Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts

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SUMMARY

The fate of mouse blastocyst tissues was examined following reconstitution of blastocysts from isolated inner cell mass (ICM) and trophectoderm differing for electrophoretic variants at the glucose phosphate isomerase (GPI-1) locus. A modified microsurgical method was used and a more sensitive enzyme assay allowed finer dissection of developing chimaeric conceptuses. In seven of nine cases, the extraembryonic ectoderm or the later ectoderm of the chorion was entirely of the blastocyst trophectoderm enzyme type, providing the first direct evidence that this tissue can be wholly derived from the trophectoderm. The two exceptions could represent contamination of the ICM with trophectoderm or might indicate some developmental lability of ICM cells. In addition, the results confirm the cell lineages of other tissues of the 7·5- to 9·5-day *pc* embryo and, for the first time, directly demonstrate the ICM origin of the parietal endoderm.

INTRODUCTION

The fate of the tissues of early mouse embryos has been studied extensively using experimental chimaeras (Gardner, 1975; McLaren, 1976; Rossant & Papaioannou, 1977 for reviews). The cell lineages outlined by Gardner & Papaioannou (1975) have been generally accepted as experimental evidence, although direct evidence is still lacking for several branches. In particular, the origin of the extraembryonic ectoderm remains controversial since, although a number of studies have indicated a trophectoderm origin for this tissue (Gardner & Johnson, 1975; Gardner & Papaioannou, 1975; Rossant, 1976), isolated inner cell masses (ICMs) have been reported to make extraembryonic ectoderm-like tissue *in vitro* (Hogan & Tilley, 1978; Wiley, Spindle & Pedersen, 1978). Copp (1979), following the fate of marked cells in intact blastocysts, showed directly that the trophectoderm does contribute to the extraembryonic ectoderm but could not show the extent of this contribution. Rossant & Lis (1979), following the fate of donor ICMs injected into whole blastocysts, found no contribution to the extraembryonic ectoderm in a large number of chimaeras but could not show directly that the host trophectoderm (and not the host ICM)

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produced all the extraembryonic ectoderm. The only means of accomplishing this is to reconstitute blastocysts with ICM and trophectoderm of different genotypes for an enzyme marker. An earlier study used this technique to examine the fate of ICM and trophectoderm at mid-gestation (Gardner, Papaioannou & Barton, 1973). This study provided evidence that all of the trophoblast of the mid-gestation embryo is derived from the trophectoderm layer of the blastocyst, with no substantial cellular contribution from the ICM. A few exceptional cases, where a minor component of ICM-type enzyme activity was apparent in the trophoblast fraction of the chimaeras, were attributed to maternal contamination or, in cases where this could be ruled out, to contamination with some presumptive ICM derivative, in particular the parietal endoderm which was routinely left with the trophoblast. The embryonic fraction, which included foetal membranes, was primarily derived from the ICM as judged by enzyme analysis, but the majority of chimaeras also had a substantial if minor contribution from the trophectoderm. This was considered most likely a trophectoderm contribution to the extraembryonic ectoderm and thence the chorion. Unfortunately, the sensitivity of the enzyme assay did not allow further dissection of the conceptuses to confirm extraembryonic ectoderm or parietal endoderm origins.

The experiments reported here were designed to confirm and extend the results of the previous reconstitution experiments as to the fate of the ICM and trophectoderm, with special reference to the origin of the extraembryonic ectoderm and parietal endoderm. A simplified method of blastocyst reconstitution was employed (Papaioannou, 1981 a, b) and a more sensitive enzyme assay (Petersen, Frair & Wong, 1978) allowed analysis of earlier stages and smaller amounts of tissue. The results indicate that the extraembryonic ectoderm can be derived wholly from the trophectoderm, and also provide the first direct evidence for ICM origin of the parietal endoderm.

