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Trophectodermal processes regulate the expression of totipotency within the inner cell mass of the mouse expanding blastocyst

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SUMMARY

Mouse blastocysts, aged 0, 2, 6 and 12 h from the onset of cavitation, were examined by transmission (TEM) and scanning (SEM) electron microscopy. In TEM sections, trophectoderm cells (TE) differed morphologically from those of the inner cell mass (ICM) by their flattened shape, paler cytosol staining and polarized disposition of both junctional complexes (apicolateral) and intracellular secondary lysosomes (SL; basal). Throughout this period of development, cytoplasmic processes, characterized by abundant SLs, cover approximately 80 % of the juxtacoelic face of the ICM. These processes are shown to be derived from the basal surface of TE cells intermediately placed between polar and mural regions. In SEM preparations of the juxtacoelic ICM surface, revealed by 'cracking open' blastocysts, the processes appear as tongue-shaped, centripetally oriented structures which terminate collectively at a central area on the ICM surface.

The potential of cultured ICMs to generate TE was demonstrated following their immunosurgical isolation from blastocysts aged up to 12 h post cavitation and by examining the sequence of ultrastructural changes associated with TE generation by ICMs from 2 h blastocysts. In contrast, the juxtacoelic cells of similarly aged ICMs observed *in situ* in ultrasections of intact embryos showed little or no evidence of totipotency expression as judged by the absence of TE characteristics. Since TE expression within presumptive ICM cells is thought to be generated by an asymmetry of cell contacts (Johnson & Ziomek, 1983), we propose that the juxtacoelic TE processes, by providing a cellular cover to the ICM, function in suppressing the expression *in situ* of ICM totipotency.

INTRODUCTION

The time at which cells belonging to the inner cell mass (ICM) of the preimplantation mouse embryo lose their capacity to express the trophectodermal (TE) phenotype occurs at approximately 3·5 days of development when the blastocyst is almost fully expanded. The timing of ICM commitment has been derived from a number of experimental approaches, including analysis of the developmental potential of microsurgically or immunosurgically isolated ICMs cultured *in vitro* (Gardner, 1972; Solter & Knowles, 1975; Handyside, 1978) or *in utero* (Rossant, 1975*b*), and assessment

of the tissue derivatives from chimaeras made by aggregating genetically labelled ICMs with other embryonic cells prior to their return to the uterine environment for subsequent development (Rossant, 1975*a*; Gardner, 1975). In contrast, cells located on the inside of 16-cell and later morulae, or ICMs within early expanding blastocysts aged up to approximately 3·3 days, show no such restriction in developmental potency when isolated and examined in the same way (Johnson, Handyside & Braude, 1977; Handyside, 1978; Hogan & Tilly, 1978; Spindle, 1978; Johnson, 1979; Rossant & Lis, 1979; Ziomek & Johnson, 1982; Ziomek, Johnson & Handyside, 1982).

The nature of the signal inducing inside cells or ICM cells to express their totipotency by generating TE has been investigated in the 16-cell morula (Ziomek & Johnson, 1981; Johnson & Ziomek, 1983) where two distinct cell populations exist: larger, less adhesive blastomeres (presumptive TE) positioned on the outside of the embryo and possessing a polar distribution of microvilli on their free apical membranes, and smaller, more adhesive inside cells (presumptive ICM) with an apolar phenotype. *In vitro* experiments have shown that inside cells will polarize to yield a phenotype similar to that of outside cells only if they are exposed, for a sufficient period, to asymmetric cell contacts (Ziomek & Johnson, 1981; Johnson & Ziomek, 1983).

These observations suggest that in the intact embryo, up until the time of commitment at around 3·5 days of development, totipotent inside cells will remain apolar and destined to form ICM so long as complete cell cover is maintained, whether provided by neighbouring inside or outside cells. Once such contacts are disturbed, either by natural cell loss in presumptive TE or by experimental manipulation, the regulative capacity of the embryo can be realized by inside cells polarizing in response to asymmetric cell contacts (Johnson & Ziomek, 1983).

This hypothesis is open to one important criticism. During cavitation, the ICM becomes displaced to an eccentric position within the embryo so that on one face it underlies the polar TE and on the other is apposed to the expanding blastocoel. Since the period of ICM totipotency extends into the period of cavitation, it is necessary to establish whether those inside cells adjacent to the blastocoel, and hence potentially exposed to asymmetric contacts, polarize and generate TE cells and, if they do not, to discover why.

Of interest in this context are observations recorded by Ducibella, Albertini, Anderson & Biggers (1975) in a study of cell junctional morphology in the developing blastocyst. They report on the presence of attenuated cell processes at the blastocoelic face of the ICM, which they propose assist in anchoring the ICM eccentrically within the blastocoel. However, a different and more important role for such processes is possible, since were they to provide sufficient cover for the ICM, they might act to restrict the expression of developmental potential. To establish such a role, it is necessary to ask whether these processes are exclusively of TE origin, whether they persist throughout

the period of ICM totipotency and to what extent do they envelop the ICM?

We have answered these questions by examining relatively synchronized populations of expanding blastocysts timed from the onset of cavitation by transmission and scanning electron microscopy and by the use of ingested horseradish peroxidase as a marker for TE cells. The data from these studies on ICMs *in situ* are compared with ultrastructural observations made on totipotent ICMs undergoing TE formation following isolation and culture *in vitro*.

MATERIALS AND METHODS

Embryo collection

Female HC-CFLP (Hacking & Churchill Ltd) or MF1 (Olac Ltd) mice were superovulated (5 i.u. PMS followed 45–48 h later by 5 i.u. hCG, Intervet) and mated overnight with HC-CFLP males; a vaginal plug indicated successful mating. Embryos were recovered by flushing oviducts with phosphate-buffered medium 1 containing 4 mg/ml bovine serum albumin (PB1 + BSA; Whittingham & Wales, 1969) or HEPES-buffered medium 2 plus 4 mg/ml BSA (M2 + BSA; Fulton & Whittingham, 1978). Embryos were cultured in medium 16 plus 4 mg/ml BSA (M16 + BSA; Whittingham, 1971) under oil in Sterilin dishes at 37 °C and 5 % CO_2 in air.

Embryo staging

Since there is variation in the timing of cavitation in embryos of identical age post-hCG or *postcoitum*, embryos were staged from the moment at which the blastocoel was first evident (Chisholm, Johnson, Warren, Fleming & Pickering, 1985). Embryos were collected at the 8- to 16-cell stage (65–72 h post-hCG) and cultured individually in drops of M16 + BSA. At 90 min intervals, each embryo was examined on a Wild inverted-phase microscope for the first sign of a blastocoelic cavity. Nascent blastocysts from each inspection period were pooled, designated 0 h blastocysts, and cultured for the required time before experimentation; in this study 0, 2, 6 and 12 h blastocysts were used.

Developmental potential of ICM

ICM potential to generate TE *in vitro* was examined in ICMs isolated from 2, 6 and 12 h blastocysts derived from CFLP mice. Removal of the zona pellucida was achieved 1 h before immunosurgery by brief exposure to prewarmed acid Tyrode's solution (pH 2·5 + 4 mg/ml polyvinyl pyrrolidone; Nicolson, Yanagimachi & Yanagimachi, 1975). The procedure used for immunosurgical removal of TE was as described by Chisholm *et al.* (1985) with exposure to rabbit antimouse species antiserum being 3 min (2 and 6 h blastocysts) or 10 min (12 h blastocysts). Since the full sequence of immunosurgery from collection of timed blastocysts to isolation of ICMs requires 1 h for completion, the ages of ICMs at isolation were 3, 7 and 13 h from the onset of cavitation.

ICMs were cultured individually in 25 μl drops of RPMI 1640 supplemented with 10% foetal calf serum (RPMI 1640 + FCS; Flow) and examined for development at regular intervals for up to 4 days using the inverted-phase microscope.

In addition, ICMs derived from 2 h blastocysts from MF1 mice were cultured for varying periods up to 48 h, prior to fixation for TEM to examine the ultrastructural changes associated with the acquisition of TE characteristics by outside cells. Timing of ICMs was from their isolation from lysed TE cells following immunosurgery.

Labelling of blastocysts with horseradish peroxidase

Intact, 0 h blastocysts were incubated in M16 + BSA containing 5 mg/ml horseradish peroxidase (HRP; Sigma Type II) at 37 °C for 1–4 h, washed briefly in two changes of M2 + BSA and immediately fixed for TEM.

Transmission electron microscopy

0, 2, 6 and 12 h blastocysts, cultured ICMs, and blastocysts preincubated in HRP were fixed in 3% glutaraldehyde in 0·1 M-cacodylate buffer (pH 7·3) for 30 min at room temperature, washed twice in buffer and postfixed in similarly buffered 1% osmium tetroxide for 30 min. Embryos were washed subsequently in distilled water, *en bloc* stained with saturated aqueous uranyl acetate for 30 min, dehydrated in a graded ethanol series and embedded in Spurr's or occasionally Araldite resin. These procedures were considered optimal following preliminary studies using different protocols, e.g. the inclusion of paraformaldehyde in primary fixation; fixation at 37 °C; fixation in buffered M16; comparing tannic acid, potassium ferricyanide and uranyl acetate for contrast enhancement of fixed membranes.

For enzyme localization, HRP-treated embryos were fixed in glutaraldehyde and incubated for 30 min in 0·1 M-Tris-HCl buffer (pH 7·3) containing 0·5 mg/ml diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0·03% H_2O_2.

Ultrathin sections were cut on an LKB Ultrotome III, stained with alcoholic uranyl acetate and lead citrate, and viewed in an AEI 6B electron microscope at 80 kV. Thick (1–2 μm) resin sections were stained with 1% toluidine blue/borax and examined and photographed on a Zeiss Photomicroscope. Thick and ultrathin sections from HRP-treated blastocysts were also viewed unstained.

Scanning electron microscopy

6 and 12 h blastocysts, without zonae, were processed for SEM according to Johnson & Ziomek (1982). In order to examine the cellular topography of the inner surface of the blastocoel, blastocysts were 'cracked open' following

critical-point drying using freshly broken glass as a knife edge, controlled either manually or with a hand-held manipulator (Singer). Specimens were viewed on a Cambridge Stereoscan 600 electron microscope.

Photography

All light microscope photographs were taken on Ilford Pan F film.

