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Axis Development and Early Asymmetry in Mammals

Review

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Introduction

Studying the formation of the embryonic axes in mammals poses a special problem because patterning of the embryo itself is a relatively late event in development. In the mouse, explicit pattern in the tissue that will generate the future organism only becomes apparent one-third of the way through a gestational period of 3 weeks. This tardiness of eutherian mammals is due to viviparity. The earliest stages of their development are necessarily preoccupied with the generation of extraembryonic tissues essential for survival in utero. This means that the conceptus, comprising both the extraembryonic and embryonic components, is already a complex structure and implanted in the uterus by the time the embryo itself begins to acquire obvious pattern. For this reason, the architecture of the conceptus as a whole cannot be ignored when considering the origin of the embryonic axes of mammals. This contrasts with the development of most invertebrates and anamniote vertebrates, where, from the moment of fertilization, embryogenesis is wholly concerned with defining the pattern of the future organism. Indeed, in many cases, this patterning begins during oogenesis when molecular asymmetries are laid down in the egg.

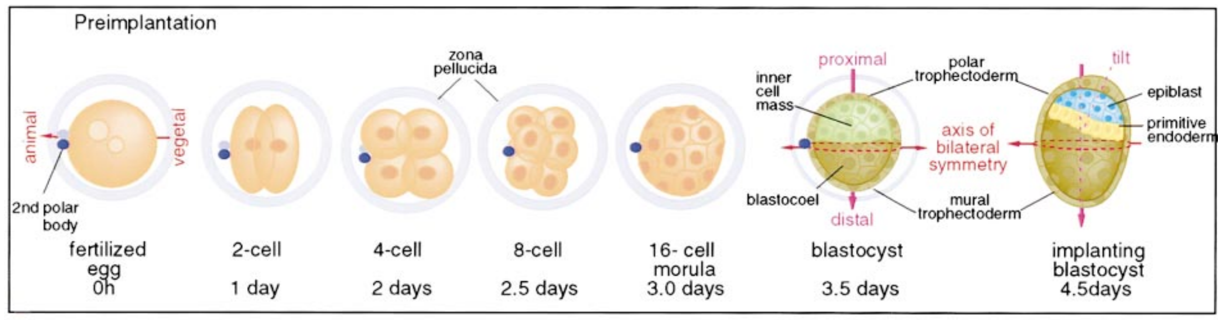
In this review, we will explore some of the characteristics of mouse development relevant to axis development. We will consider whether the pattern that develops in the mouse conceptus influences pattern that appears later in the embryo, and we will discuss what is known about how the definitive axes of the embryo (anterior–posterior [A–P]; dorsal–ventral [D–V]; and left–right [L–R]) are laid down during mammalian development.

Anatomy of Early Mouse Development

Figure 1 illustrates the first week of mouse development (see Hogan et al., 1994; Tam and Behringer, 1998, for more comprehensive review). The fertilized mouse egg is a small (approximately 80 μm in diameter) cell. It has a polarity because the polar bodies generated by the first and second meiotic divisions are sequentially extruded at the same site, where they remain tethered during ensuing cleavage (Gardner, 1997). The site of their extrusion has been called the animal pole. Although cleavage stages are prolonged, taking approximately 3 days for the egg to produce 16 cells, the zygotic genome is activated and maternal mRNA degraded at the two-cell stage, within 24 hr of fertilization.

Cleavage culminates in blastulation when two distinct tissues form: the trophectoderm (TE) and the inner cell mass (ICM). These are mutually exclusive tissue lineages, since the progeny of the TE contribute only to the trophoblast and extraembryonic ectoderm and those of the ICM to the fetus and extraembryonic mesoderm and endoderm (Gardner, 1983). The segregation of these two tissues is presaged by polarization of individual blastomeres during cleavage, first manifest at the eight-cell stage. A series of asymmetric or symmetric cell divisions ensues that results in segregation of polar cells, which form the TE, and apolar cells, which form the ICM (Johnson and Ziomek, 1981). Formation of the blastocyst gives unequivocal polarity to the conceptus with the ICM situated at one end, which we will call proximal. More recently it has been confirmed that the nascent blastocyst is also bilaterally symmetrical, being invariably oval rather than spherical (Gardner, 1997). However, this axis of bilateral symmetry does not seem to be polarized in that one end cannot be distinguished from the other (Figure 1, red arrowheads). At 4.5 days, a third tissue differentiates on the surface of the ICM. This is the primitive endoderm destined to generate only the extraembryonic parietal and visceral endoderm (Gardner, 1983). The remainder of the ICM, now known as epiblast, will give rise to the entire fetus, including the germ line and all the extraembryonic mesoderm.

Polar TE continues to divide, producing extraembryonic ectoderm that pushes the epiblast and enveloping visceral endoderm toward the distal pole of the conceptus. A cavity forms in the center of the epiblast, due to a mixture of apoptotic and survival signals from the visceral endoderm (Coucouvanis and Martin, 1995), and the epiblast becomes a cup-shaped epithelial tissue. The first morphological sign of embryonic pattern is evident 6.5 days after fertilization when gastrulation begins. Epiblast cells at one point on the rim of this otherwise apparently homogeneous epithelium undergo an epithelial–mesenchymal transformation to generate mesoderm and form the primitive streak. This point defines the posterior pole of the future embryo, and, thus, the diametrically opposite side of the cylinder is recognized as anterior. During the next 12–24 hr, the streak elongates from the rim of the cup to its distal tip. Here, at the anterior end of the streak, a specialized structure forms, which is known as the node (Figure 1). For reasons given below, the node is considered homologous to the organizer of other vertebrate embryos. The node generates axial mesendoderm, which comprises that mesoderm which will populate the midline of the embryo (prechordal plate and notochord) and the definitive gut endoderm. Until the node forms, and hence the definitive gut endoderm, the epiblast remains enveloped in extraembryonic visceral endoderm. The posterior end of the streak gives rise to extraembryonic mesoderm, while lateral plate mesoderm and paraxial mesoderm emerge from the intervening levels of the streak. The midline mesoderm from the node corresponds to what is called



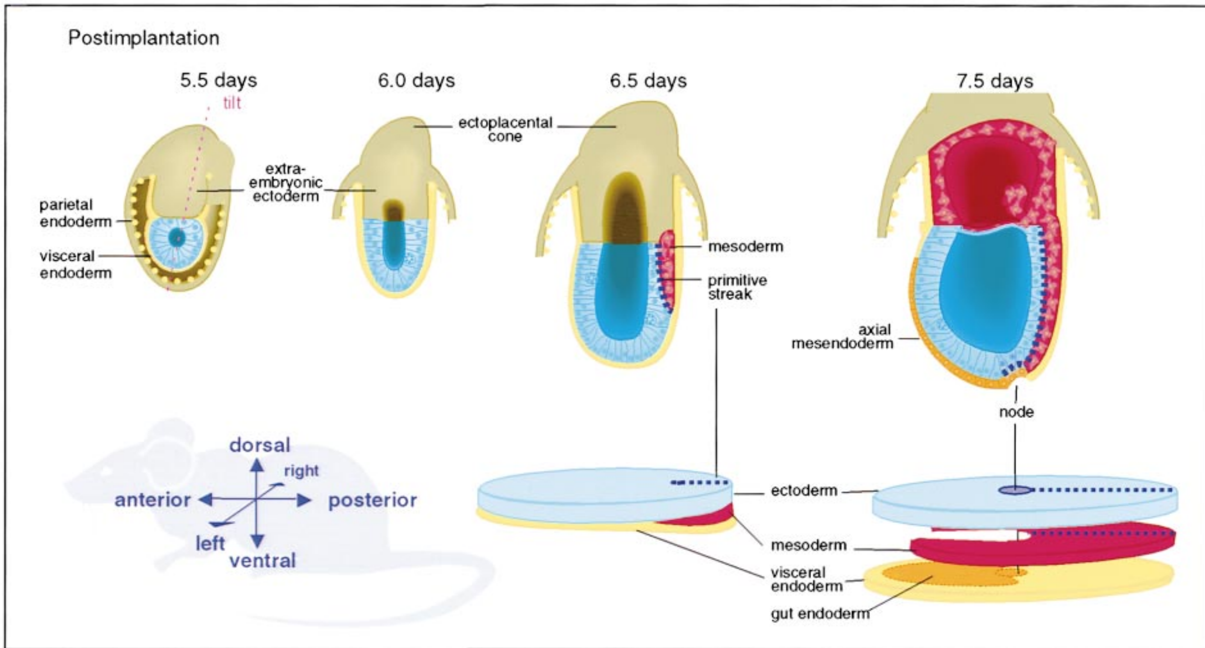


Figure 1. Diagram of the First 7.5 Days of Mouse Development from Fertilization

Preimplantation development is depicted on the top row with postimplantation development on the bottom (not drawn to scale). The germ layers of the 6.5 and 7.5 day embryos are also shown as flattened sheets for ease of comparison with other vertebrate and mammalian embryos. The major axes of the embryo are shown superimposed on the profile of an adult mouse.

