The influence of cell contact on the division of mouse 8-cell blastomeres

SUSAN J. PICKERING, BERNARD MARO*, MARTIN H. JOHNSON and JEREMY N. SKEPPER

Department of Anatomy, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK

* Present address: Institut Jacques Monod, U257 INSERM, CNRS – Université Paris VII Tour 43, 2 Place Jussieu 75251 Paris cedex 05, France

Summary

The pattern of division of polarized 8-cell blastomeres with respect to the axis of cell polarity has been compared (i) for cells dividing alone with cells dividing in pairs, and (ii) for early and late dividing cells within a pair. Cell interactions do not seem to influence significantly the overall pattern of division within the population. The only significant difference found was that the second dividing cell in a pair tended to divide in the same way as its earlier dividing companion slightly more frequently than expected. These results suggest that cell interactions immediately prior to and during division do not influence strongly the orientation and position of the division plane. In contrast, interactions between the cells within an intact early 8-cell embryo, which is subsequently disaggregated to singletons or pairs, do influence the type of progeny generated at division to the 16-cell stage, and seem to do so via an effect on the size of the microvillous region generated at the cell apex.

Key words: blastomere, division plane, cell polarity, mouse, cell contact.

Introduction

Cell diversification in the mouse early embryo seems to be established by the differential division of polarized 8-cell blastomeres. During this cell cycle, each hitherto radially symmetric blastomere acquires a polarized morphology consisting of clearly distinguishable apical and basolateral domains. This polarity is retained during division to sixteen cells so that, depending on the orientation of division with respect to the polar axis of the cell, either two polar cells or one polar and one apolar cell result (reviewed in Johnson & Maro, 1986). In the latter case, the division is said to be 'differentiative'. There is considerable variation among 8-cell embryos in the proportion of their constituent polarized blastomeres that divide differentiatively to yield one polar and one apolar descendant. In some embryos, all eight blastomeres divide differentiatively, whilst in others as few as two do so, although the average is between five and six cells (Handyside, 1981; Johnson & Ziomek, 1981b; Bałakier & Pedersen, 1982; Pedersen et al. 1986; Fleming, 1987). Whether or not a blastomere divides differentiatively is not determined randomly, since early dividing blastomeres tend to do so more frequently, thereby contributing proportionately

more cells to the inner cell mass lineage than do later dividing cells (Kelly et al. 1978; Spindle, 1982; Surani & Barton, 1984; Garbutt et al. 1987). This higher incidence of differentiative divisions among the early dividing cells is a direct consequence of their more extensive interacellular contacts (Kelly et al. 1978; Garbutt et al. 1987).

Two types of mechanism have been proposed to explain how intercellular contacts might influence division plane orientation (Fig. 1). The interaction between cells could delineate particular and distinct domains in the cell cortex that resulted in the specific positioning of either the equatorial or the polar region of the spindle, thereby orienting its axis with respect to the contact point (Fig. 1A). Alternatively, interactions between cells could lead to a deformation of cell shape as the cells pack together (Fig. 1B). Thus, the surface of the polarized 8-cell blastomere is heterogeneous with respect to its cell adhesion properties (Lehtonen, 1980; Ziomek & Johnson, 1981; Kimber et al. 1982) and only engages in intercellular contact through its basolateral surfaces. Intercellular packing could therefore deform cells in one of two ways (Fig. 1B), each with clear and different implications for the orientation of the long axis of its spindle.

A   B
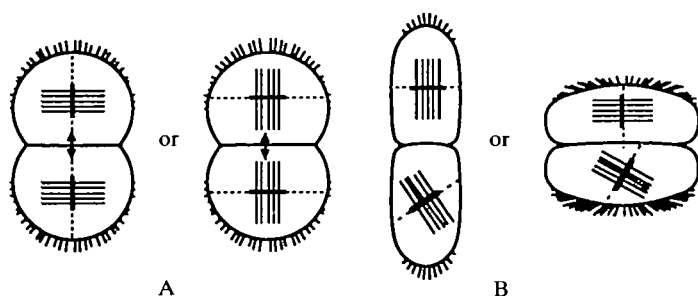
Fig. 1. Schematic outline of two types of mechanism by which cell interaction might modify the orientation of division planes. In A, cell contacts influence the orientation of the spindle, the contact area between cells favouring in its vicinity the presence of either a spindle pole or the equatorial region of the spindle. In B, cell interaction determines division plane orientation indirectly through its influence on cell shape as a result of simple geometric considerations.

Previously, we compared the incidence of differentiative divisions in a population of single polar 16-cell blastomeres with that in populations of pairs of cells and showed that the presence of a second cell did influence the phenotype of subsequent progeny and therefore, by inference, the orientation of the division that generated them (Johnson & Ziomek, 1983). Here we report the results of similar experiments with polar 8-cell blastomeres. We show that the presence of a second cell influences only marginally the pattern of division and does not influence significantly the overall incidence of differentiative divisions. However, in whole embryos, cell interactions during the 8-cell stage do exert an influence on the phenotype of the subsequent progeny and seem to do so by affecting the size of the microvillous pole developed early in the 8-cell stage.

Materials and methods

Embryo collection and culture

MF1 female mice (3–5 weeks, Cambridge animal services) were superovulated by intraperitoneal injection of 5 i.u. pregnant mares' serum (PMS, Intervet) followed 48 h later by 5 i.u. human chorionic gonadotrophin (hCG, Intervet). Mice were paired overnight with CFLP males (Interfauna) and inspected for a vaginal plug the following morning. 48 h after the injection of hCG, late 2-cell and early 4-cell embryos were flushed from the oviducts with medium 2 containing 4 mg ml^{-1} bovine serum albumin (M2 + BSA; Fulton & Whittingham, 1978) and cultured overnight in medium 16 containing 4 mg ml^{-1} BSA (M16 + BSA; Whittingham & Wales, 1969) under oil at 37°C in an atmosphere of 5 % CO_2 in air.