RESULTS

Developmental potential of ICMs

The potential of ICMs to generate TE was examined by culture *in vitro* of ICMs isolated immunosurgically from 2, 6 and 12 h blastocysts (Table 1). The criterion used for TE expression by individually cultured ICMs was the formation within 24–30 h of a vesiculated structure usually containing a cluster of inner cells and thus resembling a miniature blastocyst (Fig. 1). Vesiculated structures forming at longer incubation times can consist partly or exclusively of endoderm as assessed by light and TEM analysis (Chisholm *et al.* 1985). Embryonic bodies expressing TE morphology accounted for 77 %, 61 % and 20 % of 2 h, 6 h and 12 h ICMs respectively, following 48 h in culture. Remaining ICMs either failed to develop, possessed an ambiguous phenotype or after 48–96 h culture, generated into two-layered structures with a distinct and cohesive outer layer of endoderm-like cells (Fig. 2), some of which subsequently could become vesiculated (see above).

Table 1. *Developmental potential of isolated ICMs cultured* in vitro *for 48 h prior to phase contrast examination*

Age of ICM*	No. of ICMs	No. forming TE†	% TE development
2 h	94	70	
	98	81	
	94	70	
	—	—	
	Total 286	221	77·3
6 h	112	69	61·6
12 h	92	14	
	83	16	
	84	23	
	—	—	
	Total 259	53	20·5

* Blastocyst stage from which ICM isolated.

† TE development assessed either by vesiculation of ICM or the formation of a blastocyst-like structure.

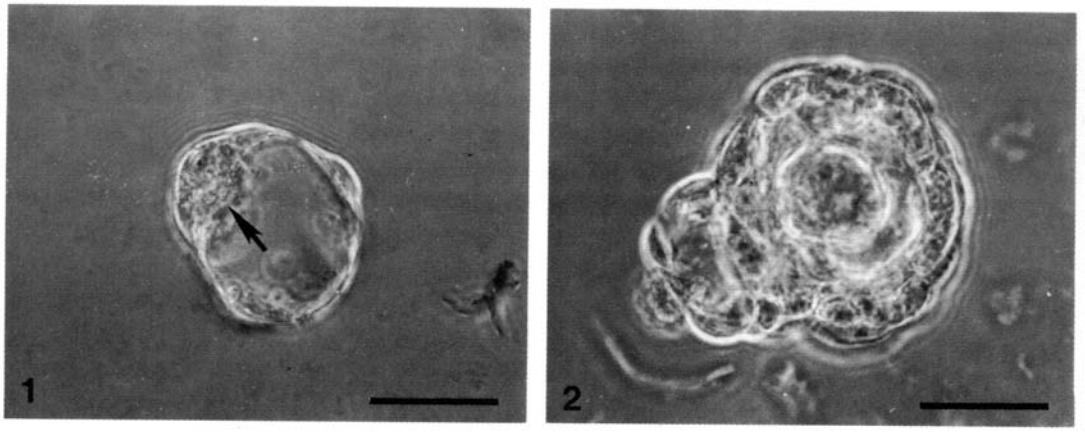

Fig. 1. Blastocyst-like vesicle containing a cluster of inner cells (arrow) derived from a 2 h ICM after 48 h in culture. Bar = 50 μm.

Fig. 2. Cavitated endodermal-like embryoid body derived from a 12 h ICM after 96 h in culture. Bar = 50 μm.

TEM morphology of timed blastocysts

Since isolated ICMs may retain the capacity to alter their developmental fate by generating TE up until 12 h postcavitation, the ultrastructure of 0, 2, 6 and 12 h blastocysts was examined to ascertain whether such expression was occurring *in situ*. A primary objective was to define any morphological differences between TE and ICM phenotypes in timed blastocysts and to discover whether those ICM cells apposed to the blastocoel cavity, and therefore potentially exposed to asymmetric cell contacts, exhibited TE characteristics. The results for the most advanced group are considered first.

12 h blastocysts

The most striking differences observed at low-power magnification between TE and ICM cells at 12 h postcavitation concern cell shape and cytoplasmic density. Both polar and mural TE cells appear elongated, with cell length in profiles through nuclei being two to three times the diameter of ICM cells

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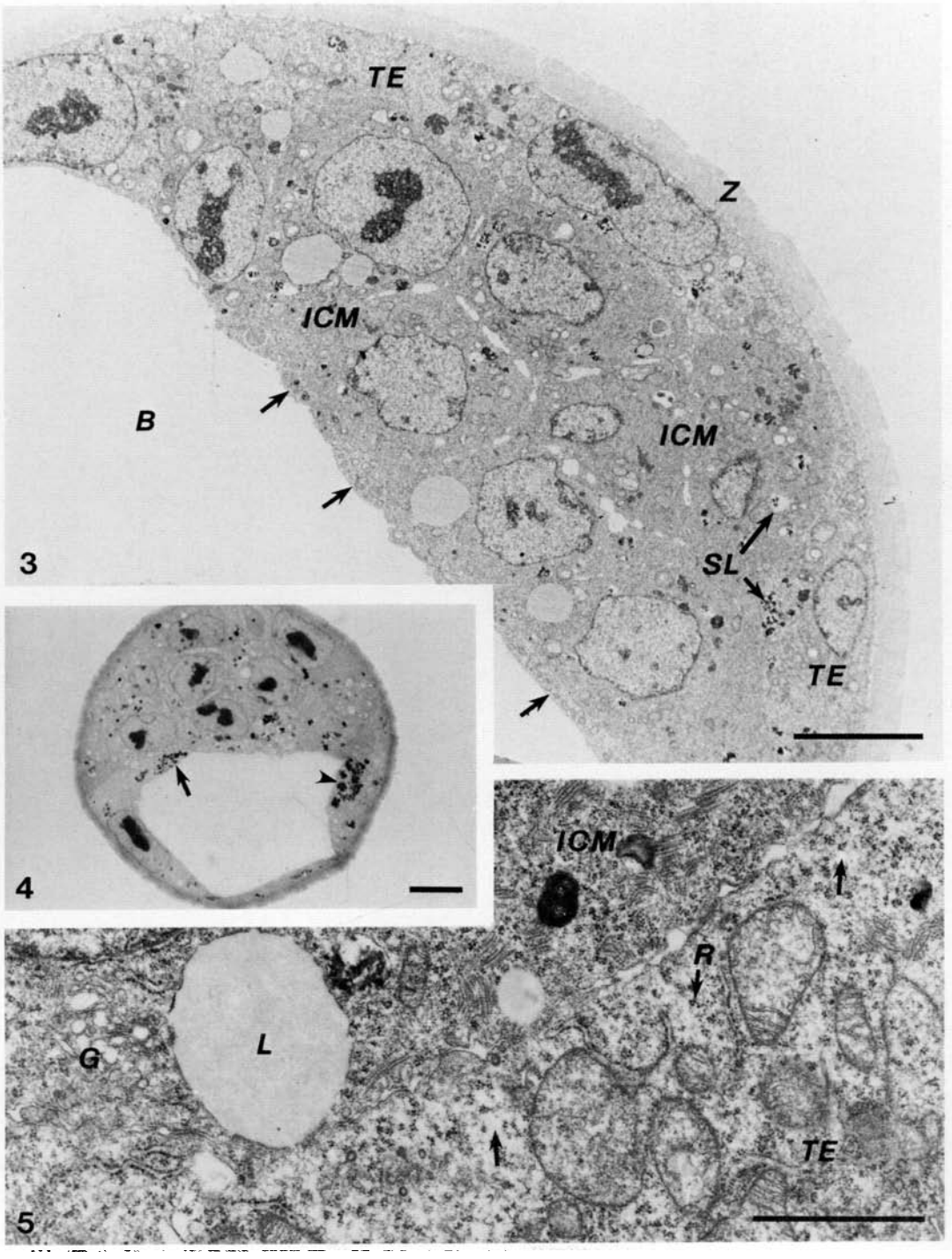
Figs 3–5. 12 h blastocysts.

Fig. 3. Low-power electron micrograph of the embryonic pole. TE cells can be distinguished from ICM cells by their attenuated shape, paler cytosol staining and basal position of secondary lysosomes (*SL*). Juxtacoelic ICM cells show no evidence of these TE features and are separated from the blastocoel (*B*) by isolated tracts of cytoplasm (arrows); *Z* = zona. Bar = 5 μm.

Fig. 4. Photomicrograph showing basal regionalization of SLs within a TE cell (arrowhead) and a juxtacoelic cell process (arrow). Bar = 10 μm.

Fig. 5. Detail of ICM and TE cytoplasm: TE cytosol contains a lower density of ribosomes (*R*) with more intervening lucent 'spaces' (arrows) compared to ICM cytosol and hence appears paler stained. *G* = Golgi body; *L* = lipid. Bar = 1 μm.


TE
Z
ICM
B
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3
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TE
5

(Figs 3, 4) and cell thickness reduced to 200–250 nm peripherally at and near the tight junction zone (Fig. 3). In contrast, ICM cells, which lie closely apposed to the inner surface of the polar TE, have a rounded appearance (5–7 μm diameter) with minimal distortion of shape due to displacement by neighbouring cells (Figs 3, 4). The attenuated TE cells also show a lesser degree of electron density of the ground cytoplasm or cytosol following conventional TEM staining with uranyl acetate and lead citrate (Fig. 3). This is shown at high magnification to be due to the reduced concentration of closely grouped clusters of free ribosomes, or polysomes, and the flocculent low-density material representative of soluble proteins precipitated by primary fixation (Fig. 5).

Further morphological differences evident between blastocyst tissues derive from the polarized condition of the TE cell membrane and intracellular components. The differences in nature and distribution of the junctional complexes have been described elsewhere (Fig. 6; Calarco & Brown, 1969; Ducibella *et al.* 1975; Magnuson, Demsey & Stackpole, 1977). In addition, TE cells exhibit a polarized distribution of certain intracellular components not previously described. In favourable sections usually through the cell centre, an accumulation of spherical, membrane-bound organelles (0·5–1·0 μm diameter), containing unstructured yet highly electron-dense deposits, is localized in the basal region of the cell (Figs 3, 4, 7). Cytochemical and TEM tracer studies have shown these structures to be secondary lysosomes (SLs) that are manufactured initially within blastomeres prior to cavitation (Fleming & Pickering, in preparation). SLs are also present within ICM cells but here they are usually less common and their distribution appears to be random (Fig. 3).

In nearly all (35/37) 12 h blastocysts examined by TEM, it was not possible to distinguish morphologically the ICM cells positioned at the interface with the blastocoel from those located either deeper within the tissue or directly

Figs 6–10. 12 h blastocysts.

