Cell fate decisions and axis determination in the early mouse embryo

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Summary

The mouse embryo generates multiple cell lineages, as well as its future body axes in the early phase of its development. The early cell fate decisions lead to the generation of three lineages in the pre-implantation embryo: the epiblast, the primitive endoderm and the trophectoderm. Shortly after implantation, the anterior-posterior axis is firmly established. Recent studies have provided a better understanding of how the earliest cell fate decisions are regulated in the pre-implantation embryo, and how and when the body axes are established in the pregastrulation embryo. In this review, we address the timing of the first cell fate decisions and of the establishment of embryonic polarity, and we ask how far back one can trace their origins.

Key words: AP axis, Blastocyst, Cell fate, Trophectoderm, Inner cell mass, Primitive endoderm

Introduction

The early development of the mouse embryo, from a fertilized egg to a gastrulating embryo, involves a tightly regulated series of lineage specification events and the simultaneous establishment of the embryonic axes (Fig. 1). These events include the formation of a blastocyst, following compaction (see Glossary, Box 1) at embryonic day (E) 3.5, when cells then go on to form either the trophectoderm (TE, see Glossary, Box 1) or inner cell mass (ICM), from which the epiblast (Epi, see Glossary, Box 1) or primitive endoderm (PrE, see Glossary, Box 1) are subsequently derived.

Given that the mouse embryo begins life as one cell, the fertilized egg must perform at least three tasks during the early phase of development. It must: (1) increase the number of cells by proliferation; (2) increase the number of cell types by differentiation; and (3) generate polarity to allow the establishment of the future body axis. How can an embryo generate multiple types of cells and polarities when it starts out as a single cell? Where do the initial signals for the induction of cell differentiation and for the generation of asymmetries come from?

Diverse mechanisms to establish early polarity are known to operate in various organisms (Kloc and Etkin, 2005; Schneider and Bowerman, 2003; St Johnston, 2005), but let us think of two extreme scenarios (Fig. 2). In one scenario, there is a determinant in a specific region of the oocyte, and this determinant becomes asymmetrically distributed after fertilization. This determinant can thus be inherited asymmetrically as the fertilized egg divides. A blastomere that receives this determinant will adopt a cell fate that is different from a blastomere that does not receive the determinant,

resulting in the generation of two cell types or of a polarity within the embryo. Bicoid in *Drosophila* (Huynh and St Johnston, 2004) and Macho-1 in ascidians (Nishida and Sawada, 2001) are examples of such determinants. In the second scenario, the embryo does not have such a localized maternal determinant, so it must make use of some other signal(s) to generate polarity and different cell types.

The current view is that the mouse embryo probably conforms to the second scenario. Fertilized mouse embryos inherit maternal RNA and proteins. Some of these maternal factors persist until the blastocyst stage, while zygotic gene expression begins at the two-cell stage (Carter et al., 2003; Wang et al., 2004). Although early development of the mouse embryo absolutely requires maternal transcripts and proteins (Li et al., 2010), there is no evidence that maternal determinants are localized. As such, the mouse embryo develops from fertilization to gastrulation through a series of cell type specifications and body axis determinants, which most probably occur in the absence of an asymmetrically localized maternal determinant.

Here, we review the mechanisms used to determine cell fate and the timing of these cell fate decisions in the early mouse embryo. We also review these cell fate decisions in relation to the establishment of the embryonic axes. As it is premature to conclude that maternal determinants are absent in mammals, we also discuss how far back we can trace the onset of cell specification and embryonic polarity in the mouse embryo.

The first cell fate decision: inner cell mass or trophectoderm

Generation and translation of inside-outside polarity

The fertilized mouse egg reaches the eight-cell stage after three rounds of cell division. The embryo then undergoes an event known as compaction in which an increase in cell-cell contact promotes cell adhesion (Fig. 1). Compaction generates apical-basal polarity in each cell of the eight-cell embryo. Proteins related to the establishment of cellular polarity, such as Ezrin, protein kinase C (PKC), Par3 (partitioning defective; Pard3 – Mouse Genome Informatics), Par6 (partitioning defective 6; Pard6a – Mouse Genome Informatics) and atypical PKC (aPKC) localize asymmetrically to the apical side of the compacted morula (Pauken and Capco, 2000; Plusa et al., 2005; Vinot et al., 2005). By contrast, proteins such as Par1 (partitioning defective 1b; Mark2 – Mouse Genome Informatics) and E-cadherin (Hyafil et al., 1980; Shirayoshi et al., 1983; Vinot et al., 2005) localize to the basolateral side of the cells. Thus, by the early morula stage, at E2.5, each cell of the compacted embryo possesses distinct apical-basal polarity.

Following this, the cells of the morula undergo further cleavages to generate the blastocyst. Given that the orientation of the mitotic spindle is random during the fourth division, both symmetric and asymmetric divisions can occur, depending on the orientation of the spindle with respect to the apical-basal polarity (Johnson and Ziomek, 1981). Two rounds or 'waves' of asymmetric cell division

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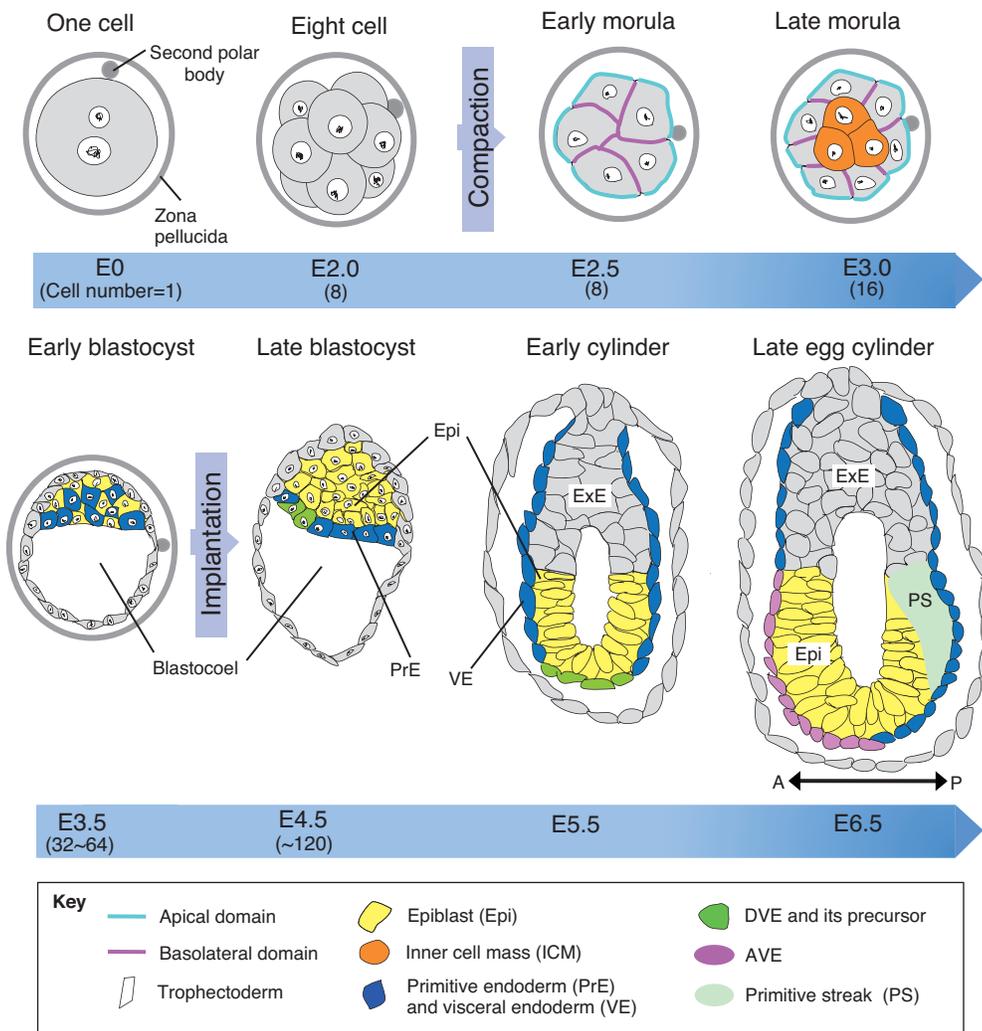
Fig. 1. Mouse embryo development from fertilization to gastrulation. The morphological changes and the cell specification events that take place from fertilization to embryonic day (E) 3.0 (top), and from E3.5 to gastrulation at E6.5 (bottom). The cell types in the embryos are color coded. AVE, anterior visceral endoderm; DVE, distal visceral endoderm; Epi, epiblast; Exe, extra-embryonic ectoderm; PrE primitive ectoderm; PS, primitive streak; VE, visceral endoderm.