dorsal mesoderm in other vertebrates and the extraembryonic and lateral mesoderm to ventral. Hence, the origin of the primitive streak is critical in defining not only the A–P but also the D–V axis. Since the orientation of the L–R axis is dependent on the orientation of the other two axes, L–R pattern also ultimately relies on where the streak forms. Not surprisingly, this morphological sequence of events suggested that pattern formation in the mammalian embryo could be explained once the cues for localizing streak formation were understood.

It is worth remembering that as well as the presence of elaborate extraembryonic tissues, mammalian embryos arrive at gastrulation in a very different state to that of other vertebrate embryos. There is a marked difference in size, there being only about 600 epiblast cells when gastrulation begins in mouse compared with several thousand cells in an equivalent stage *Xenopus* embryo. Presumably because of this, mouse embryos grow rapidly during the periimplantation period and, unlike *Xenopus*, continue to do so throughout gastrulation, epiblast cells dividing on average at least every 6 hr and in some places as fast as every 2 hr (Snow, 1977). Furthermore, unlike the predominantly coherent growth seen in pregastrula *Xenopus* embryos, the epiblast cells of the mouse intermix extensively before gastrulation (see below). These differences, together with the early activation of the zygotic genome, early degradation of maternal mRNA, and the lateness of gastrulation, make it hard to see how determinants present in the mouse egg could persist long enough directly to influence embryonic pattern. However, if embryonic pattern is dictated by the early axis of bilateral asymmetry in the blastocyst, then it is conceivable that pattern in the fertilized egg could determine the axes of the conceptus and thereby those of the embryo.

Axes of the Conceptus

In a trivial sense the proximal–distal axis of the blastocyst obviously corresponds to the D–V axis of the embryo (Figure 1). However, this is more of a geometrical coincidence than one that has meaning in terms of determining pattern formation. As stated above, the D–V axis of the embryo, with respect to where the more

dorsal tissues of the embryo originate as opposed to ventral ones, is dictated not by the shape of the conceptus but rather by where the primitive streak forms.

The first intimation that axes of the conceptus might influence the orientation of the A–P axis of the embryo came from histological studies on implanting blastocysts and early postimplantation egg cylinders (Smith, 1980, 1985). It appeared that the tilt of the 4.5 day blastocyst was preserved during implantation as a persistent bending of the ectoplacental cone to one side (Figure 1). From these static descriptive studies, it was claimed that this side corresponded to the side on which the primitive streak would form. However, when the visceral endoderm was marked with horseradish peroxidase on the side toward which the ectoplacental cone tilted and, subsequently, the site of the nascent primitive streak determined, it was found that the streak was just as likely to form on the opposite side (Gardner et al., 1992). Therefore, at present, no decisive evidence indicates that the axes of asymmetry in the postimplantation conceptus directly influence A–P pattern in the embryo itself. Moreover, the essential experiment of indelibly and clonally marking one or the other end of the 4.5 day blastocyst tilt in order to examine whether there is any relationship between the direction of the tilt and where the later A–P axis forms has yet to be attempted.

Before implantation the tilt of the 4.5 day blastocyst probably arises from the axis of bilateral symmetry described in nascent blastocysts. By tracing the location of polar bodies, this axis of bilateral symmetry has been traced back to the animal–vegetal axis of the fertilized egg (Gardner, 1997). Very unexpectedly those polar bodies, tethered by a cytoplasmic bridge to their original site of extrusion, are not randomly positioned at the early blastocyst stage. They are almost always situated in line with the blastocoelic surface of the ICM and at one or other pole of the axis of bilateral symmetry (Figure 1). This implies that in undisturbed development the animal pole of the egg maps to one pole of the early blastocyst's axis of bilateral symmetry. Furthermore, the proteins leptin and STAT3 are clearly localized to the animal pole of mouse and human oocytes and during cleavage preferentially segregate to the trophectoderm rather than ICM lineage (Antczak and Van Blerkom, 1997). Does this mean that pattern in the mammalian egg does, after all, influence pattern in the conceptus and, thereby, possibly in the embryo itself?

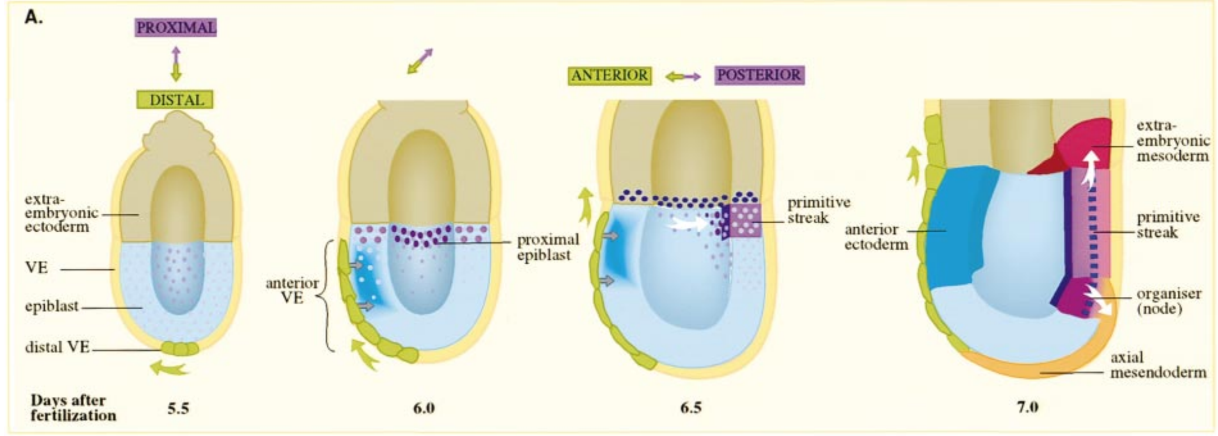
The developmental lability of the preimplantation mammalian embryo has always been taken to indicate that neither mosaicism nor graded information in the egg could be essential for dictating subsequent embryonic pattern. Recently it has been shown that fertile offspring can be produced from eggs in which a substantial volume of either the animal or vegetal pole of the egg has been mechanically removed (Zernicka-Goetz, 1998). This argues against localized determinants in the egg being required for subsequent pattern formation, although it does not necessarily rule out the presence of an influential gradient capable of regulation. Cleavage planes dictate that only at the eight-cell stage would blastomeres contain cytoplasm from different animal–vegetal levels of the egg, and it is true that the potency of all eight individual blastomeres from a single embryo has never been tested (see Gardner, 1998). However, isolated blastomeres from the two-cell stage can develop into mice, and at later cleavage stages adding, removing, or rearranging cells seems to have no effect on the pattern of the surviving embryos. Such experiments also indicate that individually cells remain totipotent up to the time of blastulation, although their position during cleavage will influence their fate—inside cells tending to become ICM and outside cells becoming TE (see Hogan et al., 1994).

