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Specification of embryonic axes begins before cleavage in normal mouse development

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This paper is dedicated to Dr Rosa Beddington, FRS, friend, former student and outstanding developmental biologist, as a tribute to her elegant work on pattern formation in mammals, and to her fortitude

SUMMARY

Studies on the development of aggregated, isolated and rearranged blastomeres have engendered the view that in mammals, unlike most other animals, egg organization has no role in the genesis of asymmetries that are essential for cellular diversification and the specification of embryonic axes. Such asymmetries are assumed to arise post-zygotically through interactions between initially naive cells. However, various findings are difficult to reconcile with this view. Here, a consistent relationship between the structure of the blastocyst and the two-cell stage in the mouse has been found using a strictly non-invasive marking technique: injection of small oil drops into the substance of the zona pellicuda. This has revealed that both the embryonic-abembryonic axis of the blastocyst and its plane of bilateral symmetry are normally orthogonal to the plane of first cleavage. This relationship was also seen when denuded two-cell conceptuses were prevented from rotating during subsequent cleavage by immobilizing them in a gel. Therefore, during normal mouse development the axes of the blastocyst, which have been implicated in establishing those of the fetus, are already specified by the onset of cleavage.

Key words: Blastocyst, Embryonic-abembryonic axis, Bilateral axis, Animal-vegetal axis, Zygote, Alginate gel, Zona pellucida, Mouse

INTRODUCTION

Despite burgeoning interest in how the body plan of the mammalian fetus is established during gastrulation, little is known about when and how its anterior-posterior (AP) axis is specified. The possibility that, as in most other species, it might normally depend on cues that are already present in the zygote is widely held to be incompatible with the marked ability of conceptuses to regulate their development during cleavage (Gardner, 1996a). However, various findings challenge the view that specification of the AP axis of the fetus is initiated only shortly before gastrulation (reviewed by Gardner, 1998; Gardner, 2000a).

Because it cavitates eccentrically, the mammalian blastocyst has an obvious axis of polarity, the embryonic-abembryonic (Em.Ab) axis, with one pole centered on the inner cell mass (ICM) and the other on the floor of the blastocoele (Fig. 1A). Although early mouse blastocysts appear roughly circular from the side, in polar view most are unquestionably elliptical (Gardner, 1997), as seems to be true also in the rat (Huber, 1915). Hence, they are prolate spheroids with two planes of bilateral symmetry parallel to the Em.Ab axis that coincide, respectively, with their greater diameter (GD) and lesser diameter (LD) (Fig. 1B). The GD in the equatorial plane is particularly interesting, as it evidently corresponds with both the animal-vegetal (AV) axis of the zygote (Gardner, 1997) and the AP axis of the future fetus (Smith, 1980; Gardner et al., 1992; Gardner, 1998; Weber et al., 1999; Ciemerych et al., 2000). It has therefore been termed the 'axis of bilateral symmetry' of the conceptus (Gardner, 1997; Gardner, 1998). This axis, unlike the Em.Ab axis to which it is orthogonal (Fig. 1B), is not discernibly polarized in the early blastocyst, although the end corresponding to the animal pole of the zygote is often evident from the persisting second polar body (Pb).

When and how the axes of the blastocyst are specified have yet to be established. Studies undertaken so far have focused on the Em.Ab axis, ascribing the siting of the blastocoele to heterogeneity in cell cycles that may be stochastic (Graham and Deussen, 1978). However, the association is tenuous and the conclusions conflicting (Surani and Barton, 1984; Garbutt et al., 1987). While the origin of the bilateral axis has not been investigated, its consistent alignment with the AV axis of the zygote seems most unlikely to be fortuitous.

The present study was prompted by finding that the mouse conceptus already has a GD and a LD during early cleavage (R. L. G., unpublished observations). Thus, from the four-cell stage onwards, conceptuses are typically elliptical when viewed with the Pb uppermost, rather than in their preferred orientation when it lies to one side. Two-cell conceptuses normally settle with both

blastomeres in the same plane and, according to the position of the Pb, also have their AV axis horizontal. Intriguingly, however, at this stage it is only the zona pellucida (ZP) that is conspicuously elliptical when the AV axis is oriented vertically (Fig. 1C,D). This change in shape of the ZP is manifest as a decrease in its diameter in the plane of first cleavage.

Marking single blastomeres gives too widespread labeling to be used for investigating the relationship between flattening of cleavage stages and the blastocyst. That the necessary resolution might be achieved by marking the ZP focally has been suggested by failure to detect obvious net rotation of conceptuses within this glycoprotein coat in time-lapse records of cleavage in vitro (R. L. G., unpublished observations). Hence, up to three specific sites were marked differentially in two-cell conceptuses from both PO (Pathology, Oxford) closed-bred and [CBA×C57BL/6] F_1 (F_1) mice by injecting one, two or three small drops of soya oil into the substance of the ZP. The distribution of the oil sites following cleavage in vitro revealed a consistent topographical relationship between the blastocyst and the two-cell stage. Thus, the Em.Ab axis of the blastocyst was typically orthogonal to the plane of first cleavage. This was also found to be the case in further two-cell conceptuses whose orientation was fixed during subsequent development in vitro by removing the ZP and embedding them in a gel.

MATERIALS AND METHODS

Recovery and culture of conceptuses

Both zygotes and two-cell conceptuses were recovered from PO and [CBA×C57Bl/6J] F_1 females mated naturally with males of the same genotype. Conceptuses were recovered, manipulated, stored at room temperature in Hepes-buffered medium and cultured in its bicarbonate-buffered counterpart in an atmosphere of 5% CO_2 in air. Initially, the medium was MTF (Gardner and Sakkas, 1993), and later KSOM (Summers et al., 1995). Conceptuses were denuded either chemically or mechanically. For chemical denudation they were exposed to acidified Tyrode's saline (Pratt, 1987) or to 0.5% Pronase (Calbiochem, USA) in phosphate buffered saline (PBS, Dulbecco A, Oxoid, UK) until the zona had become 'vanishingly thin'. Mechanical denudation was achieved by slitting the ZP repeatedly with a fine-tipped glass needle (Tsunoda et al., 1986) and then releasing conceptuses by gently aspirating them with a pipette with a tip of approx. 60 μm in diameter.

