The plane of first cleavage is not related to the distribution of sperm components in the mouse

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BACKGROUND: Marking experiments using phytohaemagglutinin (PHA) and Concanavalin A (ConA) have suggested that the first cleavage plane is related to the point of sperm entry. Because of concerns about the specificity of lectin binding, the distribution of sperm components has been investigated directly. METHODS AND RESULTS: The sperm tail could be identified in cleaving zygotes and early 2-cell stages following their permeabilization and exposure to Oregon Green Paclitaxel. At neither stage did the anterior end of the tail, which lies initially at the site of sperm entry, bear a consistent relationship to the first cleavage plane, even when it had clearly retained its original location. Moreover, using artificial insemination with MitoTracker-labelled sperm, the midpiece was found to remain associated with anterior end of the tail through to the 2-cell stage. Lectins showed no discernible binding to the fertilization cone of mechanically denuded zygotes and very strong binding to the zona pellucida. Moreover, after general labelling of zygotes with either ConA or PHA, persisting surface lectin tended to be concentrated towards the cleavage plane. CONCLUSION: The present findings challenge the claim that the sperm specifies the plane of first cleavage, and also question the methodology on which it was based. 

Key words: blastocyst axes/first cleavage plane/midpiece/mouse/sperm entry point

Introduction

Using a strictly non-invasive marking technique, the embryonic–abembryonic (Em–Ab) axis and the axis of bilateral symmetry of the blastocyst have been found to bear a consistent relationship to the structure of the 2-cell conceptus in the mouse (Gardner, 2001). Thus, the blastocyst's bilateral axis and Em–Ab axis are, respectively, approximately parallel and orthogonal to the plane of first cleavage, arguing that both axes must be specified before the 2-cell stage during normal development. Since the bilateral axis of the blastocyst is aligned with the animal–vegetal (A–V) axis of the zygote (Gardner, 1997), its specification may depend on the intrinsic polarity of the oocyte or zygote. However, because the Em–Ab axis of the blastocyst is orthogonal to the bilateral axis, information that breaks the radial symmetry of the zygote would seem to be necessary for its specification. It has been claimed that the mouse oocyte and zygote are bilaterally rather than radially symmetrical (Jones-Seaton, 1950; Dalcq, 1957). However, according to this claim, which is based on examination of fixed rather than living material, the plane of bilateral symmetry bears no consistent relationship to that of first cleavage.

Rather than being generated intrinsically, the necessary asymmetry might depend on an extrinsic factor such as the fertilizing sperm. Particularly pertinent is a recent study in which the authors claim that the plane of first cleavage corresponds with the sperm entry point (SEP) in the mouse (Piotrowska and Zernicka-Goetz, 2001). Their consequent conclusion that the fertilizing sperm has a patterning role is based on the use of subzonal injection of Concanavalin A (ConA) and of phytohaemagglutinin (PHA)-treated fluorescent microspheres to achieve focal labelling of the surface of the zygote. However, the authors make no reference to past studies on binding of these lectins to early mouse conceptuses even though awareness of two findings is of crucial relevance to the approach they used. First, both lectins bind avidly to the zona pellucida (Nicolson et al., 1975; Legge, 1991) and, given its glycoprotein composition, probably also to the cortical granule envelope that occupies the perivitelline space (Hoodbhoy et al., 2001). Second, unlike almost the entire remainder of the zygote, the fertilization cone that forms at the SEP does not discernibly bind ConA (Maro et al., 1984). If this is due to the absence of microvilli (Shalgi et al., 1978; Maro et al., 1984), it may well also apply to PHA, in which case binding of PHA-treated microspheres to the SEP will differ in nature or strength from elsewhere on the zygote's surface.

In view of these earlier findings, there are clearly serious potential pitfalls in using lectins for focally labelling the surface of the zygote with the zona pellucida left on, as was done in the experiments of Piotrowska and Zernicka-Goetz (2001). Hence, validation of the use of PHA-treated microspheres as a surface marker depends crucially on demonstrating that they retain their relative position at least through the first cleavage division. The principal assay used by Piotrowska and
Zernicka-Goetz failed to substantiate this because it entailed placing a microsphere either adjacent to, or diametrically opposite, the second polar body (PB). Given that first cleavage is usually approximately meridional, microspheres placed at either pole of the zygote should normally lie at or close to the cleavage plane. Hence, general movement of lectin-binding and other surface components towards the future cleavage plane, which has been reported both in various mammalian cells (Berlin et al., 1978; Koppel et al., 1982; Wang et al., 1994) and in sea urchin zygotes (McCaig and Robinson, 1982), would go undetected. Showing that microspheres placed diametrically opposite each other in the equatorial region of zygotes prior to first cleavage remain opposite each other thereafter would seem to be necessary for determining whether they retain their relative position.

