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Genetic identification of tissue of origin of cellular populations within the mouse placenta

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SUMMARY

The mouse haemochorial placenta is a complex mixture of maternal cells and foetal trophectoderm and inner cell mass (ICM)-derived cells. The majority of the placental tissue is assumed to be trophoblast in origin but the exact extent and localization of the ICM and maternal contribution has not previously been determined. Using embryo transfer and reconstituted blastocyst techniques, combined with isozymal and *in situ* genetic markers, we have established that about 70% of the 13 to 15-day placenta is trophectoderm-derived, 30% is maternal in origin, and 4% develops from the ICM. Nearly all of the maternal contribution was confined to the spongiotrophoblast region and all of the ICM contribution was confined to the labyrinthine trophoblast region, where it formed the foetal blood capillaries and the endodermal sinuses. Using the same genetic markers, we showed that cell suspension techniques commonly used to produce 'trophoblast' cell preparations from placenta do not enrich for trophoblast, and, indeed, that collagenase, the preferred dissociation technique for cell viability, produced cell suspensions in which ICM and maternal cells were preferentially dissociated. No method for producing pure trophoblast populations has yet been found.

Some unusually high ICM contributions to the placenta were found in reconstituted blastocyst experiments using ICMs isolated from early 3·5-day blastocysts, suggesting that these ICMs may have contributed to the trophectoderm layer of the blastocyst. These and other experiments suggest that the inner cell mass lineage may not be closed until some time after formation of the blastocyst.

INTRODUCTION

The placenta forms the interface between the foetal and maternal systems in mammalian pregnancy and consists of a complex mixture of maternal blood sinuses, and foetally derived trophoblast, mesenchyme and blood vessels (Mossman, 1937; Perry, 1981). The degree of direct contact between the maternal blood and foetal trophoblast varies considerably from species to species, but is most intimate in both mouse and man where maternal blood directly bathes the foetal trophoblast, forming the haemochorial placenta (Enders, 1965, 1983). Classical descriptive studies have delineated three distinct types of trophoblast in the murine placenta, an outer layer of trophoblast giant cells, a middle layer of relatively non-vascularized spongiotrophoblast and the innermost layer of labyrinthine trophoblast, which is infiltrated by foetal blood vessels and

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surrounded by maternal blood sinuses. All three layers are assumed to arise from the trophectoderm of the blastocyst, although this has not yet been proven experimentally. Reconstituted blastocyst experiments have shown conclusively that the ectoplacental cone and extraembryonic ectoderm of the earlier primitive-streak-stage embryo are trophectoderm-derived (Gardner, Papaioannou & Barton, 1973; Papaioannou, 1982; Rossant, Vijh, Siracusa & Chapman, 1983) and it is these tissues that are assumed to make up the bulk of the foetal component of the mature placenta. Experiments in which genetically marked inner cell mass (ICM) cells were injected into blastocysts have shown that ICM-derived cells make a measurable contribution to the chorioallantoic placenta (Gardner & Rossant, 1979), and transfer of blastocysts to genetically distinct hosts has revealed a considerable maternal contribution as well (unpublished observations). The exact localization of the ICM and maternal contributions to the different layers of the placenta remains to be established experimentally.

Trophoblast has many unusual properties presumably related to its vital role in foetal survival (Loke & Whyte, 1983). Elucidation of the importance of these properties of trophoblast would be greatly aided by the development of methods for obtaining large amounts of pure trophoblast from the mature placenta. Pure trophoblast can be obtained from the ectoplacental cone and extraembryonic ectoderm of the 7- to 8-day embryo (Rossant & Tamura-Lis, 1981), but the amount of tissue is limited. Trophoblast giant cells can be isolated by their size from later tissues (Barlow & Sherman, 1972) but no method has been developed for identifying unequivocally the diploid trophoblast components of the mouse placenta, although monoclonal antibodies specific to human trophoblast cells have been described (Lipinski, Parks, Rouse & Herzenberg, 1981). Many studies on the properties of trophoblast have used whole placenta or suspensions of placental cells prepared in a variety of ways and assumed that the results are characteristic of trophoblast (Chatterjee-Hasrouni & Lala, 1979; Toder, Blank, Drizlikh & Nebel, 1982; Müller, Verma & Adamson, 1983; Tanaka *et al.* 1983; Pavia & Stites, 1981; Razin *et al.* 1984). Morphological criteria of dubious validity have been used to quantify the trophoblast contribution, but no studies have used genetic markers to verify independently the trophectoderm origin of the supposed trophoblast cells.

