Development 129, 5803-5813

doi:10.1242/dev.00170

Early patterning of the mouse embryo – contributions of sperm and egg

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Accepted 12 September 2002

SUMMARY

The first cleavage of the fertilised mouse egg divides the zygote into two cells that have a tendency to follow distinguishable fates. One divides first and contributes its progeny predominantly to the embryonic part of the blastocyst, while the other, later dividing cell, contributes mainly to the abembryonic part. We have previously observed that both the plane of this first cleavage and the subsequent order of blastomere division tend to correlate with the position of the fertilisation cone that forms after sperm entry. But does sperm entry contribute to assigning the distinguishable fates to the first two blastomeres or is their fate an intrinsic property of the egg itself? To answer this question we examined the distribution of the progeny of early blastomeres in embryos never penetrated by sperm – parthenogenetic embryos. In contrast to fertilised eggs, we found there is no tendency for the first two parthenogenetic blastomeres to follow different fates. This outcome is independent of whether parthenogenetic eggs are haploid or diploid. Also unlike fertilised eggs, the first 2-cell blastomere to divide in parthenogenetic embryo does not necessarily contribute more cells to the blastocyst. However, even when descendants of the first dividing blastomere do predominate, they show no strong predisposition to occupy the embryonic part. Thus blastomere fate does not appear to be decided by differential cell division alone. Finally, when the cortical cytoplasm at the site of sperm entry is removed, the first cleavage plane no longer tends to divide the embryo into embryonic and abembryonic parts. Together these results indicate that in normal development fertilisation contributes to setting up embryonic patterning, alongside the role of the egg.

Supplemental data available on-line

Key words: Sperm, Egg, Polarity, Cleavage, Blastocyst pattern, mouse

INTRODUCTION

The establishment of spatial patterns and the definition of cell fate are fundamental developmental processes. In the embryos of many invertebrates and lower vertebrates these two processes are driven mainly by the segregation of morphogenetic determinants in response to spatial cues (for a review, see Gurdon, 1992). In mammalian embryos, however, such factors have thus far not been identified. Moreover, the mouse embryo has remarkable flexibility in response to developmental perturbations. Together these two facts had led to the view that patterning and subsequent cell fate might not develop with respect to any intrinsic spatial cues within the mammalian egg, and indeed led to doubts that such cues even existed (for a review, see Zernicka-Goetz, 2002).

However, we have recently learnt that early patterning of the mouse embryo in normal, unperturbed development relates to the polarity established at the very beginning of embryonic life. It appears that the first cleavage division separates the mouse zygote into two halves that have a bias to follow distinguishable fates (Piotrowska and Zernicka-Goetz, 2001; Piotrowska et al., 2001; Gardner, 2001). Specifically, one of the 2-cell embryo blastomeres cleaves ahead of its sister and tends to contribute most of its descendants to the embryonic part of the blastocyst, whereas the other, later dividing one, contributes progeny predominantly to the abembryonic part (Piotrowska et al., 2001). The plane of this first cleavage division appears to relate not only to the site of the previous meiotic division – marked by the second polar body (Plusa et al., 2002a) but also to the position of the fertilisation cone that emerges at the site where the sperm enters the egg (Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002b). The position of sperm penetration also correlates with the division asynchrony between 2-cell embryo blastomeres (Bennett, 1982; Piotrowska and Zernicka-Goetz, 2001). This is shown by the observation that the first blastomere to divide from the 2-cell to the 4-cell stage is generally the one that acquires the part of the zygote cortex at which the sperm entered the egg. Accordingly, these findings give rise to the hypothesis that in normal development of an embryo the act of fertilisation itself might contribute to setting up embryonic patterning (Piotrowska and Zernicka-Goetz, 2001; Piotrowska et al., 2001), as demonstrated in so many different species (see

Goldstein and Hird, 1996; Sawada and Schatten, 1989; Sardet et al., 1989; Roegiers et al., 1999; Vincent and Gerhart, 1987).

The role of the sperm in the early patterning of the mouse embryo has been questioned by Davis and Gardner (Davis and Gardner, 2002) who have monitored the position of sperm components taken up into the egg cytoplasm, namely the anterior part of the sperm tail and the sperm-derived mitochondria, and related these to the first cleavage. Unlike the earlier studies, they did not however mark the position of the fertilisation cone, the egg's immediate cytoskeletal response to sperm penetration, and this may help explain the discrepancy between the interpretation of data from the different groups. In addition to these two points of view it is possible that the oocyte itself possesses an endogenous polarity that is effectual only in the context of a role for the sperm.

These viewpoints could be evaluated by studying the early patterning in eggs lacking a sperm – parthenogenetic embryos. Parthenogenetically activated mouse eggs can develop into blastocysts and some even to early post-implantation stages, by which point they die owing to lack of expression of certain paternally derived genes (Barton et al., 1984; McGrath and Solter, 1984). A comparison of fertilised and parthenogenetically activated embryos could provide further insight into roles that the sperm might have. If sperm penetration contributes to a symmetry-breaking event, is its impact achieved by providing a positional cue that polarises the embryo and affects the ensuing cleavage pattern, or is it by influencing the synchrony of early cleavage divisions? It can be argued that any initial asynchrony of cleavage could be sufficient to explain differential blastomere fate. This is because the first dividing blastomere might contribute a greater proportion of smaller cells earlier and these would contribute to the embryonic part as they would be preferentially enclosed by the bigger, later dividing cells. Hence if embryo patterning arises solely from the asynchrony in the second cleavage between 2-cell blastomeres, one can expect that patterning should be normal in parthenogenetic embryos, where cleavage divisions are also asynchronous. In such a case the first blastomere to divide in parthenogenetic embryos would also be expected to contribute preferentially to the embryonic part, as in zygotes. Alternatively, if sperm entry provides a positional cue that polarises the embryo, the early patterning of parthenogenetic and fertilised embryos should differ.

To address whether 2-cell blastomeres have a tendency to follow distinguishable (embryonic and abembryonic) fates without any reference to the event of fertilisation, we have now studied development of early patterning in parthenogenetic eggs. To this end we used three different methods to activate eggs parthenogenetically – one that yields haploid eggs and two others that yield diploid eggs. Our studies reveal that both the spatial contributions of progeny of 2-cell blastomeres to the blastocyst and the consequences of asynchrony in the early cleavage divisions differ between fertilised and parthenogenetic embryos. These findings are supported by further experiments to examine the order of division and developing spatial pattern in fertilised eggs in which the cortical cytoplasm at the site of sperm penetration has been removed. Together these results implicate a role for the sperm in both of these processes and indicate that although cleavage asynchrony contributes to assigning cells to specific blastocyst regions, on its own it appears insufficient to define the blastomeres' fate.

MATERIALS AND METHODS

Eggs

Eggs were collected from F_1 (C57BL/6 × CBA) females induced to superovulate by intraperitoneal injection of 7.5 IU of pregnant mares serum gonadotrophin (PMS, Intervet) followed 48 hours later by 7.5 IU of human chorionic gonadotrophin (hCG, Intervet). Metaphase II-arrested oocytes were collected 14-15 hours after hCG injection, in PBS containing 200 IU/ml of hyaluronidase, to disperse cumulus cells, and then transferred to FHM medium (Speciality Media, Inc. Lavallette, NJ) supplemented with bovine serum albumin (BSA, 4 mg/ml) (FHM + BSA). Eggs were artificially activated 16-17 hours after hCG by treatment with 7% ethanol (in FHM+BSA) for 8 minutes. This usually resulted in 60-70% of eggs being activated. We followed two routes to obtain diploid parthenogenetic embryos. In the first group, eggs were subjected to artificial activation and then a 3 hours cytochalasin D (1 μg/ml) treatment to inhibit the extrusion of the second polar body. In the second group, artificially activated embryos were allowed to complete the second meiosis and extrude their polar body, but subsequently (1 hour after extrusion) those polar bodies were electrofused back to the eggs using an Electro Cell Manipulator (BTX 2001; BTX Inc., San Diego, CA, USA). Fusion was induced by three 90 V DC pulses, each of 35 μseconds duration, in PBS. The distance between the electrodes in the chamber was 0.75 mm. Fusion occurred within 10-15 minutes and usually resulted in approximately 80% of eggs with the polar body fused to them. In an additional series of experiments newly fertilised zygotes were marked at the fertilisation cone, which forms above male chromatin and indicates the sperm entry position, as previously described (Piotrowska and Zernicka-Goetz, 2001). Briefly, fluorescent (FITC labelled) beads (approximately 3 μm diameter; Polysciences, Inc.) were placed in FHM medium containing 300 μg/ml phytohaemagglutinin for 30 minutes and then transferred to a 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 to place the bead in contact with the membrane of the fertilisation cone. Once the bead had adhered, the micropipette was withdrawn.

Such activated or fertilised eggs were transferred into KSOM medium supplemented with amino acids (KSOM+AA) and with 4 mg/ml of BSA (Speciality Media, Inc. Lavallette, NJ) and cultured in 5% CO_2 and at 37°C to the 2-cell stage when their blastomeres were labelled. Embryos were observed under an inverted (Leica) microscope using DIC optics and micromanipulated with Leica micromanipulators using a De Fonbrune suction-force pump.

