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Nodal signaling induces the midline barrier by activating *Nodal* expression in the lateral plate

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SUMMARY

The transcription factor Foxh1 mediates Nodal signaling. The role of Foxh1 in left-right (LR) patterning was examined with mutant mice that lack this protein in lateral plate mesoderm (LPM). The mutant mice failed to express *Nodal*, *Lefty2* and *Pitx2* on the left side during embryogenesis and exhibited right isomerism. Ectopic introduction of Nodal into right LPM, by transplantation of left LPM or by electroporation of a *Nodal* vector, induced *Nodal* expression in wild-type embryos but not in the mutant. Ectopic *Nodal* expression in right LPM also induced *Lefty1* expression in the floor plate. Nodal signaling thus initiates asymmetric *Nodal* expression in LPM and induces *Lefty1* at the midline. Monitoring of Nodal activity in wild-type and *Foxh1* mutant embryos suggested that Nodal activity travels from the node to left LPM, and from left LPM to the midline.

Key words: Foxh1, Left-right asymmetry, Midline, Nodal, Mouse

INTRODUCTION

The establishment of three axes (anteroposterior, dorsoventral and left-right) is a fundamental aspect of the development of a body plan. Substantial insight has recently been achieved at the genetic and molecular levels into the generation of left-right (LR) asymmetry in vertebrate embryos (Beddington and Robertson, 1999; Capdevila et al., 2000; Wright, 2001; Hamada et al., 2002). The establishment of LR asymmetry is thought to be achieved in four distinct steps: (1) the breaking of LR symmetry in or near the node, (2) transfer of LR-biased signals from the node to the lateral plate, (3) asymmetric expression of signaling molecules such as Nodal and Lefty on the left side of the lateral plate and (4) the induction by these signaling molecules of LR asymmetric morphogenesis of visceral organs. In addition, the midline barrier must be established to separate the two sides of a developing embryo.

Nodal and Lefty (Ebaf – Mouse Genome Informatics), both of which are members of the transforming growth factor β (TGFβ) family of proteins, play important roles in several embryonic patterning events (Schier and Shen, 2000; Brennan et al., 2001; Juan and Hamada, 2001). Lefty antagonizes Nodal signaling by acting as a feedback inhibitor (Meno et al., 1999; Cheng et al., 2000; Sakuma et al., 2002). In LR patterning, genetic evidence suggests that Nodal expressed on the left side of the lateral plate acts as a left-side determinant and induces left side-specific morphogenesis of visceral organs (Oh and Li, 1997; Yan et al., 1999; Lowe et al., 2001), whereas Lefty2 (Leftb – Mouse Genome Informatics), which is induced by Nodal in the left lateral plate, restricts the timing and the region of Nodal activity (Meno et al., 2001). Nodal and Lefty have similar roles among vertebrates from the zebrafish to mouse (Sampath et al., 1997; Rebagliati et al., 1998; Bisgrove et al., 1999; Thisse and Thisse, 1999).

Despite the recent progress in our understanding of LR patterning, many important questions remain unanswered. One such question concerns the mechanism by which symmetry is broken in the first place. Breaking of symmetry in mammals appears to involve nodal flow, the leftward flow of extra-embryonic fluid in the node generated by the vortical movement of nodal cilia (Nonaka et al., 1998). Thus, nodal flow is impaired in mutant mice in which LR patterning is randomized (Okada et al., 1999). Indeed, many of the genes whose mutation results in LR patterning defects encode proteins required for the formation or motility of cilia. Furthermore, imposition of an artificial flow was able to direct LR patterning in early mouse embryos (Nonaka et al., 2002). The mechanism by which Nodal flow achieves this effect, however, has remained unclear. It is possible that the flow transports a LR determination factor toward the left side, but the identity of such a factor is unknown.

The mechanism of signal transfer from the node to the lateral plate is also unknown. Both the identity of the signal (or signals) transported from the node to the lateral plate mesoderm (LPM) and whether the signal is transferred directly from the node to the lateral plate or is first relayed to an intermediate region such as the paraxial mesoderm remain to be determined.

Another important and related question concerns the mechanism by which the expression of *Nodal* is initiated in left LPM. Although an autoregulatory mechanism involving signaling by Nodal and the transcription factor Foxh1 (previously known as Fast2) is responsible for amplification of *Nodal* expression in left LPM (Saijoh et al., 2000; Norris et al., 2002), it is not known how *Nodal* expression is initiated. Ectopic expression of Nodal in right LPM of chick embryos was not able to induce *Nodal* expression (M. Levin, PhD thesis, Harvard University, 1996), suggesting that an unknown factor other than Nodal initiates *Nodal* expression in left LPM. Bone morphogenetic protein (BMP) signaling has been proposed to regulate *Nodal* expression negatively in the chick, and a BMP antagonist such as Caronte may initiate *Nodal* expression by inhibiting BMP activity on the left side (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). However, recent evidence has suggested that BMP signaling positively regulates *Nodal* expression by inducing an EGF-CFC factor in LPM (Schlange et al., 2001; Schlange et al., 2002; Piedra and Ros, 2002). The factor responsible for the initiation of asymmetric *Nodal* expression in LPM thus remains elusive. Finally, the midline structures serve as a barrier that prevents the diffusion of asymmetric signals (Danos and Yost, 1996; Lohr et al., 1997; Meno et al., 1998), but it is unclear how the midline barrier is established and precisely how it functions. Analysis of *Lefty1* mutant mice has shown that Lefty1, a Nodal antagonist expressed on the left side of the prospective floor plate (PFP), contributes to midline barrier function (Meno et al., 1998). However, it is unknown how *Lefty1* expression is induced at the midline.

We have now studied the role of Nodal-Foxh1 signaling in LR patterning by analyzing *Foxh1* conditional mutant mice. We have also examined Nodal function by developing transplantation and electroporation systems for use with mouse embryos and applying these systems to the *Foxh1* mutant mice. Unexpectedly, Nodal-Foxh1 signaling was shown to be able to initiate *Nodal* expression in LPM and to induce *Lefty1* expression at the midline. Our results indicate that the left-sided expression of *Nodal* in LPM is initiated by Nodal produced in the node, and that *Lefty1* expression at the midline is induced by Nodal produced in left LPM. We propose that Nodal activity travels from the node to left LPM, and from left LPM to the midline.

MATERIALS AND METHODS

LPM-specific deletion of *Foxh1*

The *Lefty2-3.0 Cre* transgene was constructed by ligating the 3.0-kb upstream region of mouse *Lefty2* to a *Cre* cassette derived from pBS-Cre (kindly provided by H. Kondoh). Several transgenic lines harboring this transgene were established. To examine the specificity of *Cre* expression, we crossed each line with Cre-reporter mice harboring a *lacZ* gene that is expressed only after Cre-mediated excision (Sakai and Miyazaki, 1997). Embryos were genotyped and stained with X-gal. One transgenic line (77b) that exhibited bilateral Cre expression in the lateral plate was used in this study. Line 77b animals were crossed with $Foxh1^{+/-}$ mice to obtain heterozygotes harboring the *Cre* transgene; these $Foxh1^{+/-}$, *Lefty2-3.0 Cre* mice were then mated with $Foxh1^{flox/flox}$ animals (Yamamoto et al., 2001), and the resulting embryos were analyzed at E8.2, E9.5 and E10.5.

In situ hybridization and histology

Mouse embryos were staged on the basis of their morphology (Downs and Davies, 1993). Whole-mount in situ hybridization was performed according to standard procedures (Wilkinson, 1992). Wild-type and mutant embryos were processed in the same tube. Embryos were genotyped by PCR analysis of yolk sac DNA.

