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# The first cleavage of the mouse zygote predicts the blastocyst axis

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**One of the unanswered questions in mammalian development is how the embryonic–abembryonic axis of the blastocyst is first established. It is possible that the first cleavage division contributes to this process, because in most mouse embryos the progeny of one two-cell blastomere primarily populate the embryonic part of the blastocyst and the progeny of its sister populate the abembryonic part[1–4]. However, it is not known whether the embryonic–abembryonic axis is set up by the first cleavage itself, by polarity in the oocyte that then sets the first cleavage plane with respect to the animal pole, or indeed whether it can be divorced entirely from the first cleavage and established in relation to the animal pole. Here we test the importance of the orientation of the first cleavage by imposing an elongated shape on the zygote so that the division no longer passes close to the animal pole, marked by the second polar body. Non-invasive lineage tracing shows that even when the first cleavage occurs along the short axis imposed by this experimental treatment, the progeny of the resulting two-cell blastomeres tend to populate the respective embryonic and abembryonic parts of the blastocyst. Thus, the first cleavage contributes to breaking the symmetry of the embryo, generating blastomeres with different developmental characteristics.**

It has been shown, either by marking cells directly or by relating their position to external markers, that the majority of first cleavage divisions generate one blastomere whose progeny will primarily populate the embryonic part of the blastocyst (polar trophectoderm and deeper cells of the inner cell mass) and another whose progeny populate the abembryonic part (mural trophectoderm and the more superficial inner cell mass)[1–4]. More recently, further lineage tracing experiments have clarified this relationship and shown that, by the four-cell stage of normally developing embryos, blastomeres not only have specific fates but can also differ in their developmental potential[5,6]. Static observations and a series of micromanipulation experiments to relocate the animal pole of the mouse zygote indicate that in normal circumstances the first cleavage usually passes close to this pole[7,8]. A second marker of the first cleavage, the sperm entry site[1], reflects positioning of the spindle in relation to a slight flattening of the zygote at fertilization[9]. Together these observations raise the question of whether it is the spatial organization of the zygote with respect to the animal pole or, because cleavage orientation can reflect the random site of sperm entry, cleavage itself that influences the subsequent development of the embryonic–abembryonic axis.

To approach this question, we first developed a better method for accurate three-dimensional examination of the first cleavage orientation in a random population of zygotes. We achieved this by time-lapse observations of the first cleavage on multiple focal planes at each time point. In addition, to facilitate analysis of the position of the first cleavage plane in respect to pronuclei (and the polar body (PB)), we followed this cleavage in a newly developed transgenic line (CAG:H2B–EGFP) in which chromatin was labelled green by the expression of green fluorescent protein (GFP)-tagged histone H2B (ref. 10; Fig. 1a; Supplementary Movie 1). We found that the female pronucleus was closer to the PB than the male pronucleus in 87.5% ($n = 32$) of zygotes (categories I–III; Fig. 1b) and that in most zygotes (category I, 62.5%) the two pronuclei were aligned with the PB and the cleavage furrow formed within 30° of this plane. In 12.5% of zygotes (category II), the pronuclei were displaced from the PB by between 30° and 90° and the cleavage plane was displaced to a similar extent. In the two remaining categories, cleavage passed within 30–90° of PBs aligned with the pronuclei (category III), and within 30° of PBs not aligned with the pronuclei (category IV). We observed that this most frequent alignment of the differentially condensed[11] female and male pronuclei with the PB was maintained into prometaphase. Immunostaining of fixed zygotes to reveal CpG-methylated female chromatin showed that, in agreement with previous studies[11,12] and contrary to a recent proposition[13], the parental sets of chromosomes did not undergo mixing and adopted an arrangement consistent with our time-lapse studies that was retained into the daughter blastomeres (Fig. 1c).