The strongest argument against graded cues present in the egg being responsible for dictating the orientation of axes is that when intact eight-cell embryos are simply placed in contact with one another they will aggregate and form a single chimeric embryo. The initial aggregation and formation of a chimeric blastocyst occurs without cells from the two eight-cell embryos intermixing or sorting out (Garner and McLaren, 1974). Surely, the formation of two separate individual embryos, rather than a single chimeric one, would be the more likely outcome if each eight-cell embryo contained its own array of axial determinants inherited from the egg. It has been argued (Gardner, 1998) that the proportion of nonchimeric offspring derived from such aggregation experiments is significant, but the reasons for this nonchimerism have never been clarified. Therefore, although it cannot be ruled out that pattern in the mammalian egg normally and necessarily influences the pattern of the blastocyst, there is no experimental evidence to demonstrate that it does.

While the axes of the conceptus have not yet been shown to directly influence the embryonic ones, the importance of extraembryonic tissues in initiating embryonic pattern is becoming obvious. This is implicit from the widespread distribution of ICM clones produced by blastocyst injection and examined using an in situ lineage marker during gastrulation and early organogenesis (Beddington et al., 1989). Clonal descendants are found in all embryonic tissue types and distributed throughout the length and breadth of the embryo, showing that epiblast cells must intermix extensively prior to gastrulation. Such mixing of clonal epiblast descendants has now been shown to coincide with the epiblast becoming an epithelium on the sixth day of gestation (Gardner and Cockroft, 1998). Importantly, such cell mixing makes it unlikely that patterning information for axis formation can reside in the preimplantation epiblast. Therefore, it is more probable that it is either the extraembryonic tissues or the mother that provides the initial cues for axis specification in mammals. Influences from the mother via the uterus can probably be discounted, since it is possible to develop early somite stage embryos with normal axes from blastocysts grown in vitro (Hsu, 1979).

Origin of the D–V and A–P Axes

Undoubtedly, where the primitive streak forms is critical to the orientation of both the A–P and D–V axes. However, surprisingly, evidence has emerged recently for precocious anterior patterning in the extraembryonic endoderm (anterior visceral endoderm [AVE]) of the mammalian conceptus, in both the mouse and the rabbit, before there is any sign of primitive streak formation



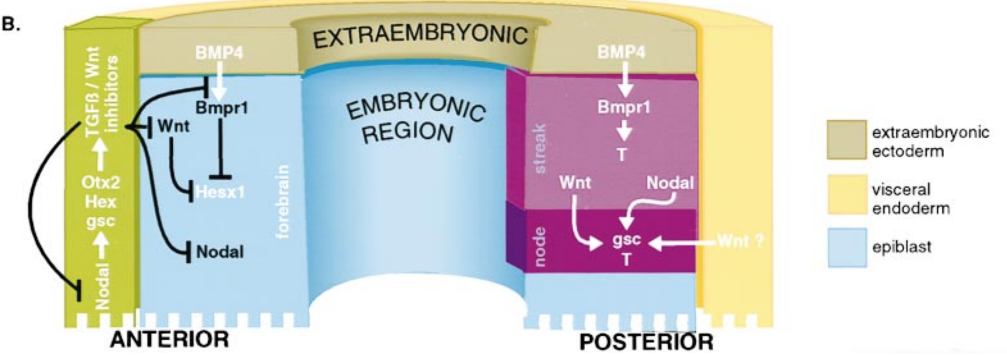


Figure 2. Cell Movements and Molecular Signals Controlling Axis Formation

(A) Changes in cell movement and gene expression that mark the conversion of the proximal–distal axis of the conceptus into the A–P axis of the embryo. At 5.5 days, distal visceral endoderm cells (green), marked by *Hex* expression, give rise only to anterior progeny, which populate the AVE and eventually move into the extraembryonic region (green arrows). The AVE induces anterior character in the underlying epiblast (turquoise). Transcripts of future markers of the primitive streak such as *Cripto* (lilac) are ubiquitously expressed in the epiblast (pale blue) at 5.5 days but restricted to the proximal rim of epiblast at 6.0 days, where other markers of the primitive streak, such as *T* (deep purple) start to be expressed. *Bmp4* (dark blue) is transiently expressed in the adjacent extraembryonic ectoderm. Caudal cell movement in the proximal epiblast (white arrow) results in the primitive streak forming opposite the AVE at 6.5 days. By 7.0 days extraembryonic mesoderm (crimson), which may be regarded as ventral in character, is produced from the posterior aspect of the streak while anteriorly the organizer (mauve) forms, which gives rise to dorsal mesoderm and gut endoderm (axial mesendoderm; orange).

(B) Possible scheme of the molecular interactions that may differentiate A from P at 6.5 days. One role of the AVE would be to provide the requisite TGFβ and Wnt inhibitors while the streak is being induced in order to allow expression of anterior markers such as *Hesx1*.

(reviewed by Beddington and Robertson, 1998). Moreover, this anterior extraembryonic endoderm has been shown to be functional in providing the embryo with anterior character. This is unexpected because years of experimental analysis in *Xenopus* and other vertebrate embryos have ascribed anterior patterning to products of the organizer—the earliest axial mesendoderm emanating from the organizer being responsible for inducing ectoderm to become neural and giving this neural tissue explicit A–P pattern (reviewed by Harland and Gerhart, 1997). Therefore, to have anterior pattern essential for complete axis formation before the streak, and hence the organizer, has formed runs contrary to traditional thinking.

Pattern and Cell Movement in the AVE

In the rabbit embryo, the AVE underlying the epiblast is morphologically distinct, being more columnar in character than the rest of the visceral endoderm before gastrulation starts (Viebahn et al., 1995). In the mouse such a morphological difference is not apparent, but the AVE can be distinguished molecularly (Figure 2A). Expression of the VE-1 antigen (Rosenquist and Martin, 1995) and of the genes *Otx2* (Ang and Rossant, 1994; Acampora et al., 1995), *Lim1*, *goosecoid*, *cerberus-related 1* (Belo et al., 1997), and *Hex* (Thomas et al., 1998) is restricted to a medial strip of the AVE underlying approximately the anterior third of the epiblast at least 12 hr before the streak has formed. As the streak forms, the most anterior extreme of the AVE in the region where the heart will develop starts to express *Mrg1* (Dunwoodie et al., 1998), and slightly more posteriorly, where it overlies epiblast fated to give rise to oral ectoderm and forebrain (Thomas et al., 1997), *Hesx1* is expressed (Thomas and Beddington, 1996). Thus, as gastrulation starts, the AVE is already a finely patterned tissue and so cannot depend on its patterning on products of the streak.

Cell lineage studies have shown that the AVE is derived from a handful of endoderm cells situated at the

distal tip of the 5.5 day conceptus, which alone express *Hex* (Thomas et al., 1998). Remarkably, these distal cells give rise only to anterior descendants (Figure 2A). Whether this anisotropic cell movement in the visceral endoderm layer is due to differences in proliferation, the orientation of cell divisions, or some kind of barrier preventing posterior movement remains to be seen. Shortly after this anterior movement of visceral endoderm cells commences it is mirrored by a posteriorward movement of epiblast cells at the embryonic–extraembryonic junction (Lawson et al., 1991). In this way the ring of gene expression in the proximal epiblast of primitive streak markers, such as *T* (Thomas and Beddington, 1996), resolves to the posterior where gastrulation begins (Figure 2A). Although cellular in nature, this "rotation," brought about by the anterior movement of distal visceral endoderm cells and the posterior movement of proximal epiblast cells, bears some resemblance to the directed movement of the oocyte nucleus in response to signals from follicle cells in the egg chamber of *Drosophila*, needed to establish the major axes of the fruit fly (Gonzalez-Reyes et al., 1995).

Recently, a mutation in *Cripto* has been described in the mouse that prevents the appropriate rotation of visceral endoderm and epiblast (Ding et al., 1998). *Cripto* is a gene encoding a member of a family of extracellular, membrane-associated proteins that contain cysteine-rich and epidermal growth factor–like motifs, and there is evidence that Cripto may be a signaling molecule that activates the MAPK pathway (Kannan et al., 1997). The product of the *one-eyed pinhead* gene in zebrafish, which when mutated results in defective anterior development and cyclopia, also belongs to this family (Zhang et al., 1998). In the mouse embryo, *Cripto* initially is expressed uniformly in the epiblast, but before gastrulation its transcripts become restricted to the region of the proximal rim of the epiblast that will give rise to the streak (Figure 2). Embryos homozygous for a loss-of-function mutation in *Cripto* do not orientate their A–P axis correctly. Instead, both AVE markers and anterior ectoderm markers such as *Hesx1* remain located distally, and extraembryonic mesoderm markers, which should be posterior, are proximal. Interestingly, although there is neither a streak nor an organizer, an ectopic AVE is present, and even more revealing, the epiblast adjacent to it assumes anterior character.