Oil-drop marking of the ZP

Fine-tipped pipettes pulled from filament capillary (TF-15, Clarks Electromedical, Pangbourne, UK) on a Brown-Flaming electrode puller (Sutter, USA) were backfilled with soya oil and connected to an injector system (IM 6, Narishige, Japan) containing heavy liquid paraffin throughout. Injections were carried out with the two-cell conceptuses immobilized by gentle suction on the tip of a pipette, and viewed by differential interference contrast optics with a ×40 water-immersion objective lens (Zeiss, Germany) and ×12.5 oculars. Up to three sites in the ZP were marked differentially. The great majority of conceptuses were first oriented so that both blastomeres were in the same plane, and then rotated until their GD was horizontal before one small oil drop was injected into the focal mid-plane of the ZP in alignment with the plane of first cleavage. Most also had two oil drops injected into the ZP over one blastomere at its furthest point from the cleavage plane (Fig. 1C,E). The conceptuses were then rotated until the single oil drop was centrally uppermost, so that the lesser diameter of the ZP could be marked with three small oil drops, also aligned with the first cleavage plane (Fig. 1D,F). In a further, more limited, series of two-cell conceptuses, only the two oil site over one blastomere was marked. A single oil drop was then injected into the ZP at the corresponding site over the other blastomere until, after extending externally, it became sufficiently large to lift the adjacent blastomere above its sister (Fig. 2A,B). After labeling, conceptuses were cultured individually to the early or expanding blastocyst stage when they were immobilized on a pipette for photography. Those with a large oil drop had this drop removed before being photographed.

Mapping the distribution of oil drop sites in blastocysts

The center of each blastocyst was located by measurement on photographic prints and a glass plate etched with a pair of orthogonal lines then placed on each print so that the point of intersection of the lines was central. Next, the orientation of the plate was adjusted until the lines were judged, respectively, to be parallel to the Em.Ab axis and the equatorial plane of the blastocyst. Finally, the locations of the oil sites were marked on the plate with an alcohol soluble marker, so that the angle of departure from the Em.Ab axis (two oils) or the equatorial plane (one and three oils) could be measured, as illustrated in Fig. 3A,B. The values recorded were the mean of measurements taken on the three separate occasions which, because only the center of the blastocyst was marked on the prints, were effectively blind. Cricket Graph (Cricket Software, Malvern Philadelphia, USA) was used to plot distributions of the three oil sites as polar graphs and Minitab Statistical Software (Minitab, Philadelphia, PA) for preparing scatter diagrams and determining correlation coefficients.

For statistical analysis of the distribution of the oil sites, blastocysts were regarded as spheres. As shown in Fig. 3G, the polar one-third of the surface of a sphere lies within the solid angle (a) of 48.2° about the polar axis, with the remaining non-polar two-thirds lying outside this angle. Correspondingly, the central one-third of the surface of a sphere lies within, and the remaining two-thirds outside, the solid angle (b) of 19.5° from the equator (Fig. 3H). Hence, blastocysts were classified according to whether their two oil site was more or less than 48° off the Em.Ab axis, and whether their one and three oil sites were more or less than 19° off the equator. Finally, the binomial expression was used to calculate the probability of obtaining up to, but not more than, the observed number of blastocysts with oil sites 'off-axis', i.e. over the non-polar two-thirds of the surface for the two oil site, or over the non-equatorial two-thirds for one and three oil sites.

Embedding conceptuses in alginate

Alginate was chosen because its gelation does not depend on heating, merely the addition of Ca^{2+}. Moreover, two- and 4-cell mouse conceptuses encapsulated in alginate have been shown to develop in vitro at a similar rate to non-encapsulated controls (Cosby and Dukelow, 1990). Medium or low viscosity sodium alginate (Sigma A2033 and A 2158, respectively) was made up at 0.3, 0.5, 0.7 or 1.0% (w/v) in KSOM and, as for standard culture of individual conceptuses, dispensed into 30 mm bacteriological dishes as drops under light paraffin oil (BDH, UK). Before use, each dish had a ruled line scratched across its base towards one side, both for aligning the drops and enabling their consistent orientation on the microscope stage for photography of the embedded conceptuses. A single conceptus was then added to each drop and either allowed to settle naturally or oriented with a fine-tipped Pasteur pipette before the alginate was induced to gel by adding a small volume of 1.5% (w/v) $CaCl_2$ in 0.9% NaCl. After about 1 minute as much fluid as possible was removed from the drop without disturbing the gel and a larger volume of fresh alginate-free KSOM added. After this rinsing process had been repeated a further three-four times over a period of several minutes, each drop was reduced until the surface of the alginate gel was closely invested by the surrounding paraffin oil. Once all conceptuses in a dish had been immobilized thus, the dish was placed on the stage of an inverted microscope (Diavert, Leitz), and oriented so that the scratch mark was strictly parallel to the *x* movement of the mechanical

