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Emerging Asymmetry and Embryonic Patterning in Early Mouse Development

Review

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Recent studies have revealed asymmetries in the mouse zygote and preimplantation embryo, well before the establishment of anterior-posterior polarity after implantation. Whether these asymmetries are causally related to embryonic patterning or are coincidental outcomes of the topology of normal development remains uncertain.

Embryonic patterning is an emergent process built on successive asymmetries in cellular and tissue organization generated as an egg proceeds from fertilization to gastrulation. Early phases of embryonic development are mostly concerned with generation of the major tissue layers through the morphogenetic processes of gastrulation and the development of axial patterning. In many species, the initial asymmetries that drive the development of the major body axes arise well before the onset of gastrulation and often derive from information laid down in the egg and reorganized at fertilization. However, in the mouse, the best-studied mammal, there is still no incontrovertible evidence of any informative molecular asymmetry in the oocyte, zygote, or preimplantation embryo that predicts the later anterior-posterior axis of the embryo established at gastrulation. Morphologically, the formation of the primitive streak around the 7th day of gestation, through which mesoderm and definitive endoderm emerge, marks the posterior side of the embryo. Once streak formation begins, the processes of germ layer formation, tissue patterning in the anterior-posterior (A-P), dorsoventral (D-V), and left-right (L-R) axes of the embryo begins to unfold by complex interactions of signals produced by different signaling centers.

It is now clear, however, that the morphological formation of the streak is preceded by other asymmetries that establish the position and polarity of the A-P axis. As early as E6.0 of development, well before any sign of gastrulation, there is already evidence of A-P polarity in the embryo, marked by expression of genes such as the transcription factor, *Hhex* (Thomas et al., 1998), in a distinct region of the visceral endoderm, the anterior visceral endoderm (AVE) (Thomas and Beddington, 1996). The AVE goes on to express a number of important inhibitors, such as *Cer1* (Belo et al., 1997), *Lefty1(Leftb)* (Meno et al., 1997), and *Dkk1* (Glinka et al., 1998), which inhibit Nodal and WNT signaling in the underlying anterior epiblast and restrict Nodal activity to the posterior, where the primitive streak will arise (Kimura et al., 2000; Perea-Gomez et al., 2002). Thus the formation of the AVE initiates the development of the A-P axis. There is now considerable evidence, from both gene expression and cell lineage tracing, that the AVE cells are initially found at the distal tip of the E5.0 embryo. A unique pattern of movement then takes place in the visceral endoderm, resulting in the displacement of the distal cells to the anterior side of the pregastrula embryo (Rivera-Perez et al., 2003; Srinivas et al., 2004; Thomas and Beddington, 1996). Formation of axial polarity thus seems to involve initially specifying a proximal-distally (P-D) oriented axis and then transforming that into an A-P axis.

These studies and more have pushed back the initiation of proven axial asymmetry in the mouse from gastrulation to as early as E5.5 and call for a critical reappraisal of whether even earlier developmental events in the oocyte, zygote, or preimplantation embryo might be involved in initiating asymmetries. Although there have been several older studies proposing that there might be cytoplasmic determinants segregating during early mammalian development (Dalcq, 1957; Mulnard, 1961), until recently the most influential view has been that processes involved in the determination of primary body axes begin after the conceptus has implanted in the uterus (reviewed by Gardner, 2001a). The foundation for this view is the well-known ability of the preimplantation mouse embryo to tolerate many kinds of experimental manipulation and still produce a normal fetus. There has also been a singular failure to find any evidence for an informative molecular asymmetry that might relate to later axis development. However, the ability of the embryo to regulate for loss or reorganization of cells does not preclude an underlying intrinsic patterning. And, of course, not finding molecular asymmetries does not mean they do not exist.

Against this kind of background, there has been a resurgence of interest recently in exploring asymmetries in the zygote to pregastrulation embryo and determining whether they are related in any meaningful way to later developing embryonic axes. In this article, we present a critical review of how asymmetries in morphological features and patterns of cellular behavior arise in the early embryo and how they may relate to the development of the body plan.

Does the Animal-Vegetal Axis of the Zygote Relate to the Axes of the Blastocyst?

The mouse blastocyst prior to implantation has three distinct lineages, the external trophectoderm (TE); the

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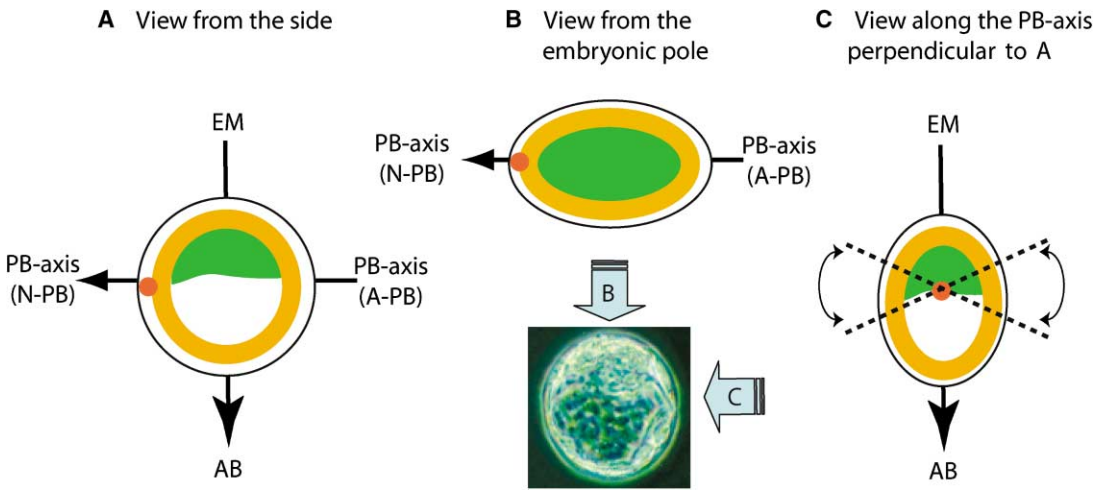

Figure 1. The Axes of the E3.5 Blastocyst

(A) Lateral view to show polar body (red) at junction of ICM and trophectoderm. The polar body (PB) axis passes across the equator of the embryo from near to (N-PB) and away from (A-PB) the polar body. Whether this axis is identical to the animal-vegetal axis of the egg is uncertain. The axis perpendicular to the PB axis marks the embryonic-abembryonic (EM-AB) axis, made apparent by the position of the ICM (green) at one end of the trophectodermal shell (brown) of the blastocyst. The zona pellucida (black) surrounds the blastocyst.