MATERIALS AND METHODS

Animals

Naturally mated, random-bred CFLP females, derived from mice originally supplied by Anglia Laboratory Animals Ltd, were used as donors for trophectoderm tissue. These mice are homozygous for the $Gpi-1^a$ allele at the glucose phosphate isomerase (GPI-1) locus. Some of the animals were maintained in a room with the light/dark cycle shifted so that the embryos were advanced by 8 h compared with the ICM donors. This facilitated isolation of the trophectoderm. Two inbred strains, AG/CamPa (Staats, 1976; Papaioannou, unpublished) and a strain derived from C57BL, were mated naturally or after superovulation by intraperitoneal injection of 2–5 i.u. PMS followed 48 h later by 2–5 i.u. HCG (Folligon and Chorulon respectively, Intervet Laboratories, Bar Hill, Cambs, England) to provide homozygous $Gpi-1^b/Gpi-1^b$ blastocysts as ICM donors.

Although both of these strains carry the homozygous-lethal A^y gene, crosses were made so as to avoid producing A^y/A^y embryos.

CFLP females in their 3rd day of pseudopregnancy following mating with vasectomized males were used as uterine recipients for reconstituted blastocysts. These females were $Gpi-1^a/Gpi-1^a$.

Microsurgery

Blastocysts were flushed from the uteri of pregnant females in PB1 + 10 % v/v foetal calf serum (FCS) (Whittingham & Wales, 1969; Papaioannou & West, 1981) at approximately 3·5 days *post coitum* (*pc*) or 3·8 days *pc* for the advanced trophectoderm donor blastocysts. The age of embryos is based on the assumption that coitus took place at the midpoint of the dark period prior to detection of a vaginal plug. ICMs were isolated either by microdissection (Gardner, 1972) or by gentle pipetting following immunosurgery to lyse the trophectoderm cells (Solter & Knowles, 1975). Heat-inactivated rabbit anti-mouse-embryo or anti-mouse-spleen serum was the source of antibody and either guinea-pig serum (Sigma) or rat serum was used as a source of complement. ICMs were stored in PB1 + 10% FCS for up to several hours until required.

Isolation of the trophectoderm and reconstitution of a blastocyst by insertion of an ICM into a trophectoderm vesicle was carried out in a single procedure, illustrated in Fig. 1, which is described in detail elsewhere (Papaioannou, 1981 b). This procedure resulted in blastocysts with a trophectoderm of CFLP origin ($Gpi-1^a/Gpi-1^a$) and an ICM of AG/CamPa or C57BL-A^y origin ($Gpi-1^b/Gpi-1^b$). One to five hours after the operation, successfully reconstituted blastocysts were transferred to the uteri of recipient CFLP foster mothers ($Gpi-1^a/Gpi-1^a$) under Avertin anaesthesia (Winthrop Laboratories, Surbiton-upon-Thames, Surrey, England).