Transplantation of LPM

Fragments of tissue (containing ~20 cells) were isolated for transplantation from the left LPM of mouse embryos at the four-somite stage. For use as recipients, mouse embryos were recovered at the two-somite stage, dissected free of decidual tissues and Reichert's membrane, and maintained in culture until manipulation. Cell clumps from donor embryos were grafted to the right LPM, left paraxial mesoderm or left LPM of the host embryos with the use of tungsten needles. The transplanted embryos were cultured for 3 hours at 37°C in 35 mm disposable dishes containing 4 ml of 50% Dulbecco's modified Eagle's medium supplemented with 50% rat serum (Lawson et al., 1986); this volume of medium was sufficient for culture of eight embryos (Sturm and Tam, 1993). A transplant remained as a mass after the culture, showed distinct density and thickness, and was easily distinguished from the host tissues.

Electroporation of a *Nodal* expression vector into LPM

The full-length cDNAs of mouse Nodal, constitutive active human ALK4 (caALK4), *lacZ* and EGFP were subcloned into the eucaryotic expression vectors pEF-BOS (Mizushima and Nagata, 1990), pcDNA3, pEF and pCX, respectively. Each plasmid was suspended in phosphate-buffered saline at a concentration of 5 mg/ml. Mouse embryos at the two-somite stage were dissected free of decidual tissues and Reichert's membrane and maintained in culture until electroporation. Electroporation was performed with a CUY21 electroporator and a pulse monitor (BTX, Tokyo). Platinum electrodes were used as an anode and a cathode and were positioned near the posterior and anterior regions of the embryo, respectively. Electric pulses were applied (14 V for 129 ms, five times) while the DNA solution (1 μl) was injected into the anterior region of left or right LPM. The electroporated embryos were cultured for 6 hours in a 50 ml disposable tube containing 4 ml of 50% Dulbecco's modified Eagle's medium supplemented with 50% rat serum (Lawrence and Struhl, 1996), a volume sufficient for the culture of eight embryos (Sturm and Tam, 1993). The tubes were rotated at 30 rpm on a roller apparatus placed in a 37°C incubator containing 5% CO_2. This culture condition is optimized for normal LR development so that left-sided *Nodal* expression in LPM is preserved in >95% of the cultured embryos. A Nodal or *caALK4* expression vector was introduced together with an EGFP expression vector by electroporation, and regions that received the vectors were confirmed by the presence of EGFP fluorescence.

RESULTS

Conditional deletion of *Foxh1* in the lateral plate

Foxh1 null mutants exhibit defects in anteroposterior patterning and in node formation (Hoodless et al., 2001; Yamamoto et al., 2001). The early mortality of the null mutants, however, has impeded characterization of the role of Foxh1 at later stages of development. We have now examined the contribution of Foxh1 to LR patterning by generating conditional mutant mice. Mutant mice harboring a flox allele of *Foxh1* ($Foxh1^{flox}$) have been described (Yamamoto et al., 2001), and, in the present study, we aimed to delete *Foxh1* in the lateral plate with the use of transgenic mice expressing the Cre recombinase in this tissue. The Cre-expressing transgene

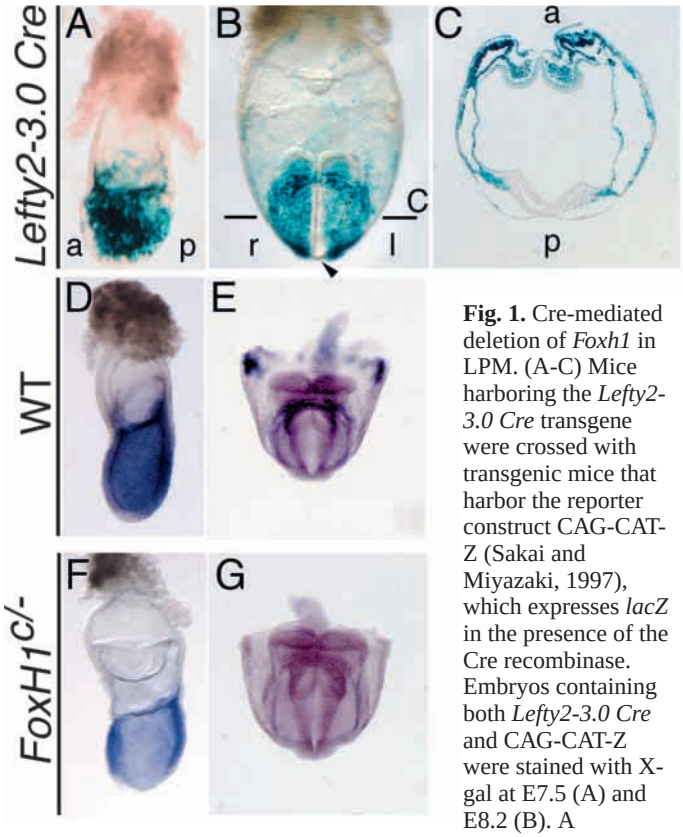

Fig. 1. Cre-mediated deletion of *Foxh1* in LPM. (A-C) Mice harboring the *Lefty2-3.0 Cre* transgene were crossed with transgenic mice that harbor the reporter construct CAG-CAT-Z (Sakai and Miyazaki, 1997), which expresses *lacZ* in the presence of the Cre recombinase. Embryos containing both *Lefty2-3.0 Cre* and CAG-CAT-Z were stained with X-gal at E7.5 (A) and E8.2 (B). A transverse section at the plane indicated in B is shown in C. At E7.5, most of the mesoderm exhibited X-gal staining whereas the ectoderm and endoderm did not. At E8.2, the anterior region of LPM, paraxial mesoderm and the heart were positive for staining, whereas the posterior portion of LPM and midline structures (arrowhead in B), including the PFP, were negative. a, anterior; p, posterior; l, left; r, right. (D-G) Whole-mount in situ hybridization analysis of *Foxh1* transcripts. Lateral views are shown for wild-type (WT) (D) and *Foxh1*$^{c/-}$ (F) embryos at E7.5 and anterior views for wild-type (E) and *Foxh1*$^{c/-}$ (G) embryos at E8.2. In the *Foxh1*$^{c/-}$ embryos, Foxh1 mRNA was not detected in the regions that were positive for X-gal staining in A-C.

Lefty2-3.0 Cre contains a 3 kb fragment of the mouse *Lefty2* promoter that directs gene expression in nascent mesoderm (Saijoh et al., 1999). One of the transgenic lines (77b) harboring this transgene was used in this study.

To confirm the expression pattern of the *Cre* transgene in 77b mice, we crossed the animals with Cre-reporter mice that express *lacZ* in response to Cre activity (Sakai and Miyazaki, 1997). Embryos harboring both *Lefty2-3.0 Cre* and the Cre-reporter *lacZ* transgene were recovered at various stages and stained for β-galactosidase activity with the substrate X-gal. Staining was first evident at embryonic day 6.75 (E6.75) in the nascent mesoderm. At E7.5, most of the embryonic region of the mesoderm exhibited staining, whereas the primitive streak, ectoderm and endoderm were negative (Fig. 1A). Mesoderm-specific staining remained evident at E8.2; however, the midline structures, including the axial mesoderm, lacked Cre activity (Fig. 1B). At this stage, the pattern of Cre activity differed between the anterior and posterior regions of the embryo (Fig. 1C). In the anterior region, X-gal staining was complete in the LPM, paraxial mesoderm, definitive endoderm and heart. In the region posterior to the node, however, Cre activity was detected only in the most lateral region of LPM. About 30 to 50% of the extra-embryonic mesoderm cells in the yolk sac and amnion were also positive for X-gal staining. At E9.5, mesoderm-derived cells in the anterior region were positive whereas the mesoderm in the posterior region was negative (data not shown). Thus, at stages later than the early somite stage, Cre activity was mostly restricted to the mesoderm of the anterior region of the embryo.