(B) View from the embryonic pole (see inset for orientation, image courtesy of Satomi Tanaka) to show how the PB axis marks the long axis of bilateral symmetry of the blastocyst.

(C) A lateral view of the blastocyst along the PB axis (the direction of view shown in the inset), demonstrating that the plane of first cleavage (dashed line), which is presumed to be associated with the polar body, may deviate over a range of angles (double arrow) around the circumference of the embryo, leading to different segregation patterns of the progeny of the first two blastomeres to opposite regions of the blastocyst. There may be a preference for this plane to be perpendicular to the EM-AB axis, although this is not clear in every study.

blastocoel, a fluid-filled cavity enclosed by the TE; and the inner cell mass (ICM), which lies at one end of the blastocoel and consists of epiblast (EPI) and primitive endoderm (PE). The blastocyst, therefore, clearly has an embryonic-abembryonic (EM-AB) axis, defined by the position of the ICM (Figure 1A). Careful observation of blastocysts has shown them to be bilaterally symmetric by E3.5 (Gardner, 1997). When viewed from the embryonic pole (the polar trophectoderm-ICM side), the blastocyst is elliptical, with the intersection of the long and short planes of bilateral symmetry defining the EM-AB axis (Figure 1B). In 1997, Richard Gardner first reported that there might be a relationship between the planes of bilateral symmetry in the blastocyst and events in the zygote (Gardner, 1997). In some strains of mice, the 2nd polar body (PB) formed during meiosis II of the oocyte remains intact and attached to the embryo throughout preimplantation development. In such strains, the PB was observed to associate with the equator of the blastocyst at the junction of the ICM and the mural trophectoderm, and was usually at one end of the long axis of bilateral symmetry (Figure 1B). Embryo manipulations of various sorts suggested that the PB remained attached to a blastomere during cleavage and underwent little displacement (Gardner, 1997). Thus it was proposed that the zygote did indeed display an axis, the animal-vegetal (A-V) axis, with the animal pole marked by the position of extrusion of the 2nd polar body, and that this axis predicted the axes of bilateral symmetry of the blastocyst (Gardner, 1997) (Figure 1B). The EM-AB axis would be orthogonal to the A-V axis and the long axis of bilateral symmetry coincident with the A-V axis (Figures 1A and 1B).

It is a common observation in all mouse embryos that, after first cleavage, the 2nd PB nearly always lies in the cleft between the two blastomeres. If there is little movement of the PB from the time of meiosis, the first cleavage must have taken place about an axis passing through the position of the 2nd PB, the so-called animal pole (Figure 1C) (Plusa et al., 2002). Removal of the animal pole randomized the plane of first cleavage and transplanting an animal pole to an ectopic site altered the plane of cleavage, suggesting that something associated with the site of meiosis was indeed important for cleavage orientation (Plusa et al., 2002). Further experiments suggested that the actual position of the plane of cleavage passing through the A-V axis was determined by the sperm entry point. Marking the fertilization cone with fluorescent beads showed a tendency for the sperm entry point to be close to the plane of first cleavage (Piotrowska and Zernicka-Goetz, 2001, 2002) The concept of sperm entry point as an important symmetry-breaking mechanism is an attractive one, since it is used in a number of other animals such as *C. elegans* and *Xenopus*. However, marking internal sperm components did not reveal the same relationship (Davies and Gardner, 2002).

Some of the assumptions about how first cleavage is oriented have been based on static observations of what is clearly a dynamic process or on experimental perturbations that may disrupt normal processes. Careful observation of undisturbed development is starting to shed new light on the events surrounding the initiation of first cleavage. When first cleavage was observed as it occurred, it rarely seemed to pass directly through the position of polar body extrusion (Gardner and Davies,

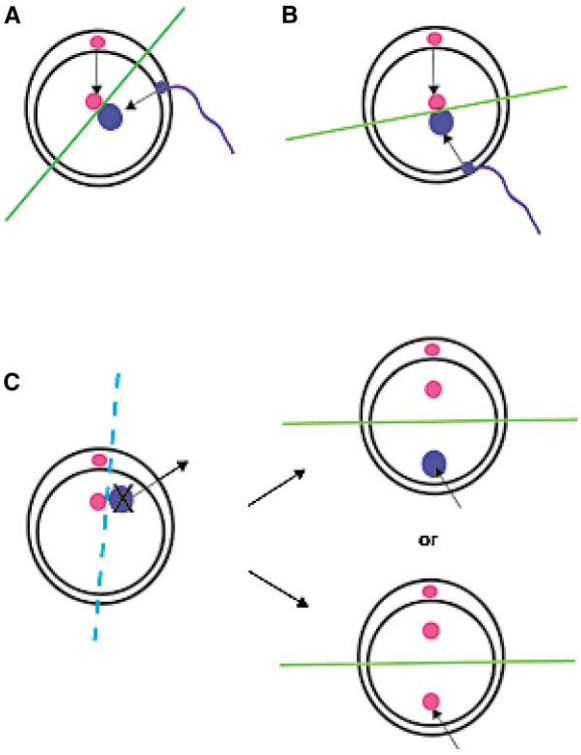

Figure 2. Pronuclear Position and Orientation of Plane of First Cleavage

(A and B) Two different zygotes in which the differing proximity of the polar body and sperm entry point lead to differing patterns of apposition of the two polar bodies (female, red; male, blue). The cleavage plane (green line) seems to consistently follow the plane of apposition of the two pronuclei.