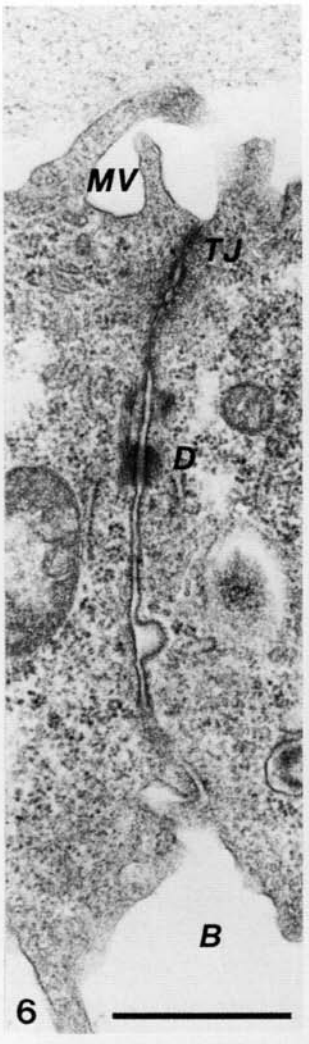
Fig. 6. TE junctional complex consisting of an apical tight junction (*TJ*) and underlying desmosomal elements (*D*). *B* = blastocoel; *MV* = microvilli. Bar = 0·5 μm.

Fig. 7. Polar TE cell with basally localized SLs. Microvilli are sparse on all TE and ICM membrane faces but aggregates may occur where three or more cells are in contact (*MV*). *C* = crystalline material; *Z* = zona. Bar = 1 μm.

Fig. 8. Juxtacoelic region of ICM containing rounded cells with an apolar disposition of SLs. The ICM is separated from the blastocoel (*B*) by isolated tracts of cytoplasm (arrowheads), one of which is rich in SLs (arrows). *L* = lipid; *M* = mitochondria. Bar = 1 μm.

Fig. 9. Juxtacoelic region of an ICM cell in which cytoplasmic process (*P*) cover is incomplete and a portion of the cell surface (bracketed by arrows) is therefore exposed directly to the blastocoel (*B*). *C* = crystalline material. Bar = 1 μm.

Fig. 10. Peripheral region of embryonic pole: a basal cytoplasmic process (arrows) from a TE cell extends over the immediate juxtacoelic surface of the ICM. *B* = blastocoel. Bar = 1 μm.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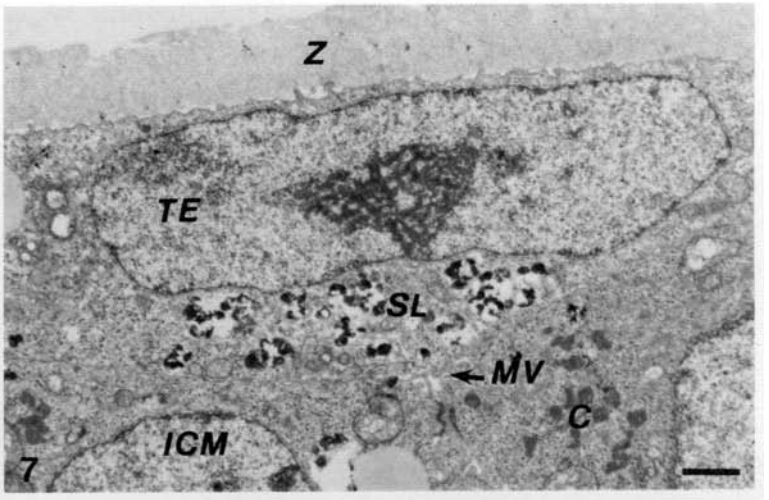
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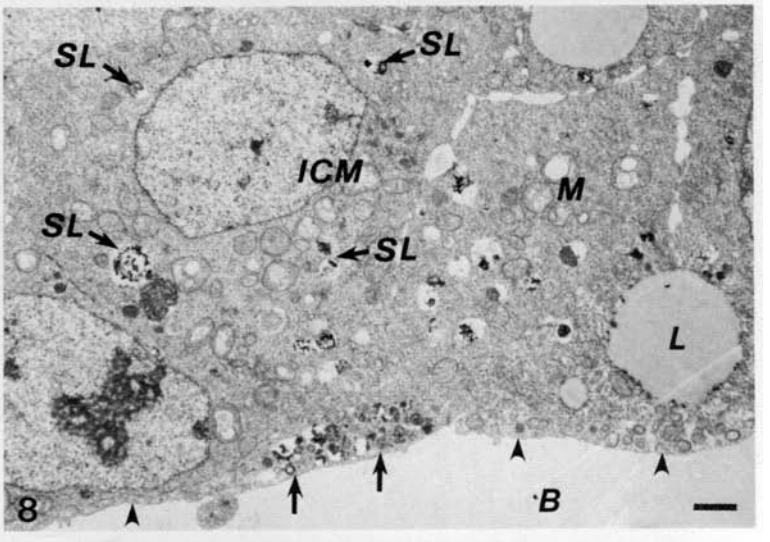
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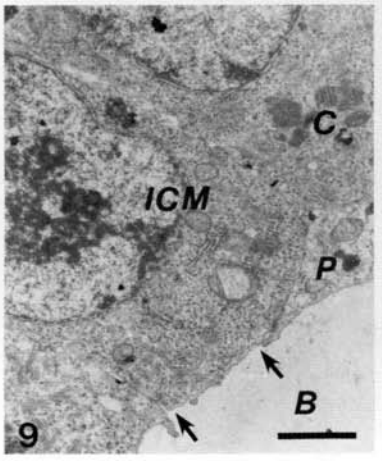
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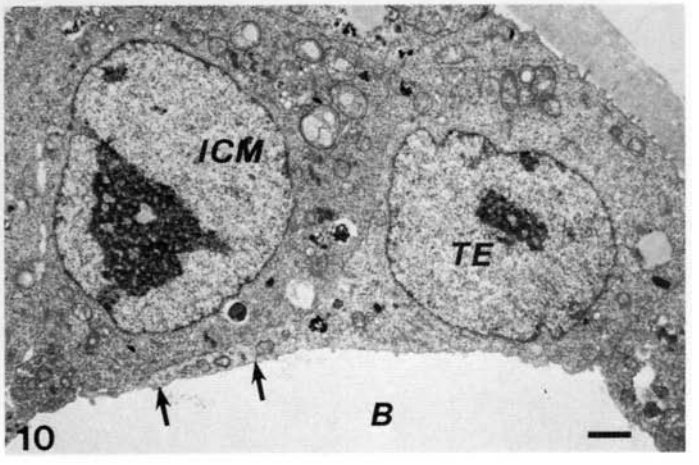
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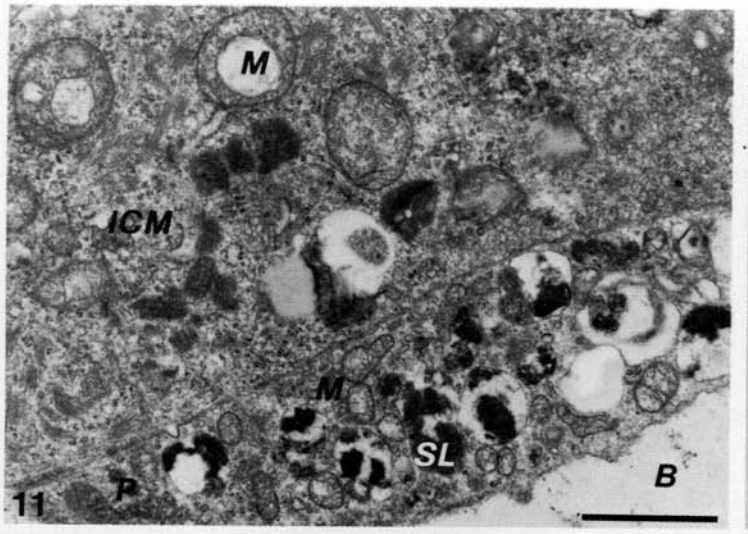

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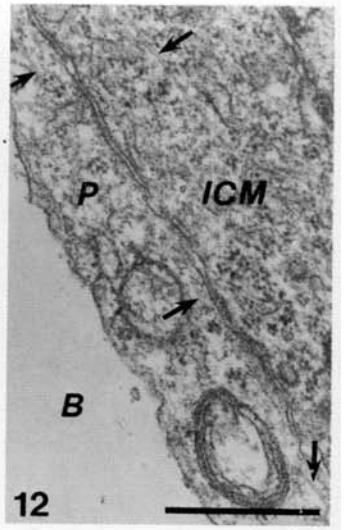

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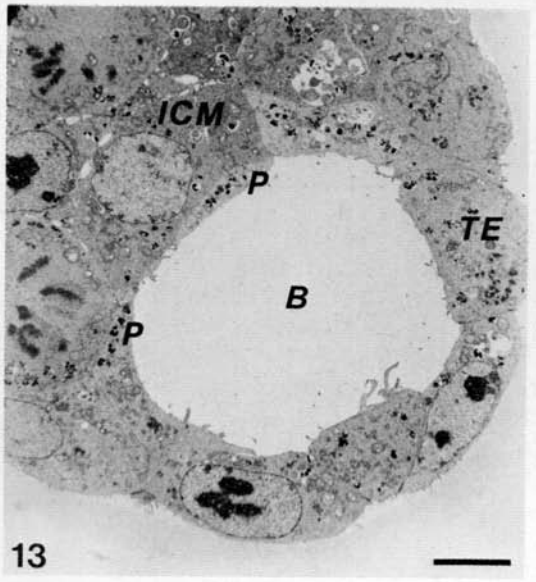

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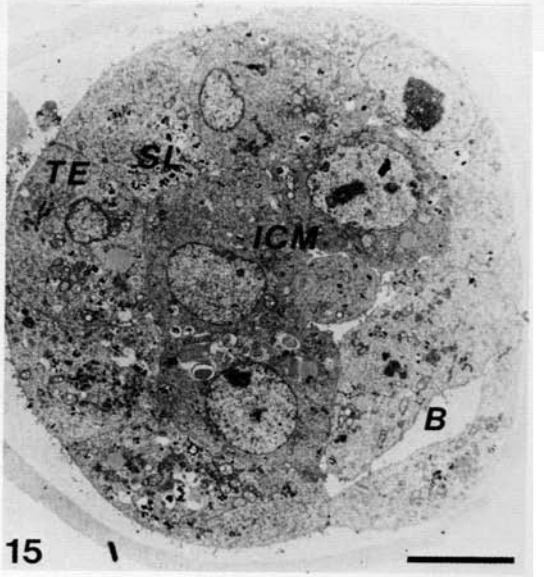

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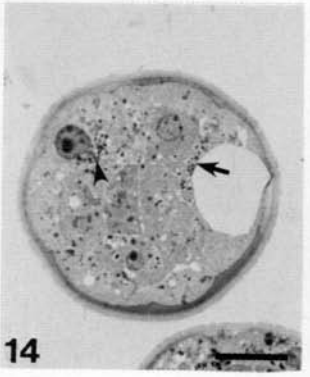
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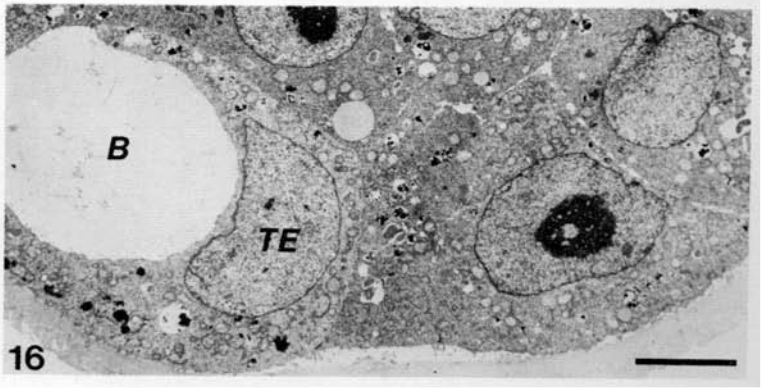

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apposed to the polar TE. All lacked any of the features which have been categorized above as trophectodermal (attenuated shape, paler cytosol, polarized SL distribution) (Figs 3, 8, 10).