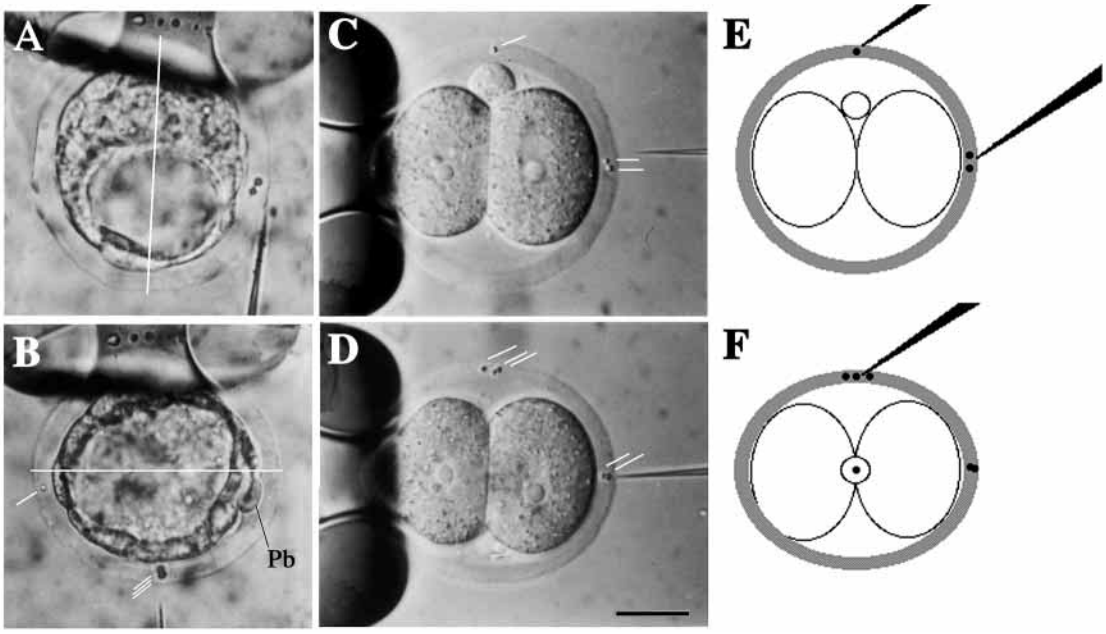

Fig. 1. (A,B) Early mouse blastocyst in side view (A) to show the Em.Ab (embryonic-abembryonic) axis, and in polar view (B) to show the bilateral axis (which has the greater diameter). The position of each oil drop in the zona pellucida (ZP) in B (and in C,D) is indicated by a single short white line. Note that, in B, the one oil site is, like the second polar body (Pb), aligned with the bilateral axis and the three oil site with the LD. (C,D) The same two-cell stage with its AV axis horizontal (C) and vertical (D). Note the location of the sites of one, two and three oil drops in the ZP and the reduced diameter of this envelope, but not of the blastomeres, in D relative to C. (E,F) Diagrams corresponding to C,D showing the siting of one and two (E) versus three oil drops (F) in the ZP. The one and two oil drops were injected into the ZP at its focal mid-plane with the AV axis horizontal, respectively, in line with and orthogonal to the first cleavage plane (E). Conceptuses were then rotated until the one oil site was centrally uppermost, and three oil drops injected into the ZP at the new focal mid-plane, also in alignment with the first cleavage plane. (A,B) Brightfield; (C,D) differential interference contrast optics. Scale bar: 30 μm.

stage. Each conceptus was then photographed in brightfield optics using ×32 objective and ×10 ocular lenses and the orientation of its first cleavage plane also sketched before the dish was cultured. Alignment and photography was then repeated for each conceptus once it had reached the blastocyst stage. Comparison of the photographic images was used to determine the relationship of the Em.Ab axis of the blastocyst to the plane of first cleavage. In blastocysts that were oriented suitably and retained a definite imprint of the first cleavage plane, the angle that the Em.Ab axis made to a line orthogonal to this plane was measured.

RESULTS

Marking of sites in the conceptus with oil drops in the ZP

Apart from rare specimens with obvious morphological anomalies, all intact two-cell conceptuses from both the PO and F_1 mice were used for marking the ZP with oil drops. The mean values of the GD and LD of the ZP in a series of 43 PO conceptuses was 96.8 μm (±0.47) and 89.4 μm (±0.47), respectively. After labeling, conceptuses were cultured to the early or expanding blastocyst stage for scoring before they shed the ZP. The proportion of labeled conceptuses scored for the distribution of marker oil drops at the blastocyst stage was 86% in the PO series and 76% in the F_2 series. The remainder either failed to cavitate within 60 hours, or formed blastocysts that were poorly integrated or which contracted before photography. Photographs were taken of all scorable

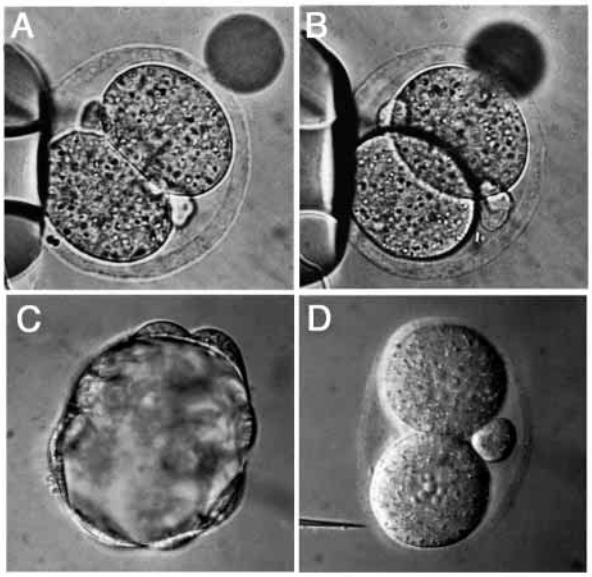

Fig. 2. (A,B) Two-cell conceptus with a single large oil drop attached to the ZP over the outer surface of one blastomere following two oil site marking of the other blastomere: (A) immediately after injection and (B) after its release and recapture when the first cleavage plane has become oblique rather than vertical. (C) Polar view of an obviously oval blastocyst developing from a denuded zygote. (D) Two-cell conceptus whose ZP was thinned by pronase at the zygote stage. Note how, relative to A, the ZP is poorly discernible and molded to the elongated shape of the conceptus.