Thus, the study of Piotrowska and Zernicka-Goetz fails to provide sufficiently rigorous validation of the use of lectins for surface marking of the zygote to show, (i) that such marks retain their position, and (ii) do not influence the cleavage plane through mediating attachment between the zygote and the overlying zona pellucida.

Given the obvious importance of establishing whether the fertilizing sperm has a role in early patterning, the present study was undertaken to investigate the relationship between its components and the first cleavage plane using methods that avoid the uncertainties associated with surface labelling of the zygote. Particular emphasis was placed on investigating this relationship in conceptuses that were not subjected to any experimental manipulation and should therefore reveal the normal situation. We find the plane of first cleavage to be almost random with respect to the SEP, and are unable to detect binding of either ConA or PHA to the SEP of mechanically denuded living zygotes. Moreover, we find not only that both lectins tend to move towards the first cleavage plane, but that the coherent labelling for ConA that persists beyond the 2-cell stage is associated with the zona pellucida rather than the surface of the conceptus.

Materials and methods

**Mice and conceptuses**

PO closed-bred albino mice were used for most of the study, and were kept under three different lighting regimes in which the dark period was 19:00–07:00, 11:00–21:00 or 03:00–11:00. Additional zygotes were obtained from matings between [CBAxC57BL/6J] F1 mice whose dark period was 03:00–11:00. All zygotes and 2-cell stages were recovered from excised oviducts, stored at room temperature and, where applicable, manipulated in synthetic oviductal medium enriched with potassium (KSOM)–HEPES (Summers et al., 1995). They were cultured at 37°C in drops of KSOM plus amino acids (Summers et al., 2000) under light paraffin oil in small bacteriological dishes that were gassed with 5% CO₂ in air.

**Inspection and manipulation of conceptuses**

Zygotes, 2-cell conceptuses and blastocysts were inspected and micromanipulated in individual hanging drops in a Pulv chamber filled with heavy paraffin oil using differential interference contrast (DIC) or epifluorescence (FM) microscopy with either a ×40 or a ×60 water immersion objective lens. For recording the location of the anterior end of the sperm tail (AnT). The second polar body (PB), which was used throughout as the marker of the animal pole, is shown with a continuous, and the first PB with a discontinuous, outline. (A–C) Non-invasive mapping of the AnT axially was done as shown with the early zygote on its side (A), and circumferentially with its animal pole uppermost (B, C). Where both PBs were anchored and intact, circumferential mapping was done by estimating the angle of departure of the AnT (α) from an axis defined by these bodies (B). Where only the second PB was intact, the angle (β) was estimated with reference to an axis defined by the second PB and its adjoining mid-body (C). Where practicable, mapping was done with respect to both axes so as to allow the fidelity with which they retained their relative orientation to be checked. These axial and circumferential coordinates were used during or after first cleavage to direct injection of a small oil drop into the periphery of the vitellus where the AnT should be, had it remained at its original location. (D, E) Measurement of the position of the AnT axially (D) and relative to the cleavage plane (E), indicated by a bold central line, in permeabilized 2-cell conceptuses. All measurements were done on enlarged images transmitted to a monitor via a low-light camera attached to the microscope. Axial measurements were made, as indicated, with a ruler so that the percentage distance of the AnT from the animal pole (ε = b/a×100) could be recorded (D). For circumferential measurements, a protractor was used to determine the angle of departure of the AnT from the cleavage plane (γ) (E). In specimens permeabilised during, rather than after, first cleavage the mid-line of the mitotic spindle or early furrow were taken as the cleavage plane. (F) For converting circumferential mapping of the AnT from degrees of arc to axial thirds of 2-cell blastomeres, the mean angles δ and ε were obtained by measurements on both blastomeres of photographs of 27 PO conceptuses (n = 54: δ = 27.6 ± 0.33 and ε = 48.5 ± 0.33) and 7 F1xF1 conceptuses (n = 14: δ =26.8 ± 0.7 and ε = 47.33 ± 0.8).

The anterior end of the sperm tail (AnT) without intervention in early zygotes, its site was first marked with an oil drop in the overlying zona pellucida, as described elsewhere (Gardner, 2001). Zygotes were then examined with their A–V axis horizontal so as to enable the proportionate distance of the oil drop from the animal pole to be estimated. Thereafter, they were reoriented with the A–V axis vertical and the approximate angle of the oil drop relative to an axis defined by the centres of the first and second PBs, or by the second PB and its midbody was noted (Figure 1A–C). Following this, each zygote was cultured separately for re-examination either during first cleavage or at the early 2-cell stage to see if the orientation of axes defined by the PB(s) appeared to have been conserved. Where conservation...
of one or both axes was indicated, a small oil drop was injected cortically according to the axial and circumferential coordinates recorded for the AnT at the early zygote stage.

