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Left-right asymmetry in the level of active Nodal protein produced in the node is translated into left-right asymmetry in the lateral plate of mouse embryos

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Abstract

Left-right (L-R) asymmetry in the mouse embryo is generated in the node and is dependent on cilia-driven fluid flow, but how the initial asymmetry is transmitted from the node to the lateral plate has remained unknown. We have now identified a transcriptional enhancer (ANE) in the human *LEFTY1* gene that exhibits marked L>R asymmetric activity in perinodal cells of the mouse embryo. Dissection of ANE revealed that it is activated in the perinodal cells on the left side by Nodal signaling, suggesting that Nodal activity in the node is asymmetric at a time when *Nodal* expression is symmetric. Phosphorylated Smad2/3 (pSmad2) indeed manifested an L-R asymmetric distribution at the node, being detected in perinodal cells preferentially on the left side. This asymmetry in pSmad2 distribution was found to be generated not by unidirectional transport of Nodal but rather as a result of L<R asymmetric expression of the Nodal antagonist Cerl2. For various mutant embryos examined, the asymmetry in pSmad2 distribution among the perinodal cells closely matched that in lateral plate mesoderm (LPM). However, autocrine-paracrine Nodal signaling in perinodal cells is dispensable for L-R patterning of LPM, given that its inhibition by expression of dominant negative forms of Smad3 or ALK4 was still associated with normal (left-sided) *Nodal* expression in LPM. Our results suggest that LPM is the direct target of Nodal secreted by the perinodal cells, and that an L>R distribution of active Nodal in the node is translated into the asymmetry in LPM.

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Keywords

Left-right asymmetry; Nodal signaling; node; Smad

Introduction

Visceral organs of vertebrates exhibit left-right (L-R) asymmetry in their positions and morphology. The mechanism responsible for generation of such L-R asymmetry is largely conserved among vertebrates, although some diversity is apparent (Tabin, 2005). Four steps are required to establish L-R asymmetric patterning in the mouse embryo (Shiratori and Hamada, 2006): (1) symmetry breaking as a result of the leftward flow generated by rotational movement of primary cilia in the node; (2) transmission of an asymmetric signal (or signals) to lateral plate mesoderm (LPM); (3) asymmetric expression of *Nodal* and *Lefty2*, which encodes a feedback inhibitor of Nodal signaling, in the left LPM; and (4) situs-specific morphogenesis mediated by asymmetric expression of *Pitx2*, which encodes a transcription factor and is regulated by Nodal signaling. Despite recent progress in characterization of this sequence of events, an important issue that has remained unresolved concerns the mechanism by which the asymmetric signal (or signals) is transferred from the node to LPM.

Nodal, a secretory protein that belongs to the transforming growth factor–beta (TGF-beta) superfamily, plays a key role in signal transfer from the node to LPM. *Nodal* is expressed bilaterally in the perinodal cells (crown cells) before its asymmetric expression begins in LPM. Mice specifically lacking *Nodal* expression in the node lose asymmetric gene expression in LPM, suggesting that Nodal produced in the node is essential for subsequent L-R patterning of LPM (Brennan et al., 2002; Saijoh et al., 2003). Several lines of circumstantial evidence suggest that Nodal protein (more precisely, the Nodal-GDF1 heterodimer) produced in the node is directly transported to LPM. First, asymmetric expression of *Nodal* and *Lefty2* in LPM is regulated by a Nodal-responsive enhancer that is designated ASE (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 2000). Second, GDF1 (growth-differentiation factor 1), a co-ligand of Nodal that increases Nodal activity (Tanaka et al., 2007), is coexpressed with Nodal in the perinodal cells and is also required for asymmetric *Nodal* expression in LPM (Rankin et al., 2000). Third, sulfated glycosaminoglycans present in the extracellular matrix between the node and LPM bind Nodal and are required for asymmetric *Nodal* expression in LPM (Oki et al., 2007). And finally, Nodal ectopically expressed in mouse (Sakuma et al., 2002) or *Xenopus* (Marjoram and Wright, 2011) embryos is able to diffuse over a long distance, such as from LPM to the midline. However, direct evidence for the transport of endogenous Nodal from the node to LPM has not been obtained to date, most likely because of technical limitations. Even if Nodal is indeed transported, however, it is unclear how it would be transported preferentially to the left side.

An asymmetric signal must be generated in or near the node, although its identity remains uncertain. The identification of this asymmetric signal and characterization of how it is transferred from the node to LPM will require identification of markers that show L-R

asymmetry on both sides at the node and elucidation of the molecular mechanisms underlying their asymmetry. Several genes, including *Cerl2* (Marques et al., 2004), *L-Plunc1* (Hou et al., 2004), and *Ablim1* (Stevens et al.), exhibit L-R asymmetric expression in the node—or more precisely, in the perinodal crown cells. However, the asymmetric expression of these genes is often dynamic, making it difficult to examine the underlying molecular mechanisms. During our analysis of transcriptional regulation of Nodal and Lefty genes, we noticed that human *LEFTY1* contains an enhancer that confers marked L-R asymmetric expression in the node. We have now examined this enhancer and have identified the signal that regulates its activity.