Foxh1$^{flox/flox}$ mice were then mated with *Foxh1*$^{+/-}$, *Lefty2-3.0 Cre* mice to obtain *Foxh1*$^{flox/-}$, *Lefty2-3.0 Cre* mice (for simplicity, these conditional mutant mice are referred to as *Foxh1*$^{c/-}$). Cre-mediated deletion of *Foxh1* in the *Foxh1*$^{c/-}$ embryos was confirmed by examining *Foxh1* expression. Whereas Foxh1 mRNA is abundant in all three germ layers of gastrulating wild-type embryos (Fig. 1D,E), *Foxh1* expression was absent in the mesoderm of *Foxh1*$^{c/-}$ embryos (Fig. 1F,G).

Right isomerism in *Foxh1* conditional mutant embryos

We first examined LR patterning in the *Foxh1*$^{c/-}$ embryos by analyzing the transcription of asymmetrically expressed genes such as *Nodal*, *Lefty1*, *Lefty2* and *Pitx2*. In wild-type embryos, *Nodal* is expressed in two domains at the early somite stage: the node and left LPM (Fig. 2A,B). In most *Foxh1*$^{c/-}$ embryos examined (31/37, 84%), however, left-sided expression of *Nodal* in left LPM was absent (Fig. 2C); in the remaining embryos (6/37, 16%), a low level of *Nodal* expression was detected in a small region of left LPM adjacent to the node (Fig. 2D,E), probably because a Nodal-positive loop was operative in this region before deletion of *Foxh1* was complete. *Nodal* expression in the node was maintained in all (37/37) *Foxh1*$^{c/-}$ embryos (insets in Fig. 2C,D). The expression of *Lefty2* apparent in left LPM of wild-type embryos (Fig. 2F) was abolished in all (16/16) *Foxh1*$^{c/-}$ embryos examined (Fig. 2G). The expression of *Lefty1* observed in the PFP of wild-type embryos (Fig. 2F) was also lost in all (16/16) *Foxh1*$^{c/-}$ embryos (Fig. 2G) (in some *Foxh1*$^{c/-}$ embryos, *Lefty1* expression was detected in a small region of PFP adjacent to the node at the two-somite stage but this expression disappeared at four-somite stage). This latter effect was unexpected given that *Foxh1* is preserved in midline structures including the PFP (Fig. 1B). The expression of *Lefty1* apparent in the node of wild-type embryos was maintained in the mutant embryos (inset of Fig. 2G). The asymmetric expression of *Pitx2* apparent in wild-type embryos (Fig. 2H,I,N-P) was abolished in two-thirds (29/43) of *Foxh1*$^{c/-}$ embryos at E8.2 (Fig. 2J) and in ~70% (18/26) of the mutant embryos between E9.0 and E10.5 (Fig. 2K,Q-S), whereas bilateral *Pitx2* expression in the branchial arch was maintained in all *Foxh1*$^{c/-}$ embryos. In the remaining mutant embryos, a reduced level of left-sided *Pitx2* expression was detected both in LPM at E8.2 (Fig. 2L) and in several organs and other structures, including the common atrial chamber, lung bud, sinus venosus, vitelline vein, common cardinal vein and gut, at later stages (Fig. 2M,T-V).

Foxh1$^{c/-}$ mice were carried to term but died within several days after birth. Examination of the visceral organs of *Foxh1*$^{c/-}$ neonates revealed various LR defects with right isomerism as the major phenotype (Fig. 3). Although the lungs of wild-type mice contain one and four lobes on the left and right sides, respectively (Fig. 3A), the lungs of most (40/45, 89%) of the