To characterize the orientation of the cleavage plane further, we performed time-lapse differential interference contrast (DIC) microscopy on zygotes of a different, wild-type mouse strain (Fig. 1d). By observing multiple focal planes we ensured that we could follow cleavage in relation to the location of markers in three dimensions in 96% of all randomly positioned zygotes. We found that the onset of cleavage furrow formation was within 30° of the PB in 70% of zygotes, 14-fold greater than at a lateral position between

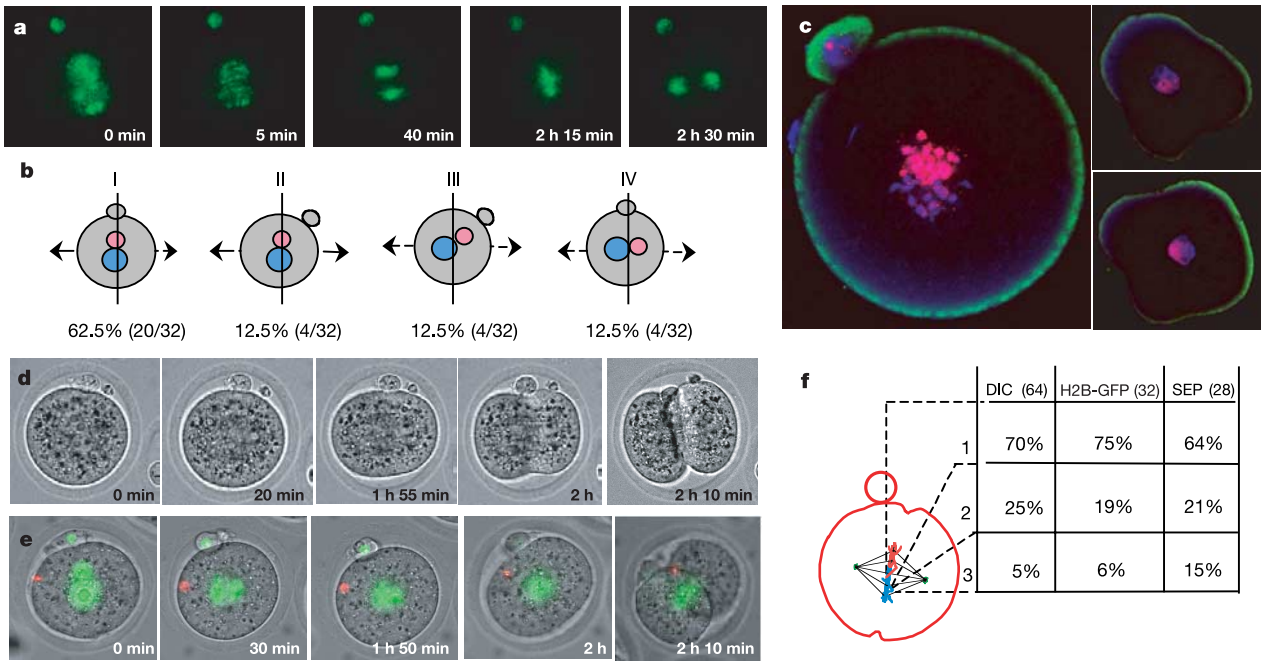


**Figure 1** Orientation of the first cleavage division. **a**, One zygote expressing H2B–EGFP from 32 embryos observed by time-lapse imaging, to note the positions of maternal and paternal chromosomes before chromosome condensation, during prometaphase, anaphase and cytokinesis. **b**, Four cleavage categories of zygotes from **a** (vertical line, cleavage plane; horizontal arrows, direction of cell separation): I, male and female pronuclei aligned within 30° of PB; cleavage passed through this plane; II, male and female pronuclei displaced from PB by 30–90°; cleavage through plane of pronuclei; III, pronuclei aligned within 30° of PB; cleavage displaced from this plane by 30–90°; IV, pronuclei not aligned with PB; cleavage within 30° of the PB. **c**, Zygotes fixed and stained to reveal maternal (CpG methylated chromatin, pink) and paternal chromatin (blue). Left, prometaphase zygote; right, optical sections through separate two-cell blastomeres. Parental chromosomes do not mix during the first cleavage and still occupy distinct territories at the two-cell stage. **d**, DIC time-lapse series of zygote with cleavage at the site of the PB. **e**, Time-lapse series of H2B–EGFP (green) labelled zygote with red fluorescent bead at sperm entry site. **a**, **c**–**e**, The diameter of the zygote is approximately 80 μm. **f**, Proportions of zygotes with cleavage plane within 30° (1), 30–60° (2) and 60–90° (3) of the PB in a DIC time-lapse series of 64, a series of 32 to follow H2B–EGFP, and a series of 28 to assess cleavage in relation to the fluorescent bead marking sperm entry.