We have been interested in obtaining pure trophoblast from the placenta to investigate both its immunoregulatory properties (Rossant, Croy, Clark & Chapman, 1983) and the methylation patterns of specific gene sequences (Chapman, Forrester, Sanford & Rossant, 1984). We have used embryo transfer and reconstituted blastocyst techniques, combined with isozymal and *in situ* genetic markers, to determine the extent of ICM, trophectoderm and maternal contributions to the different layers of the mature placenta. The results provide a detailed description of the origin of the different cell populations in the placenta, and demonstrate that the cell suspension techniques commonly used to obtain trophoblast preparations tend to select for non-trophoblast cells and often do not produce a representative sample of the component cell populations of the placenta.

MATERIALS AND METHODS

Mouse strains and collection of embryos

C3H/HeJ and BALB/c mice were obtained from the Ellerslie breeding facility of the University of Alberta. CD1 outbred mice were originally obtained from Charles River, St. Constant, Quebec and were maintained as a closed colony at Brock University. *M. caroli* outbred mice were originally obtained from Dr V. M. Chapman, Roswell Park Memorial Institute, Buffalo, N.Y. and were also maintained at Brock University. C3H/HeJ and BALB/c mice are known to differ at the glucose phosphate isomerase locus (*Gpi-1*); C3H are homozygous for the fast electrophoretic variant, $Gpi\text{-}1^b/Gpi\text{-}1^b$, while BALB/c are homozygous for the slow variant, $Gpi\text{-}1^a/Gpi\text{-}1^a$. The CD1 stocks maintained at Brock have been selected to differ at the *Gpi-1* locus, and *M. caroli* are $Gpi\text{-}1^a/Gpi\text{-}1^a$.

All embryos were recovered at the blastocyst stage of development by flushing the uteri of pregnant females with PB1 medium (Whittingham & Wales, 1969) plus 10% foetal calf serum (FCS) or Leibowitz medium (GIBCO). C3H and *M. caroli* females were induced to ovulate by treatment with pregnant mares serum gonadotrophin (PMS; 5 i.u. for inbred mouse strains and 2·5 i.u. for *M. caroli*), followed 48 h later by treatment with human chorionic gonadotrophin (hCG; 5 i.u. and 2·5 i.u. as before). CD1 females were selected for oestrus and naturally mated. In all matings, whether hormone-induced or natural, females were paired with a male of the same strain or species and blastocysts were obtained on the afternoon of the 4th day of pregnancy for *M. musculus* and on the afternoon of the 3rd day of pregnancy for *M. caroli*.

Reconstituted blastocysts

ICMs were prepared from $Gpi\text{-}1^b/Gpi\text{-}1^b$ CD1 and C3H, and $Gpi\text{-}1^a/Gpi\text{-}1^a$ *M. caroli* blastocysts by immunosurgery (Solter & Knowles, 1975). Wherever possible, fully expanded blastocysts were used but occasionally early blastocysts, in which the blastocoelic cavity was less than half the volume of the embryo, were used. ICMs were injected into microsurgically prepared trophectoderm vesicles (Papaioannou, 1982). $Gpi\text{-}1^b/Gpi\text{-}1^b$ C3H or CD1 ICMs were injected into $Gpi\text{-}1^a/Gpi\text{-}1^a$ CD1 trophectoderm vesicles while *M. caroli* ICMs were injected into $Gpi\text{-}1^b/Gpi\text{-}1^b$ CD1 trophectoderm vesicles.

Embryo transfer

Both unmanipulated and reconstituted blastocysts were transferred to the uteri of females on the third afternoon after mating to vasectomized males. $Gpi\text{-}1^b/Gpi\text{-}1^b$ C3H or CD1 blastocysts were transferred to $Gpi\text{-}1^a/Gpi\text{-}1^a$ BALB/c or CD1 females, reconstituted blastocysts were transferred to either $Gpi\text{-}1^a/Gpi\text{-}1^b$ or $Gpi\text{-}1^a/Gpi\text{-}1^a$ CD1 females, and interspecific reconstituted blastocysts were transferred to untyped CD1 females.

