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Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse

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The investigation into lineage allocation and early asymmetries in the pre- and peri-implantation mouse embryo is gaining momentum. As we review here, new insights have been gained into the cellular and molecular events that lead to the establishment of the three lineages of the blastocyst, to the determination of the origin and the fates of the visceral endoderm in the peri-implantation mouse embryo, and to the generation of cellular and molecular activities that accompany the emergence of asymmetries in the pre-gastrulation embryo. We also discuss the continuing debate that surrounds the relative impacts of early lineage bias versus the stochastic allocation of cells with respect to the events that pattern the blastocyst and initiate its later asymmetries.

Introduction

The progression of the mammalian embryo from fertilization to gastrulation involves an ordered series of lineage specifications and axial asymmetries (Fig. 1) that result, first, in the development of the blastocyst (see Glossary, Box 1), with its embryonic-abembryonic axis (Fig. 1; Box 2), and, later, in the formation of the embryo itself, with its anterior-posterior (AP), dorsal-ventral (DV) and left-right (LR) axes. In many invertebrates and vertebrates, asymmetries that are established in the egg correlate with the segregation of determinants that influence later lineage formation and axis development. However, in the mouse egg, the morphological asymmetries that exist, such as the position of the second polar body (see Glossary, Box 1) and the sperm entry point, do not clearly demarcate an asymmetric domain of, for example, signaling activity or of cell fate determinants in the fertilized mouse egg. Whether there is any instructive relationship between the asymmetry of the egg and the later asymmetry of the blastocyst and lineage allocation remains a controversial issue. It is well known that the pre-implantation mammalian embryo is highly regulative and resistant to the loss or addition of cells brought about by experimental manipulations. However, this does not preclude the existence of an, as yet, uncharacterized property that could bias developmental outcomes in the intact embryo.

Whether any early asymmetries in the mouse egg and/or blastocyst relate to the orientation of the definitive body axes is even less certain. It is now clear that the AP patterning of the gastrulating embryo is initiated prior to gastrulation by spatially localized signals that emanate from regionally patterned extra-embryonic tissues. Some of these asymmetries may be set up as early as the blastocyst stage, linking pre-implantation patterning to post-implantation morphogenesis.

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Here, we review recent experiments that define the molecular components of lineage specification in the mouse blastocyst. We also review the ongoing uncertainty and debate that surrounds the relative importance of early cleavage patterns at the two- to four-cell stage and of symmetric versus asymmetric divisions at the eight- to 16- and 16- to 32-cell stage, and the importance of final cell position in the late morula/early blastocyst for blastocyst lineage specification for the positioning of the blastocyst cavity (the blastocoel) and for the establishment of the embryonic-abembryonic axis of the blastocyst. Our critical review of the current data supports a stochastic model of lineage specification, in which cell-cell interactions and position effects reinforce and can override any underlying cell fate bias.

The asymmetries that are observed in the post-implantation development of the visceral endoderm (see Glossary, Box 1) that lead up to gastrulation are now well defined and strongly hint at the emergence of the prospective AP body axis prior to the onset of gastrulation. However, a definitive link between the asymmetries in the pre-gastrula embryo and the morphological and tissue asymmetries displayed earlier in the blastocyst has still not been established. Here, we also review the findings of recent experimental studies that help to define the events that initiate early axial patterning in the post-implantation mouse embryo.

Lineage allocation in the blastocyst

The mouse blastocyst, immediately before implantation, consists of three distinct cell groups: the trophectoderm (TE); the epiblast, which is derived from the earlier inner cell mass (ICM); and the primitive endoderm. Only the epiblast gives rise to the embryo itself, whereas the other two cell types give rise to extra-embryonic structures that support the intra-uterine development of the embryo and act as signaling sources to pattern the embryonic tissues prior to gastrulation.

Although pluripotent embryonic stem cells (ES cells) can be obtained from the epiblast of the blastocyst, other progenitor cells lines that self-renew in culture can also be derived from the blastocyst, such as trophoblast stem (TS) cells, which retain properties of the trophectoderm (see Glossary, Box 1) (Tanaka et al., 1998), and the XEN cells, which retain properties of the primitive endoderm (Kunath et al., 2005). ES cells can be converted to TS or XEN-like cells by altering the expression of appropriate transcription factors, providing a good assay for the identification of key lineage-specific factors. As we discuss below, recent progress has been made in identifying the transcription factors that specify the blastocyst lineages and their derived stem cells.

Lineage-specific transcription factors and trophectoderm specification

Cdx2, a caudal-related homeodomain protein, is a key regulator of the trophectoderm lineage. The expression of *Cdx2* in ES cells induces them to differentiate into trophoblast, and to acquire the properties of TS cells (Niwa et al., 2005). In the embryo itself, *Cdx2*

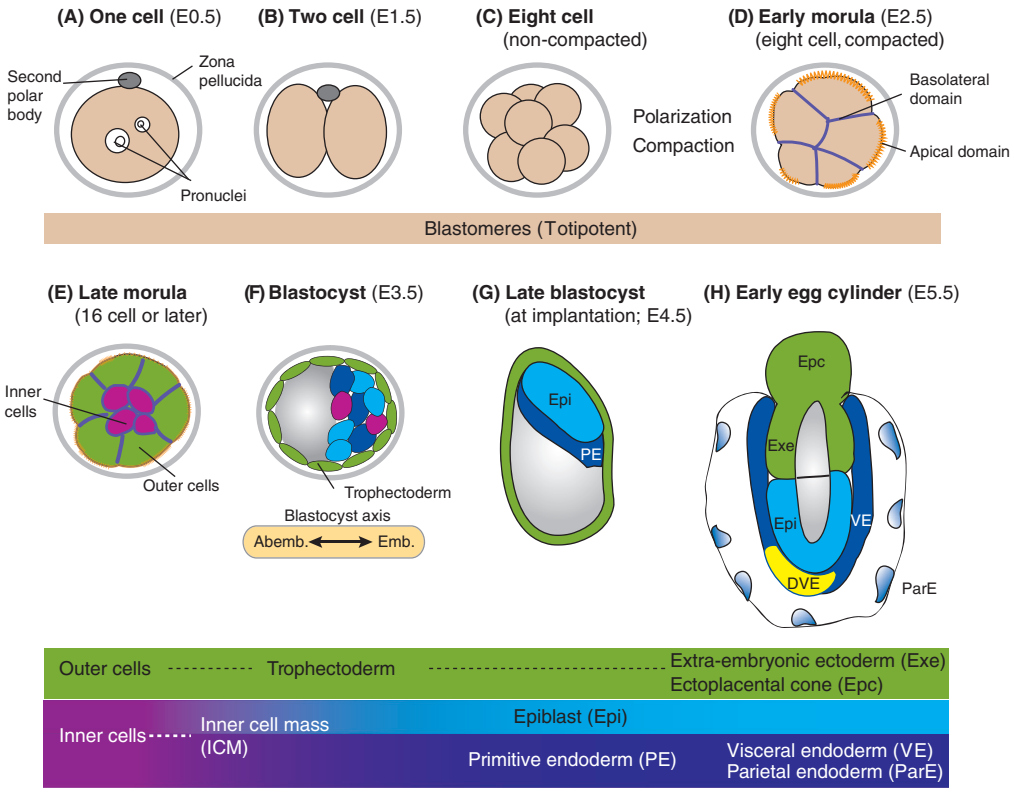

Fig. 1. Cell lineage formation from egg to egg cylinder. (**A-H**) Schematics of the morphological changes and cell lineage specification that occur in a mouse embryo, from its fertilization at embryonic day (E) 0.5 to the early egg cylinder stage (E5.5). The colored bars show the progressive allocation of totipotent blastomeres to outer and inner cells and to the trophectoderm and inner cell mass lineages. The cell types in the embryos are color coded. Abemb. ↔ Emb., abembryonic-embryonic axis of the blastocyst; DVE, distal visceral endoderm.

begins to be expressed around the eight-cell stage and gradually becomes restricted and upregulated in the outside cells of the morula ahead of blastocyst formation (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). A loss-of-function *Cdx2* mutation has no impact on the initiation of blastocyst formation (Strumpf et al., 2005), and *Cdx2* mutant cells are not excluded from the TE layer in chimeric blastocysts (Ralston and Rossant, 2008), but in embryos carrying this mutation, the outer epithelium of the blastocyst loses morphological integrity and the cells do not undergo further trophoblast differentiation (Strumpf et al., 2005). A mutation in *Eomes*, a T-box transcription factor, also arrests blastocyst development but at a slightly later stage than is found in *Cdx2* mutants (Russ et al., 2000; Strumpf et al., 2005). *Eomes* expression is reduced in *Cdx2* mutants but *Cdx2* is still expressed in *Eomes* mutants, placing the *Eomes* transcription factor downstream of *Cdx2* (Ralston and Rossant, 2008; Strumpf et al., 2005).

