RESEARCH COMMUNICATION

Nodal activity in the node governs left-right asymmetry

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***Nodal* is expressed at the lateral edges of the mouse node, but its function in this “organizer” tissue remains unknown due to the early lethality of *Nodal* mutant embryos. Here we used a genetic strategy to selectively remove *Nodal* activity from the node. Embryos lacking *Nodal* in the node fail to initiate molecular asymmetry in the left lateral plate mesoderm and exhibit multiple left-right patterning defects. *Nodal* may also act as a short-range signal to establish a functional midline barrier. Our findings confirm that the mouse node is instrumental in initiating left-right axis specification and identify *Nodal* as the key morphogen regulating this process.**

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Genetic pathways controlling invariant situs of the major internal organ systems have been described in several vertebrate model systems (for review, see Capdevila et al. 2000; Hamada et al. 2002). In all cases, expression of the TGF- β signaling molecule, *Nodal*, is restricted to the left side of the embryonic axis, where its activity is critical for specifying the embryonic left-right (LR) axis. The upstream cellular and molecular mechanisms that direct asymmetric gene expression have been extensively described in chick. However, relatively little is known about how embryonic symmetry is broken, and how the LR signaling cascade that ultimately controls the positioning and morphogenesis of the internal organs is activated in mammalian embryos.

The mouse node is a bilaminar structure found at the rostral end of the primitive streak and has equivalent secondary axes inducing properties as Spemann's organizer in *Xenopus* (for review, see Beddington and Robertson 1999). It consists dorsally of epiblast and ventrally of the most caudal aspect of the notochordal plate. Several recent studies indicate that the mouse node plays an important role in the establishment of LR asymmetry (Nonaka et al. 1998; Okada et al. 1999). Ultrastructural studies have shown that monocilia present on cells of the exposed ventral surface of the node rotate in a counterclockwise direction and have been suggested to generate a net leftward flow of extraembryonic fluid known

as the nodal flow (Sulik et al. 1994; Nonaka et al. 1998). Laterality defects documented in several mouse mutants that lack cilia, or whose cilia motility is compromised, have lead to the hypothesis that the nodal flow results in the localized accumulation of morphogen(s) on the left side of the node, thereby initiating the LR signaling cascade (Hamada et al. 2002). In keeping with this, artificially reversing the direction of nodal flow in cultured embryos is sufficient to reverse situs (Nonaka et al. 2002). Surgical ablation of the node (Davidson et al. 1999) and mutations affecting node formation (Dufort et al. 1998) both lead to embryonic laterality defects, implying that the node functions as a signaling center during LR axis formation. The node gives rise to midline structures such as the notochord and floor plate that act as a midline barrier necessary for maintaining correct laterality (Dufort et al. 1998; King et al. 1998; Meno et al. 1998). The midline barrier is proposed to prevent interference between the distinct signaling cascades on either side of the embryo. For example, in *Lefty1* mutant embryos, loss of *Lefty1* from the floor plate compromises the barrier, leading to bilateral *Nodal* expression in the lateral plate mesoderm and ultimately left isomerisms of the viscera (Meno et al. 1998).

Nodal is required in the pregastrulation embryo to establish the anterior-posterior axis (Brennan et al. 2001). *Nodal* expression at the extreme lateral edges of the ventral node (Collignon et al. 1996; Lowe et al. 1996) also make it an ideal candidate morphogen asymmetrically localized by the nodal flow to activate left-sided gene expression. However, this possibility has been impossible to test because *Nodal* mutant embryos arrest prior to primitive streak formation. Elegant studies in zebrafish have shown that a *Nodal* homolog, *Squint*, is capable of acting as a classical morphogen, influencing the fate of cells at a distance from the source of *Squint* production (Chen and Schier 2001). Here we investigated whether *Nodal* signals from the node similarly act on adjacent cell populations, by genetically removing *Nodal* activity from cells within the node. Strikingly, embryos lacking *Nodal* in the node exhibit an array of abnormalities consistent with defects in specification of the LR axis. The loss of *Nodal* within the node disrupts molecular asymmetry in the left lateral plate mesoderm (LPM). These findings strongly suggest that *Nodal* acts as a long-range morphogen. In addition, cell lineage studies demonstrate that *Nodal* acts locally to pattern the node-derived lineage, the notochord, which consequently induces the midline barrier.