(C) Experimental removal of the incoming male pronucleus and replacement by either a female or male pronucleus at the opposite side of the egg influences the plane of cleavage (blue dashed line, predicted plane; green line, actual cleavage plane; data after Hiiragi and Solter, 2004).

2003). A time-lapse study of first cleavage (Hiiragi and Solter, 2004) showed that cleavage was initiated in a plane within 30° of the site of polar body extrusion in only 50% of all zygotes examined. This is clearly still a non-random distribution, but leaves a considerable subset of embryos where cleavage did not align closely with the 2nd PB position at all. A much higher proportion of embryos showed the PB in the cleft between the two blastomeres at the 2-cell stage, suggesting that the PB can move to that position after cleavage. Such movements have been directly observed (Gardner and Davies, 2003; Hiiragi and Solter, 2004). These data cast doubt on the idea that the site of PB extrusion per se is instrumental in predicting the first cleavage plane. Hiiragi and Solter report in their time-lapse studies that the final plane of cleavage was always the same as the plane separating the two pronuclei as they congregate in the middle of the zygote (Figures 2A and 2B) (Hiiragi and Solter, 2004). Further, when zygotes were treated with Cytochalasin D and pronuclei removed and placed in different positions, in almost all cases the plane of first cleavage now respected the new plane separating the pronuclei rather than either the position of the PB or the original pronuclear positions (Figure 2C). Thus, clearly when the normal cytoplasmic architecture is disrupted, position of the pronuclei alone is sufficient to determine the plane of first cleavage. If this is the only mechanism acting in the normal zygote, why then is there still a statistically increased chance that the first cleavage plane will pass within 30° of the PB (Hiiragi and Solter, 2004) and, perhaps, the fertilization cone (Piotrowska and Zernicka-Goetz, 2001, 2002)? Hiiragi and Solter observed that sperm entry was predominantly in the upper hemisphere of the egg, close to the polar body (Hiiragi and Solter, 2004). In such zygotes, the plane of cleavage would often bisect the arc between the PB and the fertilization cone, because this bisects the shortest path to the center for the two pronuclei (Figure 2A). These embryos would then be scored as having the plane of cleavage within 30° or so of both the PB and the sperm entry point.

The relative importance of pronuclear position versus underlying asymmetries needs to be reevaluated after other experimental manipulations, such as removal or transplantation of the animal pole (Plusa et al., 2002). Recent evidence that experimental induction of shape change in the zygote alters cleavage patterns (Gray et al., 2004) should also be reevaluated to see if shape change correlates with change in pronuclear position.

Do the First Two Blastomeres Have Predictable Fates?

Although the association of the extrusion of the 2nd polar body with the site of initiation of first cleavage is not absolute, once it has become embedded in the cleft between the two blastomeres, blastomere marking experiments suggested that the PB always stays close to the boundary between the progeny of the first two blastomeres (Gardner, 1997). These progeny undergo limited clonal mixing up until the blastocyst stage. The axis passing through the PB and the equator of the blastocyst, which we will now call the PB axis, is indeed orthogonal to the EM-AB axis and coincident with the axis of bilateral symmetry. However, it was not clear whether the actual position of the plane of first cleavage bore a fixed relationship to the EM-AB axis of the blastocyst (Gardner, 1997; Figure 1C). Further studies using a variety of cell marking techniques have suggested that there is tendency for the plane of first cleavage to be orthogonal to the EM-AB axis of the blastocyst. Direct lineage marking of individual two-cell blastomeres by DiI surface labeling (Piotrowska et al., 2001) or by injection of a Cre-expressing plasmid into a Cre-reporter strain (Fujimori et al., 2003), as well as indirect labeling of the overlying zona pellucida (Gardner, 2001b), showed that the progeny of the two cells had a tendency to be distributed to the opposite ends of the EM-AB axis, with one blastomere predominantly contributing to mural TE and the PE of the ICM and the other to the polar TE over the ICM and to the EPI. However, there was never an absolute lineage segregation between the two blastomeres and, indeed, following the later fate of labeled two-cell blastomeres in the Cre-reporter marked embryos revealed that each 2-cell blastomere contributed to all cell types and regions of the postimplantation embryo (Fujimori et al., 2003). Further, the association

between the plane of first cleavage and the EM-AB axis was rarely exactly orthogonal in any study and other workers have claimed to find no necessary association between the two (Alarcon and Marikawa, 2003). The clearest separation of the progeny of the two blastomeres into embryonic and abembryonic halves came from experiments in which movement of cells during cleavage was impeded by embedding embryos in alginate (Fujimori et al., 2003; Gardner, 2001b). Since this process may also influence the normal planes of cell division and also the accumulation of the blastocoel fluid, some caution should be exercised in interpreting these results in relation to normal development.

If there is a tendency for an embryonic-abembryonic separation of the first two blastomeres, how does this occur? One possibility is that there is a difference in the probability of contributing to the embryonic versus abembryonic end of the blastocyst dependent on which blastomere divides first to the 4-cell stage. Previous studies observing embryonic cleavage patterns have predicted that early dividing cells will contribute more cells to the inner cell mass than later dividing cells (Graham and Deussen, 1978; Surani and Barton, 1984) (Garbutt et al., 1987). One study using direct lineage marking showed that the first cell to divide to the 4-cell stage tended to be associated with the embryonic pole of the blastocyst (with more ICM contribution) (Piotrowska et al., 2001). However, other studies have not found such a clear correlation (Fujimori et al., 2003). It has also been suggested that inheriting the sperm entry point may influence cell division rate and bias toward an embryonic contribution (Piotrowska and Zernicka-Goetz, 2001). There is still no clear mechanism that would reproducibly bias blastomere fate or, indeed, any evidence that clonal separation during cleavage has any causal relation to later fate. Two-cell blastomeres always contribute to all blastocyst-derived lineages in later development (Fujimori et al., 2003), and isolated 2-cell blastomeres can make normal mice (Papaioannou et al., 1989). While lineage tracing by Cre excision in intact embryos did show that individual 4-cell blastomeres could be restricted to TE fate (Fujimori et al., 2003), chimera studies have clearly shown that individual 4-cell blastomeres are capable of ICM and TE contributions (Kelly, 1977). The relationship between cell division rate and contribution to the ICM may reflect the topological constraints by which smaller cells generated from earlier cell divisions would likely end up preferentially in the inside of the embryo. Following compaction at the 8-cell stage, which generates an epithelial sphere and will restrict future cell movements, these inside cells will end up preferentially in the ICM.

