The establishment of the embryonic–abembryonic axis in the mouse embryo

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Summary

The influence of cell division order on the establishment of the embryonic–abembryonic axis (EA axis) of the mouse embryo was investigated. Aggregate embryos were constructed in which a labelled cell (or pair of cells) was combined with a group of unlabelled cells all of which were up to one cell cycle earlier or later in their progress through development to the blastocyst stage. The aggregates were cultured first to the nascent blastocyst stage and then to the expanded blastocyst stage. The positions of the progeny of the labelled cells in relation to the nascent blastocoel and to the orientation of the embryonic–abembryonic axis were recorded. It was concluded that cell division order does influence the establishment of the EA axis, early dividing cells tending to be associated with the nascent blastocoel and the site of the nascent blastocoel tending to mark the site of the abembryonic pole. However, the influence of division order was diminished by a requirement for intercellular cooperation during blastocoel formation and by a counteracting influence of division order arising from its effects on the allocation of cells to the inner cell mass.

Key words: axis, blastocoel, ICM, trophectoderm, cell cycle.

Introduction

The embryonic–abembryonic axis (EA axis) of the mouse embryo is present at the fully expanded blastocyst stage in which an outer layer of trophectodermal epithelium surrounds an eccentrically placed inner cell mass (ICM). Blastocyst formation is initiated with the appearance of a blastocoel, an event that occurs 3 days into development at about the 32-cell stage. The EA axis is important since the cells within the two constituent tissues of the blastocyst diversify according to their position with respect to it. Thus, towards the abembryonic pole the mural trophectoderm forms nonproliferative primary giant cells that initiate attachment to the endometrium, whilst the abembryonic or blastocoelic face of the ICM yields primary endoderm (or hypoblast); at the abembryonic pole, the polar trophectoderm proliferates to yield the ectoplacental cone, the secondary giant cells and the extraembryonic ectoderm, whilst the adjacent ICM forms primary ectoderm (or epiblast; Gardner, 1983). Shortly thereafter the dorsoventral and anteroposterior embryonic axes develop within the epiblast (Smith, 1980, 1985).

The establishment of the definitive EA axis is observed during the segregation within the centre of the embryo of the ICM cells from the blastocoelic fluid. Throughout the process of segregation, the two are separated by a continuous layer of trophectodermal processes derived from, and continuous with, an equatorial band of trophectodermal cells (Ducibella, Albertini, Anderson & Biggers, 1975; Fleming, Warren, Chisholm & Johnson, 1984). It is thus reasonable to suppose (Surani & Barton, 1984) that the orientation of the EA axis might depend upon the location within the late morula of the first blastocoelic fluid to be produced, this event marking the future abembryonic pole. As the blastocyst expands, the inner cells of the morula would become restricted to the opposite end of the embryo and mark the embryonic pole. If this analysis is correct, an understanding of how the EA axis forms requires an understanding of how the earliest formation of a blastocoelic cavity is regulated.

Two models for the initiation of cavity formation have been proposed, and each depends upon the observation that there is considerable temporal heterogeneity among the blastomeres within any single preimplantation embryo, some cells being as much as
a full cell cycle ahead of others (Barlow, Owen & Graham, 1972; Chisholm, Johnson, Fleming, Warren & Pickering, 1985). Surani & Barton (1984) have argued that during the transition from 16 to 32 cells those outer cells that are relatively late dividing (and which are therefore delayed with respect to faster cells) will tend to become 'stretched' by the accumulation of cells internally. Stretched cells, they argue, are less likely to undergo cytokinesis and are therefore more likely to become polynucleate. Since the cells at the abembryonic pole are known to be polyploid, Surani & Barton argue that perhaps the appearance of polynucleate cells is also indicative of premature terminal differentiation into trophoectoderm and therefore identifies the first cells capable of forming blastocoelic fluid (see also Soltynska, Balakier, Witkowska & Karasiewicz, 1985). Thus the position of these later dividing cells would also correspond to the position of the abembryonic pole.

