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The basis and significance of pre-patterning in mammals

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The second polar body (Pb) provides an enduring marker of the animal pole of the zygote, thereby revealing that the axis of bilateral symmetry of the early blastocyst is aligned with the zygote's animal-vegetal axis. That this relationship is biologically significant appeared likely when subsequent studies showed that the equator of the blastocyst tended to correspond with the plane of first cleavage. However, this cleavage plane varies both with respect to the position of the second Pb and to the distribution of components of the fertilizing sperm that continue to mark the point where it entered the egg. It also maps too variably on the blastocyst to play a causal role in early patterning. The zygote has been found transiently to exhibit bilateral symmetry before regaining an essentially spherical shape prior to first cleavage. Marking experiments indicate that the plane of bilateral symmetry of the blastocyst is aligned with, and the plane of first cleavage is typically orthogonal to, the zygote's bilateral plane. The bilateral symmetry of the zygote bears no consistent relationship either to the point of sperm entry or to the distribution of the pronuclei, and may therefore be a manifestation of intrinsic organization of the egg. Finally, the two-cell blastomere inheriting the sperm entry point has not been found to differ consistently in fate from the one that does not.

Keywords: pre-patterning; mouse; zygote; blastocyst; bilateral symmetry; axes

1. INTRODUCTION

The mouse has been the strongly preferred species for investigating early development in mammals from the time when applying the techniques of experimental embryology to this class of vertebrates first became practicable. This is particularly true regarding efforts to elucidate the basis of early patterning, a subject that for many years was dominated by two tenets. The first was that, by contrast to most other metazoa, the origin of relevant asymmetries must be sought in the evolving relationships between cells during or after cleavage rather than in the organization of the egg or zygote. The second tenet, which is consonant with the first, was that the conceptus remains radially symmetrical until after implantation, with the switch to bilateral symmetry being deferred until the primitive streak forms at the start of gastrulation. A serious challenge to this second tenet came from detailed observations by J. Smith on blastocyst to gastrula stage conceptuses that had been serially sectioned *in situ* (Smith 1980, 1985). The observations of Smith (1980, 1985) not only revealed that the mouse conceptus was bilaterally rather than radially symmetrical from the late blastocyst stage, but also suggested that its axes might specify those of the embryo proper. These intriguing findings prompted further studies which eventually showed that the first tenet was also untenable. An initial step was to confirm that the features of bilateral symmetry recorded in sectioned material could also be seen in intact conceptuses *ex utero* (Gardner 1990; Gardner *et al.* 1992). In specimens recovered as early gastrulae, alignment of the antero-posterior axis of the embryo proper with that of the conceptus could also be confirmed. However, the two axes often differed in polarity (Gardner *et al.* 1992), and this clearly conflicted with the conclusion of Smith (1985) that all three axes of the embryo accord with those of the conceptus. A possible basis for this discrepancy is that errors in determining the polarity of the antero-posterior axis are less likely when conceptuses are embedded following isolation rather than *in utero*.

Accepting the prevailing wisdom that pre-patterning could be discounted in mammals, Smith argued that bilateral symmetry must be imposed on blastocysts through exposure of different parts of their surface to the walls and floor of the uterine lumen (Smith 1980). This required them to maintain the same rotational orientation within the uterus until they attached to the luminal epithelium. It was because this appeared rather improbable that working back in preimplantation development seemed worthwhile, especially in view of an old report that *in utero*-sectioned early rat blastocysts were oval rather than circular in profile in polar view (Huber 1915). The latter was also found to be true for living early mouse blastocysts, whether freshly recovered from the uterus or grown *in vitro* from the two-cell stage, and regardless of whether the zona pellucida was present or not (Gardner 1997). It was while early blastocysts were being examined to determine their shape that two observations relating to the second Pb were made. One was that this Pb was still intact in nearly two-

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thirds of early blastocysts; the other was that its location on the surface of the trophectoderm was strikingly non-random. Typically, it lay within the central third of the Em.Ab axis which extends from above the centre of the inner cell mass to the roof of the blastocoele, and at one end of the axis of bilateral symmetry (Gardner 1997). Moreover, rather than being freely motile, the second Pb was found to be attached to the blastocyst by a thin tether whose mechanical and electrical properties, and morphology, accorded with it being the intercellular bridge that had persisted from when it was formed. This implied that the second Pb continues to mark the site of the animal pole of the zygote for as long as it survives (Gardner 1997) and, consequently, that the bilateral axis of the blastocyst is normally aligned with the AV axis of the zygote (Gardner 1997). Such a relationship seemed unlikely to be fortuitous, and this became even more improbable when subsequent use of various marking techniques revealed a conserved topographical relationship between the blastocyst and two-cell conceptus (Gardner 2001*a*; Piotrowska & Zernicka-Goetz 2001; Piotrowska *et al.* 2001). Thus, the equator of the blastocyst, and hence its axis of bilateral symmetry, tends to be approximately parallel with, and both its bilateral plane and Em.Ab axis orthogonal to, the plane of first cleavage. This means that both axes of the mouse blastocyst are normally specified before the completion of first cleavage, and poses the obvious question whether their specification might relate directly to the plane in which this cleavage occurs. Were this the case, the first cleavage plane should map reproducibly on the blastocyst, and this is not what is actually observed. Thus, this plane is not infrequently well off the equator, and can even be closer to orthogonal to it, particularly in blastocysts developing from manipulated zygotes or parthenogenetically activated eggs (Gardner 2001*a*; Piotrowska & Zernicka-Goetz 2002). This raises the further question whether there is corresponding variability in the way in which the first cleavage plane partitions the cytoplasm of the zygote. If, in spite of mapping variably on the blastocyst, first cleavage bisects the zygote in a reproducible way, then cytoplasmic localization might be discounted as a factor in early patterning. Therefore, it is important to examine how regularly the zygote is subdivided by first cleavage. This is, in principle, relatively simple to do with regard to partitioning of the cytoplasm axially since the second Pb can be used as a reference point for the zygote's animal pole.