Dissection of embryos

Embryos derived from unmanipulated and reconstituted blastocysts were dissected between 13 and 15 days of pregnancy. The entire conceptus was removed from the uterus and placed in phosphate-buffered saline (PBS). The foetus and visceral yolk sac were dissected from the placenta and flash frozen. The decidual cap overlying the placenta was removed as cleanly as possible and retained for GPI analysis. Any remaining uterine tissue, yolk sac and Reichert's membrane material was cleaned from the placenta and discarded, and a full-thickness wedge of placenta was then removed for GPI analysis. The inner layer of labyrinthine trophoblast was carefully dissected from the outer spongiotrophoblast using iridectomy scissors (Jenkinson & Owen, 1980). This procedure produced a clean preparation of the pinker labyrinthine trophoblast but usually left patches of pink labyrinthine trophoblast attached to the spongiotrophoblast. These patches were cleared away as much as possible but some contamination of spongiotrophoblast with labyrinthine tissue could not be avoided. Small samples of individual labyrinthine and spongiotrophoblast regions were frozen for GPI analysis and remaining labyrinthine and spongiotrophoblast tissues were pooled for further treatment.

Trypsin dissociation of the placental tissues was performed by mincing the tissues with fine scissors in a solution of 0·05 % trypsin in Ca^{2+}, Mg^{2+} free PBS (Smith, 1983). After incubation for 45 mins in a 37 °C water bath, the suspension was pipetted vigorously to dissociate the cells. Clumps were removed by filtration through Nitex gauze and the material remaining on the gauze was frozen and retained for GPI analysis. The cells passing through the gauze were washed, subjected to erythrocyte lysis and washed again. Viability was assessed by Trypan blue dye exclusion. Cell suspensions were then pelleted and frozen for GPI analysis. Collagenase dissociation of placental tissues (Chatterjee-Hasrouni & Lala, 1979) was performed by incubating tissues in 1 mg/ml collagenase (Worthington Type IV), 25 μg/ml DNase (Sigma) in Leibowitz medium, instead of the trypsin solution. All other steps in the disaggregation were identical to the procedure outlined above. Reconstituted blastocysts were only dissociated by the collagenase technique.

Further separation of the components of the placental layers was attempted by centrifugation over Lympholyte M (Cedarlane Laboratories, Hornby, Ont). The final cell suspensions obtained from collagenase dissociation of total placental tissue or dissected labyrinthine and spongiotrophoblast from transferred blastocysts were layered over Lympholyte M and gradients were centrifuged at 3200 r.p.m. for 20 mins at 15 °C. An interface and a pellet of cells were obtained and both were retained and frozen for GPI analysis.

Electrophoretic analysis of GPI isozymes was performed by the method of Peterson, Frair & Wong (1978). Stained nitrocellulose filter overlays were scanned in either a Camag Model 80100 Electrophoresis Scanner or a Helena Quick-Scan to quantitate the relative proportions of the isozymes.

Conceptuses derived from interspecific reconstituted blastocysts were dissected from the uterus at 15 days of pregnancy and pieces of placental tissue were fixed and processed for *in situ* DNA–DNA hydridization as previously described (Rossant *et al.* 1983).

RESULTS

GPI analysis of placental tissue after embryo transfer

Transfer of *Gpi-1*^{b}/*Gpi-1*^{b} blastocysts to *Gpi-1*^{a}/*Gpi-1*^{a} recipients was used to distinguish between maternal and foetal contributions to the various layers of the placenta. However, trophectoderm and ICM derivatives showed the same isozyme type so that their relative contributions to the placental tissues could not be determined in this experiment. GPI analysis of undissociated placental tissues derived from embryo transfer revealed a considerable proportion of maternal isozyme (Table 1). Further dissection of the placenta into labyrinthine and spongiotrophoblast showed that this maternal contribution was nearly all confined to the spongiotrophoblast region. The decidual cap was almost entirely maternally derived, as predicted, but did contain contaminating foetal tissue, presumably trophoblast giant cells or spongiotrophoblast islands.

GPI analysis of cells dissociated from the labyrinthine and spongiotrophoblast regions of the placenta by trypsin digestion showed that foetal and maternal cells were more or less equally dissociated by the procedure used (Table 1). The percentage of maternal isozyme in the spongiotrophoblast cell suspension was lower than in the undissociated matrix and significantly lower than in the untreated tissue ($P < 0{\cdot}05$). This indicates that trypsin dissociation does produce some enrichment for foetally derived cells, which are probably predominantly trophoblast in this region. However, most of the reduction in maternal isozyme was probably due to the elimination of maternally derived erythrocytes from the cell suspension.