In accordance with the observations by Ducibella *et al.* (1975), the juxtacoelic surface of the ICM is characterized by the presence of thin cytoplasmic processes, ranging from 0·1 to 3·0 μm in width which partially or totally enclose the ICM and thus separate it from the blastocoel cavity (Figs 3, 8). In most sectioned material, the juxtacoelic processes appear as short, isolated tracts of cytoplasm in close apposition to the underlying ICM and therefore are of unknown cellular origin. In such examples, adjacent tracts are either in direct contact (Fig. 8) or are variously spaced so that a portion of ICM membrane may be exposed to the blastocoel microenvironment (Fig. 9).

The cellular derivation of some juxtacoelic processes can be ascertained by examination of the ICM periphery in the region where polar and mural TE cell populations meet. Here, in favourable sections, a basal cytoplasmic process derived from a TE cell of intermediate polar–mural position can be seen to extend for a limited distance over the juxtacoelic ICM surface (Fig. 10). A similar example is shown in the photomicrograph, Fig. 4, but here the basal cytoplasmic attenuation of TE terminates in a zone rich in darkly stained SLs. The trophectodermal origin of isolated profiles of processes positioned elsewhere along the ICM-blastocoel boundary can usually be realized by virtue of their cytoplasmic features which include aggregates of SLs (Figs 8, 11) and a paler cytosol than underlying ICM (Figs 9, 10). Juxtacoelic processes may also contain closely packed mitochondria that are smaller than those within the ICM (Fig. 11) and longitudinally aligned microtubules typical of asymmetrically-shaped cells (Fig. 12).

0–6 h blastocysts

The features that distinguish TE cells from ICM cells within 12 h blastocysts (attenuated shape, paler cytosol staining, polarized junctional complexes and

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Fig. 11. 12 h blastocyst: juxtacoelic process (*P*) containing abundant SLs and a population of mitochondria (*M*) of a smaller size than those within the adjacent ICM. *B* = blastocoel. Bar = 1 μm.

Fig. 12. 12 h blastocyst: juxtacoelic process (*P*) and ICM cell with longitudinally aligned microtubules (arrows). *B* = blastocoel. Bar = 0·5 μm.

Fig. 13. 6 h blastocyst in which TE processes (*P*), rich in SLs, are shown lining the juxtacoelic surface of the ICM. *B* = blastocoel. Bar = 5 μm.

Fig. 14. Photomicrograph of 0 h blastocyst showing basal regionalization of SLs within a polar TE cell (arrowhead) and a juxtacoelic process covering the ICM (arrow). Bar = 20 μm.

Fig. 15. 0 h blastocyst in which the TE can already be distinguished from the ICM by reduced cytosol staining and basal regionalization of SLs. TE cell cover separates the ICM from the nascent blastocoel (*B*). Bar = 10 μm.

Fig. 16. Region of 0 h blastocyst where only the TE cell surrounding the blastocoel (*B*) exhibits paler cytosol staining. Bar = 5 μm.

SLs) are also present within earlier staged embryos (Figs. 13, 14, 15). However, polar TE cells in 0 h blastocysts, which at this stage of development comprise all but one or two outside cells, tend to be more cuboidal in shape (Fig. 15). Juxtacoelic processes, commonly rich in SLs and derived from TE cells, are also evident and isolate the ICM from the smaller-sized blastocoelic cavity in 0–6 h blastocysts (Figs 13, 14, 15). In a minority of 0 h blastocysts, the one or two cells comprising the mural TE are the only outside cells which can be distinguished by paler cytosol staining (Fig. 16).

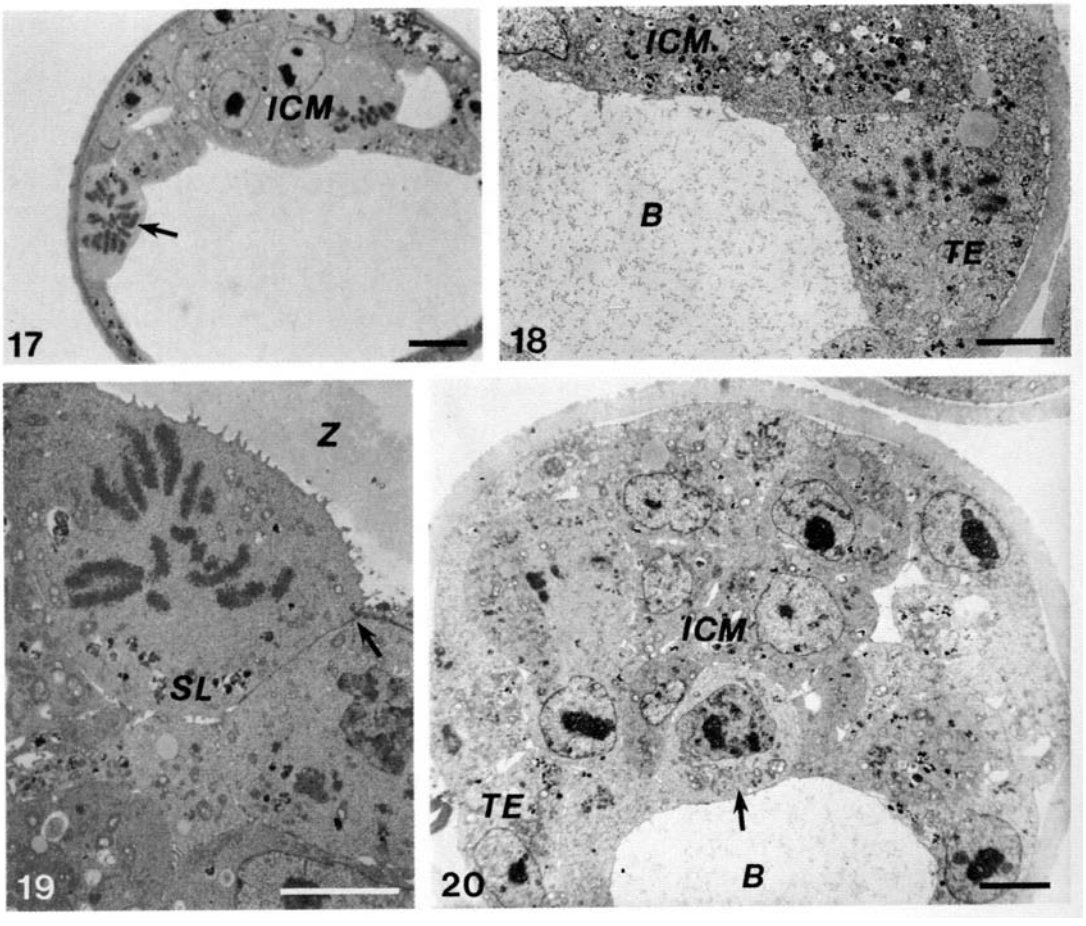


Fig. 17. Photomicrograph of 12 h blastocyst in which a TE cell of intermediate polar–mural position is mitotic (arrow) and, at this plane of section, TE processes are absent from the juxtacoelic ICM surface. Bar = 10 μm.

Fig. 18. 6 h blastocyst with a mitotic intermediate-positioned TE cell and a reduced extent of juxtacoelic process cover to the adjacent ICM. *B* = blastocoel. Bar = 5 μm.

Fig. 19. Mitotic polar TE cell from 12 h blastocyst in which both junctional contacts (arrow) and basal localization of SLs are evident. *Z* = zona. Bar = 5 μm.

Fig. 20. 0 h blastocyst in which a presumptive ICM cell (arrow) is directly exposed to the blastocoel (*B*) and exhibits pale cytosol staining of equivalent intensity to that of the TE. Bar = 5 μm.

Exceptional blastocysts with a reduced extent of juxtacoelic processes

In a small minority (8/169) of the total number of sectioned blastocysts from all age groups, a substantial portion (> 50 %) of the juxtacoelic surface of the ICM was devoid of cytoplasmic processes and hence directly faced the blastocoel cavity. Serial thick-section analysis of three such examples revealed that normal process cover to the ICM existed at other levels within the embryo. This condition was often coincident with one of the TE cells at the interface between polar and mural populations undergoing mitosis (Figs 17, 18). Such mitotic TE cells have a rounded morphology but junctional contacts are retained, thus ensuring against blastocoel collapse (Figs 18, 19). Intracellular polarity is also maintained in mitotic TE cells with SLs being localized in the basal cytoplasm (Fig. 19).