Results and Discussion

At the 4–5-somite stage, *Nodal* expression becomes transiently asymmetric in the node with increased numbers of cells expressing *Nodal* on the left versus the right side (Collignon et al. 1996). A 2.7-kb region upstream of the *Nodal* locus directs expression in the initial symmetrical domain (Fig. 1D; Norris and Robertson 1999). We further mapped this node enhancer to a 350-bp fragment that is sufficient to direct *Nodal* expression in the nodes of transgenic mice (Fig. 1E). Removal of this 350-bp sequence from the 2.7-kb transgene largely abolishes expression in the node (Fig. 1F). Weak punctate expression seen in more central ventral cells of the node potentially

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Figure 1. Characterization of the *Nodal* node enhancer. Ventral (C–F) and lateral (K) views of approximately 8.0-d embryos at the various somite (s) stages indicated. (A) Map of *Nodal* locus illustrating location of node enhancer element (red circle) and three LacZ reporter constructs used to make stable transgenic lines. Black box indicates position of exon 1 (K, Kpn I; B, Bgl II; N, Not I). (C) *Nodal* is expressed in the outermost ventral cells of the node at the 2-somite stage as assessed by the *Nodal*^{lacZ} reporter allele. Transgenic lines containing 2.7 kb (A,D) and 0.35 kb (A,E) of *Nodal* genomic sequence express β-gal reporter in the outermost ventral cells of the node. Removal of the 0.35 kb from the 2.7-kb transgene eliminates β-gal expression from the outermost cells of the node (A,F); some staining is seen in the central cells of the node. (B) Partial sequence of the minimal 0.35-kb fragment that drives *Nodal* expression in the node. Alignment of mouse and human sequences showing predicted Foxa2 binding site. Mismatches between the second and third bases and the Foxa2 consensus are compatible with Foxa2 binding (Overdier et al. 1994). (G) Whole-mount in situ hybridization showing *Cre* expression in the node of a transgenic line expressing *Cre* under the control of Tg 2.7 (the 2.7-kb node element). (H) Embryo carrying the Tg 2.7-*Cre* transgene and an allele of the *Rosa*-26 conditional reporter. Only a few cells show β-gal activity in the node (white arrow). At the 3-somite stage, *Cre* mRNA is expressed in the node (I), and *Cre* activity is detected throughout the outermost ventral cells of the node as visualized by β-gal activity (J). By the 6-somite stage, descendants of *Nodal*-expressing cells in the node are found exclusively in the notochordal plate (K'). By 9.5 d (L) and 10.5 d (M), the node descendants are found in the notochord posterior to the hindlimb. No descendants are found in the gut or floor plate.

reflects the presence of a repressor element within the 350-bp region that normally downregulates *Nodal* expression in this subpopulation. Alignment of this 350-bp minimal enhancer with the corresponding region from the human *Nodal* locus identifies a 110-bp stretch that shares 87% identity (Fig. 1B), including a predicted binding site for Foxa2 (Hnf3β), a member of the forkhead family of transcription factors expressed throughout the ventral cells of the node (Sasaki and Hogan 1993). The lack of *Nodal* expression in chimeric embryos generated from *Foxa2* null ES cells (Dufort et al. 1998), and the pronounced genetic interaction between *Foxa2* and *Nodal* (Collignon et al. 1996; Varlet et al. 1997) provide evidence that Foxa2 acts as an upstream activator of *Nodal* expression in the node.

