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Cell relationships during aggregation between preimplantation embryos and teratocarcinoma-derived cells

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SUMMARY

Cleavage-stage mouse embryos aggregate and form chimaeric blastocysts with embryonal carcinoma (EC) cells. We used scanning and transmission electron microscopy to study cell relationships during aggregate formation between 8-cell-stage embryos and F9 EC cells. Relations between heterotypic cells were similarly studied in aggregation experiments with embryos and teratocarcinoma-derived visceral (PSA5-E) and parietal (PYS-2) endoderm cells and in experiments with EC cells and endoderm cells.

The embryos and F9 cells always adhered to each other and rapidly formed compacted aggregates. Numerous microvilli and cell processes, originating from both embryo and EC cells, extended between the two cell types during adhesion and early phases of aggregation. The aggregation process involved spreading of the blastomeres on the EC cells. Frequent adherent junctions and close contacts, including possible focal gap or tight junctions were observed between the embryo and F9 cells after 3 h of culture. Apparent gap or tight junctions were infrequent during the early phases of aggregation but during further culture, extensive typical gap junctions were also seen between embryo and EC cells.

The embryos adhered only irregularly and loosely to PSA5-E and PYS-2 cells; this interaction never led to aggregate formation comparable to that seen in the experiments with embryos and EC cells. Close contacts but no gap or tight junctions could be observed between the embryo and endoderm cells. On the other hand, both PSA5-E and PYS-2 cells readily adhered to and aggregated with EC cells.

The present results suggest that microvilli and cell processes mediate membrane interactions during adhesion and early phases of aggregation between embryos and EC cells. During aggregation, blastomeres spread over the EC cells, and rapid formation of adherent junctions and close contacts, including possible focal gap or tight junctions is involved during the early phases of this process. After this initial phase, typical gap junctions are also seen between the embryo and EC cells. Interestingly, adhesive properties of embryo and EC cells differ: the former aggregate only with EC cells, whereas the latter do so also with teratocarcinoma-derived visceral and parietal endoderm cells. Mechanisms operating in the morphogenetic movement of cells in this experimental setup may be involved also in the development of the blastocyst *in vivo*.

INTRODUCTION

Cell determination and differentiation in the preimplantation mouse embryo depend on relative cell position (Tarkowski & Wróblewska, 1967; Hillman, Sherman & Graham, 1972; Kelly, 1977). Thus in the morula, the more centrally located cells tend to contribute more cells to the inner cell mass (ICM) than do the peripheral cells, which in turn tend to contribute to the trophectoderm of the blastocyst (Graham & Deussen, 1978). The movement of the cells to their different relative positions seems to depend on contact-mediated interactions (Graham & Lehtonen, 1979), apparently involving changes in cell surface structures and cytoskeletal organization (Ducibella, Ukena, Karnovsky & Anderson, 1977; Lehtonen, 1980; Lehtonen & Badley, 1980; Kimber, Surani & Barton, 1982; Pratt, Ziomek, Reeve & Johnson, 1982; Soltyńska, 1982; Sutherland & Calarco-Gillam, 1983).

Embryonal carcinoma (EC) cells, when injected into blastocyst-stage embryos, are able to contribute to a wide variety of normal tissues in the resulting chimaeric mouse (Brinster, 1974; Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975). Chimaeras have also been produced by aggregating cleavage-stage embryos (Tarkowski, 1961; Mintz, 1962), and recently this method has been used to form even embryo–EC chimaeras (Fujii & Martin, 1980, 1983; Stewart, 1980, 1982). In these experiments the embryo cells readily adhered to and aggregated with EC cells. In the resulting chimaeric blastocysts, the EC cells tended to segregate into the ICM (Stewart, 1980).

In the present study we wanted to investigate the structural basis for inside cell formation in embryo–EC aggregates. This experimental system not only provides a method for incorporating EC cells in embryos, but can also be used as a model for ultrastructural studies on interactions between heterotypic cells, as the preimplantation embryos contain characteristic paracrystalline arrays (Calarco & Brown, 1969; Lehtonen *et al.* 1983*c*) not present in other cell types.

MATERIALS AND METHODS

The supply of embryos

The embryos were obtained from natural matings and were 129 J or (C57Bl6×CBAT6T6) F_1. No obvious differences could be detected in the behaviour of the two types of embryos, and the results are therefore presented together. The embryos were collected on the third day of pregnancy (the day of the vaginal plug being the first day of pregnancy). At this time the embryos were at about 8-cell stage; embryos ranging from 7-cell stage to partially but not fully compacted 8-cell stage were used. The embryos were collected into prewarmed Mulnard's medium (1971) supplemented with 20% foetal calf serum (FCS; Flow, Irvine, Scotland) and antibiotics. The zona pellucida was removed with

acid Tyrode's solution (pH 2·5, Nicolson, Yanagimachi & Yanagimachi, 1975). In some aggregation experiments, single blastomeres isolated (Lehtonen, 1980) from the embryos were used.

Cell lines and culture

F9 embryonal carcinoma cells (Bernstine, Hooper, Grandchamp & Ephrussi, 1973), PSA5-E visceral endoderm cells (Adamson, Evans & Magrane, 1977), and PYS-2 parietal endoderm cells (Lehman, Speers, Swartzendruber & Pierce, 1974) were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % FCS on tissue-culture dishes (Nunc, Roskilde, Denmark) coated with gelatin before use. Both PSA5-E and PYS-2 are differentiated derivatives from the teratocarcinoma OTT 6050.

The cell lumps for the aggregation experiments were made as described by Stewart (1980). Briefly, the cells were trypsinized to a single cell suspension and plated on bacteriological grade dishes (Nunc). After 6–8 h, lumps consisting of 8–10 cells were collected with a micropipette and used immediately for the experiments.

Aggregation procedure

The aggregation experiments with embryos and EC or endoderm cell lumps were done in microdrops of Mulnard's medium supplemented with 20 % FCS under paraffin oil (University Pharmacy, Helsinki, Finland). A mouth-controlled micropipette was used to sandwich a lump of EC or endoderm cells between two embryos. Aggregates were fixed after 1–36 h in culture, as indicated in the Results and figure legends.

Aggregation experiments between F9 and PYS-2 or PSA5-E cells were done as those with embryos, except that the culture medium was MEM supplemented with 10 % FCS. The aggregates were fixed after 1–72 h in culture, as indicated in the Results and figure legends. To distinguish PYS-2 from F9 cells in electron microscopy, PYS-2 cells were marked with polystyrene particles (Polysciences Inc., Warrington, PA; particle diameter 1.28 μm) in some experiments. PYS-2 cell monolayers were incubated for 1–2 days in 4 ml of medium containing 10 μl of the particle solution (stock solution, $2{\cdot}2\times10^{10}$ particles/ml). To remove non-phagocytized particles, the cells were trypsinized and washed by centrifuging (7·5 min at 1500 r.p.m.) and resuspending four times in the medium. The cells were then suspended and cultured overnight in tissue-culture dishes. Thereafter the cultures were trypsinized and suspended for cell lump formation in bacteriological grade dishes. Lumps were then aggregated with F9 cell lumps as described. PYS-2 cells were marked effectively: as judged from cells growing on coverslips, over 90 % of the cells contained five or more particles.

Electron microscopy

All specimens were briefly washed with Dulbecco's saline solution (solution

'A', Dulbecco & Vogt, 1954) before fixation. For transmission electron microscopy (TEM) the aggregates were attached to poly-l-lysine-coated Petri dishes (Nunc) in Dulbecco's solution and fixed in 2·5 % glutaraldehyde in 0·1 M-phosphate buffer, pH 7·2, for 30 min at room temperature. In most experiments, the fixative was supplemented with 1 mg/ml ruthenium red (BDH Chemicals, Poole, England; Luft, 1971). After fixation the specimens were washed with 0·1 M-phosphate buffer and post-fixed in 1·5 % osmium tetroxide in the buffer. The specimens were dehydrated in ethanol and embedded in Epon 812. During dehydration, the specimens were stained *en bloc* with 2 % uranyl acetate in 50 % ethanol for 5 min. The sections were poststained with lead citrate and studied in a JEOL 100 B or 100-CX transmission electron microscope. The present TEM description on embryo-EC aggregation is based on sections from ten aggregates.

For scanning electron microscopy (SEM) the specimens were attached to poly-l-lysine-coated coverslips (diameter 12 mm) and processed as described previously (Graham & Lehtonen, 1979; Lehtonen, 1980), except that fixation with osmium tetroxide was omitted and an Aminco apparatus (American Instrument Co., Silverspring, MD) was used for critical-point drying. The specimens were coated with gold with a Jeol JFC-1100 ion sputter apparatus and studied in a Jeol JSM-C35 scanning electron microscope. The present SEM description on embryo-EC aggregation is based on 18 aggregates.