The AVE Effects Embryonic Anterior Pattern

The anterior nature of the ectoderm present in *Cripto*$^{-/-}$ embryos (Ding et al., 1998) suggests that visceral endoderm can impart anterior pattern in the absence of an organizer. Additional evidence that the AVE is important in establishing the anterior terminus of the mammalian embryo comes from several different kinds of experiments. Removal of the AVE at the earliest stages of gastrulation prevents or diminishes expression of forebrain markers (Thomas and Beddington, 1996). Conversely, recombining chick epiblast with the AVE from pre–streak stage rabbit embryos induces expression of forebrain markers in the chick epiblast (Knötgen et al., 1999). Interestingly, the anterior hypoblast from mid–streak stage chick embryos does not elicit this response in epiblast, nor does its removal prevent epiblast from expressing forebrain markers (Knötgen et al., 1999). Either the anterior chick hypoblast lacks the patterning properties of the mammalian AVE, or it functions earlier than has been tested during chick development.

Mutations in a number of genes that are first expressed in the AVE and only subsequently in epiblast derivatives affect anterior development. These include *Hesx1* (a *paired-like* homeobox-containing gene; Dattani et al., 1998), *Lim1* (a member of the LIM class of transcription factors; Shawlot and Behringer, 1995), and *Otx2* (encoding a homeodomain-containing transcription factor related to *Drosophila otd*; Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). For example, in *Otx2*$^{-/-}$ embryos at egg cylinder stages, the posterior rotation of epiblast seems to occur normally but the visceral endoderm remains distal (Acampora et al., 1998), and the resulting embryos are missing midbrain and forebrain. More complex genetic interactions are indicated by the anterior truncations observed in embryos that are heterozygous for *HNF3*β, a member of the winged helix forkhead family of transcription factors expressed throughout the visceral endoderm, and that also either lack *gsc* (yet another homeobox containing gene; Filosa et al., 1997) or are heterozygous for a mutation in *nodal* (a member of the TGFβ superfamily encoding secreted signaling molecules; Varlet et al., 1997b). On their own these mutants do not reveal whether wild-type gene function is necessary in the AVE or needed only in epiblast derivatives. However, when ES cells are injected into blastocysts they preferentially colonize epiblast and its derivatives (Beddington and Robertson, 1989), and, therefore, it is possible to make chimeras in which the extraembryonic tissues can be of one genotype and the epiblast and its derivatives of another. In this way a number of genes have been shown to be essential in extraembryonic tissues for development to proceed normally, and in a few cases this requirement has been shown to be a patterning one rather than simply nutritive.

When chimeras are made from wild-type ES cells injected into *Otx2*$^{-/-}$ morulae, anterior development is never initiated, even though the epiblast may be exclusively or predominantly wild type (Figure 3). When *Otx2*$^{-/-}$ ES cells are injected into wild-type morulae, producing predominantly mutant epiblast, anterior development is initiated correctly, but it is not maintained (Rhinn et al., 1998). Consequently, *Otx2* function seems to be required in the extraembryonic tissues, presumably in visceral endoderm where it is expressed, if anterior patterning is to commence. Interestingly, substitution of *Otx2* by its close relative *Otx1*, by recombining *Otx1* into the *Otx2* locus, also rescues only the initiation but not the maintenance of anterior patterning. In this case the *Otx1* transgene is translated normally in visceral endoderm but is only transcribed in the epiblast (Acampora et al., 1998). This curious observation may reflect natural differences in translational control between the extraembryonic endoderm and epiblast tissue lineages.

An extraembryonic requirement for the *nodal* gene, which interacts with *HNF3*β during head development, has also been demonstrated (Figure 3; Varlet et al., 1997a). Embryos that lack *nodal* fail to gastrulate, but when wild-type ES cells are injected into *nodal*$^{-/-}$ blastocysts, gastrulation proceeds. In these chimeras, embryos develop with an appropriately patterned trunk,

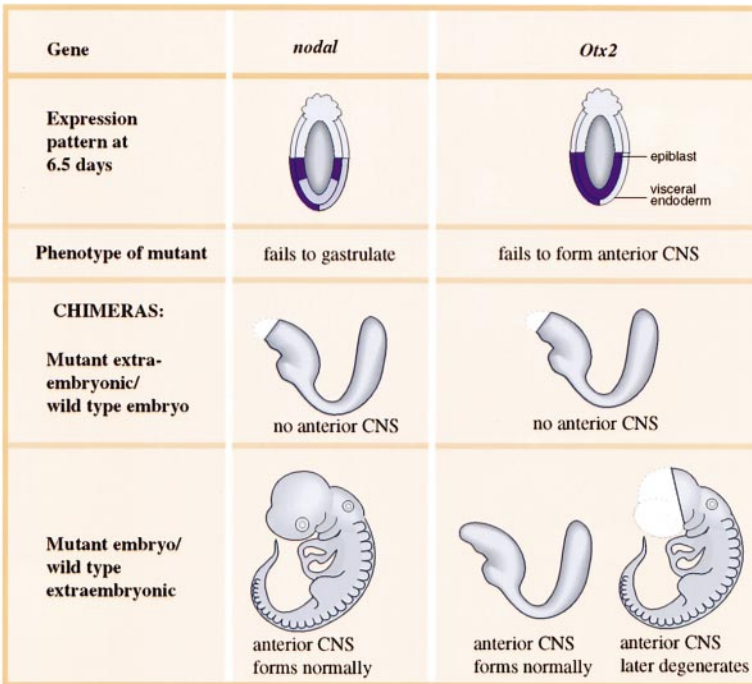


Figure 3. Visceral Endoderm Influences Anterior Pattern

The expression patterns of *nodal* and *Otx2* at the onset of gastrulation and the results from chimera experiments demonstrating that each gene is required in the visceral endoderm for normal anterior development of the embryo.

but they are missing anterior structures. The gastrulation defect is also rescued when $nodal^{-/-}$ cells are injected into wild-type blastocysts, but in this case anterior development is normal. Unlike *Otx2*, *nodal* is not expressed in anterior epiblast derivatives, but, clearly, its expression in visceral endoderm is essential if these anterior embryonic tissues are to form and be patterned correctly. Embryos that lack *Smad2*, whose transcripts are ubiquitous in the early mouse conceptus and whose product is thought to cooperate with Smad4 in order to transduce activin-like signals, make only extraembryonic (posterior) mesoderm and fail to differentiate an AVE (Waldrip et al., 1998). Again, chimeras demonstrate that absence of *Smad2* extraembryonically is sufficient to produce this phenotype. Not surprisingly, *Smad4* has also been shown to be necessary in extraembryonic tissues for the normal development and function of visceral endoderm (Sirard et al., 1998).

The nature of the TGFβ ligand or ligands whose signals need to be transduced by these Smads in extraembryonic tissues is not yet known. Activin itself is an unlikely candidate because embryos lacking activinβ_A and/or β_B subunits do not have characteristic anterior defects, and mothers deficient in activin β_B give birth to normal young (Vassalli et al., 1994; Matzuk et al., 1995a). However, embryos lacking the Activin Type IB serine-threonine kinase receptor, ALK4, fail to gastrulate, and chimeras have shown that this receptor is also required in extraembryonic tissues for normal epiblast development (Gu et al., 1998). Whatever the exact molecular pathways, it is clear that the AVE, and probably also the extraembryonic ectoderm, are essential for initiating anterior development in the embryo.