Embryo dissection

A range of developmental stages was obtained by dissecting embryos between 7·5 and 9·5 days *pc* (*post coitum* for the recipient foster mother). Decidual swellings were removed from pregnant uteri and carefully examined under a dissecting microscope for embryonic derivatives. Normal embryos were removed, washed and further dissected in PB1 + 10 % FCS according to their developmental stage. The trophoblast and parietal endoderm were removed together by separating them from the ectoplacental cone (EPC) or placenta along the lines indicated in Fig. 2 (lines a). The parietal endoderm was then removed intact by stripping Reichert's membrane and attached endoderm from the inner trophoblast surface. This was thoroughly washed and adherent trophoblast giant cells were picked off with watchmaker's forceps. The least advanced embryos were two- or three-layered, early primitive-streak-stage embryos (Fig. 2A) with variable amounts of mesoderm. The EPC was removed from these embryos, included with the trophoblast sample, and the egg cylinder
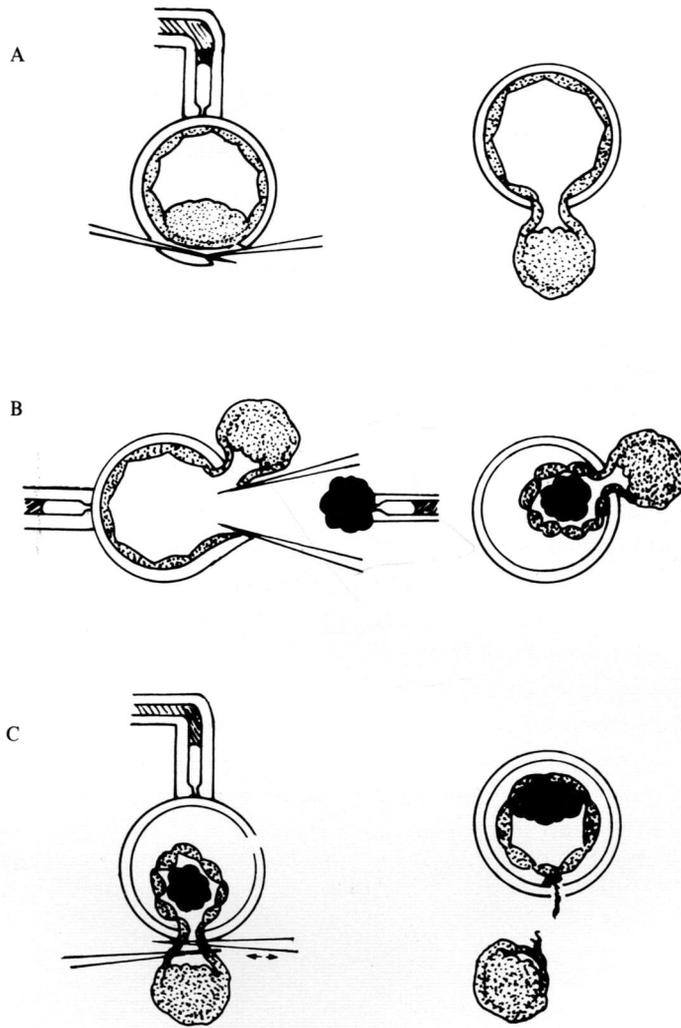
Fig. 1. Diagram of the blastocyst reconstitution procedure using Leitz micromanipulators and glass instruments. (A) A well-expanded CFLP blastocyst is held in place by suction and a slit is made in the zona pellucida directly over the inner cell mass. The blastocyst will herniate through this slit during culture at 37 °C (Copp, 1979). (B) A $Gpi-1^b/Gpi-1^b$ ICM (solid black) is injected into the herniated blastocyst using the method of Gardner (1978). (C) The extruded ICM end of the CFLP blastocyst is cut off with needles leaving a reconstituted blastocyst in the zona pellucida. (Reproduced with permission from Papaioannou, 1981b.)

was then cut into embryonic and extraembryonic portions with tungsten or glass needles along the lines indicated (Fig. 2A, lines b and c respectively). The extra-embryonic portion was separated into ectoderm and endoderm by treatment with 0·5 % w/v trypsin and 2·5 % w/v pancreatin in calcium–magnesium-free Tyrode's solution (Levak-Švajger, Švajger & Škreb, 1969) for 10 min at 4 °C,

EPC — Ex.Ec — Chorion — P.End — Troph.
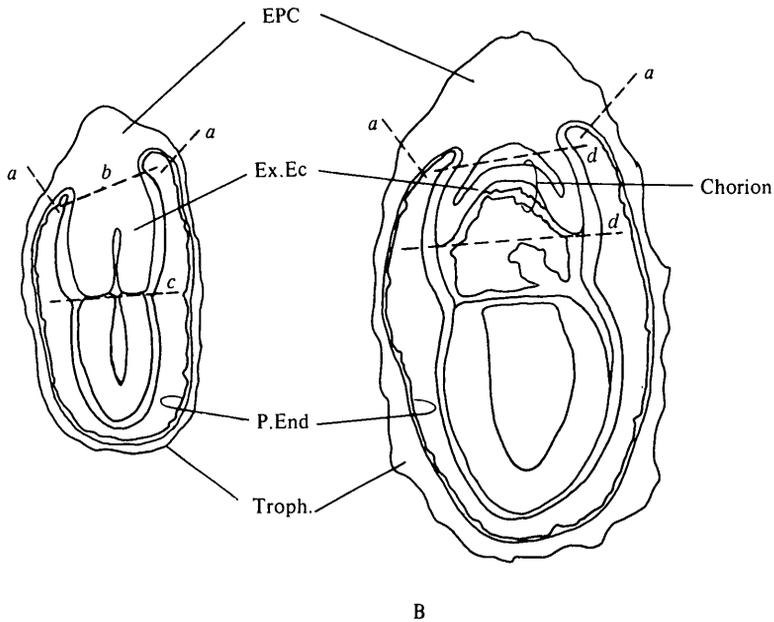
A   B