60° and 90° of the PB (Fig. 1a, f). In those zygotes in which it was located in a medial position (25%) the PB was usually drawn into the furrow as it subsequently ingressed in more than 90% of embryos. Thus, the first cleavage tended to pass close to the PB region and through the plane along which the parental genomes had aligned. We also followed the fluorescence of both GFP-tagged H2B and a bead marking the sperm entry point in time-lapse studies (Fig. 1e and Supplementary Movie 2). This showed that cleavage passed within 30° of the sperm entry position in a significant majority of zygotes (Fig. 1e, f). In addition we performed a small series of transplantation experiments to re-site the animal pole to observe by time-lapse imaging whether cleavage would then tend to pass through this new site. In almost all (six of seven) embryos the first cleavage did occur within 30° of the newly positioned PB (Supplementary Movie 3). Taken together, these studies of living embryos support and extend earlier work that showed there is a bias for the first cleavage to pass close to the attached PB[7–9] and site of sperm entry[1,9]. Recent studies have indicated that cleavage of the two-cell blastomere that inherits the PB also tends to pass close to the PB (ref. 5).

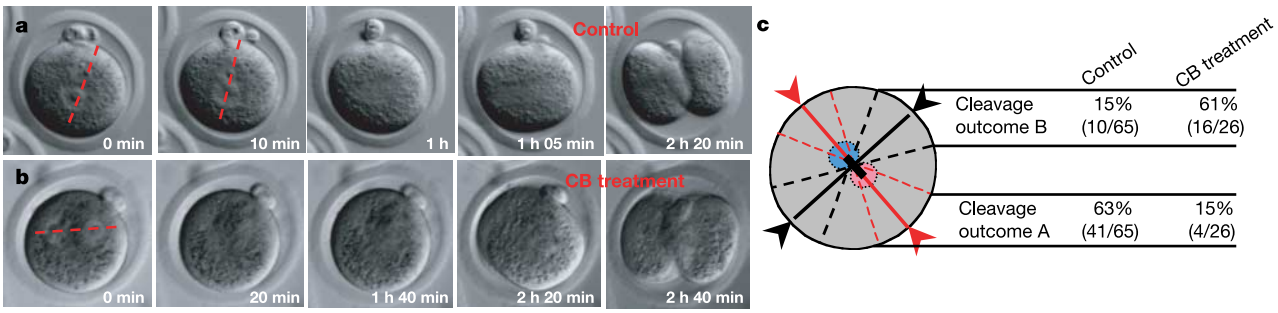


**Figure 2** Cytochalasin treatment before pronuclei migration changes the orientation of the first cleavage division. **a**, **b**, Time-lapse series of control (**a**) and cytochalasin-treated (**b**) zygotes. The diameter of the zygotes is approximately 80 μm. **c**, Diagram to show the plane represented by extrapolation of final trajectories of two pronuclei (solid red line). Cleavage within 30° of this line is represented as 'outcome A'. The solid black line represents cleavage 90° to this final path of pronuclear migration. Cleavage within 30° of this is referred to as 'outcome B'. The relative proportions of cleavages that followed these two outcomes in control and cytochalasin-treated embryos are shown.