underlie the development of the embryo from the eight-cell to the 16-cell stage and from the 16-cell to the 32-cell stage, and these result in the generation of outside cells and inside cells (Tarkowski and Wroblewska, 1967). This inside-outside polarity leads to differential Hippo signaling that is dependent on cell position within the embryo (Nishioka et al., 2009). Hippo signaling is a signaling pathway that involves a serine/threonine (Ser/Thr) kinase called Hippo in *Drosophila* and Stk3 (Ser/Thr kinase 3)/Mst in the mouse. It controls cell contact-mediated inhibition of proliferation (Pan, 2007; Saucedo and Edgar, 2007) and is activated only in the inside cells that are surrounded entirely by other cells, not in outer cells (Fig. 3A). In inner cells, Hippo activation results in the phosphorylation of the transcriptional co-activator Yes-associated protein (Yap1) and thereby prevents its translocation to the nucleus. In the absence of nuclear Yap1, its partner, the TEA domain family transcription factor 4 (Tead4), is unable to activate target genes. In the outside cells, however, Hippo signaling is downregulated because their apical side is not in contact with other cells, and an unknown apical signal may also repress the Hippo pathway. Yap1 thus remains unphosphorylated in these cells, and it therefore enters the nucleus, binds to Tead4 and activates TE-specific genes such as caudal-related homeobox 2 (*Cdx2*). Mutant embryos lacking Tead4 no longer manifest *Cdx2* expression and are unable to generate TE (Nishioka et al., 2008; Yagi et al., 2007).

ICM and TE establishment by a transcription factor network

By E3.5, two types of cells can be found in the blastocyst: TE cells and ICM cells. *Cdx2* is a functional marker for TE, whereas the pluripotency-associated transcription factors Oct3/4 (POU domain, class 5, transcription factor 1, Pou5f1 – Mouse Genome Informatics) and Nanog are functional markers for the ICM. These transcription factors show dynamic expression patterns between the morula and blastocyst stages (Dietrich and Hiiragi, 2007; Niwa et al., 2005). Of these genes, *Cdx2* is the first to show a restricted pattern of gene expression. It is preferentially expressed in the outside cells at the early blastocyst stage (at around E3.0, between the 16- and 32-cell stages), whereas *Oct3/4* is expressed uniformly at this stage (Niwa et al., 2005). The level of *Nanog* expression varies among blastomeres, but this variation is independent of blastomere position within the embryo (Dietrich and Hiiragi, 2007). There is therefore initially no apparent reciprocal relationship between *Cdx2* and *Oct3/4* expression or between *Cdx2* and *Nanog* expression. As development proceeds, however, *Cdx2* expression becomes restricted to the outside future TE cells, whereas the expression of *Oct3/4* and *Nanog* gradually becomes confined to the inside future ICM cells. This reciprocal expression pattern of *Cdx2* and *Oct3/4* is achieved by the operation of double negative-feedback loops that are mediated by the transcription factors they

> **Box 1. Glossary**
> **Compaction.** When blastomeres change from a loose arrangement to a compacted arrangement, which maximizes their intercellular contact, shortly after the eight-cell stage.
> **Egg cylinder.** A stage of mouse development characterized by a cylinder-like structure with the amniotic cavity inside.
> **Epiblast.** A group of epithelial cells that are derived from the ICM and that give rise to the three germ layers (ectoderm, mesoderm and definitive endoderm) during gastrulation.
> **Primitive endoderm.** The epithelial layer of cells that occupies the surface of the blastocoel. The primitive endoderm will contribute to the visceral endoderm and parietal endoderm of the post-implantation embryo.
> **Trophectoderm.** The outer epithelial layer of the blastocyst, consisting of polar and mural components that line the ICM and blastocoel, respectively.
> **Visceral endoderm.** The epiblast-derived epithelial layer of cells that covers the extra-embryonic ectoderm and epiblast of the post-implantation embryo. Most VE becomes the endoderm of the extra-embryonic yolk sac but a subpopulation of VE contributes to the endoderm of the embryo proper at later stages.

encode (Niwa et al., 2005) (Fig. 3B). In fact, a zygotic *Cdx2* mutant is able to form embryos containing cells that resemble TE cells morphologically but that ectopically express *Oct3/4* and *Nanog* and are negative for TE markers (Strumpf et al., 2005). Conversely, downregulation of *Oct3/4* expression or the forced expression of *Cdx2* in embryonic stem (ES) cells results in their differentiation into the TE lineage (Niwa et al., 2005). In addition, the transcription factor Gata3 (GATA-binding protein 3) promotes differentiation into the TE lineage downstream of Tead4 and in parallel to Cdx2 (Fig. 3B) (Ralston et al., 2010).

Onset of ICM versus TE fate

As mentioned above, the first cell fate decision has been thought to be based on the differential positioning of cells on the inside or outside of the embryo around the 16-cell stage (Johnson and McConnell, 2004; Tarkowski and Wroblewska, 1967). If this is the case, the initial signal that induces the first cell differentiation event would arise from the inside-outside (apical-basal) information that is generated by compaction, which begins at the eight-cell stage. However, recent studies have detected molecular differences among blastomeres as early as the four-cell stage, which indicates that the onset of the first cell fate decision may occur earlier.

The earliest molecular differences between blastomeres that have been described to date relate to histone modifications. At E3.5, the cells of the ICM and TE exhibit differences in various types of histone modifications (Erhardt et al., 2003; Reik et al., 2003). For example, the levels of H4K8ac (acetylation of histone H4 at lysine 8) and H3K4me3 (trimethylation of histone H3 at lysine 4), both of which are epigenetic marks associated with active chromatin, in the promoter region of *Oct3/4* are higher in cells of the ICM than in those of the TE (VerMilyea et al., 2009). However, the extent of H3K9me2 (dimethylation of histone H3 at lysine 9), which is a repressive epigenetic mark, in the promoter regions of *Oct3/4* and *Nanog* is lower in ICM cells than in TE cells. The promoter region of *Cdx2* shows a reverse pattern, with the levels of H4K8ac and H3K4me3 being higher in TE than in ICM cells (VerMilyea et al., 2009). Unexpectedly, the global levels of H3R17me and H3R26me (monomethylation of histone H3 at arginines 17 and 26, respectively) were found to differ among blastomeres of four-cell stage embryos that have undergone tetrahedral division patterns

(Torres-Padilla et al., 2007a). Those cells showing higher levels of H3R17me and H3R26me are biased to become ICM, whereas those with lower levels are preferentially fated to become TE. Furthermore, injection of an expression vector for Carm1 (an H3-specific arginine methyltransferase that is responsible for the generation of H3R17me and H3R26me) into one blastomere of a two-cell embryo resulted in the upregulation of *Nanog* and *Sox2* expression in the injected cell and its preferential contribution to the ICM (Parfitt and Zernicka-Goetz, 2010). Although *Carm1* mutant mice develop normally until late embryonic stages, it is possible that Carm1 may function as a maternal factor. There may be epigenetic differences between early blastomeres, but a recent transcriptome analysis has failed to detect asymmetry between blastomeres at two-cell and three-cell stages (VerMilyea et al., 2011).