Materials and Methods

Transgenes and mice

Various *lacZ* transgenes were constructed in the vector *LEFTY1pro-lacZ*, which contains the *LEFTY1* promoter linked to *lacZ*. A variety of test fragments were individually subcloned into *LEFTY1pro-lacZ* at the 5′ side of the *LEFTY1* promoter in a reverse orientation. The resulting *lacZ* plasmids were digested with appropriate restriction enzymes to remove vector sequences, and the remaining vector sequence–free DNA fragments were purified by gel electrophoresis and microinjected into fertilized mouse eggs as described (Hogan et al., 1994; Saijoh et al., 1999). Embryos were recovered at embryonic day (E) 8.2 or the indicated developmental stages and were examined for the absence or presence of the transgene by the polymerase chain reaction (PCR) and for *lacZ* expression by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) according to standard protocols. A permanent mouse line harboring hNPE7.5-*lacZ* (line 20) was established as described previously (Hogan et al., 1994); embryos derived from this line showed X-gal staining patterns identical to those observed in transient transgenic embryos. The hNPE7.5-*lacZ* transgene was transferred to various mutant backgrounds by mating of the permanent transgenic mice with *iv/iv* (Supp et al., 1997), *inv/+* (Yokoyama et al., 1993), *Nodal*$^{neo/+}$ (Saijoh et al., 2003), *Cryptic*$^{+/-}$ (Yan et al., 1999b), or *Cerl2*$^{+/-}$ (Marques et al., 2004) mice. The resulting mutant embryos were genotyped by allele-specific PCR analysis. Mice with a floxed *Foxh1* allele (Yamamoto et al., 2001) were also crossed with mice that express Cre recombinase specifically in perinodal cells (NDE-*Cre* mice) to generate *Foxh1* conditional mutant embryos (*Foxh1*$^{flox/flox}$, NDE-*Cre*) that lack FoxH1 specifically in the perinodal cells. Transgene that expresses dominant-negative ALK4 (*NDE-dnALK4-IRES-lacZ*) or dominant-negative Smad3 (*NDE-dnSmad3-IRES-lacZ*) contains two tandem copies of the node-specific enhancer (NDE) and *Hsp68* promoter linked to either *dnSmad3* or *dnALK4*, an internal ribosome entry site (IRES), and *lacZ*.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to standard procedures (Wilkinson and Nieto, 1993) with minor modifications. For two-color detection, fluorescein-labeled RNA probes were detected with alkaline phosphatase–conjugated antibodies to fluorescein and with the combination of 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (INT/BCIP) as a red substrate.

Immunostaining

Mouse embryos were recovered and fixed from 2 h to overnight at 4°C with 4% paraformaldehyde in phosphate-buffered saline (PBS). They were then dehydrated with methanol and incubated for 1 h at room temperature with 3% H_2O_2 in methanol. After rehydration with PBS containing 0.1% Tween 20, they were incubated with blocking buffer [BB: 0.5% TSA Blocking Reagent (Perkin Elmer), 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl] before exposure to rabbit polyclonal antibodies to phosphorylated Smad2/3 (pSmad2) that can detect Smad3 phosphorylated at Ser465 and Ser467, and may detect phosphorylated Smad3 at its equivalent site (Cell Signaling Technology, #3101) or chicken polyclonal antibodies to beta-galactosidase (Abcam, ab9361) at dilutions of 1:50 and 1:500 in BB, respectively. Immune complexes for pSmad2 were detected with biotinylated goat antibodies to rabbit immunoglobulin G (Vector) at a dilution of 1:200 in BB, a Vectastain ABC Kit (Vector), and Fluorophore Tyramide Working Solution (Perkin Elmer). Immune complexes for beta-galactosidase were detected with Alexa Fluor 633–conjugated goat antibodies to chicken immunoglobulin G (Molecular Probes) at a dilution of 1:500 in BB.

Whole-embryo culture

Whole embryos were cultured essentially as described previously (Yamamoto et al., 2003). E8.0 mouse embryos obtained by ICR intercrosses were recovered from the uterus, and Leichert's membrane was removed. The embryos were cultured with rotation under a humidified atmosphere of 5% CO_2 at 37°C in a Falcon tube containing Dulbecco's modified Eagle's medium supplemented with 75% rat serum. In some experiments, embryos were cultured in the additional presence of recombinant mouse Nodal (R&D Systems) at 50 mg/ml. Alternatively, the adequate volume of recombinant protein dissolved in culture medium at 50 mg/ml was injected with the use of a glass needle (Dramond) and injector (Narishige) into the para-axial mesoderm immediately below the visceral endoderm layer. Injected embryos were incubated for 30 minutes before they were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C.

Results

Asymmetric node enhancer (ANE) confers perinodal L-R asymmetry soon after the onset of nodal flow

During our analysis of human Lefty genes, we noticed that the 7.5-kb upstream region of *LEFTY1* conferred gene expression in the mouse node. Introduction into fertilized mouse eggs of the hNPE7.5-*lacZ* transgene, in which *lacZ* is linked to the 7.5-kb upstream region of *LEFTY1*, thus gave rise to X-gal staining specifically in the perinodal crown cells (Fig. 1A, B). This X-gal staining showed marked L-R asymmetry at E8.2, being predominant on the left side of the node (Fig. 1B). The expression pattern of hNPE7.5-*lacZ* depended on the stage of development, however (Fig. 1F–I). At E7.5 [the late head-fold (LHF) stage], the transgene was expressed symmetrically at a low level in the perinodal cells (Fig. 1F). The level of expression on the left side became greater than that on the right side thereafter, with L>R asymmetry being obvious at the one-somite (Fig. 1G), two-somite (Fig. 1H), and four-somite (Fig. 1I) stages. These results suggested the presence of an asymmetric node enhancer (ANE) in the 7.5-kb upstream region of *LEFTY1*.

To localize ANE more precisely, we tested various restriction fragments derived from the 7.5-kb upstream region of *LEFTY1* for the ability to confer asymmetric expression in the perinodal cells. The 1.8-kb upstream region failed to show such activity (data not shown) (Yashiro et al., 2000). In contrast, the 1.9-kb Bam-Bg and 1.0-kb BX-Bg fragments fully retained this activity when linked to the 0.5-kb minimum promoter region of *LEFTY1* (Fig. 1A, C, D), whereas the 0.8-kb Bam-BgI and 0.5-kb BgI-II fragments had lost it (Fig. 1A). The ANE was then mapped to the 0.5-kb BgI^2 region (Fig. 1A, E), which overlaps with both the 1.9-kb Bam-Bg and 1.0-kb BX-Bg regions. Whereas the 1.8-kb upstream region of *LEFTY1* contains a pair of FoxH1 binding sites (Yashiro et al., 2000), the 0.5-kb BgI^2 region, which possesses ANE activity, does not include typical FoxH1 binding sites (Fig. 1A). Mouse *Lefty1* does not possess ANE, given that the 10-kb upstream region of the gene (Saijoh et al., 1999) or a 200-kb region derived from a *Lefty1* bacterial artificial chromosome (data not shown) did not drive *lacZ* expression in the node. Furthermore, we were unable to detect an ANE-like sequence in mouse *Lefty1* that shows substantial similarity to the 0.5-kb BgI^2 region of *LEFTY1*. ANE therefore appears to be an enhancer that is specific to human *LEFTY1*.