Micromanipulation

The additional series of experiments was designed to assess the role of the position at which sperm entered the egg. Zygotes were recovered shortly after fertilisation and their fertilisation cones were labelled with a fluorescent bead as described above. Such eggs were cultured further, until the male pronucleus had migrated towards the egg centre (approximately 3 hours after fertilisation), at which stage the region of the egg that was marked by the bead was removed by micromanipulation using techniques similar to those described by McGrath and Solter (McGrath and Solter, 1983). This operation removed approximately 13% of the egg volume. The site from which the bead marking the sperm entry was removed was relabelled by attaching another fluorescent bead. In a group of control eggs another region of the cortex of the zygote (approximately 90° from the visible fertilisation cone) was removed instead.

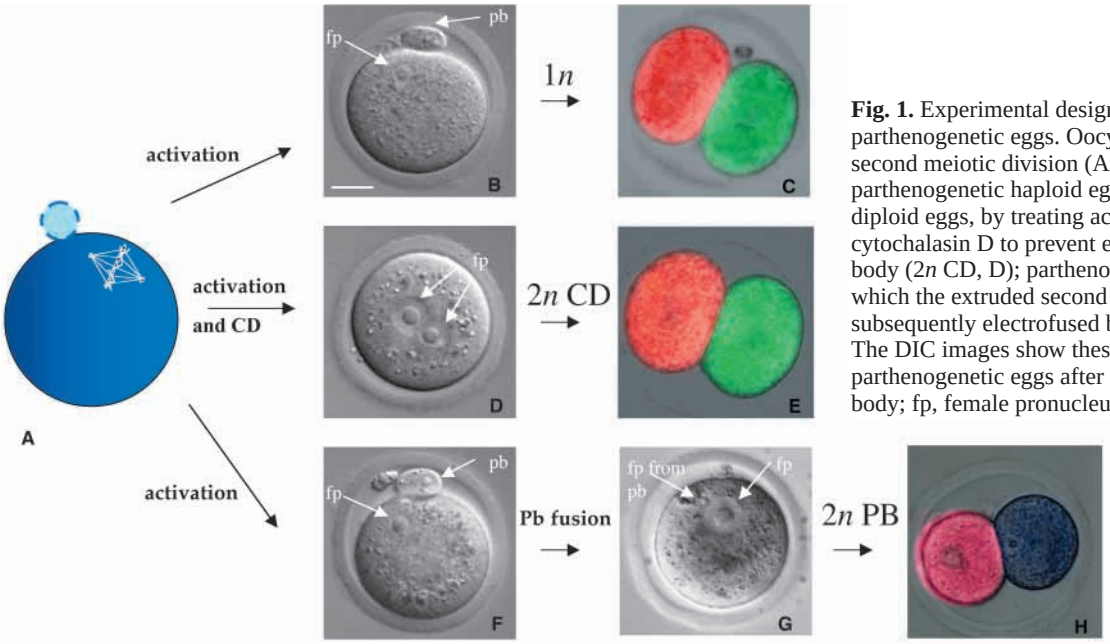

Fig. 1. Experimental design: generating parthenogenetic eggs. Oocytes in metaphase of the second meiotic division (A) were activated to obtain: parthenogenetic haploid eggs (1*n*, B); parthenogenetic diploid eggs, by treating activated eggs with cytochalasin D to prevent extrusion of the second polar body (2*n* CD, D); parthenogenetic diploid eggs in which the extruded second polar body (F) was subsequently electrofused back to the egg (2*n* PB, G). The DIC images show these three types of parthenogenetic eggs after their generation. pb, polar body; fp, female pronucleus. The merged fluorescent and DIC images (C,E,H) show 2-cell stage embryos that developed from these activated eggs in which each blastomere was labelled with a different coloured dye. Scale bar: 25 μm.

Labelling of blastomeres

DiI, DiD or DiO (Molecular Probes) was 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, which absorbed the dye. Embryos with blastomeres labelled with dyes of different colours were subsequently cultured in KSOM+AA medium in 5% CO_2 and at 37°C. They were first observed every 30-40 minutes during their 2- to 4-cell stage transition to evaluate the order of blastomere division and finally at the expanding blastocyst stage when they were analysed by confocal microscopy.

Analysis

Confocal analysis of blastocysts was performed on live embryos. Blastocysts were observed by taking optical sections every 7 μm. By examining all sections in each series, it was possible to determine the distribution of labelled cells into the embryonic part (a part including the polar trophectoderm and 'deeper' cells of the inner cell mass – ICM), abembryonic part (a part including mural trophectoderm**)** and a boundary zone between them. The boundary zone between these two parts was defined as a layer approximately one cell deep and parallel to the blastocoelic surface of the ICM as suggested in a previously described model (Piotrowska and Zernicka-Goetz, 2001). In the first analysis, blastocysts were scored depending upon the degree to which predominantly embryonic or abembryonic clones extended beyond this boundary zone according to the criteria defined in the legend to Fig. 2. The angle between the clonal border and the boundary zone was defined by examining a series of eight to ten confocal sections for each parthenogenetic blastocyst to evaluate both the position of the clonal borders and the blastocoel, which we drew as a line at the mid-points between cell boundaries, and tangential to the cavity respectively.

In a separate analysis of the distribution of descendants derived from the first versus second 2-cell blastomere to divide, we scored the cellular composition of all three parts of the blastocyst: embryonic, abembryonic and the boundary zone. Owing to the uneven labelling of cell membranes with dyes used here and lack of definition of the boundaries between the cells, it was difficult to obtain precise counts of cell numbers of intact embryos, therefore these counts should be regarded only as estimates. To be able to calculate a total number of cells derived from each (early and late dividing) blastomere in the whole blastocyst, we dissociated each of the embryos into individual cells and counted them. To this end, after confocal microscopy, we briefly exposed the blastocysts to acid Tyrode's solution to remove the zona pellucida and then we treated them with 0.5% trypsin (in Hank's buffered saline with 0.04% EDTA) for 5 minutes at 37°C before dispersing them into individual cells using thorough pipetting. Each cell in the blastocyst was either completely or substantially labelled by one of the two dyes.

RESULTS

Experimental design: generating parthenogenetic eggs

In order to gain insight into the extent to which sperm penetration acts as a positional cue for the development of pattern in preimplantation mouse embryos or whether early patterning is exclusively an intrinsic property of the oocyte, we generated three types of artificially activated eggs (Fig. 1). The first group comprised eggs that were activated by treatment with 7% ethanol (Materials and Methods) and so extruded their second polar body and therefore remained haploid. However, as haploid embryos lack half of the normal chromosome set and so could be argued to have somewhat compromised development, we used two different ways to generate diploid parthenogenetic eggs. Thus, a second group of eggs was artificially activated, but extrusion of the second polar body was inhibited by cytochalasin treatment, generating diploid embryos. Because this treatment interferes with the second meiotic division that itself might contribute to the orientation of the first cleavage, our third group consisted of activated eggs that were allowed to complete the meiotic division, but their extruded second polar bodies were then rejoined to the eggs by electrofusion.

To follow the fate of descendants derived from 2-cell blastomeres and their contribution to specific parts of the blastocyst we marked them with dyes of different colours (Piotrowska et al., 2001). The distribution of the two types of progeny was analysed by dividing the blastocysts into 3 parts: embryonic, abembryonic and a boundary zone between them

