Features of cell lineage in preimplantation mouse development

By C. F. GRAHAM\textsuperscript{1} and Z. A. DEUSSEN\textsuperscript{1}

From the Zoology Department, University of Oxford

SUMMARY

The cell lineage of the mouse was studied from the 2-cell stage to the blastocyst. Lineage to the 8-cell stage was followed under the microscope. Each cell from the 2-cell stage divided to form two daughter cells which remained attached. Subsequently, these two daughters each produced two descendants; one of these descendants regularly lay deep in the structure of the embryo while the other was peripheral. Lineage to the blastocyst was followed by injecting oil drops into cells at the 8-cell stage, and then following the segregation of these drops into the inner cell mass and trophectoderm. Between the 8-cell stage and the blastocyst, the deep cells contributed more frequently to the inner cell mass than did the peripheral cells.

INTRODUCTION

It has been often said (e.g. Mintz, 1965; Tarkowski & Wroblewska, 1967; Graham, 1976; Gardner & Rossant, 1976), and rarely shown (Hillman, Sherman & Graham, 1972; Kelly, 1977), that cell position and microenvironment influences cell fate during preimplantation mouse development. The route by which the cells take up their positions is described here.

The cell lineage has been studied in zona-contained and zona-free embryos; the latter lineage depends solely on the behaviour of embryonic cells without constraints from the zona. In zona-contained embryos it was known that the peripheral cytoplasm of 2-, 4-, 8- and 16-cell embryos (marked by oil droplets) contributed exclusively to the trophectoderm layer (Wilson, Bolton & Cuttler, 1972). The inner cell mass (ICM) was similarly shown to be derived from some interior cytoplasm in zona-contained embryos (Wilson \textit{et al.} 1972), and several cells of the 4-cell embryo were known to contribute to the ICM in cultured blastocysts (Kelly, Mulnard & Graham, 1978).

We have followed the lineage to find out if there are further regularities in the allocation of cells to the ICM and to the trophectoderm. These two tissues are biochemically distinct (e.g. Van Blerkom, Barton & Johnson, 1976) and appear committed to the formation of different cell types by the late blastocyst stage in embryos developing in the uterus (Gardner & Papaioannou, 1975).

\textsuperscript{1} Author's address: Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.
MATERIALS AND METHODS

Supply and culture of the embryos. The embryos were from natural matings and were C3H/H, A2G, 129J/Sv, and (C57BL6 x CBA)F₂. They were dissected into pre-warmed, pre-equilibrated Whitten’s medium (1971) and cultured in microdrops (approximate volume 0.05 ml) under paraffin oil (selected for absence of toxicity to cultured embryos from Boots Pure Drug Co., U.K.), in an humidified gas mixture of 5 % CO₂, 5 %O₂, and 90 % N₂ at 37 °C. The zona pellucida was removed with pronase (Calbiochem Co., U.K., technique of Mintz, 1967). Zona-contained embryos were cultured in plastic tissue culture dishes (Sterilin Ltd., Richmond, Surrey, U.K.) or 25 cm² tissue culture flasks (Falcon Plastics, Oxnard, California). Zona-free embryos were cultured in bacteriological plastic dishes (Sterilin Ltd.).

The embryos were drawn and photographed on an inverted microscope with a channelled microscope stage warmed by water from a thermocirculator (Churchill Ltd., Greenford, Middx., U.K.). The embryos were regularly observed at 20 min intervals during the periods when they were expected to divide, but they were also continuously observed if a cell was seen to start cytokinesis at one of these observation intervals. All observations on an embryo were discarded if it failed to develop into a blastocyst by late on the evening of the fourth day of development (the first day of development was the day on which the vaginal plug was found). About 10 % of the embryos which were observed from the 2-cell stage had to be discarded; almost all other embryos developed well apart from a few embryos affected by oil injection.