Previous fate mapping experiments using the lipophilic dye DiI suggest that the ventral layer of the node gives rise to multiple cell types including notochord, the

floor plate of the neural tube, and the definitive endoderm (Beddington 1994; Sulik et al. 1994; Kinder et al. 2001). Here we have exploited an in vivo cell marking system to precisely map the fate of cells expressing *Nodal* in the node. Transgenic mice were generated that express *Cre* recombinase under the control of the 2.7-kb node enhancer (Fig. 1G). These *Nodal*-*Cre* transgenic mice (Tg 2.7-*Cre*) were crossed to the *Rosa*-26 conditional reporter strain carrying a silent *LacZ* reporter allele that is specifically activated in cells expressing *Cre* recombinase (Soriano 1999). As assessed by whole-mount in situ analysis of a panel of embryos (n = 15), Tg 2.7-*Cre* directs *Cre* expression in the node at the 0-somite stage. However, β-gal activity is consistently not detected until the 2–3-somite stage, indicating an approximately 3-h time delay between *Cre* transcription and reporter activity (Fig. 1H,J). Analysis of reporter embryos at various stages of development confirms that the ventral node cells are relatively quiescent, as few descendants are labeled despite rapid growth at these embryonic stages (Bellomo et al. 1996). Although the lag in reporter activity compared to *Cre* expression makes it difficult to precisely delineate the anterior extent of notochord colonization by derivatives of the node, we only see labeled cells caudal to the hindlimb buds. Contrary to previous DiI labeling experiments, our data show that descendants of the outermost cells of the ventral node exclusively colonize the posterior notochord and do not contribute to either the floor plate or definitive endoderm (Fig. 1L,M).

To test whether *Nodal* activity is required during specification of the notochord lineage, we designed a strategy to specifically eliminate expression from the node. The 2.7-kb enhancer region was thus deleted by homologous recombination, and correctly targeted ES cells were used to generate germline chimeras (Fig. 2). Mice heterozygous for the 2.7-kb deletion (*Nodal*^{Δ/+}) are viable and fertile. Moreover, intercross matings yielded

Figure 2. Targeted deletion of the *Nodal* node enhancer. (A) Map of *Nodal* genomic locus. (B) Targeting vector designed to replace 2.7 kb of *Nodal* sequence with a single loxP site (black triangle). (C) Targeted allele. (D) Recombined allele after Cre-mediated excision of the hygro selection cassette. (E) Southern hybridization of *KpnI*-digested DNA prepared from tail biopsies of heterozygous intercross offspring at weaning. DNA was hybridized with a 5' external probe (A). (F) PCR analysis of yolk sac DNA prepared from 8.0-dpc embryos. Primers C2/D2 amplify a wild-type band of 220 bp, and primers A3/D2 amplify a 320-bp mutant band. K, KpnI; B, BglII.

Table 1. Breeding data for *Nodal*^Δ allele

A	Cross	Stage	n	+/+	<i>Nodal</i> ^{+/-}	<i>Nodal</i> ^{Δ/Δ}
	<i>Nodal</i> ^{+/-} X <i>Nodal</i> ^{+/-}	P21	110	22 (20%)	59 (54%)	29 (26%)
B	Cross	Stage	n	+/+	<i>Nodal</i> ^{+/-}	<i>Nodal</i> ^{Δ/-}
	<i>Nodal</i> ^{+/-} X <i>Nodal</i> ^{+/-}	P21	40	15 (37%)	13 (33%)	12 (30%)
	<i>Nodal</i> ^{+/-} X <i>Nodal</i> ^{Δ/-}	P1	33	8 (24%)	4 (12%)	11 (33%)
						10 (30%) ^a

P, postnatal day of development

^aAll pups die within first few hours of birth.

