

Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development

Karolina Piotrowska*, Florence Wianny, Roger A. Pedersen† and Magdalena Zernicka-Goetz§

Wellcome/CRC Institute and Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK

*Permanent address: Department of Experimental Embryology, Polish Academy of Sciences, Jastrzebiec, Poland

†Permanent address: Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, CA, USA

§Author for correspondence (e-mail: mzg@mole.bio.cam.ac.uk)

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SUMMARY

Two independent studies have recently suggested similar models in which the embryonic and abembryonic parts of the mouse blastocyst become separated already by the first cleavage division. However, no lineage tracing studies carried out so far on early embryos provide the support for such a hypothesis. Thus, to re-examine the fate of blastomeres of the two-cell mouse embryo, we have undertaken lineage tracing studies using a non-perturbing method. We show that two-cell stage blastomeres have a strong tendency to develop into cells that comprise either the embryonic or the abembryonic parts of the blastocyst. Moreover, the two-cell stage blastomere that is first to divide will preferentially contribute its progeny to the embryonic part. Nevertheless, we find that the blastocyst embryonic-abembryonic axis is not perfectly orthogonal to the first cleavage plane, but often shows some angular

displacement from it. Consequently, there is a boundary zone adjacent to the interior margin of the blastocoel that is populated by cells derived from both earlier and later dividing blastomeres. The majority of cells that inhabit this boundary region are, however, derived from the later dividing two-cell stage blastomere that contributes predominantly to the abembryonic part of the blastocyst. Thus, at the two-cell stage it is already possible to predict which cell will contribute a greater proportion of its progeny to the abembryonic part of the blastocyst (region including the blastocyst cavity) and which to the embryonic part (region containing the inner cell mass) that will give rise to the embryo proper.

Key words: Mouse embryo, Polarity, Blastocyst, Axis, Embryonic, Abembryonic, Body plan

INTRODUCTION

A growing body of evidence indicates that although the early mouse embryo has remarkable flexibility in responding to developmental perturbations, its patterning is initiated at the earliest developmental stages. Evidence for early indicators of polarity came recently from lineage tracing experiments. First, it was shown that the animal-vegetal axis of the egg could be traced by the location of the polar body at the animal pole until the blastocyst stage (Gardner, 1997; Ciemerych et al., 2000). Second, it was found that visceral endoderm precursors in the inner cell mass (ICM) located near the polar body at the blastocyst stage gave rise to progeny that occupied predominantly distal locations of the post-implantation egg cylinder (Weber et al., 1999). By contrast, the progeny of the visceral endoderm precursors in the ICM initially opposite the polar body tended to become localised proximally. Asymmetric movements of visceral endoderm cells were shown to convey this distal-proximal axis of the egg cylinder into the anterior-posterior axis of the post-implantation embryo (Thomas et al., 1998; Weber et al., 1999; Beddington and Robertson, 1999). Taken together, these studies have led to the view that the anterior-posterior axis is already anticipated by

the blastocyst stage and that it is related to the animal-vegetal axis of the egg itself.

The hypothesis that the patterning of the later mouse embryo can be related to the spatial patterning of the egg itself has been further substantiated by two recent studies. In one, Piotrowska and Zernicka-Goetz (Piotrowska and Zernicka-Goetz, 2001) marked the transitory fertilisation cone at the sperm entry position (SEP) and followed the development of such zygotes to the blastocyst stage. They found that not only was the orientation of the first cleavage related to the position of the polar body, as was previously well known, but also that it related to the SEP. Moreover, at the two-cell stage, the blastomere that inherited the SEP subsequently undertook its next cleavage division earlier than its sister. Finally, this study demonstrated that at the blastocyst stage, the SEP came to lie on or close to the border between the embryonic and abembryonic parts, thus showing that the embryonic-abembryonic axis of the blastocyst is often orthogonal to the initial cleavage plane of the zygote. In the second study, Gardner (Gardner, 2001) marked regions of the zona pellucida at the two-cell stage and then observed embryos again as blastocysts. This study has also led to the conclusion that the embryonic-abembryonic axis of the blastocyst is orthogonal to the initial cleavage.

This early 'pre-patterning' of the mouse embryo might first appear at odds with a body of evidence that demonstrates the totipotency of early embryonic cells. It has been shown experimentally that early cleavage blastomeres can contribute to both ICM and trophoctoderm lineages either in isolation or in reconstructed chimaeric embryos, demonstrating their developmentally unrestricted potential (Tarkowski, 1959; Kelly et al., 1978; Rossant, 1976). Similarly, lineage tracing studies at early stages of development have indicated that two-cell stage blastomeres contribute descendants to both the ICM and the surrounding trophoctoderm cells at the blastocyst stage, although these studies did not distinguish between contributions of early blastomeres to any specific regions of the blastocyst (Balakier and Pedersen, 1982). Together with other evidence, this has led to the view that blastomeres do not show any predisposition to develop into embryonic or abembryonic parts of the embryo until the time at which a group of ICM progenitor cells become incorporated into the inner part of the pre-implantation embryo (Tarkowski and Wroblewska, 1967; Johnson and Ziomek, 1981). These inner cells will become the ICM, whereas those on the outside will become trophoctoderm. However, because the earlier studies did not analyse contributions of early blastomeres to distinct embryonic and abembryonic regions of the blastocyst, they could not exclude the possibility that orientation of first cleavage has implications for blastocyst organisation in the intact embryo.

To reconcile the observations that early blastomeres contribute to both ICM and trophoctoderm lineages with new observations on the polarity of the early stage embryo, it has been hypothesised that one two-cell embryo blastomere gives rise mainly to polar trophoctoderm and to cells of the ICM that will form the future body, the epiblast (Piotrowska and Zernicka-Goetz, 2001; Gardner, 2001). Accordingly, it is predicted that the other two-cell blastomere develops into mural trophoctoderm and cells of the ICM that give rise predominantly to primitive endoderm, an extraembryonic tissue. This hypothesis can be tested through lineage tracing studies, provided that these do not interfere with normal developmental processes. We have therefore employed a lineage tracing method that avoids intracellular injection to enable such an analysis and re-examined the fate of the two-cell blastomeres at the blastocyst stage of development. We show that two-cell blastomeres have a strong tendency to develop into either the embryonic or the abembryonic parts of the blastocyst. Moreover, we find that the cell first to divide to the four-cell stage contributes preferentially to the embryonic part. Thus already at the two-cell stage it is possible to predict which cell will supply a greater proportion of its progeny to the embryonic part of the blastocyst and which to the abembryonic part.