Injection of oil drops. Injected oil drops were used to study the segregation of cytoplasm and to mark cells for observations on cell lineage. The oil was silicone fluid (MS 550, BDH, Poole, Dorset, U.K.), and the injection technique was based on the procedure of Wilson et al. (1972). Both pipettes were straight and held in Leitz micromanipulators (E. Leitz Instruments Ltd., Park Street, Luton, U.K.) and they were filled with heavy paraffin oil (Boots). The holding pipette was hand drawn from hard glass capillary tubes (BDH) and then flame polished to a flat blunt end with an external diameter of about 40 μm and an apparent internal diameter of about 10 μm. This pipette was controlled with an Agla all glass microsyringe (Burroughs Wellcome Co., Beckenham, Kent, U.K.) which was spring loaded. The injection pipette was made from Leitz capillary tubing drawn on an electrophysiological microelectrode pipette puller. This was arranged to form sealed pipettes. The barrel of the pipette was partly filled with the silicone oil by inserting a fine syringe and the pipette was connected to a de Fonbrune suction and force pump (C. Beaudouin, Paris, France). Under the microscope, the tip of the pipette was knocked off against a clean ground glass wedge; the final internal tip diameter was about 1 μm. All injections were made with the embryo suspended in drops of Whitten’s medium hanging from the coverslip surface of the Puliv chamber described in the Leitz
Fig. 1. Method of injecting oil drops. 

(a) Injection to mark the interior of a cell contacting five others on the exterior surface of the 8-cell-stage embryo. Note that the cell with the oil drop touches one additional cell near the centre of the embryo. Injection at the 8-cell stage. The pipette was inserted through one cell into the other along an horizontal plane.

(b) Injection to mark the interior of a cell contacting four other cells on the surface of the 8-cell-stage embryo. Injection at 4-cell stage. The pipette was inserted through one cell into the other in the same plane as that described above. The injected cell divided so that the drop usually lay in the interior half of a cell with four surface contacts. Cells with solid outlines towards the observer, and cells with dotted outlines away from the observer.

Micromanipulator Manual. The drops were surrounded by paraffin oil and the chamber and coverslip were siliconized with Repelcote (Hopkin & Williams, Chadwell Heath, Essex, U.K.). For injection, a × 20 objective was used with × 15 eyepieces.

**Positioning of the drops.** For studying the cell lineage to the 8-cell stage and for studying cytoplasmic cleavage, the injection pipette was inserted deep into the cytoplasm of a cell and the drop injected (diameter 4–8 μm). As the pipe was withdrawn, so the drop was pulled from its tip by the cell membrane.

For studying the cell lineage from the 8-cell stage to the blastocyst, the drops were aimed so that they lay by the internal surface of a cell's membrane towards the interior of the embryo. To mark cells with five surface contacts in this way, the injections were made at the 8-cell stage (Fig. 1a). To mark cells with four surface contacts at the 8-cell stage in this way, the injections were made at the 4-cell stage (Fig. 1b); this procedure reduced the possibility of damage to the embryo because the injection pipette only penetrated two cells. The position of the drop was noted every 2 or 3 h up to the late compacted 8-cell stage. Its position was described as internal if it lay in the half of the cell which was deep in the structure of the embryo. It was necessary to roll the embryo through all possible orientations to observe the positions.

About 10% of the injected cells lysed within an hour of injection. The drop appeared to have no other effect on division order or cell viability until the late blastocyst stage. At this time the drop emerged from cells in a further 10% of the embryos (data on these embryos were discarded). To make sure that the drop remained intracellular, the embryos were observed every 12 h up to the blastocyst stage on the inverted microscope. The position of the drop in the
blastocyst was observed by replacing the embryo in the chamber and rolling it through all possible orientations with the holding pipette.

**Fixation and sectioning.** For accurate serial reconstructions, the embryos were fixed at 4 °C in 1 % paraformaldehyde in 0.075 M phosphate buffer (1 h), followed by 1 % osmium tetroxide in the same buffer (1 h). The embryos were embedded in agar so that they could be easily handled. They were pipetted into a warm agar solution (1 %, w/v) in 6 % NaCl (w/v) on a slide lying on a hot plate. The agar containing the embryos was cut out when the agar had set. The block was dehydrated and embedded by standard electron microscopy techniques. It was serially sectioned at 1 μm, stained with hot 1 % toluidine blue (w/v) in aqueous 1 % (w/v) borax, and every second section was photographed.

**RESULTS**

The results are divided into two sections. First the lineage is followed to the early 8-cell stage; the relative positions of cells are noted. Second, cells in different positions relative to each other at the 8-cell stage are marked by oil drops and the segregation of oil drops is followed up to the blastocyst stage.

1. **Method of description**

Observations began at the 2-cell stage. The polar body was taken to mark the animal pole and the opposite end of the embryo was called the vegetal pole; a line joining the two poles was called the polar axis. The original position of the polar body was not known. Other terms are indicated in the legend to Fig. 2. Figure 3 illustrates an ideal cell lineage to guide the reader. During development, the polar bodies and cells moved relative to each other and to the observer; there were no fixed points. Consequently the description recorded relative movement over short time intervals and did not refer to the absolute position of cells.