Nodal^{Δ/Δ} homozygous offspring at Mendelian frequencies (Table 1A). Transcripts were examined by whole-mount in situ to confirm that removal of the 2.7-kb node enhancer element eliminates *Nodal* expression from this tissue. *Nodal* expression was unaffected during early gastrulation stages (data not shown). By early somite stages, however, node expression was greatly diminished but not completely eliminated, implicating additional as yet unmapped regulatory element(s) (Fig. 4B, see below). Alternatively, residual expression may reflect the activity of the intronic *Foxh1*-dependent feedback enhancer (Norris and Robertson 1999), which we showed to be responsible for the asymmetric expression of *Nodal* in the node (Norris et al. 2002). This feedback enhancer directs barely detectable levels of reporter expression in the node (J. Brennan, D. Norris, and E. Robertson, unpubl.). Although expression of *Nodal* in the node is markedly attenuated in *Nodal*^{Δ/Δ} embryos, robust levels of *Nodal* expression in the left LPM (Fig. 4G, see below) allow mutant embryos to develop with normal situs.

We previously documented a highly dose-dependent requirement for *Nodal* signaling during anterior-posterior axis formation (Norris et al. 2002). In these experiments, reduced levels of *Nodal* signaling in the left LPM failed to activate *Lefty2* yet still allowed activation of *Pitx2*. To test for similar dosage effects in the node, we crossed *Nodal*^{Δ/+} heterozygotes to mice heterozygous for a null allele (*Nodal*^{+/-}). We found that *Nodal*^{Δ/-} mutants were born at the correct Mendelian ratio but none survived beyond the first few hours of birth (Table 1B). Interestingly, these mutants display an array of abnormalities consistent with defects in specification of the LR axis (Table 2). For example, in wild-type animals the stomach is positioned on the left (Fig. 3A), whereas in *Nodal*^{Δ/-} mutants, positioning was randomized with 8 of 11 animals having the stomach positioned on the right (Fig. 3B). Regardless of stomach orientation, spleens in the *Nodal*^{Δ/-} mutants were invariably reduced in size compared to control littermates (Fig. 3C,D). LR patterning defects were also evident in the thoracic cavity. Normally, asymmetric branching results in the formation of

four lung lobes on the right and a single lobe on the left (Fig. 3E). However, the *Nodal*^{Δ/-} mutants consistently present right isomerisms having four lobes on the right and three or four lobes on the left (Fig. 3F). Interestingly, in human laterality syndromes, this right pulmonary isomerism is typically associated with hyposplenia (Kosaki and Casey 1998).

We used expression of *α-cardiac actin* to examine orientation of the heart tube. At 9.0 d, the direction of heart looping was randomized in *Nodal*^{Δ/-} mutants (Fig. 3G,H). At birth, the apex of the heart normally points to the left side of the thoracic cavity (Fig. 3I). However, in the *Nodal*^{Δ/-} mutants the apex of the heart points to the left, the middle (Fig. 3J), or right. Regardless of overall orientation, hearts in *Nodal*^{Δ/-} animals show multiple abnormalities, including transposition of the major arteries (data not shown) and atrial-septal defects (Fig. 3J). In all mutants examined (n = 10), the major common defect is the development of a common atrial chamber rather than separate left and right atria. This profound cardiac abnormality most likely accounts for the early postnatal lethality of the *Nodal*^{Δ/-} animals.

To understand the molecular basis of the laterality defects, we examined the expression of a number of LR marker genes by whole-mount in situ hybridization. At the early head fold stage, *Nodal* is normally expressed at the lateral edges of the notochordal plate (Fig. 4A). Although the nodes of *Nodal*^{Δ/-} embryos are morphologically indistinguishable from control littermates (Fig. 4C', data not shown), *Nodal* expression was barely, if at all, detectable (Fig. 4C). Next, we used the *Nodal*^{lacZ} reporter allele (Collignon et al. 1996) to examine the impact of eliminating *Nodal* expression in the node on the asymmetric expression of *Nodal* in the left LPM. At the 3–4-somite stage, *Nodal*^{lacZ} is expressed in the node and the left LPM (Fig. 4D). In contrast, expression of the *Nodal*^{lacZ} reporter is confined to the node and fails to be induced in the LPM of *Nodal*^{Δ/lacZ} mutants (Fig. 4E, Table 3). The absence of asymmetric *Nodal* expression in the LPM was confirmed by analyzing *Nodal* mRNA levels (Fig. 4F,H).