Neither mutation leads to complete failure to initiate the formation of the TE epithelium of the blastocyst, suggesting that there may be more players upstream of *Cdx2* and *Eomes*. Two groups have recently shown that another transcription factor of the TEA domain/transcription enhancer factor family, TEAD4, is required upstream of *Cdx2* for the formation of the TE (Nishioka et al., 2008; Yagi et al., 2007). *Tead4* mutants show a slightly more severe phenotype than do *Cdx2* mutants and fail to maintain *Cdx2* expression, placing TEAD4 currently at the top of the TE genetic hierarchy. However, unlike *Cdx2*, *Tead4* expression is not restricted to the TE lineage during pre-implantation development, making it difficult to reconcile this observation with the notion of TEAD4 having an instructive role in TE lineage specification. In *Drosophila* and mammalian cells, TEAD factors can only activate transcription in combination with a co-activator, Yorkie (Yki) in *Drosophila* or Yap (Yes-associated protein) in mammals (Vassilev et al., 2001; Zhao et al., 2008). The availability of nuclear-localized Yap could be the limiting factor in activating TEAD-dependent TE lineage specification. As such, an analysis of YAP protein localization during cleavage could be informative. As *Tead4* mutants have a more severe phenotype than do *Cdx2* and *Eomes* mutants, TEAD may activate parallel downstream pathways that are independent of Cdx2/Eomes to specify TE fate. Clearly, the transcription factor networks that drive TE formation are still not fully understood.

Prior to blastocyst formation, TE-specific factors such as Cdx2 and Eomes become restricted to the outside cells of the morula. However, the genes known to be required for specifying the pluripotent cells of the ICM, namely *Oct4* (Nichols et al., 1998), *Sox2* (Avilion et al., 2003; Nichols et al., 1998) and *Nanog* (Chambers et al., 2003; Mitsui et al., 2003), are expressed in every cell during cleavage, and are restricted to the ICM only after blastocyst formation (Fig. 2A-D). This restriction depends on Cdx2. In *Cdx2* mutants, *Oct4* and *Nanog* remain expressed in the TE (Ralston and Rossant, 2008). Thus blastocyst lineage specification begins with the activation of TE targets and repression of ICM identity in outside cells. Later, the reciprocal repression of TE targets by Oct4/Sox2/Nanog in the pluripotent lineages (Loh et al., 2006; Boyer et al., 2005), combined with the known autoregulatory properties of the *Oct4* (Chew et al., 2005) and Cdx genes (Xu et al., 1999; Beland et al., 2004), ensures the maintenance of lineage identity. In order to understand the initiation of lineage segregation at the blastocyst stage, we need to understand how factors like Cdx2 become localized to the outside cells of the morula.

Polarity and position drive trophectoderm formation

It has long been proposed that the position of cells in the developing embryo somehow influences their choice to become either ICM or TE. Initially during cleavage, all blastomeres appear to be identical

in their morphology and potential, but at the eight-cell stage, the events of compaction and polarization begin (Fig. 1; see Glossary, Box 1) (Fleming and Johnson, 1988; Johnson and McConnell, 2004). Concurrent with an increase in E-cadherin-dependent intercellular adhesion (Johnson et al., 1986), cells acquire an apical domain that is rich in proteins, such as the atypical protein kinase C (aPKC) (Pauken and Capco, 2000), the polarity protein Par3 (Plusa et al., 2005a) and the apical membrane protein ezrin (Louvet et al., 1996). However, molecules such as Lgl (lethal giant larva homolog) and the PAR polarity protein Par1 are localized exclusively in the basolateral regions of each blastomere (Vinot et al., 2005) (Fig. 2D). Adherens junctions and, later, tight junctions between cells (Fleming et al., 1989) separate the apical and basolateral domains of the blastomeres, resulting in the formation of a polarized epithelium. As cells divide from the eight- to 16-cell stage and from the 16- to 32-cell stage, the outer cells retain this polarized phenotype, whereas cells in the core of the cluster lose apical features and become morphologically apolar (Johnson and Ziomek, 1983). The outside polarized epithelium goes on to form the TE, while the enclosed apolar cells go on to form the ICM (Johnson and Ziomek, 1983).

Apolar cells have been proposed to arise by asymmetric cell divisions, in which some outer polarized cells divide such that only one daughter inherits the apical pole, whereas the remaining cell will be apolar and take up an internal position (Johnson and Ziomek, 1981). Symmetric divisions will generate two polar cells, which will stay on the outside and end up in the outer TE epithelium. A recent study that traced the complete cell lineages from the two-cell to the 32-cell stage mouse embryo has confirmed that inside apolar cells and outside polar cells can be generated from outside cells through two rounds of polarized cell divisions, and that the final cell number of the ICM versus the TE is determined by the proportion of inside to outside cells generated at each round of division (Bischoff et al., 2008). What is less clear at this time is whether the polarized cell divisions that occur lead to the differential inheritance of lineage determinants by daughters, which dictate their future fate. The specialized apical polar region could control the orientation of the mitotic spindle, and ensure the inheritance of localized determinants through asymmetric divisions, in a manner analogous to the *Drosophila* neuroblast lineage (Wodarz, 2005; Yu et al., 2006). The lineage tracing experiments just described identified a division as asymmetric or symmetric based on the location of the daughter cells after mitosis, not based on whether the anaphase plate was oriented perpendicularly or parallel to the apical domain, or according to the differential inheritance of fate-determining factors.

Could there be localized TE or ICM determinants that are segregated through polarized cell divisions? There is a close association between the acquisition of a polar phenotype and the upregulation of *Cdx2* in outer cells (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Suwinska et al., 2008). However, there is no evidence that Cdx2 protein or any other TE lineage transcription factor is subcellularly localized to the apical domain of the polarized blastomere (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). Nor is anything known about the basal localization of known negative regulators of TE fate. A recent report that *Cdx2* mRNA might be distributed asymmetrically to the polar regions in eight- and 16-cell blastomeres is intriguing (Jedrusik et al., 2008) and could provide a possible mechanism for the later rise in Cdx2 protein levels in outside cells. However, it will be necessary to monitor carefully the association of spindle plane orientation, inheritance of the polar region, the inheritance of *Cdx2* mRNA by inside/outside daughters and, most crucially, the resultant protein distribution, before concluding that this is the mechanism that initiates lineage specification.

Box 1. Glossary

Blastocyst
A vesicular mouse embryo formed 3.5 days post coitum consisting of the trophectoderm encasing an inner cell mass (ICM) and the blastocoel.

Compaction
Cellular changes associated with the formation of intercellular junctions and the flattening of the blastomeres of the morula stage embryo.

Epiblast
The epithelial tissue that develops from the ICM and gives rise to the ectoderm, mesoderm and definitive endoderm during gastrulation.

Polarization
Acquisition of morphological and molecular differences along the apical-basal axis of the cells.

Primitive endoderm
The epithelial layer of cells that lines the blastocoelic surface of the ICM.

Primitive streak
The structure that appears in the posterior region of the gastrulating embryo where the epiblast cells undergo epithelio-mesenchymal transition and ingressional movement to form the germ layers.

Second polar body
The product of second meiotic division of the oocyte, which mostly contains haploid chromosomal material and a small amount of cytoplasm.

Trophectoderm
The outer epithelial layer of the blastocyst, consisting of a polar component associated with the ICM and the mural component, which lines the blastocoel. The trophectoderm differentiates into trophoblast during post-implantation development.

Visceral endoderm
The epithelial layer of cells that envelops the extra-embryonic ectoderm and the epiblast of the post-implantation embryo.

Zona pellucida
The non-cellular covering of the oocyte, which stays with the zygote through development to the blastocyst.

It is worth remembering that Cdx2 is not at the top of the TE transcription factor hierarchy and that its expression is not initially localized to outside cells. Levels of Cdx2 and its local upregulation may depend on post-translational events that regulate TEAD/Yap complex activity (Reddy and Irvine, 2008). This concept takes us back to the first hypothesis regarding ICM/TE differentiation: the inside-outside hypothesis (Tarkowski and Wroblewska, 1967). This hypothesis is founded on the idea that inside and outside cells are in different micro-environments and could receive different levels and/or types of signaling input, depending on their degree of contact with other cells. Inside cells, by virtue of being surrounded by other cells, might perceive signaling activity differently from the outside cells, potentially leading to the post-translational modification of one or more key regulator(s), such as Yap. According to this hypothesis, the generation of the inside environment is the key factor to ensuring lineage segregation, rather than the segregation of determinants through asymmetric cell divisions. The formation of a polarized outer epithelium would still be important for ensuring the integrity of such an internal niche.