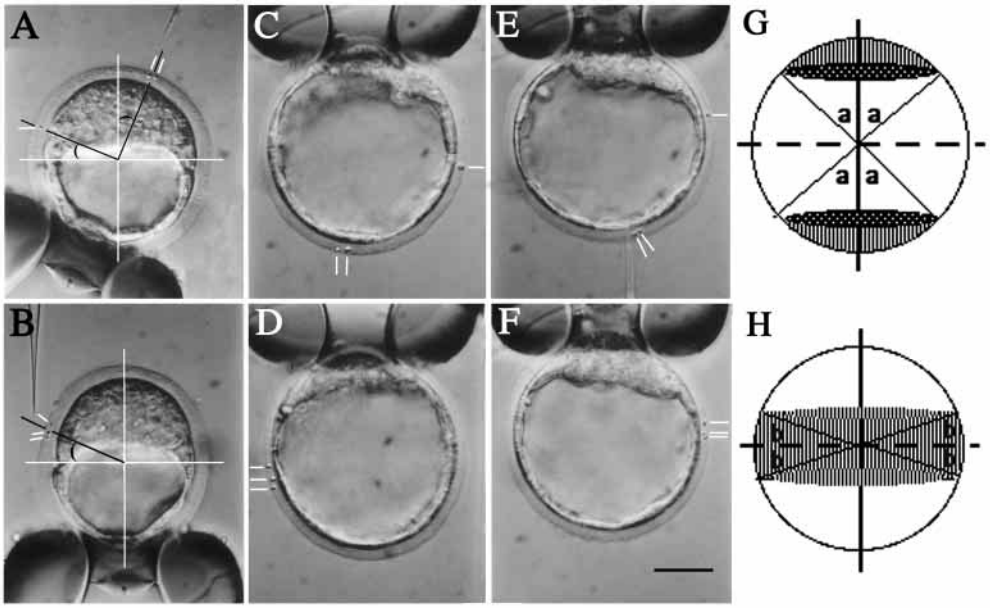

Fig. 3. (A-F) Three blastocysts developed in vitro from two-cell stage conceptuses marked with three oil sites in zona, oriented to show one and two oil sites (A,C,E). and corresponding three oil sites (B,D,F). Crossed lines are superimposed on the blastocyst in A,B to illustrate how the angles recorded in Fig. 4A-D were measured. For the two oil sites, the angle was taken as the midpoint between the two drops, and for the three oil sites, the midpoint was taken between the two outer drops. Note that the diameter of the early blastocyst is obviously greater when oriented to show the one oil (A) rather than the three oil site (B). A similar but more subtle difference is also evident in the expanding blastocyst shown in C,D. Scale bar: 30 μm. (G,H) A blastocyst as a sphere. One-third of the surface centered on the pole (G), or at the equator (H) of each hemisphere lies within solid angles a=48.2° and b=19.5°, respectively.

blastocysts so that the angle of the sites of one, two or three oil drops relative to their Em.Ab axis or equatorial plane could be determined, as illustrated in Fig. 3A,B.

The one and three oil sites typically lay over the equatorial region of blastocysts and the two oil site close to one or other pole (Figs 3, 4). The distribution of all three sites was impressively nonrandom in both PO and F_2 blastocysts (Table 1), though somewhat more variable in the latter. The three oil site was consistently the most, and the two oil site the least, circumscribed in both series (Fig. 4). Variability in location of the sites was not obviously greater in blastocysts with a large blastocoele than in those that had not yet begun to expand at the time of scoring. In the earlier blastocysts that were obviously elliptical in polar view, the one oil site was typically aligned with the GD and the three oil site with the LD (Figs 1B, 3A,B). No significant bias of the two oil site towards one or other pole of blastocysts was observed in either series of experiments, even where it had been placed over the larger or more advanced blastomere.

Effect of altering the orientation of the first cleavage plane

Explanted two-cell conceptuses lie consistently with their blastomeres side by side, raising the possibility that the orientation of the Em.Ab axis of the blastocyst is orthogonal to the direction of gravity rather than to the plane of first cleavage. Therefore, a further series of PO two-cell conceptuses had just the two oil site marked before a single oil drop was injected into the diametrically opposite point of the ZP, and enlarged until it raised the adjacent blastomere obliquely or vertically above its sister (Fig. 2A,B). This large oil drop was detached from the resulting blastocysts before they were photographed because it made them difficult to orient optimally for recording the two oil site in relation to the Em.Ab axis.

Among 41 two-cell conceptuses recovered for this series of experiments, one was discarded because it had irregular-shaped blastomeres with unusually eccentric nuclei. Two of the remaining 40 were not analyzed, one being completely degenerate and the other showing obvious cell lysis by the end of culture. The data for the remaining 38 for which the angle of the two oil site relative to the Em.Ab axis was scored are presented in Fig. 4E. These results differ significantly from expectation if the distribution of the two oil site was random, being within 48° or less of the Em.Ab axis in all except five cases ($P<4.3\times10^{-3}$). As in the larger series of experiments

Table 1. Angle of departure of labeled sites in ZP from equatorial plane (three and one oil drops) or Em.Ab axis (two oil drops)

Strain*	Marker	Sample size	Number on axis‡	Number off axis	*P* value
PO	Three oils	n=119	100	19	$P<3\times10^{-29}$
PO	Two oils	n=83	69	14	$P<3.4\times10^{-10}$
PO	One oil	n=78	48	30	$P<3\times10^{-7}$
F_2	Three oils	n=99	76	23	$P<9\times10^{-19}$
F_2	Two oils	n=99	82	17	$P<4.3\times10^{-24}$
F_2	One oil	n=99	61	38	$P<4\times10^{-9}$

*Correlation coefficients for the angle of deviation of one and two oils were 0.82 for the PO blastocysts and 0.85 for the F_2 series. For two and three, and one and three oils they were, respectively, 0.51 and 0.25 for PO and 0.43 and 0.08 for F_2 series.

‡On axis is not more than 19° from equator for the three and one oil sites, and not more than 48° from either the embryonic or abembryonic pole for the two oil site (see Fig. 3G,H).

