visceral yolk sac circulation were discernible in only 4 specimens at the time of recovery (Table 2), all the remainder having suffered arrest of development at an earlier stage. Some of the arrested foetuses showed obvious vascular congestion: others that were invested by yellowish, fragile, avascular visceral yolk sacs were pale, palpably soft, and clearly degenerating. The living 44 somite foetus recovered on the 12th day exhibited uniformly pigmented eyes (No. 13 in Tables 2 and 3; and Fig. 3). The eyes were also pigmented in both the 43 somite foetuses (no. 6, 8) recovered on this day, as well as in the 4 larger of the 13th day specimens (no. 15–18) for which crown-rump length rather than somite number was recorded.

Experimental foetuses were invariably smaller than normal for their stage of development. While none was grossly abnormal the more advanced specimens often showed aberrant flexure of the posterior region of the body (Fig. 4). This may have been related to the fact that they were unusually closely invested by their extra-embryonic membranes and, as noted earlier, within decidua which were conspicuously smaller than normal.

Three of the conceptuses showing organized development were processed for histological examination. The remaining 17 were dissected so that the genotypic composition of various component tissues could be determined by electrophoretic resolution of the allozymes of GPI. The amnion and umbilical cord were kept separate from the foetal fraction in 2 cases where they were particularly well-developed (no. 12 and 13). Separation of visceral endoderm from visceral mesoderm was unsuccessful in the presomite conceptus (no. 5) and analysis of the foetus only was attempted in 5 further specimens (nos 14, 16, 17, 19 and 20) in which the extra-embryonic membranes had so deteriorated that they were beginning to fragment. The results of the GPI analysis are given in Table 3. It is evident from this table that the allozyme characteristic of the P donor blastocyst was the only one discernible in 15 of the 16 foetal fractions in which GPI activity was recorded. The exceptional conceptus, no 17, clearly had a substantial level of contaminating

Table 3. *GPI allozymal analysis of dissected fractions of conceptuses developing from blastocysts reconstituted with P primitive ectoderms*

	Fractions analysed			
Foetus no.	Visceral yolk sac endoderm	Visceral yolk sac mesoderm	Amnion + umbilical cord (or allantois)	Foetus
1	AA	BB	BB	
2	AA + trace of BB	?BB*	BB	
3	AA	BB	BB	
4	AA	BB + trace of AA	BB	
5	AA + BB(2/1)		BB	
6	AA	BB + trace of AA	BB	
7	AA	BB	BB	
8	AA	BB	BB	
9	AA	BB	BB	
10	AA	BB	BB	
12	AA	BB	BB	BB
13	AA	BB	BB	BB
14	—	—	NA	
16	—	—	—	BB
17	—	—	—	BB + 38% AA
19	—	—	—	BB
20	—	—	—	BB

* Smeared: insufficient material for a rerun.
NA = no allozyme activity detected,
— = fractions not analysed.