Another type of molecular difference between blastomeres that was described more recently concerns Oct3/4 (Plachta et al., 2011). This study examined the intracellular behavior of Oct3/4 by monitoring the kinetics of nuclear export, import and retention of a green fluorescent protein (GFP)-tagged form of Oct3/4 in embryos at the four- and eight-cell stage. Two types of Oct3/4 behavior were observed, probably reflecting differences in the accessibility of this transcription factor to its binding sites in the genome. One type of behavior was characterized by low rates of nuclear import and export, as well as by the immobility of Oct3/4 (presumably because, in this situation, Oct3/4 is tightly bound to its target sites). Cells that manifested such behavior of Oct3/4 underwent asymmetric cell division, generating inside cells and outside cells, and preferentially contributed to the ICM. The other type of behavior was characterized by high Oct3/4 mobility (presumably Oct3/4 is not bound to its target sites in these cells), and cells manifesting this pattern of behavior underwent symmetric cell division, generating only outside cells, and showed a biased contribution to TE.
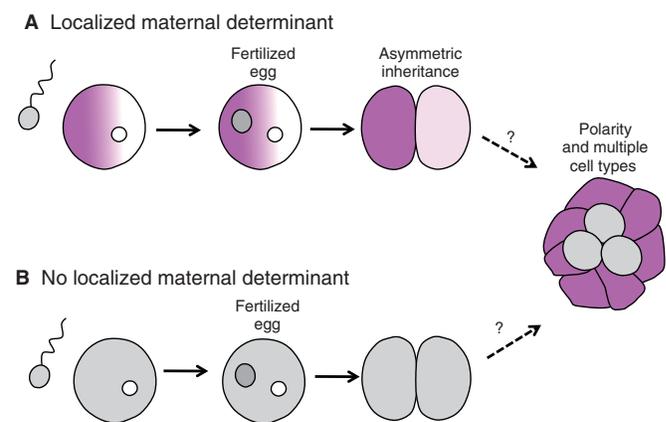

Fig. 2. Two possible mechanisms for the generation of multiple cell types and polarity. (**A**) Localized maternal determinant. In this model, a maternal determinant (pink) is unevenly distributed in the oocyte, and hence in the fertilized egg, and becomes inherited asymmetrically by daughter cells following cell division. The blastomere that receives this determinant can therefore adopt a cell fate that is different from a blastomere that does not receive the determinant. This can allow the generation of two cell types, as well as polarity, within the developing embryo. (**B**) No localized maternal determinant. In the absence of such an asymmetrically localized maternal determinant, alternatives mechanisms must exist to allow the generation of multiples cell types and polarity within the embryo.

A Hippo signaling mediates cell differentiation
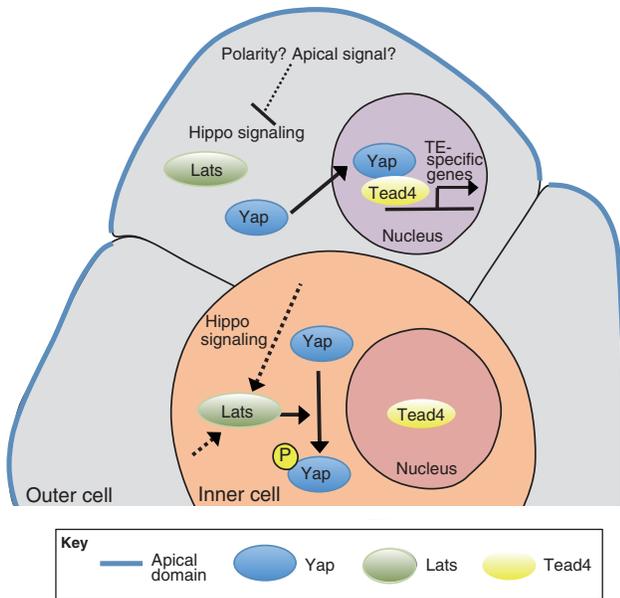
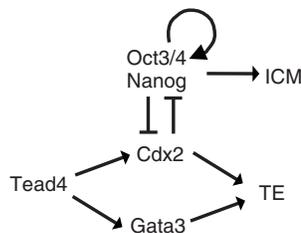**B** Transcriptional network regulating ICM and TE specification

Fig. 3. Cell position-dependent differentiation mediated by Hippo signaling. (**A**) An E3.0 mouse embryo showing inner (orange) and outer (gray) cells. In inside cells (orange), activated Hippo signaling results in the phosphorylation of Yap1 (Yes-associated protein), which prevents Tead4 (TEA domain family transcription factor 4) from functioning in the nucleus. In outside cells (gray), Hippo signaling is suppressed; unphosphorylated Yap1 thus translocates to the nucleus, where it interacts with Tead4 and regulates TE-specific target genes, such as *Cdx2* (caudal type homeobox 2). (**B**) The transcriptional network regulating ICM versus TE specification. Tead4 upregulates the expression of Cdx2 and Gata3 (GATA-binding protein 3), which together act to specify TE. A double negative-feedback loop between Cdx2 and Oct3/4-Nanog ensures that Cdx2 expression is restricted to TE cells, while the expression of Oct3/4 and Nanog is progressively confined to cells of the ICM. ICM, inner cell mass; Oct3/4 (Pou5f1), POU domain, class 5, transcription factor 1; TE, trophectoderm.

It will be interesting to examine the relationship between these two molecular differences: that in H3R17me and H3R26me at the four-cell stage; and that in Oct3/4 behavior at the four- and eight-cell stages (Fig. 4). If a direct link exists, a group of cells with higher levels of H3R17me and H3R26me would correspond to those with immobile Oct3/4. Higher levels of H3R17me and H3R26me in the genome might increase the accessibility of Oct3/4 to its targets sites, which would result in activation of its target genes. In which case, how and when is this differential histone modification generated? Why does a blastomere with low Oct3/4 kinetics preferentially undergo asymmetric division? Many questions about these findings remain to be addressed.

The second fate decision: epiblast or primitive endoderm

By E4.5, overt segregation of primitive endoderm (PrE) and the epiblast has occurred in the ICM, with the two types of cells showing clear segregation in terms of their positions and molecular markers (Fig. 1). On the one hand, the PrE comprises a layer of cells on the surface of the ICM, forming the cells that face the blastocoel cavity, and are positive for specific markers, such Gata6 and Gata4 (members of the GATA family of transcription factors). On the other hand, the epiblast, which is positive for Nanog and Oct3/4, is located inside the ICM. However, the cell fate decision of PrE versus epiblast occurs earlier than E4.5. At E3.5, two types of cells, one type positive for Nanog and the other positive for Gata6, are present within the ICM in a 'salt-and-pepper' pattern, suggesting that most ICM cells have already determined their fate as either PrE or epiblast (Chazaud et al., 2006) (Fig. 5A). Live imaging has confirmed that most of the cells positive for platelet-derived growth factor receptor α (PDGFRα), a marker for PrE, are randomly positioned in the ICM at E3.5 and will move to the surface of the ICM that faces the blastocoel cavity and eventually become PrE (Plusa et al., 2008). This scenario is further supported by the identification of low-density lipoprotein receptor-related protein 2 (Lrp2) as a marker for PrE progenitor cells and its dynamic expression pattern in the blastocyst (Gerbe et al., 2008). A minority of blastomeres in the ICM at E3.5 may remain naïve, however. Thus, some of the PDGFRα-positive cells do not express Gata6 and Gata4 (Plusa et al., 2008). Likewise, cells that are positive for the Nodal antagonist Lefty1 (left-right determination factor) at E3.5 will contribute to the epiblast, but about 20% of such cells are positive for Gata6 (instead of Nanog) at this stage (Takaoka et al., 2011). The analysis of gene expression in single cells (Guo et al., 2010) has revealed that progressive changes in molecular signature occur during blastocyst formation. *Nanog* and *Gata6* are thus co-expressed at the morula stage (eight cells) and at the early blastocyst stage (16 cells), they then undergo gradual upregulation or downregulation around the 32-cell stage, and are finally expressed in a mutually exclusive manner at the late blastocyst stage (64 cells).