MATERIALS AND METHODS

Embryos

Embryos were collected from F₁ (C57BL/6×CBA) females induced to superovulate by intraperitoneal injection of 7.5 IU of pregnant mares serum gonadotrophin (PMSG, Intervet) followed 48 hours later by 7.5 IU of human chorionic gonadotrophin (hCG, Intervet) and then mated with males of the same genotype. Two-cell stage embryos were collected 46-48 hours after hCG injection into FHM+BSA medium as

previously described (Piotrowska and Zernicka-Goetz, 2001). Eggs were collected 16.5 hours after hCG injection into PBS containing 200 IU/ml of hyaluronidase, dispersed and then transferred to FHM+BSA medium. Embryos were observed under an inverted (Leica) microscope using DIC optics and micromanipulated with Leica micromanipulators using a De Fonbrune suction-force pump.

Labelling of cells

DiI, DiD or DiO (Molecular Probes) were dissolved in virgin olive oil at 60°C allowed to cool and then used immediately. Labelling was accomplished by pressing the tip of the injection needle against the blastomere membrane, avoiding its penetration, then expelling a microdroplet against the membrane where the dye was absorbed. Labelled embryos were cultured *in vitro* in KSOM medium supplemented with amino acids (KSOM + AA) (Speciality Media, Lavallete, NJ) and with 4 mg/ml of BSA (Sigma) in 5% CO₂ and at 37°C to the blastocyst stage. Embryos were removed periodically from the culture incubator for observations which were recorded at the two-cell stage, three-cell stage, as specified in the text, and finally observed at the blastocyst stage when the ratio of the volume of the ICM to cavity was approximately 1:2 by eye. In a series of control experiments, the newly fertilised zygotes were marked at the fertilisation cone, which marks the sperm entry position, as previously described (Piotrowska and Zernicka-Goetz, 2001). Briefly, fluorescent (FITC labelled) beads (3 µm diameter, Polysciences) were placed in FHM medium containing 300 µg/ml phytohaemagglutinin for 30 minutes and then transferred to the chamber containing eggs in FHM + BSA. Individual beads were mounted on the tip of a bevelled, sharpened micropipette, which was then introduced through the zona pellucida where beads were placed in contact with the membrane of the fertilisation cone. Once the bead had adhered, the micropipette was withdrawn. Labelled eggs were transferred into KSOM medium and cultured *in vitro*, as described above, to the blastocyst stage.

Analysis

Confocal analysis of blastocysts was performed on either live embryos or embryos fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). Blastocysts were observed by confocal microscopy taking optical sections every 7.5 µm. By examining all sections in each series, it was possible to determine the distribution of labelled cells into the embryonic and abembryonic parts of the blastocyst. The boundary zone between these two parts was defined as a cell layer, one cell deep and parallel to the roof of the blastocoel cavity, as suggested in previously described models (Piotrowska and Zernicka-Goetz, 2001; Gardner, 2001). In the first series of experiments, blastocysts were scored depending on the number of cells in a clone that crossed beyond this boundary zone, according to the criteria defined in the legend to Fig. 1. We also defined the border between the progeny of the two clones as indicated in the Results section and scored the number of cells that crossed this border. In a second series of experiments, we also analysed the distribution of the progeny of the two-cell blastomeres specifically in the boundary zone. Owing to the uneven labelling of cell membranes with the dyes used here, it was difficult to obtain precise counts of cell numbers in the densely populated boundary zone of intact embryos, therefore these counts should be regarded as estimates (±1 cell). In this series of experiments, we first briefly exposed the embryos to acid Tyrode's solution to remove the zona pellucida as previously described (Zernicka-Goetz et al., 1995) and then dissociated each of the embryos by treatment with 0.5% trypsin (in Hank's buffered saline with 0.04% EDTA) for 5 minutes at 37°C dispersing them using thorough pipetting. This showed that each cell in the blastocyst was either completely or substantially labelled. These cell counts thus provide a means of estimating the contribution of early and late-dividing blastomeres to the embryonic and abembryonic parts, defined as the respective portions of the embryonic and abembryonic regions exclusive of the boundary zone (see Table 1 footnotes).

RESULTS

A method of lineage tracing that avoids intracellular injection

We wished to determine whether the two-cell blastomeres had distinguishable fates, as previously hypothesised, one contributing predominantly to the embryonic part of the blastocyst and the other to the abembryonic, a question best addressed directly by lineage tracing. However, before such a method can be used, it is important to ensure that it does not itself compromise the early cleavage divisions in ways that can influence the subsequent fate of cells. We observed that when a two-cell blastomere was microinjected intracellularly with a lineage marker such as synthetic mRNA for MmGFP, the subsequent division of that cell was slightly delayed (K. P., F. W., R. A. P. and M. Z.-G., unpublished observations). This contrasts with our experiments using cell membrane markers, where we found that the labelling itself did not change the division order, thus allowing us to recognise that inheritance of the site of sperm entry predicted the order of the second cleavage (Piotrowska and Zernicka-Goetz, 2001). We therefore attempted several alternative membrane lineage tracing methods to establish a means of marking entire cells. We focused on the use of the membrane soluble dyes, DiI, DiO and DiD, to label blastomeres avoiding any intracellular (i.e. invasive) injection procedures. These markers fluoresce at different wavelengths, thus enabling the lineages of both cells to be traced. In labelling first one cell of 100 two-cell embryos with DiI and then the other with DiD, we observed that there was an equal likelihood of the blastomeres labelled with either dye dividing first (52:48). This suggested that the division order was independent of the labelling method. In a second control experiment, we correlated the site of sperm entry (SEP) inherited by one blastomere at the two-cell stage with the most rapidly dividing cell in embryos labelled using the two dyes. We first labelled the SEP by marking the fertilisation cone of the zygote with a fluorescein-labelled phytohaemagglutinin-coated bead. After the first division, we randomly labelled first one cell with DiI and then the other with DiD. We allowed the embryos to develop to the three-cell stage and then examined the embryos to score the position of the SEP marker with respect to the second cleavage. Such doubly labelled embryos showed a high frequency (56/69, 81%) of the SEP being inherited by the earlier dividing blastomere. This compares with 75% in our previous study (Piotrowska and Zernicka-Goetz, 2001). In this group as well, the earlier dividing cell had an equal probability (28:28) of being labelled with one or the other dye. Thus, we concluded that this labelling method did not perturb the order of cleavage divisions.