RESULTS

The results are presented in three sections. First, we present observations on the aggregation process between embryos and EC cells. Second, we describe

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Figs 1–6. Scanning electron microscopy survey of aggregation between 8-cell-stage mouse embryos (E) and F9 embryonal carcinoma cell lumps.

Fig. 1. Two embryos (E) and a lump of EC cells (F9) after 3 h of culture. The F9 cell lump is relatively compact with sparse microvilli. Note the uneven distribution of microvilli in many of the embryo cells. ×630. Scale bar = 10 μm.

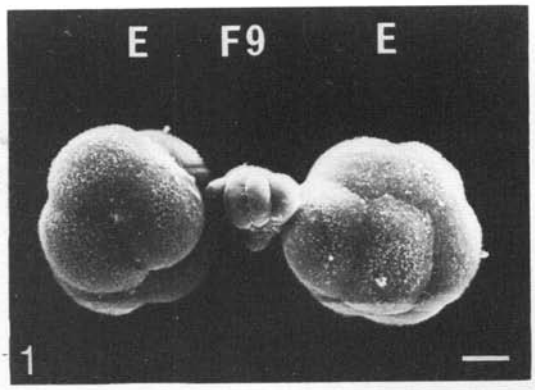
Fig. 2. More advanced aggregation in an experiment similar to that in Fig. 1. Culture time 3 h. ×620. Scale bar = 10 μm.

Fig. 3. Higher magnification of the area enclosed by the rectangle in Fig. 2. Frequent microvillous processes, apparently originating from both embryo cells (E) and F9 cells, connect the two cell types. ×5700. Scale bar = 1 μm.

Fig. 4. Two embryos and a lump of F9 cells after 4·5 h of culture. The three aggregating groups of cells are still distinguishable. ×750. Scale bar = 10 μm.

Fig. 5. Higher magnification of the area enclosed by the rectangle in Fig. 4. Note the intimate contact between the embryo cell (E) and the F9 cell. ×13 000. Scale bar = 1 μm.

Fig. 6. An aggregate consisting of two embryos and a lump of F9 cells. After 36 h of culture the F9 cells are completely engulfed by the embryo cells. As judged in light microscopy, this aggregate was at well expanded blastocyst stage. ×720. Scale bar = 10 μm.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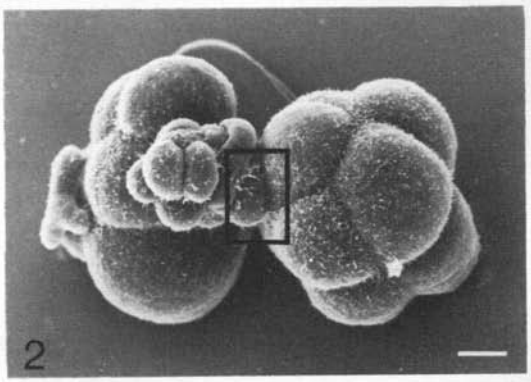
1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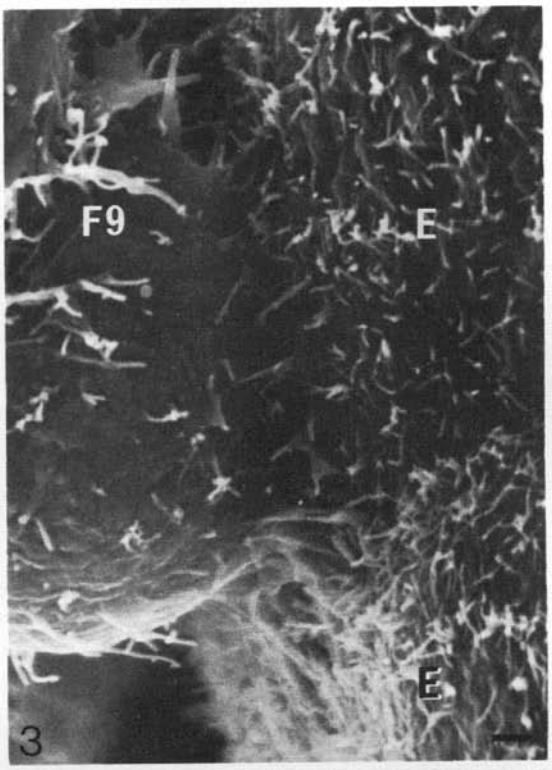
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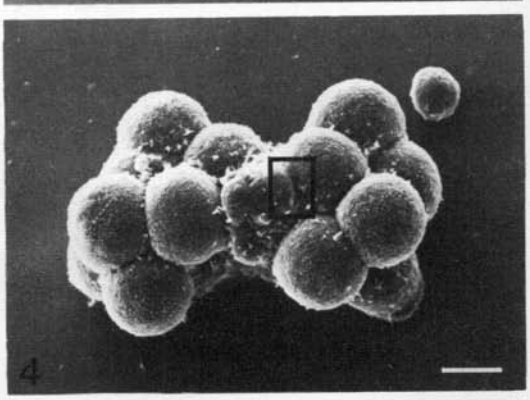
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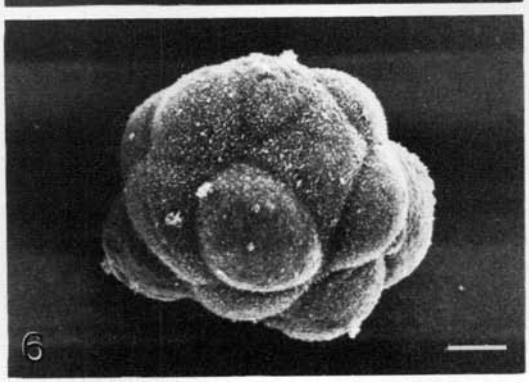
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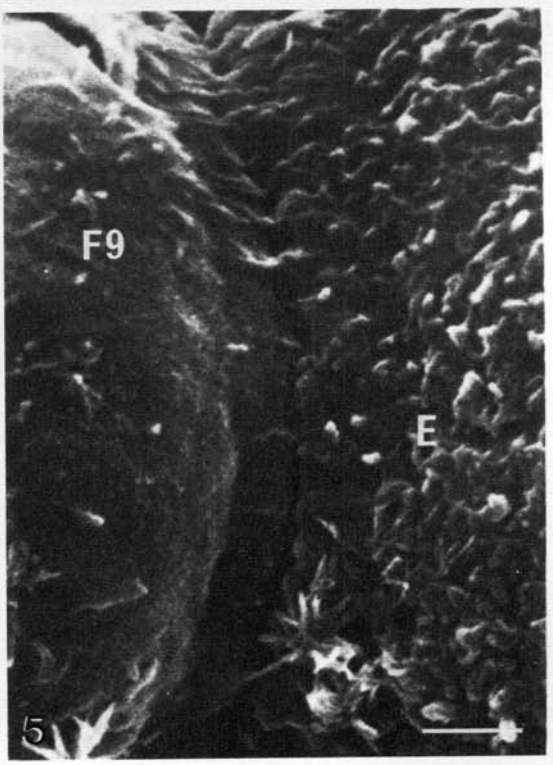
F9
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experiments on aggregation between embryos and teratocarcinoma-derived endoderm cells. Third, we present observations on aggregation between endoderm cells and EC cells.

1. *Aggregation between embryos and EC cells*

Altogether 42 aggregation experiments were performed by using 7- to 8-cell-stage embryos and F9 cell lumps. In all experiments, the two cell types adhered to each other. However, during the first hours of aggregation the extent of adhesion varied, and in three experiments, the embryos and F9 cell lumps fell apart during fixation or dehydration. In experiments continued for more than 3 h, gradual compaction of the aggregate was regularly observed.

Scanning electron microscopy was used for studying cell relationships during adhesion and aggregate formation between embryos and EC cell lumps. The two cell types adhered regularly to each other within 10 min, and aggregates with intimately apposed cell membranes were observed already after 3 h (Figs 1–3). During further culture the aggregates gradually compacted (Figs 4–6), and in light microscopy, signs of blastocoele formation could be observed during the second day in culture.