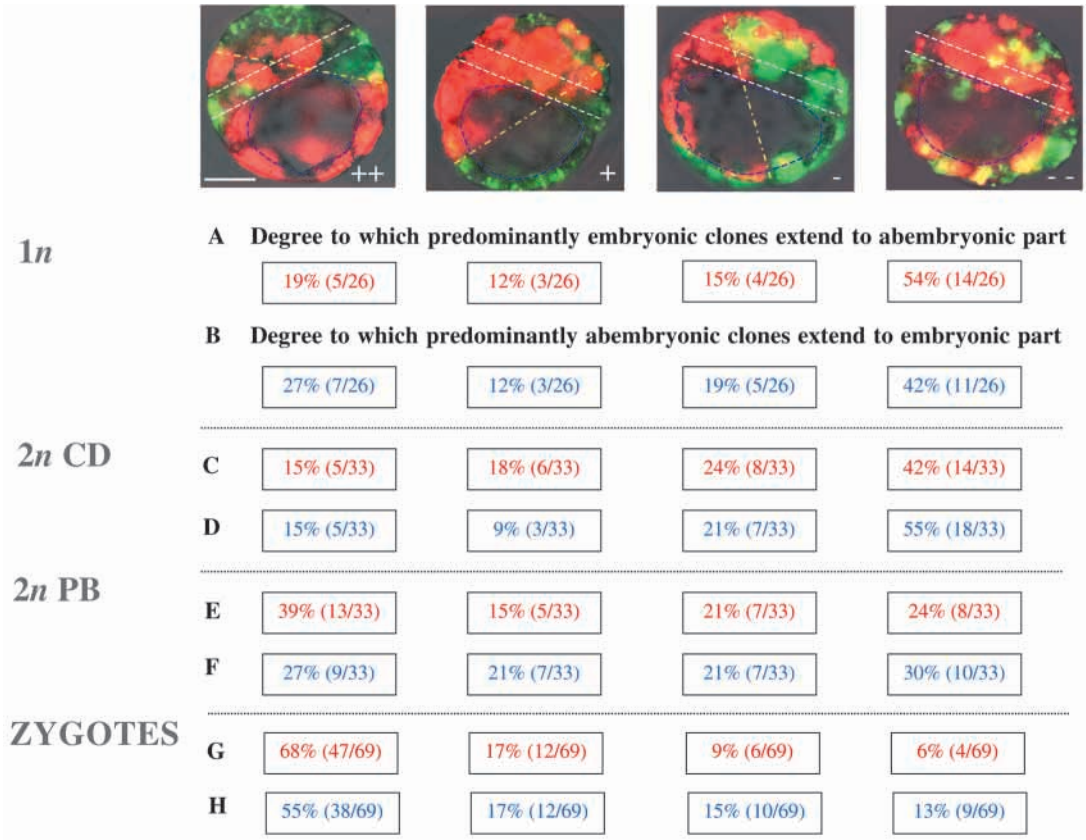

Fig. 2. Clones derived from the 2-cell blastomeres of the parthenogenetic embryos do not respect an embryonic-abembryonic boundary in the blastocyst. Blastomeres of 2-cell embryos were labelled with different coloured dyes and the distribution of the progeny of labelled cells were analysed at the blastocyst stage. The frequencies of the four categories of blastocyst scored are indicated. Blastocysts of 4 different groups of embryos: parthenogenetic haploid eggs (A,B); parthenogenetic diploid eggs treated with cytochalasin (C,D); parthenogenetic diploid eggs in which the polar body was fused back to the embryo (E,F); and fertilised eggs (G,H) [data from Piotrowska et al. (Piotrowska et al., 2001)]. Blastocysts were scored ++ if 0, 1 or 2 cells crossed the boundary zone. In cases where 3 cells crossed the boundary, blastocysts were scored +. When 4-5 cells, or more than 5 cells failed to respect the boundary they were scored – and – –, respectively. In the table, the degree to which predominantly embryonic clones extend to the abembryonic part are shown in red. The degree to which abembryonic clones extend to the embryonic part are shown in blue. The micrographs represent individual optical sections mid-way through the embryo to show the cavity, which occupies the lower half of each blastocyst. The boundary zone is marked with white dashed lines and the border of the blastocoel has been traced on to a central section and is shown projected onto each of the other sections as a blue dashed line. The clonal border is marked with a yellow dashed line. The examples shown in the micrographs are all from haploid parthenogenetically activated eggs. Scale bar: 25 μm.

defined as a layer approximately one cell deep and parallel to the blastocoelic surface of the ICM, as previously described (Piotrowska et al., 2001). 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 or the blastocoel, respectively. Two types of analysis enabled direct comparisons to be made with the distribution of progeny of blastomeres in fertilised embryos (Piotrowska et al., 2001). The first excluded cells lying in the boundary zone and assessed the extent to which clones derived from each 2-cell blastomere extended beyond it. The second analysis focused on the specific contribution of descendants of the first versus the second 2-cell blastomere to divide into each of the three parts of the blastocyst: embryonic, abembryonic and the boundary zone. We also evaluated the relationship between the clonal border (the interface between descendants of the 2-cell blastomeres, a reflection of the first cleavage plane) and the boundary zone (the morphological division of the blastocyst into its embryonic and abembryonic parts) for each embryo.

Parthenogenetic eggs differ from fertilised eggs in the fate of their 2-cell blastomeres

In the first analysis of the distribution of the progeny of 2-cell parthenogenetic blastomeres, blastocysts were classified into four categories depending upon the extent to which they conformed to the expectation (based on zygotes) (Piotrowska et al., 2001) that the embryonic part would be derived predominantly from one 2-cell blastomere and the abembryonic part from the other. The progeny of each 2-cell blastomere were scored separately according to the number of its descendants that instead of being localised in either the embryonic or abembryonic part, had come to lie in the opposite part of the blastocyst, beyond the boundary zone. Blastocysts in which the 2-cell blastomere progeny occupied predominantly either the embryonic or abembryonic part, thus having only 3 or fewer cells of the clone (up to approximately 10% of the total cell number at this stage) lying beyond the designated boundary zone, were scored as either ++ (0-2 cells beyond) or + (3 cells beyond). If more than 3 cells were found on the other side of the boundary zone, blastocysts were scored as either – (4 cells beyond) or – – (5 or more cells beyond) (Fig. 2A-H).

Haploid parthenogenetic eggs

We found that the pattern of blastocysts in haploid parthenogenotes was very variable. Thus, only 31% (8/26) and

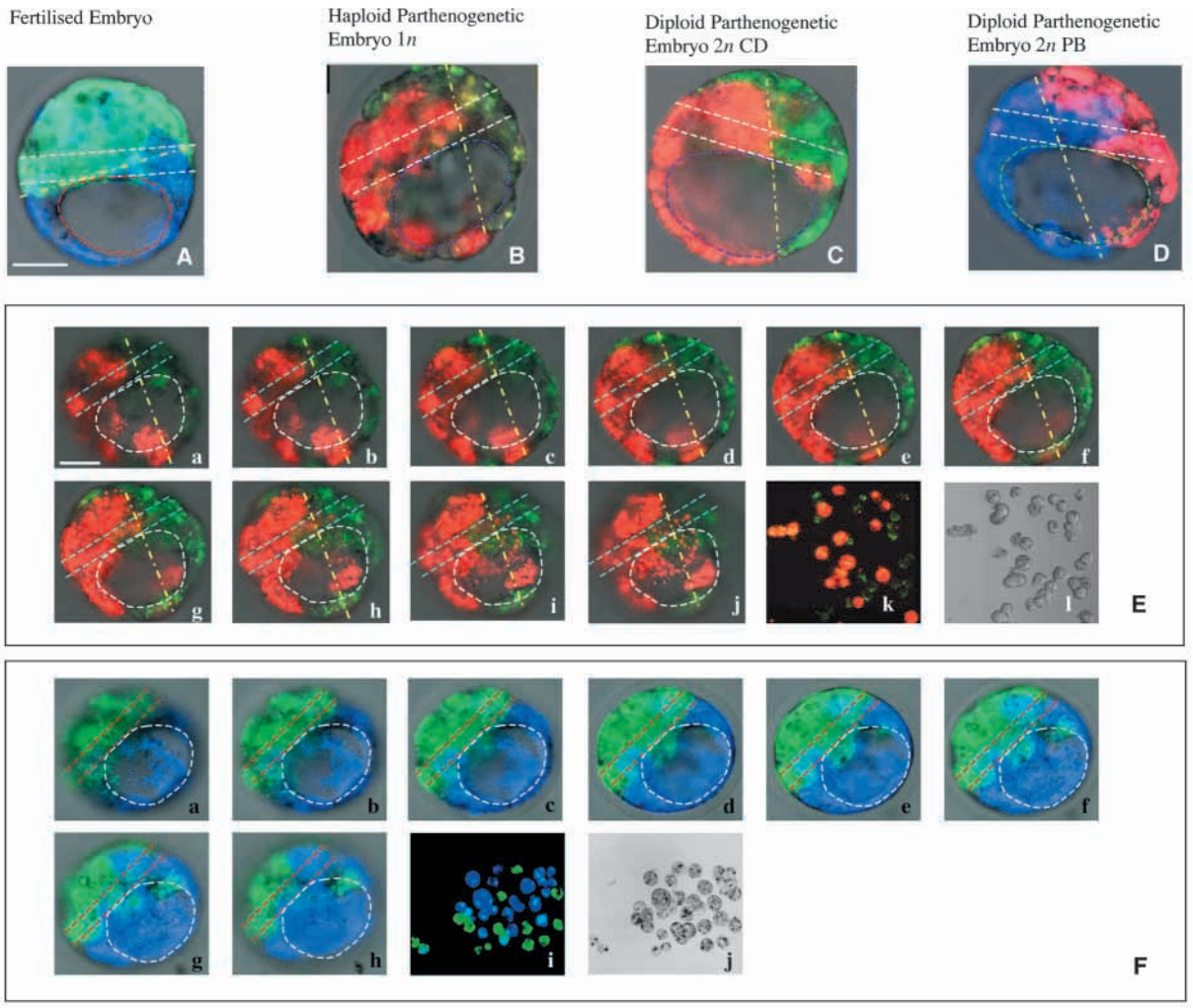

Fig. 3. Comparison of the boundaries between clones derived from 2-cell blastomeres in zygotes and parthenogenetically activated eggs. (A-D) Individual confocal sections of blastocysts in which the clonal border of the 2-cell stage progeny (yellow dashed line) is tilted with respect to the boundary zone between the embryonic and abembryonic parts (white dashed lines). Blastomeres of fertilised or parthenogenetically activated embryos labelled with different coloured dyes at the 2-cell stage and cultured to the blastocyst stage. The four examples are of the frequently found patterns of clonal distribution in fertilised eggs (A), haploid parthenogenetically activated eggs (B), diploid parthenogenetically activated eggs generated by cytochalasin treatment (C) or electrofusion (D). (E) Series of confocal sections of an individual blastocyst developed from parthenogenetic diploid cytochalasin-treated embryo. The boundary zone is marked with white dashed lines and the border of the blastocoel has been traced on to a central section and is shown projected onto each of the other sections as a red, blue or green dashed line. The clonal border is marked with a yellow dashed line. Panels a-j show individual optical sections at 7.5 μm intervals as a 'z-series'. Panels k and l show the dissociated cells of this blastocyst observed by fluorescence or DIC optics respectively. Note that all cells are labelled but not uniformly throughout. (F) Series of confocal sections of the individual blastocyst developed from a fertilised embryo. The boundary zone is marked with red dashed lines and the border of the blastocoel was traced on to a central section and is shown projected onto each of the other sections as a white dashed line. Panels a-h show individual optical sections at 7 μm intervals as a 'z-series'. Panels i and j show the dissociated cells of this blastocyst observed by fluorescence or DIC optics respectively. Scale bar: 25 μm (in A for A-D and E for E,F).