Impact of Bilaterality and Polarity of Blastocyst on Early Postimplantation Development

However they may arise, two axes of bilateral symmetry do run through the equator of the blastocyst, with the polar body marking one pole of the long axis (Figure 1). Does the existence of these axes have any influence on later development? Around the time of implantation there is another transient asymmetry observed in the blastocyst, such that the ICM/polar TE is tilted in relation to the EM-AB axis (Figure 3A). This was first observed from the careful histological analysis by Smith (Smith, 1980). Smith also recorded the fact that postimplantation embryos show a tilt of the ectoplacental cone (Figure 3C), with the ectoplacental cone (EPC)/visceral endoderm boundary being closer to the extraembryonic/embryonic boundary on one side of the embryo versus the other. She proposed that the direction of the tilt of the blastocyst was preserved in the direction of the tilt of the EPC and, further, that this angle marked the future A-P axis of the embryo (Smith, 1985). Gardner has revisited part of this hypothesis and shown that the postimplantation axis of symmetry marked by the tilt of the EPC is quite closely associated with the A-P axis of the embryo as indicated by the position of the primitive streak at gastrulation (Gardner et al., 1992). However, the direction of the tilt as often marked the anterior as the posterior of the embryo, showing that the trophoblast asymmetry relates to the orientation but not the polarity of the axis.

There are no direct data to show whether there is any relationship between the bilateral symmetry and polarity of the blastocyst, the tilt of the implanting blastocyst and the tilt of the EPC, although Cre-lineage tracing techniques should make this feasible. Short-term lineage tracing experiments at the blastocyst stage, using either injection of horse-radish peroxidase into single cells or fluorescent bead labeling of the entire polar TE (Gardner, 1996), have shown that there is a net flow of polar TE cells to the mural region and that this flow is not radially symmetric but is polarized, such that there is more net flow in one quadrant of the blastocyst than others. The flow of cells from the polar to mural TE was more often in the plane of the long axis of the blastocyst than the short axis, suggesting an association with the plane of bilateral symmetry (Gardner and Davies, 2002). However, the direction of flow was equally in the direction of or away from the position of the polar body. Thus the axis of the egg might set the position of the bilateral axis but not its directionality.

The other postimplantation asymmetry that has been proposed to relate to the polarity of the blastocyst is the asymmetric pattern of clonal growth of the early visceral endoderm derived from the primitive endoderm of the blastocyst. Superficial cells on the luminal aspect of the ICM at positions near (N-PB) and away from the polar body (A-PB) were marked by microinjecting MmGFP mRNA (Weber et al., 1999). Consistent with previous lineage analyses, descendants of the ICM cells were found in both the epiblast and the visceral endoderm of the postimplantation embryo. However, the extensive intermingling of cells in the epiblast derivatives (Gardner and Cockroft, 1998) precluded the detection of any relationship between blastocyst polarity and regionalized contributions of epiblast cells to the gastrula embryonic axis. In contrast, ICM descendants in the VE were distributed non-randomly in the pre- (E5.5) to early-streak (E6.5) gastrula embryo. In the pre-streak embryo, marked clones of VE cells showed relatively coherent growth, especially in the VE overlying the extraembryonic ectoderm. In the early-streak embryo, clones originating from N-PB ICM cells tended to colonize the distal part of the extraembryonic visceral endoderm, while those from A-PB ICM cells were found in the more proximal

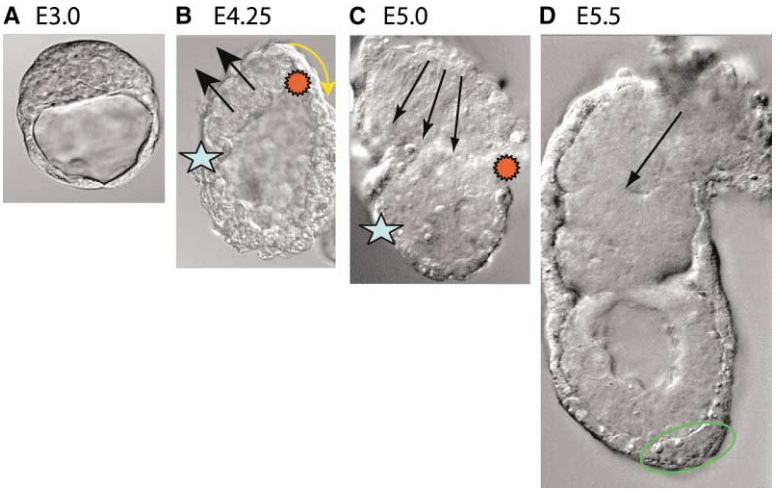

Figure 3. Morphological Asymmetry Revealed by the Tilting of the Inner Cell Mass and the Trophectodermal Derivatives

Examples of the complex tilting of the blastocyst and peri-implantation embryo.
(A) An E3.0 embryo showing slight ICM tilt.
(B) By E4.25, the embryo has a distinct tilt along the long axis of bilateral symmetry. This is the stage at which it has been reported that there is a polarized flow of polar trophectoderm to the mural regions (yellow arrow). The rest of the polar TE will proliferate and thicken above the ICM (black arrows).
(C) At E5.0, the extraembryonic ectoderm has pushed into the blastocoelic cavity (arrows), but still asymmetrically, leading to possible asymmetric displacement of the VE (marked by blue star and red dot in [B] and [C]).
(D) At E5.5, the extraembryonic tilt is still apparent and the AVE precursor is now morphologically distinct (green outline). The tilt of the extraembryonic region (arrow) does not necessarily predict the direction of the AVE migration.

regions of the extraembryonic endoderm (Weber et al., 1999). By the early-streak stage, visceral endoderm clones associated with the epiblast were more dispersed and intermingled with unlabeled cells, indicative of complex movements and migrations in this tissue. It would be informative to examine the pattern of codistribution of the N-PB and A-PB cells in the same embryo and to test the VE fate of cells in other sites of the ICM to assess the precise localization of the descendants of different ICM cell populations in the visceral endoderm.

