Ph.D. thesis

Origin of Anterior-Posterior axis in the mouse embryo.

~Baby's first steps~

(マウス胚における前後軸の起源)

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Contents

1. Abstract

In mammals, including mouse, it is generally known that three body axes are established after implantation. The anterior-posterior (A-P) axis is the first overt body axis established in the mouse embryo. The A-P axis determination becomes evident when the visceral endoderm located at the distal tip of the embryo (distal visceral endoderm, known as DVE) migrates toward the future anterior side to become the anterior visceral endoderm (AVE) at embryonic day (E) 5.5～6.0. However, when and how DVE and AVE cells are exactly specified during early development remain unknown. The direction of DVE migration is pre-determined by asymmetric expression of two Nodal antagonists, *Lefty1* and *Cerl,* at E5.5. However, it remains unknown how asymmetric expression of *Lefty1* and *Cerl* is regulated.

In this study, I found that Lefty1 gene, a Nodal antagonist known influence the direction of DVE migration, was asymmetrically expressed in the primitive endoderm of the peri-implantation embryo (E4.2-4.5). *Lefty1* expression begins in 2-3 blastomeres of the inner cell mass at E3.5. Fate mapping analysis with the *Cre-loxP* system has shown that Lefty1-positive cells at E3.5 and those at E4.2 have different fates, the former to the epiblast, while the latter will specific to a subset of VE including DVE at E5.5, indicating that *Lefty1* expression at E4.2 specifies future anterior polarity. Unexpectedly, DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5. Instead, DVE cells are fated only to the most proximal region of AVE and the neighboring VE in the lateral region, suggesting that AVE cells have multiple origins. The expression of *Lefty1* in epiblast-fated cells was shown to depend on FoxH1 binding sites, while a FoxH1-independent mechanism is involved in *Lefty1* expression in primitive endoderm

cells. *In vitro* culture of E3.5 embryos resulted in asymmetric *Lefty1* expression in the primitive endoderm similar to the one observed. Recovered from pregnant mice, therefore, the A-P axis is established without interaction with the uterus. These results suggest that the origin of the A-P axis can be pushed back to *Lefty1* expression at the peri-implantation stage.

2. Introduction

Figure1 and 2 summarize the origin of early murine tissue is thought generally.

The first two lineages to differentiate during late morula stage(embryonic day 2.5, E2.5) are an epithelial outer layer of trophectoderm (TE) and a inner cluster of cells, the inner cell mass (ICM). These two morphologically distinct cell types express different sets of genes in the early blastocyst stage. Cdx2 is expressed in the TE, Oct3 is expressed in the inner cells. At E4.5, at peri-implantation stage, a new epithelial layer known as the primitive endoderm (PrE) is formed on the blastocoelic surface of the ICM (Fig. 1). The PrE contributes to the Visceral Endoderm(VE) and Parietal Endoderm(PE). *Gata6* is a PrE-specific, *Nanog* is a Epiblast(EPI)-specific gene. The epiblast is thought to contain all the cells that will generate the actual embryo. Before the PrE emerges, the segregation and the dispersed localization of the *Gata6* and *Nanog*-expressing cells in the ICM also suggests that allocation cells to the PrE and the epiblast lineages. Also, Pre-implantation embryo harbors clearly morphologically asymmetric in embryonic-abembryonic axis. After implantation, the placenta is divided into distal and proximal portions, embryonic side is the proximal one.

At E5.5, the mouse embryo is composed of four tissues; EPI, VE, ExE, and Distal Visceral Endoderm(DVE). These cells can also be identified by molecular markers that include *Hex* (haematopoietically expressed homeobox), *Cerl* and *Lefty1*. However careful examination has revealed that the expression domains of these genes do not overlap precisely with each other. The A-P axis becomes evident when the visceral endoderm located at the DVE migrates toward the future anterior side to become the anterior visceral endoderm (AVE) at E5.5～6.0　(Beddington and Robertson, 1998, 1999). AVE

cells are responsible for the appropriate patterning of adjacent EPI, play critical role in forebrain development and initiation of gastrulation, and direct the subsequent formation of the A-P axis at E6.5.

Establishment of body axes is a critical event during embryonic development. In many organisms, including *Drosophila*, molecular asymmetries already present in the oocyte provide the initial polarity information for the subsequent development of body axes in the embryo. In the mouse, however, it has been thought that detectable molecular asymmetries develop after implantation (Beddington and Robertson, 1999).

Although several asymmetries in morphological features or cell fates are known to exist in mouse oocytes or preimplantation embryos, whether these asymmetries are related in any meaningful way to body axes at later stages of development has remained unknown (Rossant and Tam, 2004). Presently, no obvious molecular asymmetries have been identified in oocytes, zygotes, or preimplantation embryos, leaving the origin of body axes in the mouse unknown. Pre-implantation embryo harbors clearly morphological asymmetry in embryonic-abembryonic axis, but whether this axis correlates with any event in the zygote has been a subject of recent debate (Gardner, 1997, 2001; Piotrowska et al., 2001; Hiiragi and Solter, 2004; Kurotaki et al.,2007).

The A-P axis is the first overt body axis established in the mouse embryo. It was previously shown that molecular asymmetries do exist before DVE movement. The genes for two Nodal antagonists, *Lefty1* and *Cerl* (Meno et al., 1996; Belo et al., 1997, Fig.3), are expressed asymmetrically along the future A-P axis at E5.5 (Takaoka et al.,2006, Yamamoto *et al*., 2004). Expression of *Lefty1* and *Cerl* is shifted toward the future

anterior side, and influences the direction of cell movement most likely by generating a directional propulsion via local inhibition of cell proliferation in the visceral endoderm (Fig.1,3). However, it remains unknown how asymmetric expression of *Lefty1* and *Cerl* is regulated.

When is the DVE specified? DVE-specific gene; *Hex* is induced in all primitive endoderm cells at E4.5 but is transiently downregulated by BMP4 in ExE between E5.0 and E5.25, and indirectly by promoting growth of the egg cylinder, thereby attenuating the repressive influence of the BMP4 in ExE on the forming DVE at E5.5 .

This study is summarized in Figures 1, 18. I found that *Lefty1* expression was maintained in a subset of primitive endoderm cells between E5.0 and E5.25, was asymmetrically expressed in the primitive endoderm of the peri-implantation embryo (E4.2-4.5, Fig.4). *Lefty1* expression begins in 2-3 blastomeres of the inner cell mass at E3.5. Fate mapping analysis has shown that Lefty1-positive cells at E3.5 and those at E4.2 have different fates, the latter to the epiblast, while the former is specific to a subset of VE including DVE at E5.5, indicating that *Lefty1* expression at E4.2 specifies future anterior polarity (Fig.4). Unexpectedly, DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5. Instead, DVE cells are fated only to the most proximal region of AVE and the neighboring VE in the lateral region, suggesting that AVE cells have multiple origins(Fig.1). The expression of *Lefty1* in epiblast-fated cells was shown to depend on FoxH1 binding sites, while a FoxH1-independent mechanism is involved in *Lefty1* expression in primitive endoderm cells (Fig. 16). *In vitro* culture of E3.5 embryos resulted in asymmetric *Lefty1* expression in the primitive endoderm similar to the one observed. The result indicate, A-P axis formation can take place autonomously, driven by

factors intrinsic to the embryo.

These results suggest that the origin of the A-P axis can be pushed back to *Lefty1* expression at the peri-implantation stage.

3 Materials&Methods

Generation of transgenic mice

A transgenic mouse line (F32) harboring *Lefty1-9.5 lacZ* (a *lacZ* reporter that made of the 9.5-kb upstream region of *Lefty1*, including the transcription start site) was established previously (Saijoh et al., 1999). The *lacZ* transgene of F32 mice resembles *Lefty1* expression in the floor plate and left lateral plate at the early somite stage. The transgene *Lefty1-9.5 DsRed2* was constructed similarly, with *lacZ* being replaced by *DsRed2mit* (BD Biosciences). The *Hex-Venus* transgene contains the 1.2-kb upstream region of *Hex* (as the minimal promoter), the 1-kb region from intron 1 of *Hex* that has been shown to contain a DVE/AVE-specific enhancer (Rodriguez et al., 2001), and *Venus* (Nagai et al., 2002).

A transgenic mouse line harboring *Lefty1-0.7 lacZ* (a *lacZ* reporter that contains the 3.5kb upstream region of *Lefty1* juxtaposed to the region normally positioned from 8.5 to 7.8kb upstream to the transcription start of *Lefty1*) was previously established (Takaoka *et al*., 2006). The *lacZ* transgene of *Lefty1-0.7 lacZ* mice reproduces asymmetric *Lefty1* expression in the primitive endoderm and AVE. The *Lefty1-0.7FmZ* transgene activity was described previously (Takaoka *et al*., 2006). This transgene differs from the previous one, in that the two FoxH1 binding sites located in the 0.7kb sequence are mutated. The transgene *Lefty1-0.7FmCre* harbors a similar construction with *lacZ* being substituted by Cre.

The *Lefty1-BAC lacZ* transgene in made from the *Lefty1* BAC RP23-390I1, with exon 1 of *Lefty1* being replaced by *lacZ*. The highly efficient phage-based recombination system for *Escherichia coli* (Copeland et al., 2001) was used to construct the recombinant

BAC clones. BAC DNA was prepared for microinjection as described (Gong et al., 2003). Transgenic mice were generated as described previously (Saijoh et al., 1999). F32 mice were crossed with *Foxh1* null mutant mice (Yamamoto et al., 2001) to examine *lacZ* transgene expression in the absence of FoxH1.

The following *Lefty1* transgenes have been constructed from the *Lefty1* BAC clone, RP23-390I1. In the *Lefty1-BAC memVenus* transgene, the first exon of *Lefty1* has been replaced by *Venus-mem*, which encodes a fusion protein consisting of *Venus* (Nagai *et al*., 2002) and Neuromodulin N-terminal sequences(679-738) of pECFP-mem (BD Biosciences). The *Lefty1-BAC memVenus-Lefty2-memTomato* transgene is a construct, where exon 1 of *Lefty1* was replaced by *Venus-mem*, and exon 1 of *Lefty2* by *tdTomato-mem* (Shaner *et al*.,2004). In the *Lefty1-BAC CreERT2* transgene, *CreERT2* replaced the exon 1 of *Lefty1* (obtain via Addgene; Feil *et al*.,1997). The *Lefty1-BAC lacZ* transgene made from with exon 1 of *Lefty1* being replaced by *lacZ*

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Recovery of peri-implantation mouse embryos

Preimplantation embryos at stages up to E4.0 were recovered by flushing the uterus. Embryos at E4.2 and later were dissected out from the uterus. The embryos were staged on the basis of their morphology and the way they interact with the uterus.

X-gal staining and Whole-mount in situ hybridization

Transgenic embryos were stained with Xgal as described previously (Saijoh *et al*., 1999). Fluorescence in situ hybridization was performed with a method designed for peri-implantation embryos (Strumpf et al., 2005) (also see http://www.mshi.on.ca/rossant/protocols/doublefluor.html). Nuclei were stained with YOYO-1 (Molecular Probes). Whole-mount in situ hybridization was performed according to Wilkinson's procedures (Wilkinson 1992) with digoxigenin-labeled riboprobes specific for *Nodal* (Lowe *et al*., 1996), *Lefty1* and *Lefty2* (Meno *et al* 1996 and Meno *et al* 1997). Wild-type and mutant embryos were processed in the same tube. Embryos were genotyped by PCR analysis of whole embryo DNA.

In vitro culture of preimplantation embryos in hanging drops

Mouse embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5, cultured in KSOM medium (Lawitt and Bigger, 1993) until they hatched, transferred to hanging drops comprising Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, and incubated for an additional 18 h. To determine optimal conditions for hanging drop culture, we tested four different lots of fetal bovine serum that were intended for culture of mouse embryonic stem cells and one lot of rat serum prepared in-house for culture of mouse embryos. The lot of fetal bovine serum (Hyclone; catalog no. SH30070.02, lot no. AQC23292) that supported the development of embryos with a relatively normal morphology as well as expression of *Pem* in the primitive endoderm was used in this study. Incubation of embryos for a longer time (24 h) after hatching did not improve development. Under the optimal conditions (incubation in hanging drops

containing KSOM until hatching followed by further incubation in hanging drops in DMEM plus 20 % fetal bovine serum for 18 hours), embryos developed to a stage equivalent to E4.25 in terms of morphology (in particular, the tilt) and the expression pattern of *Pem*. The time of hatching was variable among the embryos in the first phase of culture in KSOM.

Mapping of the AVE-specific enhancer of *Lefty1*

F32 transgenic embryos showed specific expression of *lacZ* in the AVE between E5.5 and E6.5. To map the enhancer responsible for such an expression, we generated various smaller DNA fragments from the 9.5-kb upstream region of *Lefty1* and tested their enhancer activity with a transient transgenic assay, as described previously (Saijoh et al., 1999). All constructs contained the minimal promoter of *Lefty1* (the 3-kb upstream region). Transgenic embryos were recovered at E6.5 (to detect enhancer activity in the AVE) or at E4.5 (to detect asymmetric *lacZ* expression in the primitive endoderm). In *Lefty1-0.7Fm*, two FoxH1-binding sequences (TGTGGATT) were changed to TGTGGcag and TGTGGccc.

Immunofluorescence

Embryos were carefully staged on the basis of their morphology. They were fixed in 4% paraformaldehyde(PFA) in PBS for over night at 4℃, and washed with PBS twice, blocked in TSA blocking reagent (Perkin-Elmer) for 1hr at room temperature. Immunostaining was performed with Goat anti-GATA6 (1/50, R&Dsystems, AF1700), Mouse anti-Dab-2(1/ 100, BD Transduction Laboratories), and Alexa Fluor

568–conjugated anti-goat-immunogloblinG (IgG) antibodies (invitrogen). Washing solution used between staining steps　solution is PBS. Images were obtained with a laser scanning confocal microscope (Carl Zeiss, Inc ,LSM 510). After washes in PBS twice, nuclei were stained by 30 minutes incubation with 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI,Wako, 049-188101, 1/2000 in PBS)

Tamoxifen treatment

R26R mice were obtained from Jackson Laboratory(Soriano ,1999)and crossed with *Lefty1-BAC CreERT2* mice. We pestle 1mg Tamoxifen(Sigma, T5648), 1.25mg methylcellulose 50(Wako 135-05045) and 250 μ l water. For treatment of pregnant females, 0.5-1.0mg/body Tamoxifen was given at times indicated. For treatment of cultured embryos, 4-hydroxytamoxifen(sigma, H7904) diluted in ethanol(6mg/ml) was directly added to the embryos culture media (final concentration, 3ug/ml) for 3hr.