Despite recent advances, the exact mechanisms that link cell polarity, cell position, the effects of the local micro-environment, signaling activity and cell fate in blastocyst formation remain to be determined.

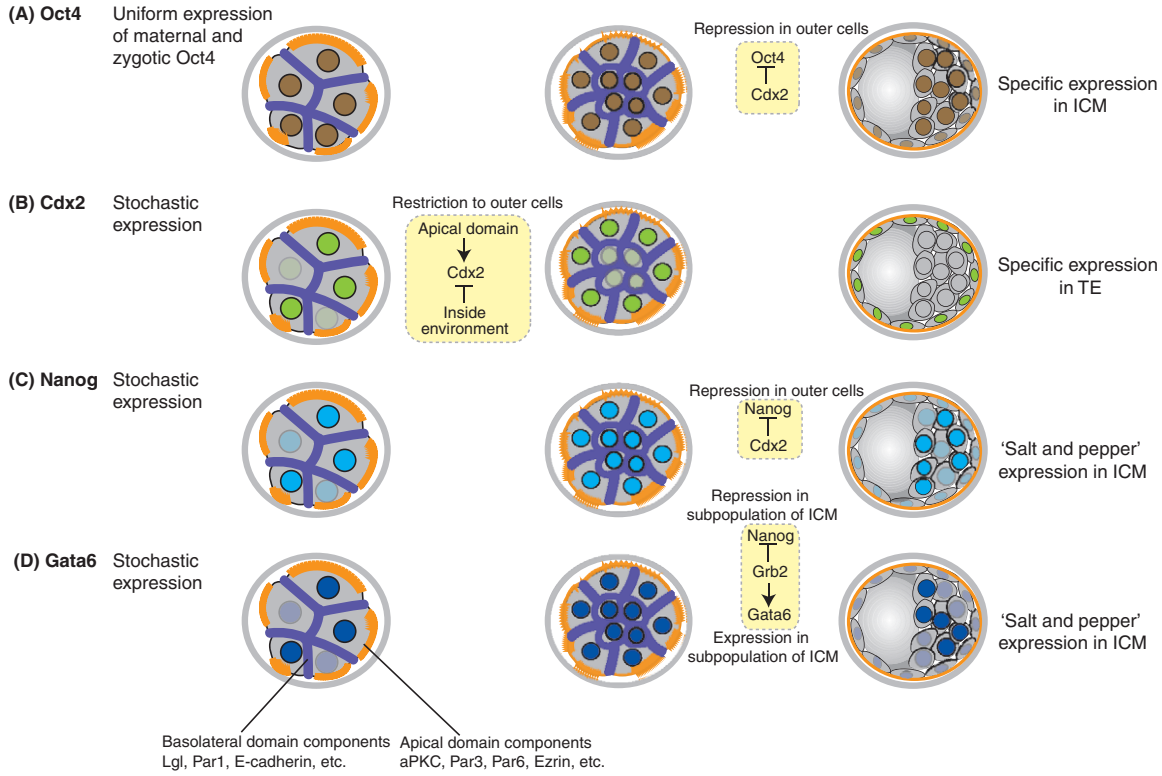

Fig. 2. Molecular players in the formation of the first lineages in the blastocyst. Four lineage-specific transcription factors, Oct4, Cdx2, Nanog and Gata6, are important for the generation of the first three lineages in the blastocyst. The initial expression of these transcription factors is not restricted to specific cell populations. Lineage-specific expression is gradually established in association with the maturation of cellular structures (such as apical-basolateral cell membrane domains, intercellular junctions, etc.) and of positive and negative interactions among the transcription factors themselves. (**A**) Oct4: Oct4 protein is observed in all blastomeres throughout early cleavage stages due to maternally encoded protein. At the eight-cell stage, all blastomeres contain Oct4. At the blastocyst stage, Oct4 is gradually downregulated in the outer trophectoderm (TE) cells by Cdx2 through direct physical interaction and transcriptional regulation. (**B**) Cdx2: Cdx2 protein is detected beginning at the eight- to 16-cell stage, its initial expression appears to be stochastic. By the early morula to early blastocyst stages, Cdx2 expression is ubiquitous but higher in outer, apically polarized cells. Restricted expression in outer TE cells is established by the blastocyst stage. (**C**) Nanog and (**D**) Gata6: Nanog and Gata6 are detected from the eight-cell stage. Both proteins are expressed uniformly in all cells until the early blastocyst stage. Nanog expression is downregulated in outer cells by Cdx2 and in a subpopulation of the ICM by Grb2-dependent signaling. By contrast, Gata6 expression is maintained by Grb2-dependent signaling. By the late blastocyst stage, ICM cells express either Nanog or Gata6 exclusively.

Primitive endoderm formation: influence of position versus gene activity

Recently, there have been new insights into how cells within the ICM of the blastocyst become segregated into the progenitors of the epiblast and primitive endoderm. Whereas cell position drives cell fate in ICM and TE formation, the converse appears to be true in epiblast versus primitive endoderm specification in the ICM: cell fate precedes and helps drives cell position. Until recently, it was thought that, at E3.5, all ICM cells were of equivalent lineage potency, with the primitive endoderm layer then forming on the surface of the ICM (Fig. 1G) by some ill-defined position-dependent mechanism. However, individual E3.5 ICM cells show the exclusive expression of either epiblast-specific genes (e.g. the transcription factor *Nanog*) or primitive endoderm-specific genes (e.g. the transcription factors *Gata4* and *Gata6*) in a 'salt and pepper' mosaic pattern prior to the appearance of the primitive endoderm layer (Chazaud et al., 2006; Gerbe et al., 2008) (Fig. 2C,D). Lineage tracing and chimera analysis has shown that the descendants of individual E3.5 ICM cells are primarily restricted in fate to one lineage or the other (Chazaud et al., 2006). These findings have led to a new model of epiblast/primitive endoderm formation that is based on an initial mosaic of two lineage progenitors at E3.5, followed by their sorting and relocation to the appropriate positions in the ICM by E4.5 (Fig. 1G) (Rossant et al., 2003). Evidence in support of this hypothesis also comes from a genome-wide expression analysis that shows that individual E3.5 ICM cells fall into two cohorts, one that is enriched for the expression of epiblast genes and the other enriched for primitive endoderm-specific genes (Kurimoto et al., 2006). One of the genes identified as being upregulated in the primitive endoderm cohort is *Pdgfra*, which encodes the platelet-derived growth factor receptor α. Plusa et al. (Plusa et al., 2008) followed the expression of *Pdgfra*-histone 2B (H2B)-green fluorescent protein (GFP) fusion protein to visualize the allocation of the primitive endoderm in live embryos. They found that *Pdgfra*-H2B was initially co-expressed in some ICM cells with epiblast factors such as Nanog. However, by E3.5, the expression of epiblast and endoderm genes became non-overlapping among the ICM cells. Videomicroscopy showed that *Pdgfra*-positive cells on the luminal surface of the ICM remained in place, whereas *Pdgfra*-positive cells that were embedded within the ICM relocated to the superficial position, or were eliminated by apoptosis, culminating in a sharp separation of epiblast and primitive endoderm by E4.5 (Plusa et al., 2008).

Signaling, in addition to cell position, is also involved in the correct specification of these lineages. In particular, active signaling through a Grb2 (growth receptor bound protein 2)-dependent pathway is necessary for the initiation of primitive endoderm gene expression (Chazaud et al., 2006) (Fig. 2D). Grb2 is an adaptor protein that links receptor tyrosine kinase activation to the downstream Ras-MAP kinase signaling pathway in a number of different contexts. In the absence of *Grb2*, no primitive endoderm forms and all cells of the ICM express *Nanog* (Fig. 2C) and are epiblast in character. It is likely that fibroblast growth factor (FGF) signaling is involved in primitive endoderm development upstream of Grb2, based on known defects in primitive endoderm development in FGF4 mutants (Feldman et al., 1995) and on the expression patterns of FGF pathway components in the early embryo (Arman et al., 1998; Chai et al., 1998). However, the exact timing and location of FGF action and its relation to early lineage mosaicism is unclear. Although recent experiments have suggested new ways of viewing the process of epiblast/primitive endoderm formation, we still lack a coherent understanding of: (1) the upstream mechanisms that lead to the initial mosaic pattern of epiblast/primitive endoderm gene expression; (2) the exact relationship between lineage restriction and gene expression; and (3) the pathways required for cells to segregate correctly to their respective positions in the ICM.