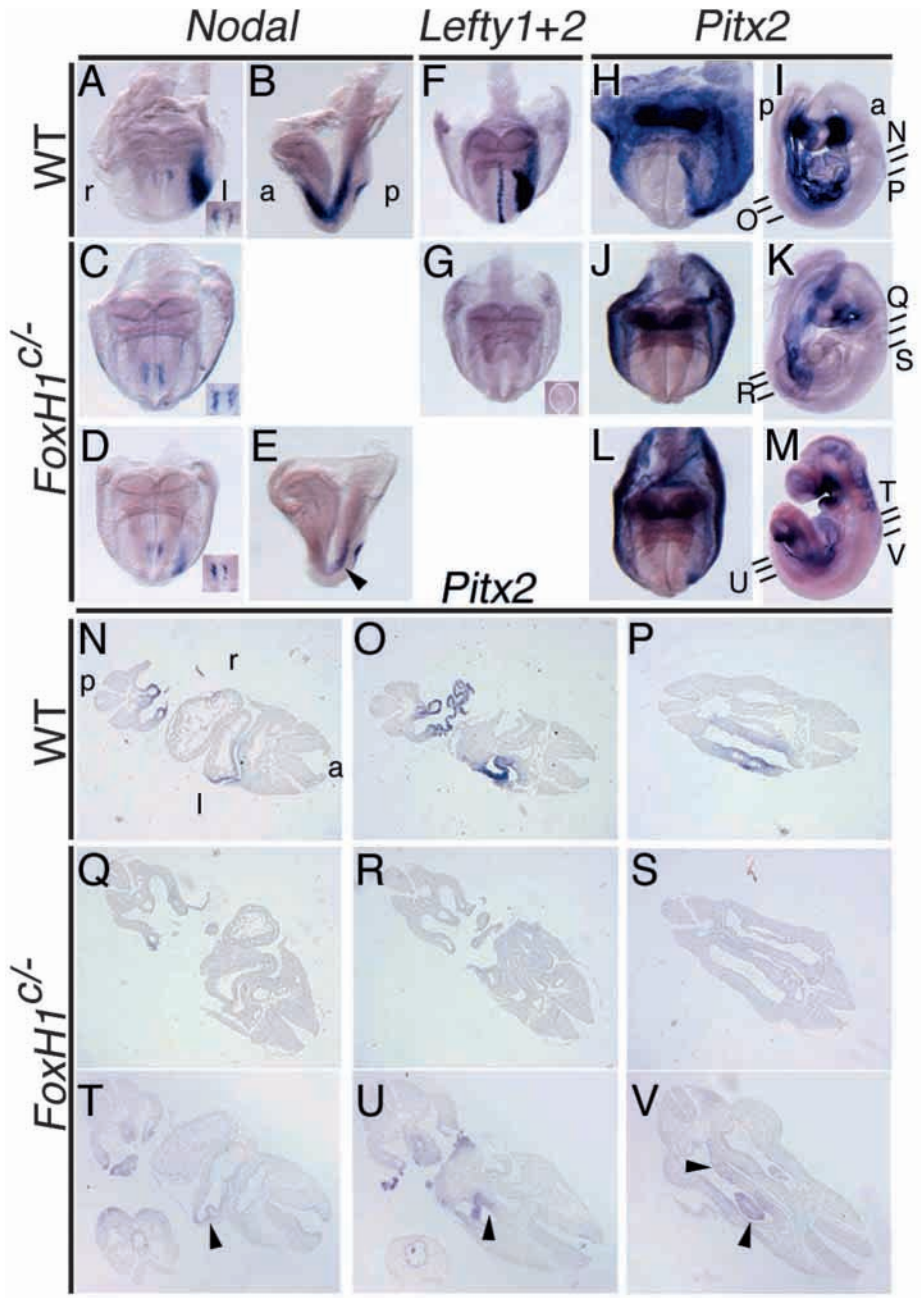

Fig. 2. Aberrant expression of *Nodal*, *Lefty1*, *Lefty2* and *Pitx2* in *Foxh1*$^{c/-}$ embryos. The expression of *Nodal* (A-E), *Lefty1* plus *Lefty2* (F,G) and *Pitx2* (H-V) in wild-type (WT) and *Foxh1*$^{c/-}$ embryos was examined by whole-mount in situ hybridization. Embryos shown in A-H,J,L are at E8.2, whereas the others are at E9.5. Transverse sections at the planes indicated in I, K and M are shown in N-P, Q-S and T-V, respectively. All E8.2 embryos are anterior views, with the exception that left lateral views are shown for B and E. The insets in A, C, D and G show the node in posterior view of the embryos. The white line in the inset of G indicates the location of the node. Most *Foxh1*$^{c/-}$ embryos fail to exhibit left-sided gene expression, although some retain *Nodal* expression in a small region of left LPM adjacent to the node (arrowhead in E) and a low level of left-sided *Pitx2* expression in various organs (arrowheads in T-V). a, anterior; p, posterior; l, left; r, right.

Foxh1$^{c/-}$ mice examined manifested right isomerism, having four lobes on both sides (Fig. 3B). A small proportion (3/45, 7%) of *Foxh1*$^{c/-}$ mice exhibited partial right isomerism, having two or three lobes on the left side and four lobes on the right side. Abnormal positioning of the great arteries, either arterial transposition (9/41, 22%) (Fig. 3P,S) or double-outlet right ventricle (31/41, 76%) (Fig. 3Q,T), was also frequently observed in *Foxh1*$^{c/-}$ mice. The position of the heart apex was reversed (toward the right; 15/41, 37%) (Fig. 3K), ambiguous (in the middle; 7/41, 17%) (Fig. 3J) or normal (toward the left; 19/41, 46%) in *Foxh1*$^{c/-}$ mice. Additional heart malformations, including ventricular septal defect without valve defects (VSD; 25/41, 61%) (Fig. 3T), atrial septal defect without valve defects (ASD; 30/41, 73%) (Fig. 3Q) and endocardial cushion defect (common atrioventricular valve plus ASD and VSD; 10/41, 24%) (data not shown), were also observed. The external morphology of the atrium was also affected, showing right isomerism (11/43, 26%) or LR inversion (13/43, 30%). The azygos vein, which is normally located on the left side (Fig. 3C), was reversed (13/43, 30%) (Fig. 3D), bilateral (14/43, 33%) or normal (16/43, 37%) in *Foxh1*$^{c/-}$ mice. Additional defects included hypoplasia of the spleen (21/40, 53%) (Fig. 3F) and the presence of the stomach on the right side (8/43, 19%) (Fig. 3H). The relative positions of left and right renal veins were reversed (14/44, 32%) or the two veins were located at the same level (12/44, 27%) (Fig. 3M). The portal vein, which normally passes dorsally to the duodenum, passed ventrally to the duodenum in *Foxh1*$^{c/-}$ mice (12/38, 32%) (Fig. 3N). These morphological defects resemble those observed with cryptic mutant mice (Yan et al., 1999), and are, in general, consistent with a lack of Nodal, the left-side determinant, in left LPM. The phenotype of *Foxh1*$^{c/-}$ mice also resemble that of the zebrafish mutant lacking Foxh1 (Chen et al., 1997; Bisgrove, 2000).

Induction of *Nodal* expression in the lateral plate requires Foxh1

Asymmetric *Nodal* expression in left LPM begins in a region adjacent to the node and expands along the anteroposterior axis. It is currently unknown how *Nodal* expression is initiated in left LPM; it may involve a positive autoregulatory mechanism, but direct evidence is lacking. To study the mechanism of *Nodal* induction, we developed a system for cell transplantation into LPM. A piece of tissue was dissected from the left LPM of a donor embryo at the four-somite stage (*Nodal* is already expressed in the entire left LPM at this stage) and was