In the above experiments we neither selected nor excluded embryos, but instead analysed 96% of all recorded zygotes on multiple focal planes at each time point. In contrast, a recent study that examined cleavage with respect to the PB on a single focal plane discarded over 50% of zygotes in which cleavage was not in a suitable position to be observed[13]. Furthermore, to examine cleavage with respect to pronuclei, these same authors selected embryos in which the pronuclei lay on the same focal plane[13]. This indicates that their data might have been biased through the selection of specific groups of embryos for analysis. It could explain why their conclusion that the first cleavage does not relate to the PB and moreover coincides with a plane between apposing pronuclei differs from the four-dimensional studies described in this paper. Such a bias might arise because in our experience zygotes position

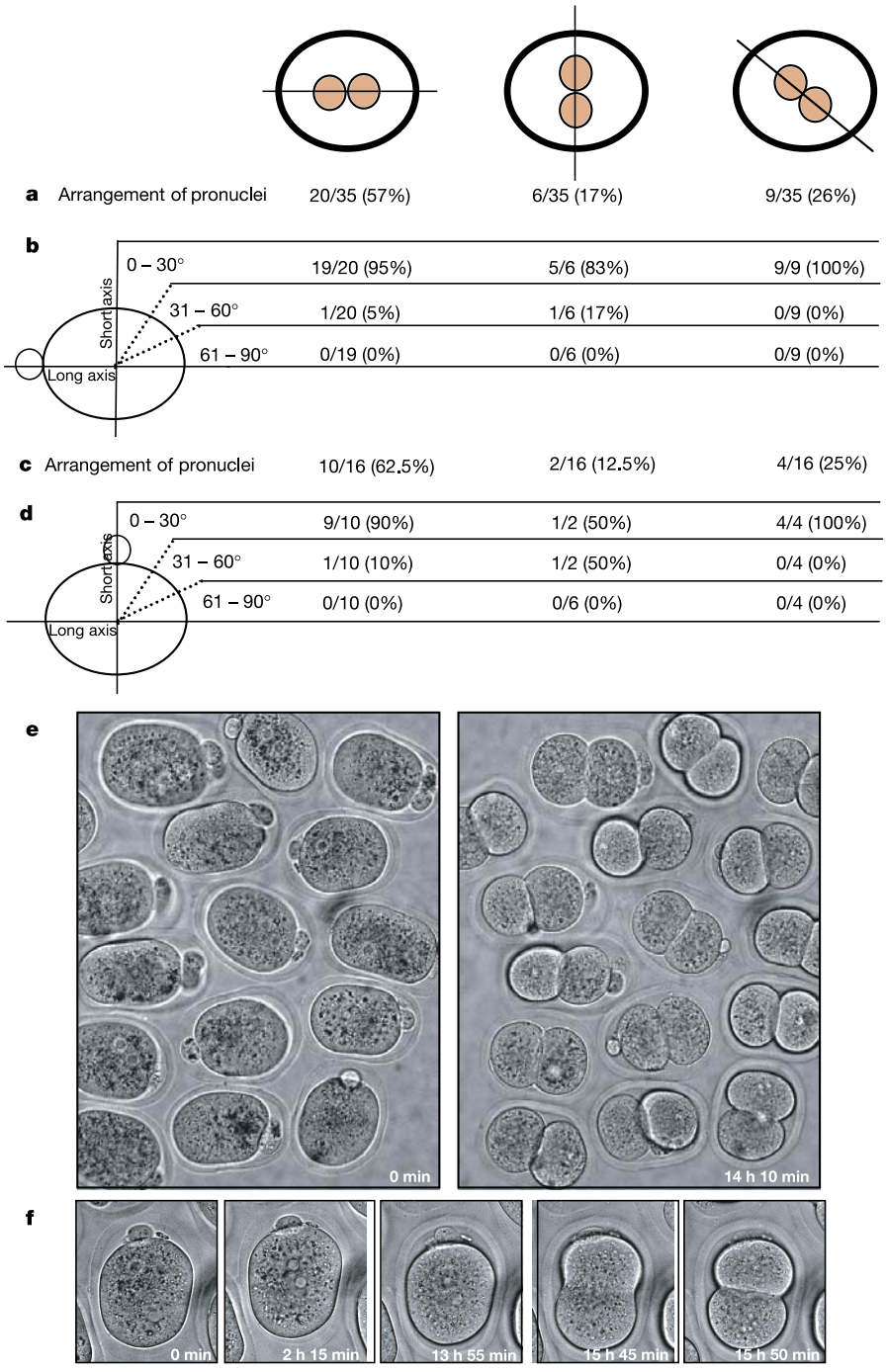


**Figure 3** Relationship between final position of pronuclei, long axis of zygote and first cleavage in experimentally elongated zygotes. **a**, **b**, PB at the end of the long axis; **c**, **d**, PB at the end of the short axis. **a**, **c**, Proportions of zygotes in which alignment of pronuclei was within 30° (left), 61–90° (middle) and 31-60° (right) of the long axis in a time-lapse series of 51 zygotes. **b**, **d**, Proportions of zygotes that showed a cleavage plane within 30° (top), 31–60° (middle) and 61–90° (bottom) of the short axis. **e**, Example of a field of experimentally elongated embryos at the beginning (20 h after hCG injection, left) and end (34 h 10 min after hCG injection, right) of a time-lapse series. **f**, One example of a zygote at five time points. The ratio between the short and long axis of this zygote was 0.83. Time points: at 0 min (beginning of the experiment 18 h after hCG injection) both pronuclei are still apart from each other; at 2 h 15 min, both pronuclei have met in the centre of the zygote; at 13 h 55 min, the last frame in which pronuclei can still be seen; at 15 h 45 min, elongated zygotes are preparing for cleavage; at 15 h 50 min, cytokinesis occurs. The diameter of the not elongated