The segregation of the epiblast and PrE lineages in the ICM is known to be regulated by fibroblast growth factor (FGF) signaling (Lanner and Rossant, 2010) (Fig. 5B). Fgf4, FGF receptor 2 (Fgfr2) and an SH2/SH3 adaptor (Grb2), which together mediate activation of the mitogen-activated protein kinase (Mapk) signaling pathway, are all necessary for PrE formation. In mouse embryos lacking Grb2, for example, all ICM cells are positive for Nanog and negative for Gata6, suggesting that they have adopted the epiblast fate at the expense of PrE (Arman et al., 1998; Cheng et al., 1998; Feldman et al., 1995; Mitsui et al., 2003; Wilder et al., 1997). Furthermore, experimental manipulation of FGF signaling can change the fate of ICM cells. Inhibitors of such signaling have thus been shown to shift ICM cells to the epiblast fate, whereas excess FGF shifts them to PrE (Yamanaka et al., 2010). In support of this, treatment of the eight-cell stage embryo with FGF signaling inhibitors eliminates PrE and expands the epiblast (Nichols et al., 2009).

Is the decision to form PrE versus epiblast dependent on cell lineage history? Does PrE originate preferentially from any of the cells generated by asymmetric cell division between the eight- and 32-cell stages, for example? Live cell tracing from the eight-cell stage onwards (Morris et al., 2010) revealed that cells internalized by the first wave of asymmetric cell division are biased toward the epiblast, whereas inner cells generated by the second wave of
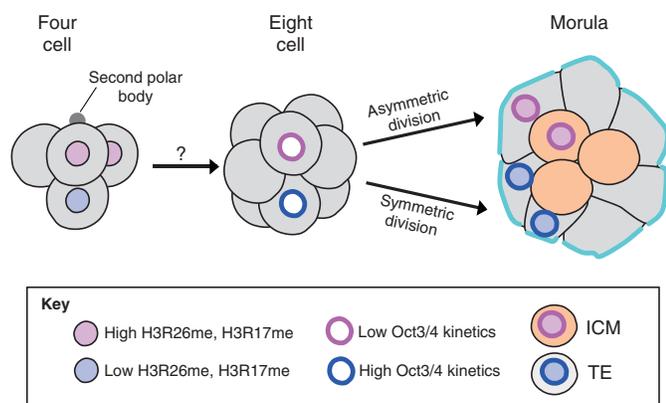
Fig. 4. Possible onset of the ICM versus TE decision. At the four-cell stage, blastomeres show differential H3R26me and H3R17me modifications: those with high H3R26me and H3R17me are biased towards becoming ICM, whereas those with low H3R26me and H3R17me are preferentially fated to become TE. At the eight-cell stage, blastomeres display differential Oct3/4 kinetics: those with high Oct3/4 kinetics preferentially undergo asymmetric division and hence contribute to mostly the ICM, whereas cells with low Oct3/4 kinetics divide symmetrically and are biased towards a TE fate. It is possible that these molecular differences are related, but this remains to be addressed. For example, do the blastomeres with high levels of H3R26me and H3R17me at the four-cell stage become the blastomeres that exhibit low Oct3/4 kinetics at the eight-cell stage? H3, histone 3; ICM, inner cell mass; Oct3/4 (Pou5f1), POU domain, class 5, transcription factor 1; R, arginine; TE, trophectoderm.

asymmetric cell division upregulated expression of *Gata6* and *Sox17*, and were heavily biased to PrE. However, a similar study based on a different lineage-tracing method detected no such apparent linkage (Yamanaka et al., 2010): inner cells generated by the first and second asymmetric cell division were found to be bipotent with regard to their ability to adopt a PrE or an epiblast fate. These discrepancies may be partly due to differences in the lineage-tracing methods employed by the studies and to the stages at which the lineage contribution was judged. In one study (Yamanaka et al., 2010), a single blastomere of the eight-cell stage embryo from the Z/EG mouse was co-injected with mRNAs for RFP reporters and Cre. RFP reporters were used to trace cell division patterns until the 32-cell stage. In order to perform long-term lineage analysis, embryos that showed colocalized expression of RFP and GFP were transferred to pseudo-pregnant females and were allowed to develop until E5.5 or E6.5. In another study (Morris et al., 2010), eight-cell stage embryos derived from a transgenic mouse that expresses a GFP cell-surface marker were cultured for ~55 hours, during which time each cell was tracked. At the end of this culture period, embryos were stained for Gata4, a PrE marker.

Although PrE progenitors and epiblast progenitors are randomly positioned in the ICM, there must be a regulatory interaction between these two types of progenitor cells (Fig. 5). For example, when a cell receives the FGF signal and becomes committed to PrE, the same cell may generate a lateral inhibitory signal to other cells. In this regard, it is interesting to note that *Fgf4* expression increases in presumably epiblast-fated cells of the 32-cell-stage blastocyst, whereas *Fgfr2* expression increases in the putative PrE-fated cells (Guo et al., 2010). Furthermore, whereas *Nanog* expression fluctuates in ES cells under conventional culture conditions (Chambers et al., 2007; Singh et al., 2007), the addition

of exogenous FGF to the culture medium inhibits *Nanog* expression in these cells (Hamazaki et al., 2006); however, the addition of FGF signaling inhibitors stabilizes *Nanog* expression (Lanner et al., 2010).

The sorting mechanism responsible for the relocation of randomly positioned PrE progenitor cells to the surface of the ICM facing the blastocoel remains unknown (Fig. 5). In this regard, *Dab2* (disabled homolog 2) mutant embryos show an interesting phenotype: PrE progenitor cells form in this mutant but are unable to move to the surface of the ICM (Yang et al., 2002). Dab2 is thought to function as a cargo-selective endocytic adaptor that links clathrin-coated endocytic vesicles to actin-based myosin VI (Morris et al., 2002). In the E4.5 embryo, Dab2, together with Lrp2 (low density lipoprotein receptor-related protein 2), is localized to the apical surface of PrE cells. The distribution of Dab2 and Lrp2 may become polarized according to embryonic-abembryonic positional information, and the polarized localization of Dab2 may facilitate directional trafficking of an unknown protein, such as a cell-adhesion protein. Such a scenario would achieve cell-autonomous cell sorting, but it may be informative to characterize the dynamics of the localization of Dab2 and Lrp2 during the movement of PrE progenitor cells to the apical surface of the ICM; Lrp2 is expressed in PrE progenitor cells at E3.5, although Dab2 is not detected at this time (Gerbe et al., 2008). In addition, there may be positional signals from the blastocoel cavity that attract migration towards the cavity. For example, Wnt9a, which is expressed in cells that surround the blastocoel cavity (Kemp et al., 2005), facilitates the repositioning of Gata4-expressing cells (Meilhac et al., 2009). Finally, cells that are incorrectly positioned during cell sorting may undergo apoptosis.

The origin of body axes: asymmetries in the blastocyst
The embryonic-abembryonic axis
Pre-implantation mouse embryos exhibit a few morphological asymmetries. For example, the sperm entry site, which is known to provide a cue for embryonic patterning in other animals, such as *Xenopus* (Gerhart et al., 1989), is located on one side of the fertilized egg. In addition, at the two-cell stage, the second polar body is located on one side between the two blastomeres. It continues to be located on one side of the embryo until the blastocyst stage (Gardner, 1997). The zona pellucida also persists as an oval rather than as a sphere. Finally, the formation of the ICM and the blastocoel cavity in the blastocyst generates the embryonic-abembryonic axis, which is perpendicular to the border between the ICM and blastocoel (see Box 2). Given that the embryonic-abembryonic axis corresponds to the long axis (the proximal-distal axis) of the embryo at the egg cylinder stage (E5.5; see Glossary, Box 1), which in turn will correspond to the dorsal-ventral axis, it can be regarded as the source of future dorsal-ventral polarity (Rossant and Tam, 2009).

