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Primitive streak formation in mice is preceded by localized activation of *Brachyury* and *Wnt3*

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Abstract

The prevalent model for the generation of axial polarity in mouse embryos proposes that a radial to a linear transition in the expression of primitive streak markers precedes the formation of the primitive streak on one side of the epiblast. This model contrasts with the models of mesoderm formation in other vertebrates as it suggests that the primitive streak is initially established in a radial pattern rather than a localized region of the epiblast. Here, we examine the proposed correlation between the expression of *Brachyury* and *Wnt3*, two genes reported as expressed radially in the proximal epiblast, with the movements of proximal anterior epiblast cells at stages leading to the formation of the primitive streak. Our results reveal that neither *Brachyury* nor *Wnt3* forms a ring of expression in the proximal epiblast as previously thought. In embryos dissected between 5.5 and 6.5 dpc, *Brachyury* is first expressed in the distal extra-embryonic ectoderm and subsequently on one side of the epiblast. *Wnt3* expression is evident first in the posterior visceral endoderm of 5.5 dpc embryos and later in the posterior epiblast. Lineage analysis shows that the movements of the proximal epiblast do not restrict *Brachyury* expression to the posterior epiblast. Our data suggest a model whereby the localized expression of these genes in the posterior epiblast, and hence the formation of the primitive streak, is the result of local cell–cell interactions in the future posterior portion of the egg cylinder rather than regionalization of a radial pattern of expression in proximal epiblast cells.

Keywords: Mouse; Epiblast; *Brachyury*; *Wnt3*; Visceral endoderm; Primitive streak; Iontophoresis

Introduction

A fundamental process during the generation of the anteroposterior axis in mammals is the formation of the primitive streak. In mice, the primitive streak forms in a localized region of the epiblast located adjacent to the extra-embryonic ectoderm where it marks the posterior site of the embryo. During gastrulation, the primitive streak serves as a conduit for the generation of mesoderm and definitive endoderm (Tam and Gad, 2004). The current model for the establishment of the anteroposterior axis and hence the primitive streak, proposes an orthogonal rotation from the proximodistal to the transverse plane of the egg cylinder at stages preceding the formation of the primitive streak (Beddington and Robertson, 1999; Lu et al., 2001; Thomas and Beddington, 1996; Thomas et al., 1998). In this model, extra-embryonic ectoderm induces the proximal epiblast to express several genes that form a ring of expression in the proximal epiblast. The expression of these genes is subsequently restricted to one side of the epiblast where the primitive streak will eventually form. The transition from a radial to a linear pattern of expression of primitive streak markers suggests that the potential for the formation of the primitive streak is initially established in the proximal epiblast (see Tam and Gad, 2004). Two hypotheses have been offered to explain this process. One hypothesis proposes that radial epiblast markers are allocated to the posterior epiblast by the posteriorward movements of proximal epiblast cells at stages preceding the appearance of the streak (Thomas et al., 1998). The second hypothesis, based on lineage studies and on tissue recombination experiments, proposes that the anterior visceral endoderm known as the AVE suppresses the expression of radially expressed genes in the anterior epiblast, limiting their expression to the posterior epiblast (Kimura et al., 2000; Thomas et al., 1998).

The movements of the distal visceral endoderm, the precursor of the AVE, have been well documented in lineage experiments

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using DiI (Thomas et al., 1998), iontophoresis (Rivera-Perez et al., 2003) or time-lapse imaging (Srinivas et al., 2004). The AVE is characterized by the expression of multiple genes that include *Hhex* and several repressors of Nodal and Wnt signaling such as *Cer1*, *Lefty1*, *Dkk1*, *Sfrp5* and *Sfrp1* (Beddington and Robertson, 1998; Finley et al., 2003; Kemp et al., 2005; Perea-Gomez et al., 2001). The study of the morphogenetic movements of the epiblast, on the contrary, has been limited to stages immediately before or after the primitive streak appears (Lawson et al., 1991). These studies have revealed an anisotropic spread of proximal epiblast cells towards the primitive streak. At the molecular level, *Brachyury* was reported to form a ring of expression in the proximal epiblast at pre-streak stages and then to localize to the posterior epiblast and primitive streak (Thomas and Beddington, 1996; Thomas et al., 1998). Recently, however, the pattern of expression of *Brachyury* was shown to form a ring of expression in the extra-embryonic ectoderm rather than the proximal epiblast (Perea-Gomez et al., 2004). The expression of *Wnt3* was also reported to form a ring in the proximal epiblast and later to be restricted to the posterior epiblast/primitive streak regions (Liu et al., 1999; Lu and Robertson, 2004).

We have previously identified molecular and morphological landmarks that allow the determination of the polarity of egg cylinder stage embryos before the appearance of the primitive streak (Rivera-Perez et al., 2003). Taking advantage of these landmarks, we have labeled anterior proximal epiblast cells at pre-streak stages and compared the distribution of labeled descendants with a systematic analysis of the expression of *Brachyury* and *Wnt3*. Our results show that *Brachyury* expression does not form a ring of expression in the proximal epiblast as originally reported (Thomas and Beddington, 1996; Thomas et al., 1998) but rather in the extra-embryonic ectoderm as reported recently (Perea-Gomez et al., 2004). In the epiblast, *Brachyury* RNA and protein are restricted to one side of the epiblast abutting the extra-embryonic ectoderm and later in the primitive streak. Similarly, *Wnt3* RNA does not form a ring of expression in the proximal epiblast. *Wnt3* expression was observed first in the posterior visceral endoderm as early as 5.5 dpc and later in both the posterior visceral endoderm and epiblast or the primitive streak. Interestingly, *Wnt3* expression was observed in the posterior visceral endoderm before *Hhex*-expressing distal visceral endoderm cells (indicative of the AVE) had shifted to one side of the epiblast. These results suggest that the symmetry-breaking event that leads to the formation of the anteroposterior axis precedes the shift of the distal AVE to one side of the epiblast. A direct comparison between the movements of anterior epiblast and the expression of *Brachyury* revealed a spatial asynchrony between these events, showing that cell movements do not account for the posterior restriction of *Brachyury* expression. Based on these results, we propose a model in which the posterior epiblast expression of primitive streak markers is produced by interactions between the epiblast and visceral endoderm region located at the epiblast/extra-embryonic ectoderm boundary. This model suggests an evolutionary conservation of the mechanisms that lead to mesoderm formation in vertebrates.

Materials and methods

Lineage analysis and mouse strains

Lineage analysis was conducted in embryos derived from crosses between *Tg(Hhex-eGFP)ARbe* homozygous males (Rodriguez et al., 2001) maintained as mixed stock and CD-1 females (Charles River Laboratories). Cells were labeled intracellularly with Lysinated Rhodamine Dextran (LDRX) and Horseradish peroxidase (HRP) using iontophoresis (Lawson et al., 1991). One to three epiblast cells were labeled per embryo. Labeled embryos were cultured in media composed of 80% rat serum in MEMα (Gibco) suspended from the lid of snap cap tubes as described (Rivera-Perez et al., 2003).