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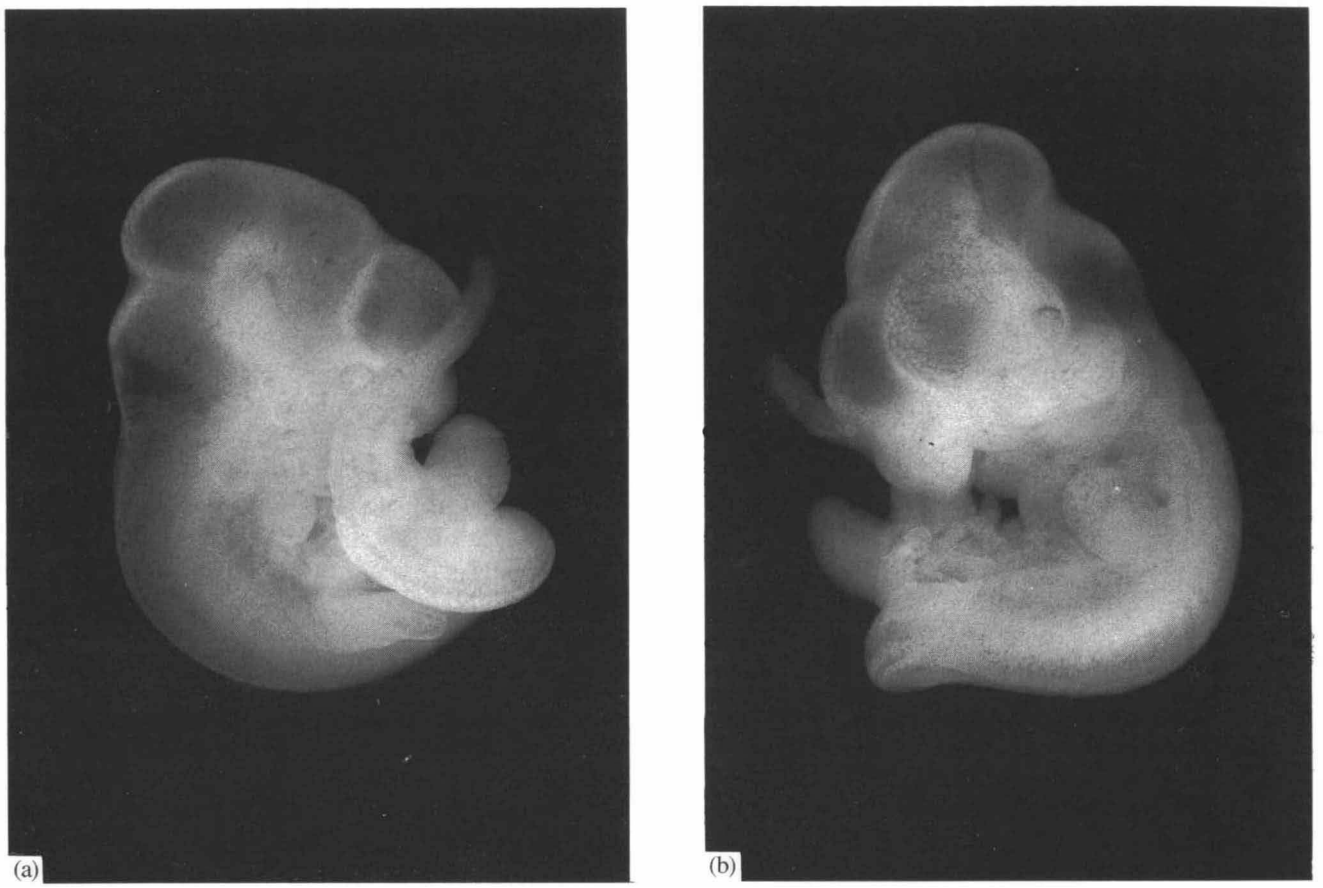

Fig. 4. Right (a) and left (b) views of a 43 somite parthenogenetic foetus obtained from a 'triple tissue' reconstituted blastocyst. This foetus which was recovered dead on the 12th day of gestation, shows markedly abnormal flexure of the posterior part of the body compared with the specimen in Fig. 3.

cells of F origin in its foetal fraction (Table 3). In 8 of the 11 conceptuses in which the visceral yolk sac endoderm and mesoderm could be run separately these fractions exhibited only the single expected allozyme. In 2 of the remaining 3 a trace of F allozyme was discernible in the mesoderm fraction. There was a trace of P allozyme in the endoderm in the third. It is noteworthy that foetus no 13 which boasted the greatest number of somites and was living at recovery was among the 8 specimens in which each of the 3 or 4 instructive fractions analysed were composed entirely of cells of the appropriate genotype (Table 3).

The task of examining serial histological sections of the remaining 3 foetuses and their placentae was complicated by the extensive post-mortem changes that had taken place in these specimens prior to their recovery and fixation. While no obvious abnormalities were apparent a deficiency of allantoic tissue was indicated by the structure of the placentae. Interestingly, this tissue was also recorded as being rather scant in 4 of the conceptuses dissected for GPI analysis (no. 2, 3, 4 and 7 in Tables 2 and 3).