Blastomeres at the two-cell stage show a predisposition to follow either an embryonic or abembryonic fate

In all subsequent lineage tracing experiments, we marked the two-cell blastomeres with different dyes, allowed the embryos to develop to the blastocyst stage, and then scored the positions of the clones with respect to the blastocyst cavity. The previously advanced model proposes that the two clones should be separated at the blastocyst stage by a boundary located within the embryonic region (containing ICM) and lying one cell deep parallel to its blastocoelic surface. But the

number of cells originating from one or other clone within this boundary zone might vary depending upon the angle of the first cleavage in relation to the blastocyst embryonic-abembryonic axis (Piotrowska and Zernicka-Goetz, 2001). Thus, we may expect and indeed observed progeny of both two-cell stage blastomeres contributing to this boundary region. To determine the distribution of progeny of the two two-cell-stage blastomeres in the blastocyst, we carried out two series of experiments. In the first series our analysis of the distribution of clones excluded cells lying in this one-cell-deep boundary zone (broken white lines on examples in Fig. 1) and scored the extent to which clones spread beyond it. We refer to regions of the blastocyst lying on either side of this boundary zone as the embryonic or abembryonic parts, according to whether they include ICM cells or the blastocoel, respectively. In a subsequent series of experiments, we specifically focused on the distribution of cells inherited from each of the two-cell blastomeres within the boundary zone itself as well as in embryonic and abembryonic parts of the blastocyst.

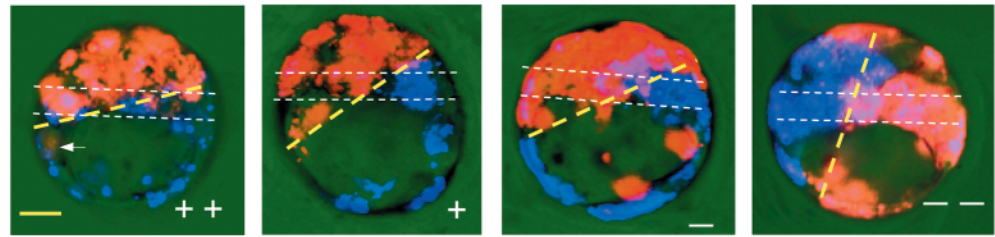
In the first series, blastocysts were classified in four groups, depending upon the number of cells that had crossed to the far side of the boundary zone and were lying in the other part of the blastocyst. If either none or a small number of cells (up to three, approximately 5-10% of the total cell number at this stage) had crossed beyond the hypothetical one cell boundary zone, blastocysts were scored as either ++ (none to two cells crossed) or + (three cells crossed). If more than three cells crossed the boundary zone, blastocysts were scored as either – (four cells crossed) or -- (five or more cells crossed) (Fig. 1; Table 1). We found that in 85% of blastocysts ($n=59/69$; categories ++ and + in Fig. 1A) only three or fewer cells from clones that come to occupy predominantly the embryonic part crossed the boundary zone into the abembryonic part. However, when we scored the fate of clones lying predominantly in the abembryonic part, we found that in 72% of the same blastocysts ($n=50/69$; categories ++ and + in Fig. 1B) three or fewer cells had crossed into the embryonic part. Thus, in the majority of embryos, most progeny of two-cell stage blastomeres came to lie in either the abembryonic or embryonic parts of the blastocyst. However, it was apparent that cells from the abembryonic part showed a greater tendency to cross the boundary zone than did their embryonic cousins (Fig. 1A,B). This could indicate that there is a greater tendency for clonal expansion, cell mixing, or other factors to distort the interface between the two clones in the abembryonic to embryonic direction.

However, it is noteworthy that in the great majority of cases, the clones remain coherent with their actual borders showing a clear spatial relationship to the boundary zone in three of our four categories (Categories ++, + and –; Fig. 1, Table 1). Thus even though the – category by definition had a greater number of cells that crossed the boundary zone than + and ++ categories, blastocysts in this category still had the majority of progeny of two-cell blastomeres occupying either the embryonic or abembryonic parts (Fig. 1, Table 1). Only in one category (–, Fig. 1) did the actual spatial orientation of the border between clones depart significantly from the boundary zone. The transverse pattern that can be seen in this latter group suggests that the orientation of the first cleavage in such embryos was parallel to the future embryonic-abembryonic

Fig. 1. Clones derived from the two-cell stage tend to occupy either embryonic or abembryonic parts of the blastocyst.

Blastomeres of two-cell embryos were labelled with DiI (red) or DiD (blue) and the distribution of the progeny of labelled cells were analysed at the blastocyst stage.

The frequencies of the four categories of blastocyst scored are indicated below. A total of 69 blastocysts were classified according to the extent to which cells derived from one blastomere comprising mainly the embryonic part (A) crossed the embryonic-abembryonic boundary zone (consisting of a region one cell deep and parallel to the roof of the blastocyst cavity, i.e. between



A Extent to which cells from embryonic part cross the boundary zone

68% (47/69)

17% (12/69)

9% (6/69)

6% (4/69)

B Extent to which cells from abembryonic part cross the boundary zone

55% (38/69)

17% (12/69)

15% (10/69)

13% (9/69)

broken white lines) into the abembryonic part and (B) vice versa. Comparison of the data in the row A and row B indicated that cells from the abembryonic part showed a greater tendency ($\chi^2 P < 0.02$, 3 d.f.) to contribute to the embryonic part than their embryonic cousins to contribute to abembryonic part. Blastocysts were scored ++ if up to two cells crossed the boundary zone (arrow). In cases where three cells crossed this boundary, blastocysts were scored +. When four cells, or five or more cells crossed the boundary the blastocyst were scored – and --, respectively. The micrographs represent individual optical sections mid-way through the embryo to show the cavity, which occupies the lower half of each blastocyst (see Materials and Methods). The broken yellow lines show the border between clones derived from each blastomere. Scale bar: 25 μ m.

Table 1. Distribution of clones between the embryonic and abembryonic parts of the blastocyst with respect to the anatomical boundary zone or clonal borders

Group (n, number of embryos in each scoring category)	Mean number of cells crossing boundary zone from embryonic part into abembryonic part	Mean number of cells crossing boundary zone from abembryonic part into embryonic part	Mean angle between clonal border and boundary zone between embryonic and abembryonic parts	Mean number of cells crossing clonal border from predominantly embryonic to abembryonic part	Mean number of cells crossing clonal border from predominantly abembryonic to embryonic part
++ group					
n=15	0	1.8	15.8°	1.0	2.1
n=10	1	0	23.6°	1.3	2.5
n=22	2	2.7	20.3°	2.3	2.2
+ group					
n=12	3	2.7	36.6°	1.6	2.25
- group					
n=6	4	2.5	34.5°	2	2.8
-- group					
n=4	5 or more	4.5 or more	55.25°	2	5.75

The boundaries and borders are defined in the text and in the legend to Fig. 1. The embryos have been placed into four groups following the criteria given in the Results and according to whether cells cross from embryonic part to the abembryonic part (excluding the boundary zone – category A of Fig. 1).

axis rather than relatively orthogonal to it, as occurs in the great majority of embryos.