embryos is approximately 80 μm.

themselves in culture dishes according to their shape. This is particularly important because zygote shape can override any influence of the region marked by the PB in relation to the orientation of the first cleavage[9]. Could it therefore be that if we were to select zygotes of a particular shape we could also observe a different relationship of the first cleavage to the position of pronuclei? If so, would circumstances in which cleavage followed a different set of cues allow us to test its independent relationship to development of the embryonic–abembryonic axis?

Treatment of zygotes with cytochalasin at concentrations that disrupt the actin cytoskeleton can interfere with the effect of the natural shape change induced by fertilization to affect positioning of the cleavage furrow[9]. We therefore examined cleavage in embryos treated with cytochalasin for 4 h at concentrations that depolymerize the actin cytoskeleton about 6 h before cleavage. In contrast with control zygotes (Fig. 2, outcome A; Supplementary Movie 4), the plane of cleavage in cytochalasin-treated zygotes (Fig. 2, outcome B; Supplementary Movie 5) lay within 30° of a plane orthogonal to the final trajectory of the pronuclei. Thus, only in drug-treated embryos do we see an outcome similar to that reported previously[13]. Indeed, we note that in these experiments, which led to the conclusion that the cleavage plane would lie between apposing transplanted pronuclei[13], zygotes must have been treated with cytochalasin to enable the necessary micromanipulations. Such a treatment would in itself be enough to influence cleavage orientation. We find that the time of cytochalasin treatment is critical to the outcome. When applied early in the course of pronuclear migration, as is necessary in pronuclear transplantation, cleavage no longer respected the position of the PB (44% cleaved within 30° of the PB, 36% between 30° and 60°, and 20% between 60° and 90°; $n = 25$). If zygotes were treated with cytochalasin once the pronuclei had migrated to the interior, as in our own experiments to transplant cytoplasm from the PB region, most cleavages (73%, 16 of 22) respected the PB.