Table 1. *GPI analysis of placental tissues after transfer of* Gpi-1^b/Gpi-1^b *blastocysts to* Gpi-1^a/Gpi-1^a *uterus*

Tissue	Enzyme treatment	No. of samples	% foetal isozyme (± S.E.M)	% maternal isozyme (± S.E.M.)
Placenta	none	20*	69 ± 3	31 ± 1
Spongiotrophoblast	none	19	64 ± 10	36 ± 6
Labyrinthine trophoblast	none	19	98 ± 1	2 ± 0
Decidua	none	5	19 ± 6	81 ± 15
Spongio matrix‡	trypsin	3†	72 ± 12	28 ± 5
Spongio. cell suspension	trypsin	3	78 ± 4	22 ± 1
Lab. matrix	trypsin	3	100	0
Lab. cell suspension	trypsin	3	100	0
Spongio. matrix	collagenase	3†	91 ± 8	9 ± 1
Spongio. cell suspension	collagenase	3	50	50
Lab. matrix	collagenase	3	100	0
Lab. cell suspension	collagenase	3	94 ± 6	6 ± 1

* Samples from individual placentae.
† Samples from 5–15 pooled placentae.
‡ Undissociated tissue remaining on Nitex gauze.

Trypsin-dissociated labyrinthine trophoblast appeared to be entirely foetal in origin, as expected from the analysis of the undissociated tissue.

Collagenase dissociation produced cell suspensions which were clearly enriched for maternally derived cells (Table 1). The undissociated matrix of the spongiotrophoblast which remained on the Nitex gauze appeared to be predominantly of foetal origin whereas the dissociated cells showed 50% maternal contribution. Even in the labyrinthine trophoblast a significant maternal contribution was detected in the dissociated cell suspension.

GPI analysis of density-separated placental cells

Collagenase digestion has been widely used to dissociate placental tissues since it is less damaging to cell surface properties than trypsin, and further separation of collagenase-derived cell suspensions on density gradients has been claimed to produce pellets enriched in trophoblast cells (Pavia & Stites, 1981). We therefore subjected cell suspensions of total placenta and separated labyrinthine and spongiotrophoblast to density step gradient separation and performed GPI analysis on both the interface and the pellet produced (Table 2). The number of samples analysed was small due to the necessity of pooling samples, but the results showed no enrichment for foetally derived cells in the pellet. In fact, in all three cases the pellet was enriched for maternally derived cells when compared with the interface fraction. The net result of collagenase dissociation followed by Lympholyte separation was to produce cell populations which were widely divergent in composition from the original tissue samples. Thus, spongiotrophoblast showed 36% maternal

Table 2. *GPI analysis of density-separated placental tissues*

Tissue	Fraction	No. of samples*	% foetal isozyme (± S.E.M., where appropriate)	% maternal isozyme (± S.E.M. where appropriate)
Placenta	interface	3	55 ± 11	45 ± 9
	pellet	3	42 ± 5	58 ± 7
Spongiotrophoblast	interface	2	50, 35	50, 65
	pellet	2	37, 38	63, 62
Labyrinthine trophoblast	interface	2	97, 94	3, 6
	pellet	2	85, 65	20, 35

* Each sample was a pool of 5–15 placentae.