Analysis of distribution of clones in these embryos revealed that indeed in the majority of embryos there was a variable angle between the plane that reflects the interface between the two clones (a consequence of the first cleavage plane) and a plane passing through the blastocoelic surface of the ICM that forms one limit of the boundary zone. In order to examine the relationship between these two planes, we measured the angle between them (Table 1). To this end we examined a series of eight to ten confocal sections for each blastocyst to evaluate the position of the clonal borders, which we drew as a line at the mid-points between cell boundaries (see examples shown by broken yellow lines in Fig. 1). The angle between these planes varied

between embryos with the mean angle (\pm s.d.) of $26.1^\circ \pm 20.7$ (data not shown). Indeed in the great majority of blastocysts the angle between these planes was less than 30° (Table 1). Thus there is a close relationship between the clonal border defined by the first cleavage plane and the embryonic-abembryonic axis, but the two are not exactly at right angles to each other as had been suggested before (Piotrowska and Zernicka-Goetz, 2001). Taken together, our results show that cells predominantly in the embryonic part of the blastocyst are derived from one two-cell-stage blastomere, and those predominantly in the abembryonic part from the other. The presence of cells derived from each of the two-cell embryo blastomeres in the boundary zone is not just a consequence of cell mixing, because the clonal border is typically well defined with few cells crossing it (see Table 1).

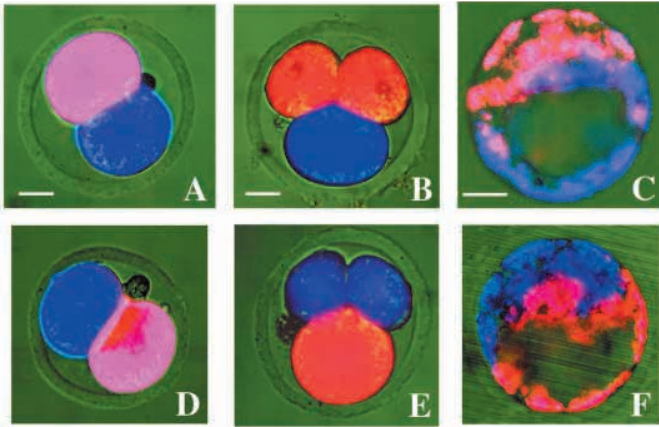


Fig. 2. The early dividing two-cell stage blastomere contributes to the embryonic part of the blastocyst. Blastomeres of two-cell embryos were labelled with DiI or DiD (left hand micrographs; A,D). At the three-cell stage it was recorded whether the earlier dividing cell had been marked with the former (red, upper panel, B) or latter (blue, lower panel, E) dye. Embryos were then allowed to develop to the blastocyst stage (right hand micrograph; C,F). The blastomere that divided first to the four-cell stage gave rise to cells in the embryonic part in 49/56 (88%) embryos in this series of experiments. Scale bar: 25 μm .

The embryonic part of the blastocyst is predominantly inhabited by the progeny of the earlier dividing two-cell stage blastomere

At the two-cell stage there is asynchrony between the blastomeres, one cell dividing ahead of the other. This division advantage has been previously correlated with inheritance of the site of sperm entry (Piotrowska and Zernicka-Goetz, 2001). We wished to determine if the cell dividing first had a tendency to follow a particular fate and contribute predominantly either to the embryonic or abembryonic part of the blastocyst. To this end we carried out a second series of experiments in which cells were labelled at the two-cell stage as before, but now we noted which cell was first to divide to the four-cell stage, then allowed the embryos to develop to the blastocyst stage. Living blastocysts were observed by confocal sectioning to determine the distribution of cells with respect to the embryonic-abembryonic and animal-vegetal axes. The blastocysts were then dissociated into individual cells to count the precise contribution of progeny of early and later dividing two-cell blastomeres (see examples of embryos in Figs 3 and 4). We analysed the embryos in two groups depending upon their final size (early blastocysts of up to 40 cells, Table 2A; expanding blastocysts from 40-69 cells, Table 2B), but because the conclusions that we draw are broadly similar for these two groups we will discuss them together.

We first note that the earlier dividing two-cell blastomere contributes overall a slightly greater number of cells (*t*-test, $P < 0.001$) to the blastocyst than its sister, on average 19 versus 16 cells in the group of early blastocysts, and 26 versus 23 cells in the expanding blastocysts (Table 2A,B). In most embryos of this series (49/56), progeny of the earlier dividing two-cell blastomere provide the dominant contribution to the embryonic part (82% and 87% in early and expanding blastocysts respectively Fig. 2; Table 2A,B). Most noticeably, in a substantial proportion of

embryos, early dividing blastomeres made an exclusive (100%, 6/49) or nearly exclusive ($\geq 90\%$, 20/49) contribution to the embryonic part. In the same embryos, progeny of the later dividing two-cell blastomere contribute the dominant portion of cells to the abembryonic part (78% and 83% in early and expanding blastocysts respectively; Fig. 2; Table 2A,B). There were also some exceptional embryos in which the later-dividing blastomere contributed predominantly to the embryonic part (7/56 embryos, Table 2C). Here also, there was a clear tendency for each blastomere to contribute to either the embryonic or abembryonic part of the blastocyst, although in these exceptional cases, the contributions were reversed. We conclude that not only does the first cleavage plane relate to the orientation of the embryonic-abembryonic axis, but also the embryonic-abembryonic axis has polarity that is usually predicted by the subsequent order of the second cleavage division.

More cells in the boundary zone are derived from the later dividing than the earlier dividing two-cell blastomere

Although two-cell stage blastomeres have a strong tendency to contribute cells into either embryonic or abembryonic parts of the blastocyst, they each supply some of their progeny to the boundary zone, a layer of cells adjacent to the blastocyst cavity. To determine the extent to which cells derived from either the earlier or later dividing two-cell blastomere contribute to the boundary zone, we counted the number of cells in this region derived from each blastomere (Table 2). This analysis showed that the later dividing blastomere contributed significantly more cells (*t*-test, $P < 0.001$) to the boundary zone (61% or 62.5% for earlier and expanding blastocysts respectively). Expressed as the fraction of total cells in the boundary zone, this indicates that approximately 7/11 cells of early blastocysts and 9/14 cells of expanding blastocysts are derived from the later dividing two-cell stage blastomere.

