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Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass

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SUMMARY

1. Inner cell mass (ICM) and trophoblast tissue were isolated from 3½-day post-coitum mouse blastocysts that were homozygous for different electrophoretic variants of the enzyme glucose phosphate isomerase (GPI). Blastocysts were reconstituted from these tissues, transferred to pseudo-pregnant recipients and allowed to develop to the early somite stage.

2. The embryo plus membranes and trophoblast were dissected and typed separately for GPI.

3. Contamination of trophoblast with maternal decidual tissue was quantified.

4. The trophoblast of the implanted embryos was almost exclusively of the trophoblast-donor GPI type. The embryos plus membranes were mainly of the ICM-donor type but most also showed a substantial proportion of trophoblast-donor type.

5. It is argued that the ICM controls trophoblast proliferation by inhibiting giant cell transformation of adjacent trophoblast cells rather than through making a significant cellular contribution.

INTRODUCTION

The trophoblast of the mouse blastocyst consists of two regions: the mural trophoblast surrounding the blastocoelic cavity and the more restricted polar trophoblast which overlies the inner cell mass (ICM). Cells of the mural trophoblast transform into a limited number of non-dividing primary giant cells during implantation (Dickson, 1963, 1966) whereas those of the polar trophoblast are believed to develop into the ectoplacental cone and a multitude of secondary giant cells (Snell & Stevens, 1966). The primary and secondary giant cells are morphologically indistinguishable and are characterized by a high content of DNA which appears to accumulate by endoreduplication of the entire genome (Sherman, McLaren & Walker, 1972) rather than by cell fusion (Chapman, Ansell & McLaren, 1972; Gearhart & Mintz, 1972).

Mural trophoblast tissue isolated from 3½-day post-coitum (p.c.) mouse blastocysts induces typical decidua in the uteri of pseudopregnant females but

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thereafter fails to proliferate, giving rise to only a few persistent, non-dividing giant cells (Gardner, 1972). These trophoblastic vesicles will proliferate only if ICM tissue is inserted into them prior to transfer (Gardner, 1971, 1973). Such combinations of tissue develop into morphologically normal conceptuses in a high proportion of cases. On the basis of this and other evidence, it was concluded that ICM tissue is essential for trophoblast proliferation during normal development (Johnson, 1972; Gardner, 1973), either to provide the cells from which the ectoplacenta develops or to interact with the overlying trophoblast by promoting division or inhibiting giant cell formation (Gardner, 1972). The present experiments were undertaken to decide between these alternative hypotheses by typing the trophoblast of implanted embryos that had developed from blastocysts reconstituted from genetically different trophoblast and ICM tissue. Isozymal variants of glucose phosphate isomerase (GPI) provide excellent genetic markers for identifying derivatives of the recombined ICM and trophoblast tissue because the enzyme is very active, ubiquitous in tissue distribution, and the paternal allele is expressed before implantation (Chapman, Whitten & Ruddle, 1971).

MATERIALS AND METHODS

Blastocysts

The $3\frac{1}{2}$-day p.c. blastocysts used for experimental reconstitution were obtained from matings of mice selected to yield *Gpi-1*^{a}/*Gpi-1*^{a} and *Gpi-1*^{b}/*Gpi-1*^{b} embryos. Some blastocysts were transferred intact to recipient females in order to determine the GPI activity of trophoblast and the degree of decidual cell contamination after implantation. Adult females were superovulated, and the conditions for recovery, manipulation, culture and transfer of blastocysts were as described earlier (Gardner, 1972), except that the medium described by Whittingham & Wales (1969) was used instead of medium M199.