Box 2. The embryonic-abembryonic axis of the blastocyst

At the conclusion of pre-implantation development, the fully expanded mouse blastocyst is distinctively partitioned into two domains: one with the inner cell mass (ICM) and polar trophectoderm; and the other with the blastocoel enclosed by the mural trophectoderm. This configuration allows the delineation of the embryonic (where the ICM is located)-abembryonic axis of the blastocyst. Following implantation, growth of the polar trophectoderm and the ICM into the blastocoel leads to the formation of an elongated structure (the 'egg cylinder', an archaic but still used term) comprising the epiblast, the visceral endoderm and the extra-embryonic ectoderm. The egg cylinder is connected via the ectoplacental cone to the uterine tissue. The embryonic-abembryonic axis of the blastocyst therefore becomes the proximal (the ectoplacental cone side)-distal (the epiblast side) axis of the post-implantation embryo. Within the ICM, an embryonic-abembryonic division of tissue compartments emerges when a layer of primitive endoderm forms on the luminal surface of the ICM, which now becomes the epiblast. The epiblast-primitive endoderm configuration heralds the arrangement of ectoderm-mesoderm-endoderm germ layers in the gastrula-stage embryo, and, by extrapolation to the early organogenesis-stage embryo, the prospective dorsal-ventral body axis of the embryo.

Mechanism of blastocyst axis formation

The formation of the blastocyst cell lineages essentially involves transforming an indeterminate expression pattern of key lineage regulators into a spatially restricted and regulated pattern, concomitant with the evolving cellular properties of the blastomeres. It is not clear whether there is a need for any kind of prepattern or lineage bias in early blastomeres to achieve this end result. However, besides the divergence of cell lineages, the blastocyst has other emergent properties. It has a clear embryonic-abembryonic axis, which is defined by the position of the ICM on one side of the blastocyst (Fig. 1F; Box 2). The second polar body, which takes up a position between the two blastomeres of the two-cell embryo (Fig. 1B), remains associated with the equator of the blastocyst (see Fig. 4A). These observations led to the suggestion that there might be some prepattern in early mouse development that determines the embryonic-abembryonic axis of the blastocyst (Gardner, 1997). Two studies that used exogenous cell lineage tracers subsequently showed that the progeny of one of the two-cell blastomeres have a strong tendency to contribute either to the embryonic half or to the abembryonic half of the blastocyst (Fujimori et al., 2003; Piotrowska et al., 2001; Plusa et al., 2005b).

There have been many studies that both support and refute that a relationship exists between the cleavage pattern (and subsequent lineage) of the first two blastomeres and the axis of the blastocyst (the so-called lineage model), with a lively debate conducted in the literature and at conferences on the relative technical merits of each successive study. Several groups, using different strains of mice and different lineage-tracing techniques, have not found any significant evidence that a relationship exists between the position of the progeny of the individual two-cell blastomeres and the embryonic-abembryonic axis (Alarcon and Marikawa, 2003; Chroscicka et al., 2004; Motosugi et al., 2005). Time-lapse movies of mouse embryos developing within the zona pellucida (see Glossary, Box 1) often show that they do not remain stationary but display considerable movement during pre-blastocyst development (Kurotaki et al., 2007; Motosugi et al., 2005). Because of this, time lapse lineage tracing has been used to observe the preferential contribution of cells to the embryonic-abembryonic axis among a fraction of embryos (Bischoff et al., 2008). Indeed, the clearest indication that a relationship might exist between the two-cell blastomere lineages and the embryonic-abembryonic axis came from studies in which mouse embryos were embedded in alginate, which inhibited the movement of blastomeres within the zona (Gardner, 2001; Fujimori et al., 2003). More recent work by the Fujimori laboratory, in which the two-cell blastomeres and their descendants were tracked over time in the living unconstrained embryo by a UV-activated fluorescent protein marker, failed to replicate any preferential contribution of the progeny of the two blastomeres to the embryonic versus abembryonic regions of the blastocyst (Kurotaki et al., 2007).

If no relationship exists between the lineage of the first two blastomeres and the later blastocyst axis in the intact undisturbed embryo, how can the observation that the second polar body adopts a consistent position from the two-cell embryo to the blastocyst be explained? And why is the location of the blastocoel restricted primarily to the progeny of one of the two-cell blastomeres when embryonic cell movement is curtailed or reduced? A mechanism, based on the mechanical constraint imposed by the zona pellucida has been proposed to explain these apparently contradictory findings (Fig. 3) (Alarcon and Marikawa, 2003; Motosugi et al., 2005; Kurotaki et al., 2007). The zona pellucida of the egg, rather than being spherical, often appears ellipsoidal (Fig. 3A), with a longer and shorter diameter (Gray et al., 2004). This shape would place physical constraints on the embryo, resulting in the blastomeres of the two-cell embryo lining up along the long axis of the zona (Fig. 3A). During successive stages of cleavages, as blastomeres get smaller, the embryo as a whole is able to adjust its position constantly within the zona, and the packing of cells becomes less constrained by the shape of the zona. Subsequently, the cavity of the blastocyst begins to form, first as secretion of intracellular vacuoles, which, when externalized, coalesce to form the expanding blastocoel. As the blastocoel expands, the zona pellucida would again impose a physical constraint on the embryo, and the ellipsoidal shape of the zona cavity would topologically favor the location of the blastocoel at one end of the long, rather than the short, axis of the

zona (Fig. 3B). When embryos are deliberately compressed into an elongated shape from the two-cell to the blastocyst stage, the blastocoel is consistently positioned at one end of the elongated blastocyst, regardless of the relationship to the original position of the first cleavage plane (Fig. 3B-D) (Motosugi et al., 2005). A computer simulation of blastocoel formation has also shown that a constraining ellipsoidal capsule could help fixing the axis of the blastocyst (Honda et al., 2008).

The role of the zona in blastocoel positioning has been tested by examining the development of zona-free embryos. In one study, removal of the zona pellucida from the morula stage onwards had no effect on the correlation between the first cleavage plane and the orientation of the blastocoel (Gardner, 2007), whereas in another study this association was lost (Kurotaki et al., 2007). Zona-free embryos develop perfectly normally, which suggests that any constraint imposed by the zona, while imposing morphological constraint on embryo development, is not relevant for the specification of cell fates or axis formation.

Although the weight of evidence suggests that any apparent difference in the lineage contribution of the two-cell blastomeres could be due to topological constraints, results from the Zernicka-Goetz laboratory have revealed some potential differences in the lineage potential of individual four-cell blastomeres. An analysis of the timing and orientation of the two- to four-cell cleavage (Piotrowska-Nitsche and Zernicka-Goetz, 2005) showed that about 80% of embryos adopt a tetrahedral four-cell arrangement. This is