We wished next to consider the relationship between the proportions of the two cell types in the boundary region and the angle of tilt displayed by the clonal border with respect to the blastocyst cavity roof. To this end, we assessed the clonal border by comparing optical sections of individual blastocysts as described above. Examples of two such series of sections are shown in Figs 3 and 4 for embryos in which the clonal border showed no tilt or a tilt of 30° respectively. We found that even when the angle between these planes is relatively small ($< 30^\circ$) an average of 36.4% ($n = 31$, considering both early and expanding blastocysts) of the cells in the boundary zone are derived from the earlier dividing blastomere (for example see Fig. 3). This compares with an average of 40.7% ($n = 18$) when the tilt is between 30° and 90° . Thus, there appeared to be no correlation between the proportion of early dividing descendants in the boundary zone and the extent of tilt, and the boundary zone was invariably a mixture of descendants from both blastomeres (Table 2A,B). However, this should be evaluated against our inability to measure the extent of tilt along the short axis of the ICM (in the vertical or *z* dimension of the confocal series) in the absence of a complete 3D reconstruction of the embryo. This can be seen, for example, in the cells in the boundary zone of the blastocyst displayed in Fig. 3 (see legend to this figure for a more detailed description). Moreover, it does not take into account the known migration of cells from the polar to the mural trophectoderm

Table 2A. Distribution of descendants of early- and late-dividing two-cell blastomeres at early blastocyst stage*

Name	Total cells	Total early	Total embryonic	Early crossing				Late crossing				Tilt			
				% Embryonic early	Early in BZ	BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	BZ to embryonic		Total BZ	%BZ late	%BZ early
1A	39	21	18	89	5	0	18	8	100	8	2	13	62	38	0
2A	33	17	11	100	3	3	16	10	70	9	0	12	75	25	8
3A	31	16	14	86	4	0	15	6	100	7	2	11	64	36	10
4A	31	16	12	100	3	1	15	9	89	7	0	10	70	30	12
5A	32	17	14	79	5	1	15	7	86	6	3	11	55	45	12
6A	36	21	19	100	2	0	15	7	100	8	0	10	80	20	12
7A	40	25	20	90	7	0	15	9	100	4	2	11	36	64	12
8A	32	20	16	100	3	1	12	7	86	6	0	9	67	33	20
9A	38	21	15	93	5	2	17	11	82	7	1	12	58	42	20
10A	34	19	16	81	5	1	15	7	86	6	3	11	55	45	24
11A	31	16	13	77	4	2	15	7	71	7	3	11	64	36	25
12A	40	24	21	86	4	2	16	8	75	7	3	11	64	36	26
13A	36	18	15	73	5	2	18	9	78	7	4	12	58	42	27
14A	40	20	16	81	4	3	20	11	73	9	3	13	69	31	28
15A	32	16	13	77	4	2	16	8	75	7	3	11	64	36	30
16A	40	20	16	81	4	3	20	12	75	8	3	12	67	33	34
17A	32	17	12	67	5	4	15	9	56	6	4	11	55	45	37
18A	38	20	18	89	2	2	18	9	78	9	2	11	82	18	37
19A	34	19	13	85	6	2	15	8	75	7	2	13	54	46	38
20A	37	20	15	87	5	2	17	10	80	7	2	12	58	42	48
21A	33	17	14	50	5	5	16	8	38	6	7	11	55	45	90
22A	40	19	15	53	8	3	21	12	75	5	7	13	38	62	‡
23A	33	18	15	67	4	4	15	7	43	7	5	11	64	36	‡
Mean	35.3	19.0	15.3	82	4.4	2.0	16.3	8.7	78	7.0	2.7	11.4	61	39	26.2
±s.d.	3.4	2.4	2.6	13.6	1.4	1.4	2.1	1.7	16.3	1.2	1.9	1.0	10.8	10.8	18.9

*Distribution of dye-labelled cells in blastocysts was determined by estimating the number of cells occupying the boundary zone (BZ) or crossing to the far side of the boundary zone and lying in the other part of the blastocyst, as described in the Materials and Methods. The number of cells derived from the early-dividing and the late-dividing two-cell blastomere were counted by dissociating each blastocyst after three-dimensional confocal imaging, also as described. The number of cells in embryonic and abembryonic parts, defined as being those portions of the embryonic and abembryonic regions exclusive of the boundary zone, was determined by subtracting the boundary zone and boundary zone-crossing cell number estimates from the total number of labelled cells (early or late) then adding the boundary-crossing cells from the opposite part. Abbreviation: BZ, boundary zone between the embryonic and abembryonic parts, consisting of a layer beginning at the blastocoel surface of the inner cell mass (ICM) and extending into the embryonic region to a depth of approximately one cell (thus including both ICM and polar trophectoderm).

‡Mixed distribution of cells prevented assessment of the tilt in these embryos

Tilt, the approximate angle between the plane of the boundary zone and the plane of the clonal border (see Materials and Methods).

(Copp, 1979; Cruz and Pedersen, 1985). However, together our findings do suggest that the pivotal point of the plane of the clonal border must lie not at the blastocoel roof, but towards the embryonic part of the blastocyst, within the boundary zone.

DISCUSSION

In this work we have used a non-perturbing lineage tracing method that showed that the progeny of the two cells of the two-cell stage mouse embryo tend to contribute to different parts of the blastocyst. Moreover, we have found that the first cell to divide to the four-cell stage contributes more descendants to the embryonic part in comparison to its later-dividing sister, which conversely contributes more descendants to the abembryonic part. Our results therefore support the main features of the recently suggested model (Piotrowska and Zernicka-Goetz, 2001; Gardner, 2001), that the first cleavage divides the egg into its future embryonic and abembryonic parts. Moreover they allow us to extend and refine this model. The previous hypotheses suggested that one of the two-cell blastomeres would have a tendency to give rise to the polar trophectoderm and the portion of the ICM that is likely to become epiblast and the other to the mural trophectoderm and

the portion of the ICM that can contribute cells not only to epiblast, but also to primitive endoderm (Fig. 5). We find that the clone of predominantly abembryonic cells does indeed extend as predicted beyond the roof of the blastocoel, contributing the majority of cells in the cell layer that borders the blastocyst cavity, but supplying relatively few of the cells that comprise the 'upper' part of the embryonic region, defined here as the embryonic part. However, the two-cell blastomere clone contributing mainly to the embryonic region also provides cells to the boundary zone, although these are fewer in number than the contributions by the other blastomere. Very few descendants of this blastomere can also be found in the abembryonic region.