The endocytosed tail of the fertilizing sperm can be identified in living late mouse zygotes or 2-cell conceptuses only exceptionally when a substantial part of it remains very close to the surface. This is in contrast to the rat where an essentially intact tail has been observed routinely at the 2-cell stage and persistent fragments are often still discernible in blastocysts (Blandau and Odor, 1952). Nonetheless, evidence that the sperm tail and mitochondrial sheath normally persist beyond first cleavage in the mouse comes from studies in which they have been labelled (Simerly et al., 1993; Cummins et al., 1997). Following various trials, it was found that the sperm tail could be identified most reliably during first cleavage and at the 2-cell stage if specimens were permeabilized and then exposed to Oregon Green 488 paclitaxel (OGPT; P 22310; Molecular Probes Inc., Eugene, OR, USA) before being examined by both DIC and FM.

The solution used to permeabilize zygotes and 2-cell stages was as described (Schatten et al., 1989), except that the concentrations of EDTA and Triton-X were raised to 1 mmol/l and 3% respectively. Initially, permeabilization was for several hours, though later in the study 1 min at room temperature was found to suffice. Thereafter, the conceptuses were rinsed briefly in an aliquot of permeabilization solution from which Triton-X and glycerol had been omitted before being transferred to a further aliquot of this rinse containing 5 mmol/l OGPT prepared in dimethylsulfoxide. After incubation in this solution for 10 min in the dark, conceptuses were transferred via a further rinse to drops of KSOM–HEPES in a Puliv chamber for scoring by microscopy.

Marking of the AnT with oil drops in the zona pellucida

Marking the location of surface features of zygotes by injecting oil drops into the zona pellucida was done as described elsewhere (Gardner, 2001). To prevent their often considerable rotation within the zona pellucida, zygotes were first incubated for 1.25–1.5 h in KSOM containing 1.1% (w/v) low viscosity sodium alginate (Sigma, Poole, Dorset, UK). After a brief rinse in KSOM–HEPES, they were exposed for up to 40 min at room temperature to a solution of 1.5% CaCl$_2$·2H$_2$O and 0.90% NaCl in analar water that had been diluted 10-fold with KSOM–HEPES so as to induce slow gelation of the alginate within the perivitelline space. Thereafter the Anita, the second PB and, where present, the first PB were marked distinctively by injecting, respectively, one small, two large, and one large oil drops into the zona pellucida or, in some cases, into the gelated perivitelline space. All zygotes were then cultured in KSOM minus alginate for harvesting during or shortly after first cleavage. Finally, they were returned to a Puliv chamber for checking from the relationship between the PB(s) and their marker oil drops whether they had rotated in the zona pellucida. Those showing no rotation had an oil drop injected into the periphery of the vitellus in alignment with the AnT marker oil drop. To prevent wrinkling during subsequent permeabilization, such conceptuses were first incubated for 15 min at room temperature in phosphate-buffered saline (Dulbecco A, Oxoid, Basingstoke, Hants, UK) supplemented with 4 mg/ml of 10 kDa polyvinylpyrrolidone (PVP) and containing 1 mmol/l EDTA to dissolve the gel in the perivitelline space. They were then permeabilized and treated with OGPT, as described earlier, before being examined microscopically.

Labelling of sperm

Sperm recovered from both vasa deferentia of PO male mice were incubated at 37°C for 10 min in 102 µl of Ca$^{2+}$/Mg$^{2+}$-free Tyrode’s saline with 10% KSOM under light paraffin oil to allow them to become well dispersed. For labelling the mitochondrial sheath that invests the midpiece (Bishop and Walton, 1960; Fawcett, 1981), 50 µl of 1 µg/ml MitoTracker Green (MT Green, M-7514; Molecular Probes Inc.) in Tyrode’s/KSOM was added to the suspension at a final concentration of 490 nmol/l, and the sperm were then incubated for a further 6–10 min. In some experiments, tetramethylrhodamine isothiocyanate (Sigma, T3163) was also included in this solution at a final concentration of 0.26–1.3 nmol/l. The labelled sperm suspension was then used for artificially inseminating female mice shortly after the end of the dark period during which they had mated with vasectomized males (Kile, 1951). The females were killed ~1 day after artificial insemination (AI) and all morphologically normal-looking 2-cell conceptuses were examined by FM. Where specific focal fluorescence was observed following labelling of sperm with MT Green alone, its location was marked in some conceptuses by injecting a small oil drop in the vitellus. These conceptuses were then permeabilized and stained for identifying the AnT. In the remaining conceptuses, the outer surface of the blastomere containing the focus was marked with a pair of oil drops in the zona pellucida before they were cultured to the blastocyst stage when the position of the marker oil drops along the Em–Ab axis was recorded. All 2-cell conceptuses obtained by AI using sperm that had been double-labelled with both MT Green and tetramethylrhodamine isothiocyanate were examined by FM to compare the distribution of red and green signals in the vitellus.