Microsurgery

All manipulations were performed with a Leitz micromanipulator. Maximal trophoblastic fragments consisting of most of the mural trophoblast were obtained by cutting the blastocyst in two close to the ICM. Isolated ICMs were freed of trophoblast by dissection with fine glass needles as before (Gardner, 1972). Trophoblastic fragments were cultured for between $3\frac{1}{4}$ and 5 h to encourage them to form vesicles. An ICM was then inserted into some of the vesicles by the technique used for transferring ICMs to the blastocoelic cavity of blastocysts (Gardner, 1971). Successfully 'reconstituted' blastocysts were cultured for $2\frac{1}{4}$–$3\frac{3}{4}$ h before transfer to recipient uteri. Other trophoblastic vesicles were transferred without prior insertion of ICM tissue.

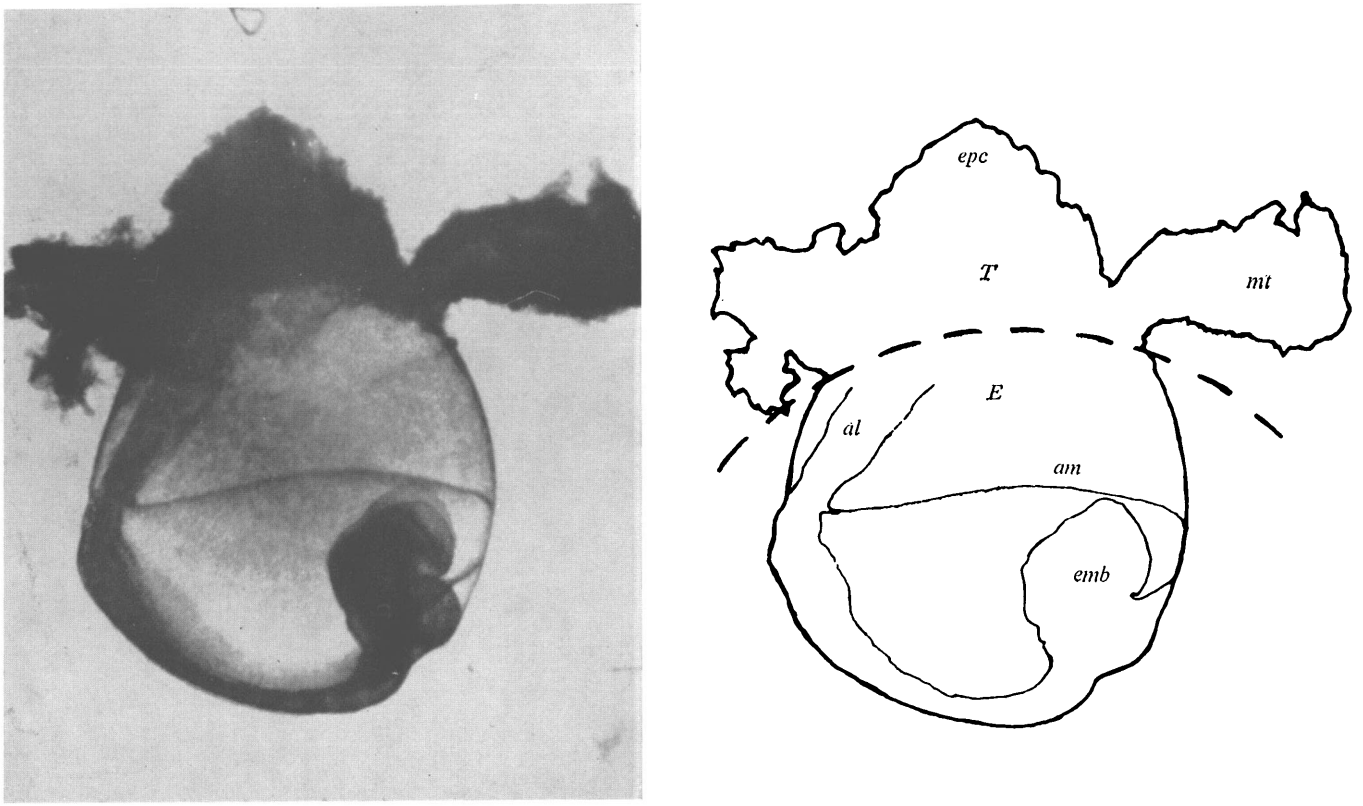

Fig. 1. Photograph of a partially dissected early somite stage embryo with diagram indicating the line of dissection into (*T*) trophoblastic fraction and (*E*) embryonic fraction; *al*, allantois; *am*, amnion; *emk*, embryo proper; *epc*, ectoplacental cone; *mt*, mural trophoblast with Reichert's membrane and attached distal endoderm.

