From fertilization to gastrulation: axis formation in the mouse embryo

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Although much remains unknown about how the embryonic axis is laid down in the mouse, it is now clear that reciprocal interactions between the extraembryonic and embryonic lineages establish and reinforce patterning of the embryo. At early post-implantation stages, the extraembryonic ectoderm appears to impart proximal–posterior identity to the adjacent proximal epiblast, whereas the distal visceral endoderm signals to the underlying epiblast to restrict posterior identity as it moves anteriorward. At gastrulation, the visceral endoderm is necessary for specifying anterior primitive streak derivatives, which, in turn, pattern the anterior epiblast. Polarity of these extraembryonic tissues can be traced back to the blastocyst stage, where asymmetry has been linked to the point of sperm entry at fertilization.

Introduction

The establishment of the anterior–posterior (A-P) axis signals the first overt manifestation of the mature body plan of the mouse embryo. Here we outline recent progress in understanding axis development in the mouse, including evidence that the blueprint for A-P patterning may be laid down at fertilization. We will consider new evidence for the translation of early polarity in the blastocyst into proximal–distal (P-D) polarity (see Figure 1 legend) in the egg cylinder, and how recent experiments support a model in which P-D polarity is converted into the A-P axis. Finally, we will discuss new information regarding the roles that the visceral endoderm (VE) and anterior definitive endoderm (ADE) play in patterning the A-P axis.

Polarity in the fertilized egg

Does early polarity in the mouse embryo relate to the later A-P axis? In organisms such as Xenopus laevis and Caenorhabditis elegans, the segregation of cytoplasmic determinants within the egg or zygote seems to have a critical role in establishing polarity, and experimental disruption of this early organization can prevent development into a normal embryo [1,2]. In contrast, the early development of the mouse embryo is highly regenerative and refractory to many different experimental disruptions, such as removal of polar cytoplasm and aggregation or removal of blastomeres. Accordingly, it has been assumed that the establishment of embryonic axes does not originate from polarity in the pre-implantation embryo.

However, recent labeling experiments indicate that asymmetry in the zygote can be linked to polarity at later stages [3••]. Marking the sperm entry point with a fluorescent bead allows this location to be followed in subsequent stages of development. Just after fertilization, the bead tends to be located at the prospective vegetal pole of the zygote (see Figure 1 legend), indicating that polar body extrusion often occurs on the side opposite to the point of sperm entry.

At the first cleavage, the bead is localized in most embryos to the cleavage plane between the two ½ blastomeres, whereas at the second cleavage, the bead usually marks the ½ blastomere that divided first. Thus, the point of entry of the sperm anticipates the orientation of the animal–vegetal axis, the plane of the first cleavage, and the timing of the second cleavage (Figure 1). These data suggest that the site of sperm fusion may provide positional information affecting the orientation and timing of blastomere cleavage, which in turn may play a key role in establishing polarity in the embryo.

Blastocyst polarity can be traced back to the sperm entry position in the fertilized egg

Previous work has demonstrated that the second polar body usually ends up at the medial region of the embryonic–abembryonic axis of the blastocyst and aligns with the bilateral axis [4] (see Figure 1 legend). Recently, the injection of green fluorescent protein mRNA into individual blastomeres has indicated that blastomeres at the eight-cell stage retain their position relative to the polar body up until the blastocyst stage [5•]. This suggests that patterning at the eight-cell stage informs development at least until the blastocyst stage.

Ironically, disturbing embryos by the removal of polar cytoplasm from blastomeres at either the two- or eight-cell stage does not prevent normal development, consistent with previous work showing that removing polar cytoplasm from the zygote results in normal embryos [6]. However, the bead marking experiments show that sperm entry position not only anticipates asymmetry at the early cleavage stages, but also, later on at the blastocyst stage,
localizes to trophectoderm cells at the border between the inner cell mass (ICM) and the blastocoel cavity, near the mid-point of the embryonic-abembryonic axis [3••].

Although it is still unclear exactly how asymmetric positioning of the ICM is achieved in the blastocyst, the consistent localization of the sperm entry position to trophectoderm cells at the embryonic–abembryonic border suggests that an early signal from the trophectoderm may position the ICM.