Table 2. LR asymmetry defects in the viscera of *Nodal*^{Δ/-} mutants at birth

	Stomach position		Spleen size		Lung lobation ^c		Heart apex direction		
	Left	Right	Normal	Small	Normal ^b	RI ^c	Left	Middle	Right
Control ^a	55/55	0	24/24	0	55/55	0	16/16	0	0
<i>Nodal</i> ^{Δ/-}	3/11	8/11	0	10/10	1/14	13/14	1/6	2/6	3/6

^aControls had one of following genotypes; Wild type, *Nodal*^{+/-} or *Nodal*^{Δ/-}^bNormal lungs had a single lobe on left and 4 lobes on right.^cRI, right pulmonary isomerism.

Figure 3. Left-right patterning defects of the viscera of *Nodal*^{Δ/Δ} deletion mutants. Organs were dissected from control and deletion pups on the day of birth. Left (L) and right (R) are as indicated in A and B. Normally the stomach is situated on the left-hand side of the body (A), but in the deletion mutants the stomach position is randomized such that half the mutants have stomachs on the right (B). The spleen is apposed to the stomach wall (C). In mutants, the spleen is greatly reduced in size (D). In the normal lung, there is one lobe on the left and four on the right (E). In the mutants, there were three or four lobes on both sides, indicating a right pulmonary isomerism (F). At 9.5 d, expression of α -cardiac actin mRNA in the heart tube illustrates normal heart looping to the right (G). In *Nodal*^{Δ/Δ} mutants, looping was randomized, with 3/7 showing rightward and 4/7 showing leftward looping (H). In normal littermates, the apex of the heart points to the left (I), yet in mutants the heart apex is ambiguously positioned such that a proportion point to the left, some to the middle (J), and others to the right. Sections through mutant hearts reveal septation defects between the two atria (J). A, accessory lobe; CA, common atrium; Ca, caudal lobe; Cr, cranial lobe; L, left lobe; LA, left atrium; LV, left ventricle; M, medial lobe; OT, outflow tract; RA, right atrium; RV, right ventricle; Vn, ventricles.

In those *Nodal*^{Δ/Δ} embryos in which *Nodal* mRNA is detected, asymmetric expression was retained in the node (Fig. 4H'). Together with the observation that deletion of the *Foxh1*-dependent enhancer leads to a loss of asymmetric *Nodal* expression (Norris et al. 2002), these findings confirm that activation of the feedback enhancer is responsible for asymmetric *Nodal* expression in the node. As suggested by the nodal flow hypothesis, asymmetric movement of fluid across the node potentially leads to local accumulation of *Nodal*, which in turn upregulates expression via the feedback enhancer. Cerberus is a secreted protein which has been shown to antagonize *Nodal* signaling in *Xenopus* assays (Piccolo et al. 1999). *Nodal* signaling on the right edge of the node is likely antagonized by the activity of the Cerberus-related factor Dante, which is expressed more strongly on the right-hand side of the node (Pearce et al. 1999). In combination, right-sided antagonism and the nodal flow potentially amplify differences in *Nodal* activity across the relatively small area of the node.