An alternative model proposes that the acquisition of developmental properties is indeed linked to the cell cycle, but that cell cycles (or some feature associated with them) are used as counting devices and that only in a particular cell cycle (the 6th or 32-cell equivalent) is competence to form a blastocoel acquired (Smith & McLaren, 1977; Chisholm et al. 1985). On this model, the first cells to reach the 6th cell cycle, that is the early dividing cells, would achieve competence first and so they would mark the abembryonic pole of the embryo. Whilst both of these models assume that temporal heterogeneity amongst cells makes an important contribution to the generation of spatial pattern, they make directly contradictory predictions about the relationship between division order and the site of blastocoel initiation.

In an attempt to resolve this problem, we have used a newly developed lineage marker (fluorescent latex beads: Fleming & George, 1986; Fleming, 1987) to label a cell (or pair of cells); we have reconstituted morulae by reaggregating the labelled cell(s) with unlabelled cells that were up to a full cell cycle ahead, a full cell cycle behind or the same age, and we have analysed the location of the labelled progeny (i) at the time of blastocoel appearance, and (ii) after blastocyst expansion. In this way, we have been able to investigate whether there is a relationship between division order and the site of nascent blastocoel formation, and whether there is a relationship between the site of nascent blastocoel formation and the orientation of the definitive EA axis that results.

**Materials and methods**

(A) **Embryo collection and culture**

Female MF1 mice (Central Animal Services, University of Cambridge) were superovulated by an injection of 5 i.u. pregnant mares' serum (PMS) followed 45–48 h later by 5 i.u. human chorionic gonadotrophin (hCG). The females were paired with HC–CFLP males (Hacking and Churchill Ltd) and checked for evidence of copulation the following morning. Embryos were collected at the 2- to 4-cell stage by flushing oviducts 52 h post hCG with prewarmed (37°C) medium 2 containing 4 mg ml⁻¹ bovine serum albumin (M2+BSA; Fulton & Whittingham, 1978). The recovered embryos were cultured in pre-equilibrated medium 16 containing 4 mg ml⁻¹ bovine serum albumin (M16+BSA; Whittingham, 1971) in Falcon culture dishes, under oil at 37°C and 5% CO₂ in air. Zona-free embryos and single blastomeres were cultured in Sterilin dishes.

(B) **Embryo manipulations**

Removal of the zona pellucida was achieved by exposure of embryos to prewarmed acid Tyrode's solution for about 20 s (Nicolson, Yanagimachi & Yanagimachi, 1975). Embryos were disaggregated by first decompacting them in prewarmed (37°C) calcium-free M2 containing 6 mg ml⁻¹ BSA for 10 to 15 min, and then pipetting them gently in and out of a flame-polished micropipette.

(C) **Latex labelling procedure**

A stock solution of carboxylated fluorescent latex micro-particles (latex; yellow-green or red Fluoresbrite, Polysciences, 0-06 μm particle diameter; 2.5% solids) was diluted 1:20 in M2+BSA. Cells were incubated in the label for 20 min, then washed in M2+BSA and cultured for up to 2 h. A preliminary series of experiments was performed to assess the effect of latex on cavitation. Aggregate embryos were constructed containing a single larger, polar 1/16 blastomere labelled with yellow–green latex and seven similarly aged 2/16 couplets either labelled with red latex or unlabelled. The aggregates were cultured until cavitation occurred and then compared for timing of cavitation and expansion. It was found that the development of the embryos containing red latex label was not significantly different from those that did not. Therefore it was concluded that labelling had not affected cavitation, a result also reported by Fleming & George (1986).

(D) **Microscopy**

Embryos were transferred to drops of M2+BSA under oil in wells of a tissue-typing slide (Baird and Tatlock), overlain with a coverslip and viewed under a Leitz Ortholux II microscope. Filter sets L2 for yellow–green latex and N2 for red latex were used. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orhomat photographic system.