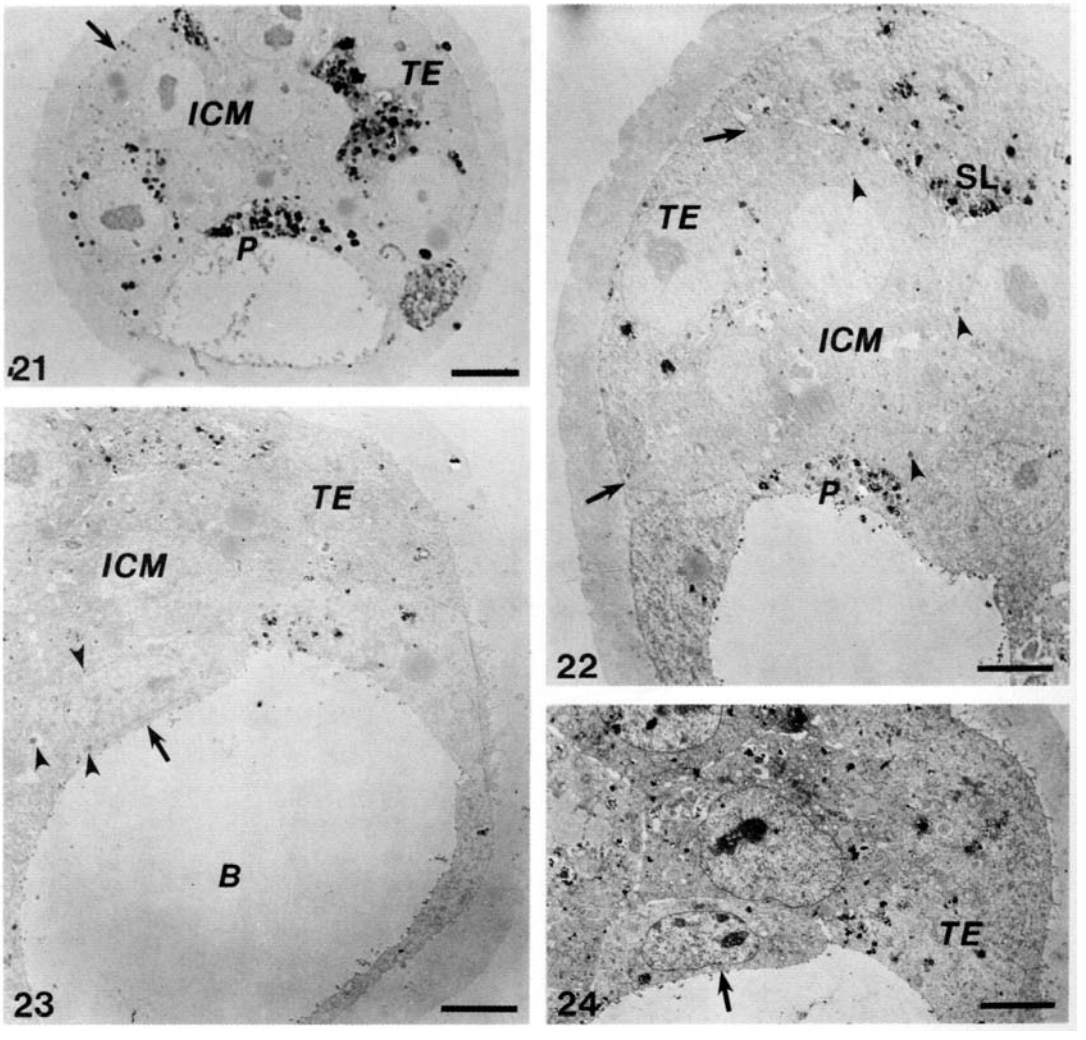
The morphology of juxtacoelic inside cells which lack trophectodermal process cover is usually consistent with the normal condition of ICM cells described above. However, in rare cases the cytosol staining of exposed presumptive ICM cells is paler than that of neighbouring cells which are completely enclosed (Fig. 20). Other trophectodermal features were not observed in juxtacoelic ICM cells exposed to the blastocoel although a limited degree of cell flattening may occur (Fig. 24).

HRP-labelled blastocysts

Zona-intact 0 h blastocysts were incubated in M16 + BSA containing HRP for 1–4 h prior to a brief wash in HRP-free medium and fixation. Localization of HRP in unstained thick and ultrathin resin sections following DAB-visualization during processing was exclusive to the TE since the establishment of zonular tight junctions blocks paracellular passage of this tracer into the embryo (Figs 21, 22, 23). The main site of endocytosed HRP in TE cells was within membrane-bound vesicular structures (0·5–1·0 μm in diameter) localized predominantly in the basal cytoplasm. Examination of adjacent stained sections revealed that these organelles correspond to the SLs described above. The juxtacoelic processes covering the ICM are particularly evident in blastocysts following HRP labelling (Figs 21, 22). Their origin from TE cells is confirmed since they contain distinct aggregates of HRP-positive SLs whereas in neighbouring ICM cells these organelles are unlabelled (Fig. 22). An identical result has been obtained using an alternative endocytotic tracer, protein A–colloidal gold (unpublished observations).

The differential staining of inside and outside cell populations that results from HRP labelling is of further advantage in recognizing cell position in sectioned material. The close proximity to the embryo surface of particular ICM cells (HRP-negative), which underlie the attenuated junctional zones between adjacent TE cells, is indicated in Figs 21 and 22.

In a small minority of HRP-treated blastocysts, trophectodermal processes were not evident on a substantial portion of the ICM blastocoelic surface at the



Figs 21–24. 0 h blastocysts labelled with HRP for 2 h (Figs 22–24) or 3 h (Fig. 21).

Fig. 21. Unstained photomicrograph in which HRP reaction product is exclusive to the TE and is predominantly confined to the basolateral cytoplasm and to a juxtacoelic process (*P*). The close proximity of an ICM cell (HRP-negative) to the embryo surface is indicated (arrow). Bar = 10 μm.

Fig. 22. Unstained electron micrograph showing basal regionalization of HRP-positive SLs within the TE and the juxtacoelic process (*P*). SLs within the ICM are HRP-negative (arrowheads). Particular ICM cells are seen close to the embryo surface beneath the junctional region of adjacent TE cells (arrows). Bar = 5 μm.

Fig. 23. Unstained section in which juxtacoelic process cover is absent and a presumptive ICM cell (arrow) is exposed to the blastocoel (*B*) and lacks HRP reactivity within its SLs (arrowheads). Bar = 5 μm.

Fig. 24. Stained section adjacent to that of Fig. 23 in which the same ICM cell (arrow) exhibits pale cytosol staining equivalent to that of TE cells. Bar = 5 μm.

Table 2. *Quantification of ICM coverage by TE processes in 6 h and 12 h blastocysts. Data derived from measurements on TEM micrographs*

Embryo No.	Length of ICM covered (μm)	Length of ICM exposed (μm)	Extent of cover (%)
6 h blastocysts			
1	29·9	1·5	95·2
2	32·0	1·7	94·9
3	11·5	10·2	53·0
4	20·0	7·6	72·5
5	9·8	8·1	54·7
6	16·7	0·0	100·0
7	21·4	3·3	86·6
8	18·1	5·8	75·7
Total	159·4	38·2	80·7
12 h blastocysts			
1	33·5	0·0	100·0
2	6·0	1·7	77·9
3	23·3	10·7	68·5
4	9·6	6·0	61·5
5	28·5	10·7	72·7
6	25·7	1·6	94·1
7	12·7	3·1	80·4
Total	139·2	33·8	80·5

plane of section examined. ICM cells thus exposed to the blastocoel when examined in adjacent stained sections were either morphologically indistinguishable from neighbouring, fully enclosed ICM cells or, alternatively, possessed a distinctly paler cytosol equivalent in staining intensity to that found within TE cells, and in some cases were slightly flattened (Fig. 24). In unstained sections, the paler category of exposed ICM cells were negative for HRP (Fig. 23) thus confirming (i) that their inside location within the embryo is genuine and not due to a tangential plane of section, and (ii) that their position is not the result of inward migration of trophectodermal cells during the period of HRP incubation. The possibility of TE cell migration occurring prior to HRP incubation cannot, however, be excluded.

Quantification of ICM coverage by juxtacoelic processes

The extent of ICM enclosure by trophectodermal processes was measured at one plane of section in eight 6 h and seven 12 h randomly selected blastocysts by recording separately the cumulative distances of covered and exposed regions of the ICM in TEM micrographs (Table 2). In both stages, when data from all embryos were pooled, the distance covered by juxtacoelic processes averaged 80 % of the ICM surface with results for individual embryos ranging from 53–100 % (6 h blastocysts) and 61–100 % (12 h blastocysts). Processes were up to 17 μm long with an average length of 6·2 ± 4·3 (S.E.M.) μm; intervals between processes were up to 9 μm long with an average length of 1·8 ± 2·2 μm.

Scanning electron microscopy

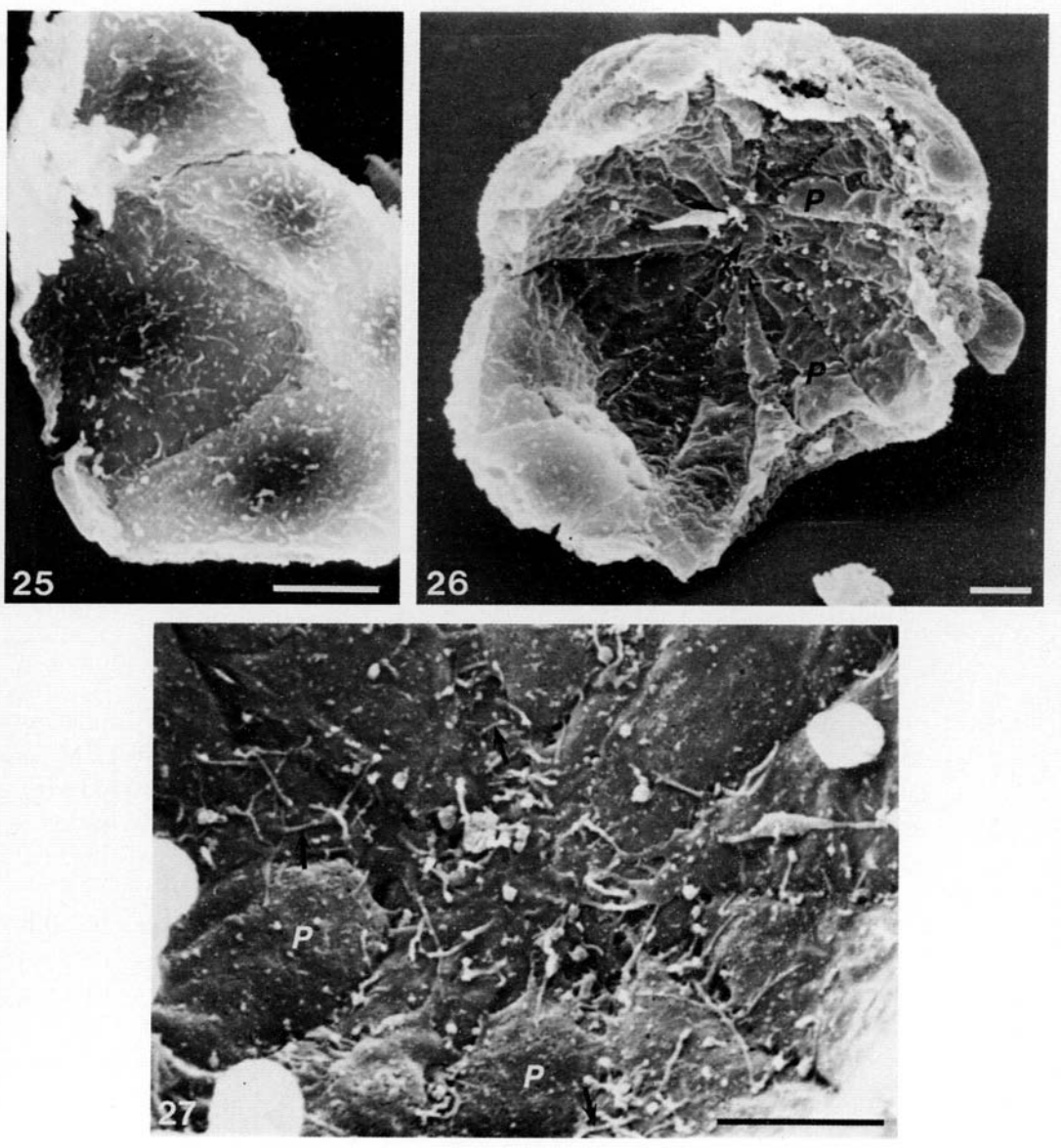
6 and 12 h blastocysts were processed for SEM and 'cracked open' following critical-point drying in order to examine the internal blastocoelic surface. The two cup-shaped fragments resulting from blastocyst fracture differ in their internal cellular topography. Fragments which were judged as thin by specimen rotation and tilting, hence corresponding to the mural or abembryonic pole of the embryo, were composed of a monolayer of polygonal-shaped cells with sparse, randomly-placed microvilli (1–4 μm long) (Fig. 25).