Brachyury (*T*) heterozygous mice were obtained from the Jackson Laboratory (B10;TFLe-*a/a*, *T tf*/++ /J, Stock Number 003879). Animals were maintained under a 12-h light cycle. The middle of the dark cycle (midnight) that occurred prior to observing a copulation plug was considered the beginning of gestation.

Embryo staging

Embryos at 5.5 and 5.75 dpc were staged using morphological and fluorescent landmarks (Rivera-Perez et al., 2003). Embryos at 5.5 dpc were characterized by the presence of tall columnar visceral endoderm cells located at the tip of the egg cylinder. These group of cells appeared as a thickening of the visceral endoderm layer and were distinguished by eGFP fluorescence in *Tg(Hhex-eGFP)ARbe* transgenic embryos. In 5.75 dpc embryos, the *Hhex*-expressing area of visceral endoderm (now AVE) was located on one side of the epiblast and its most proximal extent reached the epiblast/extra-embryonic ectoderm boundary. In embryos between 5.75 and 6.5 days of gestation (without a primitive streak), the embryonic stage was defined only as days post-coitum (dpc) since there are no reliable landmarks to establish an unambiguous developmental stage during this period of development. Embryos at 5.5, 5.75 and 6.5 dpc were dissected 12, 18 and 14 h after the middle of the dark cycle, respectively. Embryos at 6.0 dpc were dissected within 1 h before or after the middle of the dark cycle. Embryos with a primitive streak were staged according to Downs and Davies (1993).

Whole-mount RNA in situ analysis

CD-1 or Heterozygous *Tg(Hhex-eGFP)ARbe* embryos were used in whole-mount in situ hybridization. Embryos were fixed overnight in 4% paraformaldehyde prepared in PBS. After fixation, they were dehydrated in methanol series and stored in 100% methanol at −20°C. Hybridization was done at 70°C following the protocol of J. Rossant's laboratory (http://www.mshri.on.ca/rossant/protocols/html, based on Henrique et al., 1995). The *Brachyury* probe was transcribed from plasmid pSK75 (a gift from B. Herrmann). It consisted of the complete coding sequence, 108 bp of the 5′ UTR and 354 bp of the 3′UTR (Herrmann et al., 1990). We also used a 3′ *Brachyury* probe that spanned 544 bp from the *Sac*I to the *Eco*RI sites in exon 8 (Herrmann et al., 1990). The *Wnt3* probe (a gift from R. Behringer) was a cDNA fragment containing exons 3–5 (Liu et al., 1999; Roelink et al., 1990). For HRP/whole-mount in situ double staining, embryos were fixed in 4% paraformaldehyde, assayed for HRP activity (Beddington and Lawson, 1990) and then subjected to whole-mount in situ hybridization. Embryos hybridized with the *Brachyury* cDNA or *Wnt3* probes were treated for 2 min with proteinase K (10 μg/ml) and allowed to stain for 5 days in the color reaction solution. Embryos used in these experiments were littermates. Embryos hybridized with the *Brachyury* 3′ probe and those assayed for HRP activity, were treated for 5 min with proteinase K (10 μg/ml) and the color reaction conducted for 1 day or 1 h to 3 days, respectively.

Immunofluorescence staining

Embryos obtained from CD1 crosses were fixed for 1 h in 4% paraformaldehyde prepared in PBS. After fixation, embryos were washed 3

times in PBS for 10 min and incubated in blocking solution (5% goat serum, 0.5% Triton X-100 and 1% bovine serum albumin in PBS) for 1 h. Subsequently, embryos were incubated in primary antibody solution [Rabbit anti Brachyury antiserum (generated using *Xenopus Brachyury* protein, a generous gift of F. Conlon) diluted 1:2000 and Rat anti E-cadherin IgG (Zymed Cat No. 13-1900) diluted 1:500 in blocking solution] for 2 h. After primary antibody staining, embryos were washed three times in PBT (0.5% Triton X-100, 1% BSA in PBS) and incubated for 1 h in secondary antibody solution [Alexa-fluor 488 Goat anti Rabbit IgG (Molecular probes Cat. No. A11008) and Alexa-fluor 594 Goat anti Rat IgG (Molecular Probes, Cat. No. A-11007) diluted 1:500 in PBT]. Embryos were then rinsed 3 times in PBT for 10 min, once in PBSAT (0.1% Triton X-100, 0.5% BSA in PBS) for 5 min and counterstained in a 1 μg/ml aqueous solution of DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride) for 2 min. After DAPI staining, embryos were washed twice in PBSAT for 5 min and mounted in a solution containing 2.5% *N*-Propyl Gallate and 50% Glycerol in PBS.

Histology

Whole-mount hybridized embryos were rinsed in PBS, re-fixed for 1 h in 4% paraformaldehyde, equilibrated in gradients of 10, 20 and 30% sucrose/PBS and mounted in OCT. Cryosections were obtained every 7 μm and mounted in 70% glycerol/PBS.

Results

Brachyury forms a ring of expression in the distal extra-embryonic ectoderm and marks the future location of the primitive streak

Brachyury expression is known to mark the primitive streak and notochord of gastrulating and early somite stage embryos (Wilkinson et al., 1990). In addition, based on whole-mount in situ hybridization, two studies argued that the expression of *Brachyury* forms a ring of expression in the proximal epiblast of 6.0 dpc embryos followed by localization to the posterior epiblast and primitive streak by 6.5 dpc (Thomas and Beddington, 1996; Thomas et al., 1998). In contrast, a recent report described a ring of *Brachyury* expression in the extra-embryonic ectoderm rather than in the proximal epiblast (Perea-Gomez et al., 2004). This extra-embryonic pattern of expression was observed as early as 5.5 dpc and persisted into the early streak stage overlapping with *Brachyury* expression in the posterior epiblast/primitive streak at 6.5 dpc. To assess the temporal and spatial location of *Brachyury* RNA, we conducted a systematic analysis of embryos using whole-mount in situ hybridization at stages leading up to and including early primitive streak stages.