(E) **Blastomere aggregation**

Blastomeres were exposed to Ca²⁺-free M2+BSA for 5 to 10 min then aggregated in M2+BSA by nudging them together with a micropipette. If 1/8 blastomeres were incorporated in the aggregate, a brief exposure to phytohaemagglutinin (PHA; Gibco Ltd, stock solution diluted 1 in 20 with M2+BSA) was used to increase adhesiveness.
(F) Assay for cavitation
To examine isolated 2/16 pairs or their derivatives for cavitation, the cells were harvested onto glass coverslips mounted in chambers as described in Maro, Johnson, Pickering & Flach (1984). The chambers were prepared by a 5 to 10 min preincubation in 0.1 mg ml\(^{-1}\) concanavalin A (Miles) in phosphate-buffered saline (PBS; Oxooid), washed with M2+4mg ml\(^{-1}\) polyvinylpyrrolidone (M2+PVP) and then filled with M2+PVP. After a 10 min centrifugation at 500g in a Jeol GT2000 SX centrifuge, pairs were fixed in the chambers with 2% formaldehyde in PBS for 30 min, rinsed in PBS and washed for 10 min in PBS containing 50 mM-NH\(_4\)Cl. They were then stained for 10 min with Hoechst 33258 nuclear dye (Sigma; 5–19 µg ml\(^{-1}\) in PBS). After washing, coverslips were removed from the chambers and mounted in Citifluor (City University, London). Specimens were viewed on a Leitz Ortholux II microscope, using filter set A to visualize the Hoechst stain.

(G) Statistical analysis
Data were compared for significance by the \(\chi^2\) test.

Results

(A) The relationship between division order and the initiation of blastocoel formation
Blastomeres isolated from late 8-cell embryos were cultured individually and examined at hourly intervals. Any that had divided were harvested and designated 0 h 2/16 pairs. Some pairs were composed of cells of different sizes and it is known that the larger cell is almost always a polar outside cell (Ziomek & Johnson, 1981). A few such larger cells were isolated by disaggregation. This procedure was continued for up to 7 h, yielding groups of cells that varied by up to 7 h in their time of entry into the 5th cell cycle. A single large cell, or a 2/16 couplet, of known age postdivision was then labelled with latex and aggregated with seven unlabelled 2/16 couplets that were 4 h in advance (early dividing), 4 h behind (late dividing), or the same age as the labelled cells. The aggregates were then cultured until the first sign of blastocoel formation was observed (Chisholm et al., 1985), at which point they were examined as live whole mounts. In some experiments, a time difference of a blastocoel was observed (Chisholm et al., 1984). The progeny of the labelled single 1/16 cells were exclusively trophectodermal in most cases. The time at which a nascent blastocoel formed was recorded and the time interval elapsed since division of the early and the late constituent cells to the 16-cell stage was calculated. The values for this cavitation interval are shown in Table 1, together with comparable figures for zona-free but nonmanipulated embryos. There is an inherent error that could be as great as 3 h in the values for aggregate embryos, since 1/8 blastomeres were examined for division to 2/16 every 1 h and morulae were examined for evidence of cavities every 1 to 2 h. However, the error is not likely to be as large as this in most cases and the range of