Whether a direct link exists between the embryonic-abembryonic axis and the earlier morphological asymmetries is a controversial issue (see Box 2). Whereas some findings have indicated the existence of a direct relationship between the sperm entry site, the position of the second polar body at the two-cell stage and the embryonic-abembryonic axis in the blastocyst (Gardner, 1997; Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2005), others argue against such a direct interaction (Hiiragi and Solter, 2004; Kurotaki et al., 2007). Differences in the techniques used may underlie these discrepancies, but it is difficult to reconcile these two sets of findings at this time.
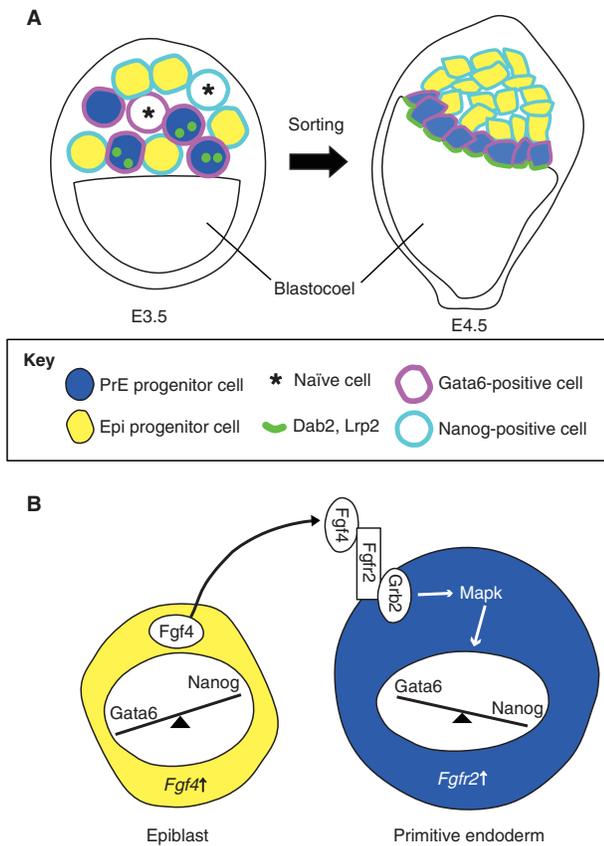
Fig. 5. Segregation and relocation of epiblast and PrE cells.
(**A**) The location of PrE progenitors and epiblast progenitors at E3.5 and E4.5. Both PrE (blue) and Epi (yellow) progenitors are randomly positioned in the inner cell mass (ICM) at E3.5. At this stage, some of the cells in the ICM are still naïve (asterisk) for Nanog or Gata6 expression. By E4.5, the PrE cells are restricted to the surface of the blastocoel cavity, whereas the Epi cells are confined to the inner region of the ICM. After PrE and epiblast allocation is completed at E4.5, Dab2 and Lrp2 are localized to the apical surface of PrE cells. (**B**) The role of FGF signaling in PrE formation. *Fgf4* expression increases in epiblast progenitor cells (yellow) from the 32-cell stage, whereas *Fgfr2* expression is upregulated in PrE progenitor cells (Guo et al., 2010). Fgf4 secreted by epiblast progenitors interacts with Fgfr2 and thereby activates Grb2 and Mapk in PrE progenitors. Activated Mapk induces the expression of PrE-specific genes, such as *Gata6*. Fgf4 simultaneously represses Nanog expression, further promoting PrE fate while inhibiting Epi fate. Dab2, adaptor protein disabled 2; Epi, epiblast; Fgf, fibroblast growth factor; Fgfr, fibroblast growth factor receptor; Gata6, GATA-binding protein 6; Grb2, growth factor receptor bound protein 2; Lrp2, lipoprotein receptor-related protein 2; Mapk, microtubule-associated protein kinase; PrE primitive ectoderm.

Origin of AP axis and the distal visceral endoderm

The anterior-posterior (AP) polarity of the mouse embryo is firmly established when the anterior visceral endoderm (AVE; see Glossary, Box 1) is formed from the distal visceral endoderm (DVE) on the future anterior side of the embryo by E6.5. Signals secreted from the AVE, such as the Nodal antagonists Lefty1 and Cer1 (cerberus 1 homolog), act on the nearby epiblast and specify it to future anterior identity (Fig. 1). The epiblast, which is located far from the AVE, escapes the AVE-derived signals and forms the primitive streak on the opposite side of the embryo (Beddington and Robertson, 1998; Thomas and Beddington, 1996).

> **Box 2. The embryonic-abembryonic axis of the blastocyst**
> As inner cell mass (ICM) cells cluster on one side of the blastocyst and because the blastocoel is located opposite this cell cluster, an embryonic (ICM side)-abembryonic (blastocoel side) polarity forms in the early embryo. The origin of this axis has been an area of intense investigation because it corresponds to embryonic polarities at later stages; for example, the proximal-distal axis of the pre-gastrulating embryo at E5.5. Thus, a question that arises is whether the embryonic-abembryonic axis is pre-determined by an unknown mechanism or whether it is stochastically generated? If a pre-pattern exists, blastomeres at very early stages (two- to approximately eight-cell stage) would be preferentially fated to the embryonic region (ICM) or abembryonic region (trophectoderm, TE). As such, this issue is closely related to another question: when does the first cell fate decision take place? There have been many reports that support or refute a correlation between the cleavage pattern of the two-cell blastomeres and the embryonic-abembryonic axis. It is not clear what might account for the discrepancies, but one possibility is the presence or absence of a mechanical constraint provided by the zona pellucida (Motosugi et al., 2005). Perhaps, the cell fate analysis performed under the conditions that most closely resemble those found physiologically is the one by Kurotaki et al. (Kurotaki et al., 2007): time lapse tracing of living unconstrained two-cell stage embryos to the blastocyst stage shows that two blastomeres equally contribute to the embryonic and abembryonic region. However, Zernicka-Goetz and her colleagues have revealed differences in the lineage potential of individual four-cell blastomeres (Torres-Padilla et al., 2007a). Furthermore, recent reports suggest there are epigenetic differences among blastomeres around the four- to eight-cell stages (Plachta et al., 2011) (see Fig. 4). These are exciting findings, but their relevance to cell fate decision needs further investigation.

Although the formation of the AVE marks the firm establishment of the AP axis, it has become increasingly apparent that AP polarity originates much earlier – at the blastocyst stage at the latest. In particular, a recent study (Takaoka et al., 2011) has shown that the origins of the AVE and DVE are different, and that cells of the DVE originate in the blastocyst. Based on these findings, it is necessary to define clearly the DVE and the AVE (Fig. 6). The DVE comprises a group of cells that express *Lefty1* and *Cer1* at the distal end of the E5.5 embryo, whereas the AVE consists of a group of cells that begin to express the same genes at the distal end after E5.5. Coincidentally, both DVE and AVE cells express the same set of marker genes, including *Lefty1*, *Cer1* and *Hhex* (Hex, hematopoietically expressed homeobox), although there is some heterogeneity among the two cell populations. Furthermore, both the DVE and the AVE migrate in the same direction, towards the future anterior side. Given these observations, the prevailing view was that AVE cells descend directly from DVE. However, recent genetic fate mapping and live imaging have revealed that they have different origins (Takaoka et al., 2011) (Fig. 6). In this study, the DVE was shown to be derived from $Gata6^+Lefty1^+$ cells that first appear around E4.0, although the generation of fully differentiated DVE cells requires additional gene expression around E5.5. By contrast, the AVE is derived from $Gata6^+Lefty1^-$ VE cells that move to the distal tip of the embryo and begin to express *Lefty1* and *Cer1* after E5.5 (see below).