Primitive Streak Induction

How the primitive streak is induced remains unsolved. In *Xenopus* the organizer is induced by the Nieuwkoop center, this inducing activity being strongest in dorsal–vegetal blastomeres (see Harland and Gerhart, 1997). Since part of the Nieuwkoop center will give rise only to yolky cells later in development, often misleadingly called gut endoderm, they may be viewed as containing extraembryonic material. Likewise in zebrafish, ectopic injection of genes expressed in the syncitial yolk layer, which does not contribute to the embryo itself, can induce or affect organizer (embryonic shield) formation and function (Feldman et al., 1998; Yamanaka et al., 1998).

As we have seen, in the mouse some of the earliest markers of streak formation, like *T*, are first expressed circumferentially in the proximal epiblast immediately adjacent to the extraembryonic ectoderm (Figure 2A). Others, such as *Cripto* and *nodal*, are initally expressed ubiquitously in the epiblast but then maintained only in this rim (Figure 2A). Subsequently, cell movements in the proximal epiblast seem to direct these cells to the posterior where epithelial continuity is lost and the streak forms (Lawson et al., 1991). If the expression of these genes is induced or maintained by extraembryonic tissues, then both the extraembryonic ectoderm and the visceral endoderm may be involved—extraembryonic ectoderm inducing gene expression in proximal epiblast and visceral endoderm providing posterior polarity. However, unlike the AVE, no genes have yet been described that are exclusively expressed in posterior visceral endoderm. Its only distinguishing feature at present is that cell lineage studies (Lawson et al., 1987) indicate that posterior visceral endoderm cells remain in situ overlying the streak throughout streak elongation and while the node becomes patent. It is possible that the AVE provides the impetus for the posteriorward movement of epiblast cells, thereby spatially coordinating A–P and D–V development. Certainly, the characteristic constriction in the conceptus seen at the level of

the proximal epiblast in several mutants, which can be rescued by the presence of wild-type extraembryonic tissues (e.g., *Otx2*, Rhinn et al., 1998; *HNF3*β, Dufort et al., 1998), indicates that growth or morphogenesis in different regions of the epiblast may be selectively influenced either by the visceral endoderm or the extraembryonic ectoderm.

Mesoderm-inducing properties of posterior visceral endoderm are implied by the fact that isolated pre– or early streak stage epiblast can only differentiate into haemopoeitic or endothelial cells, indicative of extraembryonic mesoderm formation, if they are recombined with visceral endoderm (Belaoussoff et al., 1998). This does not seem to be just a survival effect because recombining visceral endoderm and prospective forebrain ectoderm from the anterior of mid–streak stage embryos respecifies the latter to form blood and vascular tissue. This reinforces the notion that pattern in the epiblast depends critically on influences from its local environment (Beddington, 1982). With regard to mesoderm induction, it is still not clear what kind or kinds of mesodermal tissue are being induced to elicit streak formation. Anatomically, the formation of a distinct node, or organizer, occurs several hours after the streak has been induced and extraembryonic mesoderm production has already begun. Is posterior–ventral mesoderm, in the guise of extraembryonic mesoderm, induced first and the node, which will produce anterior and dorsal mesoderm, induced secondarily? Although the expression patterns of appropriate marker genes have not been compared in the same embryo at a resolution that can distinguish the expression profiles of individual cells, it would appear that *gsc*, which is subsequently expressed exclusively in the early node (Blum et al., 1992), is coexpressed with markers of ventral mesoderm, such as *Bmp4*, when the streak is first induced (Winnier et al., 1995). Therefore, the formation of the node may involve the gradual separation of organizer and streak characteristics into different cells rather than simultaneous but distinct inductive events like those invoked to explain *Xenopus* mesoderm induction (see Harland and Gerhart, 1997). What happens in mouse is not dissimilar to the initial coexpression of homologs of Gsc and T in the same cells of zebrafish embryos followed by their segregation into separate cell populations (Schulte-Merker et al., 1994). One interpretation of how the Nieuwkoop center exerts its influence in *Xenopus* is that by localizing components of the Wnt signaling pathway it selectively alters the way in which cells respond to mesoderm-inducing signals, essentially changing their competence so that they are predisposed to form more dorsal tissue (see Harland and Gerhart, 1997). Thus, sustained but localized Wnt signaling could gradually lead to node formation at the anterior end of the mouse primitive streak, leaving posterior parts competent only to produce more ventral and posterior mesoderm.

Recently it has been shown that *Bmp4* is transiently expressed in the extraembryonic ectoderm abutting the epiblast just before gastrulation begins (Figure 2; Waldrip et al., 1998) and, thereby, could induce expression in the epiblast of certain genes that will be expressed in the streak. Certainly, the absence of BMP4 causes most embryos to arrest at the egg cylinder stage and prevents expression of the primitive streak marker *T* (Winnier et al., 1995). Likewise, embryos lacking the type I BMP receptor, ALK3, form egg cylinders but fail to make mesoderm (Mishina et al., 1995). The absence of *nodal* in the epiblast also prevents gastrulation, and by the time the streak forms *nodal* is expressed only in the posterior epiblast, so it too may effect mesoderm induction (Conlon et al., 1994). Misexpression of a Wnt1 class of signal, chick *Wnt8*, can induce an additional streak in the mouse complete with a secondary node (Pöpperl et al., 1997). Moreover, the absence of *axin*, which encodes an inhibitor of the Wnt signaling pathway, results in embryos with duplicated or multiple streaks and nodes (Gluecksohn-Schonheimer, 1949; Perry et al., 1995; Zeng et al., 1997). Hence, Wnt signaling does seem to be involved in the induction of the streak and node, but there is no evidence to date demonstrating the requirement for *Wnt* gene activity, either as a competence-modifying signal or as a mesoderm inducer.

The Role of the Organizer

The mouse node expresses homologs of many of the genes expressed in the organizer of *Xenopus* (Beddington and Smith, 1993). It also gives rise to a similar repertoire of embryonic tissues, namely prechordal mesoderm, notochord, and gut endoderm (Beddington, 1981, 1984; Lawson et al., 1991), and can induce secondary axes when transplanted (Beddington, 1994; Tam et al., 1997). These attributes make the mouse node equivalent to Spemann's organizer in frogs. Therefore, it would be reasonable to suppose that, like the frog organizer, it produces tissue that neuralizes ectoderm and provides it with A–P pattern. However, we have already seen that anterior pattern evolves well before there is a node or primitive streak, and so the mouse node must be different from Spemann's organizer. Indeed, it is unable to duplicate a complete axis; secondary axes invariably have anterior truncations and lack, for example, forebrain (Beddington, 1994; Pöpperl et al., 1997; Tam et al., 1997). The anterior truncation of secondary axes induced by ectopic chick *Wnt8* could be due to the subsequent posteriorizing properties of Wnt molecules, but this would not account either for the fact that the de novo prospective forebrain marker *Hesx1* is expressed in these embryos but never in duplicated domains or that the induced and native axes are always fused anteriorly, suggesting that there was only ever a single anterior terminus. Thus, unlike Spemann's organizer, duplication of the mouse node is not sufficient to induce duplication of the most anterior structures. In this respect, it is interesting that embryos homozygous for a mutation in *HNF3*β, which lack an identifiable node, still express diagnostic markers of anterior development (Ang and Rossant, 1994; Weinstein et al., 1994). It may also be relevant that the animal cap of *Xenopus* shows signs of D–V pattern before it is contacted by products of the organizer (see Harland and Gerhart, 1997), and that distinctive anterior CNS (row 1) cells with exceptional patterning properties form early during gastrulation in zebrafish embryos, also possibly without being induced by the organizer (Houart et al., 1998).

While the node does not initiate anterior pattern in the mouse embryo, it is undoubtedly important in maintaining and extending it, and it is no accident that node

derivatives express much the same repertoire of genes as the AVE (see Beddington and Robertson, 1998). Recombining tissues produced by the node with ectoderm shows that axial mesendoderm is necessary for induction of the midbrain–hindbrain junction marker, *Engrailed*, in neurectoderm (Rhinn et al., 1998). Similarly, chimera experiments have shown that wild-type expression of *HNF3*β in node derivatives remains essential for the appropriate D–V patterning of the neural tube, normal heart morphogenesis, and aspects of L–R axis formation (see below; Dufort et al., 1998). Therefore, axial mesendoderm from the node probably initiates A–P pattern in the trunk, including the hindbrain, but serves only to maintain and refine preexisting A–P pattern further rostrally.