39% (10/26) of blastocysts had clones that occupied predominantly the embryonic or abembryonic parts respectively (++ and + categories in Fig. 2A,B). This contrasts with 85% and 72% in the comparable categories in the case of fertilised embryos [Fig. 2G-H, data from Piotrowska et al. (Piotrowska et al., 2001)]. Half (54%) of haploid parthenogenetic embryos, compared to 6% in zygotes, fell into the – – category in which the spatial orientation of the border between 2-cell blastomere clones departs dramatically from the boundary zone (Fig. 2, another example of this distribution is shown in Fig. 3B). Thus in contrast to fertilised eggs, the majority of progeny of each 2-cell blastomere in haploid parthenogenotes tended not to lie exclusively in either the embryonic or abembryonic parts of the blastocyst, but was distributed throughout the embryonic-abembryonic axis. When we measured the angle between the clonal border and the embryonic-abembryonic boundary in parthenogenetic blastocysts we also found it to be much greater (59°±25°, Table 1 and 46°±38°, Table 2; also see below) in comparison with that of fertilised embryos (26°±19°) (Piotrowska et al., 2001). Thus, this second assessment confirms that the position of the border of clones derived from each 2-cell blastomere bears no

Table 1. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributes more cells to the blastocyst*

Name (number of blastocysts)	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1*n* (14)	36.8	20.1	14.5	64	5.4	5.6	16.7	11.3	52	5.6	5.5	11.0	49	51	59.2
2*n* CD (13)	39.3	20.8	16.5	67	6.0	4.2	18.5	10.9	60	5.9	5.9	11.9	50	50	43.3
2*n* PB (22)	36.9	20.2	16.8	66	5.1	4.0	16.7	9.8	58	5.2	5.8	10.3	49	51	39.7
No SEP (14)	33.1	18.1	13.2	53	5.5	5.6	15.1	9.9	43	4.5	6.3	10.0	55	45	40.6
Con SEP (17)	31.6	17.6	13.2	84	4.2	2.4	13.9	9.2	76	4.9	2.2	9.1	45	55	30.2

*Distribution of dye-labelled cells in blastocysts was determined by estimating the number of cells occupying the boundary zone or crossing to the far side of the boundary zone and lying in the other part of the blastocyst, as described in Materials and Methods. The number of cells derived from the early- dividing and late-dividing 2-cell blastomere were counted by dissociating each blastocyst after 3D 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.

Abbreviations: BZ, boundary zone between the embryonic and abembryonic parts, consisting of a layer beginning at the blastocoel surface of the ICM and extending into the embryonic region to a depth of approximately one cell (thus including both ICM and polar trophectoderm). Tilt, the approximate angle between the plane of the BZ and the plane of the clonal border (see Materials and Methods). 1*n*, haploid parthenogenetic embryos; 2*n*, diploid parthenogenetic embryos, either from cytochalasin treatment (CD) or after electrofusion (PB); no SEP, fertilised eggs with the sperm entry point (SEP) removed; Con SEP, control fertilised embryos from which a fragment of the cytoplasm was removed from an area other than the SEP.

Mean values are indicated; for full dataset see Tables 1A, 2A, 3A, 4A and 5A presented as Supplemental data: http://dev.biologists.org/supplemental.

Table 2. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributes more or even number of cells to the blastocyst*

Name (number of blastocysts)	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1*n* (12)	31.6	14.5	11.8	43	4.0	5.4	17.1	10.4	49	5.3	6.8	9.3	44	56	45.6
2*n* CD (11)	38.3	18.5	15.4	56	5.3	4.7	19.7	11.6	60	6.0	6.8	11.3	46	54	48.4
2*n* PB (11)	34.4	15.4	14.7	36	5.1	5.1	19.0	9.5	45	5.1	9.5	10.2	50	50	30.6
No SEP (13)	31.5	14.6	13.4	51	3.5	4.3	16.8	9.2	54	5.4	6.5	8.8	39	61	55.4
Con SEP (7)	27.4	12.3	11.7	42	3.1	4.6	15.1	7.9	45	4.7	7.1	7.9	40	60	45.0

*Mean values are indicated; for full dataset see Tables 1B, 2B, 3B, 4B and 5B presented as Supplemental data: http://dev.biologists.org/supplemental.
Counts of blastocyst cells were performed as described for Table 1.

relationship to the embryonic-abembryonic axis in the majority of haploid parthenogenetic blastocysts.

Diploid parthenogenetic eggs

The spatial pattern of diploid parthenogenetic blastocysts also substantially differed from the pattern that developed after fertilisation. Specifically, in the group of embryos generated by activating the oocytes and then treating them with cytochalasin to prevent the extrusion of the second polar body (2*n* CD; Fig. 1), only a minority of blastocysts were scored within the ++ and + categories: 33% (11/33) and 24% (8/33) of blastocysts, depending whether clones occupied predominantly the embryonic or abembryonic part respectively (Fig. 2C,D). In this group of parthenogenotes, 42% (14/33) were scored in the – – category (example shown in Fig. 3C). The angle between the clonal border and the embryonic-abembryonic boundary was also greater than in fertilised eggs (Tables 1 and 2).

In diploid parthenogenetic embryos that were allowed to extrude then made to regain their second polar bodies (2*n* PB, Fig. 1), 54% (18/33) or 48% (16/33) of blastocysts were scored within the ++ and + categories, depending whether clones occupied predominantly the embryonic or abembryonic part respectively (Fig. 2E,F). In this group of activated embryos 24% (8/33) were scored in the – – category and thus showed a deviation between the spatial orientation of the clonal border and the embryonic-abembryonic boundary (example shown in Fig. 3D). There was also a substantial angle between the clonal border and the embryonic-abembryonic boundary in this group of embryos (Tables 1 and 2).

Thus, the clonal border between 2-cell blastomeres progeny in haploid and diploid parthenogenetic embryos did not tend to predict the boundary between embryonic and abembryonic parts of the blastocyst in the majority of embryos as it did in fertilised eggs. The most extreme difference in distribution of 2-cell blastomere progeny between fertilised and parthenogenetic embryos was seen in the haploid parthenogenotes and the least difference was seen in diploid parthenogenotes generated by electrofusion. Nevertheless, the distribution of embryos into analysed categories (++ and +, –, and – –) was significantly different between fertilised eggs and each of the three types of parthenogenetically activated eggs ($P<0.001$, χ^2 test 2d.f. in each case). When blastocyst patterning was assessed by measuring the variation in the tilt between the clonal border and the boundary zone it was found to be random in parthenogenotes, while in fertilised embryos it showed a non-random distribution (Piotrowska et al., 2001). Together these results indicate that the orientation of embryonic-abembryonic axial polarity is not solely an intrinsic

property of the egg itself and indicates that fertilisation plays a role in establishing it.

Parthenogenetic eggs differ from fertilised eggs in that the first 2-cell blastomere to divide does not necessarily contribute a majority of cells to the blastocyst

The differences in behaviour of clones in parthenogenetic embryos versus fertilised ones was further clarified by determining the number of cells in the blastocyst descended from each 2-cell blastomere. In fertilised eggs one blastomere cleaves ahead of the other at the 2- to 4-cell stage transition. The first dividing blastomere contributes more cells to the blastocyst (Piotrowska et al., 2001). This is not necessarily a result of the persistence of shorter subsequent cell cycles in this lineage and may be accounted for solely by the earlier division at the first cycle (Kelly et al., 1978; Graham and Deussen, 1978). Because in fertilised embryos it is the progeny of the 2-cell blastomere inheriting the sperm entry position that have been shown to maintain a division advantage, we wondered whether a similar tendency to divide earlier would be maintained in parthenogenetic blastomeres.

To assess this we analysed the number of cells in the blastocyst derived from the first and the second blastomere to divide in all three groups of parthenogenotes (Fig. 3E,F). We found that while in the majority (76%, 37/49) (Piotrowska et al., 2001) of fertilised embryos the 2-cell blastomere that divides earlier contributes more cells to the blastocyst, this was not the case for parthenogenetic embryos. Only in half (54%, 14/26) of haploid parthenogenotes did the first 2-cell blastomere to divide contribute more cells to the blastocyst (Table 1). In the remaining embryos, the 2-cell blastomere dividing first contributed either an equal number or fewer cells (Table 2). A similar conclusion was reached following the analysis of diploid parthenogenetic eggs generated by cytochalasin treatment. Again only half (55%, 13/24) of embryos showed the first 2-cell blastomere to divide to contribute more blastocyst cells (Tables 1 and 2). However diploid parthenogenetic embryos in which the second polar body had been electrofused back into the egg appeared to behave more like fertilised embryos in this respect. In this group the proportion of embryos in which the first blastomere to divide made a greater contribution to the blastocyst was higher (67%, 22/33) than in the two other groups of parthenogenetic embryos (Table 1 and Table 2). Thus fertilisation appears to have an effect on the timing of cell division that to some extent might be mimicked by the experimental manipulations to generate parthenogenetic embryos through electrofusion.