ANE is activated by Nodal signaling independently of FoxH1

To determine whether ANE activity is regulated by the canonical L-R pathway, we examined the expression of the hNPE7.5-*lacZ* transgene in various L-R mutant mice. Expression of hNPE7.5-*lacZ* was randomized in *iv/iv* embryos; X-gal staining in the perinodal cells was thus either left-sided (4/15 embryos) (Fig. 2A), right-sided (3/15 embryos) (Fig. 2B), or bilateral (8/15 embryos) (Fig. 2C, D). In the *inv/inv* mutant, on the other hand, hNPE7.5-*lacZ* expression in the perinodal cells was either right-sided (11/14 embryos) (Fig. 2E) or bilateral (3/14 embryos) (Fig. 2F). Given that nodal flow is defective in both *iv/iv* and *inv/inv* mutants, these results suggested that ANE activity is under the control of nodal flow.

We next examined whether ANE activity is regulated by the Nodal signal. We thus examined expression of hNPE7.5-*lacZ* in a hypomorphic *Nodal* mutant ($Nodal^{neo/neo}$) that fails to express *Nodal* in the node and LPM (Saijoh et al., 2003) as well as in a *Cryptic* mutant (Yan et al., 1999a). The expression level of hNPE7.5-*lacZ* on the left side was reduced in both $Nodal^{neo/neo}$ (14/14 embryos) (Fig. 2G) and $Cryptic^{-/-}$ (11/11 embryos) (Fig. 2H) mutants, giving rise to bilateral X-gal staining in the perinodal cells. These results suggested that ANE is activated in the perinodal cells on the left side by Nodal signaling.

We also examined whether the transcription factor FoxH1 is required for ANE activity. We crossed mice with a floxed *Foxh1* allele (Yamamoto et al., 2001) with mice harboring a gene for Cre recombinase that is expressed specifically in perinodal cells (NDE-*Cre* mice) in order to generate conditional mutant ($Foxh1^{flox/flox}$, NDE-*Cre*) embryos that lack FoxH1 specifically in the perinodal cells. NDE-Cre activity is already evident in crown cells at the early head-fold stage and the one-somite stage (Suppl. Fig.1), when crossed with the ROSA26-*lacZ* reporter mice (Soriano, 1999). Left-sided expression of hNPE7.5-*lacZ* was maintained in these mutant embryos (Fig. 2I), consistent with the absence of typical FoxH1 binding sites in the 0.5-kb region of *LEFTY1* that possesses ANE activity. These results thus

suggested that ANE is activated on the left side of the node by FoxH1-independent Nodal signaling.

L-R asymmetric phosphorylation of Smad2/3 in perinodal cells

Our finding that the L-R asymmetric activity of ANE is regulated by Nodal signaling suggested that Nodal activity itself may be asymmetric on the two sides of the node. To examine this possibility, we examined the distribution of phosphorylated Smad2/3 (pSmad2) in mouse embryos by immunostaining with specific antibodies that detect C-terminal phosphorylation of Smad2 and Smad3. *Nodal* expression has been shown to be bilateral in the perinodal cells until the two-somite stage, and it begins to manifest subtle (L>R) asymmetry from the three-somite stage (Collignon et al., 1996). In contrast, pSmad2 staining was already asymmetric at the LHF stage, being detected in the perinodal cells exclusively on the posterior-left side (Fig. 3A, A′). At the two-somite stage, pSmad2 staining was increased in the perinodal cells on the left side, with a portion of the left LPM adjacent to the node also being positive for pSmad2 (Fig. 3B, B′). Between the three- and four-somite stages (Fig. 3C, C′), pSmad2 staining expanded to include all perinodal cells on the left side and the entire left LPM. It was also detected in regions where *Nodal* is not expressed, including the pit cells on the left side and the prospective floor plate on the left side, suggesting that Nodal travels to these regions from the left LPM or left perinodal cells. Finally, pSmad2 staining had begun to disappear at the five-somite stage (Fig. 3D, D′). This dynamic pattern of pSmad2 staining thus coincided with that of ANE activity. The distribution of pSmad2 observed in wild-type embryos likely represents domains where Nodal is active, given that pSmad2 immunoreactivity was not detected in $Nodal^{neo/neo}$ (Fig. 3J, J′) or $Cryptic^{-/-}$ (Fig. 3K, K′) embryos.

The pattern of pSmad2 staining in the perinodal cells closely paralleled that in the LPM. In *iv/iv* embryos, pSmad2 staining in the perinodal cells was randomized, being either left-sided (4/14 embryos) (Fig. 3E, E′), right-sided (4/14 embryos) (Fig. 3F, F′), bilateral at a high level (4/14 embryos) (Fig. 3G, G′), or bilateral at a low level (2/14 embryos) (Fig. 3H, H′). In individual *iv/iv* mutant embryos, there was a close relation between the pattern of pSmad2 staining in the perinodal cells and that in LPM; for example, when pSmad2 staining in the perinodal cells was left-sided, that in LPM was also left-sided. Such a relation was also apparent in *inv/inv* embryos. Thus, in all five *inv/inv* embryos examined, pSmad2 staining in the perinodal cells was right-side dominant and that in LPM was also right-sided (Fig. 3I, I′). These results therefore suggested that asymmetry of Nodal activity in the perinodal cells is closely related to asymmetry of *Nodal* expression in LPM.