2. HOW REGULAR IS THE PLANE OF FIRST CLEAVAGE?

As the second Pb usually lies symmetrically in the groove between blastomeres in the two-cell conceptus, first cleavage is generally assumed to be accurately meridional in the mouse (Howlett & Bolton 1985). Meridional orientation of this cleavage has been attributed recently to the site of the second meiotic division that is responsible for the production of the second Pb serving to orient the mid-plane of the first mitotic spindle, possibly via the midbody that remains associated with this Pb (Plusa *et al.* 2002*a*). However, preliminary observations on cytokinesis *in vitro* revealed that the location of the second Pb following completion of first cleavage not infrequently gives a misleading impression of the fidelity with which this division is oriented parallel to the zygote's AV axis. This prompted doubts about whether it is either as accurately or as consistently meridional as has been claimed. Thus, the second Pb commonly lies at some distance from the site where furrowing is initiated, and then shifts towards or into the nascent cleavage plane as cytokinesis progresses. Because the space between the zona pellucida and the surface of the vitellus, the so-called 'perivitelline space', is restricted the second Pb tends to be squeezed towards the nascent cleavage furrow and then trapped in the interblastomeric groove on completion of cytokinesis. However, squeezing is not the only means by which the Pb can be relocated. Sometimes, it was seen to move towards the future cleavage plane at a speed that is clearly discernible in real time, even before the furrow becomes deep enough to accommodate it.

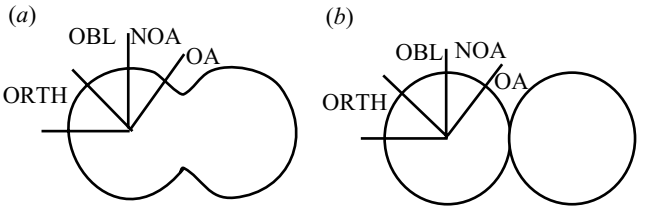

Figure 1. Diagrams of (*a*) zygote in cytokinesis and (*b*) two-cell conceptus showing the classification used in table 1 for relating the cleavage plane to the second Pb. Abbreviations: OA, on axis; NOA, nearly on axis; OBL, oblique to axis; ORTH, orthogonal to axis.

The classification system adopted for relating the cleavage plane to the location of the second Pb is illustrated in figure 1, while the data that were obtained under a variety of different conditions are presented in table 1. For observing the progress of cytokinesis directly, the original site of the second Pb was marked with a drop of mineral oil that was injected into the cortex of the zygote immediately beneath it. A second oil drop injected cortically diametrically opposite the first was used to control for spurious movement of the drop marking the second Pb. Following the progress of first cleavage directly poses various problems, particularly trying to avoid excursions in both the temperature and pH of culture drops that tend to occur during extended periods of microscopic observation. Hence, further series of conceptuses that were either recovered from the oviduct at the early two-cell stage or cultured undisturbed to this stage in a well-regulated incubator following explantation as zygotes were examined both before and after elimination of the zona pellucida with acidified Tyrode's saline. Once the Pb's location had been recorded, two blunt glass probes mounted on micromanipulators were used to loosen its adherence to the surface of the denuded conceptus (Gardner 1997). This was done to enable the point where the Pb was attached to the conceptus via the persisting intercellular bridge to be ascertained. The location of the Pb relative to the plane of first cleavage was also compared in zygotes that divided in culture with the zona pellucida off versus on. Finally, yet further zygotes had the perivitelline space gelated with the aim of preventing either active or passive displacement of the second Pb during first cleavage. This was done by incubating zygotes for up to

Table 1. Relationship of plane of first cleavage to the AV axis of the zygote.