<table>
<thead>
<tr>
<th>Type of embryo</th>
<th>Cavitation interval (mean + range in h) with respect to Early 1/16 cell</th>
<th>Late 1/16 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Aggregate embryo, all cells synchronous</td>
<td>22-1 (19-29)</td>
<td></td>
</tr>
<tr>
<td>(2) Aggregate embryo, 1/16 cell 4 h early</td>
<td>24-5 (19-26)</td>
<td>20-5 (15-22)</td>
</tr>
<tr>
<td>(3) Aggregate embryo, 1/16 cell 4 h late</td>
<td>22-5 (19-29)</td>
<td>18-5 (15-25)</td>
</tr>
<tr>
<td>(4) Aggregate embryo, 1/8 0h + (7×2/16) 0h</td>
<td>22-4 (19-29)</td>
<td></td>
</tr>
<tr>
<td>(5) Aggregate embryo, 2/16 0h + (7×1/8) 0h</td>
<td>31-6 (22-35)†</td>
<td></td>
</tr>
<tr>
<td>(6) Intact zona-free embryo</td>
<td>22-6 (11-33)</td>
<td>17-8* (18-23)</td>
</tr>
</tbody>
</table>

* Early- and late-dividing cells assessed by hourly observation of 8-cell morulae for decompaction as the first cells entered division (early cells) and for final recompaction as all cells completed division (late cells) – see Fleming, 1987.
† Only one embryo cavitated after 22 h; for remainder, range is 29–35. See also Fig. 3.

Line 5 differs from other lines \(P<0.05\); otherwise no significant differences.
errors should be similar among different groups. Moreover, the values obtained are remarkably close to those found for zona-free nonmanipulated embryos scored hourly for cavitation (Table 1, line 6 and Fig. 3). An average 5th cell cycle time of 12 h (Lehtonen, 1980; MacQueen & Johnson, 1983), taken in conjunction with a mean interval of 22 h between division to 16 cells and cavitation, means that cavitation must be occurring late in the sixth cell cycle. A similar conclusion, using quite different experimental approaches on intact whole embryos, has been reached by other workers (Smith & McLaren, 1977; Braude, 1979; Chisholm et al. 1985). When cavitation times in synchronous aggregates (line 1) are compared with those in aggregates containing an early-dividing cell (line 2), it appears that the presence of the early cell accelerates cavitation slightly with respect to the late dividing cells, but that cavitation with respect to the early cell itself is slightly delayed. In contrast, the presence of a late-dividing cell (line 3) tends to have little influence on the cavitation interval with respect to the early cells, but is associated with a shorter cavitation interval with respect to the late cell itself. Although none of these comparisons achieve significance, the observations are at least consistent with the hypothesis that the ability to cavitate is acquired as a cell reaches a particular point in a specific developmental cell cycle. The possibility that cell interaction might modify the outward expression of this ability (Prather & First, 1986) is supported by a comparison of lines 4 and 5 (Table 1) in which the temporal discrepancy between aggregated cells was increased to a full cell cycle. A single newly formed 1/8 cell did not materially retard cavitation in aggregate embryos, but cavitation in aggregates between one 2/16 pair and seven 1/8 cells was considerably delayed with respect to the age of the 2/16 pair with only a single exception.

The position of the labelled cells with respect to the nascent cavity was recorded as being adjacent (Fig. 1A,B) or remote (Fig. 1C,D). The results are recorded in Table 2. Aggregate embryos in which the labelled cell(s) was early dividing showed in two out of three comparisons a significantly higher association between the nascent blastocoel and the labelled progeny than did aggregate embryos in which the labelled cell(s) was late dividing.

(B) The relationship between the position of the nascent blastocoel and the position of the abembryonic pole

All embryos scored in the previous experiment were cultured for a further 24 h until blastocoelic expansion was completed. They were then examined as whole mounts for the position of the labelled trophodermal cells, and were assigned to one of four groups (Fig. 2) according to whether labelled trophodermal cells were present: (1) exclusively in the abembryonic half of the blastocyst (Fig. 2A,B); (2) extending from the abembryonic half over the equator into the embryonic half (Fig. 2C,D); (3) adjacent to, but not overlapping, the ICM, and (4) partly or

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Fig. 1. Superimposition of phase and fluorescence photomicrographs to show the four labelling patterns observed when a 1/16 cell or pair of cells was aggregated with seven unlabelled 2/16 pairs and cultured to the nascent blastocyst stage. Labelled progeny form (A) part of the wall of the blastocoel, (B) part of the floor of the blastocoel, or (C,D) are remote from the blastocoel. ×400.
Embryonic–abembryonic axis