We analyzed *Brachyury* RNA in embryos dissected at 5.5 ($n = 4$), 5.75 ($n = 4$), 6.0 ($n = 15$) and 6.5 ($n = 3$) dpc using a cDNA probe containing the full coding sequence and portions of the 5′ and 3′ untranslated region. All the embryos analyzed showed *Brachyury* expression in the extra-embryonic ectoderm region. This expression was restricted to the distal third of the extra-embryonic ectoderm abutting the epiblast (Figs. 1A, B). In addition, in some embryos dissected at 6.0 dpc and in embryos dissected at 6.5 dpc, *Brachyury* RNA was detected on one side of the epiblast in a region located next to the extra-embryonic ectoderm (Figs. 1A, C, D). At 6.0 dpc, the area of expression extended to approximately one third of the length and one third of the circumference of the epiblast and was restricted to embryos measuring more than 300 μm of egg cylinder length (Fig. 2). At 6.5 dpc, *Brachyury* expression was strongly expressed in the posterior epiblast/primitive streak reaching as

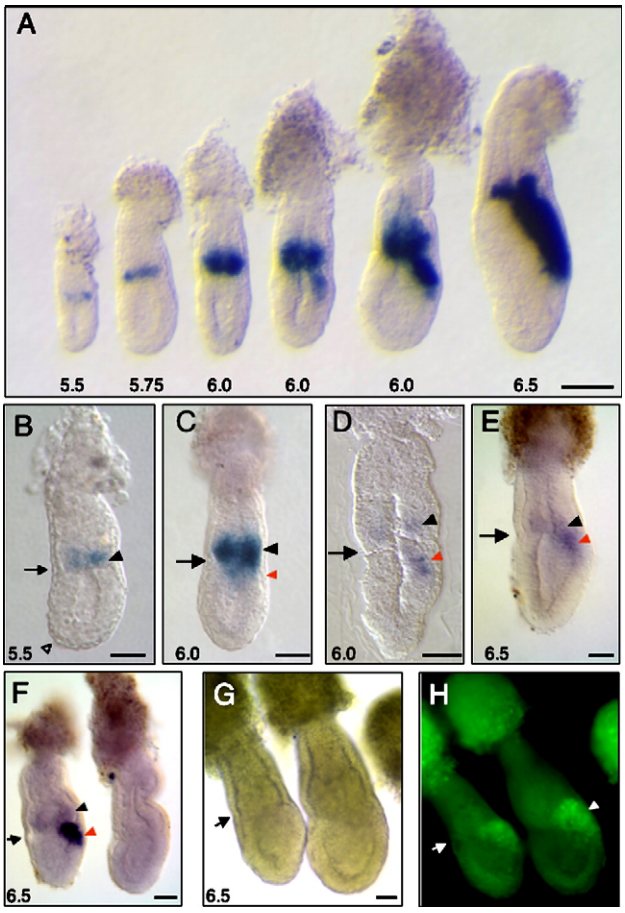

Fig. 1. Analysis of *Brachyury* RNA and protein location in post-implantation embryos. (A) Whole-mount in situ hybridization in embryos dissected between 5.5 and 6.5 dpc using a full-length *Brachyury* cDNA probe. *Brachyury* expression is observed in the extra-embryonic ectoderm in all the embryos and in both, the extra-embryonic ectoderm and on one side of the proximal epiblast in the largest embryos dissected at 6.0 and in the embryo dissected at 6.5 dpc. (B) Higher magnification of the 5.5 dpc embryo shown in panel A. *Brachyury* expression is evident only in the extra-embryonic ectoderm. The AVE can be seen on one side of the distal tip of the egg cylinder as a thickening of the visceral endoderm (open arrow). (C) Posterior view of a 6.0 dpc embryo (fourth from left in panel A) showing the radial expression of *Brachyury* in the extra-embryonic ectoderm. (D) Sagital section of a 6.0 dpc embryo showing *Brachyury* expression in the extra-embryonic ectoderm and posterior epiblast. (E) In situ hybridization using a 3′ probe that excludes the T-box. *Brachyury* RNA is detected in both the extra-embryonic ectoderm and on one side of the epiblast. (F) Embryos derived from heterozygous *T/+* crosses and hybridized with the full-length *Brachyury* probe. The embryo on the right, likely *T/T*, lacks expression in the epiblast and extra-embryonic ectoderm. (G and H) Immunofluorescence assay in 6.5 dpc wild-type embryos using Brachyury antiserum. Bright field (G) and fluorescence pictures (H) of the same embryos are shown. Brachyury protein is evident on one side of the epiblast (arrowhead) but is not discernible in the extra-embryonic ectoderm. The arrow marks the location of the epiblast/extra-embryonic ectoderm boundary. The black and the red arrowheads in panels C–F mark the location of *Brachyury* expression in the extra-embryonic ectoderm and one side of the proximal epiblast, respectively. The white arrowhead in panel H marks the location of *Brachyury* protein. Scale bar equals 100 μm in panel A, 40 μm in panels B, D and G, 60 μm in panels C and F and 50 μm in panel E.

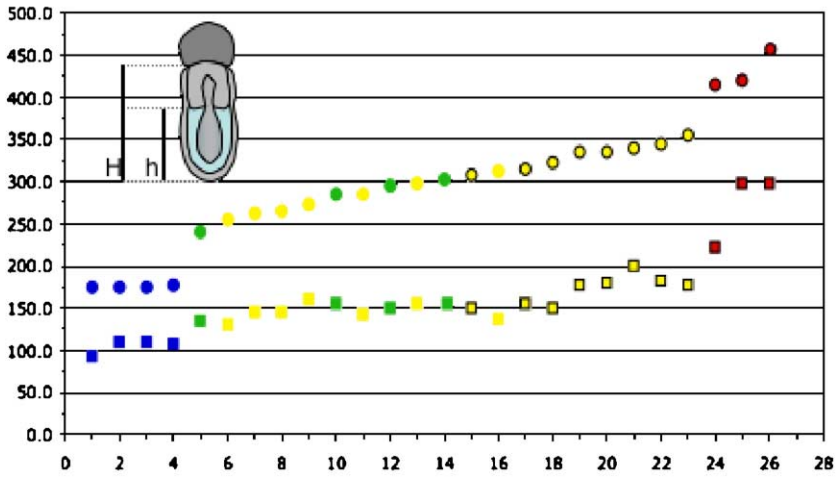

Fig. 2. Scatter plot depiction of embryonic dimensions and comparison to the onset of *Brachyury* expression in the epiblast. *Brachyury* expression in the epiblast is evident only in embryos with more than 300 μm of egg cylinder and 150 μm or more of epiblast length. Embryos at 5.5 (blue), 5.75 (green), 6.0 (yellow) and 6.5 (red) dpc have been arranged according to egg cylinder length. The egg cylinder (H) and epiblast (h) length for each embryo are depicted as circles and squares, respectively. Circles or squares lined in black represent embryos with expression in the epiblast. The length of the embryos is given in micrometers on the ordinate axis. The abscise axis represents individual embryos.

much as half of the length and circumference of the epiblast (Fig. 1A).