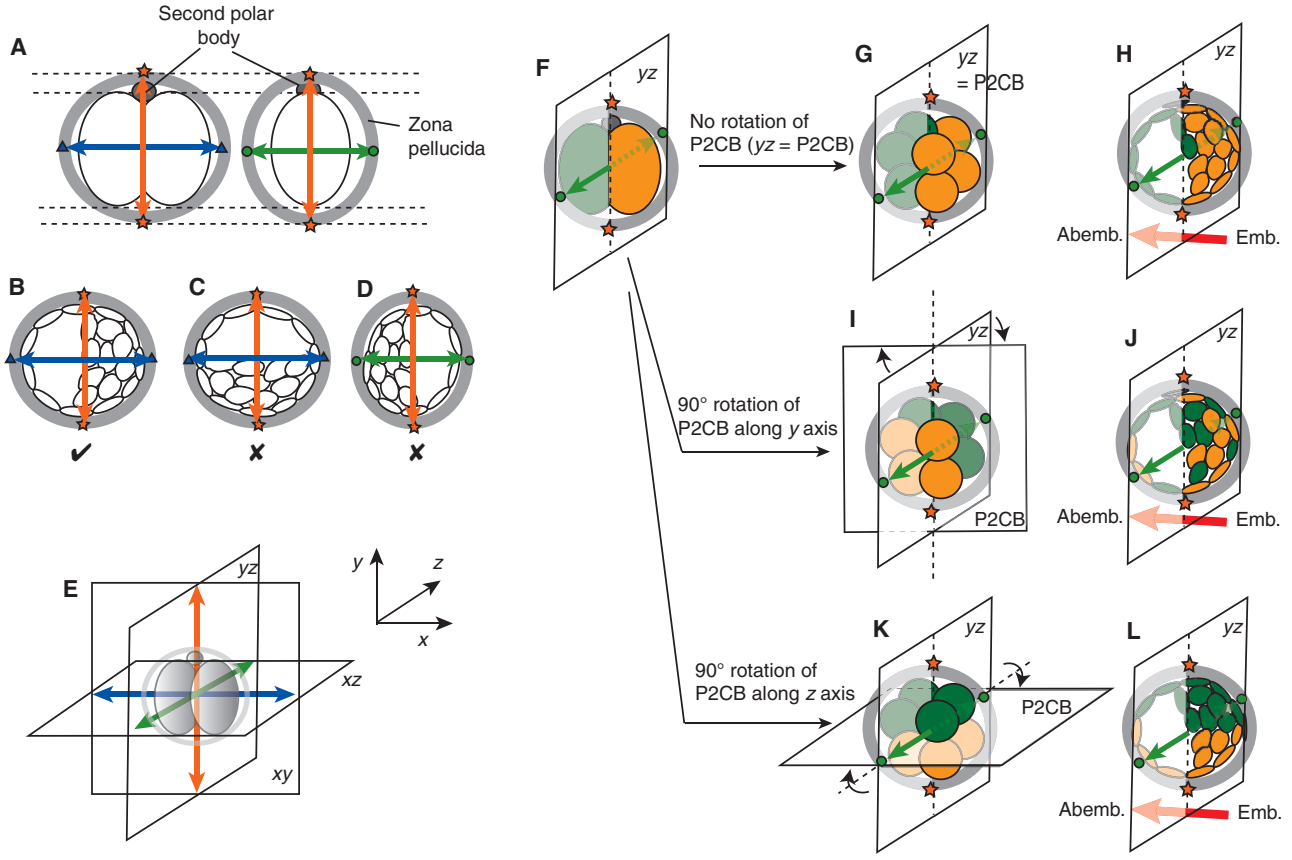

Fig. 3. The relationships among zona pellucida shape, orientation of the two-cell embryo and the embryonic-abembryonic axis of the mouse blastocyst. (**A**) In many mouse embryos, the zona pellucida is not a sphere, but a scalene ellipsoid. It has three unequal diameters: long (marked by blue arrows and triangles), medium (orange arrows and stars) and short (green arrows and circles). The two-cell stage embryo always aligns its orientation along the long axes in the zona pellucida. The broken double lines show differences in the space between the surface of the blastomere and the zona viewed at two different optical planes: left, view in the optical plane of the long and middle diameters (*xy* plane in E); right, view in the optical plane of the long and short diameters (*yz* plane in E). (**B-D**) With reference to the coordinates of the zona pellucida delineated at the two-cell stage, the abembryonic-embryonic axis in the blastocyst most frequently aligns with the longest diameter of the zona (B) and rarely with the two shorter diameters (C,D). (**E**,**F**) The two-cell embryo is visualized in 3D space with the plane of two-cell boundary (P2CB) aligned with the *yz* plane, the plane of the middle and short diameters of the zona pellucida (each blastomere and its progeny are colored green or orange). (**G**,**H**) If the embryo does not rotate during cleavage (or rotates only along the *x*-axis), this alignment is maintained through (G) the eight-cell to (H) the blastocyst stage. (H) The abembryonic-embryonic axis of the blastocyst forms perpendicularly to the *yz* plane and the P2CB. The progeny of each two-cell blastomere thus predominantly occupies either the abembryonic or embryonic domain of the blastocyst. This situation occurs in embryos in which cell movement within the zona is limited or prevented by alginate. (**I-L**) If the embryo rotates within the zona during cleavage, the P2CB will no longer be aligned with the *yz* plane (I,K). The abembryonic-embryonic axis of the blastocyst still forms perpendicularly to the *yz* plane, according to the shape of the zona pellucida, but the P2CB does not align with the abembryonic-embryonic axis (J,L). Two hypothetical examples are shown in which an embryo is rotated 90° along the *y* axis (I) or the *z* axis (K). In these situations, the progeny of each two-cell blastomere shows no predictable relationship to the lineages of the blastocyst or occupancy of specific domains. In real development, the angle between P2CB and the *yz* plane is often oriented between I and K; thus, the position of P2CB in blastocysts varies.

achieved by either the earlier-dividing two-cell blastomere dividing meridionally (M, plane of cell division parallel to that of the first cleavage), with the later division occurring equatorially (E, plane of cell division perpendicular or oblique to that of the first cleavage), in the so-called ME pattern, which is found in 42% of embryos. The other pattern of division may occur first equatorially then meridionally – the EM pattern – which is found in 39% of embryos. The later-dividing equatorial pair in the ME pattern is likely to contribute to the abembryonic region of the blastocyst in the intact embryo, whereas the earlier-dividing equatorial pair in the EM arrangement show no such bias (Piotrowska-Nitsche and Zernicka-Goetz, 2005). Embryos generated by the re-aggregation of the daughter cells that are located furthest away from the second polar body after the equatorial division of one of the two-cell blastomeres show reduced viability later in development (Piotrowska-Nitsche et al., 2005). This indicated that these so-called 'vegetal' blastomeres might have an inherently deficient potential. In the ME embryo, this 'vegetal' blastomere shows lower levels of arginine methylation of histone H3 (H3R26me) than do other blastomeres (Torres-Padilla et al., 2007a). The importance of this specific histone mark, which is associated with gene activation in lineage specification, is unclear. Ectopic expression of an arginine methyltransferase CARM1, which enhances H3R26me methylation, in a two-cell blastomere can bias the distribution of its progeny within the blastocyst, but it is not clear how this would affect cell lineage. Although expression of CARMI might have an impact on ICM fate (Torres-Padilla et al., 2007a), the endogenous level of histone methylation has yet to be correlated with lineage specification in a meaningful way. Although the vegetal blastomere of the EM embryo, like that of the ME embryo, is also less efficient in contributing to embryogenesis (Piotrowska-Nitsche et al., 2005), it displays H3R26me levels that are similar to the other three blastomeres, unlike its counterpart in the ME embryo, which is lower than its sister blastomeres (Torres-Padilla et al., 2007a). Overall, these findings suggest that this specific histone modification may not correlate consistently with cell fate or potency.

What of the possible importance of the inheritance of a specific region of the egg cytoplasm in driving trophectoderm fate, as proposed from the study of the subset of embryos that have undergone the ME pattern of cleavage up to the four-cell stage (Piotrowska-Nitsche et al., 2005)? It was proposed that the 'vegetal' blastomere of the ME embryo is biased to acquire an abembryonic TE fate (Piotrowska-Nitsche et al., 2005), although a recent study which attempted to mark the same cell, found no such bias (Alarcon and Marikawa, 2008). To support the case that, in this particular subset of embryos, this vegetal blastomere has a biased lineage fate, Jedrusik et al. (Jedrusik et al., 2008) have recently reported that its progeny shows an elevated expression of *Cdx2* at the eight-cell stage.

A priori, no pre-patterning of the egg or of the two-cell embryo is required to explain the formation of the cell lineages, the shape and the axes of the mouse blastocyst. A combination of ad hoc topological constraints and cell polarity and signaling processes that lead to the segregation of inner and outer cell populations can explain normal development. And yet, persistent clues indicate the possibility that asymmetries in the mammalian egg may have the potential to bias cell fate and morphogenesis under special conditions. Why should this be so? In many invertebrate and lower vertebrate species, it is clear that asymmetries in the distribution of cytoplasmic determinants in the egg play major roles in establishing early embryonic patterning. The early development of all of these species depends on maternally inherited factors, with zygotic transcription occurring as a later event. In mammals, by contrast, although maternal mRNAs and proteins are active in early development, maternal RNA is rapidly degraded and zygotic gene activation occurs during early cleavage and is required for blastocyst development. This switch away from the dependence on maternal inheritance was presumably accompanied by a move away from early patterning being driven by asymmetrically distributed maternal determinants to being driven by zygotic transcription. Nonetheless, the systems that allow asymmetries in the egg might persist as 'evolutionary relics' in mouse eggs, leading to the appearance of asymmetries in some embryos. These asymmetries may bias developmental pathways but can be readily overridden by processes of lineage development and blastocyst morphogenesis.