Timelapse observation

Lefty1-BAC-memVenus transgenic embryos were recovered in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 25 mM HEPES-NaOH (pH 7.2), and transferred to embryo culture medium composed of 50% fetal bovine serum (Hyclone; catalog no. SH30070.02, lot no. AQC23292), 50% DMEM buffered with 44 mM NaHCO3 (pH 7.2). Culture took place into a CO2 controlled incubator (INUG2, TOKAI HIT, Japan). Time-lapse images acquisition was done with an Olympus FV1000 confocal microscope. With samples on a glass-bottomed 35 mm plastic dish (IWAKI 11-004-008). Images were acquired on multiple positions at 30-min

intervals using the conditions described above, except for the objective lens: 40X (Olympus UPlanFl NA 0.75) and 20X(Olympus UPlanApo NA 0.70).

4. Results

Asymmetric expression of *Lefty1* in primitive endoderm of implanting embryo

We have previously shown by two-color in situ hybridization that *Lefty1* is asymmetrically expressed in the visceral endoderm of E5.5 mouse embryo, before the onset of DVE cell migration (Yamamoto et al., 2004). To elucidate the asymmetric expression of *Lefty1* relative to *Hex* expression, we generated two lines of transgenic mice: one expressing the marker protein DsRed2 in *Lefty1*-expressing cells, and the other expressing the marker protein Venus (Nagai et al., 2002) under the control of the DVE- and AVE-specific enhancer of *Hex* (Rodriguez et al., 2001). Embryos harboring both transgenes were examined for Venus and DsRed2 fluorescence at various stages of development. Venus (Hex)–positive DVE cells were located at the distal end of the embryo at E5.25 and E5.5, whereas *Lefty1*–positive cells covered a wider domain that was shifted to one side (Figure 4A). DsRed2-positive, Venus-negative cells were thus found on the prospective anterior side toward which the *Hex*-positive DVE cells will move.

We investigated when asymmetric expression of *Lefty1* begins with the use of a transgenic mouse (line F32) harboring a *Lefty1-9.5 lacZ* construct that resembles *Lefty1* expression (Saijoh et al., 1999). Unexpectedly, *Lefty1*-expressing cells (cells stained with Xgal: 5-bromo-4-chloro-3-indoyl-β-D-galactoside) were found to be distributed asymmetrically in the primitive endoderm, being present on one side only, of embryos at E4.25 (Figure 4B). They continued to be distributed asymmetrically in the primitive endoderm at E4.5 and E4.75 (Figure 4B: embryos were staged according to their morphology).

Three lines of evidence confirmed that Xgal staining patterns obtained with the *Lefty1-9.5 lacZ* transgene reflected genuine *Lefty1* expression. First, we generated a transgenic mouse line (B1) that harbors a 250-kb bacterial artificial chromosome (BAC) clone (RP23-390I1) of *Lefty1*, with exon 1 of *Lefty1* being replaced by *lacZ*. Xgal staining of embryos of this line revealed again transgene expression on one side of the primitive endoderm at E4.5 and of the DVE at E5.5 (Figure 5A). Second, a transgenic mouse line (*Lefty1-0.7 lacZ*) in which *lacZ* expression is regulated by the minimal 0.7-kb enhancer fragment of *Lefty1* manifested asymmetric Xgal staining at E4.5 (see below). Third, *Lefty1* mRNA was detected by fluorescence in situ hybridization on one side of the primitive endoderm of wild-type embryos between E4.25 and E5.5 (Figure 5B).

Localization of *Lefty1*-expressing cells in peri-implantation embryos

Expression of the *Lefty1-9.5 lacZ* and *Lefty1-0.7 lacZ* transgenes was first detected in the blastocyst at E3.5 (Figure 5B; also see Figure 12A). Expression of *Lefty1* likely begins at E3.5 because most (70%) of transgenic embryos remained Xgal-negative at this time. In the Xgal-positive embryos, stained cells were apparent in the inner cell mass (ICM), but it was not possible to determine their precise location because there are few obvious topological features at this stage. However, $Xgal^+$ cells were detected at the periphery or center of either the deep or shallow regions of the ICM (Figures 6B, 12A), suggesting that *Lefty1* expression in the blastocyst might occur randomly in blastomeres of the ICM.

Mouse embryos between E3.75 and E4.0 are bilaterally symmetric and exhibit a long axis and a short axis (Smith, 1980, 1985; Gardner, 1997). The location of *Lefty1*-positive

cells relative to the short axis and long axis was determined in *Lefty1-0.7 lacZ* embryos. At E3.75, when *Lefty1* expression was detected inside of the ICM (Figure 6A), embryos contained an average of three $Xgal^+$ cells (n = 10 embryos), and these cells showed no preferential localization. For instance, $Xgal^+$ cells were detected in equal numbers in the quadrant on either side of the short axis when viewed from the abembryonic pole (Figure 6B). In embryos at E4.0 (Figure 6A), *Lefty1*-expressing cells were mainly found near the blastocoelic surface of the ICM, where prospective primitive endoderm cells reside (Rossant et al., 2003). The $Xgal^+$ cells of each embryo were in a cluster but are still randomly distributed relative to both the long and short axes (Figure 6B; $p=0.471>>0.05$ according to Pearson's correlation coefficient test).

At E4.25, the mouse embryo develops a transient asymmetry, the tilt of the ICM along the long axis of bilateral symmetry (Smith, 1980, 1985). It has been suggested (Smith, 1985; Gardner et al., 1992) that the tilt of the ICM marks the future A-P axis. This tilt provides a landmark of the polarity of the long axis, which enables unequivocal determination of the location of $Xgal^+$ cells. Cells positive for Xgal staining were located on the "upper" side (the side closer to the "upper" edge) of the tilt in all embryos examined (n = 10) (Figure 6C, D). It is worth noting that *Lefty1* mRNA was also detected on the upper side of the primitive endoderm at E4.25 (Figure 5B). *Lefty1*-expressing cells were similarly localized to the upper side of the tilted ICM at E4.5 (Figures 4B, 5A) and E4.75 (Figures 4B, 5A, 6C). Taken together, these results suggest that *Lefty1* expression begins randomly in the ICM at E3.5, but that *Lefty1*-expressing cells become localized to a specific position within the primitive endoderm by E4.2.

Asymmetric *Lefty1* expression in the primitive endoderm does not require interaction with the uterus

The E4.25 embryo that displays asymmetric *Lefty1* expression (Figure 6C) is loosely attached to the uterus within the implantation chamber but is not fully implanted. It has been suggested that the uterus may provide positional information to the mouse embryo (for a review, Tam et al., 2001), and therefore the asymmetric *Lefty1* expression in the primitive endoderm could be dependent on the interaction of the embryo with the uterine tissues at implantation.

To test the role of implantation in the establishment of asymmetry of *Lefty1* expression domain, preimplantation embryos were cultured in vitro beyond the implantation period without being attached or exposed to the uterine environment. Embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5 and cultured in hanging drops until they developed to a stage equivalent to E4.25. *Pem*, a marker for the primitive endoderm (Lin et al., 1994), was expressed in the cultured embryos, showing that the primitive endoderm has developed normally (Figure 7C, D). In these embryos, *Lefty1* was expressed asymmetrically in the primitive endoderm. In embryos with a tilted ICM (7/13 embryos; Figure 7F), $Xgal^+$ cells were localized to the upper side of the primitive endoderm (all 7 embryos). Even in the remaining 6 embryos that did not display an obvious tilt of the ICM, $Xgal^+$ cells were asymmetrically localized to one side of the primitive endoderm (5/6 embryos: data not shown). These results strongly suggest that asymmetric expression of *Lefty1* in the primitive endoderm is acquired autonomously in the implanting embryo and does not require any interaction with the uterus.

Asymmetric expression of *Lefty1* is induced by Nodal signaling

To investigate the mechanism by which asymmetric *Lefty1* expression is achieved in the primitive endoderm and AVE, we searched for the transcriptional enhancer responsible for such an expression. A series of 5'-deletion fragments derived from the 9.5-kb upstream region of *Lefty1* was examined with a transient transgenic assay looking for enhancer activity that gives rise to *lacZ* expression in the AVE at E6.5. After testing the various *lacZ* constructs, such an enhancer activity could be mapped to a 0.7-kb region positioned between 8.5 and 7.8 kb upstream of the transcription start site (Figure 8A). The nucleotide sites of this 0.7-kb region (Figure 8B) is highly conserved among mouse, rat, and human and contains a pair of binding sequences for FoxH1 (TGTGGATT, TGTGGATT), a transcription factor that mediates Nodal signaling (Whitman, 1998; Saijoh et al., 2000). A transgene in which both putative FoxH1 binding sites in the 0.7-kb region are mutated (*Lefty1-0.7Fm lacZ*) failed to give rise to Xgal staining in the AVE at E6.5 (Figures 8A, 9B), suggesting that the enhancer activity depends on Nodal signaling. We designated this enhancer APE (asymmetric primitive endoderm enhancer).

The 0.7-kb region of *Lefty1* containing the two FoxH1 binding sites was not only active in the AVE at E6.5 but also gave rise to asymmetric Xgal staining in the primitive endoderm between E4.25 and E4.75 (Figures 8A, 9A). We also examined expression of the *Lefty1-9.5 lacZ* transgene in *Foxh1*$^{-/-}$ embryos. The transgene was inactive between E3.5 and E6.5 in the mutant embryos (Figure 9C). These results thus indicated that asymmetric *Lefty1* expression in the primitive endoderm and the AVE is induced by Nodal signaling acting at the FoxH1-dependent enhancer APE.

Lack of asymmetry in the expression of *Nodal* and of genes for Nodal effectors

Given the dependence of the identified *Lefty1* enhancer on Nodal signaling, we examined the expression of *Nodal* as well as that of genes for components of the Nodal signaling pathway, including FoxH1 and Cripto, in the mouse embryo between E3.5 and E4.75 (Figure 10). Both *Nodal* and *Foxh1* were expressed in the ICM at E3.5 as well as in both the epiblast and the primitive endoderm between E4.25 and E4.75. *Cripto* was also expressed in the ICM at E3.5 and in the epiblast between E4.25 and E4.75, but *Cripto* expression was not evident in the primitive endoderm during the latter period. *Nodal*, *Foxh1*, and *Cripto* are thus all expressed relatively uniformly between E3.5 and E4.75, with no evidence of asymmetry.

The lack of *Cripto* expression in the primitive endoderm between E4.25 and E4.75 (Figure 10) suggests that Nodal signaling may be required only for the initiation of *Lefty1* expression in the blastocyst.

Two populations of *Lefty1*-positive cells between E3.5 and E4.0

Lefty1 expression, as revealed by *Lefty-lacZ or Lefty1-memmbrane Venus* transgene, begins in fews cells (2~3) of the blastocyst at E3.5 (Fig. 12 A). These *Lefty1*-expressing cells do not express *GATA6* (Fig. 12B: 2/2 embryos), a marker for the prospective primitive endoderm, suggesting that they may contribute to the epiblast. At E3.7～4.0, the Lefty1 positive cells are GATA6 negative in most of the embryos observed (10/15 embryos) while positive for GATA6 in the remaining embryos (5/15em). At E4.2, *Lefty1* is expressed in the primitive endoderm on the upper side of the tilted embryo (Takaoka et al., 2006). Lefty1-positive cells at E4.2~E4.5 were indeed positive for GATA6 (Fig. 12B;

5/5em).

To know the relationship between Lefty1(+), GATA6(-) cells at E3.5 and Lefty1(+), GATA6(+) cells at E4.2, we performed time-lapse observation of *Lefty1-memVenus* positive cells at E3.5 for 12hr (Fig. 12C). At the end of the observation, embryos were examined for GATA6 expression with an anti-GATA6 antibody. Red arrowhead is Lefty1(+), GATA6(-). In this cell, *Lefty1* is expressed 7hr after observation began. These results suggested that Lefty1-expressing cells in future primitive endoderm differ from the ones seen expressing at E3.5.

DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5

To determine the fate of Lefty1(+) cells at various stages, we have generated transgenic mouse harboring *Lefty1* BAC, in which the coding region of *Lefty1* is replaced by *CreER^{T2}*, a tamoxifen-inducible Cre recombinase (Fig.13). Transgenic mice harboring *Lefty1 CreER^{T2} BAC* were crossed with a reporter mouse in which Cre activity induces expression of β-galactosidase (LacZ) from the ubiquitously active *Rosa26* locus. Embryos harboring both *CreER^{T2}* and *LacZ* were treated with tamoxifen (Tx) and analyzed at various stages. Transient treatment of transgenic embryos with Tx at E4.5 and E6.5 labeled primitive endoderm cells on the upper side and the AVE, respectively (Fig. 14B, C), confirming that Cre expression pattern in *Lefty1 CreER^{T2} BAC* transgenic mouse line reflect genuine *Lefty1* expression.

Lefty1 is expressed in DVE at E5.5, in AVE at 6.5 (Fig.14B). To confirm that DVE contributes to AVE, 0.5-1.0mg/body Tamoxifen was given E5.5 by feeding treatment of pregnant females, and transgenic embryos were stained with X-gal at E6.5.

Lefty1-expressing cells in DVE at E5.5 contributed to the most proximal portion of AVE and neighboring VE (Fig. 14 I). It should be noted that Lefty1-positive cells in DVE at E5.5 do not contribute to the entire region of AVE but to its most proximal region and neighboring VE in the lateral region (the remaining portion of AVE is newly formed between E5.5 and E6.5). This result suggests that DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5.

Lefty1 expression at E4.2 specifies future anterior polarity

To test the fate of *Lefty1*-expressing cells at E4.2, transgenic embryos at E3.7 were cultured in hanging drops for 12～14hr before treatment with 3 μ g/ml 4-OHTx for 3hr, then were allowed to develop *in utero,* and were analyzed at E5.5 or E6.5 (Fig.14D, D', E, E'). In these embryos, we found two different patterns of X-gal staining. The first one is DVE cells at E5.5, and proximal AVE cells at E6.5 (Fig.14 D,E). The second pattern is a X-gal-positive cells in a subset of visceral endoderm (VE) including distal tip (DVE) at E5.5 (Fig. 14D'). At E6.5, the most proximal portion of AVE, the neighboring VE in the lateral region and the extra-embryonic VE were stained with X-gal (Fig. 14E'). In addition, when pregnant females were fed with Tamoxifen at E4.2, and transgenic embryos were stained with X-gal at E5.5 and E6.5. These reproduces the results obtained by in vitro treatment (Fig.14 D,D',E,E',G,G',H,H'). These data suggested that *Lefty1* is expressed in DVE-progenitor cells, *Lefty1* expression at E4.2 specifies future anterior polarity.