2. **Division from two to four cells**

**Division of AB**

The first cell to divide to the 4-cell stage was called AB. The description is from side view AB (Fig. 2). Inside the zona the longest axis of the AB cell was initially parallel to the polar axis. During division the long axis of the AB cell changed direction so that a cell (the A cell) was formed to the right and another cell (the B cell) was formed to the left of the polar axis. Thirty-four embryos were observed. In 13 of the embryos, A was the closest cell to the animal pole (Figs. 2 and 3), while in 15 embryos it was the furthest cell from this pole. In the other six embryos the cleavage plane was equatorial. During division, the polar body frequently remained attached to A, or to B, or to both A and B, and it moved away from the apparent animal pole. The apparent rotation of the
Fig. 2. Explanation of the subsequent figures. (a) The 2-cell stage. The vertical plane is the first cleavage plane and it is meridional. The horizontal plane is described as equatorial. (b) The 3-cell stage. When viewed from side view AB (the arrow gives the direction of view) a cell (the A cell) has been formed to the right of the other new cell (the B cell). (c) The 4-cell stage. When viewed from side view AB, a cell has been formed to the left (the C cell) of the other new cell (the D cell). All subsequent figures are drawn as if from side view AB. The application of these terms to mammalian development is justified in Gulyas (1975). Note that the cleavage pattern in the mouse frequently differs in detail from that reported in the rabbit (Gulyas, 1975).

Fig. 3. The ‘ideal’ cell lineage. Division to the 8-cell stage seen from side view AB. (a) The 2-cell stage with one cell lying above the other. (b) The 4-cell stage in the same form as that in Fig. 2(b). (c) The 8-cell stage. Notice that this is a regular figure which would have a similar cell pattern if it were viewed from the left, the right, or from beneath this page. Although the ‘ideal’ cell lineage has not been observed, all 8-cell stage patterns approximate to it and it therefore serves as a model (see Figs. 5 and 7). Cells with continuous outlines are towards the observer (bold lettering), and cells with a dotted outline are away from the observer (fine lettering). The polar body, drawn with a broken line, lies between the two cell layer. The cell letters are as close as possible to the most peripheral region of the cell which they name.
AB long axis was rare in zona-free embryos (seen in one out of ten closely observed embryos). In zona-free embryos, the AB daughter formed closest to the polar body was called the A cell and in cases when the polar body fell off the designation was arbitrary (this is indicated in the figure and table legends).

**Division of CD**

The second cell to divide to the 4-cell stage was called CD. The longest axis of CD rotated with respect to a line joining the centres of the A and B cell. The rotation was in the opposite direction to the AB movement so that a cell was formed to the left (the C cell) and to the right (the D cell) of the original long axis of CD (described from side view AB, Figs. 2 and 3). In embryos without a zona, the CD daughters usually moved during cleavage. Consequently, at the completion of the CD division the cells were in a variety of patterns.

Oil drops were injected into about 20 zona-contained 2-cell embryos. The drops did not segregate according to the apparent cleavage plane or its movement and it was concluded that there was extensive cytoplasmic redistribution during this cleavage (see Discussion). This was not studied further.

**Pattern of cells at the 4-cell stage**

The cell patterns observed at the 4-cell stage are shown in Fig. 4. The patterns were classified according to the total number of cell-to-cell contacts made in each embryo; the more contacts, the more tightly packed were the cells (Table 1, Fig. 4).

The six contact arrangement did not appear regular and this was obvious in the zona-free embryos; this irregularity was due to the fact that one daughter of CD rarely sat exactly on top of the three cells of the embryo; the top cell remained closer to the other daughter of CD (Figs. 4a and 6a). The five-contact pattern was drawn as a rhomboid (Fig. 4c), but frequently it was a slightly flattened version of the six-contact pattern (Figs. 4b and 6b). In both the five-contact patterns notice that two cells have three cell contacts while the other two cells have two cell contacts. The four-contact pattern found in the form of a T in the zona-free embryos (Fig. 4e) has one cell with only one cell contact: in one case this cell was a daughter of AB and in one case it was a daughter of CD.