Peri-implantation asymmetry and axis specification

In addition to the embryonic-abembryonic axis and the ellipsoidal shape of the ICM (Fig. 1), other morphological features also reveal the asymmetry of the blastocyst. In the implanted blastocyst recovered from the mouse uterus, the ICM is often oriented in a tilted position so that the ICM has an upper and a lower side (Fig. 4A). Accompanying this tilted orientation of the ICM, the initial thickening of the polar trophectoderm also appears asymmetrical. A tilting of the ectoplacental cone away from the proximal-distal axis (Box 2) is seen in embryos at subsequent stages of post-implantation development (Fig. 4B-D). By the pre-primitive streak (E6.0) stage, the direction in which the cone tilts is aligned consistently with the orientation, but not with the polarity, of the prospective AP axis of the body, which coincides with the longer transverse diameter of the cylindrical embryo (Gardner et al., 1992) (Fig. 4D). Quite unexpectedly, the orientation of the AP axis does not always align with the longer diameter of the early embryo (Mesnard et al., 2004; Perea-Gomez et al., 2004). In the younger (E.5.5-5.75) embryo, the AP axis aligns initially with the shorter diameter (Fig. 4C), which lengthens as the embryo re-models its shape, such that the AP axis later becomes aligned with longer diameter (Fig. 4D). This re-shaping of the embryo, but not the specification of the AP axis, requires *Fgf8b* and *Wnt3* function in the epiblast (Barrow et al., 2007; Guo and Li, 2007). It would be interesting to find out whether the tilting of the ICM or the ectoplacental cone has any specific orientation with respect to the short or long transverse diameter of the cylindrical embryo during embryogenesis.

Overall, these intriguing findings beg the question of whether the long axis of the ICM, the angle of tilt of the ICM and the asymmetric position of the ectoplacental cone have any developmental relationship with each other and with any of the three primary body axes of the post-implantation embryo. The answer to this question requires lineages across the peri-implantation to gastrulation period to be traced directly. In earlier studies, single ICM cells at either end of the long axis of the ICM were marked by injection and their descendants followed in the visceral endoderm (see Glossary, Box 1) of post-implantation embryos (Weber et al., 1999). Intriguingly, clones were found to spread proximodistally in an oblique manner, suggesting that the horizontal axis of the ICM may be converted into the proximodistal axis (Box 2) of the post-implantation embryo. A similar tracking study, performed by marking cells presumably at random positions near the surface of the ICM, showed that clones span the extra-embryonic and embryonic regions of the visceral endoderm, and has revealed more diverse patterns of clonal distribution in both the proximal-distal and transverse dimensions of the cylindrical embryo (Perea-Gomez et al., 2007). It is imperative, in view of the now available tissue- and site-specific molecular markers of embryonic asymmetry in pre-gastrulation

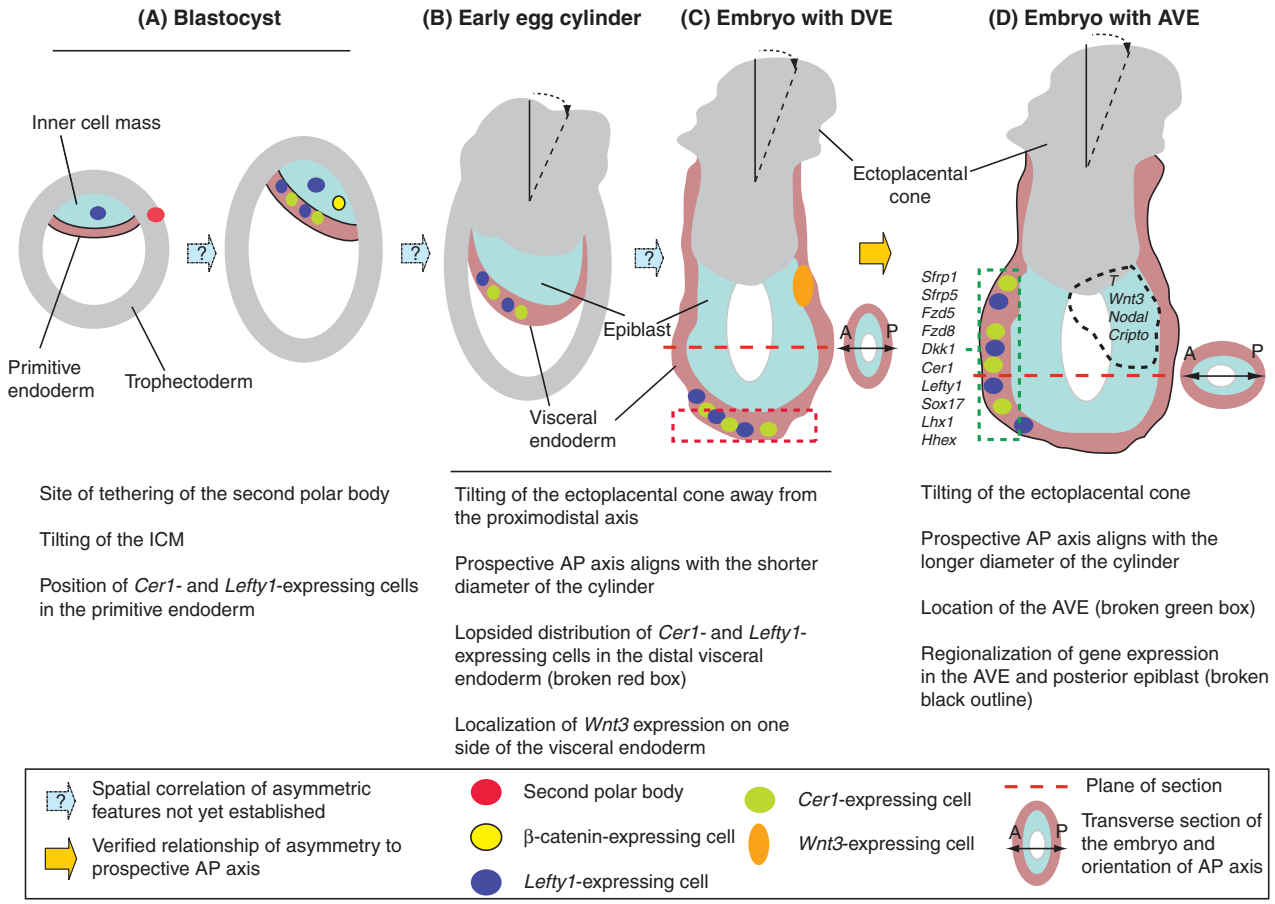

Fig. 4. The emergence of asymmetry during peri-implantation development from blastocyst to immediately before gastrulation. (**A-D**) Early pre-gastrulation stages of mouse development, with asymmetric features listed for each stage. The tilting of the ectoplacental cone from the proximal-distal axis (unbroken line) is indicated by the black broken line. Cells in the inner cells mass (ICM), the primitive endoderm and the visceral endoderm that express β-catenin, *Lefty1*, *Cer1* or *Wnt3* are color coded. Many genes that are expressed in the anterior visceral endoderm (AVE; D broken green rectangle) are also expressed previously in the distal visceral endoderm (DVE). Four examples of genes that are expressed in the posterior epiblast (D, broken black outline) are listed. In C (embryo with DVE) and D (embryo with AVE), a transverse section of the embryo is shown to illustrate the alignment of the prospective anterior-posterior (AP) axis first with the shorter and then with the longer diameter at the respective stage.

mouse embryos (Fig. 4D), to re-examine the distribution of these ICM-derived clones in the visceral endoderm to see whether there is any consistent spatial relationship between the site of origin of their precursors in the ICM and their contribution to the prospective AP body axis. It would be particularly informative to track the distribution of these cell clones throughout development from blastocyst to gastrula when it becomes possible to grow the peri-implantation embryo successfully in vitro.

Visceral endoderm: tissue patterning and emerging asymmetries

The primitive endoderm, which is formed as an epithelium initially on the luminal surface of the ICM (Fig. 1G), expands during peri-implantation development to form the parietal endoderm (which lines the luminal surface of the mural trophectoderm) and the visceral endoderm (which envelops the extra-embryonic ectoderm and the epiblast) (Fig. 1H; Fig. 4B-D). The visceral endoderm is a tissue of significant interest because of its crucial function in mediating the activity of transforming growth factor β (TGFβ) (bone morphogenetic protein and Nodal) and WNT signaling pathways, which sustain the differentiation and patterning of the epiblast (Tam et al., 2006; Tam and Loebel, 2007). Furthermore, the changes in the epithelial architecture of the endoderm, the regionalized gene expression domains (Kemp et al., 2005; Kemp et al., 2007; Kimura-Yoshida et al., 2007; Yamamoto et al., 2004; Pfister et al., 2007) and the pattern of morphogenetic movement of cells reflect a dynamic process during which structural and molecular asymmetries are translated into the AP patterning of the body axis (reviewed by Lu et al., 2001; Zernicka-Goetz, 2002; Srinivas, 2006; Tam and Loebel, 2007). Contrary to the idea that the visceral endoderm is entirely restricted to extra-embryonic fates, a small number of its descendants do contribute to the endoderm of the embryonic gut (Kwon et al., 2008).