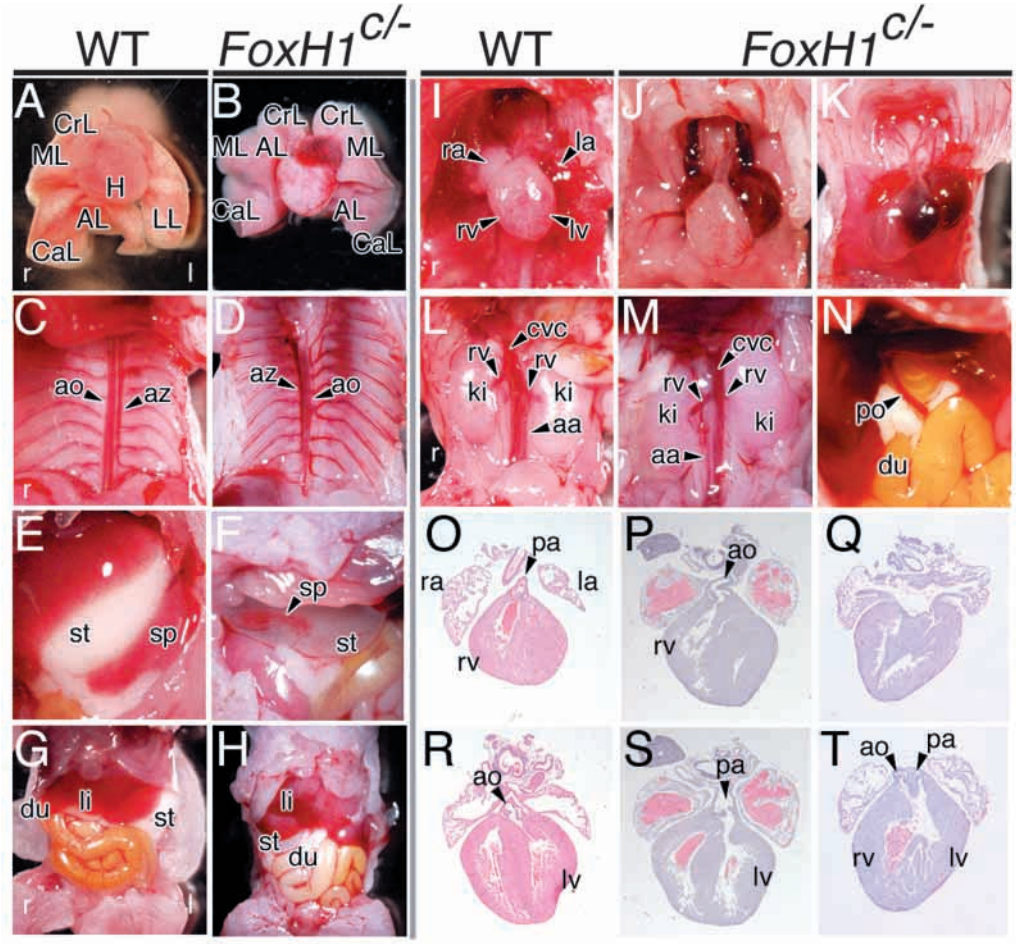

Fig. 3. LR defects in the visceral organs of *Foxh1*$^{c/-}$ mice. Visceral organs and heart sections of wild-type (WT) and *Foxh1*$^{c/-}$ neonates are shown. Genotype is indicated at the top of each column. (A,B) Lobation of the lung. In the wild-type mouse (A), the left and right lungs have one and four lobes, respectively. In most *Foxh1*$^{c/-}$ mice (B), both left and right lungs have four lobes. AL, accessory lobe; CaL, caudal lobe; CrL, cranial lobe; ML, medial lobe; H, heart; LL, left lobe. (C,D) The azygos vein (az) of the wild-type mouse is located on the left side (C). In most *Foxh1*$^{c/-}$ mice, the azygos vein is located on the right side (D) or both sides. ao, aorta. (E,F) Hypoplasia of the spleen (sp) in the *Foxh1*$^{c/-}$ mouse (F). st, stomach. (G,H) Visceral organs including the stomach are reversed in many *Foxh1*$^{c/-}$ mice (H). du, duodenum; li, liver. (I-K) The heart apex is located on the left side of the wild-type mouse (I), but is either in the middle (J) or on the right side (K) of *Foxh1*$^{c/-}$ mice. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. (L,M) The right renal vein is located anteriorly to the left renal vein in the wild-type mouse (L). In *Foxh1*$^{c/-}$ mice, the relative positions of left and right renal veins are reversed or the two veins are located at the same level (M). cvc, caudal vena cava; ki, kidney; rv, renal vein; aa, abdominal aorta. (N) Aberrant positioning of the portal vein (po) in the *Foxh1*$^{c/-}$ mouse. (O-T) Frontal sections of the heart. In most *Foxh1*$^{c/-}$ mice, the heart manifests severe malformations, including transposition of the great arteries (P,S) and double outlet of the right ventricle (Q,T). pa, pulmonary artery.

transplanted into the right LPM of a recipient embryo at the two-somite stage (*Nodal* expression is initiated but not expanded in left LPM at this stage) (Fig. 4A). When the *Foxh1*$^{c/-}$ embryo was used as a recipient, a donor LPM was transplanted into the left LPM. We then examined whether the transplanted left LPM was able to induce *Nodal* expression in the right LPM of the recipient embryo. Indeed, the transplanted left LPM induced *Nodal* expression in the recipient right LPM (3/3). Thus, *Nodal* expression in right LPM was not only apparent in the region adjacent to the transplanted left LPM but expanded along the anteroposterior axis (Fig. 4B). In most instances, the induced *Nodal* expression did not extend over the entire area of right LPM, possibly as a result of its premature termination by Lefty2 present in the transplanted left LPM (exogenous *Nodal* expression alone in the right LPM induced *Nodal* expression throughout the entire region of right LPM, as shown below). By contrast, transplantation of right LPM into the right LPM of a host embryo failed to induce *Nodal* expression in the recipient right LPM (3/3, data not shown).

We performed similar transplantation experiments with *Foxh1*$^{c/-}$ embryos. Left LPM obtained from a wild-type embryo was thus transplanted to the anterior region of left LPM of *Foxh1*$^{c/-}$ embryos. Such manipulation failed to induce *Nodal* expression in the left LPM of the host embryo (10/10) (Fig. 4F,G), indicating that the induction of *Nodal* expression in LPM requires Foxh1.

Initiation by Nodal of *Nodal* expression in the lateral plate

Our results suggested that an unknown factor derived from left LPM is able to initiate *Nodal* expression in right LPM. An obvious candidate for this factor was Nodal itself present in left LPM. To test this possibility, we introduced a *Nodal* expression vector and an EGFP expression vector into embryos at the early somite stage by electroporation. The embryos were first examined for fluorescence to locate the cells that received the vectors (Fig. 5A), and were subjected to in situ hybridization. Introduction of the *Nodal* vector into right LPM of wild-type embryos resulted in the induction of endogenous *Nodal* expression (Fig. 5B,C). *Nodal* expression induced in right LPM expanded along the anteroposterior axis and extended throughout the entire region of the right LPM (25/25) (arrowheads in Fig. 5C). Electroporation of an EGFP expression vector alone into right LPM of wild-type embryos did not give rise to such expanded *Nodal* expression except in one (1/20) case. By contrast, introduction of the

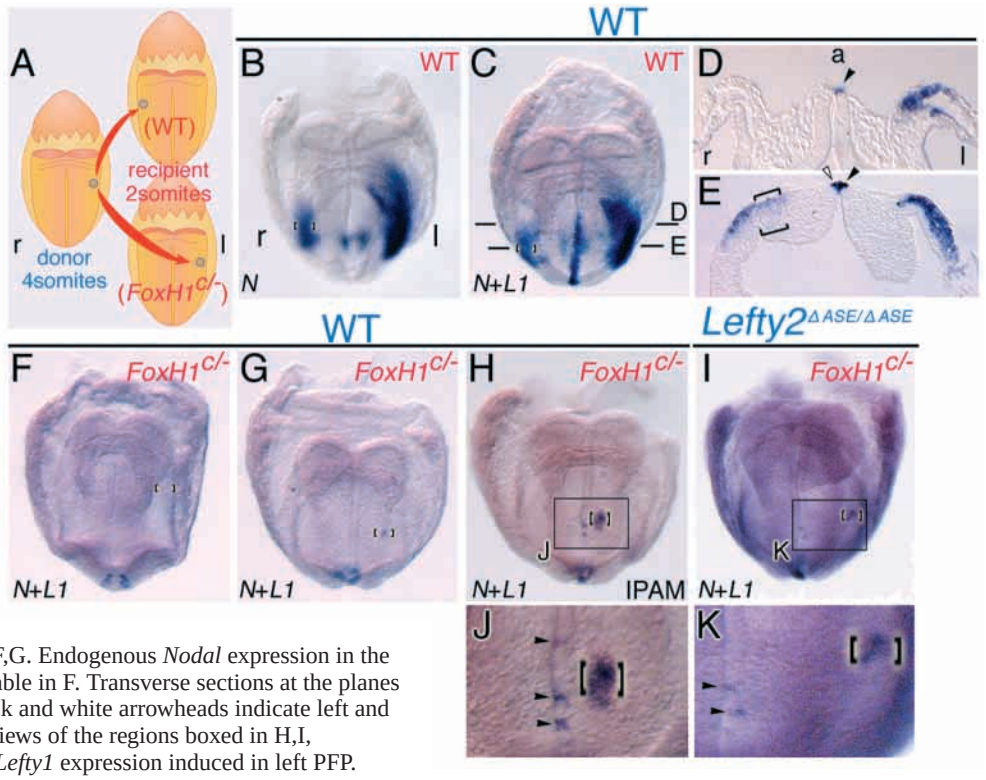

Fig. 4. Induction of *Nodal* and *Lefty1* by transplanted left LPM.
(A) Schematic representation of the tissue transplantation system.
(B-K) Expression of *Nodal* (*N* in B) and *Nodal* plus *Lefty1* (*N+L1* in C-K) was examined by whole-mount in situ hybridization 3 hours after the indicated type of transplantation. Genotypes of the recipients and donors are shown in red and blue, respectively, at the top of each panel. Donor tissue was derived from left LPM of the indicated embryos. The transplant sites in the host embryos are indicated by square brackets (B,C,E-K). Donor tissue was transplanted to the anterior region of LPM in host embryos, with the exception of the embryo shown in H,J, which received the transplant in the paraxial mesoderm (IPAM). Two representative *Foxh1*$^{c/-}$ embryos that received a transplant derived from the left LPM of wild-type (WT) embryo are shown in F,G. Endogenous *Nodal* expression in the transplant is detectable in G but undetectable in F. Transverse sections at the planes indicated in C are shown in D and E; black and white arrowheads indicate left and right PFP, respectively. (J,K) Magnified views of the regions boxed in H,I, respectively. Arrowheads in J,K indicate *Lefty1* expression induced in left PFP.