Recipients

Recipient females were either *Gpi-1*^{a}/*Gpi-1*^{a} or *Gpi-1*^{b}/*Gpi-1*^{b} CFLP random-bred mice used on the third or fourth day of pseudopregnancy following mating with sterile males.

Examination of transferred tissue after implantation

Females receiving reconstituted blastocysts or trophoblastic vesicles were killed 6 days after transfer. Females receiving intact blastocysts were killed 5 or 6 days after transfer. The decidual swellings of those that had received intact or reconstituted blastocysts were dissected in phosphate-buffered saline (pH 7·0). The implanted embryos were carefully separated using watchmakers' forceps into a trophoblastic fraction (including ectoplacental cone, giant cells, Reichert's membrane and attached distal endoderm) and an embryonic fraction (including the embryo, amnion, allantois, chorion and visceral yolk sac) as illustrated in Fig. 1. Separation was usually satisfactory in the less advanced embryos, but some chorionic and allantoic tissue was occasionally included in the trophoblastic fractions of more advanced embryos. The fractions were rinsed in saline and then placed either in marked sectors on the floor of small plastic dishes (Falcon Plastics, 35 × 10 mm) or in separate wells of micro-test plates (Falcon Plastics). Following removal of as much saline as possible with a fine pasteur pipette, 1 μl of distilled water was pipetted on to each fraction and the container frozen.

The uterine horns of recipients carrying implanted trophoblastic vesicles were fixed in Bouin's fixative and processed for histological examination as before (Gardner, 1972).

Electrophoretic separation of GPI isozymes

Electrophoresis and subsequent staining of the starch gels was based on the method of Chapman (Chapman *et al.* 1971; Chapman *et al.* 1972). The frozen tissue fragments were thawed, teased violently, and the fluid drawn by capillarity on to 1 × 5 mm or 1 × 3 mm cellulose acetate strips (Oxoid). A further 1 μl of distilled water was added to fragments that had dried out during cold storage. The strips were inserted into slits of appropriate size in starch micro-gels of 100 × 50 × 1·75 mm. Horizontal electrophoresis was carried out at 4 °C for 1$\frac{1}{2}$ h at 160 V. Blood samples from recipients and ICM and trophoblast donor females were run in addition to embryonic tissues and standards.

Trial runs using artificial mixtures of diluted kidney homogenates from adult *Gpi-1*^{a}/*Gpi-1*^{a} and *Gpi-1*^{b}/*Gpi-1*^{b} mice gave similar resolution to that described by Chapman *et al.* (1972). Both GPI isozymes could be seen clearly in stained gels even when the *Gpi-1*^{b}/*Gpi-1*^{b} homogenate comprised only 1 % of the volume of the sample.

Whole ectoplacental cones from 8$\frac{1}{2}$-day p.c. embryos of *Gpi-1*^{a}/*Gpi-1*^{a} and

Table 1. *Development of 'reconstituted' blastocysts and control trophoblastic vesicles after transfer to recipient uteri*

Transferred tissue ...	Trophoblastic vesicles	Reconstituted blastocysts
No. of recipients	4	8
No. of uterine horns	4	8
No. of reconstituted blastocysts or trophoblastic vesicles transferred	19	36
No. of decidua	15	34
No. of decidua containing embryonic derivatives	15*	21
No. of normal embryos	0	13

N.B. A further 32 'reconstituted' blastocysts were transferred to 6 additional recipients that did not become pregnant. Similarly, a further 52 trophoblastic vesicles were transferred to 8 recipients that did not become pregnant.