Cell fate allocation in the blastocyst
The means by which cells are allocated to either embryonic or extraembryonic lineages in the blastocyst (Figure 2) is not understood; however, the early establishment of cell fate may be regulated in part by the POU transcription factor Pou5f1 (also known as Oct4). Pou5f1 is expressed in all blastomeres at the four-cell stage but is downregulated in the trophectoderm by the blastocyst stage [7]. Its expression is maintained in the ICM, with highest protein concentrations accumulating in the primitive endoderm. Pou5f1 is required for ICM formation, as Pou5f1 mutant blastocysts consist only of trophectoderm [8].

Consistent with the mutant phenotype, recent studies have shown that embryonic stem (ES) cells lacking Pou5f1 differentiate into trophectoderm, whereas cells expressing a single copy of Pou5f1 maintain their pluripotential ES cell state. By contrast, a twofold increase in Pou5f1 expression causes ES cells to adopt VE or mesodermal fates [9•]. Thus, tight control of Pou5f1 levels may be important for cell fate allocation in the blastocyst. It will be interesting to learn whether the initial polarity at fertilization or the timing of cell division in blastomeres is linked to expression levels of Pou5f1.

Whereas Pou5f1 mutant blastocysts lack an ICM, the novel gene Tauhe mus (Tbn) seems to be required for survival or maintenance of the ICM [10]. Tbn-deficient embryos appear normal at the early blastocyst stage, but by late blastocyst stage consist only of trophoblast cells as a result of increased apoptosis in the ICM. Tetraploid aggregation experiments in which trophectoderm cells were wild type but ICM cells were mutant indicated that Tbn is required cell-autonomously for ICM survival.

Another gene whose role in trophoblast development has recently been uncovered is the T-box gene Eomesodermin (Eomes), which is first expressed in the trophectoderm at the blastocyst stage [11••]. Embryos lacking Eomes arrest at the blastocyst stage, and mutant trophoderm dies in culture even in the presence of the trophectoderm proliferation factor Fgf4, suggesting that Eomes is required cell-autonomously for trophectoderm development.

Polarity in the blastocyst leads to P-D polarity in the early post-implantation embryo
Recent cell-labeling experiments link the polarity in the blastocyst to later P-D polarity in the early post-implantation embryo. Previous clonal analysis has shown that of the labeled blastocyst cells injected into host blastocysts, those contributing to the epiblast do not give rise to coherent clones, but those contributing to the VE do [4]. Thus, any early patterning must occur not within the cells giving rise to embryonic lineages, but within the adjacent extraembryonic tissues.

Recent cell-marking experiments have shown that primitive endoderm cells located near the polar body at the blastocyst stage give rise primarily to clones of cells in the VE overlying the embryonic epiblast, whereas those located further from the polar body contribute preferentially to the VE overlying the extraembryonic ectoderm [12•]. These results indicate that the polarity of the animal–vegetal axis in the primitive endoderm of the blastocyst leads to P-D polarity in the VE.

P-D polarity of the extraembryonic ectoderm leads to induction of posterior genes in the proximal epiblast
In addition to the VE, the extraembryonic ectoderm, which is derived from the trophectoderm of the blastocyst (Figure 2) and is positioned just above the epiblast in the early post-implantation embryo, is important for embryonic patterning. Transplantation experiments in which distal epiblast cells grafted to the proximal epiblast give rise to primordial germ cells and extraembryonic mesoderm — cell types that normally arise from the proximal epiblast — suggest that signaling from the extraembryonic–embryonic region is important for the induction of proximal–posterior cell fates [13]. Similarly, explant culture experiments show that the extraembryonic ectoderm is able to induce primordial germ cell formation in distal epiblast [14].

Recently, genes expressed in the extraembryonic ectoderm at the egg cylinder stage have been implicated in A-P patterning. There is a clear P-D polarity in the extraembryonic ectoderm, as determined by Bmp4 expression specifically in the extraembryonic ectoderm adjacent to the epiblast [15•]. It was originally shown that Bmp4 mutant embryos display posterior truncations and fail to express T, which is normally found in the proximal epiblast immediately adjacent to the domain of Bmp4 expression [16]. Recent chimera analysis has demonstrated that Bmp4 is required in the extraembryonic ectoderm for formation of the proximal epiblast-derived primordial germ cells and allantois [15•].