Flow-mediated transport of Nodal is not responsible for the asymmetry of pSmad2 distribution among perinodal cells

Nodal mRNA is distributed symmetrically around the node at the two-somite stage (Fig. 4A), when pSmad2 distribution is already asymmetric among the perinodal cells (Fig. 4B). How then does Nodal signaling become asymmetric if the distribution of *Nodal* mRNA is symmetric? If Nodal is secreted into the node cavity where the leftward fluid flow known as nodal flow exists, then Nodal may be transported to the left side of the node and give rise to asymmetric Nodal signaling in the perinodal cells. To test this possibility, we examined

pSmad2 staining in embryos injected with medium (Fig. 4C) or recombinant Nodal (Fig. 4D) on the right side or in embryos cultured in the presence of recombinant Nodal (Fig. 4E). We found that pSmad2 staining was apparent ectopically in the right perinodal cells and para-axial mesoderm (PAM) cells after injection of embryos with Nodal in the right PAM region (Fig. 4G), whereas ectopic pSmad2 staining was not seen after injection of medium (Fig. 4F) or incubation of embryos with excess Nodal in the culture medium (Fig. 4H). These results thus suggested that, even if Nodal is secreted into the node cavity, it is unable to elicit signaling at the apical membrane of perinodal cells.

L<R expression of *Cerl2* is responsible for the pSmad2/3 asymmetry in perinodal cells

Cerl2, which encodes a Nodal antagonist, is expressed in the perinodal cells in an L<R manner (Marques et al., 2004) and was potentially responsible for the generation of L>R asymmetric Nodal activity among these cells. To investigate this possibility, we compared the timing of *Nodal* expression, *Cerl2* expression, ANE activity, and Smad2/3 phosphorylation at the node (Fig. 5). We found that the expression of *Nodal* was bilateral from the early head-fold (EHF) stage to the one-somite stage (Fig. 5A–C), but that it started to manifest subtle L>R asymmetry from the two-somite stage (Fig. 5M). ANE activity was not detected at the EHF (Fig. 5D) and LHF (Fig. 5E) stages but became manifest at the one-somite stage (Fig. 5F). Immunostaining for pSmad2 was not detected at the EHF stage but was apparent on the posterior-left side of the node at the LHF stage (Fig. 5H) in a small proportion of the embryos examined (Fig. 5M). Finally, *Cerl2* was previously shown to be expressed asymmetrically around the node by the LHF stage (Marques et al., 2004). We detected bilateral *Cerl2* expression at the EHF stage (Fig. 5J), after which *Cerl2* expression on the left side began to decline, giving rise to an L<R asymmetric expression pattern among the perinodal cells from the LHF stage (Fig. 5K, L). Therefore, among the four markers examined, *Cerl2* expression and pSmad2 distribution first exhibited L-R asymmetry, followed by ANE activity and *Nodal* expression. These results thus supported the notion that L<R asymmetric expression of *Cerl2*, rather than asymmetric *Nodal* expression, is responsible for generation of L>R asymmetry in Nodal activity among the perinodal cells.

To test this notion directly, we examined Nodal signaling in *Cerl2* mutant embryos at E8.2. In $Cerl2^{+/+}$ embryos, pSmad2 was detected only on the left side of the node (Fig. 6A, A′). In $Cerl2^{-/-}$ embryos, pSmad2 staining in the perinodal cells, as well as that in LPM, was either bilateral (2/5 embryos) (Fig. 6B, B′) or bilateral with a subtle L>R asymmetry (3/5 embryos) (Fig. 6C, C′). Similarly, expression of hNPE7.5-*lacZ* was left-sided in wild-type embryos (Fig. 6D), whereas either bilateral (4/6 embryos) (Fig. 6E) or bilateral with a subtle L>R asymmetry (2/6 embryos) (Fig. 6F) in $Cerl2^{-/-}$ embryos. Together, these results suggested that L<R asymmetric expression of *Cerl2* is responsible for L>R asymmetric Nodal activity in the perinodal cells.

Nodal expression, but not Nodal signaling, in perinodal cells is essential for L-R patterning

The asymmetric distribution of pSmad2 among the perinodal cells indicated that the level of active Nodal derived from these cells differs between the L and R sides, whereas the distribution of *Nodal* mRNA is symmetric and perinodal cells on the two sides are equally

competent to respond to active Nodal. Active Nodal produced by the perinodal cells would be expected to act on these cells in an autocrine or paracrine manner, leading to asymmetry in pSmad2 distribution. However, it remained unknown whether Nodal signaling in the perinodal cells is essential for the subsequent development of asymmetry in the LPM.

To address this question, we inhibited Nodal signaling in perinodal cells with the use either of a dominant negative form of the receptor ALK4 (dnALK4), which binds to Nodal but does not transduce the Nodal signal, or of a dominant negative mutant of Smad3 (dnSmad3) that can inhibit activity of Smad2 and Smad3 (Nakao et al., 1997) (Fig. 7). These two mutant proteins were each ectopically expressed in perinodal cells under the control of a perinodal cell–specific enhancer (NDE) derived from mouse *Nodal* (Adachi et al., 1999; Norris and Robertson, 1999). Permanent transgenic lines expressing dnALK4 or dnSmad3 were successfully established and they did not show developmental defects (data not shown). Transgenic embryos expressing dnALK4 or dnSmad3 were examined for *Nodal* expression in LPM at E8.2. In transgenic embryos expressing dnSmad3, pSmad2 staining in the perinodal cells was reduced to an undetectable level (Fig. 7M) while it was maintained in the left LPM. In those embryos expressing dnALK4, the level of pSmad2 staining in the perinodal cells was greatly reduced but was still detectable (Fig. 7E). In both types of transgenic embryo, left-sided expression of *Nodal* was maintained in LPM (Fig. 7C, K). The timing of *Nodal* expression in LPM did not appear to differ between the transgenic and nontransgenic embryos (Fig. 7H, P). These results thus suggested that, although Nodal produced by the perinodal cells is essential for L-R patterning of LPM, autocrine-paracrine Nodal signaling in the perinodal cells themselves does not play a role in this process.