Local visceral endoderm thickening marks emerging asymmetry

Changes in epithelial morphology, revealed as a local thickening of the visceral endoderm, first in the distal region and then later on one side of the pre-gastrulation embryo (Fig. 4C,D) (Kimura-Yoshida et al., 2005; Rivera-Perez et al., 2003; Yamamoto et al., 2004), are telltale signs of the acquisition of asymmetry in the proximodistal axis and the prospective AP body axis, respectively. By tracking the position of the thickened population of embryonic visceral endoderm cells, in conjunction with gene expression patterns, a

picture has emerged in which the visceral endoderm cells in the distal region of the E5.0-5.25 embryo (also called the distal visceral endoderm, DVE) contribute to the visceral endoderm cells that localize to one side of the E5.5-E6.0 embryo. The cells of the DVE then later come to reside in the prospective anterior region of the embryo, where they become known as the anterior visceral endoderm (AVE) of the early primitive streak-stage (E6.5) embryo (Rivera-Perez et al., 2003; Thomas and Beddington, 1996; Torres-Padilla et al., 2007b; Srinivas et al., 2004). At gastrulation, the primitive streak forms on the side of the embryo opposite to the AVE, thus identifying the AVE as a reliable landmark of the anterior pole of the body axis. The asymmetric localization of the visceral endoderm to one side of the embryo therefore demarcates the polarity and the orientation of the prospective AP axis (Torres-Padilla et al., 2007b). Although it has been shown that visceral endoderm are descendants of the ICM and the primitive endoderm of the blastocyst (Weber et al., 1999; Chazaud et al., 2006; Perea-Gomez et al., 2007), it remains unknown whether they derive from specific progenitor cells that are set aside early in the ICM or form de novo by an inductive/inhibitory activity of the epiblast and the extra-embryonic ectoderm.

The AVE arises from multiple progenitors

Expression profiling studies have revealed that some ICM cells express a unique set of endoderm-related genes that are characteristic of primitive endoderm (such as *Gata4*, *Gata6*, *Lrp2*, *Pdgfra*) or unique to the AVE (such as *Cer1*, *Hhex* and *Lefty1*) (Gerbe et al., 2008; Torres-Padilla et al., 2007b; Chazaud et al., 2006; Kurimoto et al., 2006; Thomas et al., 1998; Torres-Padilla et al., 2007b; Yamamoto et al., 2004; Plusa et al., 2008). Although *Hhex-GFP*-, *Lefty1-lacZ*- and *Cer1-GFP*-expressing cells are present successively in the ICM, primitive endoderm, DVE and AVE during development, it is not known whether the lineages of these cells are related. Clonal descendants of single ICM cells contribute only a fraction of the *Cer1-GFP*-expressing population that is found in the visceral endoderm, suggesting that the AVE is likely to be of polyclonal origin (Torres-Padilla et al., 2007b).

Signaling pathways establishing the DVE/AVE

The signaling activity of the Nodal-related ligands of the TGFβ superfamily is vital for embryonic patterning, especially in the AP and the left-right body axes, and for the regulation of the potency and mesendoderm differentiation of the epiblast. Nodal signaling is mediated by serine threonine kinase receptors and epidermal growth factor-CFC (crypto, FRL1, cryptic) co-receptor (crypto and cryptic), and is transduced by intracellular Smad2/3/4 pathways in conjunction with Foxh1 to activate target genes. Nodal signaling is modulated by the antagonistic activity of Lefty proteins and interacts with growth differentiation factors (GDFs) (reviewed by Shen, 2007). Mutant studies have shown that Nodal signaling and Eomes function are involved in AVE formation (Fig. 5A) (Brennan et al., 2001; Norris et al., 2002; Ding et al., 1998; Chen et al., 2006; Levine and Brivanlou, 2006; Arnold et al., 2008). Nodal induction of the AVE is mediated through the *Bmp4-Wnt3-Nodal* signaling cascade, which involves feedback activity between the extra-embryonic ectoderm and the epiblast (Fig. 5A) (Ben-Haim et al., 2006; Liu et al., 1999).

The formation of the DVE, marked by the expression of *Cer1-GFP* or *Hhex-GFP* in the visceral endoderm, is subject to a putative inhibitory activity from the extra-embryonic ectoderm. Explants of epiblast and embryonic visceral endoderm cultured without the extra-embryonic ectoderm show an expanded domain of *Cer1-GFP*

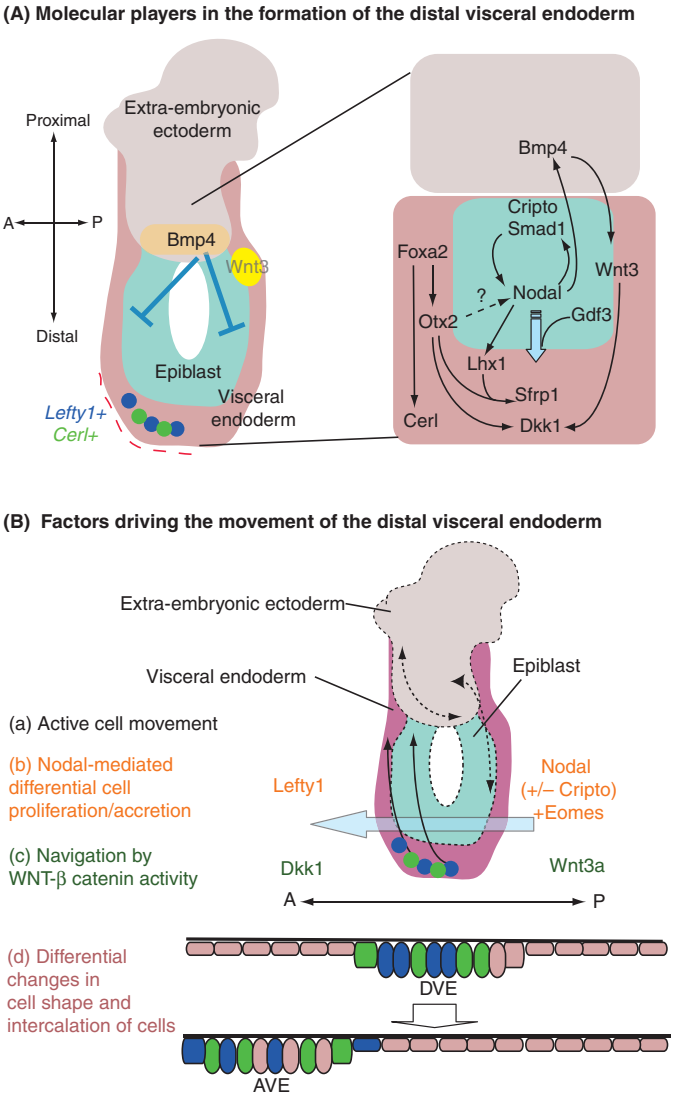

Fig. 5. The formation and movement of the distal visceral endoderm. (**A**) Schematic of a mouse embryo with discernible thickening of the distal part of the visceral endoderm (left), showing the domains of expression of the molecules involved in the formation of the distal visceral endoderm (DVE, marked by the red broken line). (Right) The cascade of molecular activity, which involves Nodal, bone morphogenetic protein (BMP) and WNT signaling in DVE formation and epiblast patterning. Arrows and bar-pointers between genes or molecules indicate positive and negative functional connections in the cascade, respectively, and not necessarily a regulatory relationship. (**B**) Factors that drive the anterior displacement of the DVE to form the anterior visceral endoderm (AVE). (a) DVE cells may move to the anterior region by active locomotion: unbroken arrows indicate the direction of movement of the DVE, and the broken double-headed arrows show the distribution of inner cell mass-derived clones in the visceral endoderm. DVE movement can also be driven by morphogenetic forces generated by: (b) differential rates of cell proliferation under the influence of Nodal signaling; and (c) graded levels of WNT signaling activity that elicit a chemotactic response. (d) A potential role of localized changes in cell shape and cell intercalation in the displacement of cells in the visceral endoderm, without an increase in cell number or the long-range movement of individual cells within an epithelium. Cell shape changes might be subject to planar signaling activity and mediated by molecular mechanisms that control cytoarchitecture. *Lefty1*- and *Cer1*-expressing cells in the visceral endoderm are colored in dark blue and green, respectively.