		relationship of cleavage plane to the zygote's AV axis			
conditions of first cleavage	number scored	on axis (%)	nearly on axis (%)	oblique to axis (%)	orthogonal to axis (%)
in vivo	55	43	51	6	0
in vitro with zona pellucida	60	27	70	3	0
in vitro without zona pellucida	42	29	59	5	7
observed *in vitro* with zona pellucida	48	27	39	19	15
in vitro with perivitelline space gelated	100	39	15	31	15

1.5 h in culture medium containing 1.1% medium viscosity sodium alginate which can readily pass through the zona pellucida and thus accumulate in the perivitelline space (Gardner 2003). Following a brief rinse to remove external alginate, zygotes were placed in medium containing 0.15% (w/v) $CaCl_2$ for 20 min at room temperature to gelate the perivitelline space. Thereafter, one or more oil drops were injected into the zona pellucida immediately above the second Pb to check that its relocation or alternatively, rotation of the entire conceptus, had been prevented. Collectively, the findings presented in table 1 show that first cleavage is accurately meridional in only a minority of zygotes. Moreover, while most often nearly meridional, it not infrequently departs very conspicuously from this orientation. This is particularly evident where gelation of the perivitelline space was used to retain the second Pb in its original, pre-cleavage location. The relative deficiency of specimens with nearly on-axis first cleavage in this series is probably attributable to their being part of a study which required the zona pellucida to be left on so that it was not possible to probe where the Pb was attached to the vitellus. Because the incidence of markedly non-meridional division was conspicuously higher in this than in all other series, the possibility that orientation of the cleavage plane was perturbed by gelating the perivitelline space cannot be neglected. However, it is difficult to envisage how coating the zygote fairly uniformly with a gel might cause such a perturbation. It is rather easier to appreciate that by not only stopping the second Pb being squeezed, but also dragged, towards the cleavage plane, this measure might be expected to reveal the latter's variability most accurately. The strong tendency for the second Pb to shift to the cleavage plane even when initially well away from it explains why it served less well as a latitudinal than a longitudinal marker for determining whether the anterior end of the sperm tail remains at its original location through first cleavage (Davies & Gardner 2002).

According to the foregoing findings, sister two-cell stage blastomeres will commonly differ in their endowment with cytoplasm from different axial levels of the zygote. Furthermore, any influence that the region of the zygote adjacent to the Pb may have on the orientation of the first cleavage spindle, and consequently the plane of cytokinesis (Plusa *et al.* 2002*a*), is either not always decisive or the active component must vary considerably in its proximity to this body. The mid-body persisting from the second meiotic division may be rather too closely associated with the second Pb to exercise such a role.

Hence, orientation of the plane of first cleavage is neither strictly fixed (Howlett & Bolton 1985) nor completely random (Evsikov *et al.* 1994) with respect to the zygote's AV axis. However, the fact that it is usually more nearly parallel than orthogonal to the AV axis raises a further question, namely what dictates which of the possible, approximately meridional planes it takes. The absence of any visible asymmetry of the zygote around its AV axis means that this is difficult to examine directly. However, it has been claimed recently that the cleavage plane accords with the site where the sperm enters the zygote (Piotrowska & Zernicka-Goetz 2001; Plusa *et al.* 2002*b*). There is, of course, a precedent for this in amphibia, although even here the relationship of the sperm entry site to the cleavage plane is quite variable (Gilbert 1997). A further problem with the mouse study is that, as discussed at length elsewhere (Davies & Gardner 2002; Gardner & Davies 2003), it lacked appropriate controls for showing that the phytohaemagglutinin-treated fluorescent latex microspheres used to mark the fertilization cone or sperm entry point retain their ancestral position through first cleavage. More importantly, no consistent relationship between the sperm entry point and first cleavage plane was found using the anterior end of the sperm tail, which lies initially at the base of the fertilization cone and typically remains peripheral, as a natural marker of the sperm entry point. This was the case despite the fact that, unlike the microspheres, the anterior end of the tail was shown to retain its relative position to the early two-cell stage in most cases (Davies & Gardner 2002).

Hence, if the choice of meridional plane for first cleavage is not specified by the sperm entry point, might it depend on conditions that are intrinsic to the egg or zygote? In this context, it is noteworthy that zygotes lose their approximately spherical shape as pronuclear development progresses, but normally regain it before they embark on first cleavage. This transient change in shape of the zygote is readily discernible when it entails elongation, usually obliquely to its AV axis (figure 2*a*). However, even zygotes that do not become obviously elongated are found to be oval rather than circular in profile when viewed in a particular orientation, typically with the animal pole uppermost (figure 2*b*). Occasionally, however, rather than being prolate and thus exhibiting bilateral symmetry, zygotes are either oblate or compressed obliquely to the AV axis. Curiously, this temporary shape change seems not to have attracted sufficient notice to have been reported in the literature, even though the mouse zygote