***Lefty1*-positive cells at E3.5 and those at E4.2 adopt different fates.**

When the transgenic embryos were treated with 4-OHT at E3.5 in vitro for 3hr, were then allowed to developed *in utero* and were finally analyzed at E6.5, the epiblast was specifically positive for LacZ, suggesting that Lefty1(+) cells at E3.5 are fated to the epiblast (Fig. 14F). When conventional Cre was used instead of $CreER^{T2}$, E6.5 embryos harboring *Lefty1 BAC-Cre* and the *LacZ* reporter allele gave rise to X-gal staining in both epiblast and VE including AVE (Fig. 14A).

These data indicate that Lefty1-positive cells at E3.5 and those at E4.2 have different fates, the former to the epiblast, while the latter will specific to a subset of VE including DVE at E5.5.

Distinct mechanisms induces *Lefty1* expression in the epiblast-fated and DVE-fated cells

We next examined transcriptional regulation mechanism that induces *Lefty1* expression either at E3.5 or E4.0. *Lefty1* expression (as revealed with various *Lefty1* transgenes or by in situ hybridization for *Lefty1* mRNA) was lost in $FoxH1^{-/-}$ embryos at both E3.5 and E4.5 (Fig. 9A; Takaoka., et al 2006), although the expression of *Nodal*, *Oct3/4* and *GATA6* remain normal at E3.5 and E4.5 (Fig. 15A). *Lefty1* expression was also absent in $Nodal^{-/-}$ embryos at both E3.5 and E4.5. These results suggest that Nodal-FoxH1 signaling is essential to induce *Lefty1* expression, in the epiblast-fated and VE-fated lineages as well. On the other hand, induction of *Lefty1* in the VE-fated cells at E4.5 can take place independently of FoxH1. The 0.7 kb region located 8.2kb upstream of *Lefty1* contains two FoxH1-binding sequences and can reproduce *Lefty1* expression from E3.5 to E6.7 (*0.7-LacZ*; Takaoka et al.,2006). When two FoxH1-binding sequences are mutated,

the corresponding *LacZ* construct (*0.7FmLacZ*) is no longer active in AVE at E6.5 and in the epiblast-fated cell at E3.5. However, *0.7FmLacZ* gave rise to X-gal staining in a subset of the primitive endoderm albeit at a low level, suggesting that it is still active in the VE-fated cells at E4.5. To confirm this, we generated transgenic mice harboring *0.7FmCre*, in which Cre is driven by the 0.7 kb region lacking the FoxH1-binding sequences. Those cell that expressed Cre at E4.5 contributed to the VE including the proximal portion of AVE and extra-embryonic VE at E6.5 (Fig. 15C), resemble in localization of cells labeled by *Lefty1 CreER*^{T2}at E4.5 (Fig. 14E',H'). These results obtained with *0.7FmLacZ* and *0.7FmCre* suggest that a FoxH1-independent signal can induces *Lefty1* expression in the VE-fated cells at E4.5.

5. Discussion

We have identified the earliest known molecular patterning in the mouse embryo. Our results thus show that the mouse embryo is already patterned along the future A-P axis around the time of implantation, the DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5. In addition, the expression of *Lefty1* in epiblast-progenitor cells at E3.5 and DVE/AVE ones at E5.5～6.5 was induced by a Nodal-FoxH1 cascade, *Lefty1* in DVE-progenitor cells at E4.5 was induced by a Nodal signaling indirectly. Furthermore, we found that establishment of asymmetric *Lefty1* expression in primitive endoderm does not require any interaction of the embryo with the uterus.

Origin of DVE

DVE cells can also be identified by molecular markers that include *Hex* (Thomas et al.,1998), *Cerl*(Belo et al.,1997), *Lefty1*(Meno et al.,1999, Perea-Gomez et al., 1999). *Hex* and *Cerl* expression is induced in some primitive endoderm cells at E4.5 but is transiently downregulated between E5.0 and E5.25, reappearing in the distal region at E5.5 (Mesnard et al.,2006, Rodriguez et al.,2001, Chazaud et al., 2006, Torres-Padilla et al.,2007). These results suggests that DVE specification occurs at E5.5.

Our results show that *Lefty1* expression begins in the implanting embryo and is maintained in a subset of primitive endoderm cells between E5.0 and E5.25, albeit at a low level (Takaoka et al.,2006, Mesnard et al.,2006, Yamamoto et al., 2009). Descendants of *Lefty1* expressing cells at 4.2 contribute to DVE (Fig.14D-E). This data suggests that DVE cells specification starts at E4.2. However, at E5.2-5.5, BMP2,4 affect DVE formation (Soares et al.,2005, Yamamoto et al.,2009). *BMP4* is expressed in

blastocyst (Coucouvanis et al., 1999). So, in half of embryos, DVE specification may take place earlier than E5.5 by BMP4. But, phosphorylated-Smad1 is localized in all primitive endoderm (Yamamoto et al., 2009). It may not to be BMPs.

Fate of DVE cells

Previously, descendants of DVE cells were thought to be the same that of AVE (Beddington et al.,1999)(Srinivas et al.,2004). However, our results indicate that DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5. These contradictory results could be due to differing experimental methods. There is a possibility that results of Srinivas and collaborators are low-resolution image.

The A–P axis of the mouse embryo is established when DVE cells migrate in one direction to form the AVE at E5.5～6.5. DVE cells contribute mainly to anterior side (Fig.14 I). Time-lapse observation of migrating AVE cells has shown that migrates cells to future anterior side (Srinivas et al., 2004). Thus, in the mutant embryo lacking Nap1, a component of WAVE complex (a regulator of the actin cytoskeleton), DVE cells fail to migrate away from the distal tip (Rakeman et al., 2006). These suggested that DVE migration is important for A-P axis formation. At E6.5, AVE cells are responsible for the appropriate patterning of adjacent EPI, play critical role in forebrain development and initiation of gastrulation, and direct the subsequent formation of the A-P axis at E6.5. It is thought that the DVE migration determines the direction of A-P axis, newly induced AVE form head formation.

How AVE is newly induced? Lefty1-0.7FmZ transgene is not expressed in DVE/AVE at E5.5/6.5. This result suggest that Nodal signaling induce Lefty1 in AVE. It

is a possible that BMP4 from ExE inhibits Nodal signaling in proximal embryonic-VE cells.

Role & biological activity of Lefty1

Lefty1, together with *Cerl*, regulates migration of DVE at E5.5 (Yamamoto et al., 2004). However, does *Lefty1* expression at peri-implantation stages have any role? Mutant mice lacking *Lefty1* can develop normal A-P patterning, although they exhibit left-right patterning defects much later, at the early somite stage (Meno et al., 1998). But this may be due to functional complementation by *Lefty2*, since *Lefty2* is expressed in ICM cells at peri-implantation stages (Takaoka et al., unpublished). To fully understand the role of *Lefty1* in A-P patterning, it is necessary to generate mutant mice lacking both *Lefty1* and *Lefty2*.

It is firmly established by the genetic and biochemical data that Lefty1 is an inhibitor of Nodal signaling. During left-right patterning, for example, by blocking Activin ReceptorII in Left-Right axis formation (Sakuma et al., 2002; Hamada et al.,2002). In this stage, Nodal is induced by a Nodal-FoxH1 positive feedback cascade. Although, in peri-implantation stage, Nodal is not induced by such a pathway (Fig. 15 B,C). Therefore, Lefty1 does not inhibit Nodal. On the other hand, Lefty1 precursor is also able to stimulate the mitogen-activated protein kinase(MAPK) signaling pathway. Possibly, Lefty1 may induce MAPK activity, and by this specify primitive endoderm cells to DVE.

At E3.5, *Lefty1* is expressed in future epiblast cells. In mouse ES cells, *Lefty1* is regulated by *Oct3/4*, *Sox2* and *Klf4* (Nakatake et al., 2006). In human ES cells, Lefty1

acts as an inhibitor of Activin/Nodal, and overexpression of *Lefty1* blocked this feedback-loop and so the induction of differentiation (Vallier et al., 2005). Therefore, it is possible that Lefty1/2 play a role in maintaining pluripotency of ICM.

How is *Lefty1* expression induced in blastocyst ?

Lefty1 expression in the blastocyst was confined in two or three cells within the ICM. Similarly, *Lefty1* is expressed only in the primitive endoderm on one side of the peri-implantation embryo. How is *Lefty1* expression restricted to two or three neighboring cells among the total of ~20 ICM cells? *Lefty1* expression at E3.5 requires FoxH1-dependent enhancer located in the upstream region of *Lefty1* gene (Takaoka et al.,2006) suggesting that such an expression is induced by Nodal signaling. One possibility is that a component of Nodal signaling pathway present in the oocyte becomes unevenly distributed during cell division after fertilization. Indeed, a similar mechanism operates in the zebrafish embryo: maternal *Squint* mRNA, a dorsal determinant, becomes unevenly distributed during cell division and is finally enriched in two blastomeres at the four-cell stage (Gore et al., 2005). However, *Nodal* and the genes for Nodal effectors such as FoxH1 and Cripto are all expressed symmetrically. Nonetheless, it remains possible that an unknown maternal factor required for *Lefty1* expression is asymmetrically inherited in a subset of blastomeres.

On the other hand, because induced Nodal pathway is FoxH1 independent, it seems unlikely that restriction of *Lefty1* expression is achieved by a self enhancement-lateral-inhibition (SELI) system (Nakamura et al., 2006), a mechanism that autonomously converts a small difference to a robust asymmetry (Takaoka et al., 2007).

Finally, It is possible that Wnt signaling induced *Nodal* in these cells. Since *Nodal* is normally expressed in *FoxH1* null embryos at E4.5 (Fig.15A), *Nodal* expression is independent of Nodal-FoxH1 signaling pathway at peri-implantation stage. One possibility would be that Wnt-canonical signaling induces *Nodal* at E3.5, because active β-catenin is localized to the nuclei of some ICM cells at E3.5 (Xie et al.,2008).

Lefty1 is expressed only on one side of the primitive endoderm at E4.2. However, *Nodal* and the genes for Nodal effectors such as FoxH1 and Cripto are all expressed symmetrically. The absence of *Cripto* expression in the primitive endoderm between E4.25 and E4.75 suggests that *Lefty1* expression is initiated at E3.5 by Nodal signaling and is maintained in the primitive endoderm lineage by a Nodal-independent mechanism. A similar mechanism regulates asymmetric expression of *Pitx2* at the early somite stage. Asymmetric *Pitx2* expression is initiated by Nodal signaling acting at FoxH1 binding sites but is maintained in the absence of Nodal in an Nkx2.5 dependent manner binding sequence located near the FoxH1 binding sites (Shiratori et al., 2001). Nodal signaling might be required only for the initiation of *Lefty1* expression.

However, given that expression of *Nodal*, *Foxh1*, and *Cripto* is uniform in the ICM at E3.5, how is *Lefty1* expression activated in some cells but not in others? It is possible that an unknown factor required for Nodal signaling is expressed in a specific and asymmetric manner. Cross talk from other signaling pathway may be also involved (Ang and Constum, 2004). For example, expression of *Hex* in AVE (and most likely *Lefty1* in DVE) is repressed by signals from the extra-embryonic ectoderm (Rodriguez et al., 2005). Similarly, it is possible that signals from the polar trophectoderm regulate asymmetric *Lefty1* expression. Although it is unknown if the polar trophectoderm is molecularly

polarized, it is interesting to note that a flow of polar trophectoderm to the mural region is polarized, such that more net flow takes place in one quadrant of the blastocyst than others (Gardner, 1996).

Implantation does not provide positional cue for A-P polarity

The uterus provides various signals to an implanting embryo, required for postimplantation development (Tam et al., 2003; Rossant and Tam, 2004). Signaling by uterus-derived factors such as HB-EGF (heparin-binding epidermal growth factor–like growth factor) is known to be activated within the embryo at the site of implantation (Das et al., 1994). Endometrial cells at the site of implantation express several signaling molecules (Paria et al., 2001). *LEFTY2* (*EBAF*) is also expressed in the endometrial cells of the human uterus (Kothapalli et al., 1997). Indeed, the axes of the embryo and the anatomic axes of the uterus appear to be related. Alignment of the A-P axis of the embryo with the transverse plane of the uterine horn (Smith, 1980) may be achieved by interaction between an implanting embryo and the uterus. Rare instances have been described of preimplantation mouse embryos developing in culture to a stage resembling the early somite stage (Hsu, 1979; Libbus and Hsu, 1980). However, the significance of these findings remains unknown, in part because the embryos were kept attached to the surface of a plastic dish. It has thus been generally believed that the uterus influences axes development in the embryo.

Our experiments involving culture of mouse embryos in hanging drops have now shown that interaction with the uterus is not necessary for establishment of asymmetric *Lefty1* expression in the primitive endoderm. The mouse embryo thus

appears capable of establishing initial A-P polarity autonomously, without positional information relative to the uterine axes. These results appear consistent with the recent finding that A-P polarity of the gastrulating mouse embryo at E6.0 is not related to any specific anatomic axis of the uterus (Mesnard et al., 2004). It is interesting to note that in marsupial, axis formation occurs before implantation (reviewered by Eakin and Behringer, 2004). Thus, marsupial embryos implant late in development, such that gastrulation takes place well before any intimate physical interaction is initiated between the conceptus and the uterus. Our findings in the mouse embryos suggest an evolutionarily conserved mechanism in mammalian development whereby axis determination is an embryo-autonomous process.

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6. Future direction and outlook

This report has shown that DVE was autonomously specified by *Lefty1*-expressing cells in primitive endoderm of mouse embryos at peri-implantation stage. However, we still have a bunch of very important questions.

First, does Lefty1 have a functional role ? To address this issue, we plan to examine the outcome of ectopic *Lefty1* expression in ICM. In addition, because the lack of Lefty1 is compensated by two other Nodal antagonists: Cerl, which is regulated independently of Lefty1, and Lefty2, a chromosomally linked gene whose expression is ectopically induced in the absence of Lefty1. A *Lefty1/2-Cerl* triple KO mouse might be used to investigate these functions.

Second, how is *Lefty1*-expression induced ? We revealed that Lefty1-expression in primitive endoderm is induced by mechanism other than Nodal signaling. Probably, *Lefty1* in E3.5 induces its own expression. We plan to perform a chimera assay. More precisely, *Lefty1/2* double KO ES cells have been established, these cells will be aggregated with *Lefty1* transgenic tetraploid embryos, in order to investigate whether there is *Lefty1*-expression in primitive endoderm or not. In the same time, we are going to perform a cis-element assay.

Finally, how does AVE newly form between E5.5 and E6.5. We should proceed to time-lapse observation of *Lefty1*, *Hex*, *Cerl* transgenic embryos harboring fluorescent reporters. In addition, these transgenes will be designed in a way that allows VE monitoring at a single cell resolution.