The cell contact patterns showed that the cells of the zona-contained embryos were more tightly packed than the cells of the zona-free embryos (more six- and five-contact patterns). Also the observation of five-contact patterns showed that cells may be in different relative positions even at the 4-cell stage, i.e. the cells within one embryo may have different numbers of cell contacts.
Fig. 4. Cell arrangements at the 4-cell stage. The patterns are classified by the total number of cell-to-cell contacts within each embryo. (a) Tightly packed six-contact pattern but note that this pattern is irregular and that the top cell lies slightly closer to the bottom right cell from which it was derived. (b) and (c), the five-contact patterns. (c) is a flattened version of (b). (d) and (e), the four-contact patterns. The (e) pattern is a T shape. (f) the three-contact pattern.

Table 1. Total cell contact data in 4-cell and 8-cell embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>Treatment (numbers)</th>
<th>6</th>
<th>5</th>
<th>4</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-cell</td>
<td>Zona-contained (38)</td>
<td>76.3</td>
<td>23.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-cell</td>
<td>Zona-free (34)</td>
<td>29.6</td>
<td>50.0</td>
<td>14.7</td>
<td>5.7</td>
</tr>
<tr>
<td>8-cell</td>
<td>Zona-contained (20)</td>
<td>0</td>
<td>75.0</td>
<td>20.0</td>
<td>5.0</td>
</tr>
<tr>
<td>8-cell</td>
<td>Zona-free (28)</td>
<td>14.3</td>
<td>32.1</td>
<td>35.7</td>
<td>17.9</td>
</tr>
</tbody>
</table>

The cell arrangements with particular total cell contact numbers are illustrated in Fig. 4. At the 4-cell stage all cell contacts within the embryo are scored. At the 8-cell stage the total cell contacts within the four-cell group from AB and within the four-cell group from CD are scored. The contacts between each four-cell group are not scored.
3. Division from the 4- to the 8-cell stage

The actual cell lineages to the 8-cell stage were irregular and they could only be described by providing drawings of each division of each of the 20 embryos which were studied in detail. Instead we note those features of the cell lineage which are common to most embryos. The morphology of the lineage is again described by the contacts which cells make with each other.

Cell-to-cell contacts within four-cell groups

One daughter of AB tended to divide to the 8-cell stage before the daughters of CD (Kelly et al. 1978). The A and the B cell divided so that the cell pattern of their four descendants at the 8-cell stage (a four-cell group) could be described by the number of cell contacts which they made with each other in exactly the same way as the 4-cell embryo was described. The pattern of the four descendants of CD were recorded similarly, and since there were no consistent differences between the four-cell group from AB and that from CD, the results were tabulated together (Table 1). During this division, the descendants of AB remained in contact with each other and the descendants of CD also cohered. The patterns were similar to those at the 4-cell stage except that the four-cell groups were less compact: for zona-contained embryos there were more five-, four-, and three-cell contact patterns than at the 4-cell stage ($P = 0.001$, $\chi^2$ on a 2 x 2 contingency table with Yates' correction), and for zona-free embryos there were more four and three contact patterns than at the 4-cell stage ($P = 0.01$, same test).

Relationship between AB and CD descendants

The AB descendants also cohered with the CD descendants, and usually one daughter of each parental 4-cell stage maintained the contacts of the parental cell. To describe this behaviour, we next introduce the terminology that the descendants of A at the 8-cell stage were called a and a, and so on for the descendants of B, C and D. The rule was that one a cell maintained the same contacts with one b, c and d cell at the 8-cell stage, as the previous contacts of A with B, C and D at the start of the 4-cell stage. The same was usually true for one b, c, and d cell (see Fig. 6). In ten zona-enclosed embryos, the rule was only broken by three out of the 40 cell pairs each formed from a single cell of the 4-cell embryo. In all the exceptional cases, the contacts of A with B, C, and D at the 4-cell stage were maintained by the a a pair with b b, c c, and d d (combined contacts). In ten zona-free embryos, the rule was only broken by five out of the 40 cell pairs each formed from a single cell of the 4-cell embryo. In two of these embryos the combined contacts of both b and both c cells did not maintain those of B and C at the 4-cell stage while in the other eight embryos the combined descendants maintained the contacts of the parental cell.

These observations show that all the cells in the embryo tend to stick
Fig. 5. Cell arrangements in three zona-enclosed embryos. (a) is embryo 6 in Table 2; (b) is embryo 10; (c) is embryo 3. Continuous outlines surround cells towards the observer (aa, bb). Dotted outlines are around cells away from the observer (cc, dd). Lettering is as close as possible to the most peripheral region of the cell which it nominates. Diagrams made from sketches drawn within the first hour of the start of the 8-cell stage.