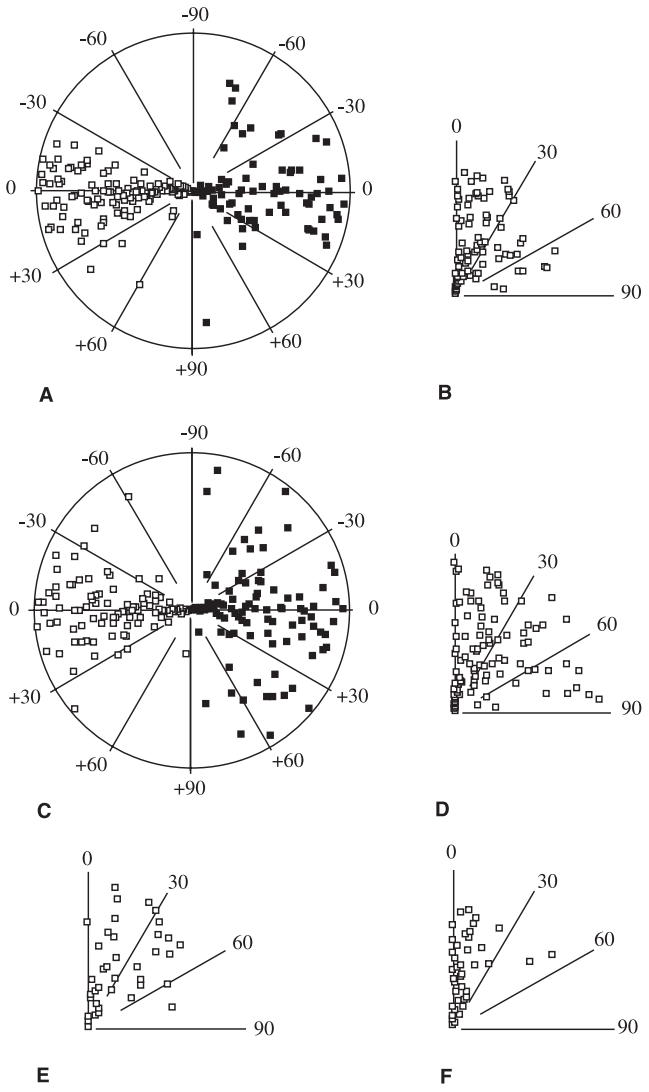

Fig. 4. Summary of the distribution of the three oil sites in polar graphic form in PO (A,B) and F_2 blastocysts (C,D). (A,C) Polar graphs showing distribution of three oil (left, white boxes) and one oil (right, black boxes) sites. The equatorial plane of the blastocyst is at 0º and its embryonic and abembryonic poles are, respectively, at –90º and +90º. (B,D) Segment of polar graph showing distribution of two oil sites relative to the Em.Ab axis (=0º). In PO blastocysts the two oil sites mapped to the embryonic hemisphere in 43 cases and to the abembryonic hemisphere in 40 cases (n=83), while in F_2 series, the corresponding values were 57 and 42 (n=99). (E) Polar graph of the distribution of two oil sites in blastocysts from two-cell conceptuses whose orientation was altered by attaching a large oil drop to the ZP. (F) Polar graph summarizing the angle of departure of the Em.Ab axis from perpendicular to the plane of first cleavage in alginate-embedded conceptuses.

described earlier, the two oil site mapped to one or other hemisphere of the blastocyst with similar frequency, being abembryonic in 20 cases and embryonic in the remaining 18.

Development of denuded two-cell conceptuses in alginate gel

The oil marking experiments described above leave unanswered the question of the extent to which the observed variability in orientation of the Em.Ab axis of the blastocyst relative to the structure of the two-cell stage is due to rotation of conceptuses within the ZP. To address this, denuded two-cell conceptuses were immobilized by embedding them in alginate gel in order to fix their orientation during subsequent culture. The aim was to ensure that, by filling the very prominent groove between blastomeres at the two-cell stage, the gel would prevent rotation of conceptuses thereafter. Both to achieve this, and to secure an enduring imprint of the first cleavage plane, the gel had to be robust enough to continue to constrict conceptuses at the site of this cleavage plane throughout subsequent preimplantation development without impairing their morphogenesis. Following initial trials using various concentrations of both medium- and low-viscosity alginate in KSOM, gels made from low-viscosity alginate at a concentration of 0.3-0.7% were used.

Determining the orientation of the Em.Ab of blastocysts relative to the first cleavage plane was complicated by three factors. First, even when conceptuses were allowed to settle on the floor of the drops before gelation was initiated, it was not possible to ensure that both blastomeres were always side by side thereafter. In cases where they were not, the extent to which the Em.Ab axis deviated from orthogonal to the plane of first cleavage could only be assessed visually rather than by measurement. The remaining two complications stemmed from the fact that the gel not infrequently detached from the floor of the drop and, being less dense than residual culture medium, floated above it. This could result not only in rotation of the drops, but could also allow conceptuses to escape from the lower surface of the gel where most were embedded. Rotation of the gel in a drop was inferred from discordance between the orientation of the plane of first cleavage at the two-cell stage and the constriction in the blastocyst, and confirmed by a shift in the orientation of extraneous fibers (see Fig. 5C,D). This complication was circumvented in later experiments by preparing oval rather than circular drops. Escape of conceptuses from the gel was indicated by the absence of a constriction and confirmed by the ease with which they moved when touched with a blunt glass probe. It was observed most commonly as a change in orientation of the Em.Ab axis of blastocysts when they were allowed to expand in culture after initial scoring. However, it could occur by the early blastocyst stage when the consequent loss of the constriction made scoring impossible. Removing all free medium from the drops on completing the rinses to remove the excess Ca^{2+} in order to preclude such escape could not be achieved without risk of damaging the gel.

The results of the embedding experiments are presented in Table 2 and Figs 4F, 5. The Em.Ab axis of the blastocyst was judged to be within 30º of perpendicular to the first cleavage plane in nearly all specimens that had to be scored visually. Among the larger number of conceptuses in which the angle could be measured, it exceeded 45º in only 5% of cases (P<7.47×10^{-10}) and was 10º or less in 58% (Figs 4F, 5).

Effect on form of the early blastocyst of removing or weakening the ZP at the zygote stage

Whereas the rate of development to the blastocyst stage was impaired by completely removing the ZP, particularly in PO zygotes, the proportion of morphologically normal early blastocysts that were oval nevertheless rivaled that among