Both the embryo and EC cells have microvillous cell processes. Characteristically, the microvilli of the embryo cells were relatively short and were often unevenly distributed (Figs 7–9). On rare occasions, the blastomeres showed microvilli up to 7 μm long (Fig. 9). The microvilli regularly reached the surface of neighbouring EC cells (Figs 3, 7–10). During the early phases of aggregation, both microvillous and non-microvillous areas of the embryo cell surface seemed to be capable of adhering with F9 cells (Figs 1–3, 7–9). During the aggregation process apparent spreading of the embryo cells over the F9 cell lump was sometimes observed (Fig. 9). The spreading and flattening of the embryo cells was particularly clear in our pilot experiments with isolated blastomeres and F9

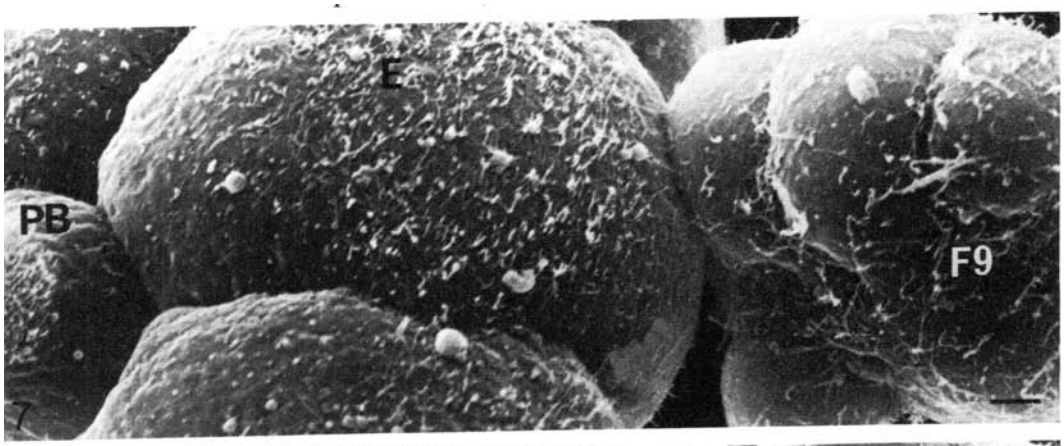
Figs 7–10. Structural details of contact areas between embryo and EC cells.

Fig. 7. An embryo (E) adhering to a lump of F9 cells, culture time 1·5 h. Note the uneven distribution of the microvilli of the embryo cells. PB, polar body. ×3100. Scale bar = 2 μm.

Fig. 8. An aggregate between an embryo (E) and a lump of F9 cells. After 3 h of culture, cell processes (arrow) and microvilli extend from the F9 cells to the embryo cell surface. Note the even distribution of the sparse microvilli of F9 cells. ×2900. Scale bar = 2 μm.

Fig. 9. An aggregate after 1·5 h of culture. Note the depression (arrow) in the surface of the embryo cell (E), apparently spreading over the F9 cell lump. In this aggregate, particularly long microvillous processes seem to originate from the embryo cell and to extend to the surface of the F9 cell. ×3700. Scale bar = 2 μm.

Fig. 10. An aggregation experiment with a blastomere isolated from an early 8-cell-stage embryo and a lump of F9 cells. Note the embryo cell (E) flattening and spreading over the F9 cell lump after 18 h of culture. Microvilli and cell processes (arrows) connect the two cell types. ×2600. Scale bar = 2 μm.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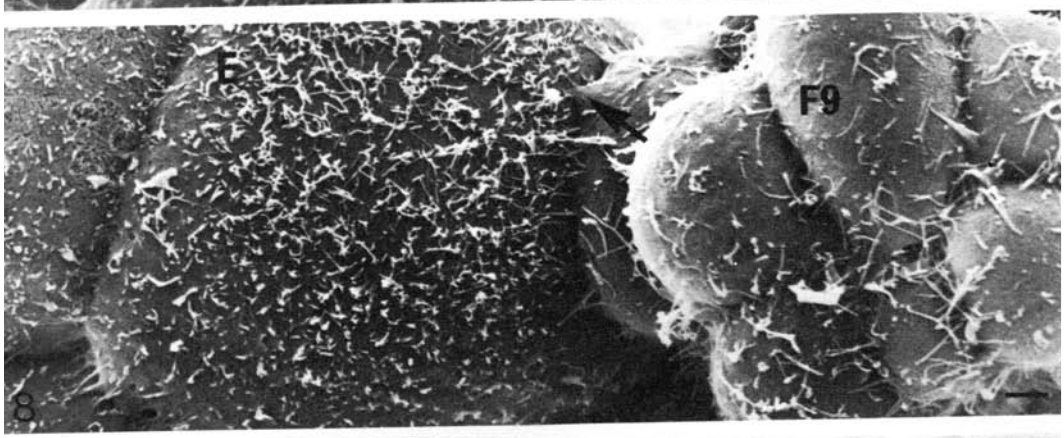
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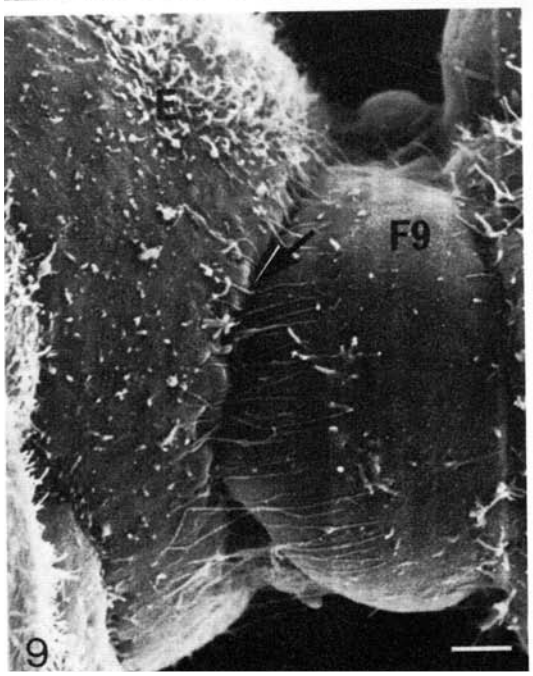
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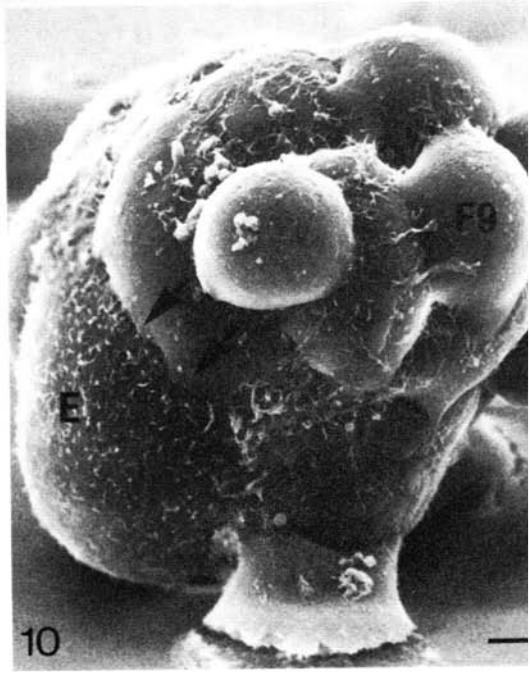
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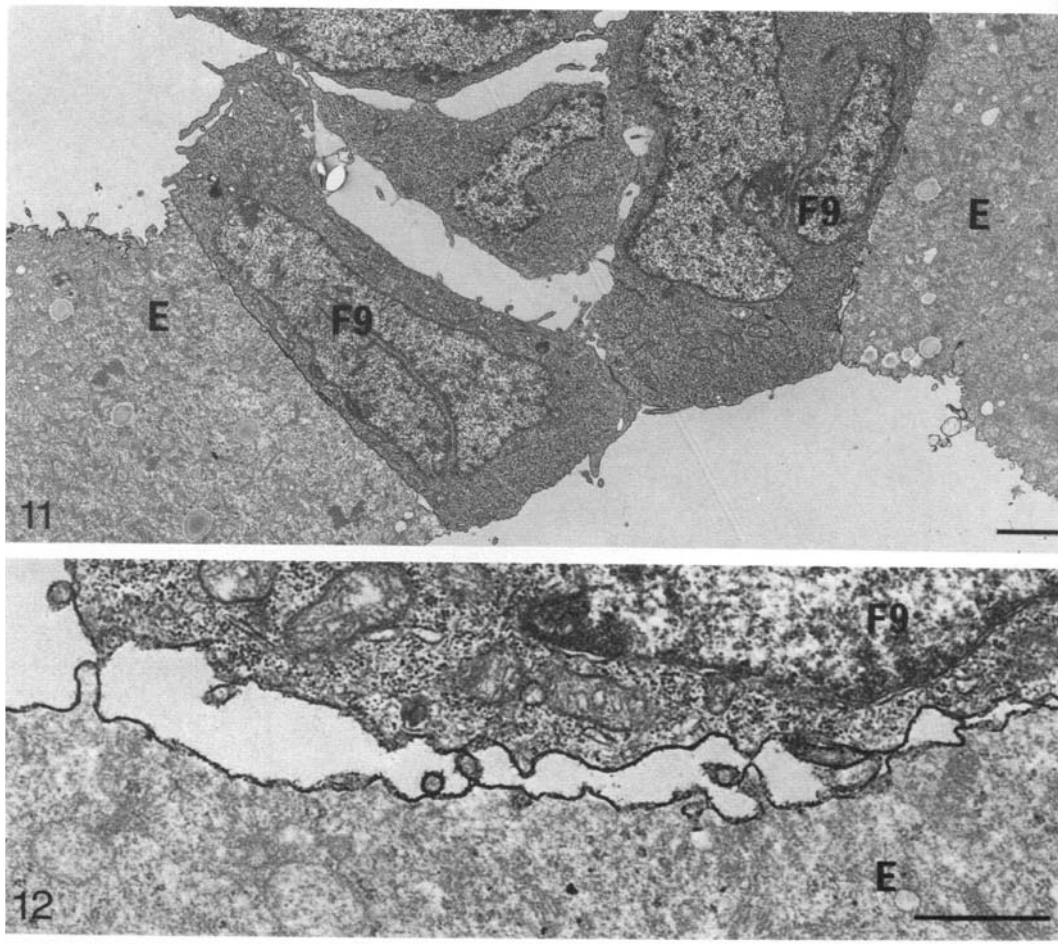


Figs 11–12. Transmission electron micrographs of early contact areas between embryo and EC cells.