* Decidua obtained following transfer of trophoblastic vesicles were examined histologically and not by dissection. All contained a few trophoblastic giant cells but no other embryonic derivatives.

Gpi-1^b/Gpi-1^b genotypes were mixed in varying proportions to check the sensitivity of the GPI assay using trophoblast tissue. No attempt was made to correct for variation in size of individual cones. Two μl of distilled water per cone were added to each sample prior to freezing and thawing. Both isozymes could be detected when the number of *Gpi-1^b/Gpi-1^b* cones comprised only 3% of the total.

RESULTS

1. *Maternal GPI contamination of implanted embryos*

One hundred and sixty 3½-day p.c. *Gpi-1^a/Gpi-1^a* blastocysts were transferred intact to the uteri of 18 *Gpi-1^b/Gpi-1^b* recipients that subsequently became pregnant. Three recipients were in the fourth day of pseudopregnancy, the remainder were in the third day. Some recipients were killed 5 days and others 6 days after transfer (7½ or 8½ days p.c.) in order to obtain a range of developmental stages comparable to that found among embryos developing from reconstituted blastocysts, which tend to develop more slowly than intact ones (Gardner, 1973, and unpublished observations). One hundred and two decidual swellings were found, 86 containing implants ranging in developmental stage from presomite to 14 somite, and including some yolk sacs without embryos. GPI was detected in the trophoblastic fraction from all but 14 implants and a maternal contribution, though frequent (54/72 or 75% were contaminated), was invariably minor as judged by the stained electrophoretograms. Hence the trophoblastic fraction yielded measurable GPI activity which was not simply due to the presence of contaminating maternal cells. No maternal GPI was detected in the embryonic fraction of 17 *Gpi-1^a/Gpi-1^a* implants recovered from

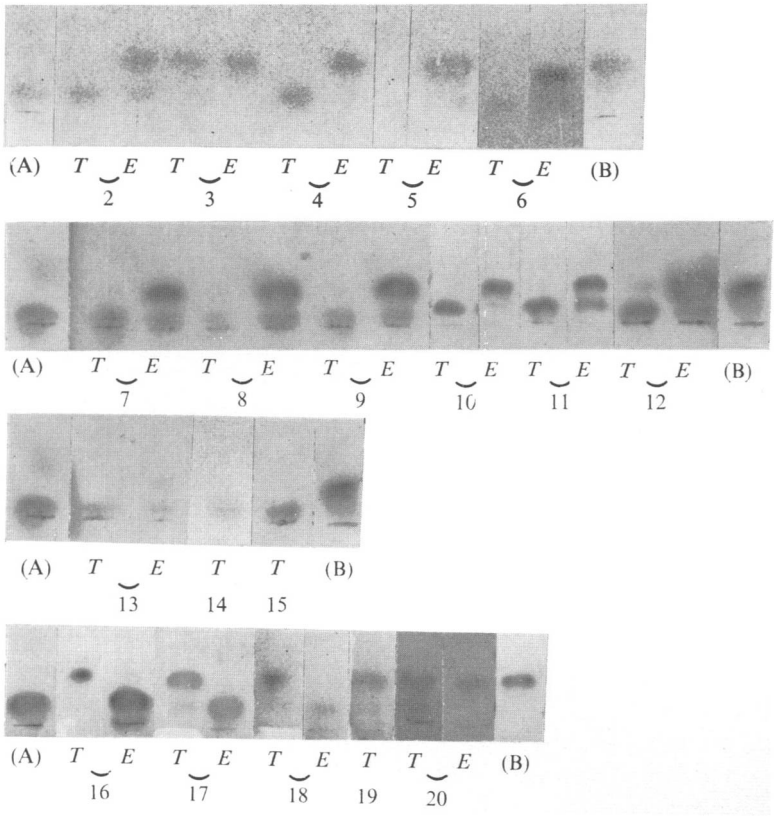

Fig. 2. Electrophoretograms of (*T*) trophoblastic fractions and (*E*) embryonic fractions developed from reconstituted blastocysts. (A) *Gpi-1*^{a}/*Gpi-1*^{a} standard; (B) *Gpi-1*^{b}/*Gpi-1*^{b} standard. Numbers correspond to embryo code numbers in Table 2. No photographs available for embryo 1.