The regionalization of the VE descendants of N-PB and A-PB ICM cells has highlighted that, in the pregastrula embryo, ICM cells from opposite ends of the long axis of the blastocyst are allocated to the distal and proximal VE populations, respectively, giving the impression that the PB axis presages the distal-proximal axis of the postimplantation embryo. There is essentially a tilted (oblique) pattern in the expansion of the VE population from blastocyst to early postimplantation (Weber et al., 1999). Given that there are also tilts associated with the formation and migration of the TE (Figure 3), it would be useful to try to correlate the two. Signals from the TE could actually be the initiators of the asymmetric growth and patterning of the VE. However, although asymmetric growth of the VE is reported to be correlated with both orientation and polarity of the axis of blastocyst symmetry, asymmetric TE growth is only associated with its orientation but is independent of the PB position. Results of lineage tracing experiments that follow both TE and VE progenitors in the same embryo are likely to be very informative.

Implantation and the Orientation of the Blastocyst in the Uterus

Up till the time when the embryo attaches to the uterus and implants within the decidual swellings, the opportunity for the maternal environment to influence the developing axes of the embryo seems limited. However, following implantation, the blastocyst is confined to a crypt-like niche between folds of the uterine mucosa (Smith, 1980). The spatial constraint of the implantation site may impact on the morphogenesis of the peri-implantation embryo. The obliteration of the uterine lumen by the growth of the endometrium and anchorage of the implanting embryo to the mesometrial wall by the polar trophectoderm restricts the option of embryonic growth to the antimesometrial direction and into the blastocyst cavity (Copp, 1981). Active proliferation of the polar trophectoderm under the influence of FGF from the inner cell mass (Tanaka et al., 1998) produces the ectoplacental cone which embeds in the uterine tissue, and a column of extraembryonic ectoderm projecting into the blastocyst cavity and carrying at its distal end the derivative of the inner cell mass—the epiblast which forms a cup-shaped epithelium. As a result, the embryonic-abembryonic axis of the blastocyst becomes the proximal (ectoplacental cone)-distal (epiblast) axis of the cylindrical pregastrula and gastrula stage embryos (Figure 4C).

During implantation, blastocysts in the same uterine horn may attach to the wall on either the right- or the left-hand side of the uterine tube (the R- and L-type) (Smith, 1980; Figures 4A and 4B). Histological examination showed that the inner cell mass may be tilted in different directions either toward the oviductal or the cervical end of the uterine horn (Smith, 1980; Figures 4A and 4B). Whether this apparent axis of bilateral symmetry of the implanted blastocyst is related to the long axis of bilateral symmetry in the E3.5 blastocyst remains uncertain. Orienting the blastocyst to the uterine axis may also be the result of remodeling of the uterine tissues at the implantation sites (Figure 4B).

It has been shown that the contact by the blastocyst triggers localized expression of the heparin binding EGF-like growth factor (HB-EGF) by the uterine tissues at the site of blastocyst attachment (Das et al., 1994). This is followed by decidual reaction of the endometrium which is associated with the proliferation and hypertrophy of the stroma. Endometrial tissues at the implantation sites express BMP-2, FGF-2, WNT4 (Paria et al., 2001), *Cox2* (Paria et al., 2000), and EGF and its receptor

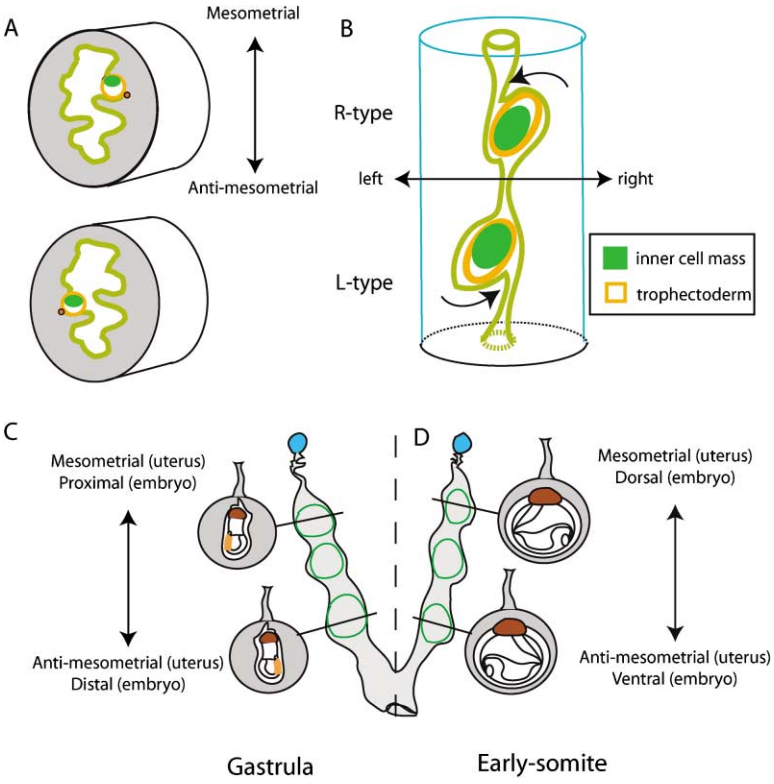

Figure 4. Orientation of the Implanting Blastocyst and the Embryonic Axes in the Uterus

(A) Blastocysts may attach to different sides (red dots) of the uterine wall at implantation. (B) The opposite orientation of the axis of bilateral symmetry shown by the direction of tilting of the inner cell mass of blastocysts implanted on the right (R-type) and the left (L-type) side of the uterine horn (after Smith, 1980, 1985) and the possible influence of local differential growth of the uterine endometrium on the orientation of the blastocyst (curved arrows). (A) is the oblique view and (B) the mesometrial views of the uterine horn. The wavy light green line represents the epithelium lining the wall of the uterine tube.