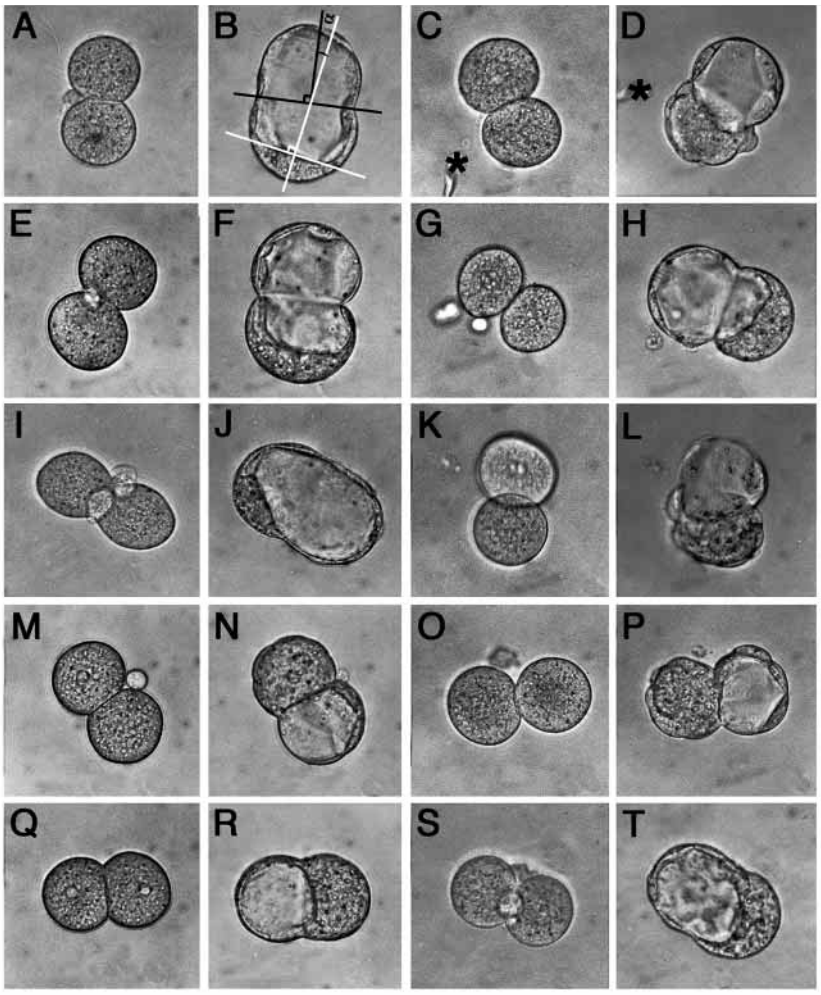

Fig. 5. Representative examples of embedded two-cell conceptuses (A,C,E,G,I,K,M,O,Q,S) and corresponding blastocysts (B,D,F, H,J,L,N,P,R,T) photographed with culture dishes in the same orientation. (B) How the angle of departure (α) of the Em.Ab axis of blastocysts from perpendicular to the constriction (plane of first cleavage) (see Fig. 4F) was measured. (C,D) Change in orientation of the tip of an extraneous embedded fiber (asterisk) showing that the gel rotated during culture. Less obvious rotation of the gel is also evident in E,F and G,H. (E) Two-cell conceptus with deep interblastomeric groove and corresponding blastocyst (F) with its Em.Ab axis accurately orthogonal to the conspicuous constriction. (G) Two-cell conceptus with deep interblastomeric groove and corresponding blastocyst (H) with Em.Ab axis clearly not orthogonal to the conspicuous constriction. (I,J) Further two-cell conceptus and corresponding blastocyst whose Em.Ab axis is more nearly parallel than orthogonal to a modest constriction. (K,L) Two-cell stage conceptus embedded with blastomeres at different vertical levels so that orientation of the Em.Ab axis relative to the constriction had to be assessed visually rather than by measurement. (M-T) Four further corresponding two-cell and blastocyst stages with the Em.Ab axis of the blastocyst orthogonal to the first cleavage plane.

untreated controls (Table 3, Fig. 2C). Adverse effects on development were less obvious in zygotes whose ZP had been attenuated by exposing it to pronase at room temperature for 9 minutes. After this treatment, which weakened the ZP so that it became molded to the shape of the enclosed conceptus (Fig. 2D), the incidence of oval early blastocysts was also similar to that in controls (Table 3).

DISCUSSION

The results of oil drop marking argue that conceptuses do not rotate freely within the ZP during development in vitro from the late two-cell to blastocyst stage. To account otherwise for such a remarkable correspondence in labeling sites between the two stages would require patterning of the conceptus to depend on information localized regionally within the ZP itself. This very unlikely possibility can be discounted because denuded oocytes form morphologically normal blastocysts following fertilization in vitro (Naito et al., 1992). Hence, the use of strictly non-invasive marking has revealed the existence of a consistent topographical relationship between the blastocyst and the two-cell stage in undisturbed mouse development. The greater dispersal of values for the one and two oil sites than the three oil site is presumably because rotation of the conceptus within its ZP is less constrained in the plane of the

Table 2. Summary of experiments on the relationship between the Em.Ab axis and first cleavage plane in denuded 2-cell conceptuses developing in 0.3-0.7% alginate gel

	Two-cell conceptuses		Blastocysts		
Gel concentration	Number embedded	Number forming blastocysts	Number scorable	Number scored visually (number on axis)*	Number scored by measurement‡ (number on axis)*
0.3%	20	17	11	3 (2)	8 (7)
0.5%	43	34	28	10 (9)	18 (18)
0.7%	24	23	19	5 (3)	14 (13)
Totals	87	74	57	18 (14)	40 (38)

*With Em.Ab axis judged to be within 30° of orthogonal to first cleavage plane.
‡Measurements displayed in Fig. 4F.

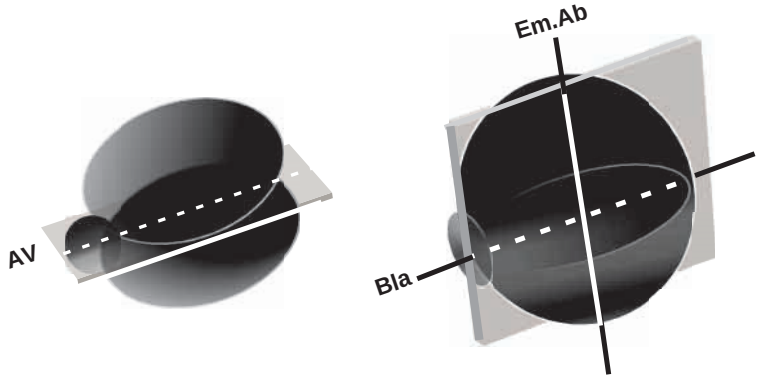

Fig. 6. The relationship of the axes and plane of bilateral symmetry of the early blastocyst to the animal-vegetal axis of the zygote and first cleavage plane. Note that while the axis of bilateral symmetry of the blastocyst is parallel to the AV axis of the zygote, both its Em.Ab axis and bilateral plane are orthogonal to the plane of first cleavage. AV, animal-vegetal axis of zygote with the second Pb marking the animal pole; Bla, bilateral axis; Em.Ab, embryonic-abembryonic axis.

GD than orthogonally to it (see Fig. 7A,B). The higher correlation coefficient for the one and two oils than the other combinations is consistent with this conclusion (see footnote to Table 1). The wider scatter of the two oil site than the one or three oil sites presumably reflects greater difficulty in accurately marking the outer extremity of a blastomere than sites on the boundary with its sister.