Table 2. Surface cell contacts of zona-enclosed embryos at the 8-cell stage

<table>
<thead>
<tr>
<th>4-8 division order</th>
<th>First AB</th>
<th>Last AB</th>
<th>First CD</th>
<th>Last CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B D A C</td>
<td>5 4 &gt; 5 3</td>
<td></td>
<td>4 3 &lt; 5 3</td>
<td></td>
</tr>
<tr>
<td>2. A B D C</td>
<td>4 4 &gt; 4 3</td>
<td>5 4 &gt; 5 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. A C D B</td>
<td>5 4 &gt; 4 3</td>
<td>5 4 &gt; 5 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. A D C B</td>
<td>5 5 &gt; 4 4</td>
<td>6 3 = 5 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. B C D A</td>
<td>5 4 &gt; 5 3</td>
<td>6 5 &gt; 5 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. B C D A</td>
<td>6 5 &gt; 5 2</td>
<td>4 4 ? 6 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. A B D C</td>
<td>5 4 &gt; 4 3</td>
<td>4 4 = 5 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. B A C D</td>
<td>5 5 &gt; 5 4</td>
<td>4 3 &lt; 5 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. A C B D</td>
<td>5 4 = 5 4</td>
<td>5 3 = 4 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. A C D B</td>
<td>4 4 &lt; 5 5</td>
<td>5 4 ? 6 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means

The symbols > and < indicates when the combined contacts of one pair of cells exceeds or is less than that of the other pair derived from the same cell at the 2-cell stage. The symbol ? means that the division order of that pair of cells is unknown and these figures were not used in calculating the means. In the column of cell division order, the solid line above cell letters indicates embryos in which several cells divided in an interval of observation.

The column marked 'First AB' gives the surface contact scores of the pair of cells derived from the first AB daughter to divide to the 8-cell stage. The other columns are labelled similarly.
Table 3. *Surface contacts of zona-free embryos at the 8-cell stage*

<table>
<thead>
<tr>
<th>4-8 division order</th>
<th>First AB</th>
<th>Last AB</th>
<th>First CD</th>
<th>Last CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ABCD</td>
<td>4 &lt; 4</td>
<td>1</td>
<td>3 &lt; 5</td>
<td>5</td>
</tr>
<tr>
<td>2. ACDB</td>
<td>4 &gt; 5</td>
<td>2</td>
<td>5 &gt; 3</td>
<td>1</td>
</tr>
<tr>
<td>3. ABCD</td>
<td>4 &lt; 5</td>
<td>4</td>
<td>5 &gt; 4</td>
<td>1</td>
</tr>
<tr>
<td>4. ADBC</td>
<td>5 &gt; 5</td>
<td>3 &lt; 4</td>
<td>5 &gt; 3</td>
<td>1</td>
</tr>
<tr>
<td>5. ACBD</td>
<td>5 &lt; 4</td>
<td>2 &gt; 3</td>
<td>6 &gt; 4</td>
<td>2</td>
</tr>
<tr>
<td>6. ACBD</td>
<td>5 &gt; 5</td>
<td>3 &lt; 4</td>
<td>5 &lt; 5</td>
<td>3</td>
</tr>
<tr>
<td>7. ABCD</td>
<td>4 &gt; 5</td>
<td>3 &lt; 4</td>
<td>6 &gt; 3</td>
<td>3</td>
</tr>
<tr>
<td>8. ACDB</td>
<td>6 &gt; 6</td>
<td>4 &gt; 2</td>
<td>5 &gt; 6</td>
<td>2</td>
</tr>
<tr>
<td>9. ABCD</td>
<td>6 &gt; 4</td>
<td>3 &lt; 5</td>
<td>5 &lt; 4</td>
<td>3</td>
</tr>
<tr>
<td>10. ACBD</td>
<td>6 &gt; 5</td>
<td>3 &lt; 5</td>
<td>5 &gt; 4</td>
<td>2</td>
</tr>
</tbody>
</table>

Means

\[
(n = 8) \begin{array}{ccc}
8.5 & 7.25 & (n = 10) 8.4 & 6.9
\end{array}
\]

The symbols are the same as those in the legend to Table 2. In the embryos numbered five to ten, the designation of A and B was arbitrary because the polar body fell off the zona-free embryos. The division order to the 4-cell stage was known and so the AB and CD distinction could be used.

together and that this adhesion does not depend on the zona. It is also shown that the cell pattern is inherited from the 4-cell stage.