Disaggregation of embryos and generation of pairs of 8-cell blastomeres
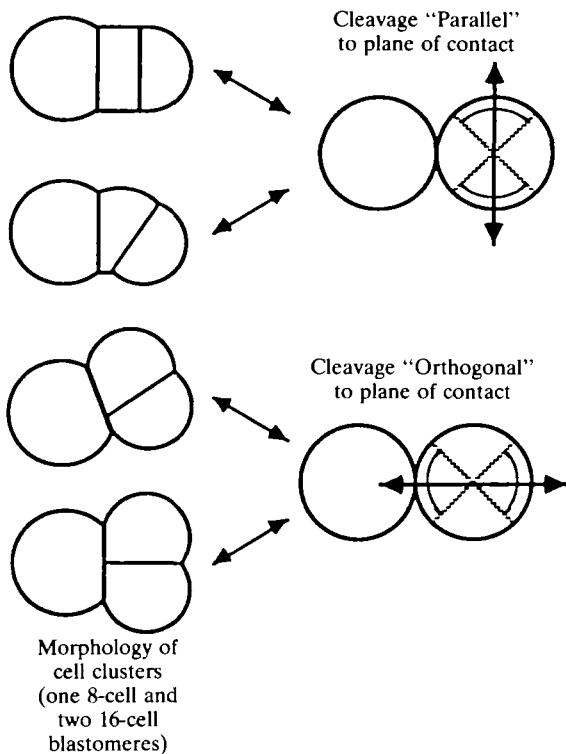
Late 4-cell embryos (60 h post-hCG) were removed from

their zonae by brief exposure to acid Tyrode's solution (Nicolson *et al.* 1975), rinsed briefly in M2 + BSA, and then placed in M2 containing 6 mg ml^{-1} BSA and free of calcium for 10–30 min during which time they were disaggregated to single 4-cell blastomeres by gentle pipetting with a flame-polished micropipette. Single 4-cell blastomeres were cultured in individual microdrops of M16 + BSA under oil at 37°C in 5 % CO_2 in air and inspected hourly for signs of division to 2/8 cell pairs. Each newly formed pair was removed to a different dish and designated 0 h old. These pairs were then cultured for a further 9 h, and then (i) left *in situ* and allowed to go through a further division undisturbed to form clusters of three or four cells, or (ii) placed in M2 + BSA free of calcium for 10 min to reverse intercellular flattening and disaggregated to two single 8-cell blastomeres after which they were returned to culture and allowed to divide to pairs of 16-cell blastomeres, or (iii) placed in M2 + BSA free of calcium for 10 min then returned to normal culture conditions without disaggregation and allowed to divide to clusters of three or four cells. The pairs in this latter group were examined 1 h after return to culture and any that had failed to reflatten on each other were discarded. Each group was again examined hourly for evidence of division, and the resulting pairs and clusters were harvested 2 h postdivision.

Isolation of pairs and single blastomeres from compact 8-cell embryos

A population of late 4-cell embryos was examined hourly and any which had completed division to 8-cells in the previous hour were isolated and designated 0 h old. Embryos intermediate in number between 4 and 8 cells were discarded. The timed 8-cell embryos were cultured for a further 9 h in M16 + BSA during which time they compacted. They were then freed from their zona as described above and placed in M2 + BSA free of calcium for 10–20 min during which time they were disaggregated to either single 8-cell blastomeres or to sister pairs still joined by the midbody from the previous division. These singletons or pairs were returned to M16 + BSA and scored hourly for division, the resulting pairs and clusters being harvested 2 h later. Any pairs which had not reflattened on each other 1 h after their return to culture were discarded.

Scoring on the dissecting microscope the division plane in each blastomere of individual pairs of 8-cell blastomeres

In five experiments, sister pairs of 8-cell blastomeres were generated from 4-cell blastomeres exactly as described above and cultured individually in microdrops of M16 + BSA for 8–9 h. Thereafter they were scored hourly for evidence of division of blastomeres. Each cell cluster was scored for its morphology and thereby for the inferred orientation of division (parallel or orthogonal as defined in Fig. 2) with respect to the plane of contact with its sister blastomere. The scoring was performed alternately and independently by two persons and in more than 98 % of cases identical scores were given over a period of 4–5 h, suggesting that (i) the scoring method was reliable, and (ii) the cells did not move appreciably with respect to each other after cell division. 95 % of divisions fell unambigu-

Cleavage "Parallel"
to plane of contact

Cleavage "Orthogonal"
to plane of contact

Morphology of
cell clusters
(one 8-cell and
two 16-cell
blastomeres)

Fig. 2. Diagram showing the different types of cell clusters observed after division of one 8-cell blastomere in a pair and the inferred orientation of the division, as used for scoring under the dissecting microscope. Similar scoring was performed on quartets of cells formed when both parent cells divided. The midbody linking two daughter cells was frequently visible as illustrated in Fig. 3B,F,H. Note that inferred division planes are not strictly parallel or orthogonal, these terms representing a range of possible orientations as indicated in the right-hand cell.

ously into one of the categories defined in Fig. 2, the remainder were ignored.

Cell fixation and immunocytological staining

Cells were placed in specially designed chambers as described in Maro *et al.* (1984) except that the chambers were coated first with a solution of $0 \cdot 1\,\mathrm{mg\,ml^{-1}}$ Concanavalin A and after the samples were placed in the chambers, they were centrifuged at $450\,g$ for 10 min at 30°C. After a recovery period of 10 min at 37°C, the cells were then washed quickly in PHEM buffer (10 mM-EGTA, 2 mM-MgCl$_2$, 60 mM-Pipes, 25 mM-Hepes, pH 6·9; derived from Schliwa *et al.* 1981), extracted for 5 min in PHEM-taxol buffer (containing $0 \cdot 6\,\mu\mathrm{M}$ taxol and 0·25 % Triton X-100), washed in PHEM buffer and fixed for 30 min with 1·8 % formaldehyde in PHEM buffer. All these steps were carried out at 30°C.