Nodal expression vector into the left or right LPM of *Foxh1*$^{c/-}$ embryos failed to induce *Nodal* (Fig. 5F). Thus, ectopic Nodal is able to induce *Nodal* expression in right LPM, but this induction requires the presence of Foxh1 in LPM.

Induction of *Lefty1* expression at the midline by Nodal produced in the left lateral plate

Lefty1 plays an essential role as the midline barrier (Meno et al., 1998). In *Foxh1*$^{c/-}$ embryos, *Lefty1* expression in the floor plate was lost (Fig. 2G) despite the fact that *Foxh1* was not deleted in the PFP (Fig. 1B). Furthermore, both *Nodal* (Fig. 2C) and *Lefty1* (Fig. 2G) expression was preserved in the node. These observations suggested that *Lefty1* expression in the floor plate might be induced by Nodal produced in left LPM. Consistent with this notion, previous studies (Chen and Schier, 2001; Meno et al., 2001) have suggested that Nodal is able to act over a long distance. We therefore tested this possibility with the use of our transplantation and electroporation systems.

Lefty1 is expressed in the PFP on the left side of wild-type embryos (Meno et al., 1997). A piece of left LPM transplanted to the right LPM of wild-type embryos was able to induce not only *Nodal* in the right LPM but also *Lefty1* in the right PFP (3/3) (Fig. 4C). Thus, *Lefty1* expression in the PFP became bilateral only at the levels where *Nodal* was ectopically expressed in right LPM (Fig. 4D,E), supporting the idea that Nodal produced in LPM induces *Lefty1* expression in the PFP. Similar experiments were performed with *Foxh1*$^{c/-}$ embryos, which retain *Foxh1* in the PFP but lack *Lefty1* expression in this region. Transplantation of left LPM from wild-type embryos to the left LPM of *Foxh1*$^{c/-}$ embryos did not result in the induction of *Lefty1* expression in the PFP (0/10) (Fig. 4F,G). However, transplantation of the left LPM to the paraxial mesoderm, a site closer to the PFP, resulted in the induction of *Lefty1* (3/4) (Fig. 4H,J). Furthermore, left LPM obtained from *Lefty2*$^{\Delta ASE/\Delta ASE}$ embryos, in which Nodal activity is increased as a result of the lack of Lefty2 (Meno et al., 2001), induced *Lefty1* expression in the PFP even when transplanted to the left LPM of *Foxh1*$^{c/-}$ embryos (4/5) (Fig. 4I,K).

Introduction of the *Nodal* expression vector into the right LPM of wild-type embryos also induced *Lefty1* expression in the PFP (18/25) (Fig. 5D,E). The spatial level of ectopic *Lefty1* expression along the anteroposterior axis of the PFP corresponded to that of ectopic *Nodal* expression in the right LPM. In most instances, *Lefty1* expression was bilateral throughout the entire PFP, while ectopically induced *Nodal* expression extended throughout the entire region of right LPM (Fig. 5E). Furthermore, introduction of the *Nodal* expression vector into the left LPM of *Foxh1*$^{c/-}$ embryos also induced *Lefty1* expression in the PFP (6/8) (Fig. 5G,H), making it unlikely that *Lefty1* was induced by secondary signals produced by the Nodal-Foxh1 pathway. In these various experiments, *Nodal* expression was never induced in the PFP either by the transplanted left LPM or by introduction of the *Nodal* expression vector into right LPM (Fig. 4B, Fig. 5B).

These results suggest that *Lefty1* expression in PFP is directly induced by Nodal produced in LPM but do not exclude an alternative possibility that it is induced by secondary factor(s) produced in LPM by a Nodal-dependent yet Foxh1-independent pathway. To test the latter possibility, we examined the effects of constitutive active ALK4 (caALK4). As expected, caALK4 was able to induce *Nodal* in the right LPM of the wild-type embryos (5/5) (Fig. 5I). However, introduction of the caALK4 expression vector into the left LPM of *Foxh1*$^{c/-}$ embryos failed to induce *Lefty1* expression in the PFP (11/11) (Fig. 5J). These results now demonstrate

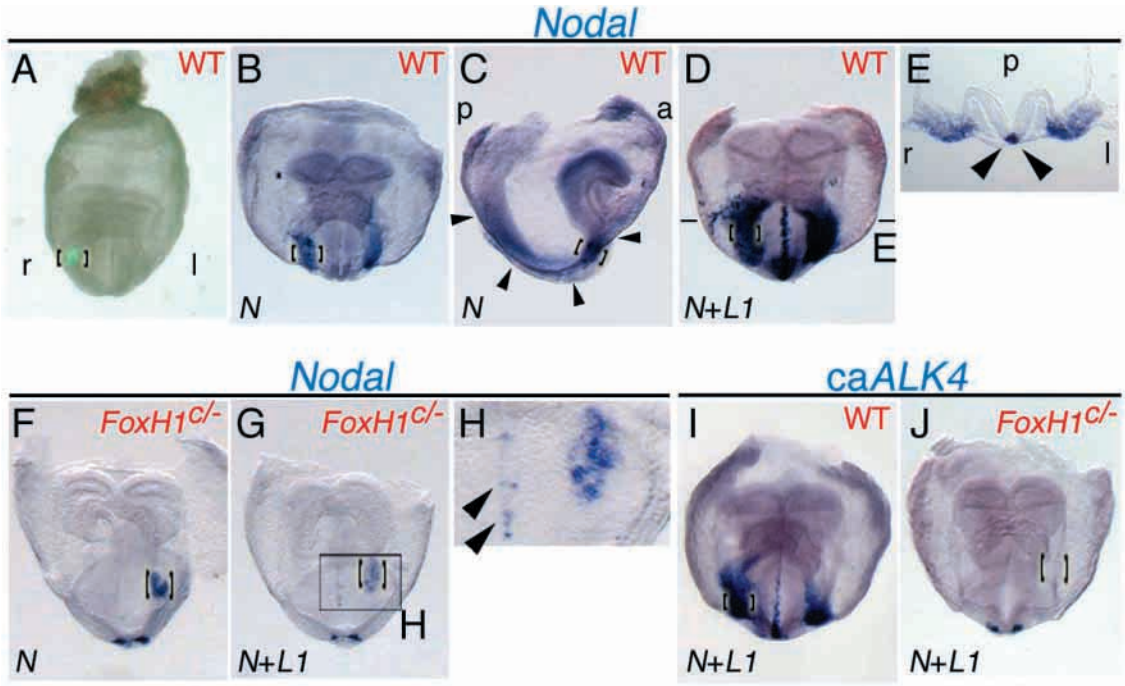

Fig. 5. Induction of *Nodal* and *Lefty1* by a *Nodal* expression vector. Expression vectors for Nodal plus EGFP (enhanced green fluorescent protein) (A-H) or those for caALK4 plus EGFP (I,J) were introduced by electroporation into the anterior region of right LPM of a wild-type (WT) embryo (A-E,I) or into the anterior region of left LPM of a *Foxh1*$^{c/-}$ embryo (F-H,J). Electroporated expression vectors are shown in blue, while genotypes of the recipient embryos are shown in red. Six hours after electroporation, expression of *Nodal* (*N* in B,C,F) or *Nodal* plus *Lefty1* (*N+L1* in D,E,G-J) was examined by whole-mount in situ hybridization. Electroporated regions, which were confirmed by the presence of EGFP fluorescence (A), are indicated by the square brackets. Anterior views are shown in (A,B,D,F-J), whereas right lateral view is shown in C. A transverse section at the plane indicated in D is shown in E. A magnified view of the boxed region indicated in G is shown in H. Arrowheads indicate *Nodal* expression in the right LPM (C) and *Lefty1* expression (E,H) induced by the *Nodal* expression vector.

that Nodal activity produced in LPM directly induces *Lefty1* expression in PFP.