Figure 4. Molecular asymmetry is not established in the lateral plate mesoderm of *Nodal*^{Δ/Δ} deletion mutants. Whole-mount in situ hybridization (A–C, F–L) and LacZ reporter staining (D, E) of 7.5-d (A–C) and 8.0-d (D–L) embryos. Lateral views with anterior to the left (A–C), caudal views (D–H), and rostral views (I–L) are shown. At the late head fold stage, *Nodal* is expressed in the node at the distal tip of the embryo (A); transverse sections through the node show that *Nodal* is expressed in the outermost ventral cells (A'). In embryos homozygous for the *Nodal*^{Δ/Δ} allele, *Nodal* expression is downregulated in the node (B). In embryos trans-heterozygous for the deletion allele and a *Nodal* null allele (*Nodal*^{Δ/Δ}), *Nodal* expression is either entirely absent or negligible in the node (C). Control embryos between 2 and 8 somites express the *Nodal*^{lacZ} reporter in the node and the left LPM (D). In embryos carrying a copy of the deletion allele and the *Nodal* null reporter allele (*Nodal*^{Δ/lacZ}), *Nodal*^{lacZ} is only expressed in the node (E). Similarly *Nodal* mRNA is expressed in the node and left LPM in control embryos (F), but in *Nodal*^{Δ/Δ} homozygotes, low levels of *Nodal* are detected in the node yet normal levels of *Nodal* expression are observed in the left LPM (G). Reduced *Nodal* expression levels are also found in *Nodal*^{Δ/Δ} nodes, and *Nodal* expression is absent from the left LPM (H'). *Lefty2* is expressed in the left LPM and the left prospective floor plate of control embryos (I) but fails to be induced in either domain in the deletion mutants (J). *Pitx2* is expressed in the head mesenchyme, body wall, and left LPM of control embryos (K, K'). In the deletion mutants, *Pitx2* is expressed in the head mesenchyme and body wall (L) but is absent from the left LPM (L'). Normal expression of *Pitx2* in the left-hand side mesenchyme of the developing heart (arrow in K') is absent in the deletion mutants (L'). llpm, left lateral plate mesoderm; n, node; nf, neural fold; nt, neural tube; pfp, prospective floor plate. Black arrows indicate *Nodal* expression domain.

Our lineage analysis shows that cells expressing *Nodal* in the node are destined to form the notochord, the tissue that induces *Lefty1* expression in the prospective floor plate (King et al. 1998). *Foxa2* and *Shh* expression

Table 3. Gene expression at the 3–5 somite stage of development

	<i>Pitx2</i>		<i>Lefty1</i>	<i>Lefty2</i>	<i>Nodal</i>		<i>Nodal</i> ^{lacZ}	
	LLPM	H. Mes	PFP	PFP + LLPM	Node	LLPM	Node	LLPM
Control ^a	14/14	14/14	1/1	4/4	7/7	7/7	4/4	4/4
<i>Nodal</i> ^{Δ/Δ}	—	—	—	—	8 ^b /8	8/8	—	—
<i>Nodal</i> ^{Δ/Δ}	1 ^b /8	8/8	0/4	0/9	2 ^b /7	0/7	6/6	0/6

LLPM, left lateral plate mesoderm; H. Mes, head mesenchyme; PFP, prospective floor plate.

^aControls had one of following genotypes; Wild type, *Nodal*^{+/Δ} or *Nodal*^{+/Δ}.

^bVery low levels of expression.

patterns demonstrate the presence of notochord and floor plate in *Nodal*^{Δ/-} mutants (data not shown). However, *Lefty1* is not expressed in the prospective floor plate of *Nodal*^{Δ/-} mutants (Table 3; data not shown), suggesting that notochord progenitors in the ventral node are not patterned correctly. Alternatively, Nodal in the ventral layer of the node may induce *Lefty1* expression in prospective floor plate precursors in the adjacent dorsal layer. Considering that deletion of the *Foxh1*-dependent enhancer leads to a loss of *Lefty1* expression in the midline (Norris et al. 2002), *Nodal*^{Δ/-} mutants may fail to induce *Lefty1* as a secondary defect associated with the loss of *Nodal* expression from the left LPM.