Discussion

Role of ANE in regulation of *LEFTY1*

We have characterized ANE, which is derived from the human *LEFTY1* gene, as the first transcriptional enhancer shown to exhibit L-R asymmetric activity in the node. This finding suggests that *LEFTY1* might be expressed in an L-R asymmetric manner in the node of human embryos. In contrast, mouse *Lefty1* is not expressed in the perinodal cells of the mouse embryo, and an ANE-like enhancer was not detected in the corresponding upstream region of mouse *Lefty1* or of *Lefty1* genes of any other mammal examined (Saijoh et al., 1999) (data not shown). Whereas expression of a Nodal antagonist (such as Cerl2) on the right side of the node is thought to be essential for L-R patterning of LPM, a Nodal antagonist would not be required on the left side of the node. As we have shown in the present study, Nodal signaling in the node cells is dispensable for L-R patterning of LPM. The *LEFTY1* gene may be activated on the left side of the node simply as a response to the increased Nodal activity. Alternatively, Lefty1 protein expressed on the left side of the node may prevent autocrine Nodal signaling in perinodal cells of the human embryo.

A typical FoxH1 binding sequence, AAT(C/A)(C/A)ACA, is not present in the 0.5-kb genomic region shown to contain ANE. Although the consensus Samd2/3 binding sequence, GTCT, is present in this region, whether this sequence alone is sufficient to confer a response to Nodal signaling or whether additional transcription factor binding sites are

involved is not known. It will be necessary to dissect ANE and identify sequence motifs essential for ANE activity in order to clarify this issue.

How is pSmad2 asymmetry induced in the node when *Nodal* expression is symmetric?

Nodal produced by perinodal cells would be expected to be secreted into the node cavity or within the embryo. Although both possibilities are formally possible, our data suggest that it is Nodal secreted within the embryo that induces phosphorylation of Smad2/3 and that Smad2/3 is therefore phosphorylated in response to autocrine-paracrine action of Nodal. This conclusion is further supported by the observed down-regulation of pSmad2 in the node of embryos lacking the Nodal coreceptor Cryptic.

We found that pSmad2 was preferentially expressed in the perinodal cells on the left side even when the distribution of *Nodal* mRNA is symmetric. How then is this asymmetry in pSmad2 distribution generated? Given that ectopic Nodal protein introduced on the right side of the node induced Smad2/3 phosphorylation on the right side, the perinodal cells on both sides are equally competent to respond to active Nodal. It is therefore most likely that the level of active Nodal derived from these cells differs between the two sides. The asymmetry in pSmad2 distribution among the perinodal cells is indirectly controlled by nodal flow, given that its pattern was influenced when the flow was impaired. However, direct transport of Nodal by the leftward flow is not likely responsible for the pSmad2 asymmetry, given that ectopic Nodal protein in the culture medium did not induce ectopic pSmad2 staining in cultured embryos. Our observations that L<R asymmetric expression of *Cerl2* precedes the asymmetry in pSmad2 distribution and that pSmad2 is distributed symmetrically in *Cerl2*$^{-/-}$ embryos suggest that the major player in induction of pSmad2 asymmetry is Cerl2, a Nodal antagonist that interacts with Nodal protein and inhibits its activity (Marques et al., 2004) and whose L<R asymmetric expression among perinodal cells is controlled by the fluid flow. Although Cerl2 may inhibit Nodal activity through its direct interaction with Nodal on the right side of the node, the L-R asymmetry of pSmad2 distribution appears more pronounced than that of *Cerl2* expression, suggesting that other mechanisms that are flow dependent but independent of Cerl2 may also contribute to the generation of pSmad2 asymmetry. For instance, the efficiency of Nodal secretion from perinodal cells may differ between the two sides of the node, possibly being influenced by flow-induced intracellular Ca^{2+} signaling.

Relation between pSmad2 asymmetry in the perinodal region and asymmetry in LPM

Whereas *Nodal* expression in perinodal cells is absolutely essential for L-R asymmetric patterning of LPM, autocrine/paracrine Nodal signaling in the perinodal cells is dispensable for this process. Therefore, targets of Nodal protein produced in the node are not perinodal cells but are most likely LPM cells. Nodal may be transported from the node to LPM via the internal route that includes PAM. Although an asymmetric distribution of pSmad2 was not detected in PAM, injection of recombinant Nodal into PAM gave rise to pSmad2 immunostaining in PAM. PAM may therefore be partially competent to respond to Nodal signaling (in that it possesses signaling components from the receptor to Smad2/3/4), although it is unable to express Nodal target genes such as *Nodal* and *Lefty* because it lacks

FoxH1. Alternatively, the pSmad2 staining induced in PAM may have been an artifact due to the excess amount of ectopic Nodal injected.

Among L-R mutant mice examined, there was a close correlation between the asymmetry of pSmad2 distribution among the perinodal cells and that of *Nodal* expression in LPM. This similarity was thus evident in the *iv/iv* mutant, in which *Nodal* expression in LPM is randomized, and in the *inv/inv* mutant, in which *Nodal* expression in LPM is reversed. Given that the level of pSmad2 likely represents the level of active Nodal derived from the perinodal cells, these results further support the notion that Nodal produced in the node is transported to LPM, where it activates *Nodal* expression via a Nodal-responsive enhancer (ASE) of the *Nodal* gene. The L>R asymmetry of Nodal activity in the node is thus translated into L>R asymmetry of the amount of Nodal that reaches LPM and subsequently into robust L>R asymmetry of *Nodal* expression in LPM through a self-enhancement lateral inhibition mechanism (Nakamura et al., 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

> Nodal activity in node is asymmetric although Nodal mRNA is bilateral. > Target of Nodal produced in the node is not node itself but lateral plate. > Successful detection of Nodal activity (phosphorylated Smad2/3) in histology.