Emphasis in this review has been placed on the importance of extraembryonic tissues in ordaining embryonic pattern. However, these tissue interactions are not just one way. The requirement for *Cripto* produced by the epiblast (Ding et al., 1998) is but one example of the reciprocal interactions that must take place between the future embryo and its surrounding extraembryonic tissues. Likewise, as node derivatives replace the AVE, without apparent disruption to gene expression patterns, information from anterior epiblast may be essential to preserve aspects of axial mesendoderm pattern. In this way, information imparted by one tissue to its neighbor may be returned in kind by that neighbor, either in the same or more elaborate form. So pattern is progressively stabilized, refined, and embellished.

Common Elements of Vertebrate Anterior Development

In *Xenopus*, head formation is thought to require the inhibition of BMP- and Wnt1-like activities while being refractory to the inhibition of FGF signaling (see Harland and Gerhart, 1997). Therefore, one role of the AVE may be to locally inhibit the induction of posterior or ventral development, for example by curtailing expression of *Bmp4* in the anterior extraembryonic ectoderm or by inhibiting BMP and Wnt activities (Figure 2B). In this respect, one might predict that anterior development should be initiated normally in mutant embryos lacking *Bmp4* or *BmprI/Alk3*. A requirement for inhibiting Wnt activity is indicated by the effects of ectopically expressing chick *Wnt8* in mouse embryos. As well as its streak duplicating activity, *Wnt8* can later caudalize nascent CNS, apparently expanding the midbrain at the expense of the forebrain (Pöpperl et al., 1997). Exactly how first the AVE and then node derivatives inhibit BMP and Wnt activities has yet to be established, but it is likely to involve secretion of inhibitory binding molecules that can protect adjacent ectoderm from caudalizing or mesoderm-inducing influences (Figure 2B). Obviously, molecules such as Cerberus, Chordin, Dickkopf, Noggin, Follistatin, and Frzb described in *Xenopus* are candidates (see De Robertis et al., 1997; Harland and Gerhart, 1997), although the phenotypes obtained in the mouse from loss-of-function mutations in these inhibitors, which so far have individually removed noggin (McMahon et al., 1998) and follistatin (Matzuk et al., 1995b), suggest that these molecules may have to operate in concert in order to be effective.

That the amphibian organizer is heterogeneous has been recognized for many years, not least in its distinct head organizer and trunk organizer activities (see Harland and Gerhart, 1997). What the mammalian embryo demonstrates is that these two organizing activities can belong to separate tissue lineages, the primitive endoderm (head organizer) and the epiblast (trunk organizer). The distinct origins of these organizing tissues in mammals may represent a specialization of viviparity, or they may indicate a more ancient dichotomy. The antiquity of mechanisms used to distinguish anterior from posterior is indicated by the existence of *paired-like* homeobox genes, which demarcate apical development in *Hydra* (Gauchat et al., 1998). In *Xenopus*, the yolky cells in the depths of the organizer may be regarded as the head organizer because they express *cerberus*, a gene encoding a BMP inhibitor, whose ectopic expression can induce secondary anterior structures (Bouwmeester et al., 1996). If these cells are equivalent to the AVE, which is known to express a *cerberus-like* gene, are they induced by the same cues that generate the trunk organizer? If they are induced and controlled in a similar way to the trunk organizer, then this is not the case for the AVE in mammalian embryos. However, the separate head and trunk organizer locations recently documented in the postimplantation mouse conceptus do not necessarily mark when symmetry is first broken to create these different organizing centers. In order to really understand how A–P and D–V pattern arise and how their patterning is coordinated in mammals, it is imperative to establish the earliest time and place that instrumental pattern is laid down. At present, although this is already known to be earlier than was supposed, its origin has yet to be pinpointed.

It must be admitted that axis development is a somewhat abstract notion. What is studied is the origin of different tissues with different properties, not the emergence of an imaginary line running the length of an organism. While a mathematician draws the imaginary line to explain what has happened, the embryo does not. In *Drosophila*, the early A–P axis has distinct termini that elaborate a spatial gradient between both termini. This gradient determines the expression boundaries of gap genes whose removal will lead to a gap phenotype, for example, intact termini but a missing midsection. In mammals no such gap phenotypes have been described for early axial pattern. Loss of part of the trunk invariably includes loss of one or other of the termini. It may be that in a rapidly growing embryo the production of a seamless axis depends more on the timetable and order of forming different tissues from each termini than generating a morphogen gradient that encompasses and dictates all axial levels. If in mammals we are dealing with sequential differentiation of specific tissues, whose geography may be continually adjusted because each tissue is reliant on the next for its further development, the problem becomes less how an axis is determined and more what asymmetries elicit the formation of different tissues.

L–R Axis Determination

The invariant nature of body situs within and across all vertebrate species implies that determination of the L–R

axis is controlled by a highly conserved pathway. In the mouse, although the left and right sides of the embryo are established when the A–P and D–V axes form, the embryonic body plan remains effectively bilaterally symmetrical for several days afterward. Only when the embryo reaches early somite stages does the first anatomical sign of an L–R difference become apparent: the primitive heart tube invariably curves to the right of the ventral midline. By the 6- to 8-somite stage, more extensive L–R asymmetry begins to emerge as the embryo starts to rotate about its A–P axis. This turning requires twisting of the embryo in an anticlockwise direction along its entire rostrocaudal axis. Axial rotation is coupled with ventral closure when the edges of the definitive gut endoderm and the most lateral aspects of the embryonic body wall grow toward each other and fuse along the ventral midline. The mechanism(s) underlying these orchestrated and directional morphogenetic events, which obviously require coordinated movement in multiple embryonic tissues, is poorly understood. Laterally asymmetric cell proliferation combined with the physical constraints imposed on the embryo by its surrounding extraembryonic membranes may provide both the impetus and directionality for some of them. Certainly, mesodermal tissue located at the most lateral edges of the embryo proliferates more on the left side than on the right (Miller and Runner, 1978).

During and after turning, organ situs patterns start to emerge. For example, the stomach and liver, which are both derivatives of the gut, come to lie on different sides of the midline. Bilaterally paired organs also acquire marked L–R differences. On the left side of the thoracic cavity, a single lobe lung primordium forms, whereas enhanced branching morphogenesis produces a four-lobed lung on the right. Interestingly, this difference is intrinsic to the developing organ because isolated left and right lung primordia in culture maintain their respective branching pattern. How do cells and tissues know on which side of the body they lie? This is an intriguing theoretical problem and one that has provoked a number of conceptual models (reviewed by Lander et al., 1998), the most influential of which is the notion that a chiral molecule is aligned along the A–P axis of the early embryo (Brown and Wolpert, 1990; reviewed by Levin and Mercola, 1998). While we still understand little about how initial polarity is determined, substantial progress has been made in identifying downstream genes that elaborate L–R pattern.

Genetic Insights into Early L–R Asymmetry

There are numerous reports of familial human situs defects (reviewed by Kosaki and Casey, 1998) that range from single autosomal recessive mutations, such as immotile cilia syndrome (ICS) that randomizes body situs, to more complex pedigrees where situs defects are confined to a subset of organs. In mouse the *inversus viscerum* (*iv*) mutation results in randomization of heart looping and body situs (Hummel and Chapman, 1959), whereas animals homozygous for the *inversion of embryonic turning* (*inv*) mutation mostly show complete inversion of embryonic turning and body situs (Yokoyama et al., 1993). In both mice and humans, assignment of L–R can be random along the A–P axis of a single individual, producing a mixture of normal and abnormal L–R pattern referred to as heterotaxia. In other cases, organs may develop as either right or left mirror images called isomerisms. Thus, genetics suggests that the L–R pathway is finely balanced with perturbations at different levels of the cascade inflicting different insults on final body situs. Interfering early tends to randomize or reverse overall situs, whereas later disturbances alter only some organs.