This surprising finding that as early as the two-cell stage mouse embryo blastomeres already follow distinguishable fates raises the question of why earlier studies (in which lineages of two cell blastomeres were traced using microinjected horseradish peroxidase as a lineage tracer) did not reveal the reciprocal fate of the two-cell blastomeres (Balakier and Pedersen, 1982). A possible explanation is that those investigators missed the reciprocal contributions to embryonic or abembryonic regions because they limited their analysis to the distinction between trophectoderm and ICM descendants of the blastomeres. It is also possible that the

Table 2B. Distribution of descendants of early- and late-dividing two-cell blastomeres at early blastocyst stage*

Name	Total cells	Total early	Total embryonic	Early crossing				Late crossing				Tilt			
				% Embryonic early	Early in BZ	BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	BZ to embryonic		Total BZ	%BZ late	%BZ early
1B	51	28	22	86	6	3	23	15	80	8	3	14	57	43	0
2B	57	26	25	84	3	2	31	16	88	13	4	16	81	19	0
3B	62	34	26	100	6	2	28	19	89	11	0	17	65	35	0
4B	47	29	22	91	7	2	18	10	80	8	2	15	53	47	4
5B	47	27	22	95	5	1	20	12	92	8	1	13	62	38	6
6B	45	22	18	94	4	1	23	13	92	10	1	14	71	29	8
7B	54	29	25	92	4	2	25	16	88	9	2	13	69	31	8
8B	49	28	24	92	4	2	21	12	83	9	2	13	69	31	10
9B	49	30	23	91	7	2	19	11	82	8	2	15	53	47	15
10B	43	24	21	90	3	2	19	10	80	9	2	12	75	25	17
11B	42	21	18	94	3	1	21	11	91	10	1	13	77	23	20
12B	43	24	20	90	4	2	19	11	82	8	2	12	67	33	20
13B	46	25	18	100	6	1	21	14	93	8	0	14	57	43	20
14B	44	20	17	88	5	0	24	13	100	9	2	14	64	36	22
15B	49	28	22	95	5	2	21	14	86	8	1	13	62	38	24
16B	47	25	20	75	7	3	22	14	79	6	5	13	46	54	26
17B	43	23	19	84	3	4	20	11	64	10	3	13	77	23	30
18B	45	26	17	88	6	5	19	14	64	8	2	14	57	43	30
19B	50	25	21	81	7	1	25	13	92	9	4	16	56	44	30
20B	52	27	22	91	6	1	25	16	94	8	2	14	57	43	35
21B	50	25	21	76	9	0	25	14	100	6	5	15	40	60	38
22B	69	35	27	78	8	6	34	22	73	12	6	20	60	40	48
23B	41	22	18	94	3	2	19	10	80	10	1	13	77	23	49
24B	46	23	18	56	7	6	23	13	54	8	8	15	53	47	90
25B	45	25	20	80	5	4	20	12	67	8	4	13	62	38	‡
26B	53	27	22	77	7	3	26	15	80	9	5	16	56	44	‡
Mean	48.8	26.1	21.1	87	5.4	2.3	22.7	13.5	83	8.8	2.7	14.2	63.5	37.5	22.9
±s.d.	6.4	3.6	2.8	9.6	1.7	1.6	3.9	2.8	11.3	1.6	1.9	1.8	10.1	10.1	20.1
Mean of combined early and expanding blastocysts ((A) and (B))															
	42.1	22.5	18.2	84.7	4.9	2.1	19.5	11.1	80.2	7.9	2.7	12.8	61.9	38.1	24.6

*Counts of blastocyst cells were performed as described for Table 1.

‡Mixed distribution of cells prevented assessment of the tilt in these embryos

Table 2C. Distribution of descendants of early and late-dividing two-cell blastomeres in exceptional (reversed) blastocysts*

Name	Total cells	Total early	Total embryonic	Early crossing				Late crossing				Tilt			
				% Embryonic early	Early in BZ	BZ to embryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	BZ to abembryonic		Total BZ	%BZ late	%BZ early
1C	31	16	13	23	8	3	15	7	29	3	2	11	27	73	38
2C	34	18	14	7	8	1	16	9	0	3	0	11	27	73	8
3C	46	20	21	10	10	2	26	11	27	4	3	14	29	71	15
4C	42	20	19	16	10	3	22	8	13	5	1	15	33	67	0
4C	43	19	16	0	8	0	24	12	8	7	1	15	47	53	10
6C	56	31	20	20	14	4	25	16	19	6	3	20	30	70	20
7C	39	18	19	32	9	6	21	7	57	4	4	13	31	69	
Mean	41.6	20.3	17.4	15.3	9.6	2.7	21.3	10.0	21.8	4.6	2.0	14.1	32	68	15.2
±s.d.	8.2	4.9	3.1	10.7	2.1	2.0	4.3	3.3	18.6	1.5	1.4	3.1	6.8	6.8	13.1

*Counts of blastocyst cells were performed as described for Table 1. Because of their reversed contribution, these embryos derive their embryonic part mainly from the late-dividing blastomere and their abembryonic part from the early-dividing blastomere.

earlier studies lacked the resolution provided by the combined use of fluorescent tracers and optical sectioning used in the present work. Our experience suggests further that intracellular microinjection of lineage tracers itself could perturb these early cleavage divisions and thus alter the fate of blastomere descendants. Unlike other cell lineage tracing methods, the approach we have used here does not require metabolic activity

of the cell nor does it introduce a metabolically active molecule that might invoke physiological reactions altering cell fate.

Despite the above limitations, previous lineage tracing methods did allow several authors to find out that the early dividing cells make a disproportionate contribution to the ICM (Kelly et al., 1978; Graham and Deussen, 1978; Surani and Barton, 1984), a conclusion that is fully supported by