Next we looked further into the effects of cell shape upon the first cleavage. Our previous studies showed that when the zygote was experimentally 'flattened', cleavage passed through the new short axis, which thus overrides the effects of the animal pole and sperm entry site[9]. Could such a shape change influence the positions of pronuclei with respect to the long and short axes of the zygote and their relationship to the cleavage plane? To address this question we changed the shape of zygotes by drawing them into a constraining micropipette and releasing them into sodium alginate such that the PB lay at one end of either the long or the short axis. We found that, independently of the PB position, pronuclei tended to align with the new long axis of the zygote (Fig. 3). In most zygotes (59%, 30 of 51) pronuclei were aligned within 30° of the long axis and only in 16% (8 of 51) were pronuclei within 30° of the short axis. Cleavage occurred within 30° of the short axis in 92% (47 of 51) of all zygotes. Thus, the division plane happens to lie on a plane between the two apposing pronuclei in zygotes whose shape overrides the influence of the PB marked region in positioning cleavage. This contrasts with the great majority of non-manipulated zygotes, in which the pronuclei align with the first cleavage plane. The positions of the pronuclei seem to be independent of the plane of cleavage because in those rare circumstances in which the pronuclei of experimentally elongated zygotes were aligned with the short rather than the long axis (16%, 8 of 51), cleavage still tended to pass through the short axis (Fig. 3). Taken together, these results suggest that it is cell shape rather than position of the pronuclei that influences the cleavage plane in these elongated embryos. Thus, we cannot confirm the previous conclusion[13] that zygotes always divide according to the plane separating the two apposing pronuclei. We saw such patterns of cleavage only within selected groups of embryos that either had been treated with cytochalasin before the migration of pronuclei or had undergone a shape change such that the pronuclei lay along the long axis.

This ability to manipulate zygotes to divide reproducibly with this alternative pattern allowed us to determine its consequences for the development of the embryonic–abembryonic axis of the blastocyst. We therefore changed the zygote shape as above and cultured embryos in medium containing sodium alginate until the two-cell stage. Embryos that divided with the PB at one end of the long axis were then lifted from the solidified medium and their blastomeres were labelled with different lipophilic dyes (DiI or DiD, Molecular Probes) applied externally. We allowed such labelled embryos to develop to blastocysts and scored the positions of the clones with respect to the blastocyst cavity by confocal sectioning (Fig. 4). In zygotes whose shape had not been changed, the two respective clones should tend to occupy embryonic (polar trophectoderm and the deeper cells of the inner cell mass) and abembryonic (mural trophectoderm) parts of the blastocyst. The