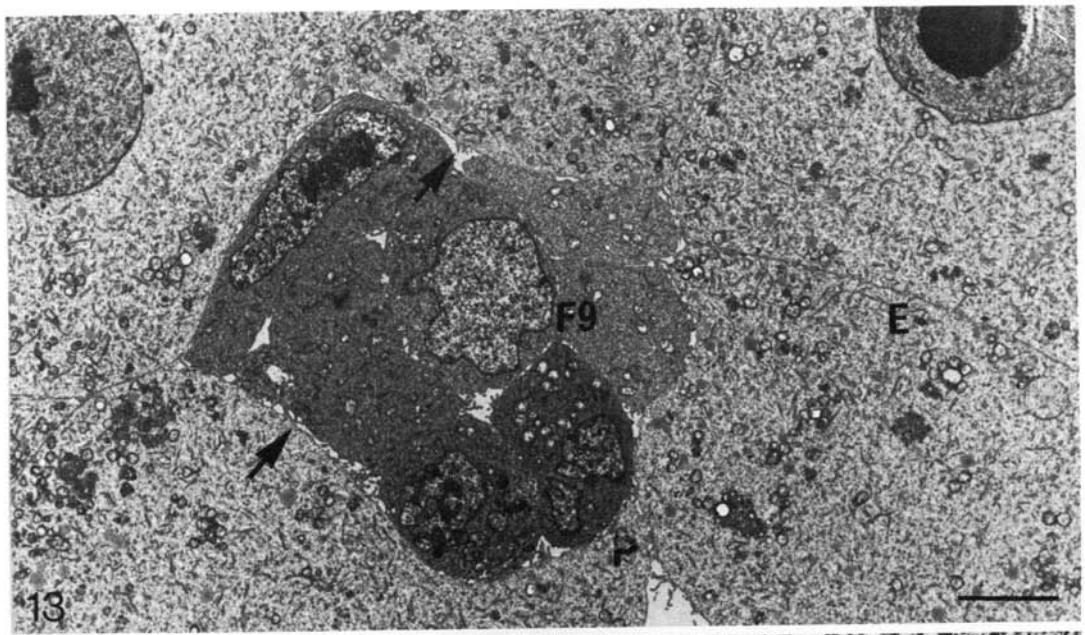
Fig. 11. After 1 h of culture the embryo cells (E) are flattening on F9 cells. Both cell types show microvilli and cell processes comparable to those in Figs 1–10. ×3900. Scale bar = 2 μm.

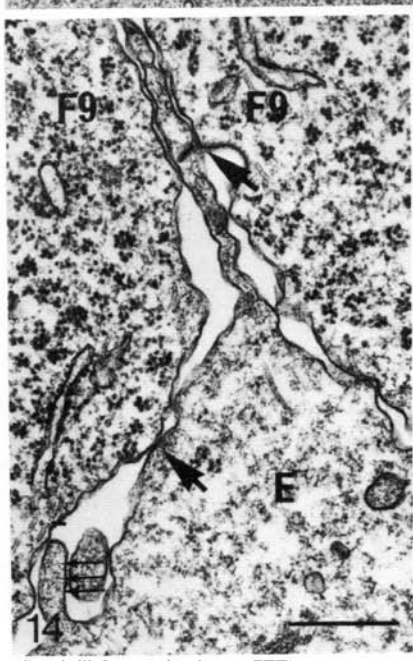
Fig. 12. Contact area between an embryo cell (E) and an F9 cell after 1 h of culture. Microvillous processes have established contacts between the two cell types at this early phase of aggregation. ×18 000. Scale bar = 1 μm.

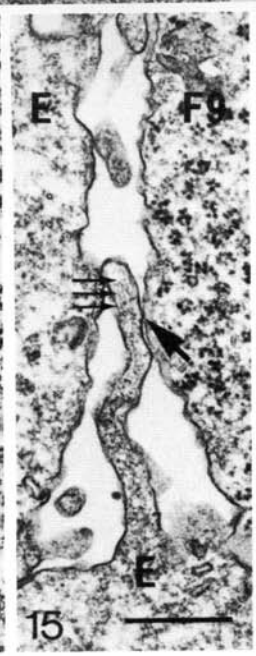
Fig. 13. Advanced aggregation and compaction between two embryos and a lump of F9 cells. At the plane of the section the F9 cells are surrounded by embryo cells (E). Note the cell process (P) of the lower left embryo cell spreading over the F9 cell lump and microvilli (arrows) of both cell types. Culture time 3 h. ×2600. Scale bar = 5 μm.

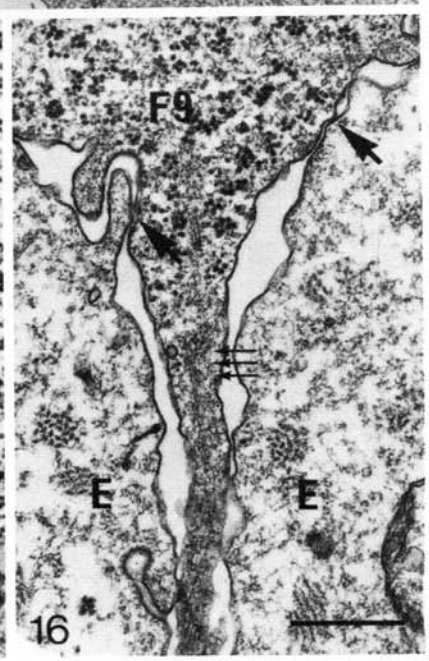
Figs 14–16. Details of contact areas between aggregating embryo and F9 cells after 3 h of culture. The embryo cells (E) as well as F9 cells show frequent microvillous processes. Ordered microfilaments (small arrows) can be seen in the processes of both cell types. Note the punctate close contacts (arrows) between the heterotypic cells. ×30 000. Scale bar = 0·5 μm.

cell lumps (Fig. 10). The microvillous cell processes of the EC cells tended to be longer than those of the embryo cells. They were usually evenly distributed, and compared with those of the embryo cells, few in number. In addition, the EC cells regularly showed microvilli up to 4 μm long (Figs 8–9), and also thicker cell processes (Figs 8, 10). The microvilli and cell processes often extended over the cell border and established contacts with embryo cells (Figs 3, 7–10).









In TEM, close contacts with an electron-lucent interphase of less than 5 nm could be seen between the embryo and EC cells already after 1 h of culture (Figs 11–12). Early contacts were often established by microvillous processes originating from both cell types (Figs 11–16). Ordered microfilaments could be seen in cell processes of both embryo and F9 cells. Adherent junctions, unclassifiable close contacts including possible focal gap or tight junctions, and typical gap junctions were observed between the heterotypic cells (Figs 19–22). We did not attempt to quantitate the different types of contacts between the embryo and EC cells. However, after 3 h of aggregation, a contact area comparable to the combined heterotypic contacts in Fig. 13 would typically show several adherent junctions, frequent close contacts including possible focal gap or tight junctions, and very few if any apparent small gap or tight junctions. Extensive typical gap junctions comparable with that seen in the 15 h aggregate of Fig. 22 were not observed during the first 3 h of aggregation. Similar membrane interactions were also seen between contiguous homotypic cells, both embryo cells (Figs 17–18) and EC cells (Figs 23–24).

2. *Aggregation between embryos and endoderm cells*

In aggregation experiments with embryos and teratocarcinoma-derived endoderm cells, six out of eight experiments with PSA5-E cell lumps and three out of fifteen with PYS-2 cell lumps resulted in loose adhesion between the heterotypic cells. No adhesion took place in the rest of the experiments, and the cell groups moved apart immediately when gently blown with a micropipette.

Despite the occasional adhering, the embryos never formed compacted

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Figs 17–24. Homotypic and heterotypic cell contacts in aggregation experiments with 8-cell-stage embryos (E) and F9 EC cell lumps.

Fig. 17. A small close contact (arrow head) between two embryo cells. Culture time 3 h. ×150 000. Scale bar = 50 nm.

Fig. 18. A gap junction between two embryo cells. Culture time 3 h. ×150 000. Scale bar = 50 nm.