In labeled early blastocysts that were bilaterally symmetrical in polar view, the one oil drop was typically aligned with the GD (=bilateral axis) and the three oil drops with the LD (Fig. 1B). This agrees with findings from an earlier study in which, before it begins to expand, the mouse blastocyst was found to be bilaterally rather than radially symmetrical about its Em.Ab axis (Gardner, 1997). Mapping of the two oil site at the blastocyst stage was not altered by attaching a large oil drop to the ZP so that the first cleavage plane was oblique or horizontal rather than vertical during subsequent culture. Hence, orientation of the Em.Ab axis of the blastocyst seems to relate to the structure of the two-cell conceptus rather than an extrinsic factor such as gravity. This conclusion is supported by results of the further experiments in which denuded two-cell conceptuses were cultured in alginate gel. Thus, according to the orientation of a persisting constriction attributable to the occupation by the gel of the interblastomeric groove at the two-cell stage, the Em.Ab axis was approximately orthogonal to the first cleavage plane in nearly all the resulting morphologically normal blastocysts (Fig. 5). However, embedding conceptuses reduced rather than eliminated the variability in the relationship of the Em.Ab to the first cleavage plane encountered in the oil marking experiments (compare Fig. 4F with 4B,D), arguing that the latter is not due entirely to rotation of conceptuses within the ZP**.** While the persisting constriction of embedded conceptuses did not obviously impair the rate of development to the blastocyst stage, a higher than usual proportion of blastocysts were poorly integrated or abnormally shaped. It might therefore have perturbed morphogenesis by restricting cell movement and thereby constraining orientation of the Em.Ab axis. Were this the case, the Em.Ab axis would be expected to be more precisely orthogonal to a marked than a

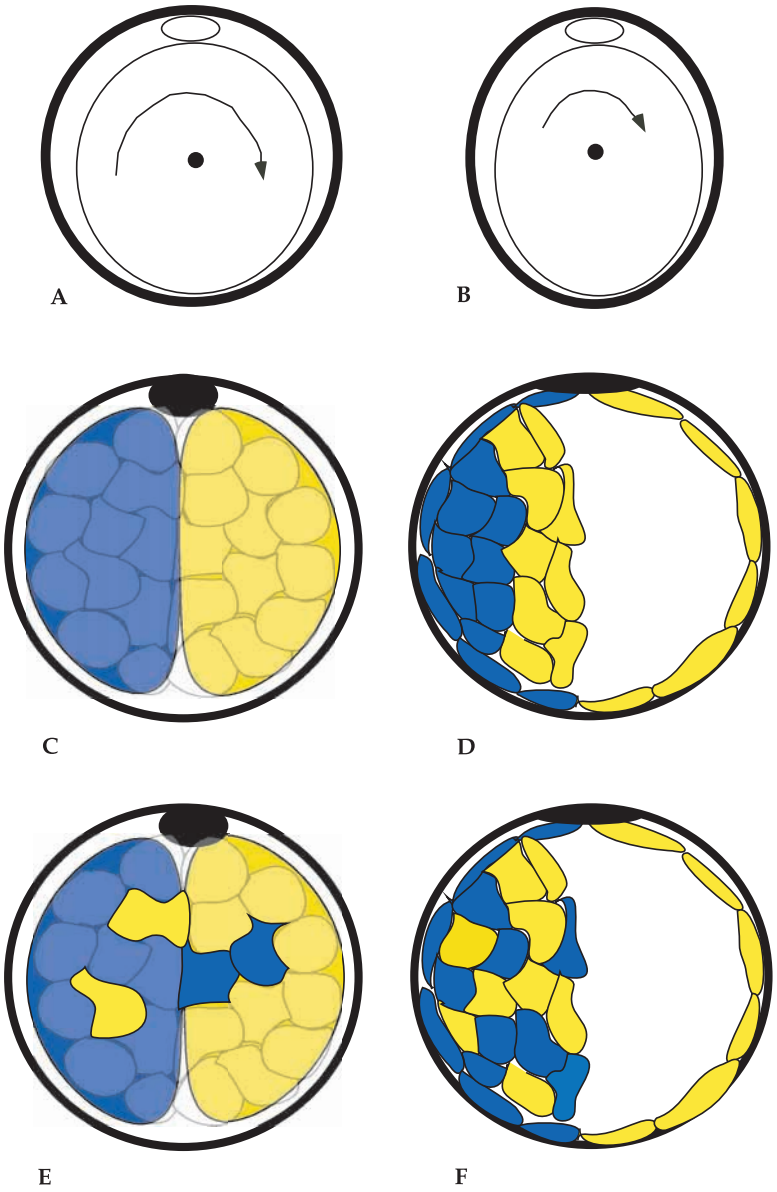

Fig. 7. (A,B) Why cleavages stages should be freer to rotate within the ZP in the plane of their GD (A) than in the plane of their LD (B). (C-F) How cells of the morula (C,E) and blastocyst (D,F) would be expected to map onto the two-cell stage if growth was coherent in both outer and inner cell populations (C,D) or in just the outer population (E,F).

modest constriction, which was not observed (compare Fig. 5B with 5H**)**.

The essential features of the conserved topographical relationship between the blastocyst and two-cell stage support the following conclusions regarding axial relationships in the early conceptus. First, while the bilateral axis of the blastocyst is aligned with the AV axis of the zygote (Gardner, 1997), both its plane of bilateral symmetry and Em.Ab axis are orthogonal to the plane of first cleavage (Fig. 6). Second, specification of both axes of the blastocyst occurs before second cleavage, and must therefore depend on information that is already present in the zygote. While cues provided by either the AV axis of the zygote or the orientation of first cleavage could specify the axis of bilateral symmetry, the Em.Ab axis would seem to require patterning information that is orthogonal to this axis. The fact that the two axes of the blastocyst bear a fixed relationship to each other (Fig. 6) implies interdependence in their specification. However, it remains to be determined whether they are specified concurrently or sequentially and, if the latter, in which order. Also to be established is whether the polarity of the two axes is specified at the same time as their orientation. No consistent mapping of the two oil drops to one rather than the other hemisphere of the blastocyst was seen (see legend to Fig. 4), even where the labeled 1/2 blastomere was obviously larger or more advanced than its sister.

Although first cleavage is described as meridional (Howlett and Bolton, 1985), it is seldom strictly parallel to the animal-vegetal (AV) axis of the zygote (Evsikov et al., 1994; R. L. G., unpublished observations). Therefore, orientation of the Em.Ab axis of the blastocyst could depend on the AV axis of the zygote or secondary oocyte, which may also diverge from each other (see Gardner, 2000a), rather than the plane of first cleavage. Such a possibility could explain why, even in embedded conceptuses, orientation of the Em.Ab axis of the blastocyst varied relative to the first cleavage plane.