In humans ICS results in randomization of total body situs, implicating a gene that acts early during the L–R pathway. ICS is caused by a mutation in a microtubule-associated dynein gene (Afzelius, 1985), a fact that informed a candidate gene approach employed to identify the product of the mouse *iv* mutation. A number of dynein genes map to the distal region of mouse chromosome 12 where the *iv* mutation resides, and one of these, a member of the class of axonemal dynein proteins, was recently shown to correspond to *iv* (Supp et al., 1997). In the original *iv/iv* mouse strain, the mutation causes a single amino acid change in a highly conserved region of the motor domain, and the gene has been named *left-right dynein* (*lrd*). As yet, there is no formal genetic proof that this missense mutation in *lrd* leads to loss-of-function of the protein in *iv/iv* mice. However, *lrd* also maps to the 600 kb deletion associated with the transgene insertion mutation *legless*, which is allelic to *iv*. Homozygous *lgl* mutants lack *lrd* and also display random body situs (Supp et al., 1997). Transcripts of *lrd* can be detected at preimplantation stages but are most abundant and symmetrically distributed in the 7.5 day mature node. Expression is then widespread, including some ciliated epithelia, although the function of later *lrd* expression does not seem to be essential because apart from situs defects *iv/iv* animals are normal and fully fertile. That randomization of the mammalian L–R axis can be due to the absence of an intracellular motor is consistent with the notion that asymmetric movement of molecules is crucial for the initial specification of L–R.

The transgene insertion causing the *inv* mutation deletes the coding sequence of a gene encoding a novel 1000 amino acid protein (Mochizuki et al., 1998; Morgan et al., 1998). Sequence analysis suggests that this is an intracellular protein whose C-terminal domain is rich in anykrin repeats. As with *lrd*, the expression pattern of *inv* is largely uniformative, since the gene is transcribed from early stages of embryogenesis, and all lineages of the early postimplantation embryo contain low levels of *inv* transcripts (Mochizuki et al., 1998). Conclusive proof that the L–R defects in *inv* are due to loss of this single gene was obtained by rescuing the phenotype of *inv/inv* embryos by introducing into them a transgene expressing *inv* cDNA under the control of a ubiquitous promoter (Mochizuki et al., 1998).

Obviously, much still has to be learned about the precise intracellular localization and function of the inv and iv proteins. Although they are essential if gene expression asymmetries are to emerge, it is not clear when they are first needed, nor why loss of one (*inv*) should lead to complete inversion of the L–R axis while loss of the other (*iv*) causes random laterality. It is also unclear how genes, and presumably their protein products, which are expressed more or less ubiquitously, can effect L–R asymmetry. Perhaps the cellular function of inv

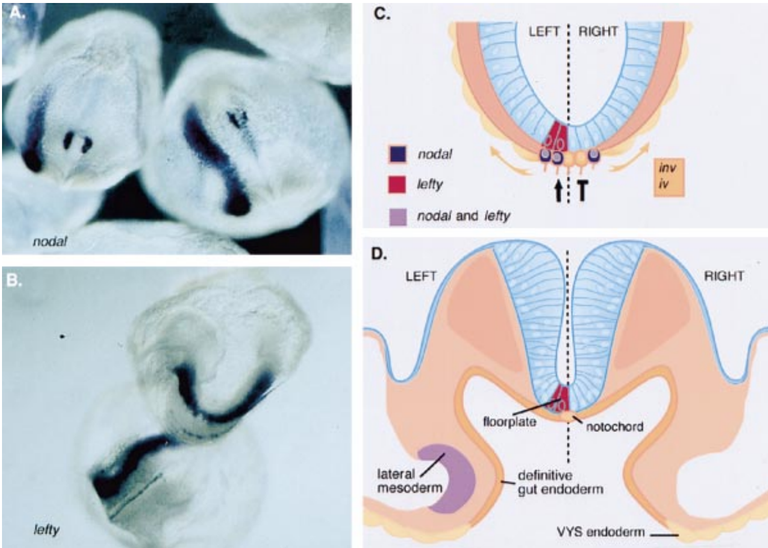


Figure 4. L–R Asymmetry in Gene Expression

(A) *nodal* expression in the node and left LPM of 8-somite stage embryos.

(B) Expression of *lefty-1* in the floor plate and of *lefty-2* in the left LPM of 8-somite stage embryos.

(C) Diagram of *nodal* and *lefty-1* expression in a cross section of the node.

(D) Diagram of *nodal* and *lefty-2* expression in the LPM and of *lefty-1* in the floor plate of an early somite stage embryo.

may be easier to decipher by studying its role in kidney morphogenesis (Yokoyama et al., 1993).

L–R Asymmetry in Gene Expression

In the chick, genes encoding a number of signaling molecules, including an activin receptor (*ActRIIa*), sonic hedgehog (*Shh*), and *nodal*, are transiently expressed on one side only of the midline during gastrulation. This asymmetric gene expression precedes any anatomical signs of laterality, and its onset is marked by *ActRII*, which is expressed first and along the right side of the primitive streak and developing node (Levin et al., 1995). Activation of this receptor, possibly by a localized source of activinβ_B, allows expression of *Shh* and *nodal* only on the left side of the node (Levin et al., 1997). In this way L–R differences start to be amplified and ultimately cause *nodal* expression in a broad stripe of lateral plate mesoderm (LPM) exclusively on the left side. In mouse, frog, and zebrafish only the asymmetric expression of *nodal* seems to be exactly like that in chick.

Mouse *nodal* is also expressed in the left LPM but seems to act in concert with two novel TGFβ family members, *lefty-1* and *lefty-2*. At very early somite stages, expression of all three signaling molecules is induced exclusively in cell populations on the left side of the embryo (Figure 4). *lefty-1* is highly expressed in the four or five cells of the left floor plate, while *nodal* and *lefty-2* are coexpressed in the left LPM fated to become the future ventral body wall (Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996, 1997). Transcripts of *lefty-2* and *nodal* appear simultaneously, and both are immediately distributed along most of the A–P axis, suggesting a rather global induction mechanism. The anterior limit of this left-sided mesodermal expression lies in the dorsal wall of the pericardial cavity and extends to a posterior boundary in the nascent mesoderm emerging from the streak (Figure 4). Just as the onset of *nodal* and *lefty-2* expression in the LPM appears to be coordinately regulated, so is their downregulation (as is that of *lefty-1*), since by the 12- to 14-somite stage their respective transcripts are barely detectable.

Another piece in the L–R puzzle is *Pitx2*, a member of the *bicoid* class of homeodomain factors, which seems to be an important and highly influential target of the *nodal* and *lefty* pathways. In frog, chick, and mouse, *Pitx2* is expressed on the left side of the axis shortly after *nodal* is detected in the LPM (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St. Amand et al., 1998; Yoshioka et al., 1998). The *Pitx2* expression domain is larger than that of *nodal*, persists longer, and includes not only the left LPM but also the left side of the developing gut tube and the heart. Both *nodal* and *lefty* can induce ectopic *Pitx2* in chick embryos, and if the right side of the axis of chick embryos is infected with retroviruses expressing *Pitx2*, both the heart and derivatives of the gut become left isomerized (Logan et al., 1998). Hence, *Pitx2* seems to act as a global executor of L–R pattern.

The mouse node and its derivatives also show asymmetric gene expression patterns. When the node first emerges, it already has a unique, bilaminar architecture. Ventrally a sheet of small cuboidal cells, each bearing a single motile cilium, constitutes the notochordal plate, while the dorsal layer comprises columnar epiblast (Sulik et al., 1994). The ventral cells give rise to notochord and definitive endoderm, while the dorsal cells contribute to the floor plate in the ventral midline of the neural tube (Figure 4; Beddington, 1994; Sulik et al., 1994). *nodal* is expressed in the ventral layer, specifically in cells at the extreme lateral edges of the notochordal plate (Collignon et al., 1996). Although initially symmetrical, by the 5- to 6-somite stage the number of cells expressing *nodal* on the left side is approximately twice that on the right (Collignon et al., 1996). This asymmetry in the node may coincide with that in the LPM, and tissue grafting experiments in chick have shown that the molecular asymmetry seen in Hensen's node is influenced by signals from lateral tissue (Pagan-Westphal and Tabin, 1998). However, unlike mouse, chick *nodal* is never expressed on the right of the node (Levin et al., 1995). Later *nodal* expression persists in the tail bud, but here its expression is symmetrical, indicating that whatever the asymmetric cues may be that regulate its

brief left-handed expression they must be short lived. The expression of *lefty-1* is particularly remarkable because it is always confined to just 4–5 cells situated only on the left side of the future floor plate (Figure 4; Meno et al., 1996, 1997).