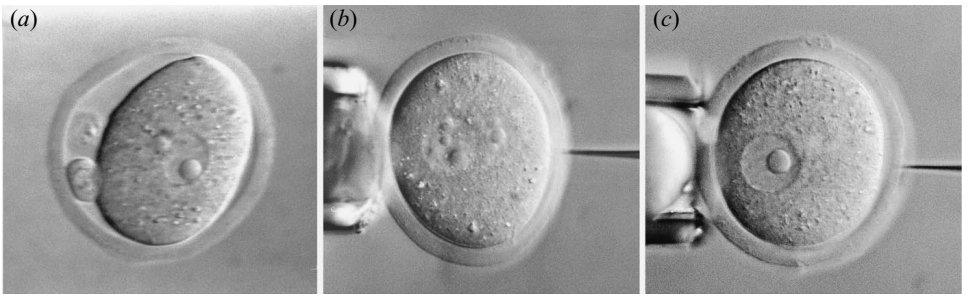

Figure 2. (*a*) Pronuclear zygote elongated obliquely to the AV axis and (*b*,*c*) examples of pronuclear zygotes oriented with the animal pole uppermost to show that they are prolate spheroids.

has been the subject of intensive investigation for many years.

3. BILATERAL SYMMETRY OF THE ZYGOTE

One difficulty in attempting to assess the significance of this transient shape change is posed by the lack of validated ways of directly marking points on the surface of the zygote. Recourse to injecting drops of mineral oil focally into the zona pellucida provided a satisfactory way of circumventing this problem for cleavage stages because the conceptus undergoes little if any net rotation within this investing membrane from the two-cell stage onwards (Gardner 2001*a*). However, trials in which this technique was used to mark the site of the second Pb in zygotes often revealed a substantial disparity between the location of this body and its oil drops by the completion of first cleavage. Consequently, for this approach to marking to be effective it was necessary to prevent such rotation. This was done by using 1.1% medium viscosity sodium alginate to gelate the perivitelline space, as described earlier (Gardner 2003). Thereafter, the entire plane of bilateral symmetry of the zygotes was ringed with individual spaced oil drops in the zona pellucida. An additional tight cluster of three oil drops was sited over the second Pb to enable prevention of rotation to be checked at any subsequent stage (figure 3*a*). An initial series of zygotes that had been marked in this way were returned to culture and the resulting blastocysts photographed to provide a record of the orientation of the oil ring. Intriguingly, the ring showed a strong tendency to align with the Em.Ab axis of the blastocyst (figures 3*b* and 4*a*). Moreover, in blastocysts that were obviously oval in polar view, the oil ring accorded with the plane of bilateral symmetry. As expected from the finding that the plane of first cleavage is usually approximately orthogonal to the blastocyst's Em.Ab axis, it was typically also orthogonal to the zygote's bilateral plane (figures 3*c*,*d* and 4*b*).

This finding shows that the zygote is, at least temporarily, bilaterally symmetrical, and suggests, moreover, that its bilateral plane specifies that of the blastocyst. If correct, this clearly raises further issues. One is the basis of the zygote's bilateral symmetry and another is how it exerts its role in patterning the blastocyst. Regarding the first, examination of a large number of zygotes has failed to reveal that either components of the fertilizing sperm or arrangement of the pronuclei bear a consistent spatial relationship to the bilateral plane. This raises the interesting possibility that bilaterality may be an intrinsic feature

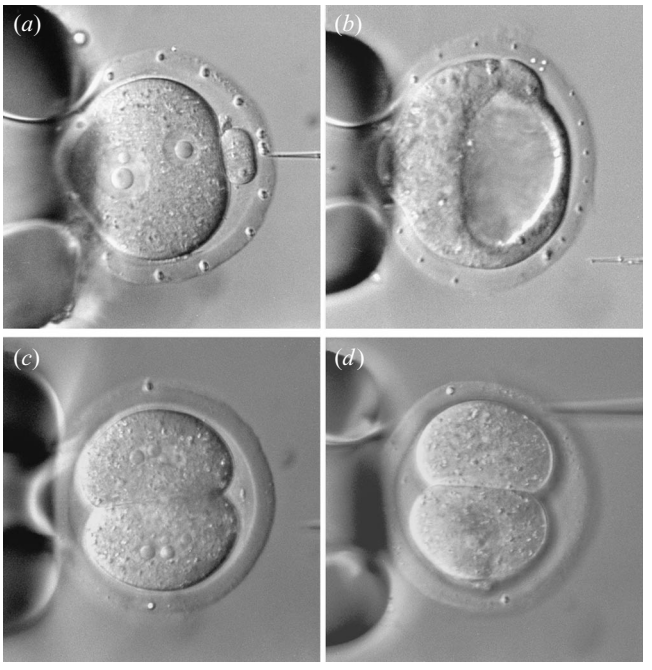

Figure 3. (*a*) Zygote with plane of prolation ringed with well-spaced oil drops in the zona pellucida and the second Pb marked with a cluster of three such drops; (*b*) side view of blastocyst showing the oil ring is aligned with its Em.Ab axis, and that the Pb still lies directly under its marker oil drops; (*c*,*d*) two-cell conceptuses with the oil ring oriented vertically, showing that the cleavage plane is approximately orthogonal to the ring whose orientation is indicated by the opposed pair of its constituent oil drops.

of the organization of the egg, whose manifestation might nevertheless be triggered by its fertilization.