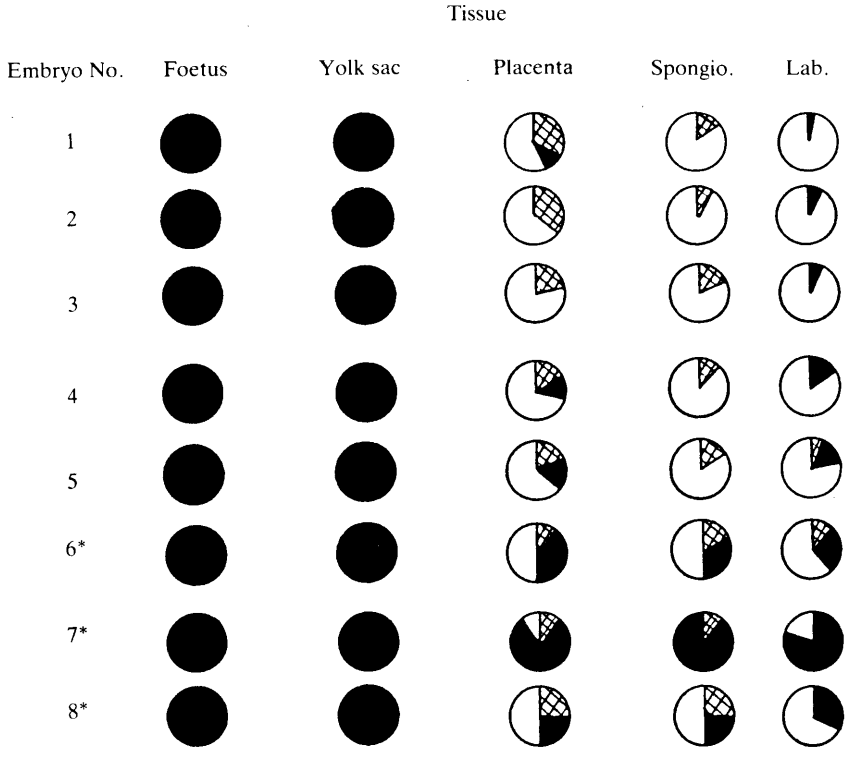
contribution in the original tissue but 61 % in the final pellet after Lympholyte separation, while labyrinthine trophoblast showed 2 % maternal contribution originally and 28 % after Lympholyte.

Intraspecific reconstituted blastocysts

Reconstituted blastocysts in which the ICM was *Gpi-1^b/Gpi-1^b* and the trophectoderm was *Gpi-1^a/Gpi-1^a* were used to determine the ICM or trophectoderm origin of the foetal component of the placenta. In the first series of experiments, such reconstituted blastocysts were transferred to *Gpi-1^a/Gpi-1^b* recipients in an attempt to distinguish the maternal, ICM and trophectoderm contributions to the placenta. Fifteen blastocysts were transferred; 13 implanted and 8 viable embryos were recovered. The results of the GPI analysis of these conceptuses are shown in Table 3. Quantitation of isozymal proportions is of necessity less accurate where hybrid enzymes are involved, and so the results are presented in diagrammatic form only. All embryos showed the expected patterns of GPI isozymes in the foetus and yolk sac, which were completely derived from the injected ICM. In embryos 1–5, the placenta was predominantly derived from trophectoderm but both ICM and maternal contributions could also be detected. Examination of the separated labyrinthine and spongiotrophoblast of these placentae revealed that the maternal contribution was mostly confined to the spongiotrophoblast, as predicted from the embryo transfers, and the ICM contribution was apparently confined solely to the labyrinthine region.

The placentae of embryos 6–8 differed from the others in showing extensive ICM contributions to all layers of the placenta including the spongiotrophoblast. Some of the ICMs used in these experiments were from early blastocysts that had just begun to cavitate, and so a further group of reconstituted blastocysts was made using only ICMs from fully expanded blastocysts. These blastocysts were transferred to *Gpi-1^a/Gpi-1^a* recipients to allow unequivocal identification of the ICM contribution. In such transfers, the presence of B isozyme in any

Table 3. *GPI analysis of reconstituted blastocysts transferred to* Gpi-1^a/Gpi-1^b *females*



*Anomalous embryos

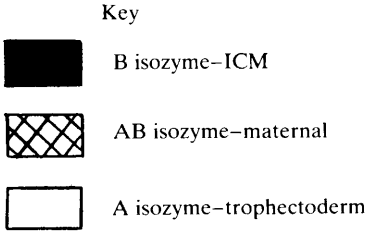


Table 4. *GPI analysis of reconstituted blastocysts transferred to* Gpi-1^a/Gpi-1^a *females.*

	Tissue (% ICM contribution)				
Embryo No.	Foetus	Yolk sac	Placenta	Spongio.	Lab.
9	100	100	6	8	23
10	100	100	3	1	18
11	100	100	3	2	n.a.
12	100	100	3	0	10
Mean	100	100	4	3	17