Eomes is also expressed in extraembryonic ectoderm at this stage, and in the posterior epiblast just before gastrulation (Figure 3). Chimeras in which extraembryonic tissue is wild type and the epiblast lacks Eomes are rescued from early lethality and express proximal–posterior genes such as T and Fgf8 in the epiblast [11••]. Thus, it is likely that Eomes expression within the extraembryonic ectoderm is required for expression of these proximal–posterior genes in the epiblast.
Figure 1

Unfertilized egg  
Fertilization  
Zygote  
Two-cell embryo  
Three-cell embryo  

4.5 dpc  
5.5 dpc  
5.75 dpc  
6.0 dpc  
6.5 dpc  
7.5 dpc
In embryos lacking Nodal expression, which is normally found in the epiblast at early post-implantation stages, expression of extraembryonic ectoderm markers such as Bmp4 and Eomes is not maintained [17••]. Expression of proximal–posterior genes such as T, Fgf8 and Wnt3 is abolished in these mutants, presumably because the extraembryonic ectoderm fails to signal to the epiblast. Thus, reciprocal interactions between the extraembryonic ectoderm and the proximal epiblast are likely to be responsible for posterior patterning of the epiblast.

Cell movements convert P-D polarity to A-P polarity in the egg cylinder as the AVE suppresses posterior fate in adjacent epiblast

Whereas the extraembryonic ectoderm seems to signal to the proximal epiblast to induce expression of proximal–posterior genes, the anterior visceral endoderm (AVE) has been proposed to counter this activity by repressing expression of these genes in the underlying epiblast as it moves anteriorward [18]. It was previously shown that a distinct subpopulation of VE cells at the distal tip of the egg cylinder expressing the homeobox gene Hex moves proximally to mark the prospective anterior side of the embryo before streak formation (Figure 3) [19,20]. Cell-labeling experiments have suggested that the movement of the distal VE cells may be part of a more global anteriorward rotation of the VE prior to streak formation [12•]; however, recent ultrastructural analyses have shown that AVE cells acquire a morphology that is distinct from all other VE cells during their anteriorward movement [21••]. This suggests that, in addition to being part of the global movement of VE, the AVE cells may detach from the epithelial sheet and move in an anteriorward direction. It is currently not understood what mechanism drives either of these processes.

Coincident with anterior movement of the AVE, a number of genes are activated in the AVE, including Dkk1, Cerl and Lefty1, which may antagonize Wnt and TGF-β signaling in the epiblast. Expression of these antagonists may act to restrict genes required for mesoderm formation such as Nodal to the posterior of the embryo [18].

While these descriptive studies have hinted at a model in which the AVE represses expression of posterior genes in the epiblast as it sweeps anteriorly, the analysis of several mutants has provided compelling evidence for it. On the one hand, in embryos lacking Smad2 [17••,22] or both
**Figure 3**

<table>
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**P-D polarity in the three lineages of the early post-implantation embryo (5.5 days post coitum)** is determined by the expression domains of Hex, which is confined to the distal VE; Wnt3, which is confined to the proximal epiblast and overlying VE; Eomes, which is expressed in the extraembryonic ectoderm; and Cripto, which is expressed throughout the epiblast. A few hours later (6.0 days post coitum), coordinated cell movements result in localization of Hex to the AVE marking the anterior side of the embryo, whereas Wnt3 and Cripto expression domains become restricted to the prospective posterior side. Concomitantly, Eomes is induced in the posterior epiblast.