To exclude the possibility that expression of *Brachyury* in the extra-embryonic ectoderm was the result of non-specific hybridization produced by the use of the full coding sequence of the gene, we repeated the whole-mount in situ hybridization experiment using a probe that included a portion of the C-terminus domain of the protein and a piece of the 3′ untranslated region but excluded the T-box. We also examined embryos obtained from crosses between *Brachyury* heterozygous (*T*/+) mice. *T* is a large deletion on chromosome 17 that encompasses the entire sequence of *Brachyury* (Herrmann et al., 1990). All 18 embryos assayed with the 3′ probe between 6.25 and 6.5 dpc showed staining in the extra-embryonic ectoderm (Fig. 1E) suggesting that *Brachyury* expression in this region of the egg cylinder was not the result of cross-hybridization produced by the presence of the T-box in the cDNA probe. In addition, from 24 embryos obtained from three litters of heterozygous *T*/+ crosses at 6.5 dpc, four embryos that did not show *Brachyury* staining in the extra-embryonic ectoderm also lacked *Brachyury* expression in the epiblast (Fig. 1F). These embryos are likely *T*/*T* homozygous, which lack the entire gene.

To corroborate our whole-mount in situ hybridization results, we analyzed the presence of *Brachyury* protein in embryos between 6.0 and 6.5 dpc using antiserum generated against *Xenopus* Brachyury. This antiserum detects Brachyury in the primitive streak and notochord of early somite stage embryos (data not shown) as expected for these stages of development according to in situ hybridization data (Wilkinson et al., 1990). Brachyury protein was detected in embryos dissected at 6.25 (20/63) and 6.5 (n = 10) but not at 6.0 dpc (n = 22). Brachyury immunofluorescence was restricted to cells located on one side of the proximal epiblast abutting the extra-embryonic ectoderm (Figs. 1G, H). We did not observe a ring of Brachyury immunostaining in the proximal epiblast. Despite the detection of *Brachyury* protein in the posterior epiblast of the older embryos analyzed, we could not discern Brachyury immunostaining in the extra-embryonic ectoderm at any of the stages assayed. These results may reflect an inability of our immunoassay to detect low levels or lack of Brachyury in the extra-embryonic ectoderm or in the younger embryos analyzed.

Epiblast cell movements do not confine Brachyury expression to one side of the epiblast

The pattern of expression of *Brachyury* was proposed to mirror the posteriorward movement of epiblast cells towards the future location of the primitive streak (Beddington and Robertson, 1999; Thomas et al., 1998). In this proposal, radial expression of *Brachyury* in the proximal epiblast is restricted to the posterior portion of the epiblast by the posteriorly directed spread of proximal epiblast cells. As shown above, *Brachyury* does not form a ring of expression in the proximal epiblast, thus the posteriorward spread of the epiblast cannot account for a shift of *Brachyury*-expressing anterior epiblast cells towards the future location of the streak. Nonetheless, there is a possibility that anteriorly located proximal epiblast cells are prevented from expressing *Brachyury* by the action of the AVE and only express the gene once they move away from it. This possibility is supported by tissue recombination or explant experiments that show that the AVE represses expression of *Brachyury* in the vicinity of AVE cells in AVE/ectodermal explants (Kimura et al., 2000; Perea-Gomez et al., 2001). To gain insight into this hypothesis, we determined whether *Brachyury*-expressing cells in the posterior epiblast of 6.25 dpc embryos descended from proximal anterior epiblast cells. We labeled anterior epiblast cells located next to the epiblast/extra-embryonic ectoderm boundary with horseradish peroxidase, cultured the labeled embryos for 12 h to approximately 6.25 dpc and then subjected them to in situ hybridization using the *Brachyury* cDNA probe (Fig. 3). Seven out of 20 labeled embryos from three independent experiments contained HRP-

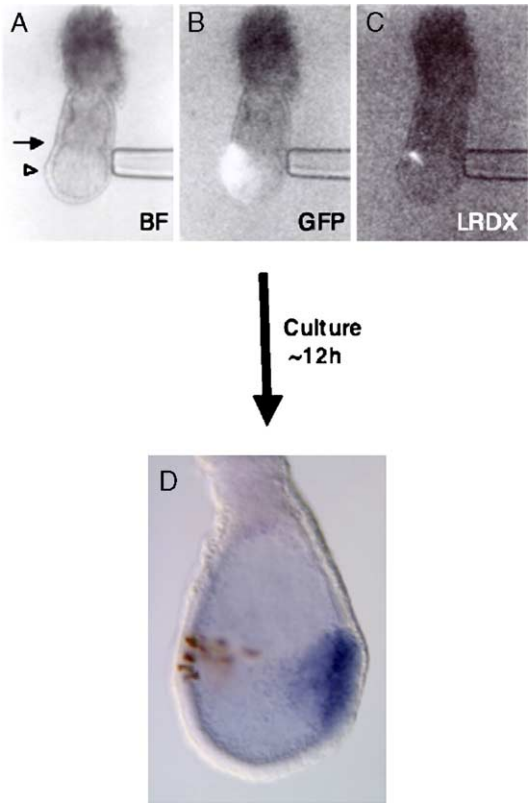

Fig. 3. Analysis of anterior epiblast cell movements and comparison with *Brachyury* expression. Proximal anterior epiblast cells of 5.75 dpc embryos were labeled with HRP and LRDX and the distribution of their descendants was compared to the pattern of expression of *Brachyury* after 12 h in culture. (A–C) 5.75 dpc embryo at the time of labeling, note the thickened visceral endoderm (open arrowhead) and the fluorescence provided by the *Tg(Hex-eGFP)ARbe* transgene (B) marking the location of the AVE. The AVE reaches the epiblast/extra-embryonic ectoderm boundary (arrow). The labeled epiblast cell is revealed by the fluorescence provided by LRDX (C). (D) Anterior epiblast descendants (stained brown) are spread around the anterior half of the epiblast while expression of *Brachyury* (purple) is visible on the opposite side of the egg cylinder. BF, Bright Field; GFP, Green Fluorescent Protein.

positive descendants, these embryos revealed *Brachyury* expression in the posterior epiblast opposite to the location of the HRP-positive cells (Fig. 3D). These results demonstrate that anterior epiblast cells or their descendants are not the predecessors of the *Brachyury*-positive cells observed in the posterior epiblast region of the labeled embryos and suggest that *Brachyury*-expressing cells in non-cultured embryos at 6.25 dpc are not descendants of the anterior epiblast.

Wnt3 expression in the posterior visceral endoderm is the first marker of anteroposterior polarity in early post-implantation embryos

Analysis of the *Brachyury* promoter has shown that *Brachyury* is a target of the Wnt signaling pathway (Arnold et al., 2000; Yamaguchi et al., 1999). Also, *Wnt3* mutants lack a primitive streak and *Brachyury* expression, showing a requirement of *Wnt3* for *Brachyury* expression in vivo (Liu et al., 1999). Since we observed restricted expression of *Brachyury* to one side of the proximal epiblast in pre-streak embryos, we wondered if *Wnt3* followed a similar pattern of expression. To determine the onset and spatial distribution of *Wnt3* expression in post-implantation embryos, we conducted an analysis of embryos dissected at 5.5 (n = 4), 5.75 (n = 5), 6.0 (n = 10) and 6.5 (n = 4) dpc.