Table 2. Relationship between the position of labelled cells and the nascent blastocoel in embryos developing a single blastocoelic cavity

<table>
<thead>
<tr>
<th>No. of embryos (%)</th>
<th>Type of labelled cell(s)</th>
<th>No. of embryos</th>
<th>Adjacent</th>
<th>Remote</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Synchronous 1/16</td>
<td>28</td>
<td>12 (61)</td>
<td>9 (39)</td>
<td></td>
</tr>
<tr>
<td>(2) Early 1/16</td>
<td>49</td>
<td>36 (74)</td>
<td>13 (26)</td>
<td></td>
</tr>
<tr>
<td>(3) Late 1/16</td>
<td>24</td>
<td>11 (46)</td>
<td>13 (54)</td>
<td></td>
</tr>
<tr>
<td>(4) Early 2/16</td>
<td>20</td>
<td>16 (80)</td>
<td>4 (20)</td>
<td></td>
</tr>
<tr>
<td>(5) Late 2/16</td>
<td>20</td>
<td>11 (55)</td>
<td>9 (45)</td>
<td></td>
</tr>
<tr>
<td>(6) 2/16 (with 7×1/8 pairs)</td>
<td>31</td>
<td>22 (71)</td>
<td>9 (29)</td>
<td></td>
</tr>
<tr>
<td>(7) 1/8 (with 7×2/16 pairs)</td>
<td>23</td>
<td>9 (39)</td>
<td>14 (61)</td>
<td></td>
</tr>
</tbody>
</table>

Lines 2 and 3: significantly different P<0.05.
Lines 4 and 5: not significantly different P>0.05.
Lines 6 and 7: significantly different P<0.05.

(C) Is there a relationship between the cell cycle and the initiation of cavitation?

An assumption underlying the second of the hypotheses that these experiments are attempting to test is that cells become competent to form a blastocoel at a particular point in a specific developmental cell cycle. It is very difficult to test this proposition directly on whole embryos because of the inherent and uncontrollable heterogeneity of cell cycling within an embryo. However, newly formed 2/16 pairs of cells can be used as a model system to approach the problem. By observing such pairs serially at regular time intervals after their formation, the time course of their progression to 4/32 quartets and to mini-blastocysts can be assessed, and the changing pattern of their morphology monitored (Fig. 3). The results of such an analysis reveal that cavitation amongst the population of 2/16 pairs and their progeny occurs not suddenly but progressively with a mean time of about 21±2 h, that is when most cells are at the late 32-cell stage, and with a range of 10 to 28 h. Indeed, the isolated cell clusters behave similarly to intact or aggregated embryos in the timing of cavitation (see Table 1). The precise cellular composition of the 4/32 quartets, e.g. four outer cells, or three outer cells plus one inner cell, or two outer cells plus two inner cells, did not appear to influence the timing of fluid accumulation, a finding that appears to be in conflict with the predictions of the first hypothesis under test, since it might have been predicted that outer cells that were forced to surround inner cells would cavitate earlier.

Discussion

Our analysis of how the EA axis is determined resolved into two questions. What is the relationship

Table 3. Position of labelled cells in expanded blastocysts in relation to their position in nascent blastocysts and their division order

<table>
<thead>
<tr>
<th>No. of embryos in which labelled cells in nascent blastocyst were</th>
<th>Adjacent to blastocoe</th>
<th>Remote from blastocoe</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position of cells in expanded blastocyst</td>
<td>Late (1)</td>
<td>Same (2)</td>
<td>Early (3)</td>
</tr>
<tr>
<td>(1) At abembryonic pole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2) Extending from abembryonic pole across equator</td>
<td>5</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>(3) Adjacent to but not overlapping ICM</td>
<td>11</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>(4) Partly or wholly overlapping ICM</td>
<td>3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>(5) Wholly overlapping ICM</td>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

Columns 4 and 8 are significantly different: P<0.001.
Columns 9 and 10 are not significantly different: P>0.05.
between cell division order and the site of nascent blastocoel formation? What is the relationship between the position of the nascent blastocoel and the position of the abembryonic pole?