For mapping the location of the AnT in permeabilized first cleavage and 2-cell stages, its location was first marked with an oil drop so that measurements could be made on the screen of a monitor that was coupled to a video camera mounted on the microscope. Each conceptus was first oriented with its A–V axis horizontal. Both the distance of the AnT marker oil from the animal pole and the total length of the A–V axis were then measured and the percentage distance of the AnT along the axis calculated (Figure 1D). The conceptus was then oriented with its animal pole uppermost and its actual or, in the case of first mitotic stages, its putative or nascent cleavage plane vertical. Finally, the angle of departure of the AnT from the centre of the cleavage plane was measured with a protractor (Figure 1E). To facilitate comparison of these findings with those of Piotrowska and Zernicka-Goetz (2001), the mean values of the angles embracing the inner and the central thirds of blastomeres were determined from measurements on both 2-cell PO and F1×F1 conceptuses (Figure 1F).

Lectin binding

For investigating binding of fluorochrome-conjugated ConA and PHA to the fertilization cone, living early zygotes were denuded mechanically using two fine-tipped glass needles held on micro-manipulators (Leitz, Luton, Bedfordshire, UK). This was done to avoid the risk of altering the surface of the zygote by exposing it to acidified Tyrode’s saline or the proteolytic enzymes that are normally used to eliminate the zona pellucida. Once denuded, zygotes were rinsed briefly in phosphate-buffered saline (PBS) before being exposed to ConA (Alexa-Fluor ConA; Molecular Probes, Inc.) at 500 or 20 µg/ml or to rhodamine isothiocyanate-conjugated PHA (Sigma, Poole, Dorset, UK) at 300 or 20 µg/ml. The surface of additional zygotes was labelled focally by injecting a small volume of 500 µg/ml ConA against it via a micropipette inserted through the zona pellucida, as described elsewhere (Piotrowska and Zernicka-Goetz, 2001). Zygotes treated thus were then cultured to the blastocyst stage.
Results

General observations on detecting components of the fertilizing sperm

In the mouse, as in most mammals, the entire fertilizing sperm is endocytosed by the oocyte (Lams and Dorme, 1908) and a conspicuous protuberance called the ‘fertilization cone’ forms where its head enters the vitellus (Austin and Braden, 1956; Austin, 1975). Before the sperm nucleus decondenses to form the male pronucleus, this protuberance can readily be distinguished from that of the nascent second PB because the AnT lies at its base (Figure 2A and B). Well into the zygote stage, the helical structure of the mitochondrial sheath which invests the anterior or midpiece region of the tail is often also apparent using DIC optics (Figure 2C and D). The AnT typically remains fairly close to the surface of the zygote after the developing male pronucleus moves towards the interior, although, in a small minority of cases, it is also carried deep inside the vitellus. Altogether, the AnT was judged to be too remote from the surface for accurate recording of the SEP in only 28 of 242 early zygotes (12%). The corresponding figures for a more limited series of late zygotes was 5/36 (14%), suggesting that little if any further change in its depth occurs between the onset of male pronuclear migration and first cleavage.

Although the tail of the fertilizing sperm is not normally discernible in living late zygotes or 2-cell conceptuses in the mouse, use of fluorescent probes has revealed that both the axoneme and the mitochondrial sheath of the midpiece clearly persist beyond first cleavage (Simerly et al., 1993; Cummins et al., 1997). Such probes are, however, unsuitable for detailed examination of sperm components because of the rate at which their signal fades. Nevertheless, when appropriately oriented relative to the microscope’s crossed polars, the sperm tail could be visualized clearly by DIC in the great majority of permeabilised cleaving zygotes and early 2-cell conceptuses (Figure 3A and B) by virtue of its birefringence (Lacey, 1989). Where difficulty was experienced, OGPT staining usually enabled the tail to be identified clearly enough by FM for it to be oriented optimally for viewing by DIC. The two ends of the tail could readily be differentiated because unravelling and splaying of its axonemal fibres progress anteriorly from its posterior end (Simerly et al., 1993). Hence, the AnT is abruptly truncated and relatively thick compared to the posterior region of the tail which is often obviously bifurcated (Figure 3B) and invariably tapered too gradually for its distal extremity to be resolved. Because of its relatively intense staining for OGPT, the location of the AnT was usually also obvious by FM.

Relationship of AnT to first cleavage plane

The position of the AnT both along the A–V axis and in relation to the plane of first cleavage was measured in series of early 2-cell conceptuses from matings of both PO and F1 mice. The results, which are summarized in Figure 4, revealed no tendency for the AnT to lie at a particular axial level or to be located close to, rather than away from, the cleavage plane. Moreover, both the orientation and location of the remainder of the tail was also extremely variable. Whilst the tail was usually discernible in only one blastomere, in some cases it could be traced to, or even through, the mid-body.

To facilitate comparison of these findings with those of others (Piotrowska and Zernicka-Goetz, 2001), the relationship of the AnT to the cleavage plane is also revealed by the frequency with which it lay in the inner, middle and outer third of the blastomere containing it (Figure 4C and F). Findings were similar for further PO conceptuses that were permeabilized for mapping the position of the AnT and determining the orientation of the remainder of the tail during metaphase or anaphase of first cleavage (Figure 5).
Does the AnT move prior to first cleavage?