Immunocytological staining was performed as described in Maro *et al.* (1984) using an antitubulin monoclonal antibody (Amersham) followed by fluorescein-labelled anti-mouse immunoglobulin antibodies (Miles) as a second layer. In order to stain chromosomes, fixed cells were incubated in Hoechst dye 33258 ($5\,\mu\mathrm{g\,ml^{-1}}$ in PBS) for

30 min. The coverslips were removed from the chambers and samples were mounted in 'Citifluor' (City University, London) and viewed on a Leitz Ortholux II microscope with filter sets L2 for FITC and A for Hoechst dye. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orthomat photographic system.

Fixation and processing for scanning electron microscopy (SEM)

Scanning electron microscopy was used to examine the surface morphology of singletons, pairs and cell clusters with special respect to the distribution of microvilli. The procedure used was modified from Johnson & Ziomek (1982). All solutions *must* be filtered immediately prior to use. Specimens selected for SEM were fixed in suspension in a drop of 3 % glutaraldehyde (Sigma) in PHEM buffer under oil for 45 min. They were removed and stored in drops of fresh PHEM buffer under oil for not more than 8 h. During this time, clean glass coverslips were coated with a freshly prepared solution of poly-L-lysine (PLL-Sigma type 1B, $1\,\mathrm{mg\,ml^{-1}}$), and were washed two or three times immediately before being placed in individual wells of a Limbro 24-well tissue culture dish containing phosphate-buffered saline (PBS, Oxoid). Pairs and cell clusters were then rinsed through 3 % glutaraldehyde in which they were pipetted on to the centre of the coverslip to which they adhered, and specimens were then dehydrated through a series of graded alcohols as follows: 20 %, 40 % and 60 % 20 min each, overnight in 70 % then 30 min each in 80 %, 90 %, 95 % and 100 % alcohol and one final change of dry absolute alcohol for a further 30 min. This alcohol has been stored over a molecular sieve for a minimum of 48 h prior to the experiment. The coverslips were then dried from 100 % ethanol via CO$_2$ in a Polaron E3000 critical-point-drying apparatus. Coverslips were mounted on stubs with Agar quick-drying silver paint (Agar Aids), left to air dry, then placed in a vacuum desiccator overnight, before being coated with a 50 nm layer of gold in a Polaron E5000 Diode sputtering system. Specimens were examined in a Jeol JSM-35CF microscope operated at 15 kV, each specimen could be seen then rotated through 90°, 180° and 270° and again tilted so that every part of the specimen was viewed thoroughly. Only in this way could the distribution of microvilli on the surface of each blastomere of a pair or cluster of cells be determined accurately.

Fixation and processing for transmission electron microscopy (TEM)

Transmission electron microscopy was used to estimate the relative proportion of the surface covered with microvilli in the three different blastomere populations used. It was necessary for the blastomeres to be embedded such that sections taken from the resulting block yielded a random sample of blastomere orientations. To achieve this, pairs or whole embryos were first disaggregated to single blastomeres as described above, then fixed in suspensions in 3 % glutaraldehyde buffered with 0·1 M-sodium cacodylate pH 7·4 for 30 min, rinsed in fresh cacodylate, postfixed with 1 % osmium tetroxide buffered in the same manner for 30 min and stored for a maximum of 24 h in drops of cacodylate under oil at 4°C. Blastomeres from each group

were collected and, with a minimum volume of accompanying fluid, were pipetted into a tiny bubble lying just underneath the surface of a solid block of agar (2 %) and being open to the atmosphere. The bubbles used to embed in this way were chosen primarily by size each holding at maximum capacity 200–300 blastomeres. Once the bubble had received its complement of blastomeres, it was filled with fresh molten agar (0·5 %) which rapidly mixed with the blastomeres and then solidified. The only bubbles used were those filled such that, in low-power cross-section, blastomeres were distributed randomly throughout the profile of the bubble. The bubble and its contents were then excised as a small agar cube from the much larger block, fixed for 10 min in the same fixative and rinsed in fresh cacodylate, pared to approximately 1 mm^2, and dehydrated through a series of graded alcohols 30 %, 50 % and 70 % 60 min each, 90 % and 95 % 2–4 h each, three changes of absolute alcohol of 1–2 h totalling at least 4 h, then overnight in 1:1 absolute alcohol:TAAB embedding resin (TAAB). The blocks were drained and transferred to fresh resin the following morning and were swirled with two further changes of fresh resin. The blocks were then embedded in flat-embedding moulds and polymerized overnight at 60 °C. Ultrathin sections were cut on a Reichert Ultracut E, and samples taken from four different levels within each block. To ensure that no blastomere was sampled more than once, at least twice the diameter of a single blastomere was allowed between each level, and each sectional plane was oriented vertically through the agar bubble. Sections were mounted on celloidin-coated, slotted copper grids and stained with lead citrate and uranyl acetate. They were viewed on a Philips EM 300 transmission electron microscope.

Quantification of surface distribution of microvilli

The percentage of the blastomere surface area occupied by microvilli was calculated by a line intersection technique. Profiles of 25 blastomeres were selected by a systematic, random sampling procedure from each of the three groups of blastomeres used (see Results). A minimum of 20 profiles was found to be necessary to bring the final estimate of microvillous area permanently within 10 % of the final mean estimated by the progressive mean technique. Profiles were recorded at a magnification of 1700 on 35 mm film with a line graticule of 2160 lines mm^{-1} included on each roll of film for calibration purposes. Each film was printed at a precise magnification of ×4860. Each blastomere profile was overlaid with a Merz type curvilinear test lattice as recommended by Weibel (1979) for use with anisotropic tissues. The microvillous region was defined as that region where the mean intervillous base spacing was less than 618 nm. A line was drawn through the base of the microvillous region of the blastomere and the profile formed was treated as a sectional spheroid. Line intersections with microvillous and nonmicrovillous regions of the blastomere profile were accumulated and the percentage of the blastomere surface area covered with microvilli was calculated using the formula: $(100 \times Iv)/(Iv+INv)$, where Iv is equal to the number of intersections with the villous region of the blastomere surface and INv is equal to the number of intersections with its nonvillous surface. The mean percent-

age villous region was calculated from the 25 profiles in each group and comparisons made between groups using one-way analysis of variance with P less than 0·05 being regarded as a significant difference.