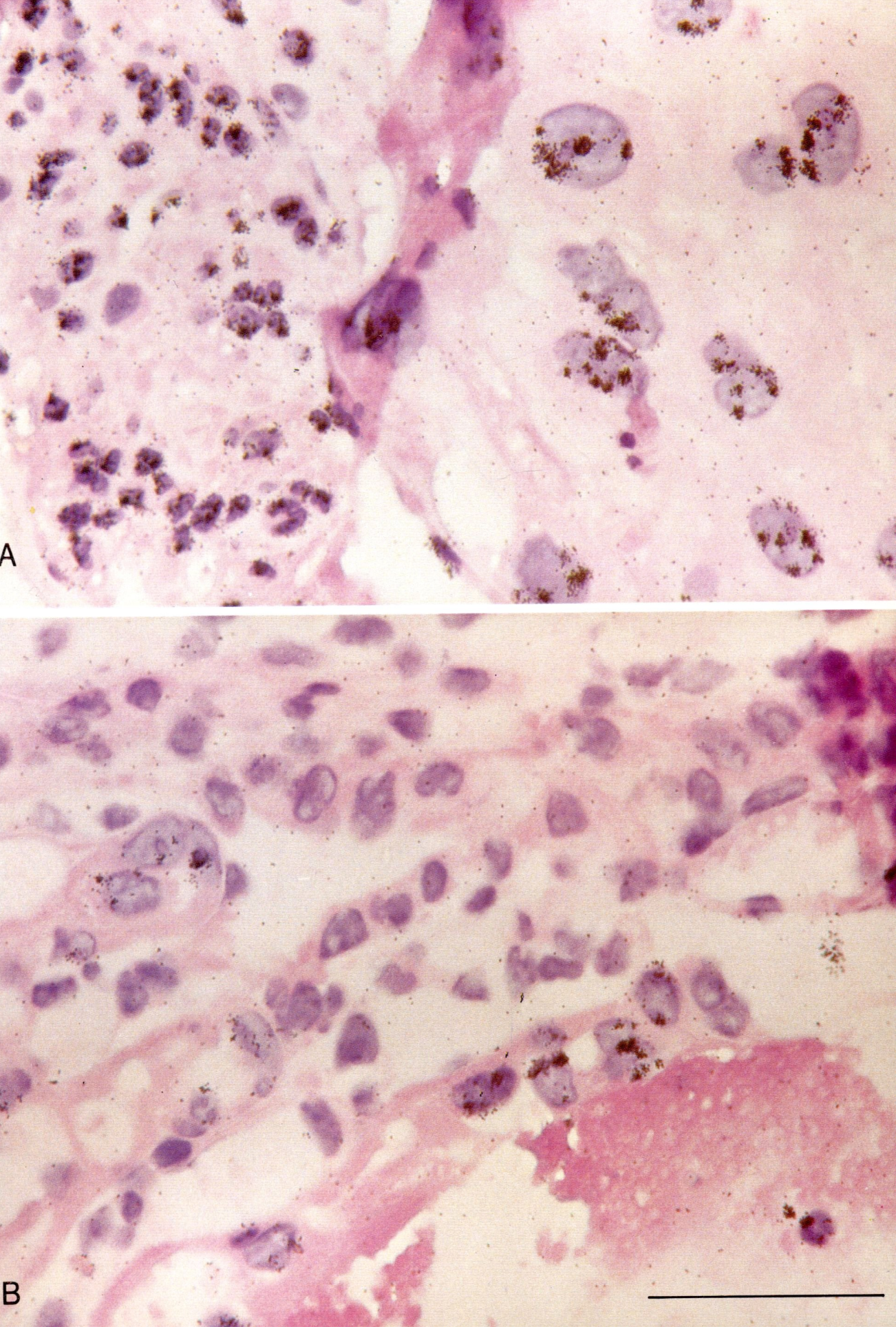
n.a. = not analysed.

tissue indicated derivation from the injected ICM. Nine blastocysts were transferred; five implants containing viable embryos were recovered. One embryo showed no ICM contribution to any tissue, including the embryo, and appears to be an intact *Gpi-1^a/Gpi-1^a* blastocyst transferred in error. The remaining four embryos showed the expected GPI distributions, and from these, it was clear that the ICM contribution to the entire placenta was small, averaging only 4 % (Table 4). Most of this contribution was confined to the labyrinthine trophoblast and may be totally confined to this tissue, since the low level of ICM contribution to the spongiotrophoblast was probably contaminating labyrinthine tissue.

Cell suspensions of separated labyrinthine and spongiotrophoblast were prepared by collagenase digestion of tissues pooled from the four embryos. Levels of ICM isozyme were higher in both undissociated matrix and dissociated cells than intact spongio- or labyrinthine trophoblast (spongiotrophoblast; 15 % in matrix, 20 % in cells: labyrinthine trophoblast; 40 % in matrix, 25 % in cells). It is clear from these results and those of the previous section that simple cell dissociation techniques do not produce pure trophoblast cell suspensions from either labyrinthine or spongiotrophoblast.