What is the significance of the axes of the blastocyst for patterning of the fetus? The bilateral axis becomes polarized by the late blastocyst stage (Smith, 1980; Gardner, 1990), possibly through anisotropic flow of cells from polar to mural trophectoderm (Gardner, 1998; Gardner, 2000b). That this axis is conserved through to the early gastrula stage is evident from recent lineage labeling of ICM cells adjacent to versus opposite the Pb (Weber et al., 1999). Interestingly, this study also suggests that, unlike the bilateral axis of the conceptus with which it is aligned (Gardner et al., 1992), the anterior-posterior (AP) axis of the nascent fetus may also depend on the AV axis of the zygote for its polarity. The obvious continuity in form between the blastocyst and egg-cylinder argues that the Em.Ab axis is also conserved through gastrulation. Not only does this axis define, if not specify, the dorsoventral axis of the future fetus but, according to recent evidence, the AP axis of the fetus may also initially be aligned with it (Beddington and Robertson, 1998; Beddington and Robertson, 1999). Hence, by revealing that both the Em.Ab and bilateral axes of the blastocyst bear a predictable relationship to the structure of the two-cell conceptus, the present study shows that two axes of the fetus are normally defined by the onset of cleavage.

While bilateral symmetry of four-cell and later stages is manifest in the shape of the conceptus as well as its investing ZP (R. L. G., unpublished observations), at the two-cell stage it is, as noted earlier, confined to the ZP itself. How can this be explained? Even when aged in vivo for 2 days, unfertilized oocytes retain an essentially spherical ZP that is easily deformed and rapidly regains its shape thereafter. In contrast, the ZP of zygotes exhibits both greater resistance to deformation and a markedly reduced capacity to recover its shape (R. L. G., unpublished observations). Hence, acquisition of bilaterality of the ZP at the two-cell stage must depend on fertilization. Changes in the ZP, referred to as 'hardening', occur after fertilization and have been attributed to its modification by products of the exocytosed cortical granules (Schmell and Gulyas, 1980). They are usually recorded as an increase in resistance to proteolysis that peaks within a few hours of sperm penetration (Krzanowska, 1972). How such 'chemical hardening' relates to changes in mechanical properties is not clear because, in the only study in which the latter was measured, comparison was limited to oocytes and conceptuses at the two-cell stage. In this study, the ZP was less deformable at the two-cell stage than in oocytes in both the hamster and mouse, despite the absence of any post-fertilization increase in its resistance to proteolysis in the former species (Drobnis et al., 1988). Providing the ZP does not harden mechanically until after completion of meiosis, its increased diameter parallel to the AV axis of the zygote is most readily explained by the presence of the second Pb. Once the ZP has been molded thus, its persisting non-spherical shape might in turn serve to hinder rotation of the encased conceptus. Nevertheless, the final hardened, molded form of the ZP is clearly not essential for acquisition of bilateral symmetry by the early conceptus (Table 3).

A further issue is the extent to which the consistent relationship between the structure of the blastocyst and two-cell stage revealed by the present investigation should be paralleled by regularities in cell lineage during normal preimplantation development. A simple scheme shown in Fig. 7C,D depicts the expected contribution of each 1/2 blastomere to the blastocyst if all their progeny remained strictly together. Since the equatorial plane of the blastocyst typically coincides with that of first cleavage, trophectoderm cells of the mural and polar hemispheres of the blastocyst should originate from different 1/2 blastomeres. Further, given that the blastocoele forms eccentrically between inner and outer cells in the mural region, superficial and deep cells of the ICM should share a common origin with the mural and polar trophectoderm, respectively (see Fig. 4C,D). Hence, the mural trophectoderm and primitive endoderm would be expected to originate from one 1/2 blastomere and the polar trophectoderm plus epiblast

Table 3. Incidence of bilateral symmetry in blastocysts developed in vitro from zygotes with ZP removed or attenuated, versus untreated controls

Genotype	Zona status	Total cultured	Number scorable*	Number oval (%)
F_2	Absent 18	13	13 (100)	
	Present	11	9	8 (89)
PO	Absent	29	12	11 (92)
	Present	11	6	5 (83)
PO	Attenuated	40	36	31 (86)
	Not attenuated	21	16	13 (81)

*Those not scorable included very retarded or poorly integrated conceptuses and, particularly among the zona-intact controls, blastocysts that were too advanced to score for bilaterality.

from the other (Fig. 4D). That at least most mural and polar trophectoderm cells originate from different 1/2 blastomeres is likely, as outer cells of the morula grow essentially like a coherent epithelium. However, the situation is very different regarding the ICM, where an initial variable stock of precursor cells is established internally by differential division of blastomeres during fourth cleavage and supplemented by further such divisions during fifth cleavage (Fleming, 1987). In view of the combined effects of growth in three rather than two dimensions and the further recruitment of cells from diverse locations in the outer layer, it is most unlikely that ICM precursors from the two 1/2 blastomeres will remain segregated. Therefore, a more realistic expectation is that while the progeny of the two 1/2 blastomeres in the trophectoderm of the early blastocyst will normally form a coherent clonal boundary that coincides roughly with the polar/mural junction, those in the ICM will show extensive and variable intermixing (Fig. 7E,F). Once the polarized flow of cells from the polar to the mural region begins, there will also be intermixing of cells within the trophectoderm (Gardner, 2000b). Such considerations could explain the failure in earlier studies to detect regularities in the distribution of labeling by conventional brightfield or fluorescence microscopy in whole mounts of blastocysts developed from two-cell conceptuses in which one 1/2 blastomere had been injected with a lineage marker (Balakier and Pedersen, 1982; Gardner, 1997). This lineage analysis needs to be repeated using fluorescent labels in conjunction with confocal microscopy, so as to enable the distribution of labeled cells in the trophectoderm to be differentiated from that in the ICM. Nonetheless, it should be borne in mind that, unlike the injection of oil drops into the ZP used here, direct cell labeling is invasive and may perturb the timing or orientation of mitoses (Cruz and Pedersen, 1985; Dyce et al., 1987; Gardner, 1996b).

Finally, it is important to note that the present findings relate to the specification of axes in undisturbed development, which, as discussed elsewhere (Gardner, 2000a), may differ from that occurring following experimental intervention.

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