Results

Incidence of conservative and differentiative cell divisions in populations of blastomeres cultured in pairs or as singletons from the early 8-cell stage

Late 4-cell embryos were disaggregated to single cells and allowed to divide to pairs of 8-cell blastomeres *in vitro*. Newly formed pairs of 8-cell blastomeres were then cultured for 9 h during which time they flattened on each other and are known to polarize opposite the point of contact with their sister cell (Ziomek & Johnson, 1980). Pairs that had not flattened on each other by 9 h were discarded. Some pairs were left in culture as compact pairs throughout. A further population of pairs was then disaggregated to single cells in medium free of calcium and returned to culture. As a control for these singletons, the remaining pairs were transferred to calcium-free medium for 10 min but these were not disaggregated and they were transferred back to culture where most reflattened on each other (any failing to do so were excluded). Each population was examined at hourly intervals thereafter in order to identify any 8-cell blastomeres that had divided to pairs of 16-cell blastomeres and any pairs of 8-cell blastomeres that had divided to clusters of three or four cells. These pairs and clusters were harvested 2 h later and processed for SEM analysis (Fig. 3). Cells were scored as being polar or apolar and the midbody remnant was used to identify sister cells in quartets of 16-cell blastomeres. Any sample in which scoring was ambiguous (between 3 and 10 % of all cells) was not included in the final data. The results from these experiments are shown in Table 1. It can be seen that the proportions of inferred differentiative (apolar/polar) and conservative (polar/polar) divisions are similar whether or not blastomeres divide in isolation or in the presence of another cell. The simplest interpretation of these results is that the presence of a companion cell has no effect on the proportions of conservative and differentiative divisions and, by inference, on the orientation of division planes of blastomeres within a pair. A random orientation of division planes is supported by the observation of spindles in a variety of different orientations with respect to the plane of contact between cells (e.g. Fig. 4).

Patterns of division in each blastomere of a 2/8 pair cultured from the early 8-cell stage

The results in section one recorded the outcome of divisions in populations of pairs or singletons. In

Table 1. *Surface phenotype as scored by scanning electron microscopy (SEM) of pairs of sister 16-cell blastomeres derived from 8-cell blastomeres*

| Origin of cells*	number of experiments	n	Surface phenotype of pairs of sister 16-cell blastomeres derived from 8-cell blastomeres	
			Polar/Polar†	Apolar/Polar‡
pair of 8-cell blastomeres	8	232	53 %	47 %
pair of 8-cell blastomeres exposed transiently to low calcium at 9 h	5	174	48 %	52 %
single 8-cell blastomeres disaggregated from a pair at 9 h	5	77	50 %	50 %

* all 8-cell blastomeres were derived from division of isolated 4-cell blastomeres.
† exposed surfaces of both cells covered totally or partially with microvilli.
‡ one of the cells had few or no microvilli, the other had microvilli on all or part of its exposed surface.

order to determine whether for any given 2/8 pair the first dividing blastomere influences the pattern of division of its partner, serial observation of 187 pairs of 8-cell blastomeres dividing to quartets of 16-cell blastomeres (374 mitoses) was undertaken at hourly intervals using a dissecting microscope. The inferred orientation of division of each cell was recorded as being either broadly parallel or broadly orthogonal to the plane of contact between them (as defined in Fig. 2). The incidence of divisions in different orien-