Various additional studies are in progress to address the issue of how bilaterality might exercise its role. One possibility that can be discounted is that it does so simply by specifying the orientation of the plane of first cleavage through imposing a corresponding change in shape on the zona pellucida. Following egg activation, the zona pellucida undergoes a process of chemical modification termed 'hardening' which is attributed to release of enzymes stored in cortical granules (Krzanowska 1972; Schmell & Gulyas 1980). Depending on how rapidly this process proceeds, prolation of the zygote might impose a corresponding change in shape on the zona pellucida that persists after the zygote becomes spherical once again. Then, regardless of orientation of the spindle, the zygote could be constrained to rotate as it elongated during cyto-

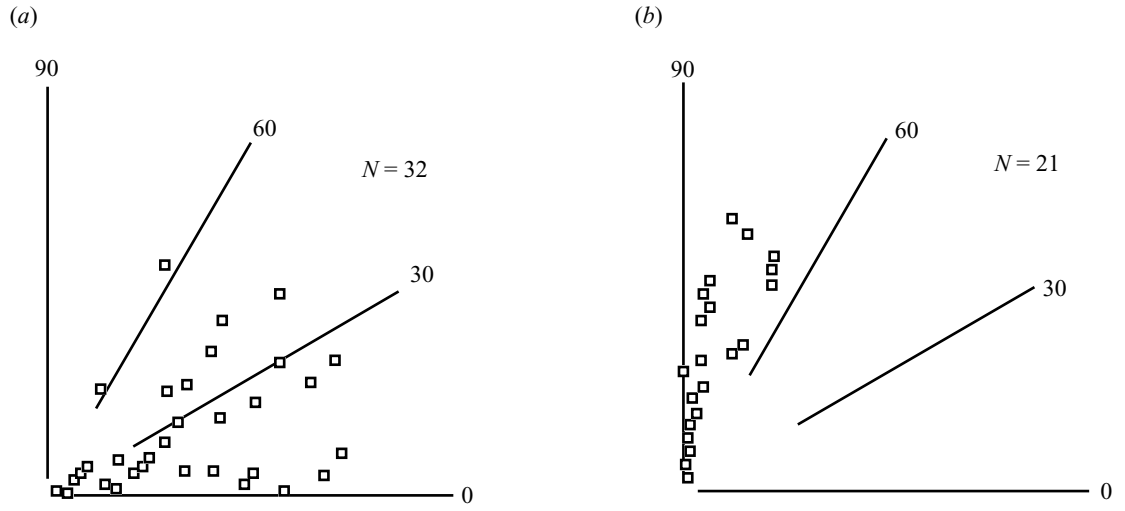

Figure 4. The angle of ring of oil drops delineating the bilateral plane of zygote relative to the Em.Ab axis of the blastocyst (*a*), and to the plane of first cleavage (*b*).

kinesis so that the plane of cleavage would appear always to be orthogonal to the plane of prolation. There is, however, the further possibility that gelating the perivitelline space while zygotes are obviously bilaterally symmetrical might impede their regaining a spherical shape prior to mitosis so that, in accordance with Hertwig's rule (Wilson 1928), the long axis of the spindle aligns with the plane of residual prolation. While it is clear that gelation of the perivitelline space does not prevent early zygotes from becoming bilaterally symmetrical, those that are already bilateral when the space is gelated show an increased tendency to remain somewhat prolate in conformity with the oil ring when pronuclei are no longer visible. To address the possibility that this might serve to orient the plane of first cleavage, further zygotes had their bilateral plane ringed without prior gelation of the perivitelline space, and were then cultured in medium plus sodium alginate until pronuclei could no longer be seen. They were then exposed to 0.15% $CaCl_2$ to gelate the perivitelline space before being examined microscopically both to confirm that the second Pb retained its position relative to its marker oil drops in the zona pellucida, and to check whether they betrayed any sign of persisting prolation. Those that were still discernibly prolate were then cultured to the two-cell stage separately from those that were not and, after checking once again that there had been no rotation in the zona pellucida, the relationship of the cleavage plane to the ring was recorded. The two planes were found to be approximately orthogonal to each other regardless of whether the zygotes did or did not retain a somewhat prolate condition shortly before cleavage. Moreover, zygotes that had regained a strictly circular profile following pronuclear membrane breakdown frequently exhibited two separate groups of chromosomes when stained with Hoechst 33342, thereby confirming that they had not yet reached metaphase. Hence, it seems that the bilateral plane of the zygote is not simply influencing orientation of the plane of cleavage by effecting a shape change that persists through to the stage of formation of the mitotic spindle. This might suggest, but does not prove, that its action does not depend on maintenance of the microtubular component of the cytoskeleton.