Interspecific reconstituted blastocysts

Reconstituted blastocysts in which the ICM was *M. caroli* in origin and the trophectoderm and uterus were *M. musculus* were used to determine the exact nature of the ICM contribution to the labyrinthine trophoblast region. GPI analysis of two such conceptuses confirmed that the foetus was entirely derived from the *M. caroli* ICM and *in situ* hybridization of a *M. musculus* satellite DNA probe to sections of placental tissue showed that unlabelled *M. caroli* ICM-derived cells were detected in the foetal capillaries and mesenchyme and in the endodermal sinuses within the labyrinthine trophoblast (Fig. 1). No ICM-derived cells were detected in the spongiotrophoblast region.


A
B

DISCUSSION

Studies on the molecular and immunological properties of mouse trophoblast have been hampered by the absence of a method for isolation of pure populations of trophoblast cells from the mature chorioallantoic placenta. It is clear from descriptive and morphological studies that both ICM and maternally derived tissues contribute to the placenta, but the extent of those contributions has not been previously established. In this study, GPI analysis of the conceptuses derived from embryo transfers revealed that roughly 30 % of the mature 13- to 15-day chorioallantoic placenta was maternally derived. When the placenta was further dissected into labyrinthine and spongiotrophoblast regions, it became clear that most of the maternal contribution was confined to the spongiotrophoblast region; the labyrinthine trophoblast region contained a very low proportion of maternally derived GPI isozyme. This was somewhat surprising since the labyrinthine trophoblast is believed to be the main area of interchange between the maternal and foetal circulations (Lala *et al.* 1983). Maternal red blood cells were not removed from the tissue samples before analysis, but some maternal blood could have been washed from the surface of the tissue. It seems likely that the penetration of maternal blood vessels into the labyrinthine region is not extensive, with interchange occurring at the junction between the spongiotrophoblast and the labyrinthine regions. The extensive maternal contribution to the spongiotrophoblast region cannot be due totally to maternal blood infiltration since cell suspensions derived from the spongiotrophoblast in which the erythrocytes had been lysed also showed extensive maternal contribution. The exact location and cellular identity of the maternal cells in the spongiotrophoblast cannot be determined by GPI analysis, but descriptive studies suggest that cords of decidual cells may infiltrate the spongiotrophoblast (Bell, 1983). Unfortunately, *in situ* marker analysis of interspecific transfers cannot resolve this issue, because *M. caroli* embryos die in the *M. musculus* uterus before the time of development of the mature placenta (Croy, Rossant & Clark, 1982).

The ICM contribution to the placenta can, however, be assessed by both GPI and *in situ* marker analysis of reconstituted blastocysts, providing a complete description of the distribution of these cells. ICM-derived cells made up only a small proportion of the total placental tissue (4 %) by GPI analysis. Further studies showed that this contribution was apparently confined to the labyrinthine region

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Fig. 1(A). Autoradiography of spongiotrophoblast region of placenta from interspecific reconstituted blastocyst, showing labelled *M. musculus*-derived trophoblast giant cells on outside and very few, if any, unlabelled *M. caroli*-derived ICM-derived cells in the bulk of the spongiotrophoblast region. A cloned probe to the *M. musculus* major satellite DNA sequence was nick-translated in the presence of [^{3}H]thymidine triphosphate and hybridized to denatured sections of placenta from interspecific reconstituted blastocysts, as described (Rossant *et al.* 1983). Accumulation of silver grains over the nucleus of a cell after autoradiography indicates hybridization of the species-specific probe. (B) Autoradiography of labyrinthine trophoblast region of same placenta, close to site of insertion of allantois, showing extensions of unlabelled ICM-derived mesenchyme, surrounded by labelled cells of trophoblast origin.

of the placenta. This finding was confirmed and extended by *in situ* analysis of interspecific reconstituted blastocysts which localized the ICM contribution to the foetal blood capillaries and mesenchyme, and the endodermal sinuses at the edge of the labyrinthine region of the placenta. No significant ICM contribution could be detected in the stromal structure of the labyrinthine region nor in any part of the spongiotrophoblast.