Fig. 2. (A) Diagram of a representative early-primitive-streak-stage embryo indicating (*a*) the lines of dissection for the isolation of the trophoblast (Troph.) and parietal endoderm (P.End.) and (*b*) and (*c*) the ectoplacental cone (EPC) and extraembryonic ectoderm (ExEc). (B) Diagram of a representative late-primitive-streak-stage embryo indicating the lines of dissection to isolate (*a*) the trophoblast, parietal endoderm, and (*d*) the ectoplacental cone and chorion.

followed by washing in PB1 + 10% FCS and gentle pipetting. The extraembryonic ectoderm was analysed separately while the endoderm from this region was included with the embryonic fraction. Late-primitive-streak-stage embryos were characterized by the presence of amnion, allantois and chorion, prior to fusion of the allantois with the chorion and EPC (Fig. 2B). The chorion was separated by cutting as indicated in Fig. 2B (lines d) and subjected to the enzyme treatment described above to isolate the extraembryonic ectoderm. The mesoderm from this separation was discarded, so the embryonic fraction consisted of the embryo plus all of the yolk sac, amnion and allantois, but not the mesoderm of the chorion.

In the most advanced embryos the allantois, chorion and EPC had fused into the chorioallantoic placenta, preventing clear demarcation or clean dissection of these areas. Thus the trophoblast sample from advanced embryos did not include the EPC and there was no extraembryonic ectoderm sample. Also, the yolk sac and amnion were analysed separately from the embryo proper.

The samples were thoroughly washed in PB1 + 10 % FCS and then frozen in a small volume of distilled water and stored at −20 °C until enzyme analysis was carried out. Only those samples that were deemed to be cleanly dissected and free from contamination with other tissue were used in the analysis.

Table 1. *Rate of implantation and development of reconstituted blastocysts with CFLP trophectoderm*

ICM strain	Method*	Vesicle† age	Implantation rate‡ No. deciduae/ No. transferred (No. of recipients)	Development – no. with:				
				Empty	G.c.§	Abnormal embryo	Normal embryo	Chimaera code
AG/CamPa	I	Syn.	15/15 (4)	10	1	1‖	3	5, 8, 16¶
	I	Adv.	25/28 (7)	19	2	0	4	4, 9, 14, 15
	M	Adv.	8/9 (2)	3	0	0	5	1-3, 11, 12
C57BL-A^y	I	Adv.	12/15 (3)	6	2	1**	3	6, 7, 10**, 13
Totals			60/67 (16)	38	5	2	15	

* I = Immunosurgery; M = mechanical dissection of ICM.

† Syn. = synchronous trophectoderm; Adv. = trophectoderm advanced 8 h compared to ICM.

‡ An additional 23 reconstituted embryos were transferred to 5 recipients that did not become pregnant.

§ G.c. = trophoblast giant cells only.

‖ This conceptus consisted of only a small amount of trophoblast enclosing a small yolk sac. It was not analysed for GPI due to its small size.

¶ No GPI analysis is available for chimaera 16 due to a technical failure.

** Chimaera 10 had abnormal folds in the yolk sac but was otherwise normal and is therefore included in the GPI analysis of chimaeras.