4. DIFFERING FATES OF SISTER 1/2 BLASTOMERES

While our findings suggest that information inherent in the organization of the egg could define the plane in which the Em.Ab axis of the blastocysts lies, additional cues are clearly needed to set both its orientation and polarity. Formation of this axis is generally assumed to depend on localization of the site of blastocoelic fluid accumulation which thereby defines where internal cells of the conceptus will retain contact with outer to form the ICM. However, the possibility has yet to be discounted that the opposite is the case, namely that local retention of contact between inner and outer cells acts to restrict the site of fluid accumulation. It is noteworthy in this context that if the polar trophectoderm herniates through a suitably placed slit in the zona pellucida to leave the ICM behind, it produces a second blastocoele (R. Gardner, unpublished data). Hence, contact with the ICM seems to prevent trophectoderm cells from participating in formation of the blastocoele.

Of relevance to the issue of specification of the Em.Ab axis are recent studies of Zernicka-Goetz and her colleagues who found a strong bias towards different fates of 1/2 blastomeres according to their division order. First Piotrowska & Zernicka-Goetz (2001) reported that the blastomere inheriting a PHA-treated fluorescent microsphere that was used to mark the sperm entry point was typically the first to divide. In a subsequent analysis of lineage from the two-cell stage, the first dividing 1/2 blastomere was found to contribute principally to the embryonic as opposed to the abembryonic hemisphere of the blastocyst in the great majority of cases (Piotrowska *et al.* 2001). In conformity with this are the results of a much earlier study which showed that the descendants of the first 1/2 blastomere to divide to the four-cell stage tended to retain a temporal advantage, and to contribute more progeny to the ICM than descendants of the second blastomere to divide (Graham & Deussen 1978). The obvious implication of the work of Zernicka-Goetz and her colleagues is that inheriting the sperm entry point

Table 2. Location at the blastocyst stage of the oil drop in the zona pellucida placed over 1/2 blastomere with sperm mid-piece.

			location on blastocyst of marker of outer extremity of positive 1/2 blastomere		
experimental series	number of two-cells with fluorescent signal	number of two-cells forming scorable blastocysts	emb. h'sphere	abemb. h'sphere	equator
old[a]	57	40	23	11	6
new	62	53	26 (14)[b]	25 (12)[b]	2
both	119	93	49 (14)[b]	36 (12)[b]	8

[a] Data from Davies & Gardner (2002).
[b] Numbers in parentheses are cases where axial rotation of the concepts within the zona pellucida could be discounted.

affects the fate of a 1/2 blastomere, possibly through endowing it with a growth advantage.

However, within batches of two-cell conceptuses the interval between division of sister blastomeres can vary from a few minutes to several hours (Kelly *et al.* 1978), and presumably it is only in cases where this interval is substantial that a temporal disparity can be maintained. Especially since the conditions of detecting and inspecting three-cell conceptuses might further delay the undivided blastomere selectively, e.g. through a disruptive effect of temporary cooling on the mitotic spindle, it is pertinent to ask whether a strong tendency for the blastomere inheriting the sperm entry point to contribute mainly to the embryonic hemisphere of the blastocyst is evident when all two-cell conceptuses are examined rather than just those with markedly asynchronous second cleavage. This was done by artificially inseminating mice that had been mated recently with vasectomized males (Kile 1951) with sperm whose mid-piece had been labelled with the fluorescent mitochondrial dye, MitoTracker Green. Conceptuses were recovered from the oviduct at the early two-cell stage when, according to our earlier findings, the mid-piece still co-localizes with the anterior end of the sperm tail (Davies & Gardner 2002). In all but a single instance, the MitoTracker signal was wholly confined to one 1/2 blastomere. Though its shape varied from rod-like to one or more less well-defined foci, it invariably occupied only a small fraction of the blastomere and, in accordance with findings on mapping the anterior end of the sperm tail, showed no tendency to lie close to rather than away from the cleavage plane. The outer extremity of the blastomere with the fluorescent signal was marked with a single oil drop in the overlying zona pellucida before the conceptuses were cultured to the blastocyst stage. The results of an initial series of experiments were reported earlier (Davies & Gardner 2002). In further experiments, the locus of the second Pb was also marked with two oil drops in the zona pellucida so that the possibility of rotation in the zona could be checked in blastocysts in which this body was still discernible. The new results accorded with the published ones in showing no significant departure from random in the frequency with which the oil drop marking the outer extremity of the MitoTracker-positive 1/2 blastomere mapped to the two hemispheres of the blastocyst (table 2). Importantly, the partitioning of the oil drop between hemispheres did not differ between specimens for which rotation in the zona pellucida between labelling and analysis could be discounted and those for which it could not. Therefore, these findings do not support the contention that the fate of two-cell blastomeres differs according to whether or not they inherit components of the fertilizing sperm that normally remain at the site of sperm entry beyond first cleavage (Davies & Gardner 2002). The possibility that fluorescent excitation of MitoTracker Green might cause sufficient damage to the blastomere containing the mid-piece to alter its fate seems unlikely because the only labelled structures that are immediately affected are paternally inherited mitochondria undergoing degradation for which they were evidently targeted during spermatogenesis (Sutovsky *et al.* 2000). Nevertheless, we are seeking to improve the sensitivity of our microscope for detecting birefringence to be able to visualize the sperm tail in living late zygotes and two-cell conceptuses without recourse to epifluorescence microscopy.