(iii) *Injection of P primitive ectoderm into F blastocysts*

Forty-three injected blastocysts were transferred to the uteri of 8 recipients. Six blastocysts were lost through failure of one recipient to become pregnant. Thirty-three of the remaining 37 implanted, 26 giving rise to normal conceptuses which were recovered on the 12th or 13th day of gestation. Twelve of the conceptuses (= 46%) proved to be chimaeric. Details of these chimaeras are provided in Table 4. In one chimaera a minor contribution of P cells to the visceral endoderm was found, while these cells were confined to derivatives of the primitive ectoderm in the remaining 11. No P cells were detectable in the visceral mesoderm of 2 chimaeras which nevertheless showed chimaerism in both the foetal fraction and amnion plus umbilical cord. While the contribution of P cells often varied markedly between the different fractions from individual conceptuses (Table 4), the mean proportion was almost identical in positive specimens of visceral mesoderm (32%), amnion plus umbilical cord (33%) and foetus (33%). Neither variation in crown-rump length nor, in specimens recovered on the 12th day, somite number showed any correlation with the extent of colonization of any particular one of the primitive ectoderm derivatives with P cells. The mean crown-rump length and somite number did not differ between the chimaeric and non-chimaeric foetuses on the 12th day. However, in specimens recovered on the 13th day the mean crown-rump length of chimaeras was significantly less than that of non-chimaeras (see footnote to Table 4).

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Table 4. *Size, somite number and contribution of donor cells in chimaeras obtained by injecting P primitive ectoderms into F blastocysts*

Chimaera no.	Recipient day pc at recovery	% P Contribution			Foetus	Somite no.	Crown rump length (mm)
		Visceral endoderm	Visceral mesoderm	Amnion & umbilical cord			
1	12th	0	3	na	23	47	6·7
2	12th	0	4	24	23	46	5·9
3	12th	0	50	46	44	48	5·5
4	12th	0	74	59	10	54	5·9
5	12th	0	58	37	30	52	5·2
6	12th	0	28	15	7	50	6·4
7	12th	0	20	23	35	47	5·9
8	13th	0	0	6	42	nr	8·4
9	13th	0	37	43	52	nr	6·4
10	13th	0	27	53	41	nr	6·7
11	13th	0	0	10	36	nr	7·2
12	13th	14	34	50	47	nr	7·0

nr = not recorded.
na = not analysed – missing.
Mean % *P* contribution in chimaeric visceral mesoderms = 31·5 ($n = 10$).
Mean % *P* contribution in chimaeric amnion and umbilical cord fractions = 33·3 ($n = 11$).
Mean % *P* contribution in chimaeric foetuses = 32·5 ($n = 12$).
12th day Mean crown rump length of chimaeras = $5{\cdot}9 \pm 0{\cdot}5$ ($n = 7$).
Mean Crown rump length of non-chimaeras = $5{\cdot}6 \pm 0{\cdot}6$ ($n = 7$) not significant.
13th day Mean crown rump length of chimaeras = $7{\cdot}1 \pm 0{\cdot}8$ ($n = 5$).
Mean crown rump length of non-chimaeras = $8{\cdot}0 \pm 0{\cdot}6$ ($n = 7$) $P < 0{\cdot}01$).

Regardless of extent of the P contribution the chimaeras showed no signs of close investment by their membranes. Furthermore, their deciduae were comparable in size to those of non-chimaeras.

4. Discussion

Triple tissue reconstitution offers a more refined way of investigating the lineage specificity of mutations or other alterations in the genetic constitution of early embryonic cells than is possible by reconstituting blastocysts from trophectoderm and ICM tissue. In the present study this new technique was used to examine the developmental potential of primitive ectoderm composed wholly of diploid P cells in conjunction with trophectoderm and primitive endoderm cells that possessed both paternal and maternal genomes. Despite the normal genetic endowment of cells in the other two lineages, in no case was development of such primitive ectoderms sustained beyond the 12th day of gestation. This suggests that the demise of P conceptuses by mid-gestation is not due entirely to deficiencies in the differentiation of their trophectodermal and primitive endodermal lineages. Rather, it seems that in the absence of a paternal genome differentiation of the primitive ectodermal lineage is also impaired. However, two factors need to be considered in critically evaluating these results. One is the frequency of normal development of appropriate control reconstituted blastocysts, and the other the efficiency of isolation of tissues from the 3 categories of donor blastocysts.