There does appear to be a relationship between the position of the earliest dividing cells and the position of the nascent blastocoel. Moreover, the time interval between division and cavitation appears to be more influenced by the presence of early-dividing than late-dividing cells. However, whilst a general relationship does appear to exist, it is by no means absolute, many individual exceptions being evident. It is possible

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**Fig. 2.** Expanded blastocysts derived from nascent blastocysts of the type illustrated in Fig. 1. In each case the position of the centre of the ICM is indicated with an arrowhead. The labelled cells may (A,B) be confined within the abembryonic half of the blastocyst, (C,D) extend across the equator to the embryonic half, or (E,F) lie in whole or in part over the ICM. ×630.
that some of these exceptions arise because cells synchronized to the 8- to 16-cell transition nonetheless became, during the ensuing period to cavitation, asynchronous again by more than the experimentally introduced 4 h difference. Marked ‘early’ cells might thereby become ‘late’ and vice versa. The only direct measurements of heterogeneity in the duration of the 5th cell cycle are those of Lehtonen (1980), who recorded a range of 11.2 to 13.9 h by time-lapse videomicrography, and MacQueen & Johnson (1983), who reported that most newly formed 16-cells isolated in the same way as reported here varied between 11 and 15 h in their cell cycles. Although the variation was less for presumptive trophectoderm cells, 80% dividing within a 2 h period, nonetheless some erosion of the 4 h experimentally introduced interval does seem to provide a plausible explanation for the lack of an absolute relationship between the positions of early-dividing cells and nascent blastocoel formation. However, this is unlikely to be the only explanation, since even when a full cycle of asynchrony was introduced by aggregating newly formed 1/8 and 2/16 blastomeres no absolute relationship between division order and site of nascent blastocoel formation was observed. Moreover, when the timing of cavitation in cell clusters derived from 2/16 pairs was examined, it too showed considerable variability (Fig. 3), albeit largely within the period of the 6th (32-cell stage) cell cycle.

A further explanation for the lack of an absolute correlation between the position (and influence) of early-dividing cells and the position of the nascent blastocoel must therefore be sought, and may lie in the nature of the process of blastocoel formation. The acquisition of a pool of fluid within the embryo requires that there be both vectorial transport of fluid and a sufficient permeability seal to retain it. The precise mechanism by which the early blastocoelic fluid forms is uncertain (Biggers, Borland & Powers, 1977; Wiley & Eglitis, 1981; Wiley, 1984; Fleming et al., 1984), but there is direct evidence that some types of vectorial transport mechanisms are developing from as early as the 8-cell stage (Fleming & Pickering, 1985; Fleming, 1987; Fleming & Goodall, 1986). The time at which a complete permeability seal forms in the embryo is controversial. Tight junctional assembly occurs as early as the 8-cell stage (Ducibella & Anderson, 1975), but does not become zonular until the late morula (Magnuson, Demsey & Stackpole, 1977; Magnuson, Jacobson & Stackpole, 1978) by which time 90% of embryos are reported to exclude lanthanum. In contrast, Smith & McLaren (1977) suggested that antibodies were not excluded from passing paracellularly into the embryo until as late as the midblastocyst stage.