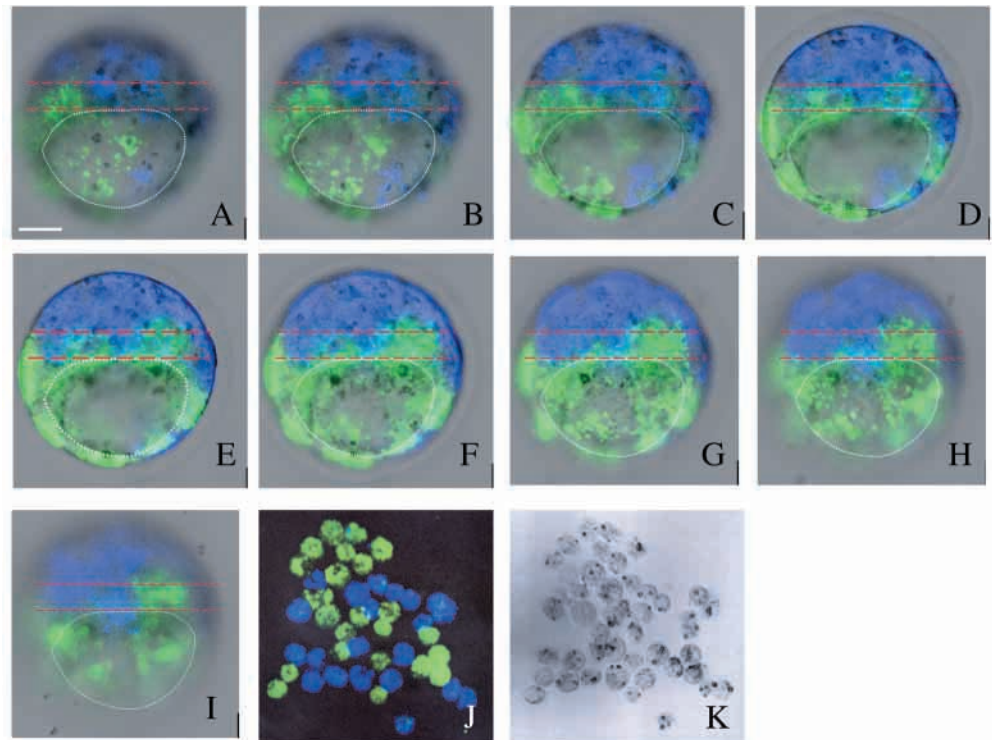


Fig. 3. Confocal sections of a blastocyst that shows correspondence of the clonal border of the two-cell stage progeny with a plane separating the embryonic and abembryonic parts. Blastomeres were labelled at the two-cell stage with dyes of different colours. The boundary zone is marked with broken red lines and the border of the blastocoel was traced on a central section and is shown projected onto each of the other sections as a broken white line. (A-I) Individual optical sections at 7.5 μm intervals in the 'z-dimension'. (J,K) The dissociated cells of this blastocyst observed by fluorescence or DIC optics, respectively.

our present findings. What properties of the more rapidly dividing two-cell blastomere might give its progeny the tendency to follow a particular developmental path? One possibility is that a division advantage per se allows cells in one lineage to initiate a developmental programme earlier than the other. Another is that as the ability to divide earlier is acquired by the two-cell stage blastomere that inherits the

SEP (Bennett, 1982; Piotrowska and Zernicka-Goetz, 2001), sperm entry may promote some other as yet unidentified developmental process to influence the final fate of the cell.

Although the progeny of each of the two-cell stage blastomeres come to inhabit either embryonic or abembryonic parts in the great majority of cases, the region between these two parts that we refer to as the boundary zone is populated by both

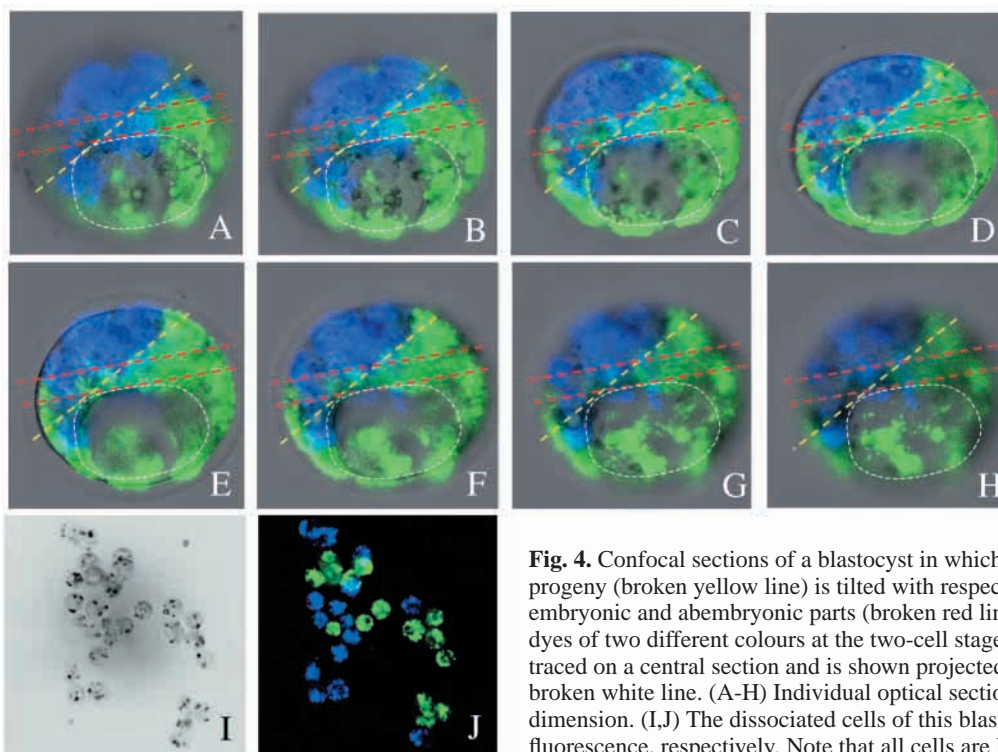


Fig. 4. Confocal sections of a blastocyst in which the clonal border of the two-cell stage progeny (broken yellow line) is tilted with respect to the boundary zone between the embryonic and abembryonic parts (broken red lines). Blastomeres were labelled with dyes of two different colours at the two-cell stage. The border of the blastocoel was traced on a central section and is shown projected onto each of the other sections as a broken white line. (A-H) Individual optical sections at 7.5 μm intervals in the z-dimension. (I,J) The dissociated cells of this blastocyst observed by DIC optics or fluorescence, respectively. Note that all cells are labelled but not uniformly throughout.