5. REVISITING BLASTOMERE POTENCY STUDIES

Might specification of the Em.Ab axis of the blastocyst depend on cues that are localized within the zygote? This is clearly compatible with the findings on the variability of first cleavage that were presented earlier. As discussed elsewhere, looking for deficiencies in the patterning of partial conceptuses rather than duplications arising from their aggregation is likely to be more informative in addressing this possibility (Gardner 2001*b*, 2002*a*). Studies on partial conceptuses have mainly entailed investigating the developmental potential of isolated blastomeres. However, while the results of such studies have consistently been interpreted as evidence against the existence of cytoplasmic localization in mammals, they suffer from an important limitation that undermines this conclusion. This is particularly true when, as is typically the case, normal development is obtained from only some rather than all blastomeres isolated from individual cleavage stages. Thus, in such studies it is impossible to carry out appropriate controls to distinguish whether failure of regulation is, as is usually held to be the case, due to limitation of cell numbers or to cell damage rather than a real restriction in developmental potential. This difficulty could be circumvented if it were possible to identify conceptuses whose pattern of cleavage was sufficiently similar to enable corresponding blastomeres to be identified in each. This would allow the development of conceptuses reconstructed from

the appropriate number of corresponding blastomeres from different conceptuses to be compared with that of those reconstructed from their 'native' set of blastomeres. Such a strategy would not only eliminate cell number as a variable, but also enable any effects on patterning of the blastomere dissociation and manipulation procedures to be controlled.

As discussed earlier, despite its marked variability, first cleavage is approximately meridional in the majority of cases. Moreover, recently, the approximately regular tetrahedral shape of many four-cell conceptuses has been found to be the product of a consistent pattern of second cleavage, with one blastomere dividing meridionally and the other approximately equatorially (Gardner 2002*b*). Preliminary findings indicate that in conceptuses with such a regular pattern of second cleavage, third cleavage is also regular, with both products of the equatorial division dividing meridionally, and both products of the meridional division dividing equatorially (J. Paul and R. Gardner, unpublished data). Not only must these observations be extended, but whether the regularity of the second cleavage depends on the regularity of the first cleavage also needs to be determined.

6. CONCLUSIONS

Despite the dramatic way in which their development has been modified to meet the demands of a particularly intimate and sophisticated type of viviparity, eutherian mammals, like most other metazoa, nonetheless normally use cues that are present before the beginning of cleavage for their early patterning. While the nature of this pre-patterning in mammals is still obscure, there are increasing indications that it is primarily maternal. Consequently, its better characterization is likely to require detailed examination and imaginative manipulation of oocytes at various stages in their maturation.

The prevailing view, engendered by the impressive regulative ability of preimplantation conceptuses, is that pre-patterning cannot be indispensable for normal development in mammals. This view, however, is not warranted on the basis of critical appraisal of available data and, moreover, fails to take account of various findings that remain unexplained (Gardner 2001*b*). Among these is why the frequency of monozygotic twinning is significantly higher following assisted than natural conception in our own species (Derom *et al.* 1987; Wenstrom *et al.* 1993; Blickstein *et al.* 1999). As this mode of twinning is associated with an increased risk of congenital malformation (Schinzel *et al.* 1979; Szymonowicz *et al.* 1986; Little & Bryan 1988; Hall 1996), the continuing expansion of assisted reproductive practices lends particular urgency to the task of ascertaining the nature and significance of pre-patterning in mammals.

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Discussion

R. G. Edwards (*Reproductive BioMedicine Online, Dry Drayton, Cambridge, UK*). Let me take you to your latest work on the cleavage planes to the four-cell stage. You say that two-cell blastomeres retain the original symmetry of the oocyte in the four-cell embryo, dividing meridionally. The other two are the result of an equatorial plane which makes one animal and one vegetal. From then on it does not matter much whether the succeeding cleavages are meridional or equatorial because one is decidedly animal and the other one is vegetal so they carry on being animal or vegetal. If the other two blastomeres which divided meridionally first, now divide equatorially, they will also inherit animal or vegetal characteristics. So one ends up in the eight-cell stage with everything being animal or vegetal. One wonders, why have polarity in the first place if this is the result of these cleavage planes?

R. L. Gardner. The short answer is that we do not know, but pre-existing polarity might actually be instrumental in ensuring the regular pattern of cleavage that you describe. It is also important to remember that a substantial minority of mouse conceptuses do not show such regular second cleavage.