The other type of internal surface, found within thicker fragments corresponding to the embryonic pole, consisted of 8–14 elongate projections tapering centripetally in a rosette-like manner from the cut edge to a central point at the base of the half-blastocyst (Fig. 26). Long microvilli and stouter cellular extensions resembling filopodia are found particularly between neighbouring projections which are either closely apposed or slightly spaced (Fig. 27). These projections are considered to be the TE processes seen in TEM, since their derivation from outside cells is evident at the cut surface of the blastocyst (Fig. 26) and an underlying cell population (ICM) is sometimes apparent between adjacent projections close to their point of covergence (Fig. 27).

TEM morphology of cultured ICMs

Totipotent ICMs derived from 2 h blastocysts were cultured for varying periods (0–48 h) from the time of isolation prior to examination by TEM to determine both the nature and timing of morphological changes associated with TE expression by the newly exposed outside cells. The extent to which some of these changes occurred during the 0–8 h culture period have been quantified by scoring cells in TEM sections (Fig. 28).

A consistent feature of isolated ICMs, including those belonging to the 0 h culture group, was the attenuated condition of the newly exposed outside cells which tend to maximize their contacts, and thereby enclose, underlying cells that usually have a more-rounded morphology typical of ICM cells *in situ* (Fig. 29). In some cases, overlapping of outside cells results in a layered profile of flattened cells (Fig. 30). Desmosomes or microfilament-based adherens junctions are not particularly evident on outside cell membranes from ICMs cultured up to 8 h, thus the process of cell attenuation and its maintenance is unlikely to depend upon structured junctional contacts. An active role for the cytoskeleton is indicated however, by the presence of cortical microtubules, oriented parallel to the basolateral membrane (Fig. 31). Attenuation of outside cells is the earliest morphological change recorded in ICMs *in vitro* and is evident in over 75 % of outside cells in newly isolated ICMs, a proportion which increases subsequently following culture (Fig. 28A). Remaining cells with at least a portion of their surface exposed to the outside were rounded and usually resembled inside cells whose envelopment by processes was incomplete



Figs 25–27. Scanning electron micrographs of 'cracked open' blastocysts.

Fig. 25. Internal (blastocoelic) surface of abembryonic fragment from a 12 h blastocyst consisting of a monolayer of polygonal-shaped cells (mural TE) with irregularly placed microvilli. Bar = 5 μm.

Fig. 26. Blastocoelic surface of embryonic pole: cell projections (*P*), derived from outside cells at the cut surface of the fragment taper centripetally towards a point (arrow) at the base of the half blastocyst. Bar = 5 μm.

Fig. 27. Higher magnification of the area of convergence of cell projections (*P*) from a 6 h blastocyst. Long microvilli (arrows) are evident between neighbouring projections behind which can be seen the cell surface of the ICM. Bar = 5 μm.

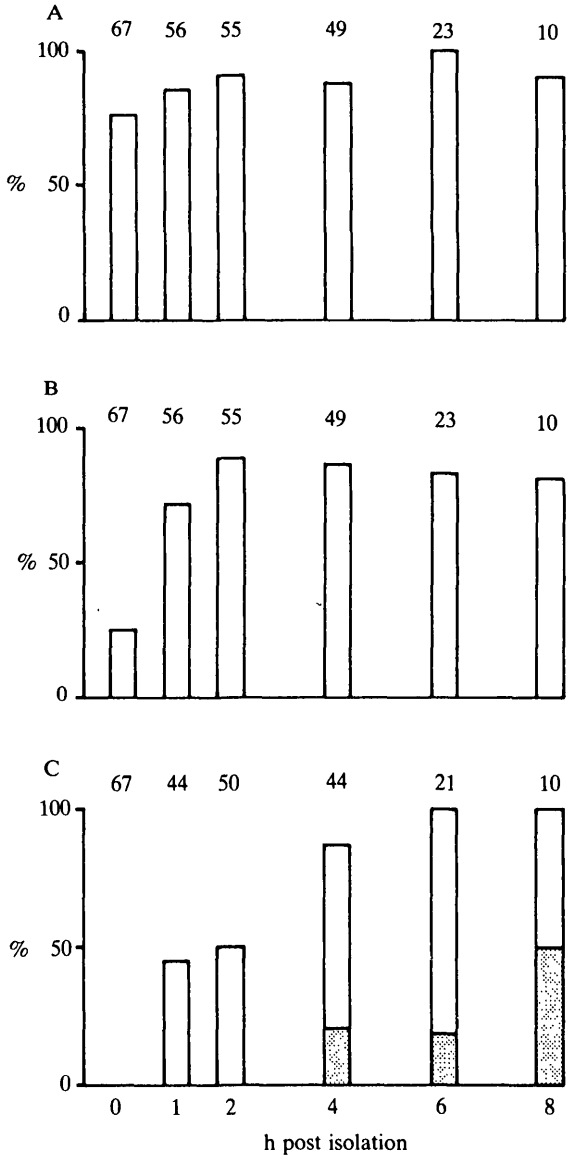


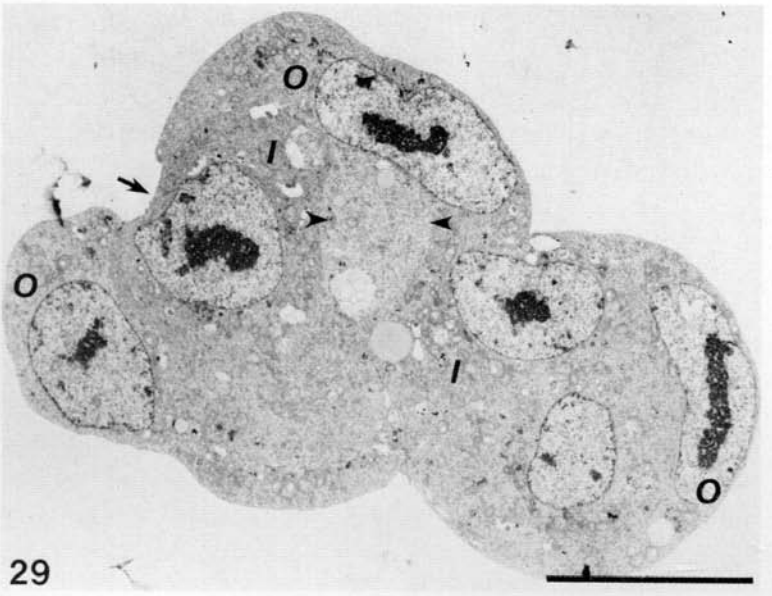
Fig. 28. Time course of morphological changes occurring in outside cells of ICMs during culture from 0–8 h post isolation. (A) cell attenuation, (B) pale cytosol staining, (C) lateral or apicolateral membrane-associated microfilament assembly. Hatched areas represent morphologically complete tight junctions with a dense plaque of inserted microfilaments and apparently fused cell membranes. The percentage of outside cells exhibiting each characteristic was determined following examination of ICMs by TEM. Figures represent total cells scored at each time point. On average, three to four cells were scored for each ICM section.

(Fig. 29). The exposed surface of partially enveloped cells differs from that of flattened outside cells by the presence of a distinct submembraneous microfilament zone which terminates at the points of intercellular contact (Fig. 32). This heterogeneous organization of the cytoskeleton was evident in 0 h cultured ICMs and is therefore distinct from the microfilament aggregation which occurs at apico-lateral contact points between attenuated cells in later cultured ICMs (see below).

Outside cells also display pale cytosolic staining within the first 2 h from immunosurgery (Figs 28B, 29, 30) and thus compare with TE cells from intact blastocysts. Underlying cells at the plane of section examined are usually darker stained thus resembling ICM cells *in situ*, but may be pale or intermediately stained especially in profiles of layered attenuated cells suggesting that at other levels within the ICM, such cells are exposed to the surface (Figs 29, 30).

The region of contact between neighbouring outside cells varies in length according to the degree of cell attenuation and is often restricted to the finger-like termini of enveloping processes (Fig. 30). In 0 h-cultured ICMs, contact zones lack an observable specialization of the submembraneous cytoskeleton (Fig. 33), but in an increasing proportion of cells from ICMs cultured for 1 h or longer, contact regions contain an accumulation of microfilaments located either at the extremities of adjacent processes or, when contacts are more substantial, at their apical limit (Fig. 34). In the majority of cells from ICMs cultured for up to 6 h, an intercellular space is present between apposed membranes at the level of microfilament enrichment. Hence such foci are not functional tight junctions but their precise localization suggests that they may represent an early stage in the formation of such junctions. Morphologically complete tight junctions with fused membranes and denser aggregates of inserted microfilaments are not seen in a majority of cells until after 8 h in culture (Fig. 35). In tangential sections of apicolateral contact points between outside cells in ICMs cultured for 4 to 6 h, dense membrane-associated microfilament aggregates appear to be focal and not zonular (Fig. 36) indicating that tight junction assembly occurs initially at localized domains of the membrane prior to extending laterally in a manner comparable with the process of formation observed in intact embyros during cleavage (Ducibella & Anderson, 1975). Similar tangential profiles of ICMs cultured for 12–48 h show tight junctions in a zonular pattern (Fig. 37).