To confirm that fertilised and parthenogenetic embryos differ in their ability to maintain the order of their blastomere divisions, we followed the first two cleavages in two such groups. In fertilised embryos, we first marked the fertilisation cone (appearing at the site of sperm entry) with fluorescent beads. In agreement with our previous observations (Piotrowska and Zernicka-Goetz, 2001), we found that the bead marked the first dividing blastomere in 47% of 30 analysed embryos (group A), was found between the two blastomeres in 26.5% of embryos (group B), and was associated with the later dividing blastomere in the 26.5% of embryos (group C). After labelling 2-cell blastomeres with dyes we found that in the great majority of embryos from group A (93%, 13/14), the first dividing 2-cell blastomere again divided first, up to the 5-cell stage. In 62.5% of embryos from group B the first blastomere to divide again divided earlier, while in group C, there was an equal chance of first and second blastomere to divide to enter the subsequent division before their sisters. In the case of parthenogenotes (1*n*) only in 60% (12/20) of embryos did the 2-cell blastomere that divided first also divide first in the next cleavage division (to the 5-cell stage). When the same group of embryos was analysed at the 8-cell stage both 2-cell blastomeres had contributed an equal number of cells in 85% (17/20) of cases. In 10% (2/20) of the remaining embryos, 5 cells were derived from the earlier-dividing blastomere and in 5% (1/20), 5 cells were derived from the later dividing one. Thus, both sets of observations lead to the same conclusion: that while in fertilised eggs there is a strong tendency for at least some of the progeny of the first blastomere to divide to maintain their division advantage this is not the case in eggs not penetrated by sperm.

The order of division alone appears insufficient to establish the fate of the 2-cell blastomeres

Our studies showed that parthenogenetic eggs differ from fertilised eggs in that the discrepancy in division times between the 2-cell blastomeres progeny was not preserved during development. Thus, we wondered whether this could account for differences in embryonic pattern. If so this would mean that the patterning develops exclusively on the basis of cleavage division order. A way to test this possibility was for us to focus our analysis on the subset of activated embryos that behaved more like fertilised eggs, in that the first blastomere to divide contributed more cells to the blastocyst. We then asked if this subset behaved similarly to fertilised eggs in showing a strong preferential contribution of the first dividing 2-cell blastomere to the embryonic part (Table 1 and Tables 1A, 2A and 3A in Supplemental data: http://dev.biologists.org/supplemental/). We found that it did not in any of the three groups of parthenogenetic eggs.

Our earlier study of fertilised eggs included an analysis of both early and late blastocysts (Piotrowska et al., 2001). If those data are reanalysed, focusing only on fertilised embryos in which the first blastomere to divide contributed a majority of the blastocyst cells, we find that on average 86% of the embryonic part is derived from this cell. This value differs statistically from the corresponding value for all three groups of parthenogenetic embryos (*t*-test, $P<0.001$). Specifically, in the group of haploid parthenogenotes, on average only 64% of the embryonic part consisted of the progeny of the first blastomere to divide (Table 1 and Table 1A in Supplemental data: http://dev.biologists.org/supplemental/). In the same group of embryos, on average only 52% of the abembryonic part consisted of progeny of the second blastomere to divide. Most noticeably, there was no single embryo in which the first blastomere to divide made an exclusive or nearly exclusive (≥90%) contribution to the embryonic part (Table 1A in Supplemental data: http://dev.biologists.org/supplemental/). This was in contrast to fertilised eggs, in which in a substantial proportion of embryos the first blastomere to divide made an exclusive (6/49) or ≥90% (14/49) contribution to the embryonic part (Piotrowska et al., 2001). In those parthenogenetic embryos in which the second blastomere to divide contributed more blastocyst cells, slightly more than

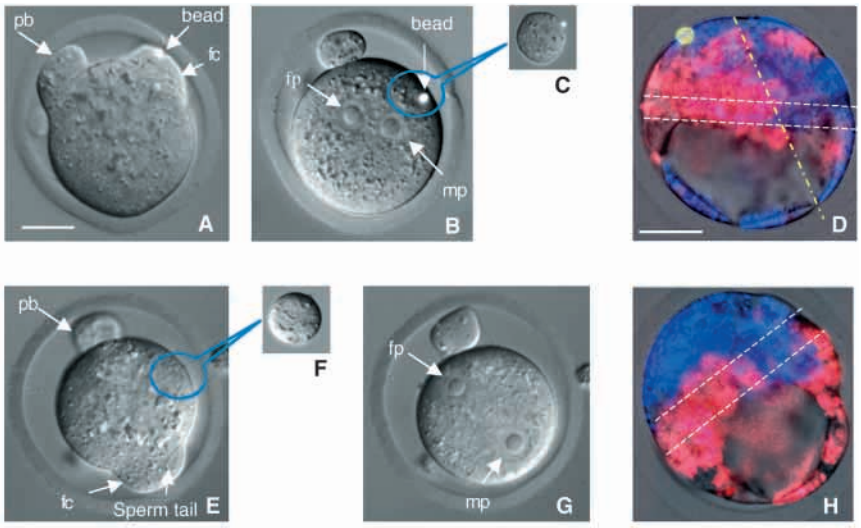

Fig. 4. Disruption of the cortical cytoplasm associated with the position of the sperm entry disturbs spatial patterning of the blastocyst. (A) Fertilisation cone (fc) of freshly fertilised eggs was marked by attaching a fluorescent bead at the time of polar body (pb) extrusion. (B) 2-3 hours later, after the male pronucleus (mp) started to migrate toward the female pronucleus (fp) at the egg centre, the cortex and the associated cytoplasm marked by the bead was removed. (C) Fragment of excised cytoplasm. Next, the site of the operation was re-labelled with another fluorescent bead (not shown). Blastomeres at the 2-cell stage were labelled with different coloured dyes and the distribution of cells was examined by the confocal sectioning at the blastocyst stage. (D) An individual section of such a blastocyst. The clonal border of the 2-cell stage progeny (marked by yellow dashed line) is tilted with respect to the boundary zone between the embryonic and abembryonic parts (white dashed lines). A fluorescent bead (pale green) is visible. (E) A control experiment in which another region of the cortex of the zygote, approximately 90° from the fertilisation cone, was removed instead. (F) Fragment of excised cytoplasm. Resulting eggs from such a manipulation (G) were labelled at the 2-cell stage as before and cultured to the blastocyst stage. (H) An individual section of such a blastocyst showing distribution of labelled cells. Scale bar: 25 μm (in A for A,B,C,E,F,G and In D for D,H).

half of the embryonic part (57%) consisted of its descendants. In this group there was only one embryo (1/12) in which the second blastomere to divide made a nearly exclusive contribution to the embryonic part (Table 1B in Supplemental data: http://dev.biologists.org/supplemental/).

This outcome was similar whether the parthenogenetic embryos were haploid or diploid. In the subset of diploid parthenogenotes treated with cytochalasin in which the first blastomere to divide contributed more cells, it contributed on average 67% of the embryonic part (Table 1 and Table 2A in Supplemental data: http://dev.biologists.org/supplemental/). In the other subset, in which the second blastomere to divide contributed more or an equal number of cells, it contributed 44% of the embryonic part (Table 2 and Table 2B in Supplemental data: http://dev.biologists.org/supplemental/). Similarly in a comparable subset of diploid parthenogenetic embryos generated by electrofusion, an average of 66% of the embryonic part was occupied by the progeny of the first blastomeres to divide when they contributed more cells to the blastocysts (Table 1 and Table 3A in Supplemental data: http://dev.biologists.org/supplemental/). Moreover, no single embryo in this group showed an exclusive (and only one ≥90%) contribution of early dividing descendants to the embryonic part.

Taken together these results indicate that unlike fertilised eggs, there is not a strong tendency for the 2-cell blastomeres of parthenogenetic eggs to follow embryonic or abembryonic fates. Moreover, this does not appear to be attributable to an exclusive role for sperm in influencing the timing of the second cleavage because even when the progeny of the first parthenogenetic blastomere to divide maintain a division advantage, they do not show such a strong predisposition to occupy the embryonic part of the blastocyst, as observed in fertilised eggs.

Removal of the cortical cytoplasm associated with the position of sperm entry in zygotes disturbs spatial patterning of the blastocyst

The above experiments indicated that fertilisation provides a bias in establishing the fate of blastomeres that is not achieved solely by an effect on the timing of cell division. Fertilisation of the mouse oocyte results in at least 3 events: introduction of the male set of chromosomes and other sperm components into the egg, global egg activation as well as localised changes at the site of the sperm entry. Because previous experiments observed a correlation between the position of the fertilisation cone at the site of sperm entry and the pattern of cleavage (Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002b), we decided to test directly the importance of localised events imposed upon the blastocyst axial organisation by sperm penetration. With this aim we surgically removed cortical cytoplasm either at the site of sperm entry (Fig. 4A-D) or, in a control group of embryos, from elsewhere on the embryo surface (Fig. 4E-H). At the 2-cell stage we labelled blastomeres, observed their order of division to the 4-cell stage, and then allowed the embryos to develop to the blastocyst.