Fig. 19. A close contact (arrow head) and an apparent small gap or tight junction (arrow) between an embryo cell (lower) and an F9 cell. Culture time 3 h. ×100 000. Scale bar = 50 nm.

Fig. 20. An embryo cell-F9 cell contact with morphological features of an adherent junction. Culture time 3 h. ×100 000. Scale bar = 50 nm.

Fig. 21. A close contact (arrow) between an embryo cell (lower) and an F9 cell inside an early cavitating blastocyst. Culture time 15 h. ×150 000. Scale bar = 50 nm.

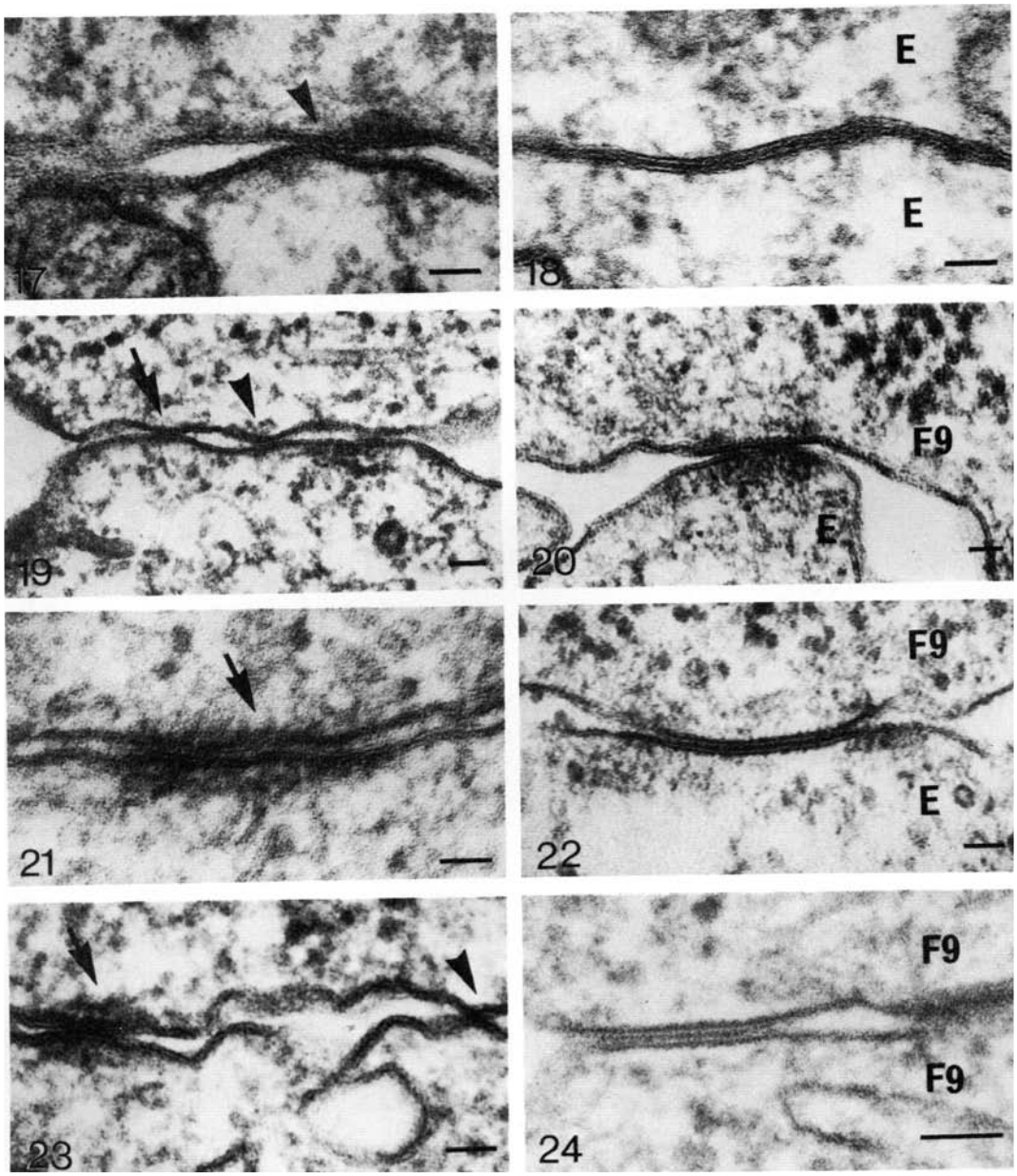
Fig. 22. A gap junction between an F9 cell and the inner face of an embryo cell covering the cavity of an early cavitating blastocyst. Culture time 15 h. ×100 000. Scale bar = 50 nm.

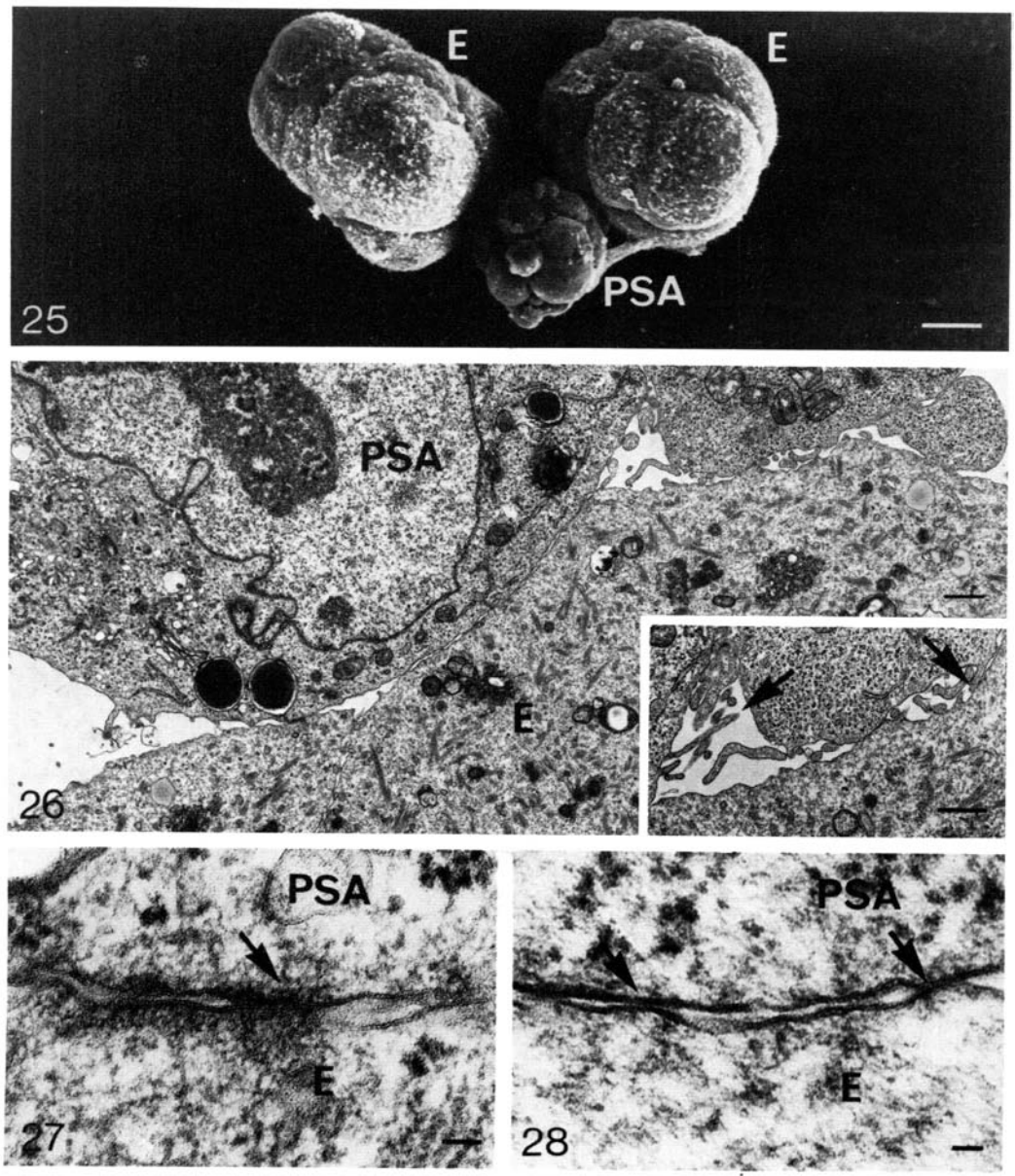
Fig. 23. Close contacts between two F9 cells. Arrow, an apparent adherent junction. Arrow head, a possible focal gap or tight junction. Culture time 3 h. ×150 000. Scale bar = 50 nm.

Fig. 24. A gap junction between two F9 cells. Culture time 3 h. ×220 000. Scale bar = 50 nm.

aggregates or chimaeric blastocysts with the endoderm cells. The endoderm cells always remained outside the embryos without interfering with their compaction or blastocyst transformation.