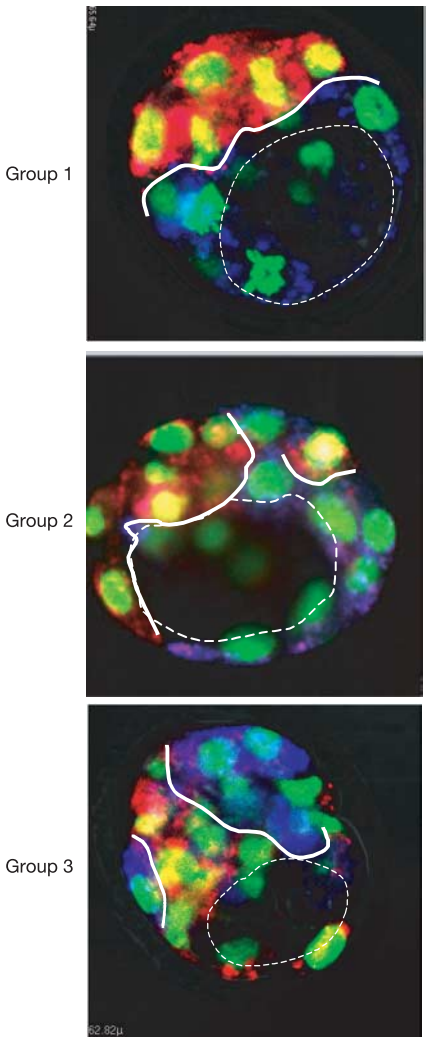


**Figure 4** Outcome of changing orientation of first cleavage on the orientation of blastocyst embryonic–abembryonic axis. Zygotes were elongated such that PB was at one end of the long axis. On their division to the two-cell stage, one blastomere was labelled with DiI (red) and other with DiD (blue). At the blastocyst stage, a total of 20 embryos were obtained and classified according to the extent to which cells derived from one blastomere comprising mainly the embryonic part crossed the embryonic–abembryonic boundary zone (a region approximately one cell deep and parallel to the roof of the blastocyst cavity) into the abembryonic part and vice versa. In group 1, up to two cells crossed the boundary zone; in group 2, three cells crossed the boundary zone; and in group 3, more than three cells crossed the boundary zone. Micrographs represent individual optical sections midway through the embryo that show the cavity (thin dashed line) and clonal boundary zone (thick solid line). The diameter of the blastocysts is approximately 80 μm.

number of cells from each clone in a boundary zone, lying one cell deep and parallel to the blastocoelic surface[3], can vary. To compare the distribution of progeny of both two-cell blastomeres between either embryonic or abembryonic parts in relation to previously published studies, we scored the extent to which clones spread beyond the boundary zone in 20 labelled blastocysts manipulated so that the PB had been at one extreme of the long axis after the first cleavage (Fig. 4 and Supplementary Fig. 1). If either no or a few cells (up to two, about 3–9% of the total mean cell number ($n = 22.25$) at this stage) had spread beyond the boundary zone, blastocysts were scored in group 1; if three cells crossed, blastocysts were scored in group 2; and if more than three, blastocysts were scored in group 3. In group 1, in 85% of blastocysts (17 of 20) two or fewer cells from predominantly embryonic clones were positioned beyond the boundary zone in the abembryonic part (Fig. 4), and in 65% (13 of 20) of the same blastocysts, two or fewer cells from predominantly abembryonic clones were located in the embryonic part (Fig. 4). In group 2, in 15% (3 of 20) of blastocysts three cells from the predominantly embryonic clone were located in the abembryonic part; in the other direction, the proportion was 20% (4 of 20 blastocysts). In group 3 there were no (0 of 20) blastocysts in which three or more cells from the predominantly embryonic clone were present in the abembryonic part. Only 15% (3 of 20) of blastocysts in this group showed the predominantly abembryonic clone extending into the embryonic part. Thus, in most embryos, most progeny of two-cell blastomeres came to lie in either the embryonic or abembryonic part of the blastocyst. A similar proportion of embryos showed this relationship in previous studies of non-manipulated embryos in which cleavage tended to pass close to the animal pole[3]. We also observed relatively little cell mixing between the two clones at the early blastocyst stage, in agreement with previous reports[14] (Supplementary Fig. 1).

Our study adds support to earlier evidence for a relationship between the first cleavage and a region close to PB[5–9]. However, this relationship of the animal pole region to cleavage orientation can be overridden by the effects of the shape upon the zygote as we have demonstrated previously[9]. We now show that, irrespective of the orientation of cleavage in relation to the pronuclei or the PB, in most embryos one two-cell blastomere populates the embryonic part of the blastocyst, and its sister populates the abembryonic part. Thus, formation of the embryonic–abembryonic axis tends to occur with respect to the first cleavage plane itself whether or not it passes close to the animal pole. Does this mean that the organization of the egg in relation to the animal pole has no influence over development other than normally coordinating the first cleavage? Recent experiments suggest that the patterns of early cleavage divisions segregate blastomeres not only with different fates but also with differing developmental properties[5,6]. This relates both to their specific position in the embryo with respect to the animal pole and the differential timing of the division of these cells. Thus, the existence of spatially distributed components of the egg working in concert with factors regulating cell division to influence development cannot be discounted. Further studies are required to identify any such putative components and to establish whether changing the orientation of the first cleavage might lead to their repositioning and indeed to the altered developmental properties of four-cell-stage blastomeres. □