J. Rossant (*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada*). How sure are you that you can determine reproducible cleavage patterns when the polar body itself seems to move?

R. L. Gardner. Our finding that the second polar body is not infrequently well away from the plane of first cleavage following gelation of the perivitelline space is a very recent one. We have therefore not yet had time to ascertain whether markedly 'off-axis' first cleavage is compatible with regular as opposed to irregular second cleavage. Moreover, it is conceivable that the site of production of the second polar body does not always correspond with one end of the physiological axis of polarity of the oocyte or zygote. It is evident, for example, that the second polar body is often well away from the first, implying that substantial axial rotation may occur between the two meiotic divisions. Whether the pre- or the post-rotational orientation more accurately reflects the physiological axis of polarity has yet to be determined.

I. Wilmut (*Department of Gene Expression and Development, Roslin Institute, Edinburgh, UK*). What can we learn from procedures that disrupt egg structure? In most mammalian cloning procedures there is neither fertilization nor the second division of meiosis. When making transgenic pigs and cattle the early zygote was centrifuged, sometimes in the presence of cytoskeletal inhibitors.

R. L. Gardner. This is difficult to answer because, at present, we have no information regarding the nature of the axial information that is present in the zygote. With respect to cloning, rates of attrition are sufficiently high in all species to raise the possibility that patterning could be perturbed by the procedures that are used. However, it is also noteworthy that in various non-mammalian species activation of the oocyte has been found to trigger cytoplasmic rearrangements that relate to patterning. Were such rearrangements to take place in mammals, and to do so only shortly before cleavage, manipulations involved in cloning and transgenesis could be completed before they occurred.

S. Frankenberg (*The Wellcome Trust/Cancer Research UK, Institute of Cancer and Developmental Biology, University of Cambridge, Cambridge, UK*). In the sperm labelling experiments, how long after disappearance of the fertilization cone was its position recorded and could the sperm already have moved through the cortex in a significant number of cases?

R. L. Gardner. The position of the anterior end of the sperm tail was marked either when or before the fertilization cone was present. In the mouse, the sperm initially attaches to the surface of the oocyte via the middle of its head. Once the head starts to be 'engulfed', motility of the tail invariably ceases before it too sinks into the cortex of the oocyte in a strictly anterior to posterior sequence. Consequently, there is no question of the tail of the fertilizing sperm being able to move actively through the cortex of the nascent zygote.

M. Zernicka-Goetz (*The Wellcome Trust/Cancer Research UK, Institute of Cancer and Developmental Biology, University of Cambridge, Cambridge, UK*). While Professor Gardner's results are interesting, we have a body of work that leads us to a different conclusion about the position of the first cleavage in the mouse. The reasons for the discrepancy between our interpretations are not clear, but may reflect the fact that the two groups are examining different parameters. By contrast to Professor Gardner we have not examined internalized components of the sperm, but we have used either a fluorescent bead or labelled sperm itself

to mark a point on the egg surface at the fertilization cone—a transient structure that forms at the sperm entry (Piotrowska & Zernicka-Goetz 2001; Plusa *et al.* 2002*a*). We find that beads placed at the fertilization cone remain attached and subsequently tend to associate with the cleavage furrow from its earliest stages of formation. Professor Gardner has claimed that beads do not stay attached to the egg surface. However, they do in our hands. He also believes that beads will always move to the cleavage furrow. This is not our experience: when we placed a bead randomly on the egg surface it showed no tendency to mark the cleavage furrow. Our most compelling evidence that the sperm entry site predicts the site of the first cleavage comes from time-lapse movies that follow eggs from the time of fertilization up to the first cleavage. Although the fertilization cone cannot itself be followed up to the time of cleavage, other markers on the egg can be followed over this time. These films demonstrate conclusively that in the majority of eggs the site of the fertilization cone is subsequently closely associated with the position of the first cleavage.

R. L. Gardner. I am afraid we disagree on several issues, particularly the relationship of the site of sperm entry to the plane of first cleavage. Without seeing the data, I am obviously not in a position to evaluate the time-lapse studies to which Dr Zernicka-Goetz refers. I should point out, however, that contrary to Dr Zernicka-Goetz's assertion, we did not make any dogmatic claims about how the phytohaemagglutinin-treated beads that she used to mark the sperm entry point behave. Rather, we drew attention to potential pitfalls arising from various published studies on binding of lectins to the cell surface, and then went on to explain why we considered that the controls she and her colleagues presented did not adequately address them. It is not appropriate to rehearse our critique here. For those wishing to make up their own minds about the issue, this critique can be found (Gardner & Davies 2003), together with Dr Zernicka-Goetz's response (Zernicka-Goetz 2003) plus an independent commentary by Professor Martin Johnson (2003), both on the web and in a hard copy.

Additional references

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GLOSSARY

AV: animal-vegetal
Em.Ab: embryonic–abembryonic
ICM: inner cell mass
Pb: polar body