*Relationship between a and a, b and b, etc.*

The other descendant of each A, B, C, and D cell rarely maintained the cell contacts of the parental cell: in only five out of 40 cases did it do so in zona-contained embryos, and in only six out of 40 cases did it do so in zona-free embryos. In zona-free embryos, this cell was formed away from the visual centre of the embryo (the visual centre was a subjective impression of the centre of gravity of the embryo, Fig. 6). To quantify this obvious arrangement, we next scored the number of contacts which each cell made with other cells on the surface of the embryo. This surface contact score was required because internal cavities begin to form between the cells during the 8-cell stage. These cavities would distort total cell contact data; it was also difficult to accurately observe internal cell contacts. Cells towards the visual centre of the embryo were surrounded by many other cells and had a high score while those at the periphery of the embryo had a low score (Tables 2 and 3; Figs. 5 and 6). Usually the daughters of each 4-cell stage cell had different scores; the scores were the same in only 17 out of 80 pairs (similar number of exceptions in zona-contained and zona-free embryos).
Fig. 6. The relationship between parental and daughter cells in zona-free embryos. Tracings from photos made within 1 h of the start of the 4-cell stage and within 1 h of the start of the 8-cell stage. (a) Six total cell contact pattern at the 4-cell stage. Note that D lies closer to C than to A or to B. The embryo divides to give an 8-cell pattern in which one daughter of each parental cell maintains the cell contacts of the parent. (b) Five total cell contact pattern at the 4-cell stage forms an 'open' 8-cell stage in which one daughter of each parental cell maintains the contacts of the parent. Note how the other daughter ‘falls out’ of the structure. Continuous line outlines cells towards the observer (bold lettering). Dotted line surrounds cells away from the observer on the bottom of the culture dish (fine lettering). Broken lines outline cells in the plane between (bold lettering). In the tracing of the 8-cell embryo, the maintained cell contacts are indicated by pairs of short parallel lines.
Fig. 7. Reconstructed late 8-cell stage embryos. (a) Embryos 1, 2, and 3 in Table 4. The 'ideal' pattern. (b) Embryo 4. (c) Embryo 5, (d) Embryo 6. (e) Embryo 7. (f) Embryo 8. These diagrams are based on the photos of serial sections of late 8-cell stage embryos. The actual appearance of the embryos is more compact and the cells do not actually lie in two planes as illustrated but they are arranged so that the external surfaces of the cells are contained by a slightly elongated sphere. Notice how widely differing surface contact scores (Table 4) can describe rather similar patterns.

Table 4. Surface contacts of zona-enclosed embryos reconstructed from serial sections

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 3</td>
<td>5 4 5 4</td>
</tr>
<tr>
<td>4.</td>
<td>5 4 6 4</td>
</tr>
<tr>
<td>5.</td>
<td>6 4 6 3</td>
</tr>
<tr>
<td>6.</td>
<td>5 3 6 5</td>
</tr>
<tr>
<td>7.</td>
<td>5 3 5 4</td>
</tr>
<tr>
<td>8.</td>
<td>6 3 5 5</td>
</tr>
</tbody>
</table>

The cell patterns of these embryos are illustrated in Fig. 7. In these embryos the cell lineage was not known. These embryos were taken from a batch of embryos which were known to be at the late 8-cell stage because some of the embryos had divided to nine or more cells. The surface cell contacts of the cells towards the reader (continuous outlines) are given in the first four columns; the surface cell contacts of the cells away from the reader (dotted outlines), are given in the last four columns.
Relationship between division order and surface contact scores

It was noticed that the first cell to divide to the 8-cell stage produced two descendants which tended to be close to the visual centre of the embryo (Fig. 8). To quantify this observation, the surface contacts of the pair of descendants from each 4-cell stage cell were summed (Tables 2 and 3). The number of surface contacts of the first pair of AB descendants to reach the 8-cell stage was greater than that of the last pair of AB descendants to reach this stage (significantly greater for zona-enclosed embryos at the 2.5% level, nine degrees of freedom, and significantly greater for zona-free embryos at the 5.0% level, seven degrees of freedom, related t test, Meddis 1975). The mean surface scores of the first pair of CD descendants were greater than those of the last pair of CD descendants but this difference was not significant.