Electrophoretic analysis

Starch gel electrophoresis of the samples was carried out with 12% w/v electrostarch using a pH 6·4 Tris-citrate buffer system as before (Gardner *et al.* 1973). Diluted blood samples of known GPI-1 genotype were used as standards. Maximum enzyme sensitivity was obtained by staining the gels essentially as described by Petersen *et al.* (1978) except that rectangular nitrocellulose filters (Sartorius, pore size 0·45) were used in place of circular overlays, and petroleum jelly was substituted for vacuum grease (modifications of J. D. West, personal communication). This procedure permits the resolution of GPI-1 isozymes from a single mouse oocyte (Petersen *et al.* 1978). Clear, strong bands of activity were usually visible after 1-5 h staining. Proportions of two bands in double-banded samples were estimated by eye.

RESULTS

Although the implantation rate was high (Table 1), most decidual swellings were devoid of recognizable embryonic tissue or contained only a small clump of trophoblast giant cells. One conceptus (not further analysed) consisted of an empty parietal yolk sac and trophoblast. Another was normal except for folding

Table 2. *GPI-1 analysis of tissues dissected cleanly from reconstituted embryos*

Chimaera code	Age*	Developmental stage	EPC/ trophoblast	Extra-embryonic ectoderm	Parietal endoderm	Yolk Embryonic‡	sac	Amnion
1	7·5	Early primitive streak	—	A	—	B		
2	7·5		A	A	—	B		
3	7·5		A	A	—	B		
4	8·5		A	A	—	B		
5	7·5		A	A	—	B		
6	7·5		A	B	—	B		
7	7·5	Late primitive streak	A+B(10:1)	A+B(4:1)	—	B		
8	7·5		A	A	—	B		
9	8·5		A	A	B	B		
10§	7·5		A	—	B	B		
11	8·5	7 somites	A	—	B	B	B	←
12	8·5	8 somites	A	—	—	B	B	←
13	9·5	21 somites	A	—	B	B	B	B
14	9·5	> 21 somites	A+B(15:1)	—	A+B(1:5)	B	B	B
15	9·5	> 21 somites	A+B(15:1)	—	B	B	B	B

* In days *pc* for the recipient foster mother.

† A = GPI-1AA, B = GPI-1BB, A+B = GPI-1AA and GPI-1BB (approximate ratio in parentheses).

‡ See Materials and Methods for an explanation of the composition of this portion in the different stage embryos.

§ Chimaera 10 had abnormal folding in the yolk sac.

of the visceral yolk sac (chimaera 10). Fifteen of 60 decidual swellings, or 25 %, contained normal embryos of various developmental stages. This is somewhat lower than the 38 % (13/34) normal development of embryos from reconstituted blastocysts obtained by Gardner *et al.* (1973). The difference is probably a reflexion of technical failures accompanying the development of the new technique. Later experiments in the present series were more successful than the earlier ones (Papaioannou, 1981*b*), indicating some technical improvement was being made.

A breakdown of the experiments by genotype and method of isolation of ICM, and by the relative age of the trophectoderm donor blastocyst, is also provided in Table 1. The technical importance of these variables is discussed elsewhere (Papaioannou, 1981*b*). Each combination produced some normal chimaeras. Although the numbers are probably too small for meaningful comparisons to be made, the difference in normal development between immunosurgically isolated ICMs and mechanically isolated ICMs is notable and could reflect a detrimental effect on ICM survival of some batches of immunosurgery reagents.

The GPI-1 analysis of 15 chimaeras, including the one with folding in the yolk sac, is presented in Table 2. Samples from chimaera 16 were inadvertently lost before analysis. Of the 14 EPC/trophoblast samples analysed, 11 contained only the GPI-1 type appropriate to the trophectoderm donor and the other 3 were predominantly of trophectoderm type with minor bands of ICM-type GPI-1 activity (chimaeras, 7, 14, 15). Seven of nine extraembryonic ectoderm samples were exclusively trophectoderm type, while one had a minor ICM component and one was exclusively ICM type (chimaeras 7 and 6). Only six of the more advanced embryos yielded sufficient parietal endoderm tissue for GPI-1 analysis. These were all of the ICM GPI-1 type except for one which also had a minor component of trophectoderm type (chimaera 14). The embryonic portions of all 15 chimaeras, including the yolk sac and amnion, showed GPI-1 activity only of the original ICM type.