Lefty1 expression begins in a subset of blastomeres in the ICM at E3.5 (Takaoka et al., 2006; Takaoka et al., 2011). Most of these $Lefty1^+$ cells are $Gata6^-$ (some are $Gata6^+$, but this heterogeneity most probably reflects the fact that some ICM cells at this stage are
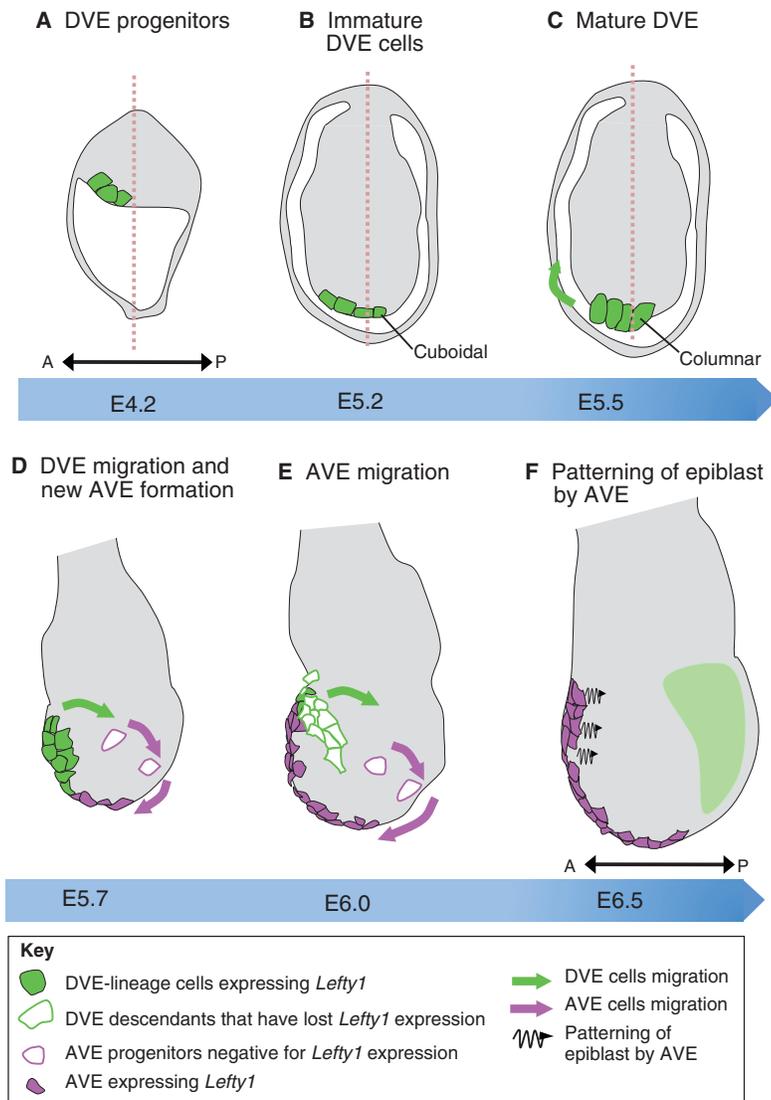
Fig. 6. Origins of the dorsal and anterior visceral endoderm. The origins and movements of the DVE and the AVE in pregastrulation embryos. The DVE lineage (green) and the AVE lineage (purple) cells are indicated; solid colors indicate cells that express *Lefty1* (left-right determination factor), whereas outlined colors indicate cells that do not. (**A**) At E4.2, DVE-fated cells (green) that express *Lefty1* are found on one side of the primitive ectoderm (PrE). (**B**) Immature DVE cells, which maintain *Lefty1* expression but are negative for other DVE markers, are found at the distal tip of the embryo, but are slightly dislocated to one side (probably the future anterior side) at E5.2. (**C**) At E5.5, immature DVE cells have matured, and they express *Lefty1* and other DVE markers. The green arrow represents DVE cells migrating to the future anterior side of the embryo. (**D**) At E5.7, DVE cells have migrated towards the proximal side via the future anterior side. As DVE migrates away from the distal tip, VE cells negative for *Lefty1* expression (open magenta shapes) move to the distal tip and become AVE. (**E**) At E6.0, DVE cells that have reached the embryonic/extra-embryonic junction migrate laterally and lose expression of DVE markers, including *Lefty1*. AVE cells that are generated at the distal tip also migrate towards the proximal side, while new AVE cells continue to be generated at the distal tip. (**F**) At E6.5, AVE is fully formed and occupies one side (the future anterior side) of the embryo. AVE, anterior visceral endoderm; DVE, distal visceral endoderm.

still naïve, as described above). However, these cells rapidly lose *Lefty1* expression and eventually contribute to the epiblast. The biological relevance of the epiblast-fated Lefty1+ cells is unknown, but they may be required to induce the subsequent *Lefty1* expression in Gata6+ cells at E4.0 or to pattern the epiblast before gastrulation. A second wave of *Lefty1* expression takes place in Gata6+ cells around E3.75 to E4.0. These Lefty1+ cells become asymmetrically located at the upper side of the PrE in the tilted embryo at E4.2 (Takaoka et al., 2006) (Fig. 6) and will contribute to the DVE at E5.5 (Takaoka et al., 2011). It is likely that *Lefty1* expression is maintained throughout the cell lineage that gives rise to the DVE. Asymmetrically located Lefty1+ cells can be generated by culturing blastocysts in vitro (Takaoka et al., 2006). Furthermore, these in vitro-generated Lefty1+ cells contribute to the DVE when the cultured blastocyst is returned to a pseudo-pregnant female (Takaoka et al., 2011). These observations suggest that initial AP polarity is specified autonomously without information from the uterus.

Given that the Gata6+Lefty1+ cells in the blastocyst contribute to the DVE, the origin of AP polarity can be traced back at least to these Lefty1+ cells in the blastocyst. It has been reported that *Cer1* expression also initiates in a subset of PrE cells of the E4.25

expanded blastocyst, possibly in the same cells that are positive for *Lefty1* expression (Torres-Padilla et al., 2007b). The next important issue concerns how future DVE cells are selected among PrE progenitor cells in the blastocyst: that is, how *Lefty1* expression is restricted to a subset of PrE progenitor cells. *Lefty1* expression in the PrE at E4.5 and in the DVE at E5.5 depends on the Nodal-responsive Foxh1 (forkhead box H1)-dependent enhancer located in the 5′ upstream region of *Lefty1* (Takaoka et al., 2006). If *Lefty1* expression in the earlier blastocyst also depends on this enhancer, Nodal signaling may contribute to the regulation of *Lefty1*, given that it regulates *Lefty1* and *Lefty2* expression during left-right patterning at a later stage by constituting a reaction/diffusion-type regulatory system (Nakamura et al., 2006). In this regard, it is interesting to note that the Foxh1-dependent enhancer of *Nodal* is active in a subset of ICM cells in the blastocyst (Granier et al., 2011) and that Furin and Pace4 (Pcsk6 – Mouse Genome Informatics), secreted protein convertases that can process Nodal precursor to its active form, are already active at the blastocyst stage in the ICM (Mesnard et al., 2006).

Additional asymmetries have also been described in the peri-implantation embryo. For example, nuclear localization of β-catenin is detected in a subset of epiblast cells at E4.5 (Chazaud et

al., 2006). Furthermore, the Wnt/β-catenin-dependent enhancer (PEE) of *Nodal* can mark a subset of blastomeres in the blastocyst (Granier et al., 2011). There may thus be an asymmetry at the level of canonical Wnt signaling within the blastocyst and the implanting embryo, although its relevance remains to be determined. It will be interesting to learn how each asymmetry relates to the other.

Establishment of the AP axis
Behavior of the DVE and newly generated AVE

Although *Lefty1* expression is maintained in the DVE-fated cells, Lefty1^{+} prospective DVE cells before E5.5 do not express other DVE markers such as *Cer1* and *Hhex* (Mesnard et al., 2006), although *Cer1* has been detected prior to this (Torres-Padilla et al., 2007b). Expression of *Cer1* and *Hhex* begins in DVE cells at E5.5 (Mesnard et al., 2006). The complete differentiation of DVE cells may therefore require the expression of additional DVE-specific genes. Interestingly, DVE cells change their shape between E5.0 and E5.5: Lefty1^{+} prospective DVE cells at E5.0 are cuboidal, whereas DVE cells at E5.5 are columnar. Fully specified DVE cells at E5.5 begin to migrate unilaterally towards the proximal side. When they reach the embryonic/extra-embryonic junction, they migrate back in the lateral-distal direction and rapidly lose expression of *Lefty1* (and of other DVE markers) (Takaoka et al., 2011).