However, the establishment of a nascent blastocoel need not require either that all cells can secrete fluid vectorially (in principle a single competent cell might suffice) or that a complete and permanent permeability seal should exist (a local seal among two or three cells could suffice). Moreover, a blastocoel could form when the leakage of fluid out through an
imperfect or episodic seal was more than offset by the vectorial inward transport of fluid. Since the emergence of a blastocoel is influenced by a balance between variables, one at least of which requires interaction between adjacent (though not necessarily synchronous) cells, it is perhaps not surprising that even in aggregates of highly synchronized cells (e.g. Fig. 3 or Table 1, line 1), a wide range of initial cavitation times is observed. Likewise, in aggregates of highly asynchronous cells (e.g. Table 2, lines 6 and 7), the lack of an absolute relationship between division order and the site of nascent blastocoel formation is not unexpected.

The results therefore suggest that there is indeed an influence of division order on the site of nascent blastocoel formation, early-dividing cells being more often involved. However, the nature of the process whereby the blastocoel is formed, requiring as it does cooperation amongst cells, means that this influence is not the sole determinant of blastocoel position. It is possible that a larger group of early-dividing cells within the embryo, such as may occur in situ in many undisturbed embryos, may have a more decisive effect.

The presence of labelled cells in nascent blastocysts enabled us to examine the relationship between the position of the nascent blastocoel and that of the abembryonic pole. There was a significantly different pattern of distribution along the EA axis of the progeny of cells located near the nascent blastocoel from that of cells remote from the nascent blastocoel, but again the relationship was not absolute. It is known that, in the expanding blastocyst, trophectoderm cells pass out from the polar region to the abembryonic region (Copp, 1979; Cruz & Pedersen, 1985; Dyce, George, Goodall & Fleming, 1987). Such a movement could explain why in some embryos (13/42) labelled cells remote from the nascent blastocoel nonetheless distributed progeny abembryonically after expansion. However, such a mechanism cannot explain how labelled cells initially adjacent to the nascent blastocoel became located adjacent to the ICM. The implication is that the ICM cells became anchored to those trophectoderm cells adjacent to the nascent blastocoel, and that the blastocoel spread around them. Why should this happen in 28 of the 87 such embryos analysed (Table 3, column 4)? It is possible that the explanation may lie in a quite different influence that division order has on cell behaviour in the early mouse embryo. There is clear evidence that early-dividing cells contribute proportionately more progeny to the ICM than do late-dividing cells (Kelly, Mulnard & Graham, 1978; Surani & Barton, 1984; Garbutt, Johnson & George, 1987). The major part of this preferential contribution occurs during division from the 8- to 16-cell stage, but a significant second contribution occurs in about 25% of cases at the 16- to 32-cell transition (Fleming, 1987; Garbutt et al. 1987). It is by the differentiative division of outer polar 1/8 or 1/16 cells that ICM cells (or their precursors) are generated, the apicolateral portion of each dividing cell remaining outer and polar (presumptive trophoblastic), while the basolateral portion remains inner and apolar (presumptive ICM). A residuum of each cleavage furrow, the midbody, can and does remain as a physical connection between daughter cells for up to two cell cycles (Balakier & Pedersen, 1982; Goodall & Johnson, 1984; Pedersen & Balakier, 1986). Therefore early-dividing 1/16 polar cells that did contribute one daughter cell to the ICM might remain anchored by the residual midbody to the nascent ICM during at least the early stages of blastocoel expansion. In those embryos in which a significant cellular contribution to the ICM occurs at the 16- to 32-cell transition, there would be several midbodies to engage in such an anchoring, and this might therefore account for our observations.

The experiments reported here were aimed at elucidating the relationship between division order and the establishment of the EA axis, based on different predictions arising from the two hypotheses outlined in the Introduction. Neither hypothesis is sustained unequivocally. The notion that late-dividing cells mark the abembryonic pole is not supported at all, indeed the results argue against such an idea. The results offer more support for the hypothesis that early division of cells influences the site of the abembryonic pole, but indicate that such an influence is not exclusive, partly because of the nature of the process of blastocoel formation itself, and partly because a confounding effect may be at work deriving from the acknowledged influence that early division of cells has on the allocation of cells to the ICM.

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