## Methods

Zygotes were collected from $F_1$ (C57BL/6 × CBA) females induced to superovulate as described previously[3] and then mated with males of the same strain. In experiments in which chromatin was detected by fluorescence, zygotes were collected from CAG:H2B–EGFP[10] transgenic mice mated with males of the same strain. Zygotes were collected 23 h after injection with human chorionic gonadotrophin (hCG) as described previously[3]. In experiments examining the significance of the actin cytoskeleton in the subsequent process of spindle orientation, we treated zygotes with 1 or 5 $\mu g\,ml^{-1}$ cytochalasin B for 3–4 h starting 18–19 h after injection with hCG.

Time-lapse imaging was performed with either a Nikon inverted microscope with a Princeton Instruments camera using IP lab software or with a Leica inverted microscope with a Hamamatsu Orca camera using Openlab software (Improvision). Embryos were placed in 0.8 ml of M2 medium under paraffin oil in a glass-bottomed dish on a heating stage at 37 °C. Images were recorded at 5-min intervals for up to 12 h. The positions of pronuclei were measured with Volocity software (Improvision) and described as the coordinates of the centre of each pronucleus ($x_1$, $y_1$, $z_1$ and $x_2$, $y_2$, $z_2$). To define the division plane we used the coordinates of the centres of two blastomeres by the end of cytokinesis ($x_3$, $y_3$, $z_3$ and $x_4$, $y_4$, $z_4$). The angle between division plane and pronuclear position was then calculated from the equation

$$\theta = \arccos \frac{((x_2 - x_1)(x_4 - x_3) + (y_2 - y_1)(y_4 - y_3) + (z_2 - z_1)(z_4 - z_3))}{\sqrt{((x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2((x_4 - x_3)^2 + (y_4 - y_3)^2 + (z_4 - z_3)^2))}}$$

To label the sperm entry point the procedure was as described previously[1], except that red rather than green fluorescent beads were used so that they could be distinguished from H2B–GFP. Animal pole transplantation was performed as described previously[8]. Zygotes were treated with 1 $\mu g\,ml^{-1}$ cytochalasin D for 30–40 min immediately before transplantation.

Immunostaining was performed on embryos fixed with 3.7% paraformaldehyde in phosphate-buffered saline and processed as previously[8]. The shapes of zygotes were changed and their resulting dimensions measured as described previously[9]. Zygotes were elongated 18–20 h after hCG injection such that the mean ratio between the short and the long axis was 0.7 (range 0.6–0.9).

In experiments to monitor the effect of changing the position of the first cleavage on the development of the embryonic–abembryonic axis, zygotes were elongated 25–27 h after hCG injection. They were subsequently cultured in medium containing sodium alginate for the next 17–18 h and then lifted from it when they divided to the two-cell stage. Both blastomeres of these embryos were subsequently labelled: one blastomere with DiD, the other with DiI. The labelling and subsequent culture was performed as described previously[3]. Lineage tracing experiments were performed on the H2B–GFP transgenic line to facilitate analysis of the distribution of cells at the blastocyst stage. Blastocysts were observed by confocal microscopy and images were captured at 7-μm intervals. By examining all sections (nine or ten) in each series it was possible to determine the distribution of cells labelled by specific dyes in the embryonic (polar trophectoderm and deeper inner-cell mass cells) and abembryonic (mural trophectoderm) parts of the blastocyst. The boundary zone between these two parts was defined as a cell layer about one cell deep and parallel to the roof of the blastocoel cavity, as previously[3].

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