and *Hhex-GFP* expression. This is in contrast with explants that are recombined in culture with the extra-embryonic ectoderm, in which GFP expression is restricted to the region furthest away from the extra-embryonic tissue (Rodriguez et al., 2005; Richardson et al., 2006). The inhibitory activity appears to come from the prospective posterior part of extra-embryonic ectoderm, which is on the same side as the *Wnt3*-expressing visceral endoderm (Rivera-Perez and Magnuson, 2005) but opposite to the lopsided *Cer1* and *Lefty1* expression domain in the DVE (Yamamoto et al., 2004) (Fig. 4C; Fig. 5A). The ablation of the posterior extra-embryonic ectoderm from the embryo leads to an expansion of the *Cer1-GFP* expression to the posterior visceral endoderm (Richardson et al., 2006). *Cer1*-GFP expression becomes localized to the visceral endoderm, when the embryonic fragment (epiblast + visceral endoderm) is co-cultured with the posterior extra-embryonic ectoderm, but not with anterior extra-embryonic ectoderm (Richardson et al., 2006). The inhibitory activity of the extra-embryonic ectoderm is diminished by the knockdown of *Bmp4* (Soares et al., 2008), indicating that BMP signaling and/or downstream gene activity have a role in this inhibition, which underpins the downregulation of *Cer1-GFP* in the visceral endoderm outside of the DVE and AVE (Torres-Padilla et al., 2007b). The inhibitory activity of the extra-embryonic ectoderm is expected to diminish with the increase in distance between it and the DVE. Thus, a likely cause of failed AVE formation in *Nodal* mutants is a lack of sufficient epiblast growth that enables the DVE to stay outside of the range of this inhibition (Mesnard et al., 2006). This inhibitory function of the extra-embryonic ectoderm could be related to WNT signaling, because embryos with a gain of WNT function mutation of the adenomatous polyposis coli (*Apc*) gene fail to form the AVE (Chazaud and Rossant, 2006).

Cellular and molecular mechanisms of DVE translocation

Results of embryological studies and mutant analysis have pointed to several mechanisms that might act synergistically to translocate the DVE to the AVE (Fig. 5B). A time-course study that tracked the movement of *Hhex-GFP* expressing visceral endoderm cells has provided compelling evidence that DVE cells are actively moving (Srinivas et al., 2004) (Fig. 5Ba). The visceral endoderm cells adopt the morphological features of migratory cells and display a concerted pattern of locomotion towards the region of the prospective AVE. The displacement of DVE cells could also be driven by regional differences in the rate of accretion of cells (Fig. 5Bb). The proliferation and accumulation of cells in a local region of the epithelium with a high level of Nodal signaling (Yamamoto et al., 2004) may generate the propulsive force required to displace cells in the adjacent region to other parts of the epithelium. It has been estimated that, within 10-15 hours, the number of *Cer1-GFP*-expressing visceral endoderm cells nearly doubles. This is thought not to be brought about solely by cell multiplication but also by de novo activation of *Cer1* (Torres-Padilla et al., 2007b). Whether an overall doubling of the cell population in a localized region of the embryo is sufficient to drive the rapid displacement of cells from the distal to the anterior visceral endoderm is, however, questionable. Experimentally, cells in the visceral endoderm may be directed to move from regions of high (where there is elevated cell proliferation) to low Nodal activity (Fig. 5B) (Yamamoto et al., 2004) and, consistent with Nodal having such a role, DVE cells do not move when Cripto activity is lost (Ding et al., 1998). Interestingly, restoring an effective level of Nodal activity, by raising the Cripto activity partially above the null level (as in *Cripto*-hypomorphic mutants) or by reducing the activity of Nodal antagonist in embryos that totally lack Cripto (i.e. *Cer1*$^{+/-}$*;Cripto*-null), allows the formation and translocation of the DVE to become the AVE (Liguori et al., 2008; D'Andrea et al., 2008). AVE formation may therefore be accomplished, at least in part, by a Cripto-independent Nodal pathway that is sensitive to Cer1 inhibition.

WNT signaling activity is also implicated in driving the translocation of the visceral endoderm (Fig. 5Bc), although the molecular details remain vague. The DVE is displaced away from region of high WNT activity towards a region where WNT signaling is lowered by dickkopf 1 (DKK1, a secreted factor that blocks the function of the WNT co-receptor LRP6) (Kimura-Yoshida et al., 2005). In *Otx2* mutants, DVE translocation is impaired, but can be rescued by the expression of *Dkk1* from the *Otx2* locus or by the lowering of WNT signaling through a reduction of β-catenin gene dose (Kimura-Yoshida et al., 2005). High up in this genetic cascade of the WNT-mediated morphogenetic activity is likely to be *Foxa2*, which, in addition to being essential for AVE formation, regulates genes such as *Otx2* and those of WNT and Nodal/BMP antagonists (*Dkk1*, *Cer1*) in the visceral endoderm (Kimura-Yoshida et al., 2007). In the *Foxa2*-null embryo, β-catenin expression is elevated in the visceral endoderm, as it is in the *Otx2*-null embryo, suggesting that excess WNT activity has an impact on the translocation of the visceral endoderm. It has also been noted that, during DVE translocation, visceral endoderm cells in the distal region flatten, whereas those in the anterior region acquire a tall columnar morphology (Srinivas, 2006). In this regard, localized changes in cell shape and packing density in the visceral endoderm in response to planar signaling activity may also lead to an apparent translocation of the DVE (Fig. 5Bd).

Translocation of the DVE to the anterior region therefore involves active cell migration (Srinivas et al., 2004), which is brought about by concerted changes in cell shape and local neighbor relationship (Srinivas, 2006; Rakman and Anderson, 2006), and by the displacement of cells via extrinsic morphogenetic forces (Yamamoto et al., 2004; Kimura-Yoshida et al., 2005).

A pre-set landscape for anterior-posterior axis specification?

With respect to the prevailing notion that the direction of DVE displacement delineates the orientation of the AP body axis, it remains unresolved whether this is determined by a stochastic mechanism or by pre-set molecular or environmental parameters that specify this body axis. The consensus is that at an early stage, when *Lefty1* and *Cer1* expression is detected in the DVE, the domain of both genes is already shifted to one side (Fig. 4C). This asymmetry is believed to herald the direction of visceral endoderm translocation (Yamamoto et al., 2004; Torres-Padilla et al., 2007b). *Wnt3* expression is localized to the posterior visceral endoderm even before the asymmetric *Lefty1* and *Cer1* expression pattern is observed in the DVE (Rivera-Perez and Magnuson, 2005). These observations imply that there has been an even earlier developmental process that pre-sets this asymmetry for AP axis specification.

In the blastocyst and peri-implantation embryo, *Lefty1-lacZ* expressing cells localize asymmetrically: on one side of the blastocyst ICM, in the primitive endoderm on the upper side of the tilted ICM and on one side of the egg cylinder, all prior to their asymmetric expression in the DVE (Takaoka et al., 2006) (Fig. 4A,B). An asymmetric distribution of β-catenin-expressing cells in the ICM is also observed (Fig. 4A) (Chazaud and Rossant, 2006). It is not known how this asymmetric localization of *Lefty1*- or β-

catenin-positive cells is related to the long axis of the ICM or to the initial tilting of the ICM prior to the formation of the primitive endoderm. *Cer1*-expressing cells (as visualized by GFP reporter, mRNA and protein expression) in the primitive endoderm do not localize to any specific regions (Perea-Gomez et al., 2007). However, *Cer1* activity, as revealed by GFP fluorescence, appears to be uneven in the primitive endoderm and tends to be stronger in the visceral endoderm on one side of the tilted ICM (Fig. 4A). Potentially, the regionalization of the *Lefty1*- and *Cer1*-active cells in the visceral endoderm prior to the formation and movement of the DVE might be a manifestation of an underlying asymmetric pattern that foreshadows the orientation and polarity of the AP body axis. As the current data are primarily correlative, such inference is at best conjectural. In blastocysts cultured in vitro over the period of implantation, *Cer1-GFP* and *Lefty1-lacZ* positive cells localize unevenly in the primitive endoderm. If this pattern reflects the acquisition of embryonic asymmetry and the specification of the body axis, such developmental capacity would have to be inherent to the embryo and not acquired by the act of implantation.

Conclusions

Embryogenesis requires the generation of diverse cell types and the orderly assembly of these cells into an organized body plan. During this process, asymmetries of anatomical and/or molecular characteristics emerge within cells, tissues and the whole embryo. Some of these asymmetries are relevant to cell fate, such as the radial asymmetry that differentiates outer and inner cells of the morula. Some of them drive cell rearrangements, such as the morphogenetic processes that accompany the formation of the anterior visceral endoderm by the anterior migration of the distal visceral endoderm. Others may be incidental partners in influencing axis formation, such as the non-spherical shape of the zona pellucida. The challenge ahead is to determine whether developmentally relevant asymmetries influence lineage allocation and to translate our knowledge of morphological asymmetries into molecular mechanisms.

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