(C and D) The opposite orientation of the anterior-posterior axis of (C) the gastrula (primitive streak in orange, ectoplacental cone in brown) and (D) the early somite stage embryos in the same uterine horn (placenta in brown).

(Cai et al., 2003). Subsequently, *Cox2* and EGF expression shifts from around the blastocyst to the tissues on the mesometrial side of the uterus where the placenta will be formed (Paria et al., 2000). These changes in gene expression pattern are consistent with the site of active proliferation first of the mural trophoblasts and later the polar trophoblasts at the ectoplacental cone (Sutherland, 2003). Induction of the decidual reaction can be elicited by local application of IGF-1 and HB-EGF (Paria et al., 2001), raising the possibility that the blastocyst-derived IGF-1 (Lin et al., 2003) may activate the expression of HB-EGF in the uterine tissues, which initiates the cascade of molecular and cellular activity of decidual reaction. Localized expression of these factors could cause differential growth of the uterine tissues, remodeling the architecture of the implantation sites and impacting the orientation of the implanted embryo in the uterus. The shape of the blastocyst may be molded mechanically by the uterine tissue to create the apparent uniform morphology of embryos that have implanted on the same side of the uterine wall. However, the passive reorientation and modeling of the blastocyst cannot account for the orientation of the axis of bilateral symmetry associated with the two types of implantation (Smith, 1980). If the antiparallel orientation of the blastocyst axis associated with the sidedness of implantation can be verified, it may point to the existence of some intrinsic functional asymmetry of the mural trophectoderm of the blastocyst that could determine how it implants in the uterus.

Notwithstanding the gaps in our knowledge of the establishment of the embryonic axes in the mouse, the emerging picture reveals a complex interplay between the orientation and the polarity of the blastocyst, the anterior-posterior (A-P) axis of the gastrula and the uterine environment. By the gastrula stage, the orientation of the three primary body axes of embryo has been shown to bear a predictable relationship to the three anatomical planes of the uterine tube (Smith, 1980, 1985; Snell and Stevens, 1966). The dorsal side of the embryo is facing the mesometrial (placental) aspect and the ventral side is facing the antimesometrial pole (Figure 4D). Regarding the A-P and the left-right (L-R) body axis, they tend to align with the transverse and the longitudinal plane of the uterine tube, respectively. However, the polarity of these two body axes can be completely opposite in neighboring embryos: the anterior of one embryo may point to the left-hand side and the other to the right-hand side of the uterine tube, and the left side of the embryo could be facing the oviductal pole in one and the cervical pole in the other (Figure 4D).

Cell Movement of the Visceral Endoderm Reveals Axis Reorientation in the Pregastrula

The first clear sign of the future A-P axis of the embryo is the asymmetric expression of genes like *Hhex* and *Hesx1* in a subset of the visceral endoderm (VE) on one side of the pregastrula embryo. Analysis of *Hhex* expression pattern in the pregastrula embryo revealed a progressive shift of the expression domain in the visceral endoderm from the distal to the prospective anterior side in the E5.5 to E6.0 embryos (Rivera-Perez et al., 2003; Srinivas et al., 2004; Thomas et al., 1998). This finding forms the foundation of the contemporary model of the establishment of the axis polarity of the gastrula-stage mouse embryo. It is envisaged that the anterior

pole of the future A-P axis is re-aligned from the proximal-distal plane to the transverse plane of the cylindrical pregastrula embryo (Thomas and Beddington, 1996).

The movement of the AVE precursors from the distal to the prospective anterior region of the pregastrula has been confirmed by tracking the positions of distal VE that have been marked by dye or histochemical label (Thomas and Beddington, 1996; Rivera-Perez et al., 2003; Yamamoto et al., 2004) and of *Hhex-GFP*-expressing cells by real-time imaging (Srinivas et al., 2004). These studies have revealed several interesting insights into the pattern and the mechanism of VE cell movement. The asymmetrical movement of the distal VE cells has been postulated to be propelled by the morphogenetic force generated by the differential expansion of the VE cell population due to regional differences in the cell proliferation. As a result, cells are displaced away from regions of high cell proliferation toward regions of low proliferative activity. Experimentally, the direction of cell displacement may be manipulated by ectopic expression of Nodal, the Nodal antagonists (encoded by *Cer1* and *Lefty1*), or the dominant-negative form of *Cdk2* (Yamamoto et al., 2004). An interesting observation is that an asymmetric expression domain of *Cer1* and *Lefty1*, as compared with *Hhex*, in the distal region before the onset of VE movement, seems to predict the direction of cell movement by local suppression of nodal activity (Yamamoto et al., 2004). How this asymmetric antagonistic activity is initiated in the embryo is not known.

Positioning the A-P Axis in the Pregastrula Embryo

Detailed study of AVE gene expression, combined with lineage tracing, has revealed dynamic relationships between the shape of the embryo, the position of the AVE markers, and the axis of the uterus (Mesnard et al., 2004; Perea-Gomez et al., 2004; Rivera-Perez et al., 2003; Smith, 1985). The radially symmetric E5.5 embryo becomes ellipsoid in the transverse plane and develops a long and a short transverse axis by E6.0. In view of the similar topographical features, it would be intuitive to predict that the long axis of the pregastrula predicts the later orientation of the A-P axis of the gastrulating embryo. Two recent studies have shown that this is not the case (Mesnard et al., 2004; Perea-Gomez et al., 2004). In the pregastrula, AVE genes (*Cer1*/*Cer1-GFP, Hhex*/*Hhex-GFP*, and *Gsc*) and the posterior epiblast markers (*Nodal, Fgf8, Evx1*, and *T*) were initially expressed at opposite ends of the short rather than the long axis in the transverse plane of the embryo. This finding suggests that the AVE moves from the distal region of embryo along the meridian of the short axis and not the long axis.