Wnt3 expression was detected in all embryos analyzed (Fig. 4A). At 5.5 dpc, *Wnt3* RNA was observed in a small area of visceral endoderm overlying the epiblast at the boundary between epiblast and extra-embryonic ectoderm

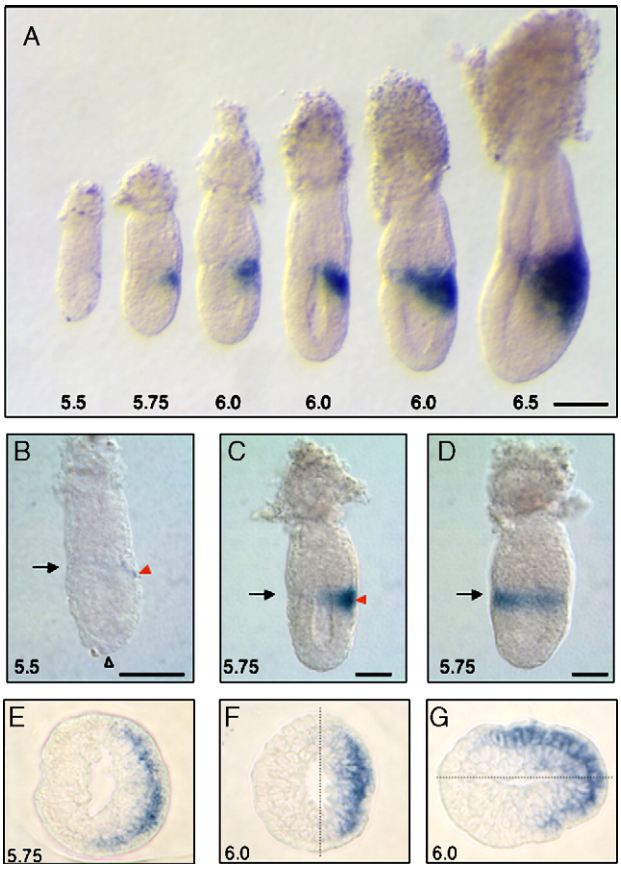

Fig. 4. Whole-mount *Wnt3* in situ hybridization in embryos dissected between 5.5 and 6.5 dpc. (A) *Wnt3* RNA is detected on one side of the egg cylinder at the boundary between the epiblast and extra-embryonic ectoderm. Expression is evident in a small area of visceral endoderm in 5.5 dpc embryos and increases in larger embryos forming a crescent that extends to about half the length of the epiblast and tapers anteriorly around the circumference of the egg cylinder. (B) Higher magnification of the 5.5 dpc embryo shown in panel A. The area of *Wnt3* staining is visible on the visceral endoderm layer (red arrowhead). The AVE is located distally and can be distinguished by a thickening of the visceral endoderm (open arrowhead). (C and D) Side (C) and posterior view (D) of the 5.75 dpc embryo shown in panel A. *Wnt3* is expressed on one side of the egg cylinder and its expression is aligned with the shorter axis of the embryo. (E–G) Cross-sections of the 5.75 (E) and the two largest 6.0 dpc embryos (F, G) shown in panel A. *Wnt3* RNA is evident in the visceral endoderm of the 5.75 dpc embryo and in both the posterior epiblast and overlying visceral endoderm in the embryos dissected at 6.0 dpc. In the smaller 6.0 dpc embryo sectioned (F, fourth from left in panel A), *Wnt3* expression is aligned with the short axis of the egg cylinder. In the largest 6.0 dpc embryo sectioned (G, fifth from left in panel A), expression associates with the long axis of the egg cylinder. The arrow points to the location of the epiblast/extra-embryonic ectoderm boundary. The dotted line in panels F–G marks the long axis of the embryo. Scale bar equals 100 μm in panel A, 80 μm in panel B and 60 μm in panels C and D.

(Figs. 4A, B). In these embryos, the *Hex*-expressing visceral endoderm cells indicative of the future AVE were located at the distal tip of the epiblast (Fig. 3B). These results show that *Wnt3* expression in the posterior visceral endoderm precedes the distal to proximal movements of the AVE. At 5.75 dpc, the area of *Wnt3* expression was clearly evident on one side of the egg cylinder abutting the epiblast/extra-embryonic ectoderm boundary (Figs. 4C, D). This area of expression extended to one third of the length of the epiblast half of the egg cylinder and encompassed half its circumference. In cross-sections, the area of expression was restricted to the visceral endoderm layer in one of two embryos analyzed (Fig. 4E) but included both the posterior epiblast and overlying visceral endoderm of the second embryo sectioned (not shown). In embryos dissected at 6.0 dpc, *Wnt3* RNA spanned one third of the length of the epiblast and about two thirds of the circumference of the egg cylinder (Fig. 4A). Expression included both the posterior epiblast and overlying visceral endoderm (Figs. 4F, G) in four of five embryos sectioned but was restricted to the visceral endoderm layer of one embryo (not shown). *Wnt3* expression was oriented either parallel to the long axis of the egg cylinder in the smaller embryos sectioned (Fig. 4F) or perpendicular to the long axis in the larger 6.0 dpc embryos (Fig. 4G). At 6.5 dpc, the expression of *Wnt3* extended to half the length of the epiblast and overlying visceral endoderm and tapered anteriorly encompassing the whole circumference of the egg cylinder (Figs. 4A and 5). In these embryos, however, expression of *Wnt3* in the anterior portion of the egg cylinder was confined to the anterior visceral endoderm layer overlying the extra-embryonic ectoderm and proximal to the location of the AVE. This expression is evident in cross-sections of 6.5 dpc embryos (Figs. 5B, C). The expression of *Wnt3* in the epiblast was confined to the posterior epiblast encompassing about half the circumference of the epiblast (Fig. 5C).

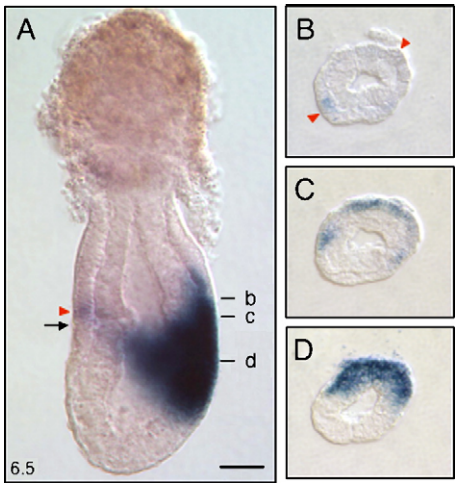

Fig. 5. Analysis of *Wnt3* expression in 6.5 dpc embryos. (A) *Wnt3* expression covers one side of the egg cylinder spanning half of the length of the epiblast and tapering anteriorly around the circumference of the egg cylinder. In the anterior portion of the egg cylinder, *Wnt3* RNA is evident in the visceral endoderm covering the extra-embryonic ectoderm proximal to the AVE (arrowhead). (B–D) Cross-sections of the embryo shown in panel A, the approximate location of the sections is marked in panel A. In the most proximal sections (B and C), *Wnt3* expression is restricted to the visceral endoderm (arrowheads). In the most distal section (D), *Wnt3* expression is observed in approximately half of the circumference of the epiblast and overlying visceral endoderm. Arrow points to the location of the epiblast/extra-embryonic ectoderm boundary. Scale bar equals 50 μm.