In conclusion, I'll say that I don't know how does baby take first steps, but I reached took a higher step in my researcher's life.

7. Publication List

学術論文名、著書、学協会、討論会、国際会議等での研究発表題名及び特許・発明等の名称	発表論文誌の名称、巻、頁、発表年（印刷中または投稿中の場合は、その旨を記入してください。） 発表学協会、討論会、国際会議等の名称及び発表年月日	共著者又は共同発表者氏名（本人を含め上から発表順に記入してください。）
Lefty1 expression in blastocyst specifies the future anterior-posterior polarity of the mouse embryo	投稿準備中	Katsuyoshi Takaoka, Masamichi Yamamoto, Hiroshi Hamada
The mouse embryo autonomously acquires anterior-posterior polarity at implantation.	Developmental Cell vol.10 pp : 451-459 ,2006	Katsuyoshi Takaoka, Masamichi Yamamoto, Hidetaka Shiratori, Chikara Meno, Janet Rossant, Yukio Saijoh, Hiroshi Hamada
Origin of body axes in the mouse embryo.	Current Opinion in Genetics & Development vol.17 pp : 344-350, 2007	Katsuyoshi Takaoka, Masamichi Yamamoto, Hiroshi Hamada
The mouse embryo autonomously acquires anterior-posterior polarity at implantation.	Annual report of Osaka university, 100papers: 24 selected graphics vol.8 p.55 2006-2007	Katsuyoshi Takaoka, and Hiroshi Hamada
Antagonism between Smad1 and Smad2 signaling regulates formation of the distal visceral endoderm in the mouse embryo.	Jounal of Cell Biology , vol.184(2) pp:324-334 2009	Masamichi Yamamoto, Hideyuki Beppu, Katsuyoshi Takaoka, Chikara Meno, En Li, Kohei Miyazono, Hiroshi Hamada
Removal of maternal retinoic acid by embryonic CYP26 for correct Nodal regulation during early embryonic patterning.	Submitted	Masayuki Uehara.,Kenta Yashiro, Katsuyoshi Takaoka, K.,Masamichi Yamamoto, Hiroshi Hamada
The mouse embryo autonomously acquires anterior-posterior polarity at implantation.	20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress 6 月 2006 年(ポスター)	○Katsuyoshi Takaoka, Masamichi Yamamoto, Hidetaka Shiratori, Chikara Meno, Janet Rossant, Yukio Saijoh, Hiroshi Hamada

The mouse embryo autonomously acquires anterior-posterior polarity at implantation.	Mouse Molecular Genetics Meeting 8-9月2006年(ポスター)	○Katsuyoshi Takaoka, Masamichi Yamamoto, Hidetaka Shiratori, Chikara Meno, Janet Rossant, Yukio Saijoh, Hiroshi Hamada
Origin of Anterior-Posterior axis in the mouse embryo	The 1st Mouse Development Workshop 6月2008年(口頭)	○Katsuyoshi Takaoka, Masamichi Yamamoto, Hiroshi Hamada
「前後軸の起源〜Lefty1の発現制御機構から探る〜」	遺伝情報DECODE・冬のワークショップ（転写研究会共催）1月2007年(口頭)	○高岡勝吉、山本正道、白鳥秀卓、目野主税、Janet Rossant,西條幸男、濱田博司
マウス胚における前後軸の起源	遺伝情報DECODE・冬のワークショップ（転写研究会共催）1月2009年(口頭)	○高岡勝吉、山本正道、濱田博司
「前後軸の起源」	第40回日本発生生物学会、第59回日本細胞生物学会合同大会、5月2007年(口頭)	○高岡勝吉、山本正道、白鳥秀卓、目野主税、Janet Rossant,西條幸男、濱田博司
「臓側内胚葉のBMPシグナルはDVEの形成及びNodalシグナルの制御をしている」	第28回分子生物学会年会, 12月2005年(ポスター)	○山本正道、別府秀幸、高岡勝吉、宮園浩平、目野主税、濱田博司
「遠位臓側内胚葉はBMP、ACTIVIN、ExEシグナルのIの相互作用により形成される」	第40回日本発生生物学会、第59回日本細胞生物学会合同大会　5月2007年(ポスター)	○山本正道、別府秀幸、高岡勝吉、宮園浩平、目野主税、濱田博司

8. Acknowledgement

I am deeply grateful to Pr. Hiroshi Hamada whose help and suggestions were an inestimable value for my study. I am also indebt to Masamichi Yamamoto, Yanick Botilde, and all laboratory members for their comments and enormous contribution to my work. I would also like to express my gratitude to my family for their moral support and warm encouragement. Finally, I would like to thank Japan student services organization and CREST(Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation for the grant that allowed me to complete this study.

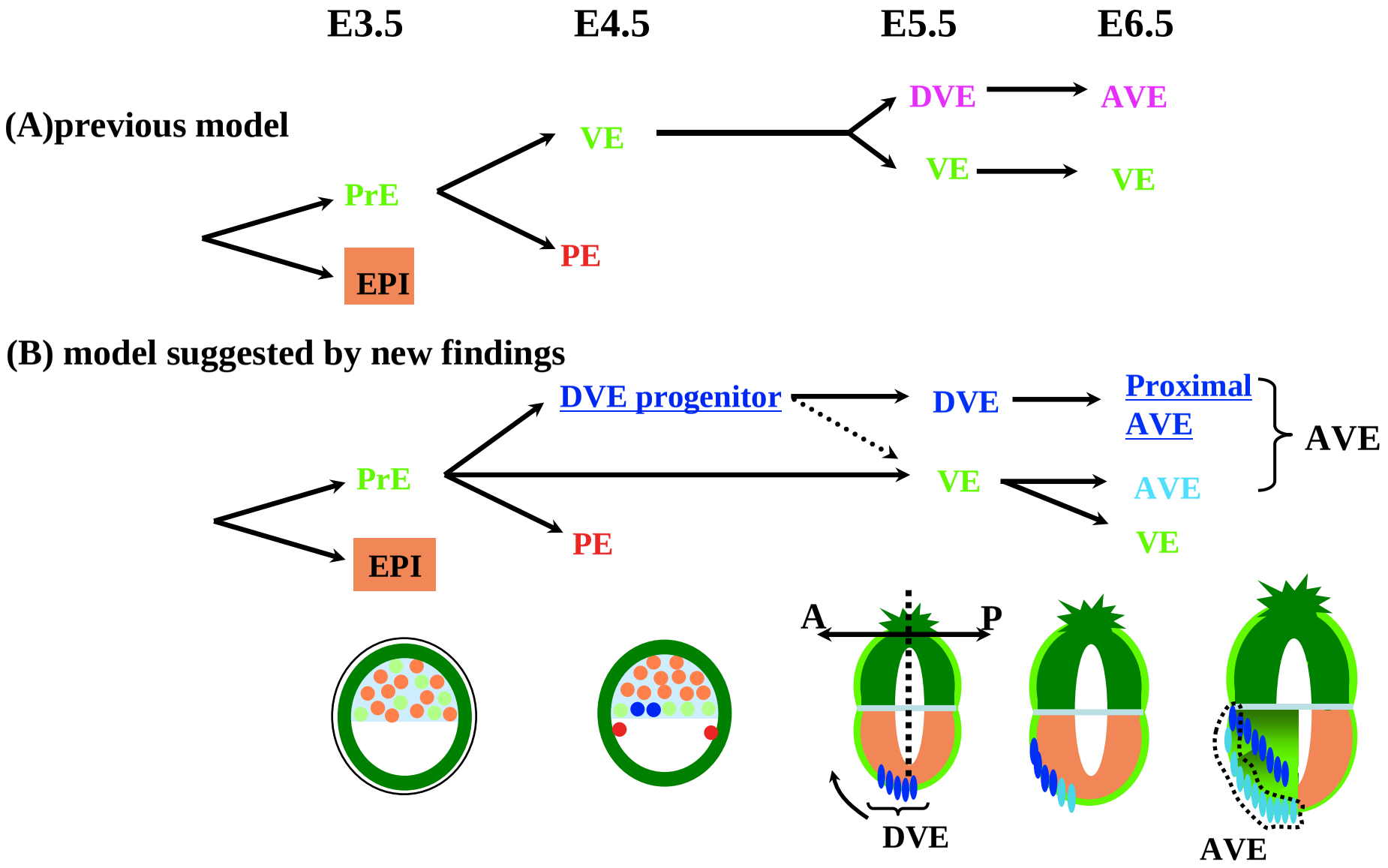


Figure 1 Schematic model of DVE induction and migration.

TE:Troph Ectoderm, PrE:Primitive Endoderm, EPI:Epiblast, VE:Viceral Endoderm, PE:Parietal Endoderm, DVE:Distal Visceral Endoderm, AVE:Anterior Visceral Enderm, A-P: anterior-posterior axis

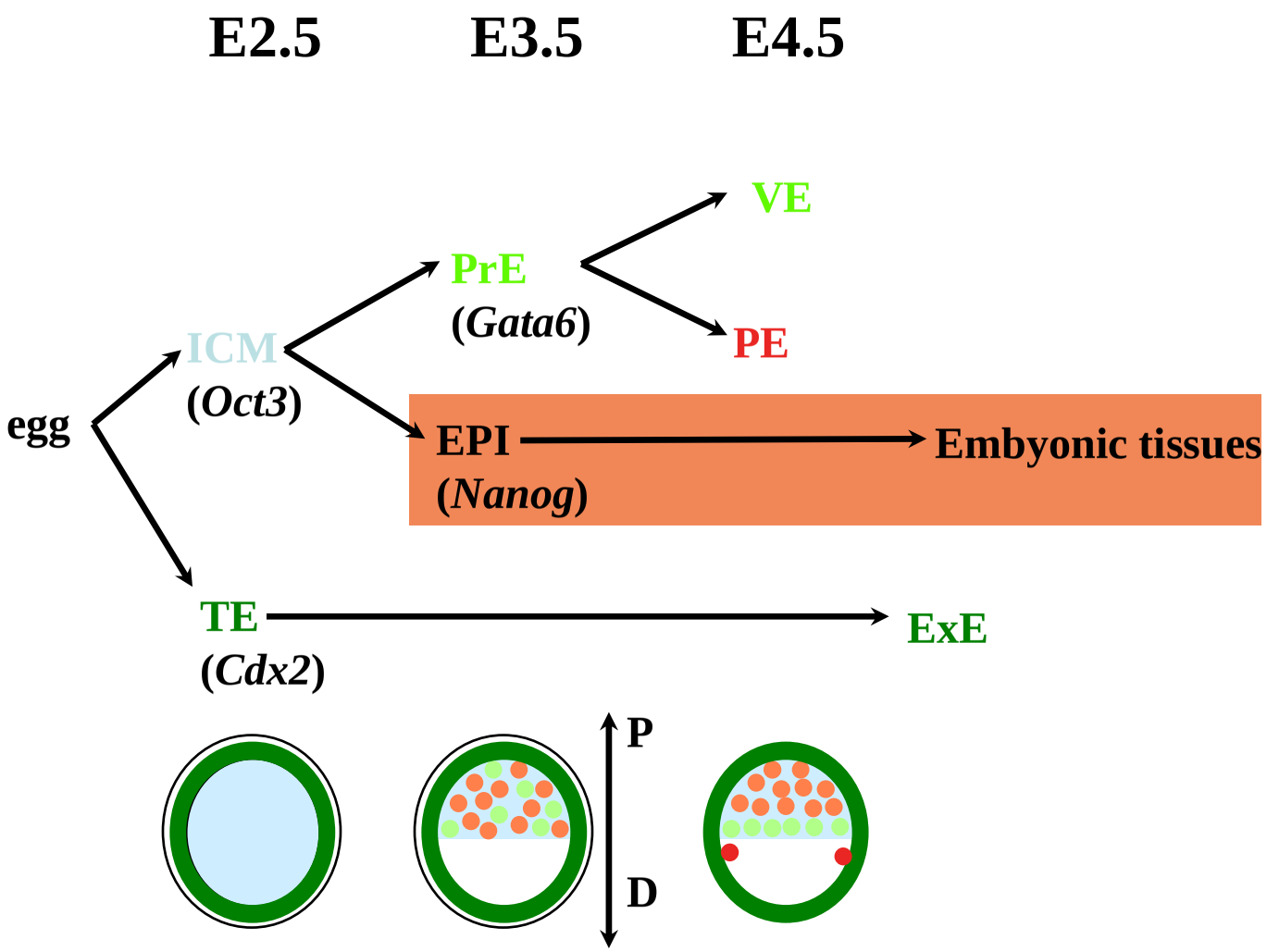


Figure 2 **Schematic diagram showing the derivation of tissues in mouse embryos.**

ICM: Inner Cell Mass, TE:Troph Ectoderm, PrE:Primitive Endoderm, EPI:Epiblast, VE:Viceral Endoderm, PE:Parietal Endoderm, ExE: Extraembryonic ectoderm P-D:proximal-distal axis