Despite the fact that the AVE expresses the same set of genes and migrates in the same direction as does the DVE, the origin of the AVE differs from that of the DVE (Takaoka et al., 2011). AVE is thus newly formed from Gata6^{+}Lefty1^{−} VE cells. These AVE progenitor cells are initially located in a more proximal region at E5.5, move towards the distal side and begin to express AVE markers when they reach the distal end (Fig. 6). This scenario was previously suggested by fate-mapping analysis with HRP and by time-lapse observation of cells labeled with a Hhex-GFP transgene (Perea-Gomez et al., 2001; Rivera-Perez et al., 2003), but it was only clearly demonstrated by recent genetic fate-mapping and live imaging studies (Takaoka et al., 2011). AVE formation is inhibited by signals that originate from the extra-embryonic ectoderm, most probably bone morphogenetic protein (BMP) (Yamamoto et al., 2009). Thus, AVE progenitors may begin to express AVE markers when they reach the distal end because it is the only region that is negative for the BMP signal in the embryo proper (Yamamoto et al., 2009). Newly formed AVE cells also migrate towards the proximal side, following DVE, and they occupy the future anterior side of the embryo at E6.5.

DVE and AVE migration: driving forces and cellular mechanisms

How is the migration of the DVE and the AVE regulated? What might be the driving forces of such migration and are there instructive signals that attract the cells?

DVE cells change their shape and become columnar by the time they start to migrate (Migeotte et al., 2010; Rivera-Perez et al., 2003; Takaoka et al., 2011). Given that the VE is a monolayer of cells, DVE cells must migrate through the surrounding VE cells rather than on top of them (Srinivas et al., 2004). Migrating DVE cells acquire a snail-like morphology with a highly elongated lamella terminating in two lateral horn-like protrusions; these filopodium-like protrusions are polarized in the direction of cell movement (Migeotte et al., 2010). DVE cells retain cell-cell junctions during migration and display the hallmarks of a collective cell movement (Migeotte et al., 2010; Srinivas et al., 2004). In a mutant mouse that lacks the Rho GTPase family member Rac1

(RAS-related C3 botulinum substrate 1), DVE cells are unable to change their shape and to form the filopodium-like structures, and they fail to migrate away from the distal side of the embryo. Rac1 appears to act autonomously within DVE by activating the Wiskott-Aldrich syndrome protein family verprolin-homologous protein (WAVE) complex: specific deletion of *Rac1* in VE cells phenocopies the *Rac1*-null embryo and that a hypomorphic mutation that disrupts the gene for Nck-associated protein 1 (Nap1; Nckap1 – Mouse Genome Informatics), a component of the WAVE complex, often results in impaired DVE migration (Rakeman and Anderson, 2006). When DVE cells reach the embryonic/extra-embryonic junction, they do not go beyond the junction but rather migrate back in the lateral-distal direction (Fig. 6). A recent study (Trichas et al., 2011) has suggested that there are regional differences in F-actin and dishevelled 2 (Dvl2) protein localization between embryonic VE and extra-embryonic VE, and that these differences may explain why DVE cells do not pass the embryonic/extra-embryonic junction.

What might be the driving force of DVE cell migration? As the involvement of Rac1 and the WAVE complex suggests, time-lapse observations (Takaoka et al., 2011) have shown that DVE migration is an active and rapid process (it takes 5 hours for a DVE cell to move from the distal tip of the embryo to the embryonic/extra-embryonic junction). The driving force may be provided by an attractant (or attractants) that is expressed at the proximal side of the egg cylinder. Indeed, dickkopf 1 (Dkk1), an antagonist of Wnt signaling, is expressed in the region immediately proximal to the DVE and is able to attract DVE migration (Kimura et al., 2000). Although the DVE still migrates normally in *Dkk1* mutant embryos, a *Dkk1* transgene can rescue the DVE migration defects that are present in an *Otx2* (a paired-type homeobox gene) mutant mouse (Kimura-Yoshida et al., 2005), suggesting that *Dkk1* is a target of the Otx2 transcription factor, which itself provides the driving force for this migration. Nodal signaling stimulates cell proliferation in pre-gastrulation embryos. Cell proliferation regulated by Nodal signaling, either in the VE (Yamamoto et al., 2004) or in the epiblast (Stuckey et al., 2011), may provide an additional driving force, although it is not clear how it would do so. Yamamoto et al. (Yamamoto et al., 2004) have reported that cell proliferation is inhibited in the AVE by Nodal antagonists, but this has not been observed by Stuckey et al. (Stuckey et al., 2011). This discrepancy may result from the differences in the methods employed to detect cell proliferation.

Finally, what determines the direction of DVE cell migration? The DVE must move from the distal tip towards the proximal side, but it could do so by taking different routes or directions with respect to the future AP polarity. Formally, one can think of two alternative scenarios. In the first scenario, the embryo is already pre-patterned with respect to its future AP polarity before the DVE starts to migrate. DVE cells thus already 'know' in which direction to migrate. In the second scenario, there is no pre-patterning at the time of DVE migration. As long as the DVE undergoes collective migration towards the proximal side of the embryo, it would not matter which route it takes because the side of the embryo that receives the DVE would become the future anterior side.

In reality, the expression domains of the Nodal antagonists Lefty1 and Cer1 are already shifted toward the future anterior side before the DVE starts to migrate. As described above, *Lefty1* expression domains continue to localize asymmetrically, shifted towards the future anterior side, between E4.5 and E5.5 (Takaoka et al., 2006). *Cer1* is expressed in the PrE at E4.75 and is more strongly expressed on one side of PrE. As the embryo grows, *Cer1*

expression progressively decreases, but re-appears at the distal end of the embryo between E5.25 and E5.5 (Torres-Padilla et al., 2007b). Furthermore, these proteins are able to attract DVE migration: DVE has thus been shown to migrate towards ectopic expression sites of Lefty1 or Cer1, or of both proteins (Yamamoto et al., 2004). Similarly, as mentioned above, Dkk1 can attract DVE cells (Kimura-Yoshida et al., 2005). *Dkk1* expression domains change dynamically before and after DVE migration, but they may show a transient asymmetry with regard to future AP polarity. However, DVE still migrates unilaterally towards the proximal side in mutant mice that lack Lefty1 or Cer1 (or both proteins) (Perea-Gomez et al., 2002) or in those that lack *Dkk1* (del Barco Barrantes et al., 2003). This lack of a migration phenotype might be due to functional redundancy between these attracting signals. Alternatively, the signals may simply confer to DVE cells a bias to the direction of their migration, with the DVE still being able to achieve collective migration towards the proximal side without such a bias.

Functions of the DVE and AVE

The role of the AVE in patterning the AP axis of the early embryo is well established. AVE cells located at the future anterior side of the egg cylinder secrete several signaling molecules, including Nodal inhibitors, such as Lefty1 and Cer1, as well as Wnt inhibitors, such as Dkk1. These signals act in concert on the

neighboring epiblast and specify it to adopt the future head identity, whereas the region of the epiblast on the opposite side, located away from the AVE, does not receive such AVE-derived signals and adopts the future tail identity. A series of elegant manipulation experiments have thus revealed that an embryo that lacks the entire VE is not able to form head identity, whereas an AVE that is implanted into such an embryo is able to induce such an identity (Kimura et al., 2000).