When we analysed the distribution of blastocyst cells between the embryonic and abembryonic parts with respect to the boundary zone, only 37% (10/27) of embryos in which cortical cytoplasm associated with sperm entry was removed fell into the ++ and + category when the embryonic part was analysed, and 52% (14/27) when the abembryonic part was considered. This compared with 75% (18/24) and 79% (19/24), respectively, of control manipulated embryos. When the sperm entry-associated cortex was removed, 30% (8/27) of blastocysts scored in the – – category, compared with only 4% (1/24) in the control group. Thus while the control manipulated eggs are similar to non-manipulated fertilised eggs, in that descendants of the 2-cell blastomeres tend to occupy either the embryonic or abembryonic parts, this tendency is lost following surgical removal of cortical cytoplasm around the site of sperm entry.

An analysis of the timing of the division of the 2-cell blastomeres' progeny led to a similar conclusion. Out of 27 blastocysts developed from eggs with the sperm entry cortical cytoplasm removed, only half (14/27) had a greater number of

cells derived from the first dividing blastomere (Tables 1 and 2; Table 4A and 4B in Supplemental data: http://dev.biologists.org/supplemental/). Also there was no tendency of this first 2-cell blastomere to divide to contribute the majority of the embryonic part. Thus on average only 53% of the embryonic part was derived from the first blastomere to divide when this contributed a greater proportion of the blastocyst cells and 51% when it contributed a lesser portion (Tables 1 and 2). These results indicate that in fertilised embryos lacking cortical cytoplasm around the sperm entry there was a roughly equal possibility of the embryonic part being developed from either 2-cell blastomere. By contrast, when a sector of cortical cytoplasm was removed from the sperm entry site, the tendency for the first dividing 2-cell blastomere to contribute more cells to the blastocyst remained. Although not as dramatic as in non-manipulated embryos it was still seen in 71% (17/24) of blastocysts (Tables 1 and 2). In those control manipulated blastocysts in which there were more cells derived from the first dividing 2-cell blastomere, it contributed on average 84% of the embryonic part (Table 1; Table 5A in Supplemental data: http://dev.biologists.org/supplemental/). This figure is similar to a contribution of 86% of the embryonic part by the first dividing blastomere in non-manipulated fertilised embryos (Piotrowska et al., 2001). Moreover, within this control group, the earlier dividing 2-cell blastomere made an exclusive or nearly exclusive contribution to the embryonic part in 47% (8/17) of embryos (Table 5A, in Supplemental data: http://dev.biologists.org/supplemental/). This was not observed in the embryos with the sperm entry-associated cortical cytoplasm removed. Thus despite the surgical manipulation of control eggs there is relatively little loss of normal embryo patterning. We conclude that the disruption of the cortical cytoplasm specifically in the region of sperm penetration does indeed influence the pattern of blastocyst development.

DISCUSSION

In the majority of fertilised mouse eggs the first cleavage gives cells that show a tendency to follow distinguishable fates – one divides first and contributes predominantly embryonic cells while the other shows predominantly an abembryonic fate (Piotrowska et al., 2001). Here we show that such tendency of two halves of the egg to follow these different destinies is missing in embryos that develop without sperm penetration. Moreover, whereas in fertilised embryos the first 2-cell blastomere to divide contributes more cells to the blastocyst, the first dividing blastomere in parthenogenetic embryos does not necessarily do so. Both of these observations point to specific events associated with sperm penetration that participate in biasing the fate of 2-cell embryo blastomeres.

Because the plane of the first cleavage division correlates with both the position of the previous meiotic division – the animal pole (Plusa et al., 2002a) and the position of the fertilisation cone that marks the sperm entry (Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002b), the question arises of the extent of the role of the oocyte, and that of the sperm in setting up early embryo patterning. It could be argued that allocation of the progeny of 2-cell blastomeres in diploid parthenogenetic blastocysts differs from that in normal embryos because their animal pole has been perturbed by either cytochalasin treatment or electrofusion. This could disturb factor(s) at the animal pole that influence the first cleavage orientation. For this reason we also examined two experimental situations in which the animal pole was not perturbed. But here again we found that development of embryonic-abembryonic patterning in relation to the first cleavage was lost. The first of these was in haploid parthenogenetic eggs. In this case, the second meiotic division was not perturbed and the resulting polar body subsequently came to lie between the 2-cell blastomeres. Thus the first cleavage divided the egg along the animal-vegetal axis as it did in fertilised eggs. Yet the two blastomeres did not follow predominantly embryonic or abembryonic fates. This suggests that the division of the embryo into its two distinct parts cannot exclusively depend on the information provided by the animal pole alone. That the failure of such embryos to develop normal polarity is related to the absence of fertilisation rather than haploid development per se, is indicated by experiments in which the cortical cytoplasm at the sperm entry position was surgically removed from fertilised eggs. In such surgically manipulated eggs, the first cleavage also continued to respect the spatial information provided by the animal pole, but it no longer divided the zygote into blastomeres with embryonic or abembryonic destinies. If cortical cytoplasm was removed in control embryos at approximately 90° to the sperm entry site then the first cleavage did tend to divide the egg into these two blastocyst parts. These experimental situations indicate that when sperm does not participate in development, the tendency for the embryo to be partitioned into embryonic and abembryonic parts at the first cleavage is lost.

Can we determine whether sperm polarises the embryo directly or exerts its effect through the order of cell division? Our findings that the patterning of the fertilised and parthenogenetic embryos are different and yet both show asynchronous early divisions seem at first sight to indicate that the sperm penetration may have a directly polarising effect. However, we have unexpectedly observed that, unlike fertilised eggs, the progeny of the first blastomere to divide in parthenogenetic eggs show no tendency to retain their division advantage. The one exceptional group in this respect are parthenogenetic eggs subjected to electrofusion. At present we cannot conclude whether the ability of one 2-cell blastomere to retain a division advantage in this group of eggs relates to a response to the electric field or the process of fusion that is mimicking some aspect of sperm penetration. Regardless of the explanation, this ‘electrofused’ group of parthenogenotes is similar to the other groups in showing no strong tendency for progeny of the first dividing 2-cell blastomere to occupy predominantly the embryonic part of the blastocyst as occurs in fertilised eggs. This is despite the net difference in number of progeny derived from the early dividing versus later dividing 2-cell blastomere in parthenogenetic embryos being often similar to fertilised ones. This therefore suggests that the order of cleavage itself might not be the sole factor responsible for assigning cells to the embryonic and abembryonic parts. It does not however preclude the possibility that in fertilised eggs the sperm might contribute to defining embryonic pattern by influencing the order of cleavage.

Taken together, our results provide a strong support for previous findings that fertilising sperm has a role in early

patterning of the embryo in normal, unperturbed development (Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002b). They do not support the notion of Davis and Gardner (Davis and Gardner, 2002) who concluded that since they could not detect a relationship between the localisation of sperm components within the egg and the first cleavage plane, fertilisation plays no role. Davis and Gardner (Davis and Gardner, 2002) also criticised the use of lectins to mark the region of the fertilisation cone in previous experiments, arguing that this method should preferentially mark the zona pellucida. However, they overlooked the observations that even zona-denuded eggs could be marked in this way and the marker still remained close to the cleavage plane in the majority of embryos (Plusa et al., 2002b). Their references to the Concanavalin A and phytohaemagglutinin binding properties of the zona pellucida and the egg surface (excluding the fertilisation cone) do not discredit the observation that the beads remained bound to the plasma membrane. While the precise mechanism for bead attachment is difficult to ascertain, it may have been due to initially weak affinities that are at later stages enhanced by recruitment of lectin-binding receptors from adjacent membrane. Davis and Gardner (Davis and Gardner, 2002) were also inaccurate in their criticism of control experiments to ensure that surface markers maintained their relative positions. Contrary to their assertion, a control with microspheres placed randomly at the equator of the egg as well as adjacent to, or diametrically opposite, the second polar body was performed (Piotrowska and Zernicka-Goetz, 2001). Furthermore, the validity of such markers was demonstrated by Plusa et al. (Plusa et al., 2002b) through the use of double labelling techniques to mark independent sites and thereby show that only the bead attached to the fertilisation cone and not one attached to a random position, tends to mark the first cleavage plane. The possibility cannot be ruled out that pressure placed on the fertilisation cone by positioning the marker bead could itself have influenced the plane of cleavage. Nevertheless, beads positioned in this way did come to lie on the boundary between the embryonic and abembryonic parts in the majority of blastocysts suggesting that 2-cell blastomeres have a tendency to follow distinguishable fates, a finding confirmed by lineage tracing studies (Piotrowska and Zernicka-Goetz, 2001; Piotrowska et al., 2001). Perhaps then the differences between the conclusions reached by ourselves and Davis and Gardner lie in different parameters that were scored by the two groups; cortical events and the fertilisation cone, on the one hand and internal events, localisation of sperm components taken up by the egg, on the other.