In view of the foregoing results, it was important to find out whether the AnT normally remains at the site of sperm entry up to or beyond first cleavage, and can thus serve as an enduring natural marker for it. Initially, the possibility was explored that the site of the AnT could be marked non-invasively by injecting oil drops into the substance of the zona pellucida (Gardner, 2001). However, it was found that before the 2-cell stage, unlike thereafter, conceptuses often rotated extensively in the zona pellucida. Hence, as outlined in Figure 1A–C, the position of the AnT was mapped both latitudinally and longitudinally with references to the PB(s) in a series of early zygotes in which it lay away from either pole. On recovery from culture during or after first cleavage, those conceptuses whose PBs appeared to have retained their position had an oil drop injected into the periphery of the vitellus where the AnT should be, had it remained at its original location. It is important to note that the oil injection was done before conceptuses were permeabilized so that the tail was not discernible. The AnT was scored as having not moved circumferentially if it was within 15° of the marker oil drop and as having not moved axially if it was within 10% of the length of the axis from the drop. Movement was recorded as modest if the AnT and its marker oil were less than 40° apart circumferentially and 20% apart axially, and as substantial whenever it exceeded these values.

According to this assay, the AnT remained at its initial location in the majority of cases, and seldom lay far from it (Table I). Importantly, even when it had remained at its original location it showed no greater tendency to lie closer to the cleavage plane than when it had not. Thus, it was remote from the cleavage plane in 5/8 2-cell conceptuses, 2/2 anaphases, and 1/1 metaphases in which it lay near the equator and had not shifted circumferentially.

However, particularly in cases where substantial displacement circumferentially was recorded, it was not possible to decide whether this was genuine or due to rotation or displacement of the PBs. Therefore, a further series of early zygotes was cultured for up to 1.5 h in medium containing 1.1% low viscosity sodium alginate, and then exposed to Ca²⁺ so as to induce gelation of the alginate that had entered the perivitelline space. The positions of the PB(s) and AnT were then marked differentially by injecting oil drops into either the zona pellucida or perivitelline space, after which the zygotes were cultured until they had embarked on first mitosis or reached the early 2-cell stage. They were then examined for rotation in the zona pellucida by checking the relationship of the PBs to their marker oil drops. Specimens showing no evidence of
Cleavage plane not related to sperm entry

Figure 5. Axial (A, C) and circumferential (B, D) location of the anterior end of the sperm tail (AnT) at metaphase (A, B) and anaphase (C, D) of first cleavage in PO conceptuses. The filled parts of the histograms in B and D relate to specimens in which the AnT lay within the equatorial third of the animal–vegetal (A–V) axis.

Table I. Relationship between location of anterior end of the sperm tail (AnT) during or after first cleavage and in the early zygote: non-invasive mapping

| Stage and no. analysed | Circumferential disparity between AnT and oil | Axial disparity between AnT and oil |
|------------------------|---------------------------------------------|-----------------------------------|
|                        | None | Modest | Marked | None | Modest | Marked |
| 2-cell (n = 17)         |      |        |        |      |        |        |
| First anaphase (n = 7)  |      |        |        |      |        |        |
| First metaphase (n = 4) |      |        |        |      |        |        |
| Total (n = 28)          |      |        |        |      |        |        |

*In two cases the AnT and its marker oil drop were just separated by the cleavage furrow.

*In this specimen, the AnT was diametrically opposite its marker oil drop, suggesting that the first and second polar body had been confused.

rotation had an oil drop injected into the periphery of the vitellus immediately beneath the oil marking the original site of the AnT. They were then incubated at room temperature for 15 min in PBS/PVP with EDTA to dissolve the alginate in the perivitelline space before being permeabilized and stained for scoring. From the results shown in Figure 6 for analysis at the 2-cell stage, it is clear that distribution of the AnT relative to the A–V axis and first cleavage plane is similar to that seen without gelation of the perivitelline space (Figure 4). However, even with gelation when movement of the PBs could be discounted, cases of obvious disparity between the initial and final location of the AnT were not uncommonly encountered (Table II). Evidently, while normally remaining at its original location through first cleavage the AnT is unquestionably sometimes displaced from it.

**AI with MT Green-labelled sperm**

Females were killed ~24 h after insemination and their oviducts were dissected for recovery of 2-cell conceptuses which were inspected by FM for MT Green-labelling. Fluorescence invariably took the form of a cluster of foci which was confined to one blastomere and showed no tendency to be located near the cleavage plane (Figure 7A–C). Some 2-cell conceptuses were permeabilized and stained with OGPT for locating the sperm tail after an oil drop had been injected into the vitellus to mark the site of fluorescence. The remainder of the conceptuses were cultured to the blastocyst stage after the outer surface of the fluorescing blastomere had been marked with oil drops in the zona pellucida.