To the late 8-cell stage

Despite this irregularity, at a superficial glance the 8-cell embryo often appears to have the simple cell pattern illustrated in Fig. 3(c). To find out if a simple cell pattern was established during the 8-cell stage, late 8-cell stage embryos were taken from the uterus and fixed immediately. In these embryos, the lineage to the 8-cell stage was unknown but the pattern could be described by surface contact numbers (Fig. 7, Table 4). The cells can be fitted to pairs which alternated in contact numbers in 29 out of 32 cases. However, these patterns were only slightly simpler than the patterns at the early 8-cell stage; in three of these eight embryos the pattern was the alternating five, four surface contacts illustrated in Fig. 3(c). In all other embryos, and in the embryos whose lineage was traced, there were cells with exceptionally many (six or more) or exceptionally few (three or less) surface contacts.

4. From the 8-cell stage to the blastocyst

We next followed the segregation of cytoplasm in cells with particular surface contacts in zona-contained 8-cell embryos, during their development to the blastocyst stage (see Materials and Methods). The number of surface contacts of the injected cell was noted and the position of the drop in the late compacted 8-cell embryo recorded (Table 5, Fig. 9). Drops which moved to the peripheral half of the cell usually contributed to the trophectoderm irrespective of the cell's surface contacts (16/17 cases). The drops which remained in the internal half of the cell segregated to the ICM in five or more surface contact cells (11/12 cases), but rarely did so in four or less contact cells (3/12 cases). These two proportions were significantly different (P = 0.01, $\chi^2$ on a 2 x 2 contingency table with Yates' correction for small samples, Bailey, 1959).
Table 5. Distribution of oil drops

<table>
<thead>
<tr>
<th>Surface contacts</th>
<th>Location in compacted 8-cell stage</th>
<th>Tissue location in the blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Internal</td>
<td>ICM 1</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 2</td>
</tr>
<tr>
<td>3</td>
<td>Internal</td>
<td>ICM 2</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 5</td>
</tr>
<tr>
<td>4</td>
<td>Internal</td>
<td>ICM 7</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 6</td>
</tr>
<tr>
<td>5</td>
<td>Internal</td>
<td>ICM 1</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 6</td>
</tr>
<tr>
<td>6</td>
<td>Internal</td>
<td>ICM 3</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 3</td>
</tr>
<tr>
<td>7</td>
<td>Internal</td>
<td>ICM 1</td>
</tr>
<tr>
<td></td>
<td>External</td>
<td>External 0</td>
</tr>
</tbody>
</table>

The drops were injected at either the 4-cell or 8-cell stages (see Materials and Methods), and their position was observed in the late compacted 8-cell stage embryo.

**DISCUSSION**

We would like to have maps of the position of each cell's membrane at all times in development. Our intermittent observations only record the more obvious features of morphogenesis and these are discussed below.

1. **Cytokinesis and the cleavage of cytoplasm**

There is no obvious relationship between the orientation of the membranes which separate the two daughter cells and the cleavage of the cytoplasm. First,
the orientation of the cleavage plane appears to change in the 5–10 min between
the start of cell constriction and the completion of a dividing line between the
two cells. This movement is probably real because the orientation of the
cleavage plane frequently changes with respect to the observer, with respect to
the polar body, and with respect to oil drops in neighbouring cells. Second, oil
drops move from one half of the cell to the other during division, and their
distribution in the two daughter cells does not correspond to the cleavage of
cytoplasm in the plane of the membrane which finally separates the two
daughter cells. Third, oil drops placed internally in the cytoplasm often move
away from this position (8- to 16-cell stage, this study; see also Wilson et al.
1972). None of these oil drop movements can be simply explained by floating.
These observations suggest that there is considerable cytoplasmic redistribution
during cleavage; we do not know if the redistribution is regular.
2. Daughter cells and relative positions

Cell division frequently gives rise to two daughter cells whose relationship with other cells differs. Only one of these cells usually maintains the cell contacts of the parent cell while the other is usually formed away from the centre of the embryo and has fewer surface cell contacts. This process is seen in the generation of the five, four, and three total cell contact patterns at the 4-cell stage (Table 1). It occurs again during division to the 8-cell stage and this regular feature of the lineage does not depend on the zona because it is seen in zona-free embryos (Tables 2 and 3, Figs. 4, 6). One interpretation is that one half of the dividing cell is held in place by a cytoskeleton or non-fluid cell membrane which is constrained by the cell contacts of the parental cell.

It was also observed that related cells cohered through several cell divisions. Such cohesion between related cells has been previously observed (Kelly, 1978; Garner & McLaren, 1974; Mintz, 1964a, b, 1965). This cohesion of related cells could be due to special features of inherited cell contacts (e.g. the mid body), or it could be due to the absence of mechanisms of cell migration at this stage of development.