Where then does the ‘cue’ provided by sperm entry act? Since the partitioning of the embryo into its future embryonic and abembryonic parts by the first cleavage can be disturbed by changes in the cortical cytoplasm associated with the site of sperm entry, it can be concluded that the cue provided by sperm does not reside exclusively within, nor is it directly associated with, the male pronucleus. However, the relative positioning of the male and female pronuclei may be important for spindle orientation as their two sets of chromosomes do not mix for the first division cycle (Mayer et al., 2000). If this were the case, it is possible that a potential secondary effect of micromanipulation could be to disrupt the position of the male pronucleus in relation to the female counterpart and thereby disrupt patterning. We are unable to exclude this possibility. However as manipulating even control embryos could interfere with the position of male and female pronuclei, it seems more likely that the disruption of patterning following removal of the fertilisation cone site reflects rather the importance of the localised events at this site. Our study does not allow us to distinguish whether it is a response of the egg at the site of sperm entry itself or a local concentration of specific sperm components (Hewitson et al., 2002) that is important for early patterning. Either of these in turn might be reflected in the changes of organisation of the egg cortex we see after sperm entry that may be in some way analogous to the rearrangements of the cortex of the *Xenopus* egg that occur upon sperm penetration **(**Vincent and Gerhart, 1987; Gerhart, 1991).

Could it be that besides the information provided by the animal pole to orient the first cleavage, the egg has an additional role in establishing embryo polarity? We note that there is some patterning, although significantly reduced, in diploid parthenogenetic embryos that have been subjected to electrofusion. Does this mean that the egg has some inherent patterning and if so is it partially activated by electrofusion as it would be by fertilisation? We certainly take into account this possibility and at present cannot determine the extent to which the role of the sperm is to activate pre-existing elements of polarity in addition to provide a ‘cue’ that breaks the egg’s symmetry.

It is difficult to be certain of the later developmental consequences of the failure of the first cleavage to partition cells between embryonic and abembryonic parts in parthenogenetically activated embryos. This is because such embryos normally die shortly after implantation because of lack of expression from the paternally derived genes (McGrath and Solter, 1984; Barton et al., 1984). However, given that when a male pronucleus is transplanted to a parthenogenetic egg the resulting embryo can develop to term (Mann and Lovell-Badge, 1984) the early patterning events we describe here are unlikely to be essential for further development. Indeed, the mouse embryo is highly regulative in its development and thus there are likely to be means of countering spatial perturbations imposed at early developmental stages. Hopefully an analysis of the re-establishment of patterning following its perturbation should give us valuable clues to the mechanisms that operate in normal egg development.

We thank David Glover, Stephen Frankenberg, Jonathon Pines, Roger Pedersen and Chris Graham for their comments on the draft of this manuscript. M. Z. G. is grateful to the Wellcome Trust for their support. K. P. thanks Marie Currie for the Fellowship.

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Table 1A. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributed more cells to the blastocyst – 1*n

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	49	27	17	76	7	7	22	17	59	8	4	15	47	53	50
2	49	28	19	74	9	5	21	15	67	6	5	15	60	40	45
3	32	17	10	60	5	6	15	11	45	6	4	11	45	55	90
4	32	18	15	73	4	3	14	8	63	5	4	9	44	56	54
5	37	19	16	63	5	4	18	11	64	5	6	10	50	50	67
6	35	18	14	50	6	5	17	11	55	4	7	10	60	40	89
7	27	16	13	85	4	1	11	6	83	4	2	8	50	50	35
8	20	12	7	86	3	3	8	6	50	4	1	7	43	57	65
9	33	18	11	64	6	5	15	10	50	6	4	12	50	50	73
10	34	19	12	83	5	4	15	12	67	5	2	10	50	50	42
11	45	25	19	68	5	7	20	13	46	8	6	13	38	62	‡
12	35	18	15	53	6	4	17	10	60	4	7	10	60	40	80
13	37	20	13	31	6	10	17	13	23	5	9	11	55	45	80
14	50	26	22	27	5	15	24	15	0	8	16	13	38	62	0
Means	36.8	20.1	14.5	64	5.4	5.6	16.7	11.3	52	5.6	5.5	11.0	49	51	59.2
±s.d.	8.7	4.6	4.0	18	1.5	3.5	4.3	3.2	20	1.5	3.7	2.4	7	7	25.3

*Distribution of dye-labelled cells in blastocysts was determined by estimating the number of cells occupying the boundary zone or crossing to the far side of the boundary zone and lying in the other part of the blastocyst, as described in Methods. The number of cells derived from the early-dividing and 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. Abbreviations: BZ, boundary zone between the embryonic and abembryonic parts, consisting of a layer beginning at the blastocoel surface of the 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; s.d., standard deviation. Tilt, the approximate angle between the plane of the boundary zone and the plane of the clonal border (see Materials and Methods).

Table 1B. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributed more or even number of cells to the blastocyst – 1*n***

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	32	15	11	45	5	5	17	10	50	6	6	11	45	55	‡
2	24	11	3	33	5	5	13	13	62	3	2	8	63	38	90
3	28	13	10	50	3	5	15	10	50	5	5	8	38	63	‡
4	30	15	12	42	5	5	15	8	38	5	7	10	50	50	85
5	27	10	9	44	5	1	17	9	89	4	5	9	56	44	25
6	32	14	12	58	2	5	18	11	55	7	5	9	22	78	‡
7	31	15	12	50	6	3	16	10	70	3	6	9	67	33	‡
8	32	15	12	42	4	6	17	11	45	5	7	9	44	56	75
9	30	13	13	85	1	1	17	7	86	9	2	10	10	90	10
10	46	21	20	30	4	11	25	15	27	7	14	11	36	64	70
11	28	14	13	8	6	7	14	7	0	2	12	8	75	25	10
12	39	18	15	33	2	11	21	14	21	8	10	10	20	80	0
Means	31.6	14.5	11.8	43	4.0	5.4	17.1	10.4	49	5.3	6.8	9.3	44	56	45.6
±s.d.	5.8	2.9	3.9	18	1.7	3.2	3.2	2.6	26	2.1	3.6	1.1	20	20	37.8

*Counts of blastocyst cells were performed as described for Table 1A.
‡Mixed distribution of cells prevented assessment of the tilt in these embryos

Table 2A. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributed more cells to the blastocyst – 2*n* CD*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	32	18	13	100	4	1	14	8	88	7	0	11	36	64	10
2	39	20	20	25	7	8	19	8	0	4	15	11	64	36	43
3	37	21	18	83	6	0	16	8	100	5	3	11	55	45	35
4	43	22	16	75	7	3	21	13	77	7	4	14	50	50	45
5	40	21	17	71	6	3	19	11	73	6	5	12	50	50	8
6	39	21	14	71	6	5	18	12	58	7	4	13	46	54	20
7	42	22	14	79	7	4	20	14	71	7	3	14	50	50	19
8	40	21	15	67	6	5	19	14	64	5	5	11	55	45	50
9	43	23	17	65	6	6	20	13	54	7	6	13	46	54	70
10	41	22	14	86	5	5	19	15	67	7	2	12	42	58	60
11	47	24	26	31	8	8	23	9	11	4	18	12	67	33	89
12	35	18	15	53	6	4	17	9	56	5	7	11	55	45	44
13	33	17	15	67	4	3	16	8	63	6	5	10	40	60	70
Means	39.3	20.8	16.5	67	6.0	4.2	18.5	10.9	60	5.9	5.9	11.9	50	50	43.3
±s.d.	4.2	2.0	3.5	21	1.2	2.4	2.4	2.7	27	1.2	5.1	1.3	9	9	24.8

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

Table 2B. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributed more or even number of cells to the blastocyst – 2*n* CD*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	31	15	15	60	3	3	16	7	57	6	6	9	33	67	67
2	42	20	20	60	6	2	22	8	75	8	8	14	43	57	90
3	36	17	13	54	6	4	19	11	64	6	6	12	50	50	75
4	34	16	14	36	4	7	18	11	36	5	9	9	44	56	45
5	36	18	14	71	5	3	18	12	75	5	4	10	50	50	0
6	34	17	11	64	6	4	17	11	64	6	4	12	50	50	19
7	43	21	17	76	6	2	22	14	86	6	4	12	50	50	10
8	42	20	17	59	4	6	22	14	57	7	7	11	36	64	80
9	50	25	22	36	7	10	25	15	33	6	14	13	54	46	50
10	35	17	12	50	5	6	18	13	54	5	6	10	50	50	‡
11	38	18	14	50	6	5	20	12	58	6	7	12	50	50	72
Means	38.3	18.5	15.4	56	5.3	4.7	19.7	11.6	60	6.0	6.8	11.3	46	54	48.4
±s.d.	6.0	2.8	3.4	13	1.2	2.4	2.7	2.5	16	0.9	2.9	1.6	6	6	32.6

*Counts of blastocyst cells were performed as described for Table 1A.
‡Mixed distribution of cells prevented assessment of the tilt in these embryos