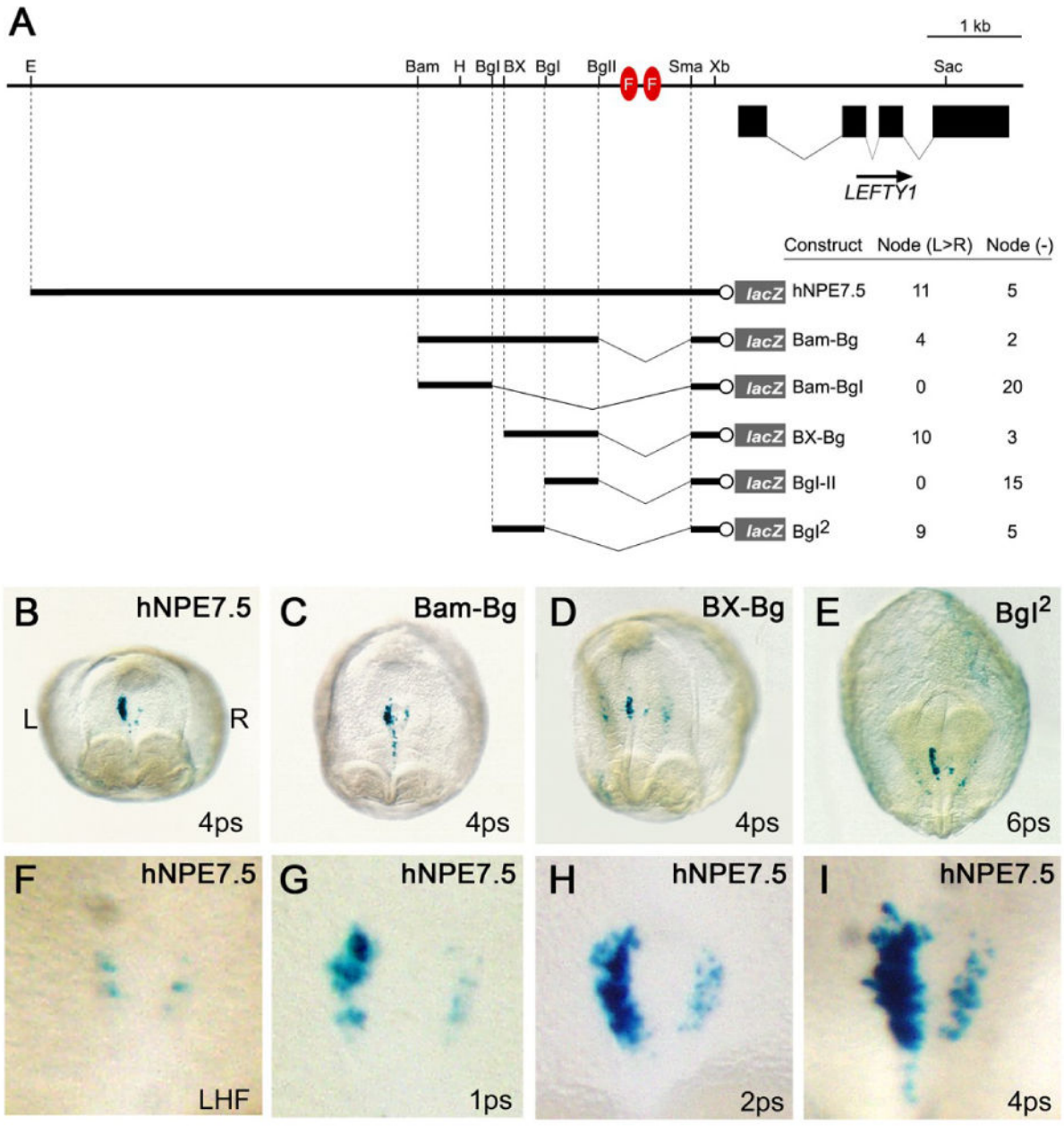

Figure 1. ANE is an L-R asymmetric node-specific enhancer

(**A**) Various restriction fragments of human *LEFTY1* were linked to *lacZ*, and the enhancer activity of each fragment was examined in transgenic mouse embryos. The number of embryos showing L>R expression or no expression (−) of the transgene in the node at E8.2 is summarized. The approximate location of FoxH1 binding sites is indicated by red ovals. Restriction sites: E, EcoRI; Bam, BamHI; BgI, BglI; BX, BstXI; BgII, BglII; Sma, SmaI; Xb, XbaI; Sac, SacI. (**B**–**E**) Ventral views of X-gal–stained embryos harboring the indicated *lacZ* transgenes. (**F**–**I**) Magnified views of the node region of X-gal–stained embryos harboring hNPE7.5-*lacZ*. The L-R axis and developmental stage (ps, pairs of somites) is indicated for each embryo.