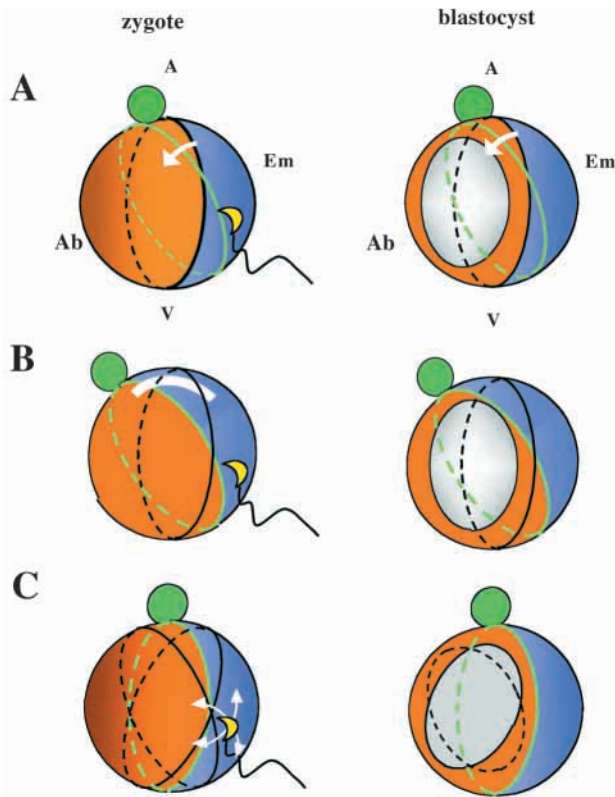


Fig. 5. Several possible models depicting the relationship between the first cleavage plane and the boundary between embryonic and abembryonic parts of the blastocyst. (A) Model by Piotrowska and Zernicka-Goetz (Piotrowska and Zernicka-Goetz, 2001). The left-hand diagram indicates the position of the first cleavage that divides the zygote into two (orange and blue) cells. The first cleavage plane (outlined in black) is set in relation to the animal pole (marked by the polar body shown in green) and the SEP (the sperm head is shown in yellow). Depending on whether cleavage passes on one or other side of these structures, its actual position with respect to the animal pole might vary by several degrees (arrow, plane outlined in green). The two-cell stage blastomere that inherits the SEP tends to divide first to produce cells that tend to populate the embryonic part of the blastocyst (right-hand diagram). The first cleavage plane is reflected in the blastocyst as the border (black disc) between the embryonic region (Em (blue); polar trophoctoderm and ICM destined to become epiblast) and the abembryonic region (Ab (orange); mural trophoctoderm and ICM that will contribute cells to primitive endoderm). Blastocoel in grey. The precise position of this border may reflect variations in the first cleavage plane (arrow leading to green disc). (B) Variation on the model in A in which factors influencing both the embryonic-abembryonic axis and the first cleavage plane are inter-dependent. In this model, the first cleavage plane of the zygote, and hence the clonal border of the blastocyst stage (outlined in green), is set by the relative positions of the polar body and SEP. The morphology of the blastocyst defined by a plane separating the embryonic and abembryonic parts (outlined in black) is depicted as being independently set by factor(s) (white arc) present in the animal pole cytoplasm. (C) Variation on the model in A in which the animal pole marked by the polar body is the primary determinant of the cleavage plane of the zygote or clonal border of the blastocyst (outlined in green). The displacement of the plane separating embryonic and abembryonic parts (outlined in black) is depicted as being influenced by the variation in the position of sperm entry (arrows).

types of cells. This does not seem to be so much a consequence of cell mixing in this region of the embryo as suggested (Gardner, 2001), because most of the cells in the clones derived from one or the other two-cell blastomere are separated by a distinct clonal border. Rather it appears to be because the clonal border, which in turn reflects the initial cleavage plane, shows some variation in its angular position and does not necessarily lie exactly perpendicular to the embryonic-abembryonic axis. Our findings suggest that the point about which the plane of the clonal border is tilted must lie not at the blastocoel roof, but towards the embryonic region, within the boundary zone. This provides an explanation of why within this boundary zone, the clone derived from the later dividing blastomere (which will give predominantly the abembryonic part) contributes a greater number of cells. A similar tilt was observed by Gardner (Gardner, 2001), though it was attributed mainly to the limitations of the approach used (i.e. owing to rotation of the embryo within its zona pellucida). Such a tilt is accommodated by the previously advanced model that postulated the boundary between the embryonic and abembryonic parts of the blastocyst as not always being parallel to the cavity, but sometimes displaced at an angle to it (Piotrowska and Zernicka-Goetz, 2001; Fig. 5A). This would be the case if the initial cleavage plane passed on one or the other side of the polar body or the SEP. Because the polar body is known to be a marker of the first cleavage plane, we sought to determine whether there was any relationship between the angular displacement of the clonal border from the embryonic-abembryonic axis and the position of the polar body. We found that the polar body was clearly preserved in the 17 of the blastocysts within this study. In 14 out of these 17 embryos, the polar body lay at or very close to the border between the two clones. This demonstrates that the plane

defined by the clonal borders at the blastocyst stage is indeed a reflection of the first cleavage plane. If the polar body is associated with the first cleavage plane, what, then, might explain the angle this plane with respect to the embryonic-abembryonic axis? We suggest one possible mechanism is that a factor (yet unknown) could pre-exist within the egg bearing some geometric relationship to the polar bodies and could specify orientation of the embryonic-abembryonic axis (Fig. 5B). It is also possible that variation in the position of the SEP along the animal-vegetal axis could contribute to the variation in the orientation of the embryonic-abembryonic axis, while remaining in close proximity to but not directly on the first cleavage plane (Piotrowska and Zernicka-Goetz, 2001) (Fig. 5C). Alternatively, there might be other stochastic factors operating in the embryo, for example random variation in the point where the blastocoel cavity is initiated, which could modulate the position of the blastocoel roof relative to the clonal boundary of the two-cell blastomeres. We wish to emphasise that these hypotheses are speculative and that currently we do not favour one over another.

What consequences might this distribution of cells in the boundary zone have for subsequent development? This of course will depend upon the period for which the boundaries are maintained in the absence of cell mixing and whether signalling events might occur between the cell types. Although our data show there to be relatively little cell mixing during cleavage, in agreement with Garner and McLaren (Garner and McLaren, 1974), the embryo becomes morphogenetically

active as it reaches the blastocyst stage and this can shift the relative positions of cells. For example, cells will also naturally migrate across the boundary as the blastocyst develops. In the trophectoderm lineage, this takes the form of a flow of cells from the polar to mural trophectoderm (Copp, 1979; Cruz and Pedersen, 1985).

Recent observations suggest that once embryos reach the blastocyst stage, the position of cells within subregions of the blastocyst have an important bearing on their subsequent fate (Weber et al., 1999). ICM cells that border the early blastocyst cavity were often found to contribute to primitive endoderm. Moreover, cells that are precursors for visceral endoderm located near the polar body tend to become located distally in the egg cylinder, whereas progeny of those located opposite the polar body become located proximally (Weber et al., 1999). Could it be that these visceral endoderm precursors located in the boundary zone initially nearby the polar body and their counterparts opposite the polar body originate from different blastomeres and so behave differently? The tilted clonal border indicates that this is a possibility, although further studies would be needed to clarify this issue. Our present observations suggest that while primitive endoderm cells might have dual origins (from both the earlier and later dividing blastomeres), much of the embryo proper could be derived from ICM cells contributed by the first dividing two-cell embryo blastomere. Because of the tilt, it can be predicted that some four-cell blastomeres will have more exclusive fates as they occupy distinct positions along the embryonic-abembryonic axis. Thus, our findings add to the increasing evidence that the polarity of the embryo not only has its origins in the preimplantation period, but also that two blastocyst axes become recognised by the first cleavage.

The precise role played by sperm entry in this process is still unclear. The cell inheriting the SEP has a division advantage, but the division advantage of this cell may be secondary to some other consequence of sperm entry that could influence development. In organisms such as *C. elegans* and *Xenopus*, sperm entry is known to reorganise directly the distribution of cytoplasmic constituents within the egg, such that they will be asymmetrically inherited by different blastomeres and so influence cell fate (Goldstein and Hird, 1996; Gerhart et al., 1989; De Robertis et al., 2000). Although the mechanism by which sperm entry influences fate in the mouse embryo is not yet understood, our observations point towards an underlying order in preimplantation development that has previously been unanticipated. However, the process is not determinative and fate is not fixed at these early stages (Zernicka-Goetz, 1998). The mouse embryo remains endowed with regulative abilities that can generally overcome developmental perturbations.

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