Nodal activity travels from left LPM to the PFP

Our results suggest that Nodal ectopically produced in LPM may diffuse over the relatively long distance to the PFP and there induce *Lefty1* expression. We next examined whether the PFP indeed receives Nodal signals from left LPM with the use of a *lacZ* transgene whose expression is strictly dependent on Nodal signaling. This transgene, *(n2)7-lacZ*, contains seven tandem repeats of a Foxh1 binding site and its expression is induced by the Nodal-Foxh1 pathway (Saijoh et al., 2000; Sakuma et al., 2002). X-gal staining of transgenic embryos harboring *(n2)7-lacZ* revealed *lacZ* expression in the PFP predominantly on the left side as well as in left LPM (Fig. 6A,B). Given that *Nodal* is not expressed in the PFP, Nodal produced elsewhere (either in left LPM or the node) must have traveled to the PFP. By contrast, X-gal staining of *Foxh1*$^{c/-}$

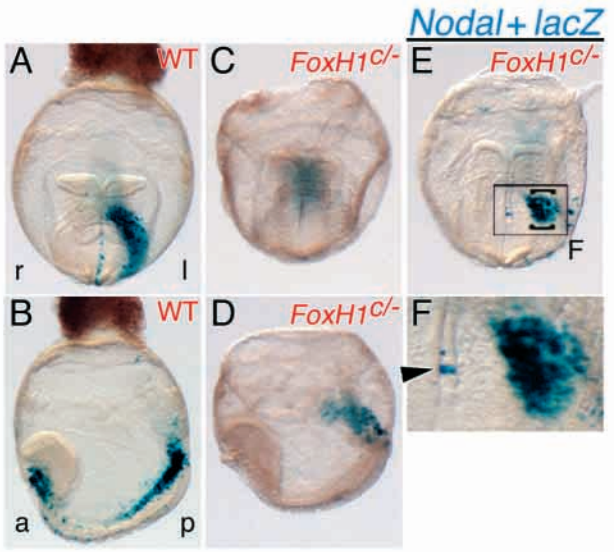

Fig. 6. Monitoring of Nodal activity in mouse embryos with a Nodal-responsive transgene. Expression of the Nodal-responsive transgene *(n2)7-lacZ* was examined in wild-type (WT) (A,B) and *Foxh1*$^{c/-}$ (C,D) embryos. In the *Foxh1*$^{c/-}$ embryo, X-gal staining in left LPM and PFP is lost whereas that in the allantois remains (C,D). Expression vectors for *Nodal*, *EGFP* and *lacZ* were also introduced into the left LPM of a *Foxh1*$^{c/-}$ embryo harboring the *(n2)7-lacZ* transgene and, 6 hours later, the embryo was stained with X-gal (E,F). The region that received the expression vectors is apparent from the EGFP fluorescence and X-gal staining in left LPM (indicated by the square brackets). X-gal-positive region in left LPM failed to expand due to the absence of Foxh1 in LPM. A magnified view of the boxed region indicated in E is shown in F. The arrowhead in F indicates X-gal staining in the PFP that was induced by Nodal. Anterior views are shown in A,C,E, whereas left lateral views are shown in B,D.

embryos harboring the *(n2)7-lacZ* transgene revealed that *lacZ* expression was abolished in the left LPM and PFP, although staining in the allantois and at the base of the allantois remained (10/10) (Fig. 6C,D). Similarly, another Nodal-responsive *lacZ* reporter gene, *Lefty2 ASE-lacZ*, that contains the asymmetric enhancer (ASE) of *Lefty2* also gave rise to X-gal staining in the PFP in addition to the left LPM of wild-type embryos (Saijoh et al., 1999); however, this transgene was inactive in the PFP of *Foxh1*$^{c/-}$ embryos (11/11) (data not shown). *Foxh1*$^{c/-}$ mice lack *Nodal* expression in left LPM but that in the node is unaffected (Fig. 2C-E). Furthermore, they retain *Foxh1* in the PFP (Fig. 1B,C). Finally, introduction of the *Nodal* expression vector into left LPM of *Foxh1*$^{c/-}$ embryos harboring the *(n2)7-lacZ* (7/7) (Fig. 6E,F) or *Lefty2 ASE-lacZ* (2/2) (data not shown) transgene resulted in expression of *lacZ* in the PFP. Together, these results suggest that Nodal synthesized in left LPM, not Nodal produced in the node, travels to the PFP and activates the Nodal-responsive *lacZ* transgenes.

DISCUSSION

Our results indicate that Nodal signaling induces the initiation and expansion of asymmetric *Nodal* expression in LPM as well as initiates *Lefty1* expression at the midline. They also provide insight into the mechanism by which asymmetric signals are transferred between structures during LR patterning. On the basis of the present and previous data, we propose the following scenario (Fig. 7). First, Nodal produced in the node travels from the node to left LPM, where it initiates asymmetric *Nodal* expression (alternatively, Nodal may act on left LPM indirectly). Second, Nodal protein produced in the small region of left LPM adjacent to the node diffuses along the anteroposterior axis, resulting in the expansion of *Nodal* expression within left LPM. Third, Nodal produced in left LPM also travels toward the midline, where it induces the expression of *Lefty1*, the product of which is crucial for midline barrier function. Thus, according to this scenario, two critical events of LR patterning, asymmetric expression of *Nodal* in LPM and *Lefty1* expression at the midline, are established by diffusion of Nodal followed by Nodal signaling. Foxh1 is required at least for the induction of *Nodal* in left LPM.

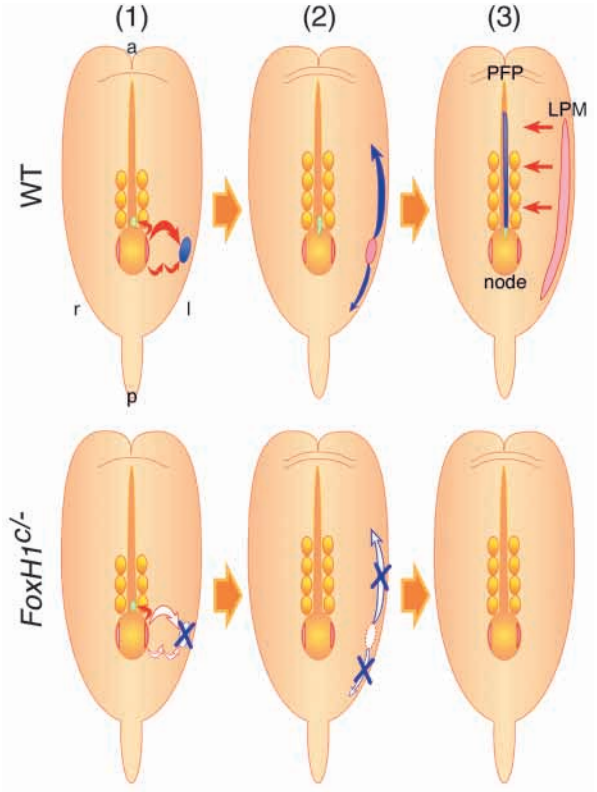

Fig. 7. Model for the movement of Nodal activity during LR patterning. Three events involving Nodal are illustrated. Blue represents the domains that receive Nodal signals and in which Foxh1 is active. Red indicates the domains in which *Nodal* is expressed. Arrows indicate the directions of signal transfer. In the wild-type embryo (top row), Nodal produced in the node travels to left LPM and initiates *Nodal* expression in a small region adjacent to the node (1). At this stage, *Lefty1* is expressed in a small region of PFP adjacent to the node (green). *Lefty1* expression in this domain may be induced by Nodal produced in the node. (2) Nodal produced in the small region of left LPM diffuses along the anteroposterior axis in left LPM and thereby induces the expansion of *Nodal* expression. (3) Nodal produced in left LPM travels to the entire PFP region along the AP axis (blue), where it induces *Lefty1* expression. In *Foxh1*$^{c/-}$ embryos (bottom row), Nodal produced in the node is unable to initiate *Nodal* expression in left LPM because of the absence of Foxh1 in LPM. As a result, *Nodal* expression in left LPM and *Lefty1* expression in PFP are absent.