**Foxa2** (also known as **HNF3ß** and **Lhx1** (also known as **Lim1**) [23••] in the VE, the AVE fails to form and the epiblast adopts a completely proximal–posterior identity in response to widespread expression of T, Fgf8 and **Nodal**. On the other hand, in embryos lacking **Otx2** [24,25] or **Cripto** [26], global P-D polarity is established, but the distal VE fails to move proximally [27]. Accordingly, the A-P axis is misaligned, with the distal epiblast adopting anterior fates and posterior genes such as **T** expressed radially in the proximal part of the epiblast. **Otx2** is normally expressed throughout the VE, but recent experiments have shown that expression of **Otx2** specifically in the distal VE cells by transgenic rescue can restore proper alignment of the A-P axis in **Otx2** mutant embryos [21••].

Furthermore, germ-layer explant experiments have shown that AVE tissue cannot induce expression of anterior neuroectoderm markers or earlier pan-neural markers [28••]. However, the AVE can repress the expression of posterior markers such as **T** and **Cripto** in ectoderm explants, demonstrating that its primary role is to restrict expression of posterior genes involved in mesoderm induction, thereby allowing the anterior epiblast to remain receptive to later neural induction and patterning. **Otx2** appears to be essential for this repression, because AVE explants lacking **Otx2** are unable to repress **T** and **Cripto** expression in the ectoderm [21••].

Significantly, new data indicate that embryos lacking **Nodal** function do not express any anterior or posterior markers in either the epiblast or VE. Although **Nodal** is required in the epiblast for maintaining gene expression within the extraembryonic ectoderm and for mesoderm induction, it is also necessary for activating the **Smad2** pathway in the VE, which in turn causes expression of AVE genes required for anterior development [17••].

**Mesoderm induction in the posterior epiblast is independent of AVE patterning**

As AVE patterning precedes primitive streak formation, streak formation should not affect AVE patterning. Indeed, recent work has confirmed that AVE patterning occurs independently of primitive streak formation. **Wnt3** is expressed in the proximal epiblast and the adjacent proximal VE at the egg cylinder stage (Figure 3), and subsequently becomes restricted to the posterior epiblast and VE coincident with AVE movement [29••]. **Wnt3** mutants express AVE markers such as **Lhx1** and **Cerl**, but fail to express streak markers such as **T** and **Fgf8**. The epiblast expresses undifferentiated ectoderm markers, including **Otx2** and **Pou5f1**. **Bmp4** expression is normal in **Wnt3** mutants, suggesting that the signaling properties of the extraembryonic ectoderm are unaffected.

In addition to being expressed in the trophectoderm and extraembryonic ectoderm, **Eomes** is expressed in the posterior epiblast before gastrulation (Figure 3). The early defects associated with lack of **Eomes** in the trophectoderm and extraembryonic ectoderm are rescued in chimeric embryos with normal extraembryonic tissues but mutant embryonic tissues, resulting in expression of proximal–posterior markers such as **T** and **Fgf8** in the epiblast, as well as AVE markers such as **Hex1**. However, absence of **Eomes** in the epiblast abolishes mesoderm formation. Thus, in this context, the AVE can develop normally in embryos that lack a primitive streak [11••]. Similarly, **Mesd** mutants lack mesoderm formation. In these embryos, proximal epiblast genes such as **T** and **Fgf8** fail to be expressed at the primitive streak stage, but AVE markers such as **Cerl**, **Lhx1** and **Mrg1**, and extraembryonic markers such as **Bmp4** are expressed (B Holdener, personal communication).

Thus, **Eomes**, **Wnt3** and **Mesd** are required for mesoderm formation, but are not responsible for the induction of AVE...
genes. Nodal seems to perform this function by signaling from the epiblast to the VE through Smad2 [17••]. Nodal is also necessary for the induction of mesoderm, as mutant embryos fail to express Wnt3 and Eomes. This places Nodal at the apex of a signaling mechanism that regulates both anterior and posterior development. Although Nodal signals may function in a tissue-specific manner in the epiblast to activate Wnt3 and Eomes expression, it is also possible that the induction of these genes by Nodal is indirect — via the maintenance of gene expression in the extraembryonic ectoderm, which has been shown to be capable of inducing posterior genes in the epiblast. Further work will be required to clarify this mechanism.