tations is shown in Fig. 5. The incidence of parallel (equivalent to differentiative) divisions in second-dividing blastomeres after the first division had been parallel was not significantly different from the incidence of first divider parallel divisions ($t = 2\cdot08$), and the incidence of orthogonal divisions (equivalent to conservative) in second-dividing blastomeres after the first division had been orthogonal was not significantly different from the incidence of first divider orthogonal divisions ($t = 1\cdot85$). However, the patterns of division planes in the two groups of second-dividing cells did differ significantly from each other ($t = 6\cdot27$), suggesting that when the first division was parallel the chance of the second also being parallel was increased slightly, with a corresponding tendency for orthogonal first divisions to be followed by an increased frequency of second orthogonal divisions. In these experiments, it was also noted that the mean time interval to first division and between first and second divisions (Table 2) does not differ significantly statistically between the two groups ($t = 1\cdot88$ for time
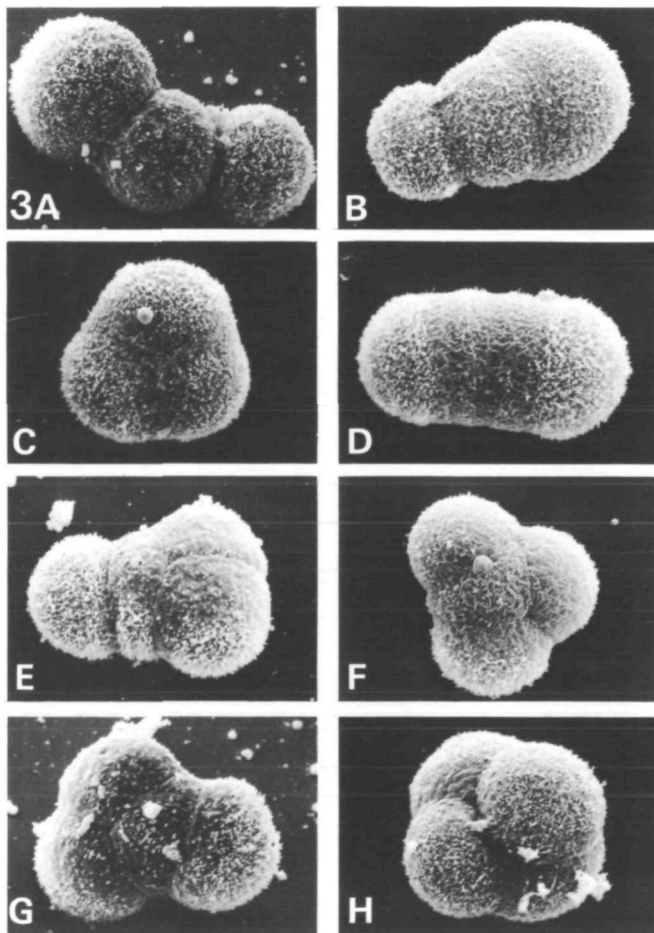
Fig. 3. Examples of pairs of 8-cell blastomeres examined by SEM that have divided to yield various cell clusters. (A) Cluster consisting of one polar 8-cell blastomere, one apolar 16-cell blastomere and one polar 16-cell blastomere, the latter two having formed by division across the axis of polarity of the parent 8-cell blastomere (differentiative). (B,C) Clusters consisting of one 8-cell blastomere (right and top respectively) and a pair of polar 16-cell blastomeres derived by division of a parent cell along the axis of polarity (conservative). (D,G) Cluster consisting of two pairs of 16-cell blastomeres, each made up of one polar and one apolar cell, and each derived by a differentiative division. (E,F) Clusters each consisting of three polar and one apolar 16-cell blastomeres that were assumed to have arisen by the division of one parent cell differentiatively and one conservatively. (H) Cluster in which all 16-cell blastomeres are polar and which were assumed to have arisen by two conservative divisions. All ×1000.
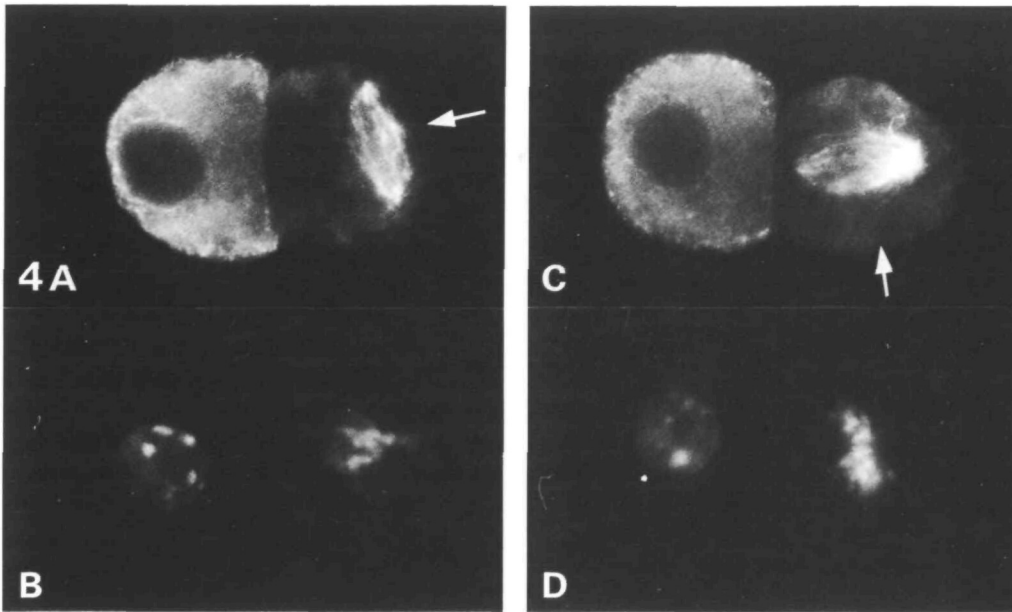
Fig. 4. Two pairs of 8-cell blastomeres stained for tubulin (A,C) and with Hoechst 33258 to stain chromatin (B,D) showing examples of spindles in different orientations with respect to the plane of contact between cells. The predicted positions of the cleavage furrows are indicated with arrows: orthogonal (A,B) and parallel (C,D).
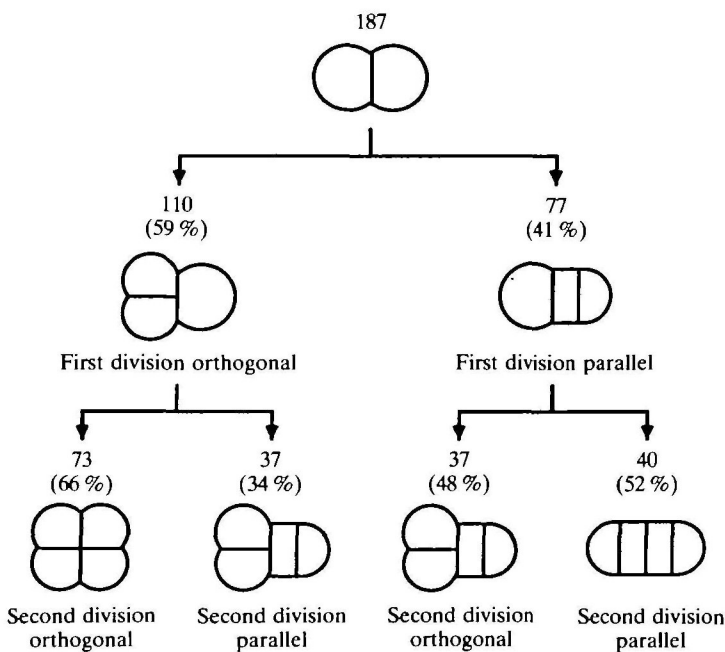
Fig. 5. Schematized patterns of division in pairs of 8-cell blastomeres giving rise to quartets of 16-cell blastomeres. The number of clusters actually observed is given in each case and the percentage of each type of division is indicated. Note that parallel and orthogonal are used as defined in Fig. 2.

to first division and 1·61 for interval between divisions).

A division parallel to the plane of contact should correspond for most cells in pairs of 8-cell blastomeres to a differentiative division (apolar/polar), since the long axis of polarity in most cells is itself orthogonal to the contact plane; similarly, a division orthogonal to the contact plane should correspond in

most cases to a conservative division (polar/polar). The clusters derived in these experiments were harvested in groups corresponding to the categories indicated in Fig. 5, and prepared for examination by scanning electron microscopy (Table 3). In 88 % of cases, orthogonal divisions were found to have been conservative and, in 82 % of the cases, parallel divisions were differentiative, a reasonable correspondence. Moreover, the incidence of orthogonal divisions by first divider cells in this experiment did not differ significantly from the incidence of conservative divisions recorded in Table 1 for singleton or paired cells.