Just over one fifth of control reconstituted blastocysts developed normally. This is substantially lower than the rate of normal development encountered in initial trials when the viability of the technique was being assessed (Gardner, 1988, and unpublished observations), and in earlier control reconstitutions using F ICM and trophectoderm tissue from blastocysts grown *in vitro* (Barton *et al.* 1985). The present experimental reconstitution results using P primitive ectoderm were also poorer in two respects than those reported by Barton *et al.* (1985) for blastocysts reconstituted from P ICM and F trophectoderm. First, the frequency with which blastocysts that implanted yielded foetuses was much lower, being only 32 as opposed to 74%. Second, the foetuses were more variable in somite number in the present study, even when larger P ectoderms or aggregated pairs of smaller ones were used. Such variability was not seen in earlier trial 'triple tissue' blastocyst reconstitution experiments (Gardner, 1988*b*, and unpublished observations). The relatively poor rate of development of both control and experimental reconstituted blastocysts observed in the present study is unlikely to have been due to the late pre-implantation shipment of primitive ectoderm donor embryos *in vivo* by rail since the majority of F embryos in the 4 recipient autopsied later in gestation yielded normal foetuses. Hence, it probably reflects adverse effects of the more extensive

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manipulation that is necessary with triple tissue reconstitution on embryos that have been rendered fragile by earlier culture. Nevertheless, approximately 15 normal viable foetuses would have been expected from the experimental reconstituted blastocysts in the present study had these rivalled controls in their developmental potential.

Chimaerism can be detected occasionally in tissues of later conceptuses or the young developing from reconstituted blastocysts and is most readily explained by failure to achieve complete separation of primitive endoderm from primitive ectoderm or ICM tissue from trophectoderm in donor blastocysts (Gardner, 1988*b*; Gardner *et al.* 1973; Papaioannou, 1982). It is a more common problem using triple tissue reconstitution because of the greater amount of tissue dissection that this technique entails. It requires monitoring since the development of P primitive ectoderms would be expected to be enhanced by the presence of contaminating F cells. However, chimaerism was detected in only a minority of the tissue fractions analysed in both control and experimental reconstitutions undertaken in the present study. Most notably, it was not seen in any of the 4 instructive tissue fractions from the most advanced conceptus that had developed from blastocysts reconstituted using P ectoderm (no. 13, see Fig. 3 and Tables 2 and 3).

Judging by somite number, the presence of ocular pigmentation and, in one case, persistence of a heartbeat and visceral yolk sac circulation, several foetuses had progressed further in development in the present study than in the earlier one in which P ICMs were inserted into F trophectoderms (Barton *et al.* 1985). However, the difference amounted to a few hours at most. Hence, switching the primitive endodermal as well as trophectodermal lineage from a P to an F origin does not markedly improve the development of pathenogenetic foetuses since even unmanipulated P blastocysts can attain the 25 somites stage (Kaufman, 1983).

P foetuses derived from both types of reconstituted blastocyst were invariably small for their stage of development. Furthermore, the more advanced specimens recorded in the present study occupied conspicuously smaller decidua than normal and were also very tightly enveloped by their extra-embryonic membranes. This may account for the abnormal flexure of the posterior part of the body of many such foetuses (Fig. 4) which otherwise looked normal in external appearance. No internal defects could be seen in the 3 foetuses that were serially sectioned, although the extent of post-mortem deterioration was such that only gross anomalies would have been recognizable. Interestingly, a deficiency of allantoic tissue was noted during dissection of 4 experimental conceptuses. In addition, the labyrinthine region of the placenta also seemed to be poorly developed in the specimens that were sectioned. Taken in conjunction with the contracted state of the foetal membranes, these observations indicate that a paternal genome may be required in cells of an extra-embryonic derivative of the primitive ectoderm.