Nodal produced in the node may initiate *Nodal* expression in left LPM

How is asymmetric *Nodal* expression initiated in LPM? Our transplantation and electroporation experiments with mouse embryos revealed that ectopically expressed Nodal induced endogenous *Nodal* expression in right LPM. Nodal is thus able to initiate *Nodal* expression in LPM, suggesting the possibility that Nodal produced in the node travels to left LPM and there initiates *Nodal* expression. Previous observations are also consistent with this idea. First, *Nodal* expression in the node (the perinodal region) begins earlier than that in left LPM (Collignon et al., 1996). Second, asymmetric *Nodal* expression in left LPM begins in a small region adjacent to the node. Third, asymmetric *Nodal* expression in left LPM is controlled by a left side-specific enhancer designated ASE (Adachi et al., 1999; Norris et al., 2002; Norris and Robertson, 1999), the most critical elements of which are two Foxh1-binding sites (Adachi et al., 1999; Saijoh et al., 2000). These Foxh1-binding sites act as a Nodal-responsive element, suggesting that *Nodal* is regulated by a positive autoregulatory mechanism. Fourth, components required to mediate Nodal signaling (such as ALK4, ActRII, Cryptic, Smads and Foxh1) are all expressed in LPM on both sides. Fifth, in various mutant mice with LR defects, asymmetric *Nodal* expression in LPM is always absent when *Nodal* expression in the node is abolished (Lowe et al., 2001; Saijoh et al., 2003). Finally, the role of Nodal produced in the node has been more convincingly demonstrated by Brennan et al. (Brennan et al., 2002). Thus, mutant mice specifically lacking Nodal in the node fail to initiate asymmetric *Nodal* expression in LPM, revealing that *Nodal* expression in the node is indeed essential for asymmetric gene expression in left LPM (Brennan et al., 2002). Nonetheless, it

remains to be seen whether Nodal coming from the node directly acts on left LPM to initiate *Nodal* expression. Other factors such as GDF1 may also be involved in signal transfer from the node to LPM, as GDF1 is expressed in the node and the lack of GDF1 results in the loss asymmetric *Nodal* expression in LPM (Rankin et al., 2000).

Foxh1 is expressed bilaterally in LPM at the early somite stage when *Nodal* is expressed in left LPM (Saijoh et al., 2000), and may function in both the initiation and amplification of *Nodal* expression in left LPM. Although it is difficult to distinguish these two processes experimentally, Foxh1 is implicated in both by our observation that the transplantation of left LPM to *Foxh1$^{c/-}$* embryos failed to induce *Nodal* expression even in the cells adjacent to the transplant site.

If Nodal synthesized in the node acts on left LPM, what might prevent Nodal activity from traveling toward the right side? Nodal flow, the leftward flow of extra-embryonic fluid in the node generated by vortical movement of the cilia (Nonaka et al., 1998), may transport Nodal preferentially toward the left side. Indeed, the role of nodal flow in LR patterning was recently demonstrated by testing the effects of artificial fluid flow in embryos (Nonaka et al., 2002). Although ciliated cells can be found in the organizer region of non-mammals, including the chick (Essner et al., 2002), fluid flow may not be generated there. Coincidentally, ectopic introduction of Nodal into the right LPM failed to induce endogenous *Nodal* expression (M. Levin, PhD thesis, Harvard University, 1996). Thus, a different mechanism may operate in the chick for the transfer of asymmetric signals from the node to left LPM.

Nodal protein produced in left LPM induces *Lefty1* expression at the midline

The midline structures, including the floor plate and notochord, are required to separate the two sides of the embryo (Danos and Yost, 1996), with Lefty1 being critical for midline barrier function (Meno et al., 1998). Our observations now suggest that *Lefty1* expression in the PFP is induced by Nodal produced in left LPM. First, *Foxh1$^{c/-}$* embryos, which lack *Nodal* expression in left LPM but retain it in the node, fail to express *Lefty1* in the PFP, suggesting that Nodal produced in the node is unable to induce *Lefty1* expression in the PFP. Second, and more importantly, transplanted left LPM or a *Nodal* expression vector introduced into right LPM induced *Lefty1* expression in the PFP of wild-type embryos but not in that of *Foxh1$^{c/-}$* embryos. Third, introduction of constitutively active ALK4 into the left LPM of *Foxh1$^{c/-}$* embryos was unable to induce *Lefty1* expression in PFP, excluding a possibility that an unknown factor produced by a Nodal-dependent yet Foxh1-independent pathway induces *Lefty1*. The idea that *Lefty1* expression is induced by LPM-derived Nodal is also consistent with previous observations. Comparison of the kinetics of *Lefty1* and *Nodal* expression thus revealed that *Lefty1* expression in the PFP is preceded by *Nodal* expression in left LPM (C. M. et al., unpublished data). Furthermore, *Nodal* is not expressed in the PFP. Finally, mutant mice lacking a component of the Nodal signaling pathway, such as the co-receptor Cryptic, fail to express *Lefty1* in the PFP as well as *Nodal* in left LPM (Yan et al., 1999).

After Nodal produced in left LPM travels to the midline and induces *Lefty1* expression, Lefty1, which is also able to travel over long distances (Sakuma et al., 2002), might then be expected to diffuse toward the LPM. Lefty1 that reaches the right LPM would render it incompetent for Nodal signaling and prevent *Nodal* expression there. Lefty1 that reaches left LPM, together with Lefty2 produced in the left LPM, may contribute to rapid repression of *Nodal* expression in this region. Midline barrier function is abolished in mutant mice that lack Lefty1, resulting in bilateral expression of *Nodal* and *Lefty2* (Meno et al., 1998). We previously suggested that, in the absence of Lefty1, an unknown left-side determinant travels across the midline and reaches the right LPM, where it induces the expression of *Nodal* and *Lefty2* (Meno et al., 1998). Our data now suggest that this left-side determinant is most likely Nodal.

Although our results indicate that *Lefty1* expression at the midline is induced by Nodal produced in left LPM, it is not clear whether this expression depends on Foxh1. *Lefty1* expression is lost even in the least severe type of *Foxh1*-null mutant (Yamamoto et al., 2001). However, this effect may be secondary to misspecification of the midline cells in the absence of Foxh1 (Hoodless et al., 2001; Yamamoto et al., 2001). Our previous analysis of the transcriptional regulatory elements of *Lefty1* by transgenic approaches (Saijoh et al., 1999) suggested that the 1.2 kb region immediately upstream of *Lefty1* is sufficient for its asymmetric expression in the PFP. Although this 1.2 kb region contains three potential binding sites for Foxh1, mutation of these sequences did not impair the PFP-specific expression of *Lefty1* (Y.S. and H.H., unpublished). Nodal signaling that induces *Lefty1* in the PFP therefore may not involve Foxh1.

Overall, our results obtained with *Foxh1$^{c/-}$* mice suggest that Nodal activity travels from the node to left LPM, and from left LPM to the midline. A direct test of this conclusion will require visualization of the behavior of Nodal in mouse embryos.

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