Three conclusions can be drawn from these results; first, the expression of *Wnt3* does not form a ring of expression in the epiblast. Second, *Wnt3* expression in the posterior visceral endoderm precedes its expression in the epiblast and third, *Wnt3* expression in the posterior visceral endoderm provides the first landmark of the anteroposterior axis in early post-implantation embryos.

Discussion

Brachyury expression is restricted to the posterior epiblast and extra-embryonic ectoderm

The expression pattern of *Brachyury* has traditionally been considered a hallmark of primitive streak formation in mice (Wilkinson et al., 1990). Therefore, the report that *Brachyury* was expressed radially in the proximal epiblast (Thomas and Beddington, 1996; Thomas et al., 1998) suggested that the potential to form the streak initially resided in proximal epiblast cells (Tam and Gad, 2004). Our results show that *Brachyury* forms a ring of expression in the extra-embryonic ectoderm and not in the proximal epiblast. In addition, evidence from whole-mount in situ hybridization and immunofluorescence demonstrates the presence of *Brachyury* RNA and protein on one side of the proximal epiblast in embryos at pre- and early streak stages. Therefore, these results confirm those of Perea-Gomez et al. (2004) and suggest that the initial steps to form the primitive streak begin on a localized region of the proximal epiblast rather than reflecting the transformation of a radial to a linear specification program established in the proximal epiblast.

Wnt3 expression is restricted to the posterior epiblast and provides the first landmark of the anteroposterior axis in the early post-implantation embryo

Wnt3 was reported to form a ring of expression in the proximal epiblast of 6.25 dpc embryos at stages before the appearance of the streak and later in the posterior visceral endoderm and epiblast at the onset of primitive streak formation (Liu et al., 1999; Lu and Robertson, 2004). In our experiments, we did not observe a ring of expression of *Wnt3* in the epiblast, instead we observed expression of *Wnt3* in the posterior epiblast and overlaying visceral endoderm. At 6.5 dpc, the pattern of expression of *Wnt3* tapered from about half the length of the epiblast and overlying visceral endoderm in the posterior region to the anterior visceral endoderm overlying the extra-embryonic ectoderm. The circumferential expression of *Wnt3* in the visceral endoderm layer, could have led to the belief that there is radial expression of *Wnt3* in the proximal epiblast.

Two additional observations have emerged from our study. First, we noted that expression of *Wnt3* is restricted to the visceral endoderm layer in the earliest embryos dissected and subsequently spreads to the adjacent epiblast. *Wnt3* expression also precedes the expression of *Brachyury* in the epiblast. Thus, *Wnt3* signaling in the posterior visceral endoderm may direct the formation of the primitive streak in a non-cell autonomous manner. Second, we observed expression of *Wnt3* in the posterior visceral endoderm of embryos dissected at 5.5 dpc. In these embryos, morphological and fluorescent landmarks indicated that the AVE was still located at the tip of the epiblast. The movement of the AVE to one side of the epiblast is considered the symmetry-breaking event that leads to the establishment of the anteroposterior axis in post-implantation embryos (Beddington and Robertson, 1999; Rossant and Tam, 2004; Thomas et al., 1998). In our experiments, *Wnt3* expression in the posterior visceral endoderm precedes the movements of the AVE to one side of the epiblast. These data suggest that the symmetry-breaking event that establishes the anteroposterior axis in mice occurs before the AVE shifts to one side of the epiblast.

The movements of the proximal epiblast towards the future location of the primitive streak are not mirrored by the expression of primitive streak markers

The allocation of primitive streak markers such as *Brachyury* was proposed to be a consequence of movements of proximal epiblast cells towards the future location of the primitive streak (Beddington and Robertson, 1999; Lu et al., 2001; Thomas et al., 1998). In our experiments, *Brachyury* expression was observed in posterior epiblast cells at a time when labeled anterior epiblast cells were still restricted to the anterior half of the circumference of the egg cylinder. These data show that *Brachyury*-expressing cells in the posterior region of the epiblast are not descendants of anterior epiblast cells at 6.25 dpc. Similarly, since the expression of *Wnt3* is restricted to the posterior side of the epiblast, epiblast cell movements do not appear to account for its posterior restriction.

Another gene known to form a ring of expression in the proximal epiblast is *Nodal*. *Nodal* expression initially covers the whole epiblast at 5.5 dpc and then is found in the proximal and later posterior epiblast (Conlon et al., 1994). The transition of *Nodal* expression from the proximal to the posterior epiblast does not appear to be a consequence of posteriorward movements of the proximal epiblast. Evidence for this assertion is provided by the comparison of the pattern of expression of *Nodal* with the distribution of β-galactosidase staining in a heterozygous *Nodal-LacZ* transgenic line (Perea-Gomez et al., 2004). Because of the stability of the β-galactosidase protein, the cells that express *Nodal* (β-galactosidase and *Nodal* positive) can be distinguished from those that no longer express the gene (β-galactosidase positive and negative for *Nodal* expression). In these experiments, the activity of β-galactosidase was detected as a ring in the proximal epiblast while the expression of *Nodal* was confined to the posterior epiblast. Therefore, the posterior restriction of *Nodal* expression in these embryos is due to changes in the location of the expression of the gene rather than by cell movement (Perea-Gomez et al., 2004). Taken together, these data suggest that the posteriorward movements of proximal epiblast do not trans-