There are several reasons why the rules of cell division and cell contacts do not predict the structure of the 8-cell embryos which develop from known 4-cell embryo structures. First, cells sometimes make new contacts as they elongate during division (Fig. 8). Second, the form of each four-cell group is variable. We believe that the form of any four-cell group depends on its division order to the 8-cell stage with respect to the other four-cell group within the same embryo, but our small number of intermittent observations make it impossible to detect this regularity. There are 48 possible combinations of lineage, division order, and orientation, in the change from a six contact pattern zona-free 4-cell stage to the 8-cell stage.

3. Formation of the ICM

We cannot describe actual cell lineages from the 2-cell stage to the blastocyst stage. It is invalid to use the surface scores of the daughters of an A cell, for instance, to calculate the contribution of the A cell to the ICM; the reason is that we have experimental evidence (unpublished) which shows that the formation of inside cells by one parental cell may affect the chances of neighbouring parental cells contributing to the ICM. Instead various regularities of ICM formation are discussed.

The zona-contained 8-cell stage embryo had on average 3-4 cells with five or more surface contacts and 91-6% of these, on average, contributed at least a single cell marked with an oil drop to the ICM. They contained on average 6-7 cells with four or more surface contacts and on average 25% of these contributed a single cell marked with an oil drop to the ICM.

It follows that the ICM will usually be formed by at least four cells of the
8-cell embryo. However, the 16-cell stage embryo rarely contains more than two inside cells (Barlow, Owen & Graham, 1972), and some of those four cells must therefore contribute to the ICM between the 16- and 32-cell stage. In any particular embryo, the chances are that the ICM is principally formed from daughters of the five surface contact cells. It is not surprising that this occurs because such cells lie deep in the structure of the 8-cell embryo; such deep cells have been previously described at the 8-cell stage (Mulnard, 1967). Each of the cells of the 8-cell embryo also seem to contribute to the trophectoderm; in this and previous work it has been found that almost all cells marked by an oil drop at the periphery contribute to the trophectoderm (Wilson et al. 1972).

It is probable that the processes leading to ICM formation begin at the 2- to 4-cell division and continue beyond the 8-cell stage. At the 4-cell stage in zona-free embryos, one cell frequently lies slightly away from the visual centre of the embryo and this cell is often a daughter of the last cell to divide (Fig. 6a, b). Consequently the A and B cells tend to be closer to the visual centre and at the 8-cell stage there is a tendency for the first pair of descendants of AB which reach this stage to have the greatest number of cell contacts (Fig. 8 and Table 2); they tend to lie deep in the embryo, and it is therefore probable that they make a disproportionately large contribution to the ICM. Similarly the first cell to divide to the 8-cell stage is usually derived from AB and it makes a disproportionately large contribution to the ICM in embryos which are each reconstituted from all the cells of a single dissociated embryo (Kelly et al. 1978).

4. Irregular cell lineages

The lineage to the 8-cell stage varied from one embryo to another and these observations contrast with the invariance of lineage attributed to other animal phyla (Reverberi, 1971). A variety of cell arrangements has also been observed in briefly cultured 8-cell mouse embryos (e.g. Lewis & Wright, 1935; Ducibella & Anderson, 1975), and so it is unlikely that irregularity is due to the particular mouse strains and culture conditions used in this work.

It might be supposed that the cytoplasm is simply divided by cell division and that a cell is formed in an internal or an external position solely because its component molecules were internal or external in the 2-cell embryo. The redistribution of oil drops at the 2- to 4-cell division does not support this simple view. An alternative possibility is that the cytoplasm and cells move to internal or external positions in response to continuous interactions with neighbouring cells, and these cell interactions would then be the mechanism by which the cells in the embryo are exposed to different environments (Graham & Kelly, 1977).
CONCLUSIONS

The cell lineage to the blastocyst has the following features:

1. Each parental cell at the 2-cell stage produces two daughter cells which cohere.
2. These two daughters divide and each forms two descendants.
3. One of these descendants usually lies deep in the structure of the embryo and one of these descendants is usually towards the periphery of the embryo.
4. These patterns are produced by zona-free as well as zona-contained embryos and do not depend on containment by the zona.
5. Between the 8-cell stage and the blastocyst, the deep cells contribute more frequently to the inner cell mass than do the peripheral cells.

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REFERENCES


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