In SEM and TEM, microvillous processes, originating from both the embryo and endoderm cells, were seen between the adhering cells (Figs 25–26). During subsequent culture, close contacts (Figs 27–28) but no gap or tight junctions were seen between the heterotypic cells.





Figs 25–28. Interaction between embryos and teratocarcinoma-derived endoderm cells.

Fig. 25. Aggregation experiment with two embryos (E) and a lump of PSA5-E visceral endoderm cells (PSA). After 4·5 h of culture the embryos have only loosely adhered to the PSA5-E cell lump. ×760. Scale bar = 10 μm.

Fig. 26. Aggregation experiment with two embryos (E) and a lump of PSA5-E cells (PSA) after 3 h of culture. There is no compaction-like adhesion between these two cell types. ×5200. Insert is from a section adjacent to that shown in Fig. 24. Note the microvillous processes of PSA5-E cells (arrows). ×6600. Scale bar = 1 μm.

Figs 27–28. Close contacts (arrows) between embryo cells (E) and PSA5-E cells (PSA) after 3 h of culture. Fig. 27, ×100 000; Fig. 28, ×78 000. Scale bar = 50 nm.

3. *Aggregation between endoderm cells and EC cells*

As the two endoderm cell types showed only a very limited capacity to adhere to the embryo cells, we also tested their ability to aggregate with F9 EC cells. F9 cell lumps adhered to and aggregated with PSA5-E cell lumps in all 17 cases and with PYS-2 cell lumps in 48 of 51 cases. In these experiments, PSA5-E cell lumps appeared relatively compact. Close apposition and compaction-like adhesion took place between lumps of PSA5-E and F9 cells (Fig. 29). PYS-2 cell lumps, on the other hand, stayed relatively loose, and no compaction-like adhesion between the lumps of PYS-2 and F9 cells could be observed. Instead, the PYS-2 cells often appeared to migrate on the F9 cell lump (Fig. 30). In TEM, close contacts were observed between PYS-2 and F9 cells after 3 h of culture (Fig. 31). Abundant deposits of matrix material, apparently preventing membrane contacts, were found between PYS-2 and F9 cells after 1–3 days of culture (Fig. 32).

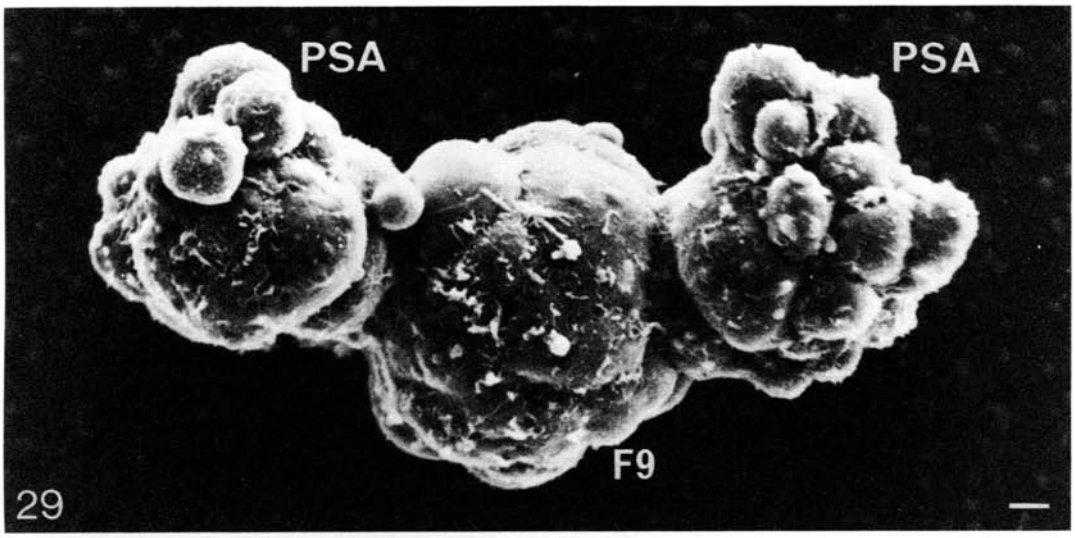
DISCUSSION

One of the central questions in the differentiation of the blastocyst is how the segregation of cells into inside and outside positions is regulated. Here we describe cell relationships during experimental inside cell formation in embryo–EC aggregates. The behaviour of the different cell types in our aggregation experiments is summarized in Table 1.

Previously it has been shown that embryo cells aggregate with various EC cells (F9, PC13, PSA-1, AT805) but not with other cell types, such as teratocarcinoma-derived parietal endoderm cells (PYS) and fibroblastoid cells (Stewart, 1980; Fujii & Martin, 1980; Ogou, Okada & Takeichi, 1982). Our present observations confirm these results: embryos readily aggregated with F9 EC cells but they adhered only irregularly and loosely to teratocarcinoma-derived endoderm cells, and adhesion with endoderm cells never led to formation of aggregates or chimaeric blastocysts. On the other hand, F9 cells readily aggregated with both PSA5-E visceral endoderm and PYS-2 parietal endoderm cells. Differencies in the cell surface topography may be associated with the differential behaviour of the cells in our experiments. Like the inner cells in the

Table 1. *Summary of interactions between 8-cell-stage embryos and teratocarcinoma-derived cells*

	8-cell embryo	F9 cell lump
8-cell embryo	aggregation and compaction	aggregation and compaction
F9 cell lump	aggregation and compaction	aggregation and compaction
PSA5-E cell lump	loose or no adhesion	compaction-like adhesion
PYS-2 cell lump	loose or no adhesion	adhesion, PYS-2 migrate on F9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PSA
F9
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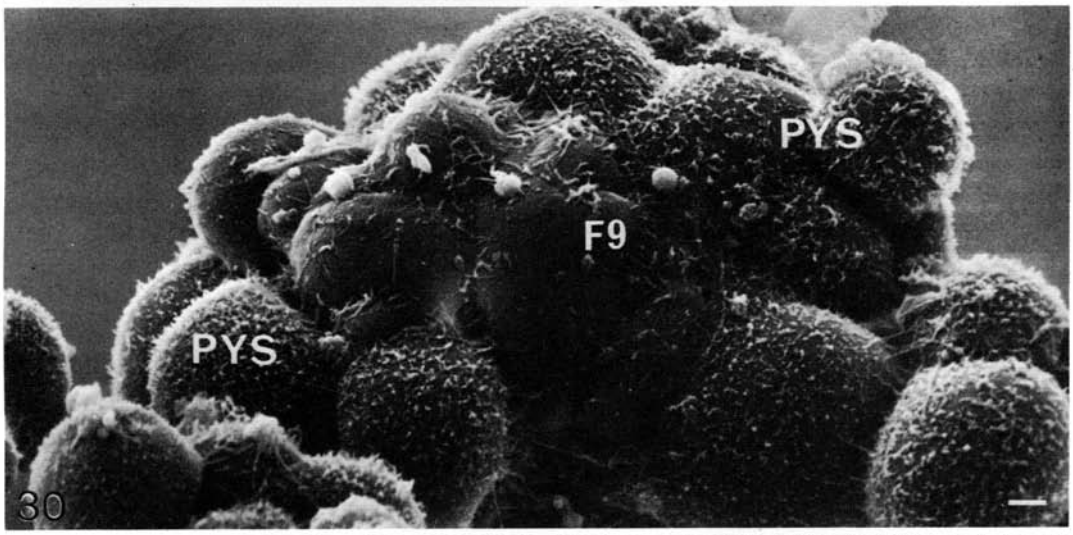
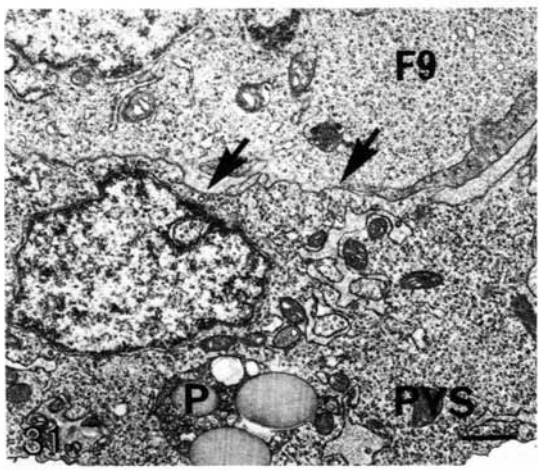
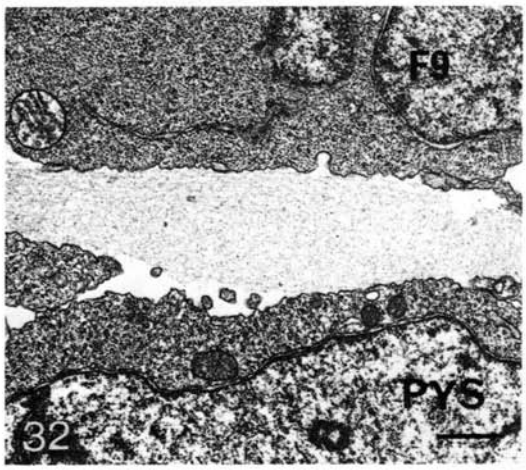

PYS
F9
PYS
30


F9
P
PYS
31


F9
PYS
32

late morula (Johnson & Ziomek, 1982; Surani & Handyside, 1983), F9 cells lack dense microvilli, and they tend to segregate into an inside position when aggregated with more microvillous embryo or PYS-2 cells. The molecular basis for these different recognition and adhesion phenomena is not known, but it is probably connected with the adhesion molecules described in EC and embryo cells (Hyafil, Morello, Babinet & Jacob, 1980; Yoshida & Takeichi, 1982; Damsky *et al.* 1983; Shur, 1983; Shirayoshi, Okada & Takeichi, 1983). There seems to be cell-type specificity in these molecules, and EC and embryo cells apparently share adhesion systems not present in fibroblastic or PYS cells (Ogou *et al.* 1982; Damsky *et al.* 1983).