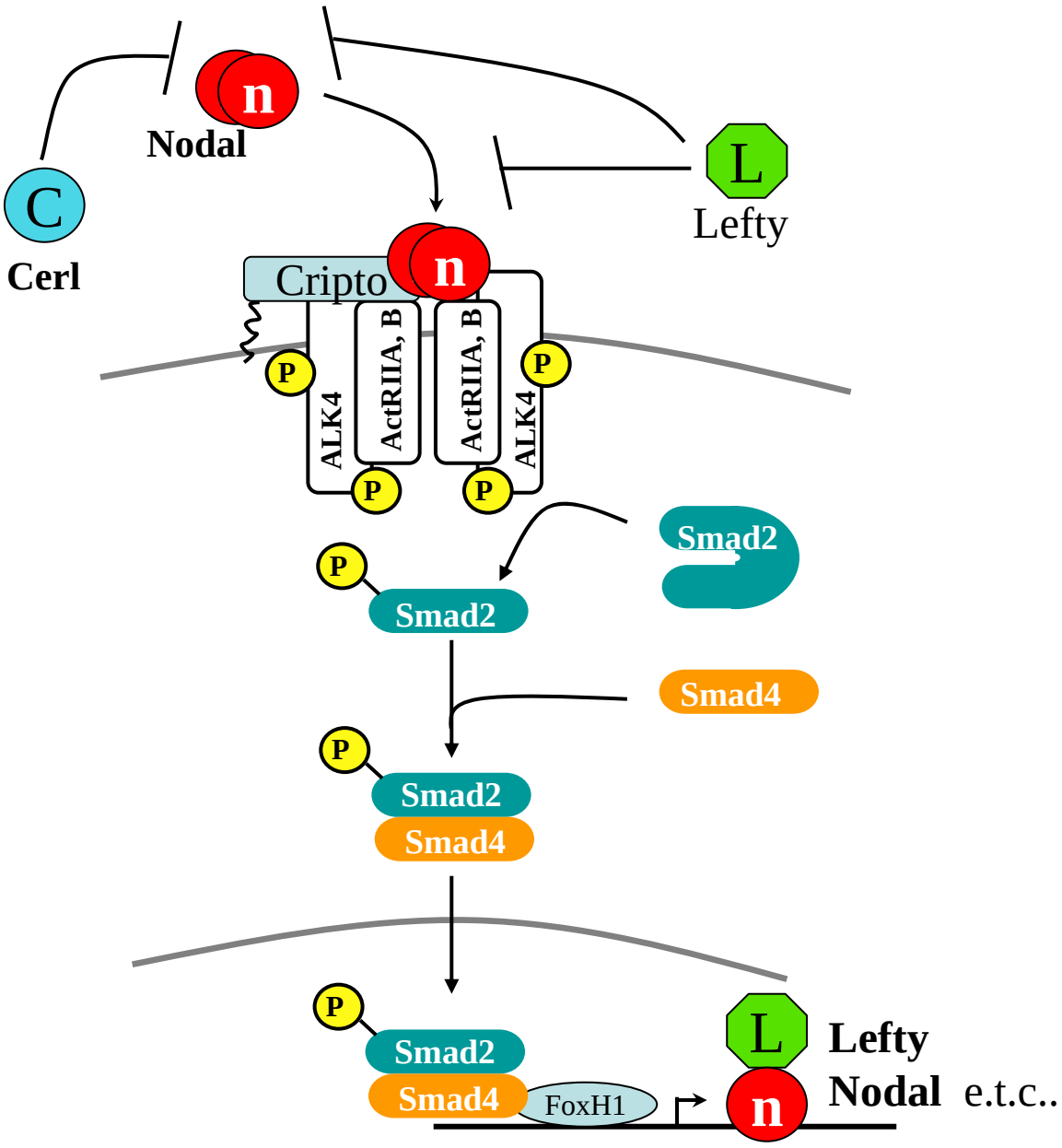


Fig.3 Schematic outline of the Nodal signaling pathway

Nodal ligands can bind to an Cripto(EGF-CFC coreceptor) in a complex with type I receptor (ALK4) and type II receptor (ActRII or ActRIIB) dimers. Cerberus and Lefty proteins are soluble antagonists that can interact with Nodal ligands; Lefty proteins can also interact with Cripto to inhibit their function. Receptor activation leads to the phosphorylation of the type I receptor by the type II kinase, as well as phosphorylation of Smad2 (or Smad3). Activated Smad2 or Smad3 associates with Smad4 and translocates to the nucleus. Within the nucleus, activated Smad2-Smad4 complexes interact with the winged-helix transcription factor FoxH1 on target promoters.

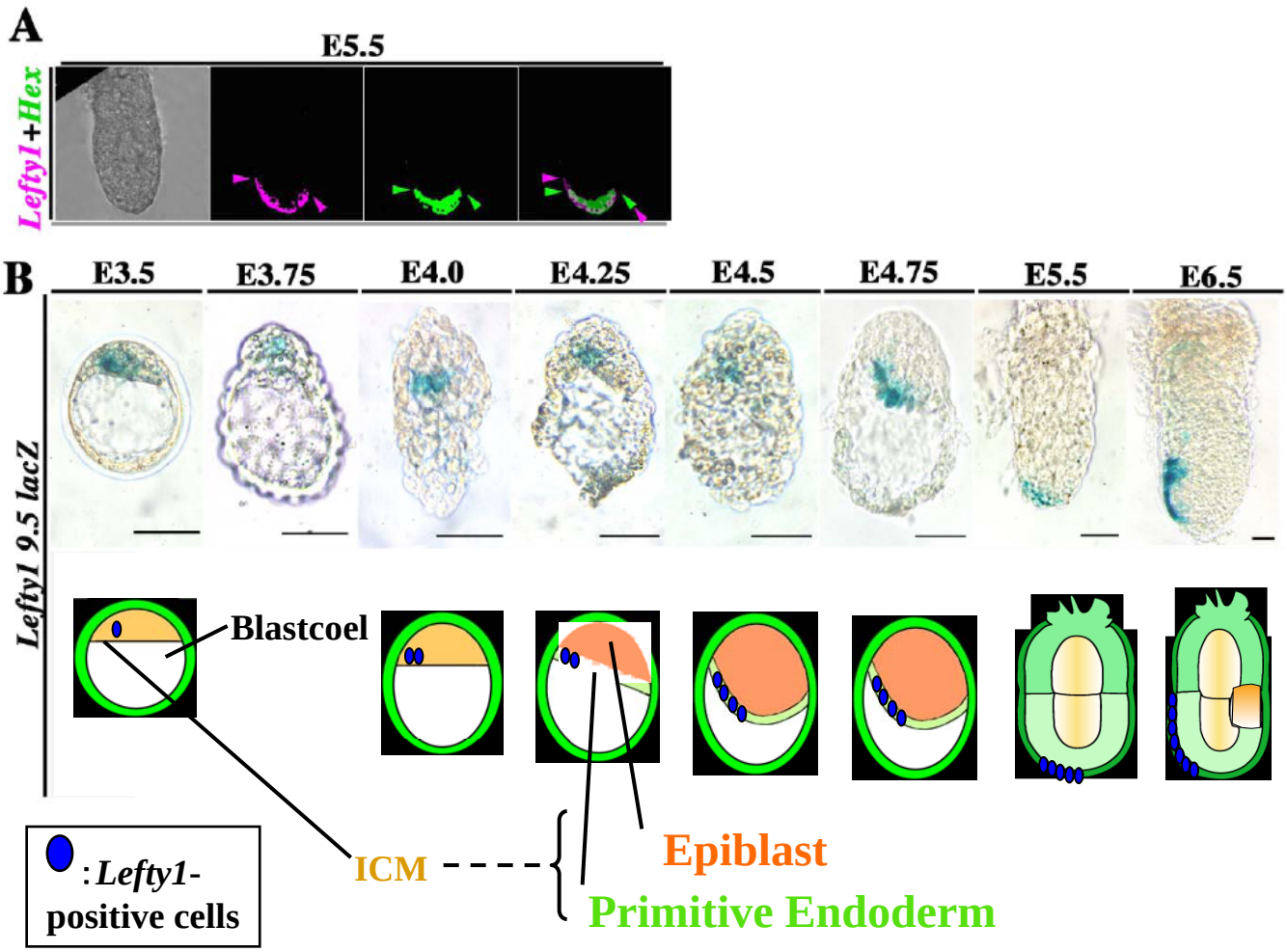


Figure 4. Asymmetric expression of *Lefty1* transgenes in peri-implantation mouse embryos
(**A**) Mouse embryos harboring two transgenes, *Lefty1-9.5 DsRed2* and *Hex-Venus*, were recovered at E5.25 or E5.5, and the fluorescence of DsRed2 (magenta) or Venus (green) was examined separately. Merged fluorescence images and phase-contrast images are shown in the right and left panels, respectively. Arrowheads delineate the regions of expression of each transgene.
(**B**) Mouse embryos harboring the *Lefty1-9.5 lacZ* transgene were recovered at the indicated stages and stained with Xgal. Photographs of Xgal-stained embryos (upper panels) are accompanied by illustrations showing the locations of $Xgal^+$ cells (shown in magenta in lower panels). Between E4.25 and E5.5, $Xgal^+$ cells are located asymmetrically in the primitive endoderm. Embryo stages were judged as follows: E3.5, blastocyst with the zona pellucida; E3.75, hatched blastocyst with the embryonic-abembryonic axis elongated and with long and short axes; E4.0, blastocyst with more pronounced long and short axes; E4.25, implanting embryo with the tilt; E4.5, implanted embryo with a flat shape and showing formation of the decidua; E4.75, fully implanted embryo in which the ICM has grown into the blastocoel cavity. Scale bars, 50 μm.

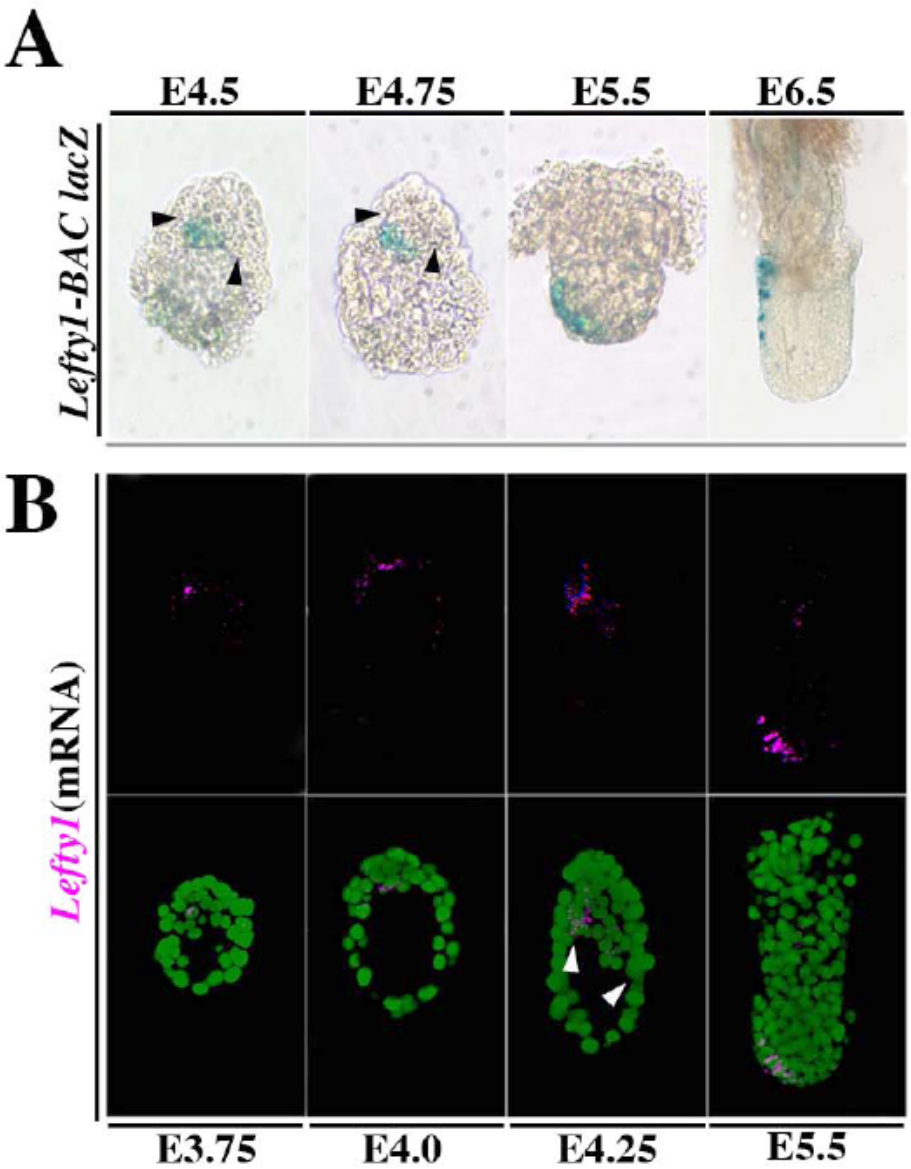


Figure 5. Asymmetric *Lefty1* expression as revealed by a *Lefty1* transgene and by in situ hybridization

(**A**) Mouse embryos harboring a *Lefty1-BAC lacZ* transgene were recovered at the indicated stages and stained with Xgal. Arrowheads indicate junctions between the primitive endoderm and the trophectoderm.

(**B**) Fluorescence in situ hybridization of whole-mount preparations of mouse embryos at the indicated stages with a probe specific for *Lefty1* mRNA (magenta). Nuclei were counterstained with the dye YOYO-1 (green). The distribution of *Lefty1* mRNA was similar to that of Xgal staining in *Lefty1-lacZ* transgenic embryos. At E4.2, for example, when the tilt of the ICM is apparent, *Lefty1* mRNA was localized to the "upper" side of the tilt, as were $Xgal^{+}$ cells in *Lefty1-lacZ* transgenic embryos. Arrowheads indicate junctions between the primitive endoderm and the trophectoderm.

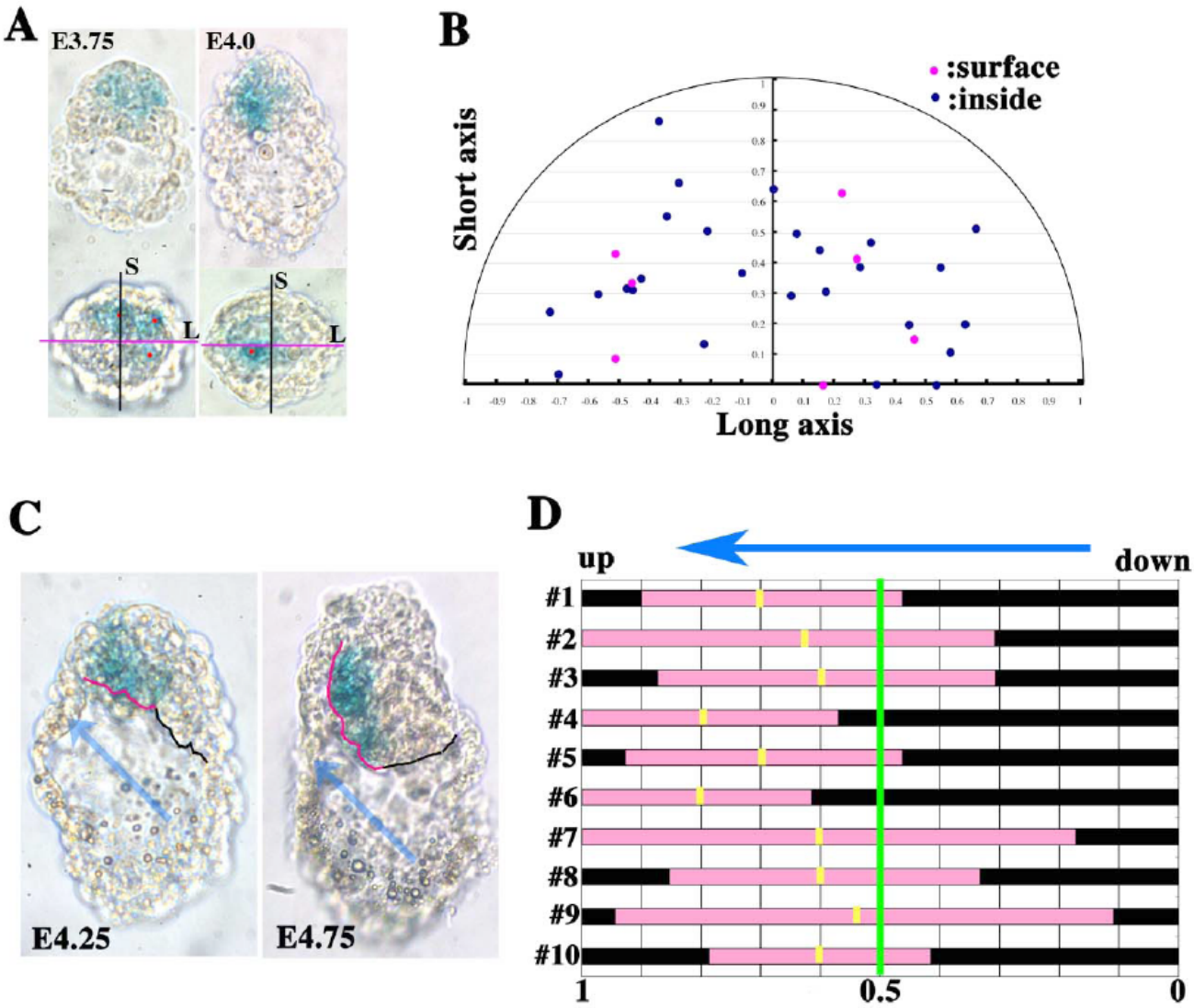


Figure 6. Localization of *Lefty1*-expressing cells in the mouse embryo

(**A**) Transgenic mouse embryos harboring the *Lefty1-0.7 lacZ* transgene were recovered at the indicated ages and stained with Xgal. The positions of $Xgal^+$ cells were examined by observing embryos from various angles. $Xgal^+$ cells were detected inside of the ICM at E3.75 and at the surface of the ICM facing the blastocoel at E4.0. In some embryos at these stages, $Xgal^+$ cells were found in both locations. Lateral views of representative embryos are shown in the upper panels, whereas the long (L) and short (S) axes in the view from the abembryonic pole are indicated in the lower panels.