By the initiation of gastrulation, the A-P markers are oriented as predicted along the long axis of the embryo, but the pregastrula embryo undergoes dynamic shape changes in this window of development—the difference in the length of the long and the short axis is progressively reduced and the embryo first loses and then regains the ellipsoidal shape. By tracking the displacement of labeled visceral endodermal cells relative to *Cer1-GFP*-expressing AVE, it was shown that the repositioning of the AVE to a different axis of bilateral symmetry does not require wholesale cell movement in the visceral endoderm (Mesnard et al., 2004) or in the epiblast (Perea-Gomez et al., 2004). Rather, it is more likely to be caused by the changes in the shape of the embryo. The short axis of the pregastrula is converted into the long axis of the gastrula by tissue remodeling, which produces an illusion that the expression domains of genes associated with the AVE and the posterior epiblast shift from the opposite ends of the short to the long axis (Perea-Gomez et al., 2004). The orientation of the prospective A-P axis therefore has remained constant despite the shifts in the axes of bilateral symmetry as the pregastrula embryo remodels its shape.

Accompanying these complex shape changes are shifts in the alignment of the axes of the embryo and the uterine horn (Figure 5). In E6.0–6.25 pre-streak embryos where the polarity of the A-P axis can be identified by the expression of the AVE and posterior epiblast genes,

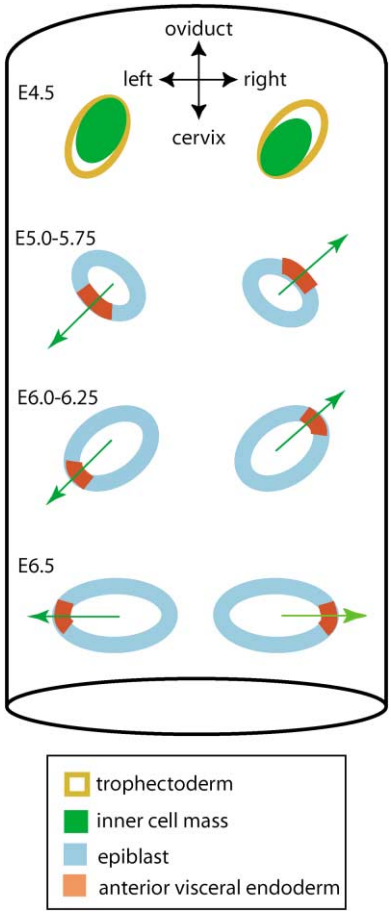

Figure 5. Embryonic Asymmetry and Axis Orientation during Immediate Postimplantation Development

The oblique orientation of the axis of bilateral symmetry of the implanting blastocyst (E4.5) relative to the uterine axes (Smith, 1980) and the re-alignment of the prospective anterior-posterior axis (green arrow) from the oblique orientation to the transverse axis of the uterine tube during the transformation of the long and the short axes of the pregastrula embryo at E5.5–5.75, E6.0–6.25, and E6.5 (after Mesnard et al., 2004, and Perea-Gomez et al., 2004). The antiparallel orientation of the blastocyst axis at E4.5 may underpin the opposite orientation of the anterior-posterior axis of the E6.5 gastrula stage embryo in the transverse plane of the uterus.

the A-P axis is still not aligned perfectly with either the longitudinal or the transverse axis of the uterine horn. Alignment of the A-P embryonic axis to the transverse uterine axis only occurs when the embryo commences gastrulation (Mesnard et al., 2004) (Figure 5). The remodeling and realignment of the embryonic axes seemed to take place in embryos that were grown in vitro, although the embryos develop abnormal shapes in culture (Perea-Gomez et al., 2004), making an overriding role for the uterus unlikely. Nevertheless, it is possible that there is normally a fine-tuned interplay between embryo and the uterine environment resulting in consistent orientation of the embryo in utero with respect the uterine axes.

The mechanism underlying the opposite polarity of the A-P axis of different embryos implanting in the same uterine horn (Figures 4C, 4D, and 5) is not known. To account for variation in the polarity of the A-P axis among the embryos, some form of positional cue may have to be provided to the embryo at stages earlier than the onset of VE movement or reorientation of the axis. It is possible that intrinsic information is endowed in the asymmetry of the blastocyst during embryogenesis and that the direction of the A-P axis reflects the random orientation to the uterine axis as the blastocyst implants (Figures 4A and 4B). Alternatively, the local interaction of the mural trophectoderm with the uterine tissue may provide the necessary positional cue for axis orientation. The role of the embryo-uterine interaction in the generation of asymmetry in the embryonic body plan warrants further investigation.

Establishment of the Definitive A-P Axis of the Body Plan

Analysis of gene expression in the pregastrula embryo reveals that, concurrent with the anterior movement of the distal visceral endoderm, cells in the epiblast display a posterior regionalization of the expression domain of genes that are characteristic of the posterior epiblast and the primitive streak. Expression of genes such as *Wnt3, T, Fgf8, Evx1, Cripto*, and *Nodal* are found in the posterior epiblast of the pre-streak embryo opposite to the *Hhex*- and *Cer1*-expressing visceral endoderm on the anteriorside (Brennan et al., 2001; Ding et al., 1998; Liu et al., 1999; Perea-Gomez et al., 2001; Thomas and Beddington, 1996). In mutant embryos that fail to shift the expression domain of the AVE genes, the expression of *Brachyury* and *Wnt3* also fails to localize to a posterior position (Kinder et al., 2001).