In normal embryos *in vivo*, segregation of the first inside cells begins at the 8- to 16-cell stage (Barlow, Owen & Graham, 1972). Active cell movements, including spreading of blastomeres on one another, seem to be involved in this process *in vivo* (Soltyńska, 1982). *In vitro*, similar spreading movements occur during aggregation of two embryos (Burgoyne & Ducibella, 1977), and isolated 8-cell-stage blastomeres are capable of spreading on lectin-coated agarose beads (Kimber & Surani, 1982). Microvillous cell processes have been suggested to have a role in the morphogenetic movements of the cleavage-stage cells (Ducibella & Anderson, 1975; Ducibella *et al.* 1977; Graham & Lehtonen, 1979; Lehtonen, 1980). The formation of cell processes and spreading of blastomeres in the preimplantation embryos have been described by several authors (Soltyńska, 1982; Kimber *et al.* 1982; Surani & Handyside, 1983). In the present experiments, microvillous cell processes established contacts between embryo and EC cells during adhesion and early phases of aggregation. Both microvillous and nonmicrovillous areas of the embryo cell surface seemed to be capable of participating in this process. The role of the microvillar poles (Reeve & Ziomek, 1981) in this phenomenon remains open. Subsequently, during compaction of the aggregate, larger cell processes extended over the cell borders, and embryo cells spread on the EC cells which contribute to the inside cells in the chimaeric aggregate (Stewart, 1980; the present study). This experimental model thus closely mimicks inside cell formation *in vivo*.

Figs 29–32. Interaction between lumps of teratocarcinoma-derived endoderm cells and F9 EC cells.

Fig. 29. An aggregate between two lumps of PSA5-E cells (PSA) and a lump of F9 cells after 3 h of culture. Note the compaction-like adhesion between the lumps. ×2600. Scale bar = 2 μm.

Fig. 30. An aggregate between two lumps of PYS-2 cells (PYS) and a lump of F9 cells after 3 h of culture. The PYS-2 cells seem to migrate on the F9 cell lump. Note the abundant microvilli of the PYS-2 cells. ×2500. Scale bar = 2 μm.

Fig. 31. An agggregate between PYS-2 cells (PYS) and F9 cells after 3 h of culture. Note the close contacts (arrows) between the heterotypic cells. P, polystyrene particle (see Materials and Methods). ×7300. Scale bar = 1 μm.

Fig. 32. An aggregate between PYS-2 cells (PYS) and F9 cells after 3 days of culture. A layer of matrix material separates the two cell tpyes. ×8600. Scale bar = 1 μm.

The mechanism of cell movements during the morula stage involves changes in the organization of the blastomere cytoskeleton. For instance, ordered filaments and bundles of filaments, apparently actin-containing microfilaments (Lehtonen & Badley, 1980), are present in the flattening external cells and in cell protrusions of morula-stage cells (Soltyńska, 1982). In the present embryo–EC aggregation experiments, similar arrangements of the cytoskeleton were observed, and adherent junctions, known to be associated with microfilament bundles, were rapidly formed between the embryo and EC cells. The differential behaviour of the two types of endoderm cells when aggregated with EC cells may also be associated with differences in their cytoskeletal organization. In the present experiments, the PSA5-E visceral endoderm cell lumps showed compaction-like adhesion with EC cell lumps, whereas the PYS-2 parietal endoderm cells appeared to migrate on the EC cell lump. *In vivo*, the visceral endoderm cells, forming a stationary epithelial layer, express only intermediate filaments of cytokeratin type (Lehtonen, Lehto, Paasivuo & Virtanen, 1983*b*), whereas the migratory parietal endoderm cells express both cytokeratin and vimentin filaments (Lane, Hogan, Kurkinen & Garrels, 1983; Lehtonen *et al.* 1983*b*). In addition to the differential expression of intermediate filaments, there are striking differences between the EC cells and their differentiated derivatives in their organization of vinculin-containing adhesion plaques (Lehtonen, Lehto, Badley & Virtanen, 1983*a*).

Formation of specialized junctions is apparently a prerequisite for blastocyst differentiation. Thus, ionic coupling and intercellular transfer of dye (Lo & Gilula, 1979; McLachlin, Caveney & Kidder, 1983), as well as gap and tight junctions (Ducibella & Anderson, 1975; Magnuson, Demsey & Stackpole, 1977) have been demonstrated between the compacting blastomeres of 8-cell-stage embryos. In the present experiments, frequent adherent junctions and close contacts, including possible focal gap or tight junctions were observed between embryo and EC cells after 3 h of aggregation. Apparent gap or tight junctions were infrequent during the early phases of aggregation but during further culture, also extensive typical gap junctions were found between embryo and EC cells. Gap junctions were not seen between embryo and EC-derived endoderm cells. Consistently with our observations, morula cells have been shown to co-operate metabolically with EC cells (Gaunt & Papaioannou, 1979). EC cells co-operate metabolically also with many differentiated cell types, though in some cases at a very low level (Gaunt & Papaioannou, 1979; Lo & Gilula, 1980; see, however, Nicolas, Jakob & Jacob, 1978). It is clear that there is selectivity in metabolic co-operation between heterotypic cells (Gaunt & Subak-Sharpe, 1979). Our present results would be consistent with the suggestion that upon differentiation the EC cells lose both their capacity to aggregate and to form gap junctions with embryo cells; more direct evidence for this suggestion might be gained from experiments with retinoic-acid-treated F9 EC cells (for references, see Lehtonen *et al.* 1983*a*). Gap junctional communication may thus be involved in

the interactions leading to the incorporation of EC cells into chimaeric embryo–EC blastocysts.

CONCLUSIONS

The aggregation process between preimplantation embryos and teratocarcinoma-derived cells has the following features:

1. During adhesion and early phases of aggregation, microvilli and cell processes establish contacts between embryo and ECcells.
2. During the early phases of aggregation, frequent adherent junctions and close contacts are seen between embryo and EC cells. Apparent gap or tight junctions are infrequent at this stage but during further culture, extensive typical gap junctions are also seen between the two cell types.
3. During aggregation, blastomeres spread over the EC cells.
4. Embryo cells adhere only irregularly and loosely with teratocarcinoma-derived visceral and parietal endoderm cells.
5. Both visceral and parietal endoderm cells aggregate with EC cells.
6. The aggregates between visceral endoderm cells and EC cells differ morphologically from those between parietal endoderm cells and EC cells.

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REFERENCES

ADAMSON, E. D., EVANS, M. J. & MAGRANE, G. G. (1977). Biochemical markers of the progress of differentiation in teratocarcinoma cell lines. *Eur. J. Biochem.* **79**, 607–615.

BARLOW, P. W., OWEN, D. A. S. & GRAHAM, C. F. (1972). DNA synthesis in the preimplantation mouse embryo. *J. Embryol. exp. Morph.* **27**, 431–445.

BERNSTINE, E. G., HOOPER, M. L., GRANDCHAMP, S. & EPHRUSSI, B. (1973). Alkaline phosphatase activity in mouse teratoma. *Proc. natn. Acad. Sci., U.S.A.* **70**, 3899–3903.

BRINSTER, R. L. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. *J. exp. Med.* **140**, 1049–1056.

BURGOYNE, P. S. & DUCIBELLA, T. (1977). Changes in the properties of the developing trophoblast of preimplantation mouse embryos as revealed by aggregation studies. *J. Embryol. exp. Morph.* **40**, 143–157.

CALARCO, P. G. & BROWN, E. H. (1969). An ultrastructural and cytological study of preimplantation development of the mouse. *J. exp. Zool.* **171**, 253–284.

DAMSKY, C. H., RICHA, J., SOLTER, D., KNUDSEN, K. & BUCK, C. A. (1983). Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. *Cell* **34**, 455–466.