A possible role for the VE in patterning the posterior epiblast

The VE plays a crucial role in anterior development, and recent data provide hints that it may also be important in patterning the posterior of the embryo. As mentioned above, Wnt3 expression initially in the proximal epiblast and VE at the egg cylinder stage becomes restricted to posterior epiblast and VE just before gastrulation (Figure 3) [29•]. In addition, explant culture experiments have shown that early streak stage VE can cause anterior ectoderm to differentiate into hematopoietic cells, which normally arise from the posterior streak [30].

Furthermore, it has recently been shown that Bmp2 is expressed at elevated levels in the posterior VE overlying the primitive streak during gastrulation and is required for proper primordial germ cell and allantois formation [31]. Amnionless, a novel type I transmembrane protein expressed throughout the VE during gastrulation, is required for forming the middle primitive streak derivatives [32]. The extracellular domain of Amnionless contains regions of homology to the Bmp-binding cysteine-rich domains in Chordin, possibly implicating it in modulation of Bmp signaling; however, it is not yet clear exactly how Amnionless might interact with the Bmp pathway. Much work remains to be done to elucidate the role of the VE in posterior patterning.

The anterior primitive streak gives rise to the ADE, which patterns the anterior neur ectoderm

Although the AVE appears to repress posterior signals in the epiblast, it is unable to pattern the neur ectoderm or cause formation of anterior embryonic structures. The node, which forms at the anterior primitive streak at late gastrula stages, is a classical ‘organizer’, which is capable of inducing a secondary trunk axis in transplantation experiments [33]. Like the AVE, however, it is unable to induce secondary anterior structures even when node precursor cells are transplanted from an early gastrula stage [28••], and node ablation fails to abrogate formation of anterior structures [34,35]. Recent transplantation experiments have demonstrated, however, that a mixed graft of the AVE and the anterior epiblast plus the anterior streak can induce anterior neural genes [28••].

During gastrulation, the ADE arises from the anterior streak region before node formation and notochord extension, and moves anteriorly to displace the AVE and underlie the prospective neur ectoderm [36,37]. It expresses many of the same genes as the AVE, such as Hex and Gerl, making it an attractive candidate tissue from the anterior streak for patterning the anterior epiblast [38••]. Indeed, at late gastrula stage, removal of the rostral region of the anterior midline tissue, which includes the ADE together with prechordal plate and axial node derivatives, results in truncation of the anterior neur ectoderm [39]. This supports a model in which the AVE primes the epiblast for anterior patterning, and the ADE and other anterior streak derivatives pattern the anterior neur ectoderm.

Recent genetic studies support this model. For example, embryos lacking the forkhead DNA-binding protein Foxh1/2 (also known as FAST), which is thought to bind Smad2 in response to Nodal signaling, fail to form anterior streak derivatives although the AVE is specified [40]. These embryos lack anterior CNS structures, showing that the ADE and/or other anterior axial tissues arising from the anterior streak are required for anterior patterning.

Further support comes from embryos lacking the RING-domain protein Arkadia (Ark), which is ubiquitously expressed but has been shown by chimera analysis to be required in extraembryonic lineages for node formation [41••]. Ark-deficient embryos are able to specify an AVE, but it is displaced anteriorly by a population of cells that lacks expression of ADE or mesendodermal markers, consistent with failure of Ark mutants to form a patent node. Presumably as a result of this mis-specification, the underlying epiblast fails to maintain expression of anterior neur ectoderm markers. Ark interacts genetically with Nodal in the mouse [41•], and its ability to induce anterior endodermal markers in Xenopus ventral marginal zone explants is blocked by cer-S, a Nodal antagonist [42•].

It has previously been shown that chimeric embryos in which extraembryonic tissues lack Nodal expression but embryonic tissues are wild type develop anterior truncations [43]. In addition, cells lacking Smad2 are unable to colonize the ADE in chimeras, but can contribute to other anterior streak derivatives [44•]. Together, these data suggest that Smad2 may be required in the anterior streak to receive Nodal signals from the overlying VE in order to segregate ADE from other anterior streak derivatives.