The anterior displacement of the AVE precursors results in the anterior positioning of a source of antagonistic activity to the nodal and Wnt signaling activities that promote posterior development (Perea-Gomez et al., 2002) (Mukhopadhyay et al., 2001, 2003; Yamamoto et al., 2004). The relocation of the VE cells therefore breaks the radial symmetry and creates an axis of bilateral symmetry in the epiblast along the definitive A-P axis. It converts the radial gradient of signaling activity into a linear one by the regionalization of the source of ligands and antagonists to opposite sides of the embryo (Figures 6A and 6B) (Agius et al., 2000; Robertson et al., 2003; Vincent et al., 2003). Fate-mapping of the pre- and early-streak embryo shows that the regionalization of cell fates in the epiblast seems to follow the presumptive

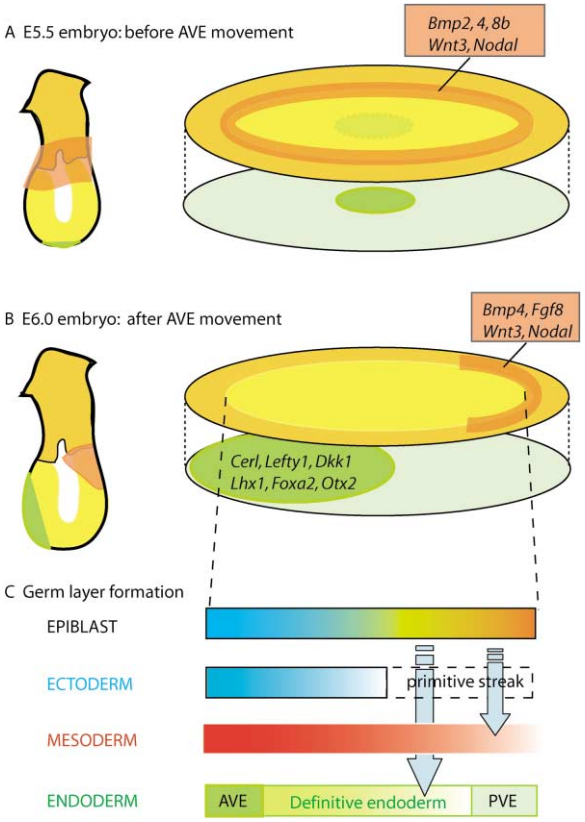

Figure 6. Establishment of the Anterior-Posterior Axis and Tissue Patterning at Gastrulation

(A) The proximal-distal distribution of signaling and antagonistic activities in the E5.5 embryo is equivalent to a peripheral-to-central gradient of signaling activity in the discoid model of the embryo. This establishes a radially symmetrical embryonic pattern showing a precocious specification of the prospective anterior pole of the embryonic axis in the center of the epiblast. The footprint of the anterior visceral endoderm is marked by the shadow in the epiblast layer.

(B) After the anterior displacement of the source of antagonistic/modulating activity and the posterior regionalization of the source of signaling activity, the polarization of the signaling gradient in the sagittal plane of the E6.0 pre-streak embryo specifies the orientation and the polarity of the anterior-posterior embryonic axis and the germ layer fates of the epiblast cells along this axis. Genes that are expressed in (A) the proximal epiblast-extraembryonic tissue border and (B) the anterior visceral endoderm (AVE, the green area) and the posterior tissues (the orange arch) are listed.

(C) The transformation of the two-dimensional blueprint into a three-dimensional body plan is accomplished during gastrulation, by an orderly control of the morphogenetic movement of the mesoderm and endoderm progenitors through the primitive streak, the organization of new tissue layers, and the expansion of the ectodermal progenitors that remain in the epiblast. Cells in the germ layers and their progenitors in the epiblast are shown in matching color codes. The graded pattern represents the region-specific fates or lineage potency of cells at different anterior-posterior positions in the germ layer. PVE represents the posterior visceral endoderm underneath the primitive streak, which is yet to be replaced by the definitive endoderm by late gastrulation.

gradient of Nodal/BMP/WNT activity: the ectodermal progenitors in the area close to the AVE, the mesodermal progenitors in the posterior region close to the source of ligand, and the endodermal progenitors in between

the two (Lawson et al., 1991; reviewed by Lawson, 1999, Tam and Behringer, 1997, and Tam et al., 2003). Modulation of signaling activity in the epiblast by the visceral endoderm and the extraembryonic tissues may therefore be instrumental in the specification of tissue lineages and the regionalization of the germ layer precursors in the epiblast of the gastrula embryo (Tam and Gad, 2004).

Gastrulation: The Rendition of Embryonic Patterning

During the development of the blastocyst to the prestreak embryo, the consolidation of the axes of symmetry and the progressive increase in tissue complexity generates a blueprint of the topographical relationship of the precursors of the three definitive germ layers—the ectoderm, the mesoderm, and the endoderm. Since this blueprint is mapped on an epithelium (the epiblast), which is a two-dimensional representation, the major objective of gastrulation is the rendition of this blueprint into a three-dimensional body plan (Figure 6C). The breaking of asymmetry following the anterior movement of the AVE transforms a radially symmetric pattern of signaling information into one with A-P polarity that heralds the complex process of germ layer formation. Understanding how this initial asymmetry is developed is therefore key to understanding the establishment of the body plan. It is certainly important to determine whether architectural asymmetries in the zygote and the blastocyst may influence the orientation of the primary body axes of the postimplantation embryo, since this would affect the search for molecular initiators of asymmetry. At this point there is still no clear evidence that any preimplantation asymmetries play a causative role in driving later lineage restrictions or axial patterning, but several tantalizing hints, such as the early non-random growth of VE clones and the tilting of the TE-ICM complex, do suggest that blastocyst asymmetries should be considered in modeling early postimplantation axis development. Whether any blastocyst asymmetry is directly influenced by the so-called animal-vegetal axis of the zygote is currently less certain. In the absence of any clear evidence of segregation of molecular determinants in the egg cytoplasm to different regions of the developing preimplantation embryo, it seems that most examples of apparent asymmetries may be coincidental outcomes of normal preimplantation development. Future studies will need to link the pre- and postimplantation asymmetries by lineage tracing and molecular marking across the peri-implantation boundary.

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