DUCIBELLA, T. & ANDERSON, E. (1975). Cell shape and membrane changes in the eight-cell mouse embryo: prerequisities for morphogenesis of the blastocyst. *Devl Biol.* **47**, 45–58.

DUCIBELLA, T., UKENA, T., KARNOVSKY, M. & ANDERSON, E. (1977). Changes in cell surface and cortical cytoplasmic organization during early embryogenesis in the preimplantation mouse embryo. *J. Cell Biol.* **74**, 153–167.

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167–182.

FUJII, J. T. & MARTIN, G. R. (1980). Incorporation of teratocarcinoma stem cells into blastocysts by aggregation with cleavage-stage embryos. *Devl Biol.* **74**, 239–244.

FUJII, J. T. & MARTIN, G. R. (1983). Developmental potential of teratocarcinoma stem cells *in utero* following aggregation with cleavage-stage mouse embryos. *J. Embryol. exp. Morph.* **74**, 79–96.

GAUNT, S. J. & PAPAIOANNOU, V. E. (1979). Metabolic co-operation between embryonic and embryonal carcinoma cells of the mouse. *J. Embryol. exp. Morph.* **54**, 263–275.

GAUNT, S. J. & SUBAK-SHARPE, J. H. (1979). Selectivity in metabolic cooperation between cultured mammalian cells. *Expl Cell Res.* **120**, 307–320.

GRAHAM, C. F. & DEUSSEN, Z. A. (1978). Features of cell lineage in preimplantation mouse development. *J. Embryol. exp. Morph.* **48**, 53–72.

GRAHAM, C. F. & LEHTONEN, E. (1979). Formation and consequences of cell patterns in preimplantation mouse development. *J. Embryol. exp. Morph.* **49**, 277–294.

HILLMAN, N., SHERMAN, M. I. & GRAHAM, C. (1972). The effect of spatial arrangement on cell determination during mouse development. *J. Embryol. exp. Morph.* **28**, 263–278.

HYAFIL, F., MORELLO, D., BABINET, C. & JACOB, F. (1980). A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell* **21**, 927–934.

JOHNSON, M. H. & ZIOMEK, C. A. (1982). Cell subpopulations in the late morula and early blastocyst of the mouse. *Devl Biol* **91**, 431–439.

KELLY, S. J. (1977). Studies on the developmental potential of 4- and 8-cell stage mouse blastomeres. *J. exp. Zool.* **200**, 365–376.

KIMBER, S. J. & SURANI, M. A. H. (1982). Spreading of blastomeres from eight-cell mouse embryos on lectin-coated beads. *J. Cell Sci.* **56**, 191–206.

KIMBER, S. J., SURANI, M. A. H. & BARTON, S. C. (1982). Interactions of blastomeres suggest changes in cell surface adhesiveness during the formation of inner cell mass and trophectoderm in the preimplantation mouse embryo. *J. Embryol. exp. Morph.* **70**, 133–152.

LANE, E. B., HOGAN, B. L. M., KURKINEN, M. & GARRELS, J. I. (1983). Co-expression of vimentin and cytokeratins in parietal endoderm cells of early mouse embryo. *Nature* **303**, 701–704.

LEHMAN, J. M., SPEERS, W. C., SWARTZENDRUBER, D. E. & PIERCE, G. B. (1974). Neoplastic differentiation: characteristics of cell lines derived from a murine teratocarcinoma. *J. Cell Physiol.* **84**, 13–28.

LEHTONEN, E. (1980). Changes in cell dimensions and intercellular contacts during cleavage-stage cell cycles in mouse embryonic cells. *J. Embryol. exp. Morph.* **58**, 231–249.

LEHTONEN, E. & BADLEY, R. A. (1980). Localization of cytoskeletal proteins in preimplantation mouse embryos. *J. Embryol. exp. Morph.* **55**, 211–225.

LEHTONEN, E., LEHTO, V.-P., BADLEY, R. A. & VIRTANEN, I. (1983*a*). Formation of vinculin plaques precedes other cytoskeletal changes during retinoic acid-induced teratocarcinoma cell differentiation. *Expl Cell Res.* **144**, 191–197.

LEHTONEN, E., LEHTO, V.-P., PAASIVUO, R. & VIRTANEN, I. (1983*b*). Parietal and visceral endoderm differ in their expression of intermediate filaments. *EMBO J.* **2**, 1023–1028.

LEHTONEN, E., LEHTO, V.-P., VARTIO, T., BADLEY, R. A. & VIRTANEN, I. (1983*c*). Expression of cytokeratin polypeptides in mouse oocytes and preimplantation embryos. *Devl Biol.* **100**, 158–165.

LO, C. W. & GILULA, N. B. (1979). Gap junctional communication in the preimplantation mouse embryo. *Cell* **18**, 399–409.

LO, C. W. & GILULA, N. B. (1980). PCC4azal teratocarcinoma stem cell differentiation in culture. III. Cell-to-cell communication properties. *Devl Biol.* **75**, 112–120.

LUFT, J. H. (1971). Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Rec.* **171**, 347–368.

MAGNUSON, T., DEMSEY, A. & STACKPOLE, C. W. (1977). Characterization of intercellular junctions in the preimplantation mouse embryo by freeze-fracture and thin-section electron microscopy. *Devl Biol.* **61**, 252–261.

MCLACHLIN, J. R., CAVENEY, S. & KIDDER, G. M. (1983). Control of gap junction formation in early mouse embryos. *Devl Biol.* **98**, 155–164.

MINTZ, B. (1962). Formation of genotypically mosaic mouse embryos. *Am. Zool.* **2**, 432.

MINTZ, B. & ILLMENSEE, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. natn. Acad. Sci., U.S.A.* **72**, 3583–3589.

MULNARD, J. G. (1971). Manipulation of cleaving mammalian embryos with special reference to a time-lapse cinematographic analysis of centrifuged and fused mouse eggs. *Adv. Biosci.* **6**, 255–277.

NICOLAS, J. F., JAKOB, H. & JACOB, F. (1978). Metabolic cooperation between mouse embryonal carcinoma cells and their differentiated derivatives. *Proc. natn. Acad. Sci., U.S.A.* **75**, 3292–3296.

NICOLSON, G. L., YANAGIMACHI, R. & YANAGIMACHI, H. (1975). Ultrastructural localization of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263–274.

OGOU, S.-I., OKADA, T. S. & TAKEICHI, M. (1982). Cleavage stage mouse embryos share a common cell adhesion system with teratocarcinoma cells. *Devl Biol.* **92**, 521–528.

PAPAIOANNOU, V. E., MCBURNEY, M. W., GARDNER, R. L. & EVANS, M. J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. *Nature* **258**, 70–73.

PRATT, H. P. M., ZIOMEK, C. A., REEVE, W. J. D. & JOHNSON, M. H. (1982). Compaction of the mouse embryo: an analysis of its components. *J. Embryol. exp. Morph.* **70**, 113–132.

REEVE, W. J. D. & ZIOMEK, C. A. (1981). Distribution of microvilli on dissociated blastomeres from mouse embryos: evidence for surface polarization at compaction. *J. Embryol. exp. Morph.* **62**, 339–350.

SHIRAYOSHI, Y., OKADA, T. S. & TAKEICHI, M. (1983). The calcium-dependent cell–cell adhesion system regulates inner cell mass formation and cell surface polarization in early mouse development. *Cell* **35**, 631–638.

SHUR, B. D. (1983). Embryonal carcinoma cell adhesion: the role of surface galactosyltransferase and its 90K lactosaminoglycan substrate. *Devl Biol.* **99**, 360–372.

SOLTYŃSKA, M. S. (1982). The possible mechanism of cell positioning in mouse morulae: an ultrastructural study. *J. Embryol. exp. Morph.* **68**, 137–147.

STEWART, C. (1980). Aggregation between teratocarcinoma cells and preimplantation mouse embryos. *J. Embryol. exp. Morph.* **58**, 289–302.

STEWART, C. L. (1982). Formation of viable chimaeras by aggregation between teratocarcinomas and preimplantation mouse embryos. *J. Embryol. exp. Morph.* **67**, 167–179.

SURANI, M. A. H. & HANDYSIDE, A. H. (1983). Reassortment of cells according to position in mouse morulae. *J. exp. Zool.* **225**, 505–511.

SUTHERLAND, A. E. & CALARCO-GILLAM, P. G. (1983). Analysis of compaction in the preimplantation mouse embryo. *Devl Biol.* **100**, 328–338.

TARKOWSKI, A. K. (1961). Mouse chimaeras developed from fused eggs. *Nature* **190**, 857–860.

TARKOWSKI, A. K. & WRÓBLEWSKA, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *J. Embryol. exp. Morph.* **18**, 155–180.

YOSHIDA, C. & TAKEICHI, M. (1982). Teratocarcinoma cell adhesion: Identification of a cell-surface protein involved in calcium-dependent cell aggregation. *Cell* **28**, 217–224.

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