These experiments demonstrate that neither the timing of division nor the orientation of division of the first blastomere to divide in a pair seem to be influenced significantly by the companion blastomere. There is a slight influence of the pattern of the first division on the pattern of the second.

Incidence of conservative and differentiative cell divisions in blastomeres taken from compacted late 8-cell embryos

We know from previous work on nonmanipulated embryos of the strain used in this laboratory that the average numbers of outside and inside cells in a 16-cell embryo are 10·8 and 5·2 (Fleming, 1987). However, if the cells within an intact 8-cell embryo divided in the same way as those described above, the resulting 16-cell embryo would only contain between 3·3 and 3·8 inside cells. We therefore harvested newly formed intact 8-cell embryos from a population of dividing 4-cell embryos and cultured them for 9 h during which time they compacted. They were then disaggregated in calcium-free medium either to single

Table 2. *Timing of division of the cells in pairs of 8-cell blastomeres*

Cleavage plane orientation of first division*	n	Mean ± s.d. time interval (in h)	
		between division to 8-cell stage and division of first cell	between division of first cell and division of second cell
Orthogonal to plane of contact	110	10·6 ± 2·1	1·2 ± 1·3
Parallel to plane of contact	77	10·0 ± 2·0†	1·5 ± 1·5†

* as scored under the dissecting microscope
† the two groups do not differ statistically

Table 3. *Correlation between the results from scoring divisions on the dissecting microscope and from examination of the same cell clusters by scanning electron microscopy (SEM)*

Orientation of division as scored on the dissecting microscope	Surface phenotype of the same pairs of sister 16-cell blastomeres examined by SEM	
	Polar/Polar*	Apolar/Polar†
Orthogonal to plane of contact	83 (88 %)	11 (12 %)
Parallel to plane of contact	14 (18 %)	64 (82 %)

*exposed surfaces of both cells covered totally or partially with microvilli
† one of the cells had few or no microvilli on its exposed surface

cells or to pairs connected by the residual midbody from their previous division and were returned to culture. Any pairs that had not reflattened on each other within 1 h were discarded, as were any cells that had divided within 1 h. Thereafter, cells were scored at hourly intervals for division to the 16-cell stage. Two hours after division, cells were fixed and processed for SEM. An analysis of polarity in the progeny of such experiments yielded the results shown in Table 4. As in the first set of experiments, the presence of a companion cell prior to and throughout division did not significantly affect the proportion of blastomeres dividing differentiatively. However, this proportion (around 72 %) is significantly higher than that found in the cells cultured as

natural couplets throughout the 8-cell stage (around 50 %, see Table 1), and indeed would give an inside cell number of between 5 and 6, corresponding to that observed by Fleming (1987 – see above).

These results suggest that the blastomeres examined in the two populations of cells derived by different routes differ in some way that depends upon the pattern of intercellular contacts present during the development of polarity at the early 8-cell stage but which does not depend on the pattern of intercellular contacts present immediately prior to and during division.

The surface area covered by the pole of microvilli differs in cells with different origins

Since it is known that polar cells at the 16-cell stage inherit all or part of the pole of the mother 8-cell blastomere, a simple explanation for the above observations would be that the surface area covered by the microvillous pole may differ in the two populations, being smaller in 8-cell blastomeres derived from intact embryos. This possibility was examined using three types of late eight cells. Some pairs of 8-cell blastomeres formed by division of isolated 4-cell blastomeres were cultured for 9 h and then disaggregated to single cells. Some newly formed whole 8-cell embryos were cultured for 9 h, then disaggregated to single cells. Finally, other newly formed pairs of 8-cell blastomeres were aggregated together in clusters of four (to yield a total of eight cells) and allowed to flatten and polarize over a 9 h period as in control intact embryos. These aggregate embryos were then

Table 4. *Surface phenotype as scored by scanning electron microscopy (SEM) of pairs of sister 16-cell blastomeres derived by division of 8-cell blastomeres isolated from compacted 8-cell embryos*

Origin of cells*	number of experiments	n	Surface phenotype of pairs of sister 16-cell blastomeres	
			Polar/Polar†	Apolar/Polar‡
pair of 8-cell blastomeres	3	200	33 %	67 %
single 8-cell blastomere	3	309	25 %	75 %

* all 8-cell blastomeres were derived by disaggregation of 9 h old compacted 8-cell embryos
† exposed surfaces of both cells covered totally or partially with microvilli
‡ one of the cells had few or no microvilli on its exposed surface