DISCUSSION

Copp (1979), using melanin granules to mark the polar trophectoderm cells of blastocysts, provided the first direct evidence that these cells could contribute to the extraembryonic ectoderm, although the method did not allow determination of the extent of the contribution. The trophectoderm origin of this tissue had been previously indicated by a number of less direct studies of cell potential in chimaeras and *in vitro* (Gardner & Johnson, 1975; Gardner & Papaioannou, 1975; Rossant & Ofer, 1977; Rossant Gardner & Alexandre, 1978). The present study provides the first direct evidence that the entire extraembryonic ectoderm and later the ectoderm layer of the chorion can be derived from the trophectoderm of the blastocyst. There were two exceptional chimaeras. In one the extraembryonic ectoderm was exclusively of the ICM enzyme type, whereas the ICM component was minor but substantial in the other. It is possible that the ICMs used to make chimaeras 6 and 7 (which were part of the same experiment) did in fact still contain labile cells capable of making trophectoderm (Handyside, 1978; Rossant & Lis, 1979) or, alternatively, that the immunosurgery was not successful in killing all the trophectoderm cells in the preparation of these ICMs. Gardner *et al.* (1973) showed that intentional contamination of ICMs with trophectoderm prior to reconstitution could result in a chimaera with a large part of the trophoblast derived from the contaminated 'ICM' component. This is a possible explanation corroborated by the minor contribution of ICM-type activity in the EPC/trophoblast fraction of chimaera 7.

Although it has been suggested that the primitive endoderm arises from the trophectoderm, the weight of evidence favours an ICM origin of this tissue (Rossant & Papaioannou, 1977 for review) and it has been assumed that the parietal and visceral endoderm share a common origin. Studies with interspecific rat/mouse chimaeras have shown that rat ICM cells can contribute to the parietal endoderm (Gardner & Johnson, 1973; Rossant, 1976), and cell-marking

studies with mouse blastocysts gave no indication that polar trophectoderm, at least, made any contribution to parietal endoderm (Copp, 1979). The present study provides the first direct evidence that most, if not all, of the parietal endoderm is derived from the ICM of the blastocyst. The single exceptional sample from chimaera 14 had a minor band of trophectoderm-type enzyme. This could represent a genuine contribution from still-labile trophectoderm cells in the reconstituted blastocyst, or it is possible – since the trophectoderm was obtained from an advanced blastocyst – that some parietal endoderm had already formed from the ICM and spread onto the trophectoderm before the latter was isolated. However, the increasing difficulty in isolating the parietal endoderm free from contaminating trophoblast in the older, more advanced chimaeras makes it likely that this activity is due to contamination from trophoblast.

The additional data bearing on the fate of the ICM confirm previous studies of fate and potential as to the ICM origin of the embryonic ectoderm and visceral endoderm, as well as the extraembryonic visceral endoderm of the egg cylinder, and all of the foetus, yolk sac and amnion of the later conceptus (Gardner *et al.* 1973; Gardner & Papaioannou, 1975; Rossant, 1975*a, b* 1976; Rossant *et al.* 1978; Gardner & Rossant, 1979). On the whole, the trophectoderm origin of the EPC and later trophoblast is also confirmed by the present study. A possible explanation of the exceptional chimaera 7 has been given. The other two chimaeras which showed a minor band of ICM-type enzyme activity in the trophoblast (chimaeras 14 and 15) were well-advanced embryos, and Reichert's membrane had greatly expanded and begun to thin (Dickson, 1979). It seems likely that the ICM enzyme activity in the trophoblast of these chimaeras was due to inadvertant contamination with parietal endoderm.

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