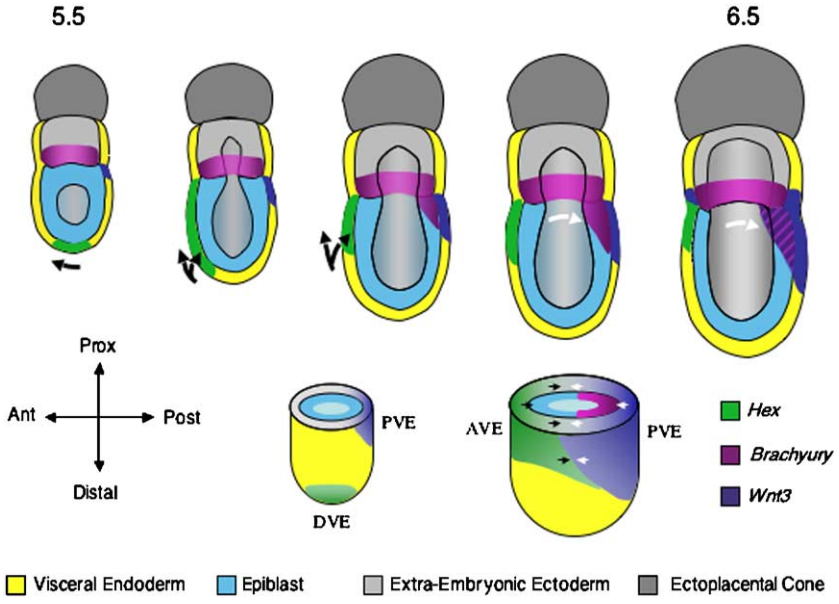

Fig. 6. Proposed model of the morphogenetic and molecular events occurring between 5.5 and 6.5 dpc. At 5.5 dpc, *Wnt3* expression is present in the posterior visceral endoderm while *Hex*-expressing cells indicative of the AVE are still located at the tip of the egg cylinder. By 5.75 dpc, the *Hex*-expressing distal visceral endoderm cells have reached the epiblast/extra-embryonic ectoderm boundary and cover the anterior half of the epiblast (Rivera-Perez et al., 2003; Srinivas et al., 2004). The AVE cells express multiple antagonists of Wnt signaling and likely prevent the spread of Wnt signaling to the AVE and anterior epiblast. The localized expression of *Wnt3* first in the posterior visceral endoderm and later in the posterior epiblast results in the activation of *Brachyury* in the posterior epiblast and subsequent formation of the primitive streak on one side of the epiblast.

form a radial pattern of expression of primitive streak markers into a linear one at stages before the appearance of the streak.

The movement of the distal visceral endoderm towards one side of the epiblast prompted researchers to propose that this region of visceral endoderm, the AVE, restricts the radial expression of *Brachyury* to the posterior epiblast by active suppression of *Brachyury* expression in the proximal anterior epiblast (Kimura et al., 2000; Thomas et al., 1998). The lack of expression of *Brachyury* in the proximal anterior epiblast contradicts this hypothesis. The AVE may instead prevent the expression of *Brachyury* in the anterior epiblast by blocking the spread of *Wnt3* signaling from the posterior region of the egg cylinder to the anterior epiblast (Fig. 6). Support for this hypothesis is provided by the analysis of *Wnt3* expression in *Lpp3* mutant embryos (Escalante-Alcalde et al., 2003). *Lpp3* mutants have expanded or duplicated primitive streaks. In addition, analysis of AVE markers revealed that, in about 30% of *Lpp3* null embryos, the AVE remains at the distal tip of the epiblast. In these embryos, *Wnt3* expression has expanded to the anterior epiblast. These data suggest that misallocation of the AVE to the distal epiblast, leads to ectopic expression of *Wnt3* in the proximal anterior epiblast resulting in expansion or duplications of the primitive streak.

A Nieuwkoop-like center in mice? Posterior visceral endoderm versus extra-embryonic ectoderm

The Nieuwkoop center in amphibians is an area of the dorsal–vegetal region of the blastula that can induce an organizer in a non-cell autonomous manner without contributing cells to the induced axial structures (Harland and Gerhart, 1997). In mice, the location of a Nieuwkoop-like center has been predicted to reside in two extra-embryonic tissues, the posterior visceral endoderm and the extra-embryonic ectoderm (Bachvarova, 1996; Conlon and Beddington, 1995). Wnt signaling is known to induce an organizer in *Xenopus* (Fagotto et al., 1997) or a primitive streak in misexpression experiments in the chick (Skromne and Stern, 2001). These experiments suggest that Wnt signaling mediates the effects of the Nieuwkoop center. In our experiments, the localized expression of *Wnt3* in the posterior visceral endoderm suggests that this region of the pre-streak embryo is the equivalent to the Nieuwkoop center in mice. In a recent study, the non-phosphorylated form of β-catenin, indicative of Wnt activity, was found in the posterior epiblast and overlying posterior visceral endoderm (Mohamed et al., 2004). These data suggest that *Wnt3* activity may lead to the generation of the active form of β-catenin in the posterior visceral endoderm and epiblast. In chick embryos, the first indication of non-radial distribution of the nuclear form of β-catenin is found in the Koller's sickle (Roeser et al., 1999). The Koller's sickle has been proposed to be the Nieuwkoop center equivalent in chick (Callebaut, 2005 and references herein). This structure is located adjacent to the region where chicken *Brachyury* is first expressed in the epiblast (Knezevic et al., 1997). Hence, these data suggest that localized Wnt signaling in extra-embryonic tissues, namely the posterior visceral endoderm in the mouse and Koller's sickle in the chick, precedes the activation of *Brachyury* in the epiblast and subsequent appearance of the primitive streak in amniotes.

In chick embryos, the Nieuwkoop center equivalent has also been proposed to reside in the posterior marginal zone of the pre-primitive streak embryo at stage XII (Bachvarova et al., 1998). Topologically, the equivalent of the chick posterior marginal zone in the mouse is the posterior extra-embryonic ectoderm. This region of the mouse embryo is thus a candidate Nieuwkoop center equivalent in mice. In support of this hypothesis, two recent articles have proposed that the extra-embryonic ectoderm is responsible for induction of the primitive streak. In *Elf5* mutants, the apparent lack of extra-embryonic ectoderm and absence of *Brachyury* expression in half of the mutant embryos has been interpreted as proof of the inductive role of extra-embryonic ectoderm in primitive streak formation (Donnison et al., 2005). Also, based on experiments in which the egg cylinder is severed at the epiblast/extra-embryonic ectoderm boundary and on experiments in which extra-embryonic ectoderm cells are grafted to the distal tip of the epiblast, the extra-embryonic ectoderm has been proposed to be the source of signals that induce a streak (Rodriguez et al., 2005). Both studies, however, rely on the expression of *Brachyury* as a marker of the primitive streak. Our results and those of Perea-Gomez et al. (2004) clearly demonstrate that *Brachyury* is not an exclusive marker of the primitive streak or mesoderm in mice; therefore, caution should be taken on the interpretation of these and other experiments in which *Brachyury* is used as a primitive streak or mesodermal marker.

Regardless of whether the posterior visceral endoderm, the extra-embryonic ectoderm or a synergy of both tissues results in the induction of the primitive streak in mice, functional evidence to support their role in primitive streak formation remains to be provided.

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References

Arnold, S.J., Stappert, J., Bauer, A., Kispert, A., Herrmann, B.G., Kemler, R., 2000. Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mech. Dev. 91, 249–258.

Bachvarova, R.F., 1996. Anterior–posterior polarization and mesoderm inducing factors in the pre-gastrula mouse embryo. Comparison to chick and frog embryos. Adv. Dev. Biol. 4, 147–191.