The TGF-β family member *Lefty2* is a downstream target of Nodal signaling in the left LPM. As for *Nodal*, *Lefty2* expression requires activation of the *Foxh1*-dependent enhancer (Saijoh et al. 2000). As predicted by the loss of Nodal signaling in the left LPM of *Nodal*^{Δ/-} mutants, *Lefty2* expression is not activated in the LPM of mutants (Fig. 4J). Nodal also induces expression of the homeobox gene *Pitx2* in the left LPM (Shiratori et al. 2001). *Pitx2* null mutant embryos resemble the *Nodal*^{Δ/-} mutants described here, and similarly exhibit pulmonary right-sided isomerisms, atrial septal defects, and hypoplasia (Gage et al. 1999; Kitamura et al. 1999; Lin et al. 1999; Lu et al. 1999). *Pitx2* is not expressed in the left LPM of *Nodal*^{Δ/-} mutants, accounting for the laterality defects seen in some of the visceral organs (Fig. 4K,L). In contrast to the *Pitx2* mutants, cardiac looping is randomized in *Nodal*^{Δ/-} mutants. Thus downstream targets of Nodal signaling, other than *Pitx2*, likely control some aspects of organ laterality such as cardiac looping.

Transduction of Nodal signaling often requires the presence of a Nodal coreceptor of the EGF-CFC family on the recipient cell (Yeo and Whitman 2001). Animals carrying a null mutation in the EGF-CFC gene, *Cryptic*, exhibit laterality defects resembling those described here for *Nodal*^{Δ/-} embryos (Gaio et al. 1999; Yan et al. 1999). *Nodal* expression in *Cryptic* mutants is unaffected in the node but fails to be induced in the left LPM. We suggest that *Cryptic* selectively amplifies Nodal activity in the node. Another possibility is that *Cryptic* allows cells to sense a long-range Nodal signal in the LPM. Loss of GDF-1, a TGF-β ligand expressed in both the node and the LPM, also causes laterality defects (Rankin et al. 2000). *GDF-1* expression is unaffected in the *Nodal*^{Δ/-} mutants (data not shown). An intriguing possibility is that Nodal and GDF-1, coexpressed in the node, may form heterodimers with distinct properties. Such an interaction has not yet been shown for GDF-1, but Nodal is capable of forming heterodimers with another TGF-β ligand, *Bmp7* (Yeo and Whitman 2001). Alternatively, cross-talk between Nodal and GDF-1 signaling pathways in the node and/or LPM may enhance activation of downstream targets.

The ability of Nodal to act as a diffusible morphogen relaying a signal from the node to the LPM is unexpected, because previous studies suggest that the mature processed protein is unstable (Constam and Robertson 1999). However, Squint, a Nodal homolog in zebrafish, can signal over 6–8 cell diameters (Chen and Schier 2001). Moreover, studies of *Lefty2* mutant mice suggest that long-range Nodal signaling activity is normally restricted by the feedback inhibitor *Lefty2* (Meno et al. 2001). The mechanism for long-range activity is thought to be diffusion involving endocytosis (Sakuma et al.

2002). Alternatively, long-range Nodal signaling could involve a relay mechanism involving other unidentified molecules.

Materials and methods

Transgenic and targeted mouse strains

Transgenes were generated by introducing *Nodal* genomic sequences into the hsp68lacZpA reporter construct (Sasaki and Hogan 1996). Tg 2.7 contains a 2.7-kb BamHI-SpeI fragment located approximately 7 kb upstream of *Nodal* coding exon 1. Tg 0.35 contains a 350-bp BglII fragment from Tg 2.7. Transgenic mice were produced as previously described (Norris and Robertson 1999).

The targeting construct for the node enhancer deletion (*Nodal*^Δ) contains a 4.1-kb (EcoRI-SpeI) 5' homology arm and a 2.8-kb (BamHI-BamHI) 3' homology arm. The homology arms were cloned into a plasmid containing a loxP-flanked hygro selection cassette. The hygro cassette was excised from targeted ES cell lines by transient transfection of the pMC13-cre plasmid. Mice containing the Δ2.7 mutation were genotyped using the following primers: A3 (TGAATCATGACCATCCTCC), C2 (CTGAGTGCTGGGATTATACC), and D2 (TTGCGAGCAATAGTCTCAGC).

Histology and RNA in situ hybridization

Embryos were genotyped prior to whole-mount in situ analysis by yolk sac PCR analysis.

Probes and protocols used for whole-mount in situ were as described (Norris et al. 2002).

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