A quantitative evaluation of the time of occurrence of intracellular polarity within outside cells was prohibited by their attenuated shape, which resulted in a high proportion of cells being sectioned through enveloping processes. Qualitative observations reveal that polarity was less well defined within cultured ICMs than within TE cells during cavitation but when present consisted of secondary lysosomes and other endocytotic organelles being localized preferentially in the perinuclear basal cytoplasm. Polarity was not



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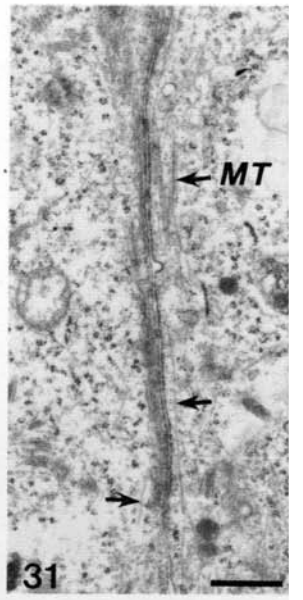

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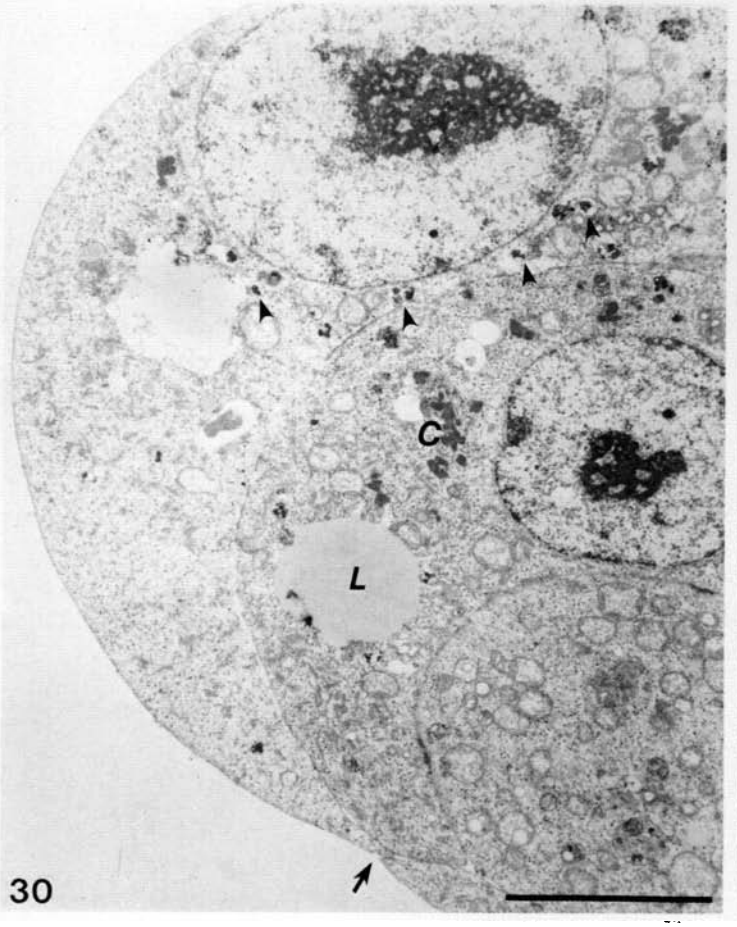

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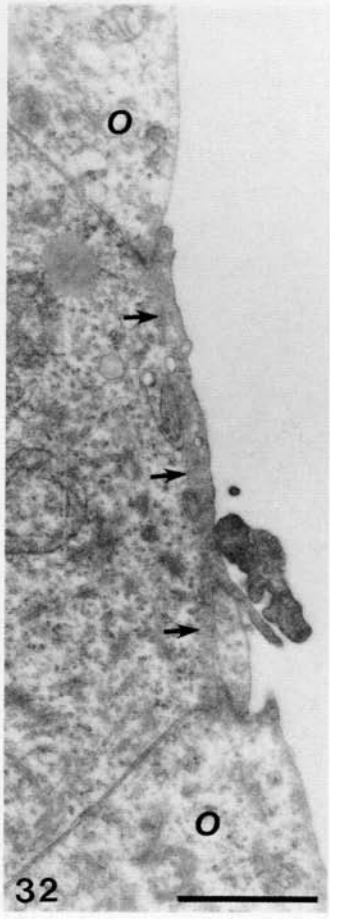

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detected in ICMs cultured for 0 or 1 h, was found in relatively few cells from ICMs cultured for 2–8 h (Fig. 30) and was most frequently seen in ICMs cultured for 12 h or longer (Fig. 38). The formation of an apical microvillous pole on outside cells was not observed throughout the culture period studied.

In vesiculated stages (ICMs cultured for 24–48 h) outside cells were further distinguished firstly by prominent bundles of intermediate filaments which often inserted into basolateral desmosome plaques (Fig. 37) and secondly by the synthesis of a pale amorphous matrix in various regions of the cytoplasm (Fig. 35) of equivalent substructure to extracellular matrix found on basolateral cell surfaces.

DISCUSSION

The TEM and SEM studies on normal and HRP-labelled intact embryos have demonstrated that throughout at least the first 12 h of cavitation, most of the juxtacoelic surface of the ICM is covered by cytoplasmic processes. The processes are exclusively of TE origin since (a) they frequently contain an abundance of SLs, a feature characteristic only of TE basal cytoplasm; (b) in tracer studies, SLs belonging to both TE cells and juxtacoelic processes invariably accumulate HRP whilst those within ICM cells do not; (c) membrane continuity between a juxtacoelic process and a TE cell of intermediate polar-mural position is sometimes apparent whereas no such continuity between ICM cells and processes has been observed, and (d) processes viewed by SEM in 'cracked open' blastocysts are derived exclusively from outside cells at the cut surface of the specimen.

The SEM image of the blastocoelic ICM surface is particularly informative

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Figs 29–32. Cultured ICMs.

Fig. 29. 2 h cultured ICM with outside cells (*O*) attenuated and slightly paler in staining than inside cells (*I*) of a more rounded shape. Arrow indicates region where outside cell envelopment is incomplete thus partially exposing an inside cell (see Fig. 32). One apparent inside cell is paler stained (arrowheads) suggesting that at other levels of the ICM, it may be exposed to the surface. Bar = 10 μm.

Fig. 30. Region of 2 h cultured ICM showing a layered arrangement of flattened cells with a gradation in staining intensity. The outermost cell is devoid of surface microvilli but contains SLs (arrowheads) and other endocytotic organelles localized in the basal perinuclear cytoplasm. Contact between outside cells is restricted to the thin termini of enveloping processes (arrow). *C* = crystalline material; *L* = lipid. Bar = 5 μm.

Fig. 31. Tangential section of region of contact between two outside cells from a 4 h cultured ICM showing bundles of microtubules (*MT*, arrows) aligned parallel to the apposed membranes. Bar = 0·5 μm.

Fig. 32. Partially exposed surface of apparent inside cell (see Fig. 29) from a 0 h cultured ICM showing a prominent terminal web (arrows) between regions of cell contact with outside cell processes (*O*). Bar = 1 μm.

with regard to the spatial organization of the processes, which are arranged in radial symmetry with each tongue-shaped projection tapering inwards towards an area of convergence at the centre of the ICM surface. Adjacent processes are either contiguous or are spaced by small gaps through which can be seen regions of underlying ICM cells. This interpretation is compatible with the TEM image in which the cross-sectioned processes usually appear as isolated portions of cytoplasm that collectively cover approximately 80% of the ICM surface.

Juxtacoelic processes in mouse blastocysts were first described by Ducibella *et al.* (1975). These authors considered that the most likely function of the processes was to stabilize the position of the ICM with respect to the polar TE. Such a role is supported by the observation by Johnson & Ziomek (1982) that debris from dead TE cells was often retained on one particular face of ICMs examined by SEM following immunosurgery, suggesting that processes may not always be removed by this treatment and that their association with the ICM may be tenacious. However, based on current data on the phenotypic characteristics of presumptive TE and ICM cells, and on the cellular mechanisms by which these lineages are preserved, we now propose that juxtacoelic processes perform a more fundamental role in the preimplantation developmental programme, that of restricting the expression of totipotency of ICM cells during early cavitation. Our proposition is founded upon the following observations.

Firstly, Ziomek & Johnson (1981) have shown, using FITC-Con A and SEM analyses, that inside cells from the 16-cell stage of development which have undergone a change of lineage following experimental manipulation do so by the acquisition of a polarized distribution of microvilli, a morphological

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Figs 33–38. Cultured ICMs.

Fig. 33. Contact region devoid of cytoskeletal specialization between outside cells from a 0 h cultured ICM. Bar = 0·5 μm.

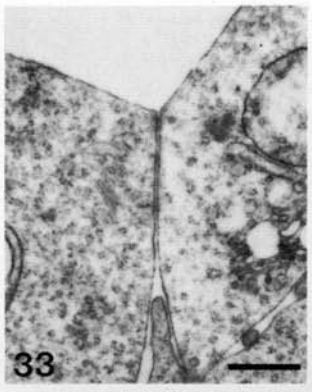
Fig. 34. Contact region between outside cells from a 2 h cultured ICM. An intercellular space is evident between apposed cells but a membrane-associated microfilament zone is organized apically (arrows). Bar = 0·5 μm.

Fig. 35. Region of outside cells from vesiculated ICM after culture for 48 h. Tight junctions are present with a normal morphology; an amorphous matrix (*MA*) accumulates freely in the cytosol. *SL* = secondary lysosome. Bar = 0·5 μm.

Fig. 36. Tangential section of contact regions between outside cells from a 4 h cultured ICM showing focal assembly of tight junctions (*TJ*, arrows) containing dense microfilament aggregates. Bar = 0·5 μm.

Fig. 37. Tangential section of contact regions between outside cells from a vesiculated ICM cultured for 48 h. Tight junctions (*TJ*) appear zonular whilst in the cytoplasm densely stained bundles of intermediate filaments (*IF*) are present which may insert into desmosome plaques (*D*) located on the basolateral membrane. *MA* = cytoplasmic matrix. Bar = 1 μm.