This analysis of undissociated tissues showed that simple dissection of the two layers of the placenta did not produce pure trophoblast derivatives; maternal and ICM-derived cells were always present as minority contaminants. Further, maternal cells were almost entirely confined to the spongiotrophoblast while ICM-derived cells were entirely confined to the labyrinthine region. Simple dissection could be useful for some studies in which the only aim is to separate uterine and foetal components, but not for studies requiring pure trophoblast. Various dissociation techniques have been used to obtain supposed trophoblast cell suspensions from either unseparated placenta or separated labyrinthine and spongiotrophoblast, and we assessed the trophoblast contribution to such preparations by GPI analysis. Erythrocyte lysis was performed on all preparations to remove some of the maternal contamination. However, neither trypsin nor collagenase dissociation produced a marked enrichment for trophoblast in either the labyrinthine or the spongiotrophoblast. Trypsin digestion, while not producing obvious trophoblast enrichment, did produce cell suspensions that were similar in genetic constitution to the intact tissues. However, the milder and less destructive collagenase treatment produced cell suspensions in which non-trophectoderm-derived cells were more prevalent than in the original tissues. This was most marked in the cell suspensions of the spongiotrophoblast of embryo transfer conceptuses, where maternally derived cells were preferentially dissociated, but it was also apparent in the labyrinthine trophoblast of reconstituted blastocysts where ICM-derived cells were present in a higher proportion in the final cell suspension than in the intact tissue.

Further separation of undissected placenta and dissected labyrinthine and spongiotrophoblast by passage over Lympholyte provided no greater enrichment for trophoblast. Indeed, the pellet which has been reported to be enriched for trophoblast (Pavia & Stites, 1981) showed the least trophoblast contribution of all. This is most dramatically demonstrated by the pellet obtained from the labyrinthine tissue which shows nearly 30 % maternal cell contribution while the intact labyrinthine tissue showed a negligible maternal contribution. Thus, the combination of collagenase digestion plus Lympholyte separation provides an efficient means for enriching for maternal cells but is a poor method for producing trophoblast cell suspensions. It is not clear why collagenase selects for maternal cells while trypsin does not, but it seems likely that trophoblast cells are more tightly bound together than the maternal cells and are thus not as readily disrupted by the gentle collagenase treatment. A previous study on the origin of decidual cells showed a similar non-representative tissue dissociation by collagenase (Gambel, Rossant, Hunziker & Wegmann, 1985).

We have been unable to date to find any cell dissociation and separation technique which does produce viable pure trophoblast cell populations (unpublished), although further density-gradient separation of collagenase-treated spongiotrophoblast can produce pure maternal cell populations (I. Athenassakis & T. G. Wegmann, personal communication). In the absence of any tissue-specific marker which can unequivocally identify trophoblast in mature mouse placenta, it is clear that attributing a particular property, for example, immunosuppression, to the trophoblast component of the placenta by assessing the properties of either intact tissue or cell suspensions is of dubious validity.

The experiments on reconstituted blastocysts also provided some apparently anomalous results. Three conceptuses showed abnormally high contributions of the ICM-derived cells to the trophoblast layers of the placenta. Although trivial explanations for these results, such as contamination of ICMs with unlysed trophectoderm or incomplete enclosure of the ICMs by trophectoderm cannot be excluded, it is also possible that the injected ICM cells contributed to the trophectoderm layer of the blastocyst prior to implantation. These anomalous conceptuses arose in experiments in which some of the injected ICMs were from early cavitating blastocysts. All previous experiments on reconstituted blastocysts have used ICMs from mature blastocysts only (Gardner *et al.* 1973; Papaioannou, 1982; Rossant *et al.* 1983), and have shown no ICM contributions to postimplantation trophoblast tissues, with the odd exception (Papaioannou, 1982). Early ICMs can form trophectoderm derivatives when isolated from the blastocyst environment (Handyside, 1978; Hogan & Tilly, 1978; Spindle, 1978; Rossant & Lis, 1979*b*). This was presumed to represent their potential and not their fate, since similar early ICMs never contributed to trophectoderm derivatives after blastocyst injection (Rossant & Lis, 1979*a*). However, the present results with reconstituted blastocysts, where no host ICM prevents contact of donor ICM and host trophectoderm, support the earlier suggestion of Handyside (1978) that the ICM of the early blastocyst may contribute progeny to the trophectoderm layer. Experiments currently under way using short-term cell lineage markers (G. K. Winkel, Y. Cruz and R. A. Pedersen, personal communication) should resolve the question of exactly when the ICM cell lineage becomes closed in the intact blastocyst.

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