(**B**) Summary of the positions of $Xgal^+$ cells relative to the long and short axes of embryos at E3.75 (n = 11) or E4.0 (n = 8) determined as in (**A**). Blue dots and pink dots denote $Xgal^+$ cells located inside or at the surface of the ICM, respectively. (**C**) *Lefty1-0.7 lacZ* transgenic embryos at E4.25 and E4.75 stained with Xgal. The tilt of the ICM is indicated by the blue arrow. $Xgal^+$ and $Xgal^-$ populations of the primitive endoderm are indicated by red and black lines, respectively.

(**D**) Summary of the locations of $Xgal^+$ cells along the tilt of *Lefty1-0.7 lacZ* embryos (#1 to #10) at E4.25. Red and black bars represent $Xgal^+$ and $Xgal^-$ cells, respectively. The midpoint of the $Xgal^+$ region is shown by the yellow bar.

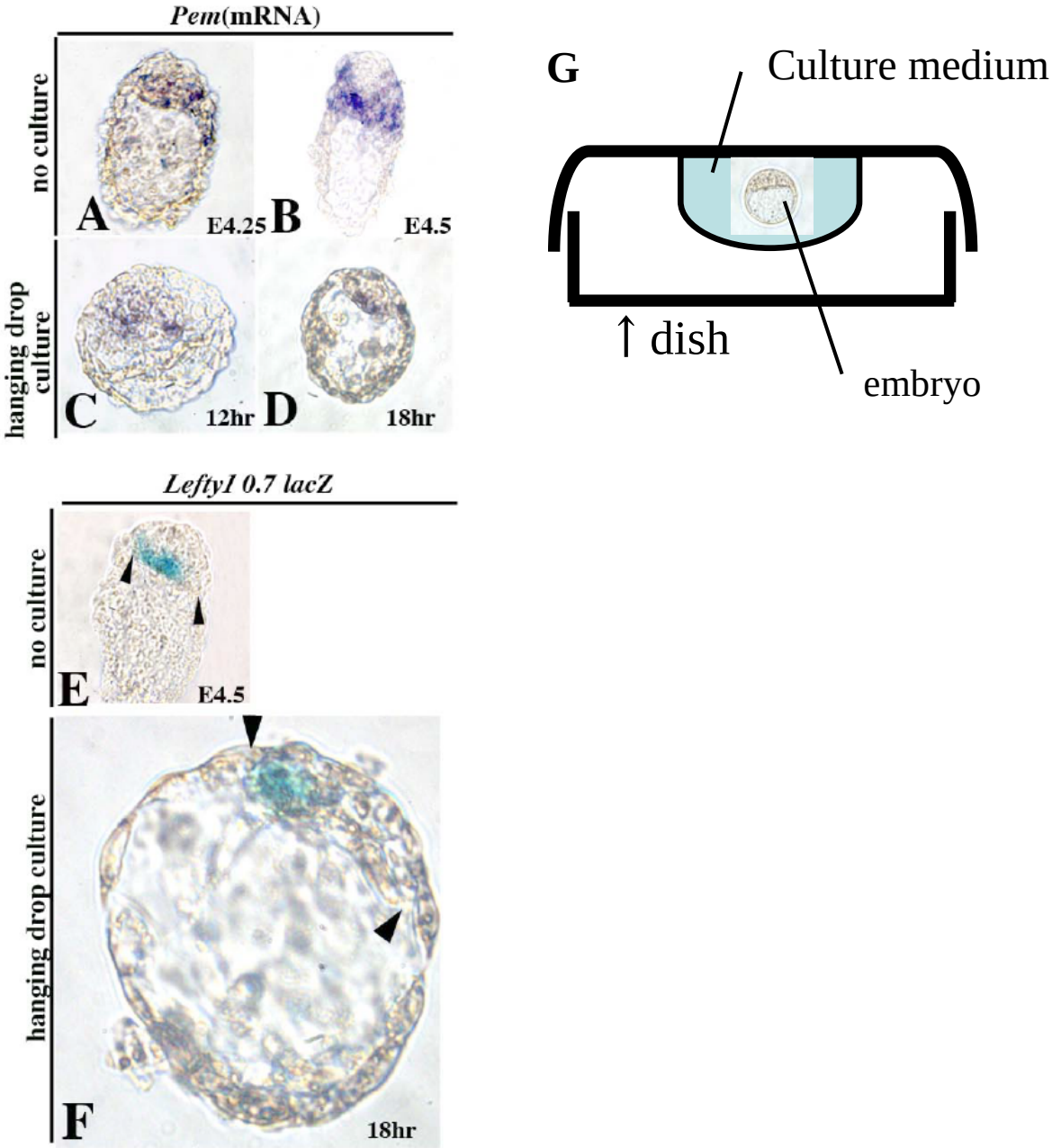
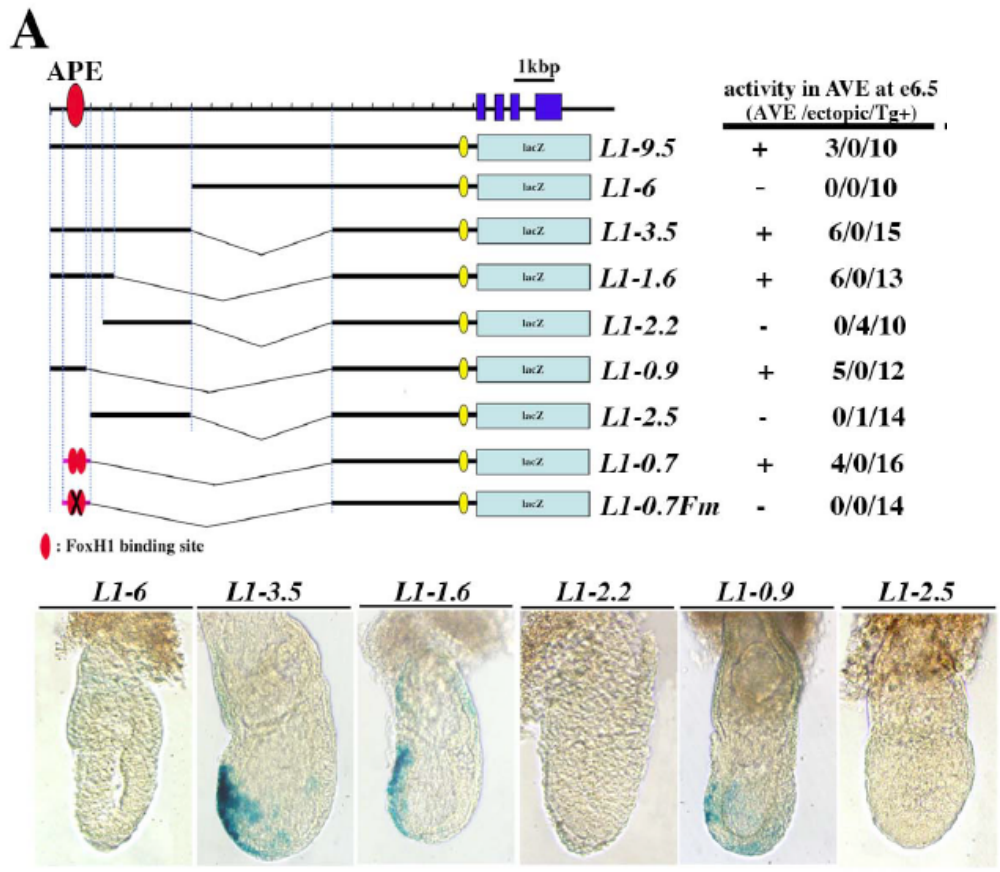


Figure 7. Asymmetric expression of *Lefty1* in cultured mouse embryos

(**A-D**), Wild-type embryos were recovered at E3.5 and cultured in hanging drops for 12 or 18 h after hatching as described in Experimental Procedures. The cultured embryos (**C, D**) were examined by in situ hybridization for expression of *Pem*, a marker for the primitive endoderm. Non-cultured embryos at E4.25 (**A**) or E4.5 (**B**) were examined for comparison.

(**E, F**), Transgenic embryos harboring *Lefty1-0.7 lacZ* were recovered at E3.5 and cultured in hanging drops for 18 h after hatching. The cultured embryos were stained with Xgal to detect expression of *Lefty1-0.7 lacZ* (**F**). Non-cultured embryos at E4.5 (**E**) were examined for comparison. Arrowheads in (**E, F**) indicate junctions between the primitive endoderm and the trophectoderm. (G) overview of hanging drop culture.



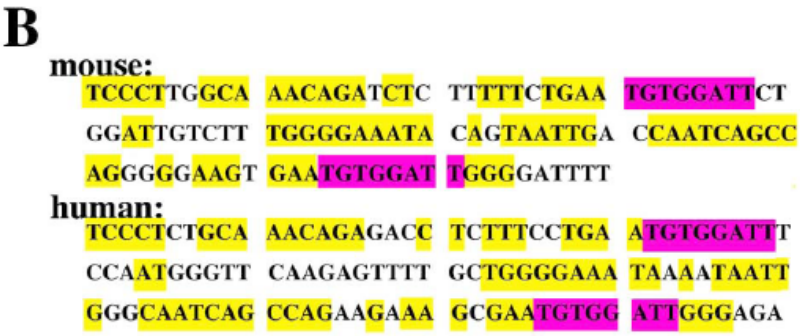


Figure 8. Mapping of the enhancer responsible for asymmetric *Lefty1* expression

(**A**) Nine *lacZ* constructs containing portions of the 9.5-kb upstream region of *Lefty1* (L1) were tested with a transient transgenic assay for their ability to give rise to *lacZ* expression in the AVE at E6.5. Representative Xgal-stained embryos harboring the indicated constructs are shown in the lower panels. Results are summarized above as: number of embryos with Xgal staining in the AVE/number of embryos with ectopic Xgal staining/total number of transgenic embryos. For two constructs (*Lefty1-0.7 lacZ* and *Lefty1-0.7Fm lacZ*), transgenic embryos were also recovered at E4.5 and examined for expression of *lacZ* in the primitive endoderm (PE); results are summarized as for analysis of expression in the AVE. The enhancer (designated APE) that confers *lacZ* expression in the AVE was mapped to the 0.7-kb region of *Lefty1* in *Lefty1-0.7 lacZ*. Two FoxH1 binding sequences present in this 0.7-kb region are indicated by red ovals. Yellow ovals indicate the transcription start site of *Lefty1* and the dark blue boxes indicate exons of *Lefty1*.

(**B**) Nucleotide sequences of the 0.7-kb region of mouse *Lefty1* that contains the APE and their conservation in human *LEFTY1*. The conserved FoxH1 binding sequences and other conserved sequences are shown in magenta and yellow, respectively.