Gpi-1^b/*Gpi-1*^{b} recipients $7\frac{1}{2}$ or $8\frac{1}{2}$ days p.c., nor in the embryonic fraction of 30 *Gpi-1*^{b}/*Gpi-1*^{b} implants recovered from *Gpi-1*^{a}/*Gpi-1*^{a} recipients.

In accord with the observations of other workers (Chapman *et al.* 1972; Gearhart & Mintz, 1972) no evidence was found of the heterodimeric variant expected if maternal cells fuse with those of the trophoblast.

2. *Development of transferred trophoblastic vesicles*

Trophoblastic vesicles were transferred to recipient uteri to determine the effectiveness of exclusion of ICM cells. Four of the 12 recipients contained decidua at autopsy 6 days later; amongst these the rate of implantation was high (Table 1). None of the 15 decidua contained presumptive ICM derivatives, and all were characterized by a central chamber, eosinophilic material and a few trophoblastic giant cells, as in a previous study (Gardner, 1972).

Table 2. *GPI in trophoblastic and embryonic fraction of implanted embryos developed from 'reconstituted' blastocysts*

Experimental series			Embryo code no.	Description of implanted embryos	Gpi-1 phenotype	
					Trophoblastic fraction	Embryonic fraction*
I	Trophoblast	*Gpi-1^a/Gpi-1^a*	1	Normal somite stage	A (weak)	B + very weak A
	ICM	*Gpi-1^b/Gpi-1^b*	2		A	B + weak A
	Recipient	*Gpi-1^a/Gpi-1^a*	3		B + weak A	B
			4		A	B
			5		A	B + weak A
			6		A	B + A
			7		A	B + A
			8		A (weak)	B + A
			9		A	B + A
			10		A	B + A
			11		A + very weak B	B + A
			12		A + weak B	B + A
			13	Trophoblastic mass + small yolk sac	A	B + A
			14	Trophoblastic mass only	A	—
			15	Trophoblastic mass only	A	—
II	Trophoblast	*Gpi-1^b/Gpi-1^b*	16	Normal somite stage	B + very weak A	A + very weak B
	ICM	*Gpi-1^a/Gpi-1^a*	17	Single trophoblast, twin embryonic fractions†	B + weaker A	A
	Recipient	*Gpi-1^a/Gpi-1^a*	18	Trophoblast mass + small yolk sac	B	A (trace)
			19	Trophoblast mass only	B + trace A	—
III	Trophoblast	*Gpi-1^a/Gpi-1^a*	20	Normal somite stage	B + weak A	B
	ICM fragment‡	*Gpi-1^b/Gpi-1^b*				
	Recipient	*Gpi-1^a/Gpi-1^a*				

* Refers to normal embryonic fraction as illustrated in Fig. 1, or to parts of this such as empty yolk sac in abnormally developing embryos.

† One was a headfold-stage embryo with allantois, the other an empty yolk sac vesicle.

‡ ICM together with overlying trophoblast.

3. *GPI activity in trophoblastic and embryonic fractions developed from reconstituted blastocysts*

Twenty-one decidual swellings contained identifiable embryonic derivatives (Table 1). Two embryos were small and degenerate and were not analysed. Thirteen of the remainder were morphologically normal embryos varying between the late presomite and 8-somite stage. A further embryo had a single ectoplacental cone but twin yolk sacs (one containing a normal embryo and the other empty), and the last five each consisted of a mass of trophoblast cells with or without an enclosed yolk-sac vesicle (Table 2).