In each of 17 conceptuses that were scored at the 2-cell stage, the oil drop marking the site of the MT Green fluorescence was
Table II. Relationship between location of the anterior end of the sperm tail (AnT) during or after first cleavage and in the early zygote: oil in zona pellucida marking after gelation of the perivitelline space

| Stage and no. analysed | Circumferential disparity between AnT and oil | Axial disparity between AnT and oil |
|------------------------|----------------------------------------------|-----------------------------------|
|                        | None                          | Modest | Marked | None | Modest | Marked |
| 2-cell (n = 12)         | 8                             | 1      | 3      | 7    | 2      | 3      |
| First metaphase–anaphase (n = 9) | 3 | 4 | 2 | 6 | 1 | 2 |
| Total (n = 21)          | 11                            | 5      | 5      | 13   | 3      | 5      |

*The AnT and its marker oil drop were just separated by the cleavage furrow.

Figure 6. Axial (A) and circumferential (B) mapping of anterior end of the sperm tail (AnT) in 2-cell conceptuses developed from early zygotes whose perivitelline space had been gelated with alginate. The filled parts of the histogram in B relate to specimens in which the AnT lay within the equatorial third of the animal–vegetal (A–V) axis.

by the anterior region of the sperm tail, implying that, notwithstanding its deterioration, the mitochondrial sheath invariably remained near or over the part of the tail that it originally invested. Among 57 PO conceptuses that were returned to culture after the outer surface of the MT Green–positive blastomere was marked with oil in the zona pellucida, 40 formed scorable blastocysts. The oil mapped to the embryonic hemisphere in 23 of these blastocysts, to the abembryonic hemisphere in a further 11, and was too close to the equator to be assigned to either hemisphere in the remaining six.

In 12 of 15 2-cell conceptuses obtained from AI with double-labelled sperm in which red fluorescence due to tetramethylrhodamine isothiocyanate was detected, it co-localized very precisely with the MT Green signal (Figure 7D–I).

Each of the remaining three conceptuses also had an additional small focus that exhibited only red fluorescence. This focus was near the cleavage plane in two cases and remote from it in the third.

**Binding of ConA and PHA to the zygote**

All findings reported here were based on examination of at least 12 specimens. Before being exposed to either lectin, early zygotes from matings between PO or F1 mice were denuded of the zona pellucida mechanically with micro-needles so as to avoid chemical modification of their surface by the reagents that are normally used to remove this envelope. Relative to the remainder of the surface of the zygote, the fertilization cone showed essentially background fluorescence with both ConA and PHA (Figure 8A, B and E, F), even when exposed to the very high concentration of the lectins used by Piotrowska and Zernicka-Goetz (300–500 µg/ml) at which non-specific binding may occur (Konwinski et al., 1977). When the isolated zonae pellucidae were exposed to the lectins, they fluoresced very intensely throughout (Figure 8C, D and G, H). When part of the vitelline surface of early zygotes was labelled by restricted subzonal injection of ConA this sometimes led to such strong adherence between the surface of the vitellus and the zona pellucida as to cause the zygote to lyse during subsequent mechanical exzonation. In zygotes that were cultured within an intact zona pellucida, much of the ConA or PHA was endocytosed by the early 2-cell stage and, regardless of the original site of labelling, what remained on the surface tended to be concentrated towards the cleavage plane (Figure 8I, J). All zygotes focally labelled with ConA that were cultured to the blastocyst stage showed a persisting coherent patch of fluorescence. However, following mechanical denudation or spontaneous hatching of such blastocysts, the patch was invariably found to be associated with the zona pellucida. This was obvious even without recourse to removing the zona pellucida if labelled conceptuses were rotated during examination at any stage before blastocyst expansion when the perivitelline space disappears (Figure 8K). In no case was coherent fluorescence found to persist beyond the 2-cell stage at the surface of the conceptus itself.

**Discussion**

Dependence of the plane of first cleavage on the SEP would seem to require a component of the fertilizing sperm that is
Figure 7. (A–C) Combined brightfield/epifluorescence photographs of three 2-cell conceptuses obtained following artificial insemination (AI) with MitoTracker (MT) Green-labelled sperm. (D–F, G, H) Two 2-cell conceptuses from AI with a double-labelled sperm photographed using differential interference contrast optics (D, G), and fluorescence microscopy for MT Green (E, H) and for rhodamine (F, I). Note the coincidence of the cluster of MT Green and rhodamine fluorescent foci which is away from the cleavage plane in both cases.