Similarly, embryos mutant for the Hex gene have forebrain defects [38••]. In addition to its early expression in the AVE, Hex is expressed specifically in the midline ADE, and chimera experiments indicate that it is indeed required in the embryonic lineages for anterior patterning. Although some defects in the formation of prechordal and notochordal plate tissues are observed in Hex mutants, expression of Foxa2 and Lhx1 is preserved in the node, and the notochord expresses Foxa2. The loss of only forebrain
tissue — as opposed to truncations, including mid or hindbrain territories that characterize Foxa2 and Lhx1 mutants, in which notochord is not formed [45–47] — suggests that the ADE, which begins to displace the AVE at early streak stages, may function together with prechordal plate to pattern the anteriormost neural tissue, and subsequently may coordinate with later ingressing node-derived structures to pattern the mid and hindbrain.

Conclusions

Rapid progress has been made in unraveling the cellular and molecular basis of early axis patterning; however, much remains to be learned. It will be important to understand how the sperm entry point is linked to embryonic polarity. It is possible that local cytoskeletal reorganization of the actin cortex is responsible — perhaps through the localized uncapping of barbed ends of actin filaments, which stimulates actin polymerization and membrane protrusion. The positioning of the mitotic spindle is thought to be linked to actin architecture, and this may be the mechanism by which the cleavage planes are oriented in the zygote. How the temporal division advantage in the cleavage-stage embryo translates into differential gene expression and the establishment of cell fate remains to be explored.

Animal–vegetal polarity in the blastocyst appears to be translated into P-D polarity at the egg cylinder stage, but how this occurs is still unknown. We now appreciate that coordinated global cell movements accompany the molecular patterning seen in the early post-implantation embryo; however, we have little understanding of the basis of the morphogenetic program that elicits this stereotyped movement in these cells. For example, it will be interesting to uncover whether differential cell division or active migration is responsible for AVE movement. It will also be of interest to understand how gastrulation-stage interactions between the VE and the epiblast control specification of the anterior streak derivatives, and how these derivatives in turn contribute A-P pattern to the neurectoderm.

Finally, many of the experiments reviewed here were inspired by the work of our friend and colleague Rosa Beddington. Her insightful predictions [17] about the importance of “the reciprocal interactions that must take place between the future embryo and its surrounding embryonic tissues” provided the conceptual framework for what we now understand to be the means by which patterning in the mouse embryo “is progressively stabilized, refined, and embellished”.

Update

A new paper [49], published in the same issue of *Genes and Development* as [40], further characterizes the Foxd11/2 mutant.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- **of outstanding interest**


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39. The role of Hex, a homeodomain protein expressed in the AVE and ADE, has been studied by targeted mutagenesis. Loss of Hex does not prevent AVE formation, but development of the AVE and axial mesendoderm is compromised. Chimaera studies firmly establish that Hex expression in the AVE is required to promote forebrain development, and provide the first concrete evidence that signals from the AVE are essential for establishment of rostral pattern within the central nervous system.


This paper describes the identification of a novel RING domain containing protein, Arkadia (Ark), that is required for correct specification of anterior streak derivatives. Ark-deficient embryos fail to form a node or definitive endoderm. Chimaera experiments show that Ark, which is ubiquitously expressed in the embryo, is required in the extraembryonic lineages for node specification, providing the first evidence that signals from the extraembryonic cells are required to specify the mammalian organizer. The authors uncover a genetic interaction between Ark and Nodal, leading them to propose that Ark modulates Nodal signaling between the VE and epiblast, and influences cell fates in the anterior streak.

Overexpression of the Xenopus Arkadia homolog and Xnr-1 is shown to result in the formation of mesendoderm versus mesodermal fates. The Xnr-1 antagonist Cer-S blocks this activity. These experiments underscore the involvement of Ark in conserved TGF-β1 signaling pathways responsible for induction of the vertebrate organizer.


This study uncovers an essential requirement for Smad2 in specification of the definitive endoderm during gastrulation. Chimera studies using LacZ-marked Smad2-deficient cells show that Smad2 cells are excluded from the gut endoderm. Thus in mouse, TGF-β signaling pathways that are mediated by activated Smad2 complexes are responsible for recruiting distal streak cells into an endodermal versus mesodermal fate.