6A

C

E

B

D

F
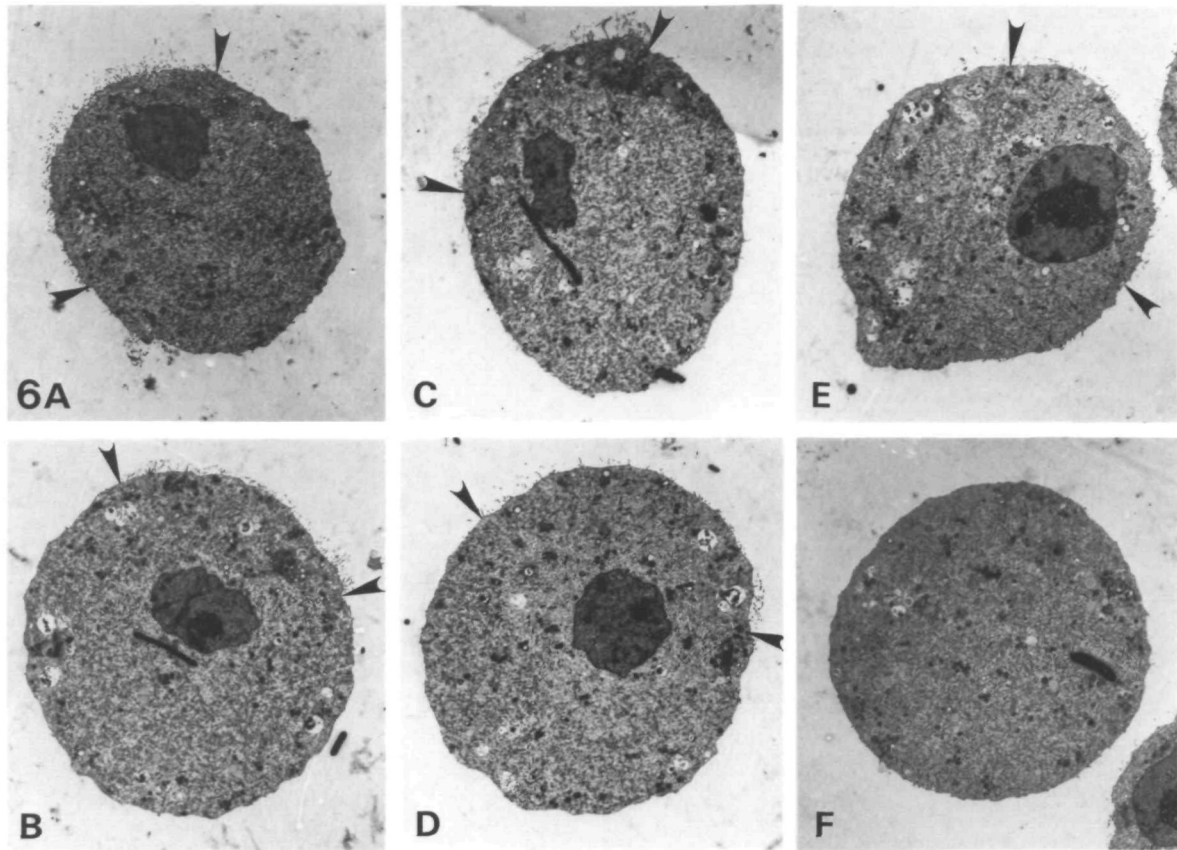
Fig. 6. Transmission electron microscope profiles showing sections through individual blastomeres used for scoring the presence of a microvillous region, the boundary of this region being indicated in each case here by arrowheads. (A,B) Sections from blastomeres originating from control whole 8-cell embryos. (C) Section of blastomere originating from an embryo reconstituted from four pairs of 8-cell blastomeres. (D,E) Sections of blastomeres originating from pairs of 8-cell blastomeres. (F) Section of a blastomere originating from a control whole 8-cell embryo and showing no microvillous region on its sectioned surface. All ×1800.

also disaggregated to single cells. The single cells from all three groups were fixed immediately for transmission electron microscopy, embedded in a random suspension and sectioned so that 25 different profiles could be analysed in each group (Fig. 6). It was possible in this way to make an estimate of the relative proportion of the surface covered by microvilli in each group (Table 5). The value for blastomeres from pairs of 8-cell blastomeres differs significantly from those for blastomeres derived from normal or reconstructed embryos ($P < 0.001$, one-

way analysis of variance). The distribution patterns of the microvillous profiles in the three groups of blastomeres are shown in Fig. 7.

This result suggests that it is irrelevant whether 8-cell blastomeres are formed by the division of an isolated 4-cell blastomere or by the division of blastomeres within an intact 4-cell embryo, but that their subsequent culture in the presence of either one or seven 8-cell blastomeres does influence microvillous distribution. If pairs of 8-cell blastomeres are cultured for the same time in isolation rather than aggregated

Table 5. *Proportion of cell surface covered with microvilli in isolated 8-cell blastomeres of different origins as scored by transmission electron microscopy*

Origin of isolated 8-cell blastomeres*	Number of cells	Percentage of the surface covered in microvilli (mean ± s.d.)
Compacted 8-cell embryos	25	24·1 % ± 3·3 %
Compacted 8-cell embryos constructed from four newly formed pairs of 8-cell blastomeres	25	24·6 % ± 3·2 %
Pairs of 8-cell blastomeres	24	49·0 % ± 3·2 %

* all disaggregated to single cells and fixed at 9 h after division from the 4-cell stage
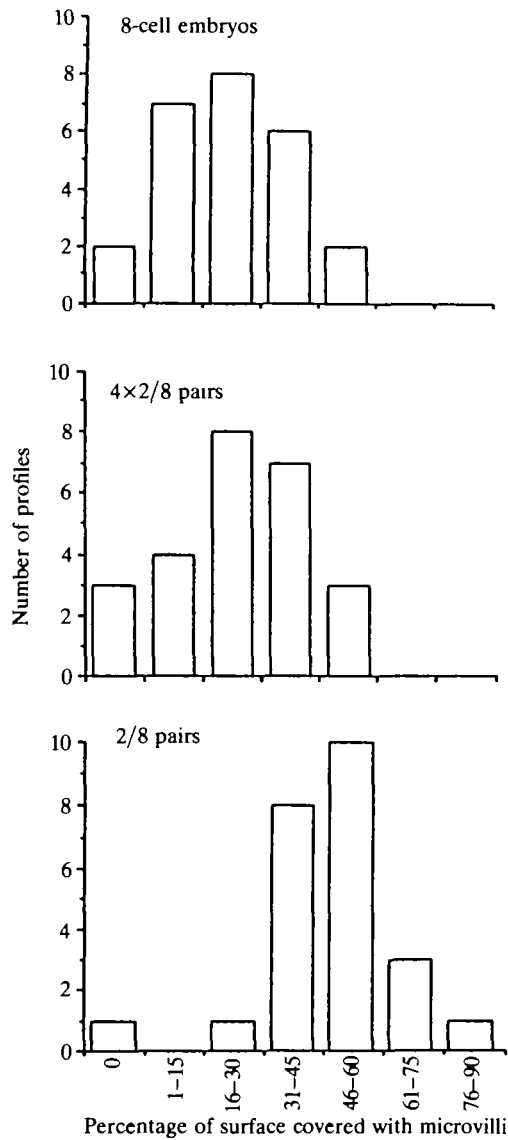
Fig. 7. Distribution profiles of the proportion of the surface covered with microvilli for three different populations of blastomeres. The distribution profiles for blastomeres from control 8-cell embryos and from reconstituted (four pairs of 8-cell blastomeres) embryos are of a similar shape and distribution, whereas that for blastomeres originating form pairs of 8-cell blastomeres is the same shape but shifted to the right with the majority of profiles now falling in the range between 31 and 60 %.

as an 8-cell embryo, the proportion of their surface covered with microvilli is increased significantly. It is interesting to note that the percentage of the surface area covered by the microvillous pole corresponds to the incidence of conservative divisions in each group of cells (Tables 1, 4 and 5) consistent with the notion that the orientation of division planes in all cells of this stage may indeed be random with respect to the axis of polarity.