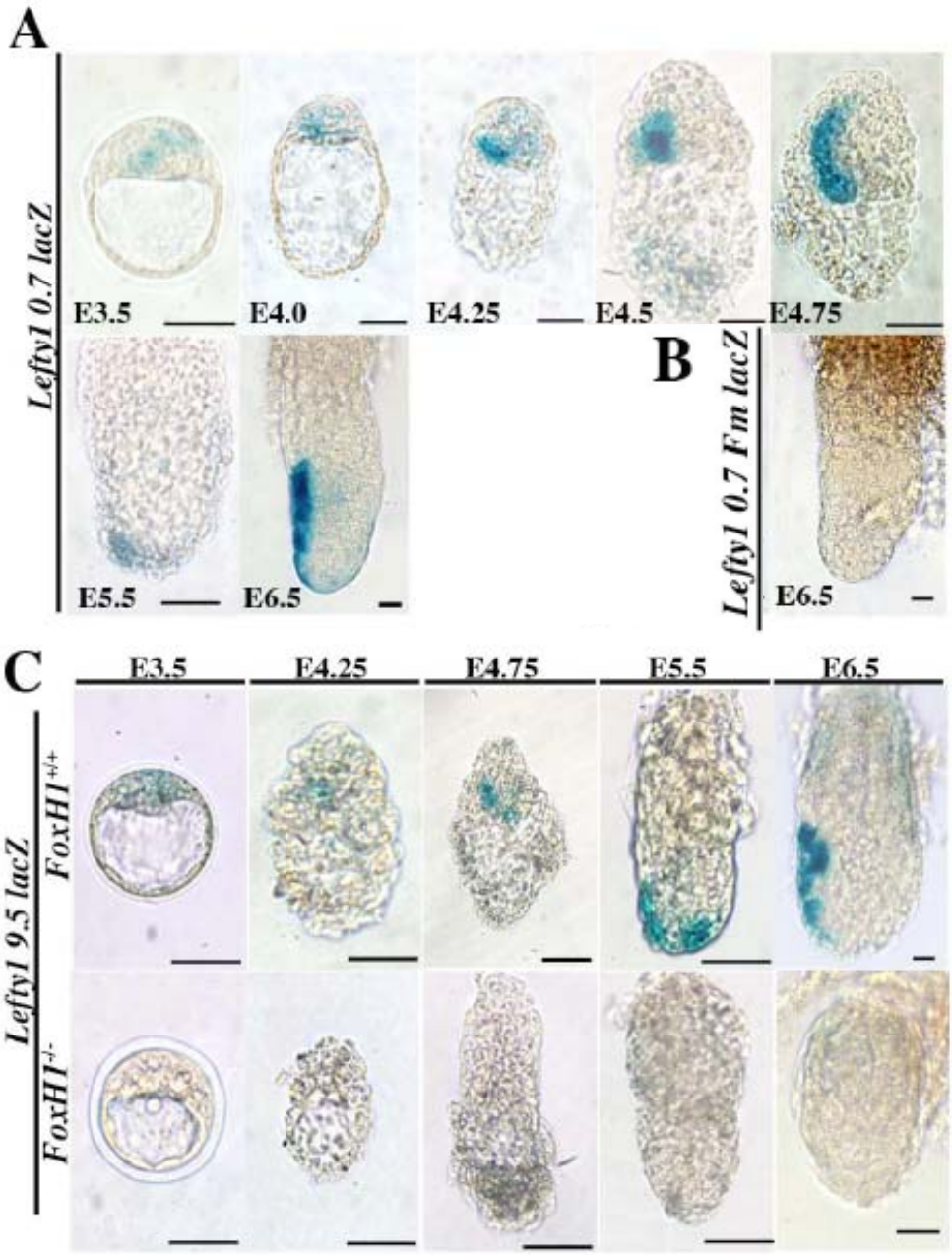


Figure 9. Asymmetric *Lefty1* expression in the primitive endoderm induced by Nodal signaling

(**A**) Embryos of a permanent transgenic mouse line harboring the *Lefty1-0.7 lacZ* transgene were recovered at the indicated stages and stained with Xgal. Scale bars, 50 μm.

(**B**) Transient transgenic embryos harboring *Lefty1-0.7Fm lacZ* were recovered at E6.5 and stained with Xgal. Scale bars, 50 μm.

(**C**) Expression of the *Lefty1-9.5 lacZ* transgene in *Foxh1*$^{+/+}$ or *Foxh1*$^{-/-}$ embryos at the indicated stages. Scale bars, 50 μm.

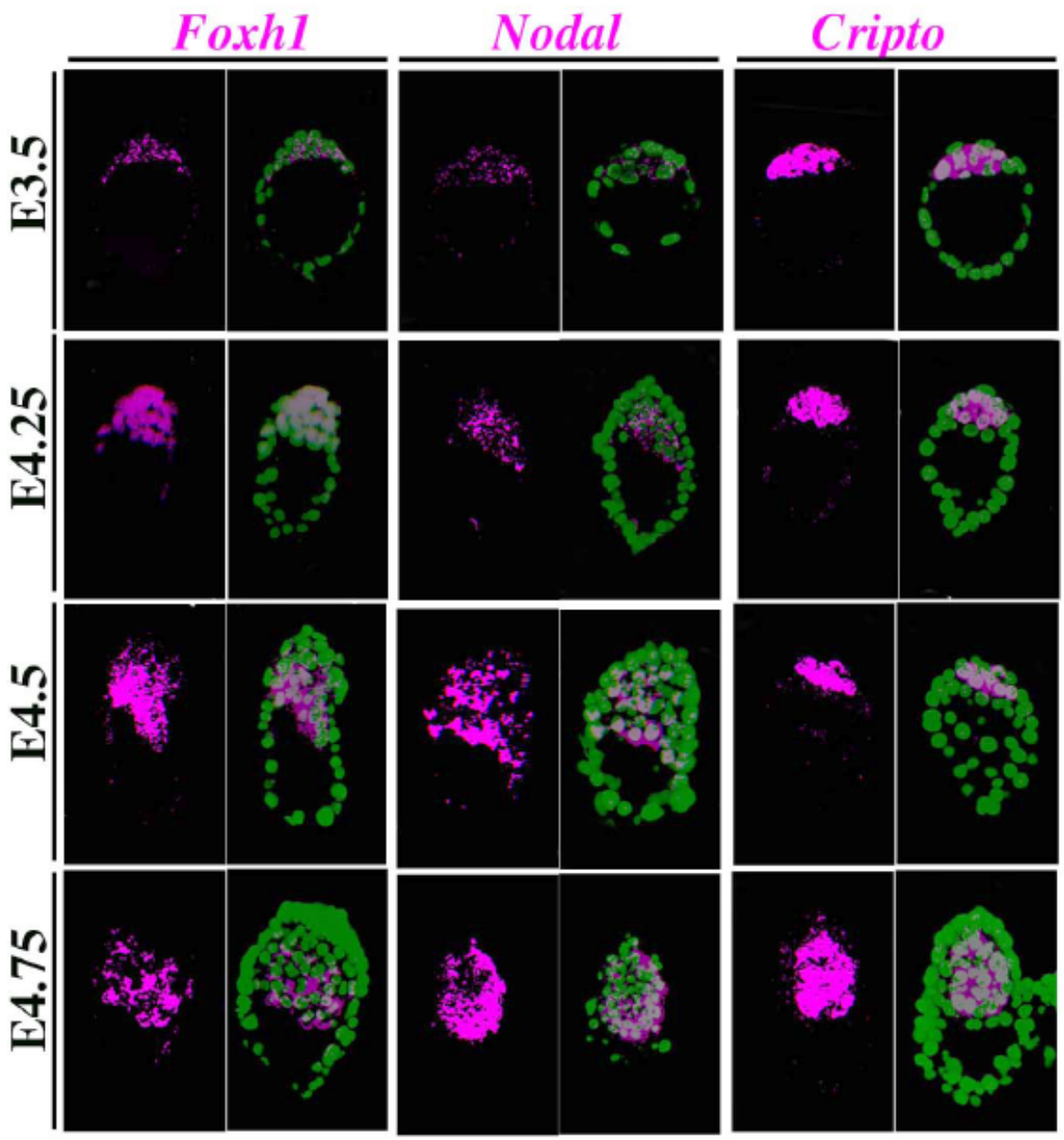


Figure 10. Lack of asymmetry in the expression of *Nodal, Foxh1, and Cripto* in mouse embryos between E3.5 and E4.75

Expression of *Nodal*, *Foxh1*, and *Cripto* in mouse embryos was examined at the indicated stages by fluorescence in situ hybridization. Signals for the mRNA of each gene are shown in magenta, with YOYO-1 fluorescence in nuclei being shown in green.

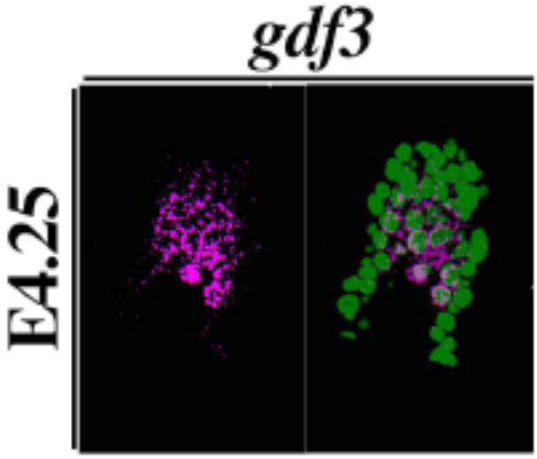


Figure 11. Expression of *Gdf3* in the Peri-Implantation Embryo
Expression of *Gdf3* in mouse embryos was examined at the indicated stage by fluorescence in situ hybridization. Signals for the mRNA of each gene are shown in magenta, with YOYO-1 fluorescence in
nuclei being shown in green.

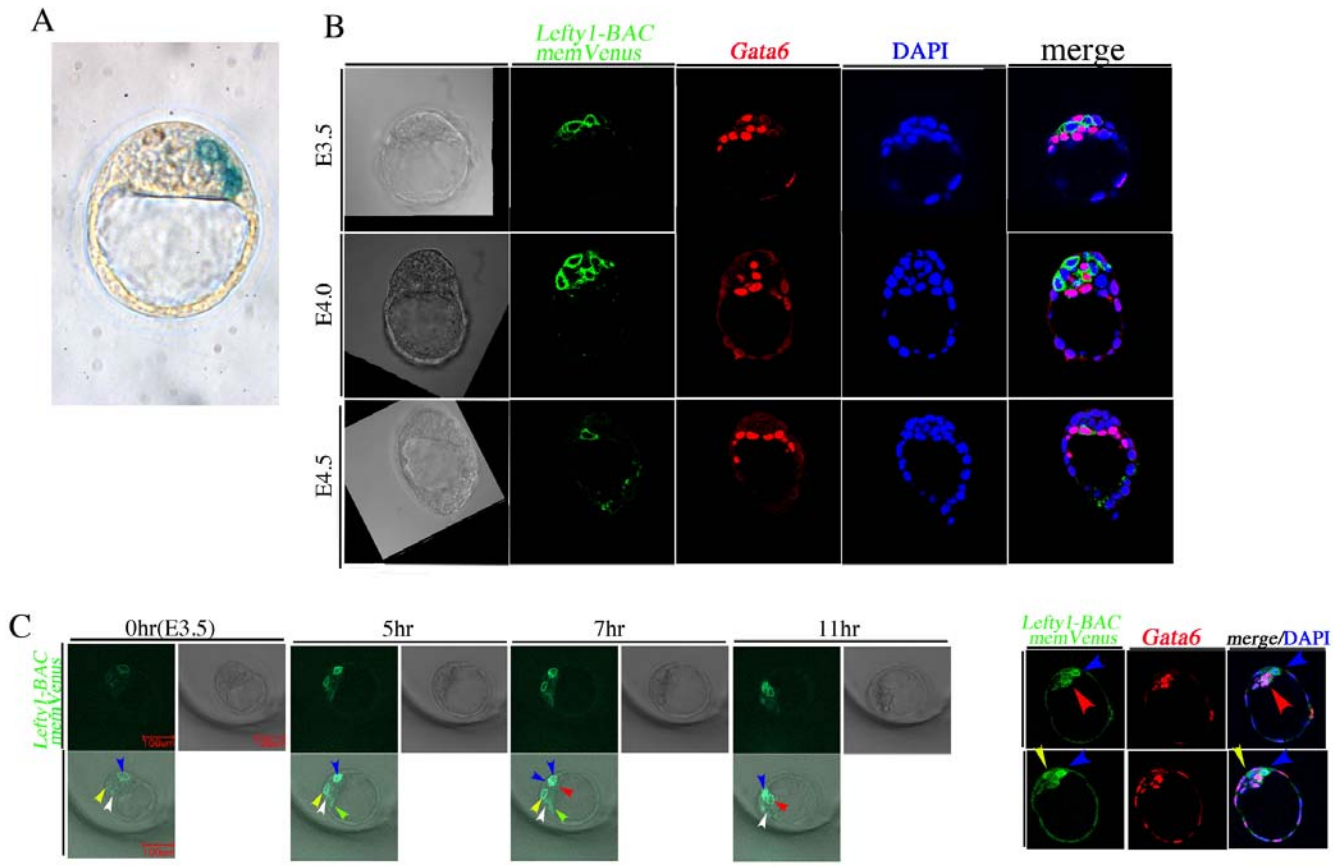


Figure 12. Characterization of *Lefty1*-positve cells at E3.5 and E4.2.
(A), X-gal staining of E3.5, Mouse embryos harboring the *Lefty1-0.7 lacZ* transgene, Lefty1-expression begins in a fewc ells of ICM (2～3) at E3.5. (B), immnunolocalisation of GATA6 in *Lefty1-BAC-memVenus* embryos at the indicated stages. At E3.5, GATA6(magenta) is negative in Lefty1-positive(green) cells. At E4.0, positive cells for Lefty1 can be either positive or negative for GATA6. At E4.5, GATA6 signal is present in Lefty1-expressing cells. (C), Timelapse observation of *Lefty1-BAC memVenus* at E3.5 for 12hr. After observation, performed GATA6 immunofluorescent. (red arrowhead: GATA6(+), blue, yellow: GATA6(-), white: unknown)

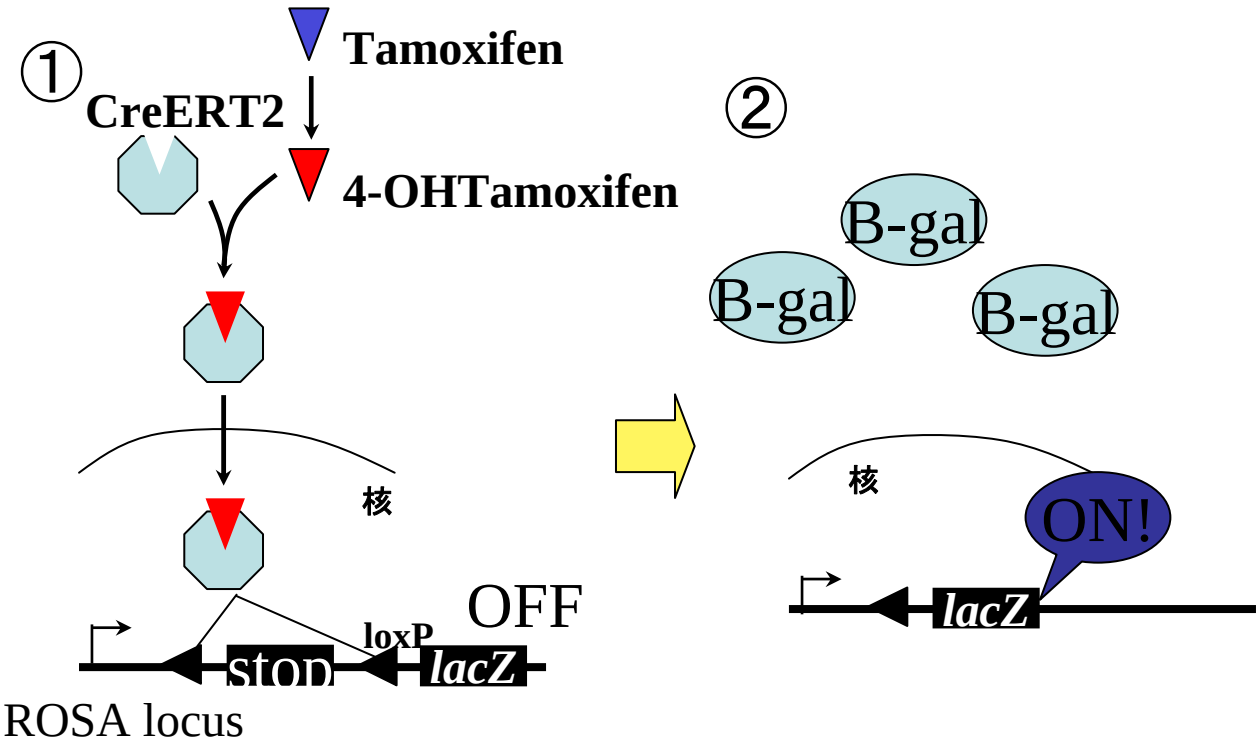


Figure 13. Schematic of CreERT2-loxp system

①The CreERT2 fusion protein is transcribed under the control of a tissue-specific promoter and translated in the cytoplasm. Interaction of the estrogen receptor ligand binding domain the Cre in the cytoplasm until ligand binding at which time the activated protein translocates to the nucleus where it can mediate recombination at loxP sites in the genome. ②lacZ expression starts.