Hitherto, the distribution of components of the fertilizing sperm have not been investigated systematically either during or after first cleavage in the mouse. This is presumably because they are harder to discern in this species than in the rat (Blandau and Odor, 1952). Nevertheless, use of permeabilization in conjunction with OGPT staining gave clear enough resolution of the tail to allow its anterior extremity to be distinguished from its progressively unravelling posterior region in both late zygotes and 2-cell conceptuses. Moreover, AI with MT Green-labelled sperm enabled localization of the mitochondrial sheath which surrounds the anterior part of the tail in intact sperm (Fawcett, 1981). In both PO and F1×F1 early 2-cell stages the AnT was more often remote from, than near to, the plane of first cleavage, regardless of its position along the A–V axis (Figure 4). This was also true for additional series of PO conceptuses examined during first cleavage (Figure 5), both at metaphase when the plane of cytokinesis may not yet have been specified in all cases, and during anaphase when it clearly must have been (Rappaport, 1974). Furthermore, both the direction in which the remainder of the tail was oriented and its overall shape were highly variable, effectively discounting the possibility that any part of it bore a consistent relationship to the plane of first cleavage.

Most importantly, where the AnT was superficial enough for its position to be mapped strictly non-invasively at the early zygote stage and this site could later be marked confidently with oil, it was found in the majority of cases to have remained at its original location through to, and even beyond, first cleavage.
Since the AnT lies initially at the SEP (Figure 2), the latter must normally bear the same relationship to the plane of first cleavage as the former. That instances of obvious disparity between the AnT and its marker oil occurred following gelation of the perivitelline space argues that these were due, at least partly, to occasional shift in the position of the AnT rather than the PB(s) used to map its location. Such shifts might depend on proximity of parts of the tail to microtubule organizing centres orchestrating pronuclear migration.

In each of 17 early 2-cell conceptuses in which the main focus of the MT Green signal was marked with an oil drop, the drop was found to be close to the anterior region of the sperm tail. Moreover, where the mitochondrial sheath was identifiable directly by DIC, it invariably lay by or over the anterior region of the tail (e.g. Figure 3A, inset). In view of its consistent co-localization with the AnT, separate mapping of the relationship of the midpiece to the plane of first cleavage was considered unnecessary. Parenthetically, the above findings do not accord with an early claim that the midpiece mitochondria become dispersed throughout the cytoplasm of the mouse zygote before it reaches an advanced pronuclear stage (Gresson, 1940).

Where a vitelline focus of red fluorescence was seen in 2-cell conceptuses following AI with double-labelled sperm, it almost invariably co-localized precisely with the MT Green fluorescence. Hence, the mitochondrial sheath seems the most likely candidate for the superficial patch of fluorescence in zygotes and cleavage stages from mice that had been artificially inseminated with fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-labelled sperm (Gabel et al., 1979).

Apart from the mitochondrial sheath and the remainder of the tail, another internal component of the mouse sperm is the perforatorium which, being adjacent to the nucleus, must also lie initially at the SEP. This structure proved impossible to identify unequivocally either by DIC or FM, although Cummins et al. claimed to be able to distinguish it by FM following direct microinjection of MT Green-labelled sperm or their components into the cytoplasm of oocytes (Cummins et al., 1997). If, despite our failure to recognize it in early 2-cell stages after AI with MT Green-labelled sperm, it was nonetheless labelled sufficiently to contribute to the observed fluorescent foci, then it too must invariably have been associated with the anterior end of the sperm tail. In addition, the protein(s) equatorin, which is associated with a ladder-like
structure in the pouch formed in the equatorial region of the sperm head between the persisting parts of the outer and inner acrosomal membranes (Manandhar and Toshimori, 2001), has also been found to enter the oocyte on sperm penetration. While initially adjacent to the developing pronucleus, it later becomes separated from it and is typically associated with one blastomere at the 2-cell stage, but is said to be undetectable thereof. Although the location of the residual equatorin-positive body relative to the cleavage plane has not been mapped systematically, it is remote from this plane in the 2-cell conceptus in which it is illustrated (see Manandhar and Toshimori, 2001, Figure 3E).

Hence, none of the components of the sperm that enter the oocyte has been found to bear a sufficiently consistent relationship to the plane of first cleavage to be instrumental in its specification. A further possibility to consider is whether, following its fusion with the oocyte, part of the surface membrane of the fertilizing sperm might continue to mark the site of sperm entry in an enduring way. This would run counter to general experience with membrane fusion where complete mixing seems inevitably to be the eventual outcome. From available data, this also appears to be true for fertilization (Gaunt, 1983). Possibly because it is organized maternally rather than paternally, the first mitotic spindle is unusual in being barrel-shaped and anastral in the mouse (Schatten et al., 1989). It is therefore conceivable that its mode of orientation and, in consequence, the process of specification of the plane of first cleavage, could differ from that in other species. There is, however, no reason to suppose this might be the case. Nevertheless, according to the results of non-invasive mapping, the AnT often continues to mark the SEP despite its very variable relationship to the first cleavage plane.