The results of the GPI analysis of the 19 implants are illustrated in Fig. 2 and summarized in Table 2. The GPI of the trophoblastic fraction dissected from 13 implanted embryos was exclusively of the original trophoblast donor type (Table 2, I and II). The trophoblastic fraction of five embryos displayed an additional minor band of the ICM donor type (embryos 11 and 12) or of ICM and/or maternal type (embryos 16, 17 and 19). The trophoblastic fraction from a single embryo (no. 3) was predominantly of ICM type with a minor contribution of the trophoblast and/or maternal cell type.

The GPI of the embryonic fraction from four embryos was exclusively of ICM type. The remaining 15, however, also showed a second band of the trophoblast donor type which, though variable, was sometimes almost as strong as that of ICM type (Table 2, Fig. 2).

4. *Development of blastocysts reconstituted from trophoblastic vesicles and minimal ICM fragments*

The exceptional embryo (no. 3) with mainly ICM-type GPI in its trophoblast may have developed from a reconstituted blastocyst whose ICM had not been freed of all trophoblast cells during dissection. Some ICMs with the trophoblast intentionally left intact were used for reconstitution with trophoblastic vesicles to test this possibility. Almost all of these reconstituted blastocysts, unlike those obtained using isolated ICMs, had extruded this minimal ICM fragment after postoperative culture. Seven were nevertheless transferred to two recipients in which seven decidual swellings were found 6 days later. Only one decidual swelling had recognizable embryonic tissues, containing a normal 8 somite embryo with trophoblastic GPI almost exclusively of the ICM fragment type (embryo 20, Fig. 2 and Table 2).

DISCUSSION

The present results confirm those of an earlier study showing that a high proportion of $3\frac{1}{2}$-day blastocysts reconstituted from isolated ICM and trophoblast can implant and develop into morphologically normal conceptuses (Gardner, 1971). They also provide valuable data on the tissue of origin of the trophoblast, embryo and membranes of the implanted embryo.

The trophoblastic fraction of the majority of implanted embryos (13/19) showed only one band of GPI activity which corresponded to that of the trophoblast donor type. The remaining six embryos yielded two bands – that appropriate to the trophoblast donor genotype predominating in all but one (no. 3). The second band was definitely of ICM origin in three cases. It was of uncertain origin in the further 3. A minor contribution of ICM type activity could be due to inclusion of possible ICM derivatives such as chorionic or allantoic tissue in the dissected trophoblast; also the distal endoderm was routinely included with the trophoblastic fraction in our dissections (see Fig. 1). A maternal contribution to trophoblastic GPI is also to be expected (see Results, section 1), and finally, contamination of donor ICMs with trophoblast cells could lead to an apparent ICM contribution, probably explaining the exceptional result shown by embryo no. 3. Nevertheless, it is evident that most, if not all trophoblast cells of the postimplantation embryo are mitotic descendants of trophoblast cells of the $3\frac{1}{2}$-day blastocyst.

The major GPI activity of the embryonic fractions was the ICM donor type. However, all but four had a second band of the trophoblast donor type which represented a substantial proportion of the total GPI activity in several cases (Fig. 2). Failure to exclude all ICM cells from the donor trophoblastic vesicles is unlikely since none of the 15 implants that developed from such vesicles showed any evidence of proliferation or of ICM derivatives. In an earlier study only 2 out of 53 maximal trophoblastic vesicles contained ICM derivatives (Gardner, 1972). The inclusion of substantial amounts of ectoplacental tissue with the embryonic fraction during dissection also seems unlikely since the boundary between the two regions is clear (Grobstein, 1950; Kirby, 1971*a*). However, the trophoblast of the mouse blastocyst might actually make a cellular contribution to the developing egg-cylinder and, were this the case, the boundary chosen could be arbitrary. Indeed, Dalcq has suggested on the basis of cytochemical observations that the endoderm of the rat embryo originates from the trophoblast (see Dalcq, 1957, fig. 43*k*). Finally, the presence of GPI of trophoblast or ICM type in tissues other than their presumptive derivatives might indicate the existence of occasional labile cells within these tissues.