Table 3A. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributed more cells to the blastocyst – 2*n* PB*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	38	21	14	86	7	2	17	13	85	4	2	11	64	36	32
2	47	24	21	67	6	3	23	12	75	7	7	13	46	54	56
3	36	19	16	75	3	4	17	10	60	7	4	10	30	70	24
4	31	16	12	67	4	4	15	9	56	6	4	10	40	60	88
5	43	22	22	64	5	3	21	10	70	6	8	11	45	55	50
6	46	24	24	63	7	2	22	9	78	6	9	13	54	46	0
7	34	19	15	87	4	2	15	10	80	5	2	9	44	56	20
8	32	17	13	77	5	2	15	10	80	4	3	9	56	44	0
9	28	15	12	75	4	2	13	8	75	4	3	8	50	50	0
10	34	20	13	69	7	4	14	10	60	4	4	11	64	36	35
11	35	22	14	50	5	10	13	11	9	5	7	10	50	50	43
12	34	18	13	54	5	6	16	11	45	5	6	10	50	50	40
13	31	16	15	53	5	3	15	8	63	3	7	8	63	38	84
14	53	28	30	53	8	4	25	10	60	5	14	13	62	38	35
15	43	22	19	42	7	7	21	12	42	5	11	12	58	42	87
16	39	21	19	42	6	7	18	8	13	6	11	12	50	50	27
17	35	21	15	73	5	5	14	10	50	5	4	10	50	50	87
18	43	24	19	53	7	7	19	12	42	5	9	12	58	42	71
19	42	24	20	90	5	1	18	11	91	6	2	11	45	55	0
20	24	14	11	55	3	5	10	6	17	4	5	7	43	57	‡
21	32	20	17	88	1	4	12	7	43	7	2	8	13	88	39
22	32	17	15	80	3	2	15	8	75	6	3	9	33	67	26
Means	36.9	20.2	16.8	66	5.1	4.0	16.7	9.8	58	5.2	5.8	10.3	49	51	39.7
±s.d.	6.9	3.5	4.6	15	1.7	2.2	3.8	1.8	23	1.1	3.4	1.8	12	12	30.3

*Counts of blastocyst cells were performed as described for Table 1A.
‡Mixed distribution of cells prevented assessment of the tilt in these embryos

Table 3B. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributed more or even number of cells to the blastocyst – 2*n* PB*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	46	20	22	14	7	10	26	11	9	6	19	13	54	46	17
2	38	19	14	71	7	2	19	12	83	5	4	12	58	42	0
3	41	20	15	53	8	4	21	13	69	5	7	13	62	38	51
4	38	18	16	31	6	7	20	10	30	6	11	12	50	50	30
5	24	10	10	30	4	3	14	6	50	4	7	8	50	50	35
6	38	15	16	38	5	4	23	10	60	7	10	12	42	58	69
7	28	11	15	0	5	6	17	6	0	2	15	7	71	29	35
8	32	16	13	15	5	9	16	10	10	4	11	9	56	44	25
9	29	14	15	73	2	1	15	6	83	6	4	8	25	75	41
10	34	14	14	7	5	8	20	11	27	4	13	9	56	44	34
11	30	12	12	67	2	2	18	9	78	7	4	9	22	78	0
Means	34.4	15.4	14.7	36	5.1	5.1	19.0	9.5	45	5.1	9.5	10.2	50	50	30.6
±s.d.	6.5	3.6	3.0	26	1.9	3.1	3.5	2.5	32	1.5	4.9	2.2	15	15	20.3

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

Table 4A. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributed more cells to the blastocyst – NO SEP*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	31	17	13	54	6	4	14	8	50	4	6	10	60	40	‡
2	38	21	14	50	7	7	17	12	42	5	7	12	58	42	72
3	39	22	15	27	8	10	17	11	9	5	11	13	62	38	17
4	27	14	12	25	4	7	13	7	0	4	9	8	50	50	17
5	41	21	16	63	7	4	20	12	67	6	6	13	54	46	‡
6	31	16	12	25	5	8	15	10	20	4	9	9	56	44	15
7	30	17	12	58	5	5	13	9	44	4	5	9	56	44	80
8	32	19	14	86	4	3	13	10	70	4	2	8	50	50	‡
9	34	19	15	47	6	6	15	9	33	4	8	10	60	40	‡
10	27	14	10	80	4	2	13	9	78	4	2	8	50	50	0
11	36	21	13	85	6	4	15	12	67	5	2	11	55	45	15
12	27	14	11	55	5	3	13	8	63	3	5	8	63	38	90
13	34	18	13	23	6	9	16	11	18	4	10	10	60	40	40
14	37	20	15	60	4	7	17	11	36	7	6	11	36	64	60
Means	33.1	18.1	13.2	53	5.5	5.6	15.1	9.9	43	4.5	6.3	10.0	55	45	40.6
±s.d.	4.6	2.8	1.7	22	1.3	2.4	2.1	1.6	24	1.0	2.9	1.8	7	7	32.3

*Counts of blastocyst cells were performed as described for Table 1A.
‡Mixed distribution of cells prevented assessment of the tilt in these embryos
SEP, sperm entry position

Table 4B. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributed more or even number of cells to the blastocyst – NO SEP*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	33	16	12	50	3	7	17	11	36	7	6	10	30	70	54
2	32	16	14	64	3	4	16	9	56	6	5	9	33	67	42
3	31	15	13	46	2	7	16	10	30	6	7	8	25	75	60
4	33	14	16	38	2	6	19	8	25	7	10	9	22	78	‡
5	27	13	11	36	4	5	14	8	38	4	7	8	50	50	84
6	42	17	18	33	5	6	25	12	50	7	12	12	42	58	‡
7	25	11	9	67	3	2	14	8	75	5	3	8	38	63	20
8	33	16	15	73	5	0	17	8	100	5	4	10	50	50	20
9	30	14	12	33	4	6	16	10	40	4	8	8	50	50	40
10	30	14	12	67	3	3	16	11	73	4	4	7	43	57	85
11	30	13	12	33	4	5	17	10	50	4	8	8	50	50	89
12	31	15	15	47	4	4	16	7	43	5	8	9	44	56	82
13	32	16	15	80	3	1	16	8	88	6	3	9	33	67	33
Means	31.5	14.6	13.4	51	3.5	4.3	16.8	9.2	54	5.4	6.5	8.8	39	61	55.4
±s.d.	4.0	1.7	2.4	17	1.0	2.3	2.8	1.5	23	1.2	2.7	1.3	10	10	26.4

*Counts of blastocyst cells were performed as described for Table 1A.
‡Mixed distribution of cells prevented assessment of the tilt in these embryos
SEP, sperm entry position

Table 5A. Distribution of descendants of first and second dividing 2-cell blastomeres when first blastomere contributed more cells to the blastocyst – control NO SEP*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	23	12	10	100	2	0	11	6	100	5	0	7	29	71	0
2	26	14	10	100	4	0	12	8	100	4	0	8	50	50	24
3	26	15	14	86	3	0	11	5	100	4	2	7	43	57	28
4	26	14	8	88	3	4	12	11	64	4	1	7	43	57	52
5	26	16	12	92	3	2	10	7	71	4	1	7	43	57	48
6	32	18	16	69	3	4	14	7	43	6	5	9	33	67	54
7	28	16	9	100	3	4	12	12	67	4	0	7	43	57	50
8	24	15	13	92	3	0	9	4	100	4	1	7	43	57	11
9	36	20	17	82	4	2	16	9	78	6	3	10	40	60	28
10	26	14	10	90	4	1	12	8	88	4	1	8	50	50	18
11	45	25	17	82	9	2	20	16	88	3	3	12	75	25	27
12	33	18	16	75	3	3	15	8	63	6	4	9	33	67	35
13	37	19	15	87	6	0	18	10	100	6	2	12	50	50	20
14	45	27	21	90	5	3	18	12	75	7	2	12	42	58	26
15	37	22	13	62	6	8	15	12	33	6	5	12	50	50	64
16	29	15	9	33	5	7	14	10	30	5	6	10	50	50	18
17	38	20	15	93	5	1	18	12	92	6	1	11	45	55	11
Means	31.6	17.6	13.2	84	4.2	2.4	13.9	9.2	76	4.9	2.2	9.1	45	55	30.2
±s.d.	6.8	4.1	3.6	17	1.7	2.4	3.2	3.1	24	1.1	1.9	2.1	10	10	17.8

*Counts of blastocyst cells were performed as described for Table 1A.
SEP, sperm entry position

Table 5B. Distribution of descendants of first and second dividing 2-cell blastomeres when second blastomere contributed more or even number of cells to the blastocyst – control NO SEP*

Name	Total cells	Total early	Total embryonic	% Embryonic early	Early in BZ	Early crossing BZ to abembryonic	Total late	Total abembryonic	% Abembryonic late	Late in BZ	Late crossing BZ to embryonic	Total BZ	%BZ early	%BZ late	Tilt
1	28	13	11	55	3	4	15	10	60	4	5	7	43	57	35
2	28	10	13	38	3	2	18	8	75	4	8	7	43	57	64
3	20	10	7	57	3	3	10	6	50	4	3	7	43	57	43
4	37	17	16	19	3	11	20	11	0	7	13	10	30	70	42
5	31	15	15	27	3	8	16	8	0	5	11	8	38	63	0
6	25	12	12	50	3	3	13	6	50	4	6	7	43	57	79
7	23	9	8	50	4	1	14	6	83	5	4	9	44	56	52
Means	27.4	12.3	11.7	42	3.1	4.6	15.1	7.9	45	4.7	7.1	7.9	40	60	45.0
±s.d.	5.6	2.9	3.4	15	0.4	3.6	3.3	2.0	33	1.1	3.7	1.2	5	5	24.8

*Counts of blastocyst cells were performed as described for Table 1A.
SEP, sperm entry position