Overall, when mapping of the AnT, and by association its midpiece and possibly the perforatorium, relative to the cleavage plane is compared with that of the PHA-treated microspheres that Piotrowska and Zernicka-Goetz (2001) used to mark the SEP, a striking disparity is evident. The microspheres most often mapped close to the cleavage plane during first cleavage and to the inner third of the positive blastomere thereafter. In contrast, the AnT was in the majority of cases remote from this plane both during and after first cleavage (Figures 4 and 5). A tendency for the SEP to be orthogonal to the cleavage plane rather than aligned with it could be explained if the long axis of the first mitotic spindle lies in the plane in which the two pronuclei come together. Regardless, it is clear that the present findings offer no support for the notion that the plane of first cleavage is related to the SEP in the mouse. For reasons enumerated earlier, we suggest that Piotrowska and Zernicka-Goetz (2001) have been misled through the use of lectins as markers. The present study has not only confirmed earlier findings that ConA does not bind detectably to the SEP and does so avidly to the zona pellucida (Maro et al., 1984; Legge, 1991), but has revealed that this is also true for PHA. This, in conjunction with evidence from both somatic cells and zygotes of movement of bound lectins towards the cleavage plane (Berlin et al., 1978; Koppel et al., 1982; McCaig and Robinson, 1982; Wang et al., 1994), suggest that the association between the first cleavage plane and SEP reported by Piotrowska and Zernicka-Goetz (2001) is artefactual.

A further implication of the study by Piotrowska and Zernicka-Goetz (2001) is that following surface labelling of the zygote, ConA persists as a coherent patch on the surface of the conceptus through to the blastocyst. If true, this would provide a very valuable way of fate-mapping between the zygote and blastocyst stage. However, no evidence was provided that the patch persists on the conceptus rather than in the overlying zona pellucida, which is necessary in view of the marked propensity of blastomeres to endocytose this lectin (Handyside, 1980). Our own findings reported here have revealed that enduring coherent ConA labelling is entirely in the zona pellucida, and that little of the lectin associated with the vitellus remains on its surface beyond the first cleavage. For the purposes of their experiments it would not matter if the ConA patch were in the zona pellucida, providing the conceptus did not rotate within it. However, while the conceptus usually undergoes little or no rotation within the zona pellucida from the 2-cell to blastocyst stage (Gardner, 2001), this is clearly not true before first cleavage (T.J.Davies and R.L.Gardner, unpublished observations).

Although the present findings offer no support for the claim that the fertilizing sperm is instrumental in specifying the plane of first cleavage, they do not discount the possibility that it might play other roles in early patterning. Gabel et al. did not follow up their observations to ascertain whether the fate of the blastomere with the patch of fluorescence differed consistently from that of its sister (Gabel et al., 1979). Here, it was found that the blastomere with the MT Green fluorescence tended to contribute more often to the embryonic than the abembryonic hemisphere of the blastocyst. However, the bias was much less striking than reported in two recent studies implying a link between precocious division and embryonic polar fate of the blastomere inheriting the SEP. In the first, in which a PHA-treated microsphere was placed at the SEP, the microsphere was found on the first blastomere to divide in 75% of 3-cell conceptuses (Piotrowska and Zernicka-Goetz, 2001). In the second study, the earlier dividing blastomere was found to contribute predominantly to the embryonic hemisphere in >80% of blastocysts (Piotrowska et al., 2001). It should be noted, however, that the interval between division of the two blastomeres varies greatly, so there is a risk of bias in limiting observations to specimens with an enduring 3-cell stage (Kelly et al., 1978). Various differences between the two blastomeres of 2-cell conceptuses have been documented in the mouse. For example, it has been claimed that 2-cell blastomeres are unequal in size and that the larger of the two is the first to divide (Lewis and Wright, 1935), but this has not been substantiated by subsequent work. Differences in dry mass occur early in the second cycle and the disparity increases as it progresses (Abramczuk and Sawicki, 1974). Sister 2-cell blastomeres seem also to differ in the time at which nucleoli acquire staining properties thought to be indicative of onset of RNA synthesis (Engel et al., 1977), and in the duration of DNA synthesis (Luthardt and Donahue, 1975).
Apart from the microvillus-free region immediately overlying the second meiotic spindle (Johnson et al., 1975; Eager et al., 1976), there seem to be no obvious differences axially in the likelihood of attachment of the fertilizing sperm to the oocyte in the mouse. However, in the absence of discernible radial asymmetry in the living mouse oocyte, whether the site of sperm attachment is completely unrestricted orthogonally to the A–V axis is uncertain. Hence, the possibility has yet to be excluded that both the site of sperm entry and the plane of first cleavage depend on organization of the oocyte.

Note added in proof

Plusa and colleagues (Plusa et al., 2002) claim that PHA-treated fluorescent microspheres behave similarly when used to label denuded as well as zona-intact zygotes. However, neither in these experiments, nor in another in which IVF with Alexa Fluor-labelled sperm was used to mark the sperm entry point, is evidence presented that either marker retains its ancestral position through first cleavage.

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