Fig. 38. Outside cell from 12 h cultured ICM without apical microvilli but showing a basal polarization of endocytotic organelles and SLs (arrows). Bar = 1 μm.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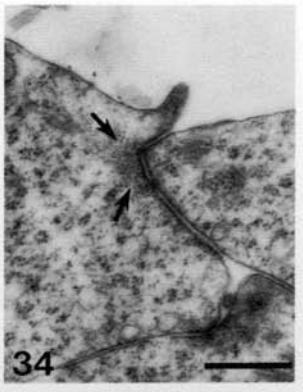
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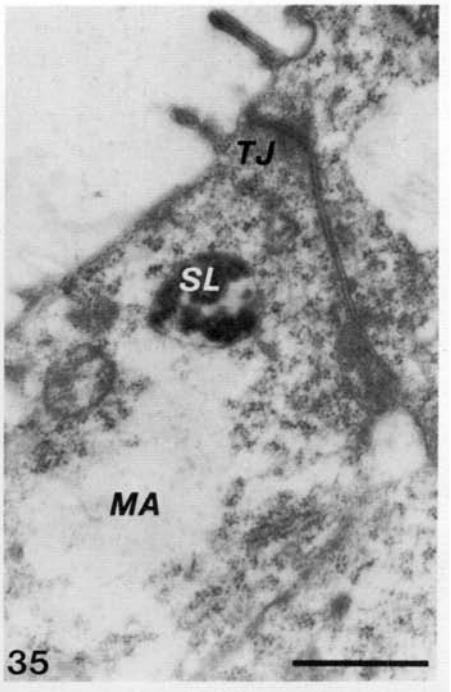
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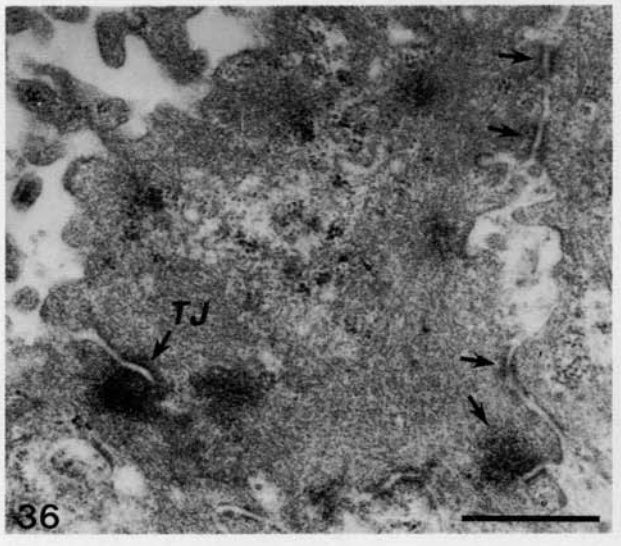
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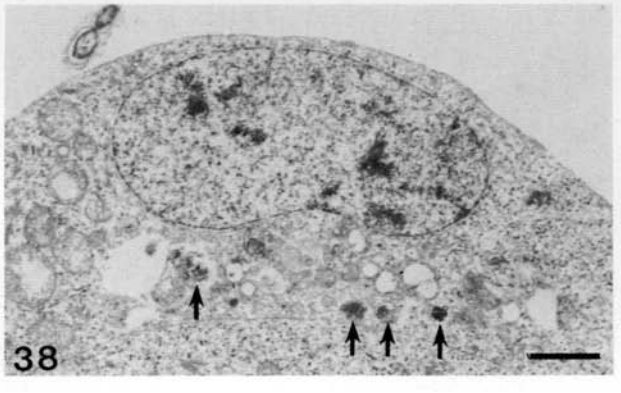
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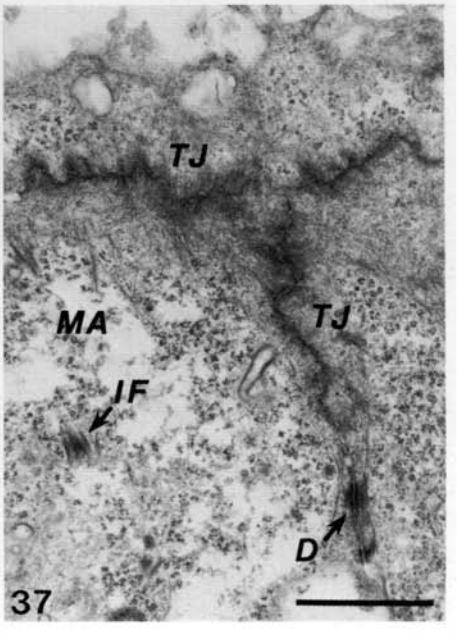
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characteristic of outside blastomeres. The mechanism controlling the developmental fate of inside 1/16 cells has been shown to reside in the nature of their cell associations; microvillous polarization will only occur when cell contacts are asymmetric and a critical proportion of the plasma membrane is exposed to outside conditions for a sufficiently prolonged period (Johnson & Ziomek, 1983).

Secondly, the mechanism controlling ICM totipotency remains the same at the blastocyst stage as that operating on inside cells at the 16-cell morula stage. Our results together with those reported previously on ICM developmental lability following exposure to outside conditions after immunosurgery (see Introduction), demonstrate that in early expanding blastocysts aged up to 12 h post cavitation, the ICM retains the capacity to generate TE.

Thirdly, ICM cells within intact blastocysts, in contrast to those exposed by immunosurgery, show little evidence of lineage transformation. During cavitation, the apical surface of the TE is less microvillous than that of outside cells at the 16-cell stage but TE polarity is evident with respect to the localization of junctional elements between cells and the basal regionalization of SLs. TE cells are further differentiated from ICM cells by their attenuated shape and paler cytosol staining. Thus the criteria by which a change of lineage by ICM cells may be detected are somewhat altered from those applicable to inside cells of earlier staged morulae. This has been shown in the time-course study of TE formation in outside cells from isolated 2 h ICMs in which the sequence of ultrastructural changes was found to be cell attenuation, paler cytosolic staining, acquisition of structured junctional complexes and finally a less coherent polarity of intracellular endocytotic organelles. Since the near absence of such TE characteristics within juxtacoelic ICM cells *in situ* is coincident with the provision of extensive cell cover by enveloping TE processes, we conclude that the processes are responsible for suppressing expression of ICM totipotency. The reduced frequency (20%) of TE development by ICMs isolated from 12 h blastocysts suggests that symmetrical contacts are maintained until and beyond the time of ICM commitment. This has been confirmed following observations on later staged blastocysts in which TE processes are retained until at least 20–24 h postcavitation, immediately prior to primary endoderm delamination (J. Chisholm, personal communication).

The maintenance of ICM cover by juxtacoelic processes during cavitation must be compatible with the mechanism of TE cell proliferation that accommodates blastocoel expansion (Copp, 1978, 1979). The rare examples where process enclosure was either reduced or absent at the section plane examined often coincided with one of the intermediate-positioned TE cells being mitotic. Such cells were rounded in shape, lacked observable processes yet retained evidence of SL basal polarity. It is unlikely that process withdrawal during mitosis has any lasting effect on the proposed function of juxtacoelic

processes since both the period of cytokinesis and the overall extent of ICM exposure (given that all process-bearing TE cells do not divide simultaneously) would be insufficient to generate a stable change in ICM phenotype. As the absence of TE cover was an exceptional observation, we suggest that process withdrawal during mitosis is followed rapidly by the formation of a new basal cytoplasmic extension from an adjacent polar TE cell in a manner similar to the *in vitro* envelopment of apolar 1/16 cells by larger, polar 1/16 cells (Ziomek & Johnson, 1981).

Amongst the small proportion of blastocysts in which TE processes were either sparse or absent at the plane of section examined, a few contained a single exposed ICM cell (its interior position confirmed by absence of reactivity following HRP incubation) with a paler cytosol than surrounding cells and occasionally a more flattened appearance. This condition has been shown in the TEM study on cultured ICMs to be an early consequence of exposure to outside conditions with a majority of outside cells attenuating and possessing a pale cytosol within 1 h of isolation (Fig. 28A,B). The occurrence of these characteristics in ICM cells *in situ* cannot be interpreted as evidence for a transformation of lineage since the subsequent increments in TE formation detected in cultured ICMs (apicolateral microfilament assembly and junction formation, endocytotic polarity) were never encountered. Furthermore, the evidence available on lineage transformation during cleavage stages argues against such changes occurring in ICM cells with 1–2 h of exposure. Microvillous polarization of inside 1/16 cells is labile during the whole course of the fifth cell cycle (Ziomek & Johnson, 1981) and only becomes irreversible following cell division (Johnson & Ziomek, 1983), and even 1/8 apolar cells attain stable polarity only after 4–5 h (Ziomek & Johnson, 1980). Therefore the induction of stable polarity, and hence expression of totipotency, appears to take longer for inside 1/16 cells than for 1/8 cells. This is not surprising since the molecular differentiation of inside cells towards an ICM fate may be a progressive phenomenon, as judged, for example, by their changing pattern of synthesis of ICM-specific polypeptides (Handyside & Johnson, 1978), and hence a change of fate might require a proportionately greater molecular reorganization the later it is initiated. Thus it would seem that transient fluctuations in the extent of cell cover provided by TE processes can result in a limited and labile response by a minority of juxtacoelic ICM cells but need not ultimately lead to a change in developmental fate under normal conditions.

The earliest change noted in ICMs following isolation, that of core cell envelopment by cells exposed at the surface, must represent a near immediate response to TE lysis since at the completion of immunosurgery (which requires about 1 h) the majority of outer cells are already attenuated. This observation is compatible with the SEM image of recently isolated ICMs consisting of compacted cell clusters (Johnson & Ziomek, 1982). The rapidity of envelopment underlines the importance of the extent of cell contact as the

mechanism controlling lineage differentiation and aptly demonstrates the early embryo's capacity to regulate its development (Johnson, 1985). The synchronous assembly of a well-defined submembraneous terminal web at exposed apices of inner cells is suggestive of their active involvement in maintaining their enclosed condition.

The consistent differentiative feature of paler cytosol staining of mouse TE cells has not been reported previously, although Calarco & Brown (1969) refer to staining differences between unspecified cells. Since this condition is not evident in precavitation stages and is specific in its localization in cavitating embryos, we do not consider it to be an artifact of fixation or processing. Paler staining is based upon a lower density of free ribosomes and precipitated soluble proteins, and probably results from a more hydrated state of the cytoplasm at the time of fixation. The earliest developmental stage at which differential staining is detected is within 0 h blastocysts where occasionally only those TE cells surrounding the nascent blastocoel are affected. Thus a correlation exists between reduced cytosol staining and cells assumed to be involved in fluid accumulation.

Two mechanisms have been proposed for the method of blastocoel formation and subsequent expansion in mammalian embryos. Biggers, Borland & Powers (1977) have suggested that the entry of water into the blastocoel is by passive bulk flow and occurs in direct response to the passage of particular solutes which are actively transported into the cavity by means of a Na^+, K^+-ATPase pump localized on the basolateral plasma membrane of TE cells. The alternative pathway proposed by Wiley & Eglitis (1981) invoked the migration of fluid-filled vacuoles (lipid?) to the juxtacoelic surface of the TE where water is generated by a metabolic process and passed into the cavity. Our data, indicating a diluted cytosol within the earliest detected fluid-transporting cells, therefore support the argument that blastocoel expansion occurs by bulk flow at the molecular level, but not decisively so. Resolution of this question will require direct labelling of fluid components prior to their intracellular localization during cavitation.

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