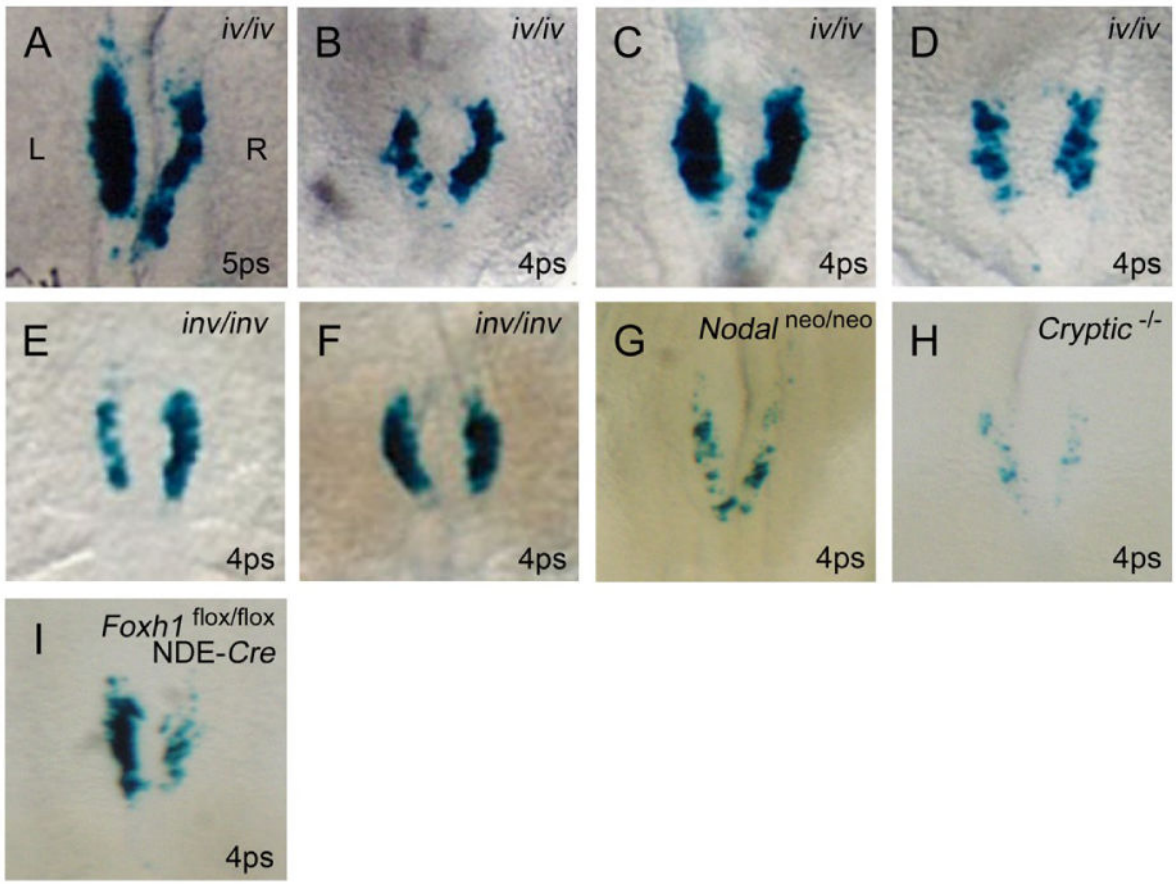

Figure 2. ANE is regulated by Nodal signaling but not by FoxH1

The expression patterns of hNPE7.5-*lacZ* around the node of the indicated mutant mouse embryos were determined at the indicated developmental stages. X-gal staining in the node was left-sided (**A**, **I**), right-sided (**B**, **E**), bilateral (**C**, **D**, **F**), or down-regulated and bilateral (**G**, **H**).

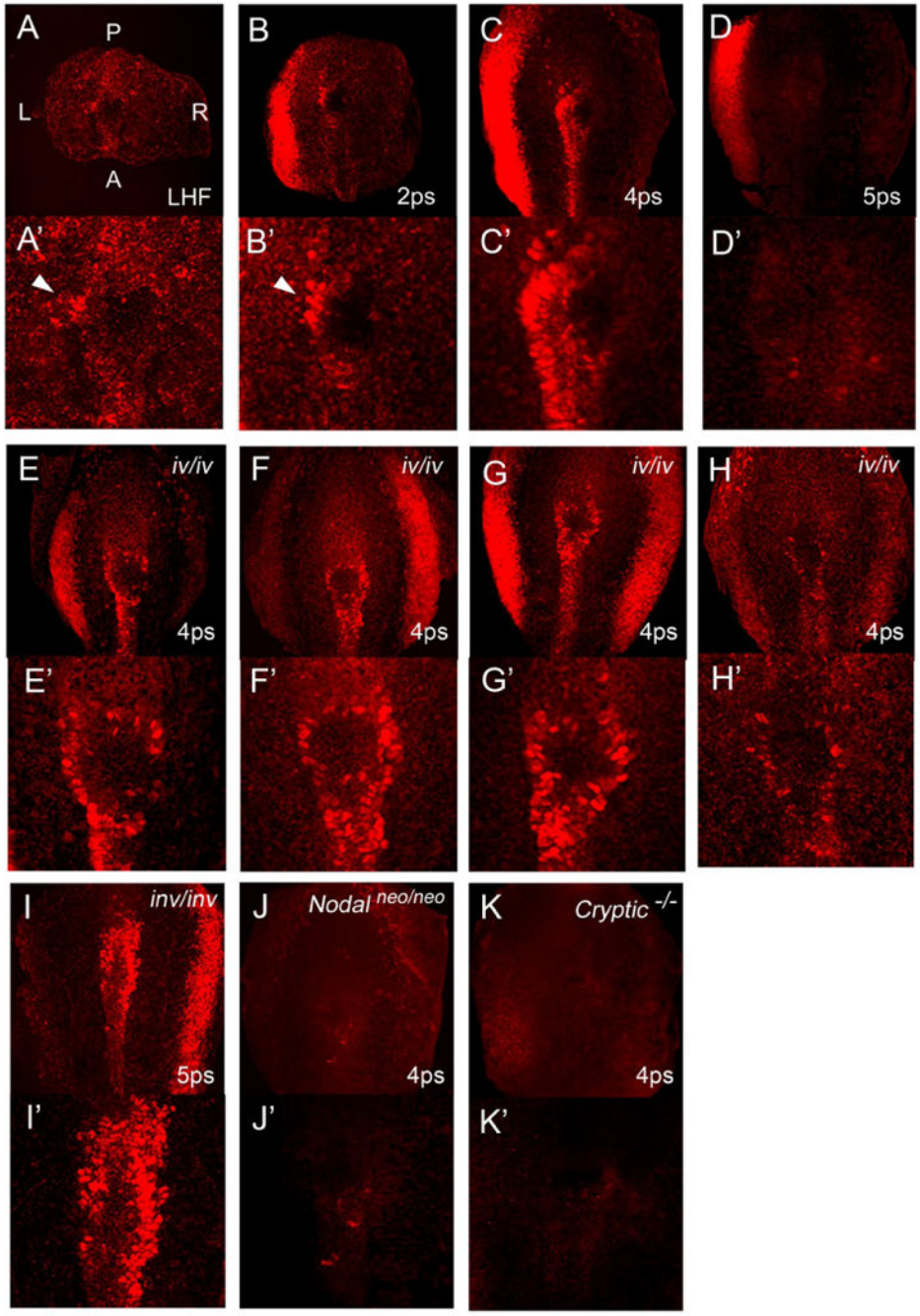

Figure 3. Asymmetry of pSmad2 distribution around the node correlates with that in LPM