Discussion

The phenotype, properties and fate of each newly formed 16-cell blastomere appears to depend upon whether it receives during division a part of the cytocortical polar domain of its parent 8-cell blastomere (Johnson & Ziomek, 1981b; Johnson et al. 1988). Cells that inherit all or part of this domain are polar and will contribute to the trophectoderm lineage, whilst cells lacking (or perhaps inheriting a very small part of) this domain are apolar and contribute almost exclusively to the inner cell mass (Johnson, 1986). 8-cell blastomeres that divide differentiatively produce one polar and one apolar cell, and the proportion of blastomeres so dividing in any 8-cell embryo will determine the ratio of polar to apolar cells in the 16-cell embryo derived from it. Since there is evidence to suggest that, *in situ*, regulation of the pattern of differentiative divisions might occur (see Introduction), and that this regulation is influenced by the pattern of intercellular flattening between blastomeres, we have attempted to determine how intercellular flattening might exert its influence.

We showed previously that pairing a polar 16-cell blastomere with a companion cell influenced the orientation of its division plane (Johnson & Ziomek, 1983) and, in this paper, we used a similar experimental design to examine whether the same may also be true of the polar 8-cell blastomere. Late 8-cells have been cultured through division either in couplets or in isolation and the resulting progeny scored for the incidence of differentiative divisions. Unlike the case of 16-cell blastomeres, the presence of single companion cell immediately prior to and during division did not have any significant effect on the incidence of differentiative divisions in the first polar 8-cell blastomere in a pair to divide. However, the second to divide did tend to do so in the same orientation as its companion cell slightly more frequently than expected. This result is reminiscent of Albrecht-Buehler's observations (1977) where, in cell lines, sister cells are often the mirror image of each other. Our results suggest that contact between cells does not systematically direct the orientation and position of the developing spindle by identifying in some way a preferred reference point (Fig. 1A). Cell interaction must therefore influence whether a cell divides differentiatively by some other mechanism.

In the experiments on the effects of the presence of a companion cell, two different experimental approaches were used. In the first, cells were cultured singly or in pairs throughout the 8-cell stage, whereas in the second, cells were cultured to the late 8-cell stage as intact embryos, and then disaggregated to singletons or sister pairs prior to their division. Although within each experimental strategy there

was no difference in the proportion of differentiative divisions when single and paired cells were compared, with the latter experimental procedure differentiative divisions occurred more frequently in both single and paired cell populations. Thus it would appear that it is the pattern of contact during the first part of the 8-cell stage that exerts the major influence subsequently on the proportion of differentiative divisions, not those operating at the time of division itself. Moreover, we have also shown that there is no intrinsic difference in age since last division between those cells that divide differentiatively and those that divide conservatively.

The greater proportion of the cell surface occupied by the microvillous pole, the higher will be the probability that a division plane, however oriented, would cut it. Our results show clearly that cells cultured as pairs throughout the 8-cell stage have a proportionately larger microvillous surface than do cells cultured in groups of eight. The method we have used is satisfactory for accurately quantifying microvillous proportions, but it is important to point out that the result obtained could be explained because the populations of polar cells in the two groups either differed in the relative size of their poles (poles in the couplet group being approximately twice the size) or differed in the proportion of cells in each population that had polarized (the population of couplets having only half the incidence of polar cells found in a population of singletons and therefore having more cells totally covered in microvilli giving more microvillous profiles). This latter explanation of the results can be discounted by consideration of the distribution of values for the proportion of the surface covered with microvilli in each group (Fig. 7). These data provide no evidence for a subpopulation of non-polarized cells in the group of pairs of 8-cell blastomeres. We therefore conclude that polar size in blastomeres cultured as pairs is indeed approximately twice that found on blastomeres from intact embryos. Such a conclusion provides an explanation for the lower incidence of differentiative divisions observed in cells cultured as pairs rather than groups of eight. Why should cells incubated as pairs for 9 h develop larger polar regions of microvilli than do cells incubated for the same period in groups of eight? Clearly, the major difference between these two conditions is the extent of possible intercellular contacts experienced by the cells. We know that cell contact patterns early in the 8-cell stage determine the axis of polarity and influence the rate at which polarity develops (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981a; Johnson et al. 1986). It seems that an early component of the cellular response to contact with other blastomeres involves a local change in the organization of cytoskeletal and cytoplasmic elements that secondarily spreads beyond the immedi-

ate confines of the contact zone (Johnson & Maro, 1986; Houliston et al. 1987). Clearly, the more extensive the contact area, the greater will be the spread of response, and proportionately less of the surface will remain in a microvillous state to serve as the basis of a pole. Indeed, we know that if early contact is complete, the whole surface being apposed to other cells, then the enclosed cell becomes completely nonmicrovillous and apolar (Johnson & Ziomek, 1981a). The results here describe an intermediate state. This explanation also clarifies the result of Garbutt and her colleagues (1987) showing that early forming and flattening cells allocate more progeny to the inner cell mass (ICM) lineage, since early flattening cells will make more extensive contacts and occupy a deeper position in the embryo leading (as predicted by the present results) to the retention of a smaller polar area of microvilli and the observed higher incidence of differentiative divisions. Thus, the division patterns of 8-cell polar blastomeres, like those of 16-cell polar blastomeres, are influenced by intercellular contacts, but the mechanisms involved in each case appear to be different. In the case of 8-cells, contact patterns affect primarily the size of the microvillous pole that develops, and the consequences for division pattern arise only secondarily. For 16-cell blastomeres, in which a stable axis of polarity is present throughout, the effects of cell interaction on division patterns seem to operate mainly by effects on cell shape (Johnson & Ziomek, 1983).

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