Playing out L–R Pattern

Given that *iv* and *inv* must be at or near the top of the L–R pathway, how is their influence translated into L–R pattern? In *inv/inv* embryos, the expression of *nodal*, *lefty-1*, and *lefty-2* is essentially reversed (Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996, 1997). Sometimes only the anterior boundary of *nodal* expression is reduced, but this seems sufficient to uncouple the direction of heart looping from that of axial rotation (Collignon et al., 1996); the heart tube now loops to the left, indicating that normally *nodal* promotes right-handed looping. This is reminiscent of the redirected heart looping seen in chick embryos when *nodal* is ectopically expressed in the right LPM (Levin et al., 1997).

The situation in *iv/iv* animals is more confusing. One would have predicted that molecular asymmetry in the LPM would either be lost or randomized, but in fact four different patterns of gene expression are seen. Approximately, equal numbers of embryos show expression of *nodal*, *lefty-1*, and *lefty-2* on the left side, the right side, both sides, or neither side (Lowe et al., 1996; Meno et al., 1997). Why the loss of *lrd* can upset the L–R program in such a way that four different effects on LPM gene expression result is unclear, but it may be relevant that reversing the A–P axis of the node in chick embryos can phenocopy *inv* in terms of the spectrum of *nodal* expression patterns produced (Pagan-Westphal and Tabin, 1998).

Compound heterozygotes for mutations in *nodal* and *HNF3*β, which have a high incidence of laterality defects, also show altered *nodal* expression. In these embryos it is expressed bilaterally and somewhat abnormally in the LPM (Collignon et al., 1996). *lefty-1* mutants also result in situs defects, which can be traced back to disturbed expression of *lefty-2* and *nodal* in the LPM: *lefty-2* expression changing first, closely followed by alterations in *nodal*. Transcripts of both genes appear in the right LPM and are most abundant anteriorly. Thus, Lefty-1 activity is required in 4–5 cells of the midline in order to restrict *nodal* and *lefty-2* to the left LPM (Meno et al., 1998). Once again ectopic expression of these TGFβ molecules leads to *Pitx2* appearing on the wrong side of the embryo, and left isomerisms appear later in the thorax. Interestingly, *ActRIIA*-deficient mice display right isomerisms (Oh and Li, 1997), and expression of dominant-negative forms of the activin receptor in frogs blocks asymmetric *Pitx2* expression (Ryan et al., 1998). Thus, *Pitx2* expression is almost certainly controlled by Nodal and Lefty presumably operating via the activin signal transduction pathway (see below). However, the absence of situs defects in chimeras containing almost exclusively *nodal* mutant cells (Varlet et al., 1997a) indicates that in the LPM Nodal and Lefty-2 may serve the same function. Wherever it has been studied, the sidedness of *Pitx2* expression seems always to follow that of *nodal* and *lefty*, and therefore these genes collectively are seen as the important executors of L–R pattern. Since the elevated proliferation rate in the left LPM that correlates with normal anticlockwise axial rotation occurs instead on the right in *iv/iv* embryos (Miller and White, 1998), one function of these genes must be normally to selectively increase growth on the left side of the embyro.

Circumstantial evidence also implicates mouse *snail*, a zinc finger transcription factor. In the chick, *cSnr-1* is expressed in the right LPM in a domain very similar to that of *nodal* in the left (Issac et al., 1997). Antisense experiments have placed *cSnr-1* downstream of *nodal*, and the simplest model to explain this reciprocal pattern of gene expression is that Nodal acts to repress expression of *cSnr-1* on the left side (Issac et al., 1997). Mouse *snail* also appears to be more strongly expressed in mesoderm on the right side (Sefton et al., 1998), but because loss-of-function mutations in *snail* arrest development at early postimplantation stages (Jiang et al., 1998), it is not known whether mouse *snail* can influence situs. Nor is it known whether mouse *snail* expression is perturbed by the *iv* and *inv* mutations. Another possible target of TGFβ signaling may be *Zic3*, a putative zinc finger transcription factor, whose mutation can cause heterotaxia or situs inversus in humans (Gebbia et al., 1997).

Nodal, Lefties, and the Midline Barrier

In the developing node, asymmetry of *nodal* in the notochordal plate precedes asymmetry of *lefty-1* in the overlying prospective floor plate cells. The node is clearly instrumental in inducing *lefty-1* because its midline expression is absent in embryos lacking *HNF3*β that have no node (Dufort et al., 1998). However, *nodal* itself is unlikely to be the sole inducer of *lefty-1* because of the apparent lack of situs defects in chimeric *nodal* mutant embryos (Varlet et al., 1997a). In the chick, the SHH pathway has been implicated in regulating *nodal*, because LPM expression of *nodal* is completely blocked and heart looping randomized by application of anti-SHH antibodies (Pagan-Westphal and Tabin, 1998). However, genetics suggests that *shh* is not required to establish normal situs either in mammals (Chiang et al., 1996) or zebrafish (Schauerte et al., 1998).

What then mediates the asymmetric expression of *nodal* and *lefty* genes? Are *nodal* and *lefty-2* coordinately regulated, or does one induce the other? Misexpression of either mouse *lefty* gene in chick embryos can induce expression of the endogenous *nodal* gene, albeit only at certain developmental stages (Yoshioka et al., 1998). Since *nodal* cannot induce itself in this way, Nodal and Lefty probably signal via different receptor complexes, as predicted by their divergent signaling domains. However, the chick homolog of *lefty* has never been tested in these experiments, and in such overexpression assays it is always possible that an overabundance of Lefty protein binds unphysiologically to TGFβ receptor complexes to activate them. It is also possible that Lefty acts in a different way because Lefty molecules lack the conserved cysteine residue normally used to form active dimers (Meno et al., 1996, 1997). Therefore, it could instead be an extracellular antagonist of TGFβ signaling, blocking ligands or their receptors. Indeed, in *Xenopus* animal caps, overexpression of chimeric molecules comprising the *Bmp2* prodomain joined

to the *lefty* mature domain leads to direct neuralization, indicating that BMP signaling has been blocked. However, it may be too simplistic to extrapolate this finding to the normal situation because Lefty proproteins are poorly processed (Meno et al., 1996, 1997), and the identity of the prodomain of TGFβ molecules can alter the half-life of the mature ligand after processing (Constam and Robertson, 1999). The relationships between *nodal* and *lefty* signaling may become clearer when the regulatory elements governing their expression are characterized and the receptor complexes through which they signal are more accurately defined.

It has been proposed that the primary role of Lefty-1 is to locally inhibit molecules on the left from diffusing across the midline, *lefty-1* being expressed when the midline is only two cells thick, consisting of a monolayer of floor plate cells and a monolayer of mesendoderm (Figure 4). Superficially, this is an attractive hypothesis, but it does not fit easily with the reality of a rapidly growing embryo and a cast of signaling molecules that probably act only as short-range signals (Jones et al., 1996; Reilly and Melton, 1996). Nodal and Lefty-2 produced by the LPM are a long way away from the midline, and the likelihood of their passive diffusion to the right side of the embryo must be remote (Figure 4). If Lefty-1 is blocking other signals, we do not yet know what they are. The only gene later expressed with L–R asymmetry in gut endoderm is *Pitx2*, but its transcripts are on the same left-hand side as those of *lefty-1* in the floor plate.

Some key ingredients of the L–R pathway have been identified, but we are still a long way from understanding exactly what they do or how they interact with each other. Again the first molecular signs of asymmetry and the results from overexpression assays in chick do not tell us when the L–R axis is established. If anterior pattern is already present in the conceptus before gastrulation, is it possible that the mammalian L–R axis is also a legacy from extraembryonic tissues? This may only be clear when the intermediates translating lrd and inv function into asymmetric Lefty and Nodal expression are identified.

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