Bachvarova, R.F., Skromne, I., Stern, C.D., 1998. Induction of primitive streak and Hensen's node by the posterior marginal zone in the early chick embryo. Development 125, 3521–3534.

Beddington, R.S.P., Lawson, K.A., 1990. Clonal analysis of cell lineages. In: Copp, A.J., Cockroft, D.L. (Eds.), Postimplantation Mammalian Embryos: A Practical Approach. Oxford Univ. Press, Oxford, NY, pp. 267–292.

Beddington, R.S., Robertson, E.J., 1998. Anterior patterning in mouse. Trends Genet. 14, 277–284.

Beddington, R.S., Robertson, E.J., 1999. Axis development and early asymmetry in mammals. Cell 96, 195–209.

Callebaut, M., 2005. Origin, fate, and function of the components of the avian germ disc region and early blastoderm: role of ooplasmic determinants. Dev. Dyn. 233, 1194–1216.

Conlon, F., Beddington, R., 1995. Mouse gastrulation from a frog's perspective. Semin. Dev. Biol. 6, 249–256.

Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B., Robertson, E.J., 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120, 1919–1928.

Donnison, M., Beaton, A., Davey, H.W., Broadhurst, R., L'Huillier, P., Pfeffer, P.L., 2005. Loss of the extraembryonic ectoderm in Elf5 mutants leads to defects in embryonic patterning. Development 132, 2299–2308.

Downs, K.M., Davies, T., 1993. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development 118, 1255–1266.

Escalante-Alcalde, D., Hernandez, L., Le Stunff, H., Maeda, R., Lee, H.S., Gang Jr., C., Sciorra, V.A., Daar, I., Spiegel, S., Morris, A.J., Stewart, C.L., 2003. The lipid phosphatase LPP3 regulates extra-embryonic vasculogenesis and axis patterning. Development 130, 4623–4637.

Fagotto, F., Guger, K., Gumbiner, B.M., 1997. Induction of the primary dorsalizing center in *Xenopus* by the Wnt/GSK/beta-catenin signaling pathway, but not by Vg1, Activin or Noggin. Development 124, 453–460.

Finley, K.R., Tennessen, J., Shawlot, W., 2003. The mouse secreted frizzled-related protein 5 gene is expressed in the anterior visceral endoderm and foregut endoderm during early post-implantation development. Gene Expression Patterns 3, 681–684.

Harland, R., Gerhart, J., 1997. Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. 13, 611–667.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., Ish-Horowicz, D., 1995. Expression of a Delta homologue in prospective neurons in the chick. Nature 375, 787–790.

Herrmann, B.G., Labeit, S., Poustka, A., King, T.R., Lehrach, H., 1990. Cloning of the T gene required in mesoderm formation in the mouse. Nature 343, 617–622.

Kemp, C., Willems, E., Abdo, S., Lambiv, L., Leyns, L., 2005. Expression of all Wnt genes and their secreted antagonists during mouse blastocyst and postimplantation development. Dev. Dyn. 233, 1064–1075.

Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S., Matsuo, I., 2000. Visceral endoderm mediates forebrain development by suppressing posteriorizing signals. Dev. Biol. 225, 304–321.

Knezevic, V., De Santo, R., Mackem, S., 1997. Two novel chick T-box genes related to mouse Brachyury are expressed in different, non-overlapping mesodermal domains during gastrulation. Development 124, 411–419.

Lawson, K.A., Meneses, J.J., Pedersen, R.A., 1991. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development 113, 891–911.

Liu, P., Wakamiya, M., Shea, M.J., Albrecht, U., Behringer, R.R., Bradley, A., 1999. Requirement for Wnt3 in vertebrate axis formation. Nat. Genet. 22, 361–365.

Lu, C.C., Robertson, E.J., 2004. Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior–posterior patterning. Dev. Biol. 273, 149–159.

Lu, C.C., Brennan, J., Robertson, E.J., 2001. From fertilization to gastrulation: axis formation in the mouse embryo. Curr. Opin. Genet. Dev. 11, 384–392.

Mohamed, O.A., Clarke, H.J., Dufort, D., 2004. Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. Dev. Dyn. 231, 416–424.

Perea-Gomez, A., Rhinn, M., Ang, S.L., 2001. Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. Int. J. Dev. Biol. 45, 311–320.

Perea-Gomez, A., Camus, A., Moreau, A., Grieve, K., Moneron, G., Dubois, A., Cibert, C., Collignon, J., 2004. Initiation of gastrulation in the mouse embryo is preceded by an apparent shift in the orientation of the anterior–posterior axis. Curr. Biol. 14, 197–207.

Rivera-Perez, J.A., Mager, J., Magnuson, T., 2003. Dynamic morphogenetic events characterize the mouse visceral endoderm. Dev. Biol. 261, 470–487.

Rodriguez, T.A., Casey, E.S., Harland, R.M., Smith, J.C., Beddington, R.S., 2001. Distinct enhancer elements control Hex expression during gastrulation and early organogenesis. Dev. Biol. 234, 304–316.

Rodriguez, T.A., Srinivas, S., Clements, M.P., Smith, J.C., Beddington, R.S., 2005. Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. Development 132, 2513–2520.

Roelink, H., Wagenaar, E., Lopes da Silva, S., Nusse, R., 1990. Wnt-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to int-1/Wnt-1 and is normally expressed in mouse embryos and adult brain. Proc. Natl. Acad. Sci. U. S. A. 87, 4519–4523.

Roeser, T., Stein, S., Kessel, M., 1999. Nuclear beta-catenin and the development of bilateral symmetry in normal and LiCl-exposed chick embryos. Development 126, 2955–2965.

Rossant, J., Tam, P.P., 2004. Emerging asymmetry and embryonic patterning in early mouse development. Dev. Cell 7, 155–164.

Skromne, I., Stern, C.D., 2001. Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo. Development 128, 2915–2927.

Srinivas, S., Rodriguez, T., Clements, M., Smith, J.C., Beddington, R.S., 2004. Active cell migration drives the unilateral movements of the anterior visceral endoderm. Development 131, 1157–1164.

Tam, P.P.L., Gad, J.M., 2004. Gastrulation in the mouse embryo. In: Stern, C.D. (Ed.), Gastrulation. From Cells to Embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 233–262.

Thomas, P., Beddington, R., 1996. Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. Curr. Biol. 6, 1487–1496.

Thomas, P.Q., Brown, A., Beddington, R.S., 1998. Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development 125, 85–94.

Wilkinson, D.G., Bhatt, S., Herrmann, B.G., 1990. Expression pattern of the mouse T gene and its role in mesoderm formation. Nature 343, 657–659.

Yamaguchi, T.P., Takada, S., Yoshikawa, Y., Wu, N., McMahon, A.P., 1999. T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes Dev. 13, 3185–3190.