What, then, is the role of the DVE? Recent studies suggest that the DVE is not required for de novo generation of the AVE but is essential for the proper migration of the newly formed AVE as a result of its initiation of the global movement of VE cells. Thus, when DVE that had been labeled with a *Hhex-GFP* transgene was surgically removed at E5.5, *Cer1*-expressing (AVE) cells were subsequently generated successfully but failed to migrate anteriorly (Miura and Mishina, 2007). Similarly, when Lefy1^{+} (DVE) cells at E5.5 were genetically ablated, AVE cells were newly generated but were unable to migrate (Takaoka et al., 2011). In mutant mice that lack the Nodal signaling component Cripto (cryptic family 1), the AVE forms in the absence of a DVE but fails to migrate (Chu and Shen, 2010; Ding et al., 1998; Kimura et al., 2001). An examination of the behavior of the entire VE by time-lapse imaging has revealed that VE cells other than DVE and AVE cells also change their positions between E5.5 and E6.5 (Takaoka et al., 2011). Interestingly, these VE cells begin to move concomitantly
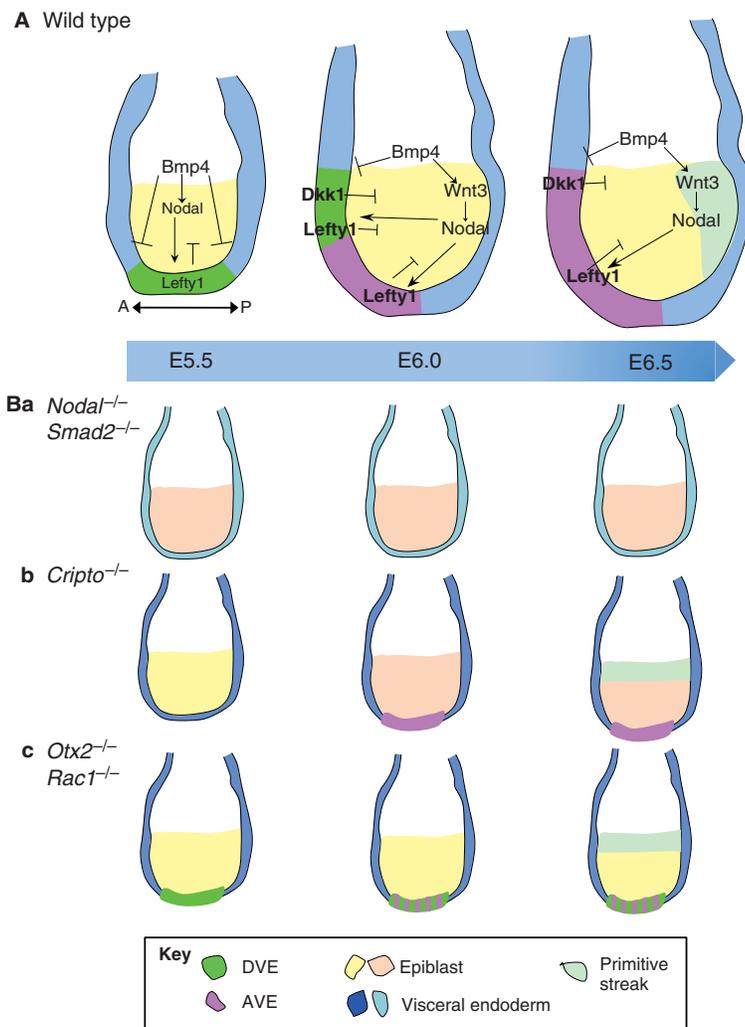

Fig. 7. Three groups of mouse mutants that show impaired AVE formation. (**A**) Behaviors of DVE-lineage and AVE-lineage cells are shown for the wild-type embryo, together with main signaling events. At E6.0 and E6.5, signals from the AVE (and possibly DVE) specify the nearby epiblast by inhibiting Nodal signaling and Wnt signaling. On the opposite side far from the AVE, Nodal and Wnt signaling remains active, which results in the formation of the primitive streak (light green). (**B**) Three groups of mouse mutants defective in AVE formation are illustrated. Yellow and orange indicate normal and abnormal epiblast, respectively. Dark and light blue indicate normal and abnormal visceral endoderm, respectively. (**a**) The DVE and AVE fail to form throughout E5.5 and E6.5. (**b**) The DVE does not form at E5.5. AVE is newly formed at the distal tip at E6.0 but fails to migrate. As a result, cells positive for *Brachyury* expression (which marks primitive streak-like cells) form at the proximal side of the embryo. (**c**) The DVE forms at the distal tip of the embryo at E5.5 but fails to migrate. Cells positive for DVE/AVE markers remain at the distal tip at E6.0 and E6.5. As a result, cells that express primitive streak markers are found at the proximal side of the embryo. AVE, anterior visceral endoderm; Bmp4, bone morphogenetic protein 4; Cripto, cryptic family 1; Dkk1, dickkopf homolog 1; DVE, dorsal visceral endoderm; Lefty1, left-right determination factor; Otx2, orthodenticle homolog 2; Rac1, RAS-related C3 botulinum substrate 1; Smad2, MAD homolog 2.

with or shortly after DVE migration, suggesting that DVE guides the migration of AVE by initiating the global movement of VE cells (Fig. 6). In support of this notion, the genetic ablation of Lefty1+ DVE cells in E5.5 embryos also impaired the global movement of VE cells (Takaoka et al., 2011).

DVE cells may have an additional role in AP patterning. Given that they express the same signals as do AVE cells, DVE cells may pattern the nearby epiblast just as the AVE does. Whether epiblast patterning requires the continuous action of both the DVE and the AVE between E5.5 and E6.5 or only the action of AVE remains to be tested. It is also possible that the DVE progenitors may act at an early stage to 'pre-pattern' the epiblast prior to implantation.

Reinterpretation of mouse mutants with defective AVE formation

Given that the DVE and the AVE have only recently been shown to have different origins, it is necessary to reinterpret the previously described phenotypes of mouse mutants. Thus, it is more appropriate to assess such phenotypes on the basis of four criteria: (1) whether the DVE forms; (2) whether it migrates; (3) whether the AVE forms; and (4) whether the AVE migrates (supplementary material Table S1; Fig. 7). In light of these criteria, the phenotypes of the following mutants need to be re-interpreted. In *Nodal*- and *Smad2*-null mutants, in which the VE and epiblast are not correctly specified (Brennan et al., 2001; Mesnard et al., 2006; Waldrip et al., 1998), both the DVE and AVE fail to form. The formation of the DVE (and also most probably of the AVE) is also impaired in BMP signaling mutants, because BMP signaling is required for the differentiation of the PrE to VE around E4.5 (Yamamoto et al., 2009). In the *Cripto*-null mutant, the DVE does not form at E5.5. *Cer1*-expressing cells (most likely AVE) do form in this mutant at E6.0~E6.5 but they fail to migrate anteriorly and remain at the distal tip of the embryo (Chu and Shen, 2010; Ding et al., 1998), indicative of a role for the DVE in guiding AVE migration. In other null mutants, such as in *Otx2*−/− (Perea-Gomez et al., 2001), *Rac1*−/− (Migeotte et al., 2010) and *Prickle1*−/− (Tao et al., 2009) embryos, the DVE forms at the distal dip at E5.5, but fails to migrate. Cells positive for *Cer1* (DVE, AVE or both) remain at the distal tip of the embryo at E6.0 and E6.5.

Conclusion

The generation of multiple lineages within the early mouse embryo and the establishment of embryonic polarity is a multistep process. Compaction, which begins at the eight-cell stage, generates the inside-outside polarity in the embryo, which in turn is responsible for the first cell fate decision that dictates whether cells will become ICM or TE. However, there may be molecular differences among blastomeres as early as the four-cell stage, and the onset of the first cell fate decision may take place earlier. The AP body axis is firmly established when the DVE migrates to the future anterior side of the egg cylinder. However, the DVE originates from a subset of cells in the late blastocyst, suggesting that the origin of AP polarity can be traced back to the peri-implantation stage. At this time, however, the origin of the first cell fate decision and that of AP polarity remain unknown, and one cannot completely exclude the presence of a maternal determinant(s) in the oocyte that plays a role in establishing AP polarity.

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