Some ICM tissue is essential for the proliferation of trophoblast after implantation (Gardner, 1971, 1972, 1973). The present results demonstrate that the ICM makes little or no cellular contribution to the trophoblast of the implanted embryo. Hence the ICM must control trophoblast proliferation in a different way – for example, by promoting division or inhibiting giant transformation of some cells (Gardner, 1972).

Giant cell formation begins at the abembryonic pole of the implanting blastocyst very early on the fifth day of gestation, reaches the edge of the ICM by noon, but does not extend beyond the latter point to involve the trophoblast cells overlying the ICM (Dickson, 1966). Polyploidy has been detected in some cells at this time, even in blastocysts cultured *in vitro* (Barlow, Owen & Graham,

1972; Barlow & Sherman, 1972), hence transformation does not depend on maternal factors. Barlow & Sherman (1972) suggest that formation of the blastocoele provides the stimulus for polyploidization of the mural trophoblast. However, this hypothesis does not explain the formation of a large number of morphologically indistinguishable secondary giant cells that occurs slightly later in development in circumstances in which involvement of the blastocoele is out of the question.

We prefer to regard giant cell transformation as the normal path of differentiation of all trophoblast cells of the blastocyst not in contact with the ICM. Trophoblast cells in contact with ICM cells would be prevented from transforming and thus could give rise to the ectoplacental cone by continued mitotic activity. It is noteworthy that, according to Duval (1892), growth of the ectoplacental cone occurs mainly by cell division in deeper regions. He likened the process to renewal of adult epidermis by divisions in the Malpighian layer of the skin. Furthermore, the cells show a graded increase in nuclear size (and hence presumably in DNA content, Barlow & Sherman, 1972) from base to periphery of the cone. This is consistent with the hypothesis that continued division occurs only in those cells adjacent to the ICM or its derivatives. The progeny of these cells that are pushed peripherally would no longer be inhibited, and could thus undergo progressive transformation to form the secondary giant cells that eventually surround the entire conceptus (Snell & Stevens, 1966). Viewed thus, the identical morphology of the primary and secondary giant cells is readily explained.

A consequence of the above scheme would be the dependence of continued proliferation of the trophoblast on continued development of the ICM. This could be important in ensuring that the development of the trophoblast was coordinated with that of the embryo it supports. Such growth control may indeed extend to later stages of pregnancy. The mitotic activity of ectoplacental tissue declines considerably by the 8th day of gestation, but increases again thereafter (Avery & Hunt, 1969; Barlow & Sherman, 1972). This secondary rise seems to occur after the base of the ectoplacenta comes into intimate contact with 2 presumptive ICM derivatives, the chorion and allantois (Snell & Stevens, 1966).

Reconstituted blastocysts lack embryonic polar trophoblast because they are obtained by inserting ICM tissue into vesicles of mural trophoblast. The fact that many develop normally shows that at $3\frac{1}{2}$ days p.c. at least some of the mural trophoblast cells are not yet committed to form giant cells. This argues against the existence of regional determination within the trophoblast of the $3\frac{1}{2}$-day blastocyst. Giant cells are not in fact evident until some hours after the blastocyst is orientated with its ICM directed mesometrially. Orientation may occur by means of the ICM migrating round the inner surface of the trophoblast (Kirby, Potts & Wilson, 1967; Jenkinson & Wilson, 1970; Gardner, 1971; Kirby, 1971*b*). Hence it may be the position which the

ICM finally occupies that determines the location of future ectoplacental and mural trophoblast.

We cannot at present exclude the hypothesis that in the intact blastocyst embryonic polar trophoblast proliferates independently of the ICM, and that the latter is necessary only when presumptive mural trophoblast is called upon to support development. However, we consider it unlikely.

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