Marked disparities in chimaerism between the extra-embryonic mesoderm and foetus have been reported which suggest that they may originate from separate pools of primitive ectoderm cells (Gardner & Beddington, 1988). Hence a series of blastocyst injections were undertaken with P primitive ectoderms to see if there were any effects on development that could be correlated with high levels of chimaerism in one or other derivative of this tissue. No such effects were found. The primitive ectodermal chimaeras compared favourably with non-chimaeras in size, somite number and condition at recovery on the 12th day of gestation, but were significantly smaller when recovered one day later. Furthermore, there was no evidence of greater selection against P cells in one primitive ectoderm derivative than another, their mean contribution being close to $\frac{1}{3}$ in foetus, the mesoderm of the visceral yolk sac, and in the amnion plus umbilical cord.

Post-implantation conceptuses composed wholly of P or G cells differ most strikingly from normal in their almost complete lack of polar trophectoderm derivatives (Surani *et al.* 1990). Since P or G ICMs can support the development of extra-embryonic ectoderm and ectoplacental cone tissue from vesicles of F trophectoderm (Barton *et al.* 1985), this defect must reside in the trophectoderm lineage in conceptuses lacking a paternal genome. Poor development of the visceral as well as the parietal yolk sac in such conceptuses (Barton *et al.* 1984; Surani *et al.* 1984) and the very strong selection against P or G cells in these components in aggregation chimaeras (Clarke *et al.* 1988; Thomson & Solter 1988*b*; Nagy *et al.* 1987; Surani *et al.* 1987, 1988) suggest the primitive endodermal lineage is also defective. The present findings argue that normal development of the primitive ectodermal lineage is also precluded by the absence of a paternal genome. Interestingly, the most advanced P foetuses die approximately when P cells decline most precipitously in most foetal tissues in chimaeras (Surani *et al.* 1990). However, the immediate cause of death of P foetuses in conceptuses developing from reconstituted blastocysts in which defects in the trophectodermal and primitive endodermal lineage can be discounted has not been established. Obviously, it could depend on anomalous differentiation in an extra-embryonic derivative of the primitive ectoderm rather than in the foetus itself. The experiment in which P primitive ectoderms were transplanted into F blastocysts failed to resolve this issue.

When combined with those of F embryos cells from preimplantation P or G embryos can participate in the development of viable fertile mice (Stevens, 1978; Kaufman, 1983; Anderegg & Markert, 1986; Otani *et al.* 1987; Paldi *et al.* 1989). However, the resulting chimaeras are unusual in several respects. For example, they tend to show a high perinatal mortality

(Stevens *et al.* 1977; Stevens, 1978; Surani *et al.* 1977; Kaufman, 1983), and those that survive are usually smaller than normal, their weight being inversely correlated with the level of contribution of P cells (Paldi *et al.* 1989). In addition, where the distribution within foetuses or offspring of P cells has been examined systematically, such cells have been found to be consistently underrepresented in certain organs and tissues compared with others. Thus, whereas a contribution of P cells is commonly observed in brain and melanocytes, it is exceptional in liver and skeletal muscle (Nagy *et al.* 1989; Fundele *et al.* 1989, 1990; Surani *et al.* 1990). Overall, it seems from the chimaera data that absence of a paternal genome may reduce the general fitness of cells in the primitive ectodermal lineage rather than exert any very specific effects on their differentiation. A striking exception is the formation of oocytes with normal developmental potential where P and G cells rival F cells in performance in chimaeras (Stevens, 1978; Kaufman, 1983; Nagy *et al.* 1989).

Further progress in accounting for the defects in the various cell lineages of diploid conceptuses deprived of one or other parental genome will depend on the identification and characterization of genes that are subject to imprinting. This in turn may provide clues as to why this unusual pattern of gene regulation evolved, and whether it is peculiar to mammals or more widespread among vertebrates.

Finally, although triple tissue reconstitution of blastocysts is a complex and exacting procedure, it clearly provides an effective way of examining various aspects of cell lineage that are not amenable to investigation by conventional blastocyst reconstitution or other manipulations on pre-implantation embryos.

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