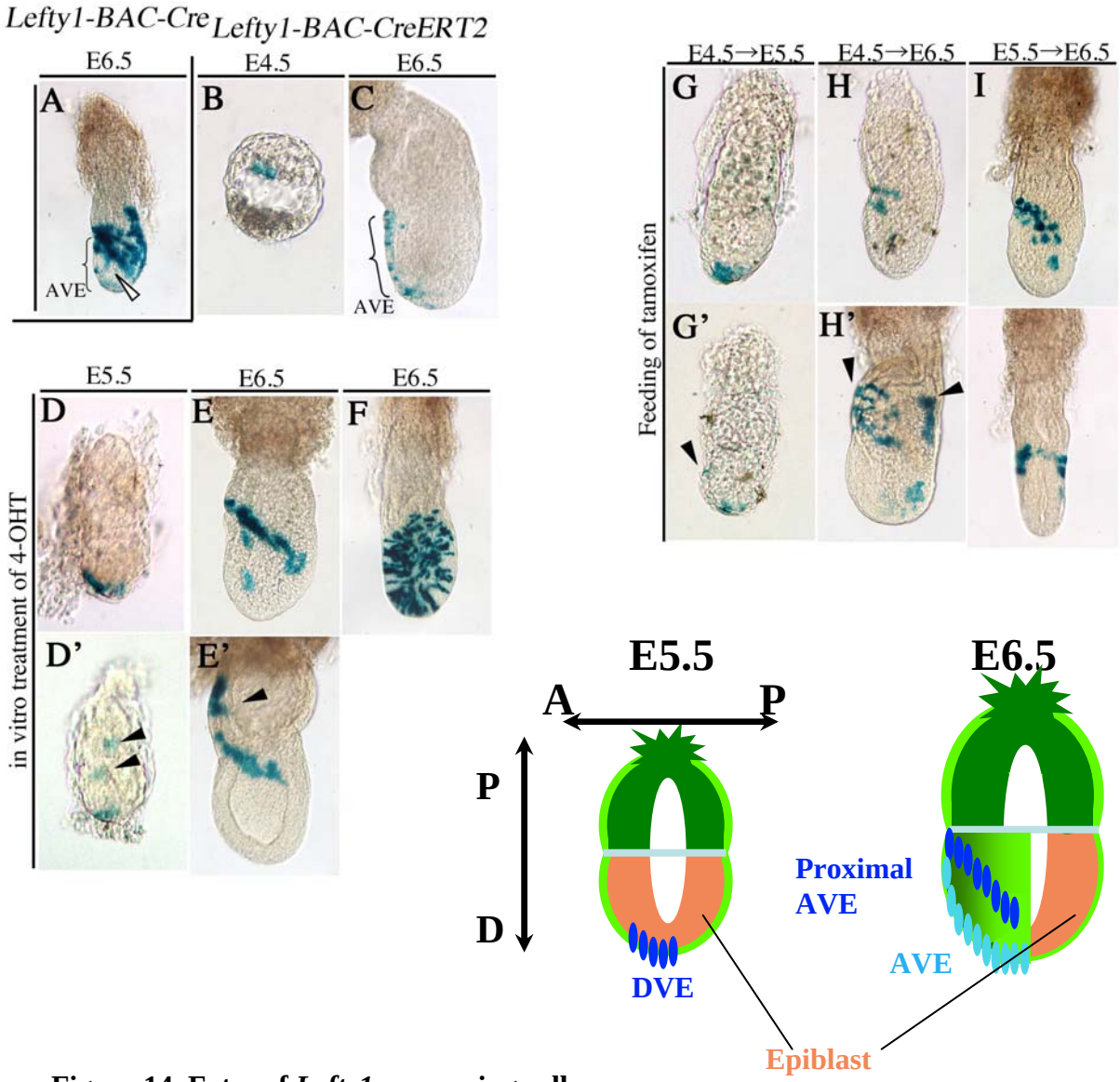


Figure 14. Fates of *Lefty1*-expressing cells.

(A), X-gal staining showing activation of *Rosa26R* reporter allele by the *Lefty1-BAC Cre* transgene at E6.5. Recombination occurs throughout a subset of epiblast (arrowhead) and VE including AVE. *Lefty1-BAC CreERT2* mice were crossed to *Rosa26R* mice(C-I). At E4.5, after 4-hydroxytamoxifen (4-OHT) treatment for 10hr, Xgal staining of transgenic embryos revealed on one side of the primitive endoderm (B). At E6.5, after 5hr treatment, Xgal staining can be seen at AVE (C). The X-gal staining of E5.5, transgenic embryos had been recovered at E3.7, treated with 4-OHT in vitro for 3hr after hanging drop cultured 12～14hr. (D, D') and E6.5 (E, E'). Recovered transgenic embryos at E3.5-3.7 were treated with 4-OHT in vitro for 3hr, and analyze at E6.5 (F). Pregnant females were fed with Tx at E4.5, and recovered embryos analyzed at E5.5(G, G') and E6.5(H, H'). Feeding at E5.5, and analyzed at E6.5(I).

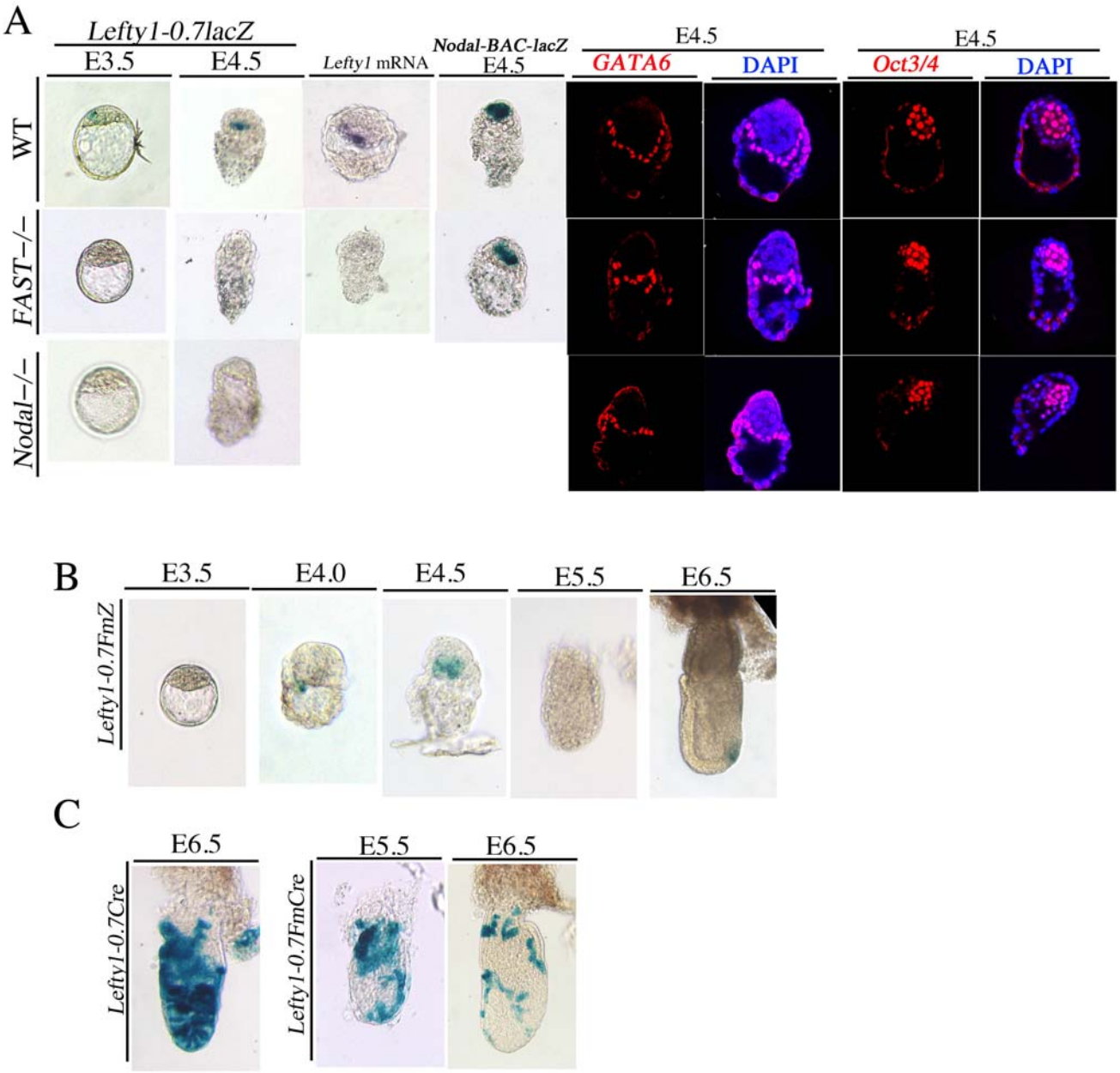


Figure 15. Transcriptional regulation of *Lefty1* in the epiblast-fated and primitive endoderm-fated cells.

(A) Expression of the *Lefty1-0.7lacZ, Nodal-BAC lacZ* transgene, Lefty1 mRNA, GATA6, Oct3/4 in WT or *Foxh1*$^{-/-}$ or *Nodal-/-* embryos at the indicated stages.

(B) Transgenic embryos harboring *Lefty1-0.7Fm lacZ* were recovered at indicated stages and stained with Xgal.

(C) *Lefty1-0.7 Cre* mice were crossed to *Rosa26R* mice, and recovered embryos were stained with Xgal at E6.5.

(D) *Lefty1-0.7Fm Cre* mice were similarly analyzed at E5.5 and E6.5.

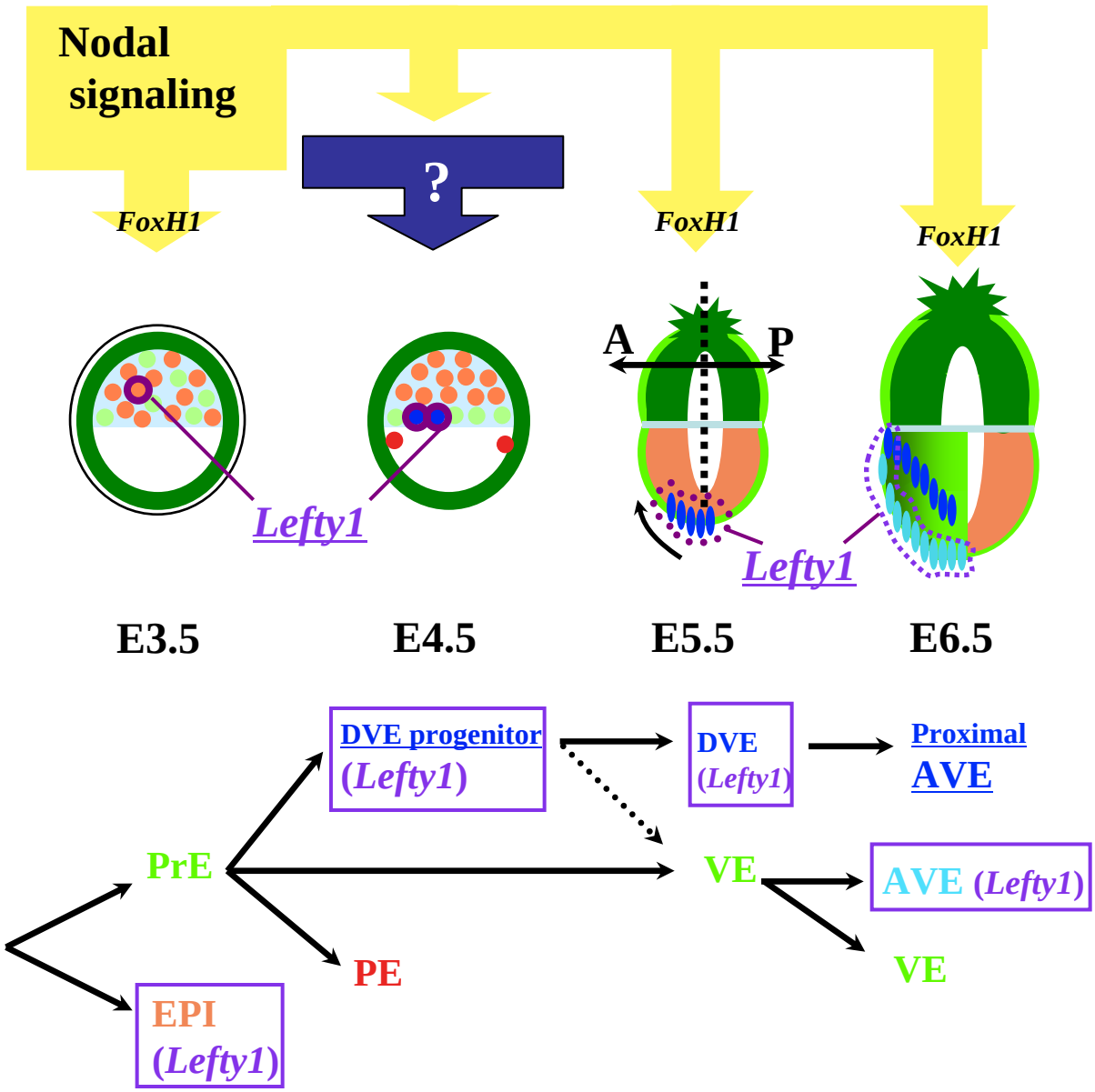


Figure 16. Fate of *Lefty1*-expressing cells and *Lefty1*-expression mechanism

- **DVE specification start at E4.2.**
- **DVE cells at E5.5 do not contribute to the entire region of AVE at E6.5.**
- **Distinct mechanisms induces *Lefty1* expression E3.5, 5.5, 6.5 and E4.5.**