(**A**–**K**) Ventral views of pSmad2 immunostaining in wild-type or the indicated mutant embryos at the indicated developmental stages. (**A′**–**K′**) The node region of the embryos in (**A**) through (**K**), respectively, is shown at higher magnification. Arrowheads in (A′) and (B′) indicate pSmad2-positive cells at the node.

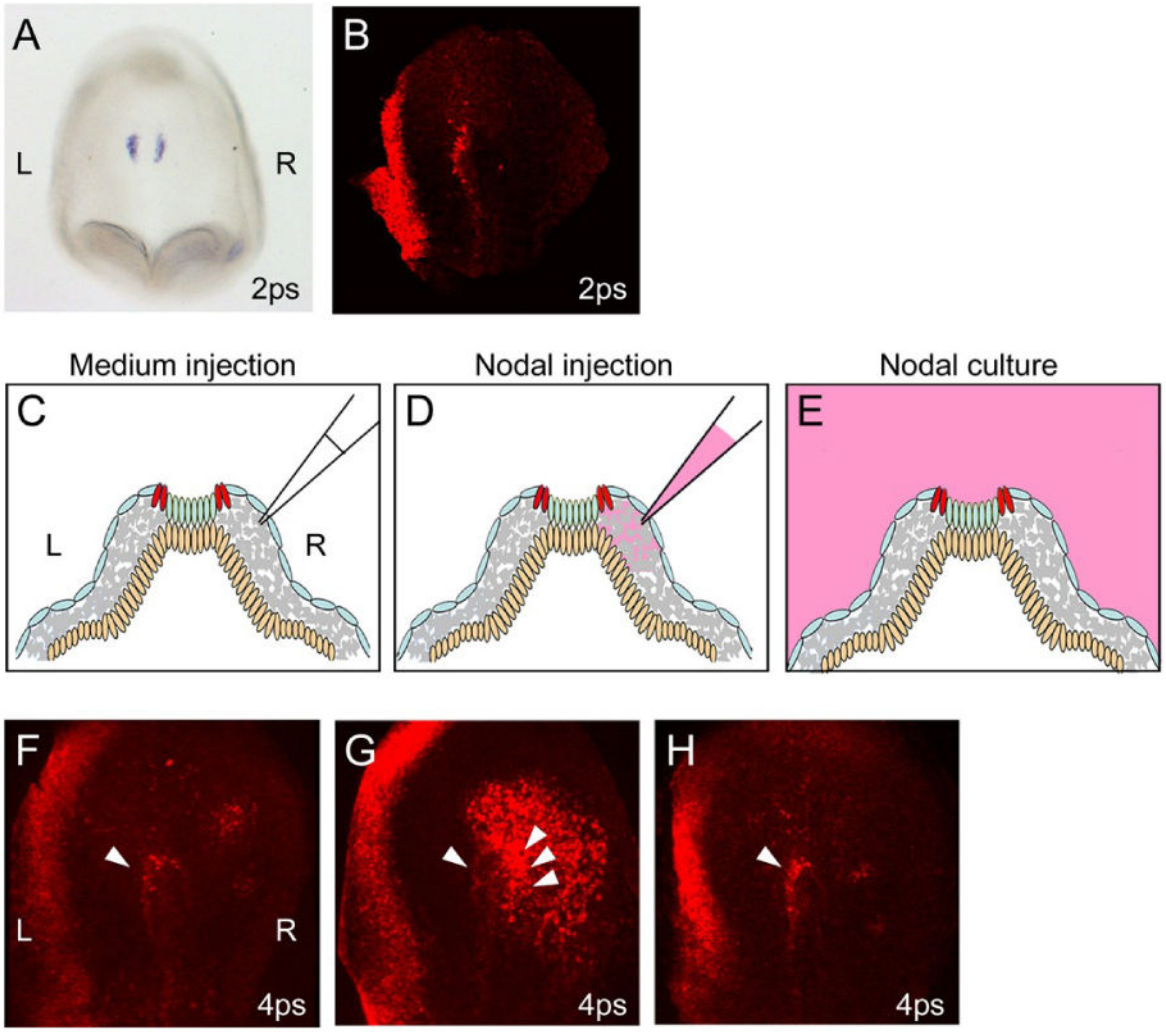

Figure 4. Phosphorylation of Smad2/3 in perinodal cells is induced by internally secreted Nodal

(**A**) In situ hybridization analysis of *Nodal* mRNA in the node at the two-somite stage. (**B**) Immunostaining of pSmad2 at the two-somite stage. (**C**–**E**) Experimental protocol. Transverse schematic representations of E8.0 embryos are shown. Sky blue, endoderm; gray, mesoderm; yellow, ectoderm; red, perinodal cells expressing *Nodal*; pink, recombinant Nodal. (**F**, **G**) Immunostaining of pSmad2 in embryos that were recovered at the three-somite stage, injected with medium (**F**) or recombinant Nodal (**G**), and cultured for 30 minutes before they were fixed with 4% paraform aldehyde. (**H**) Immunostaining of pSmad2 in an embryo that was recovered at the three-somite stage and cultured in the presence of recombinant Nodal to the four-somite stage. Arrowheads in (**F**) through (**H**) indicate pSmad2-positive cells at the node.

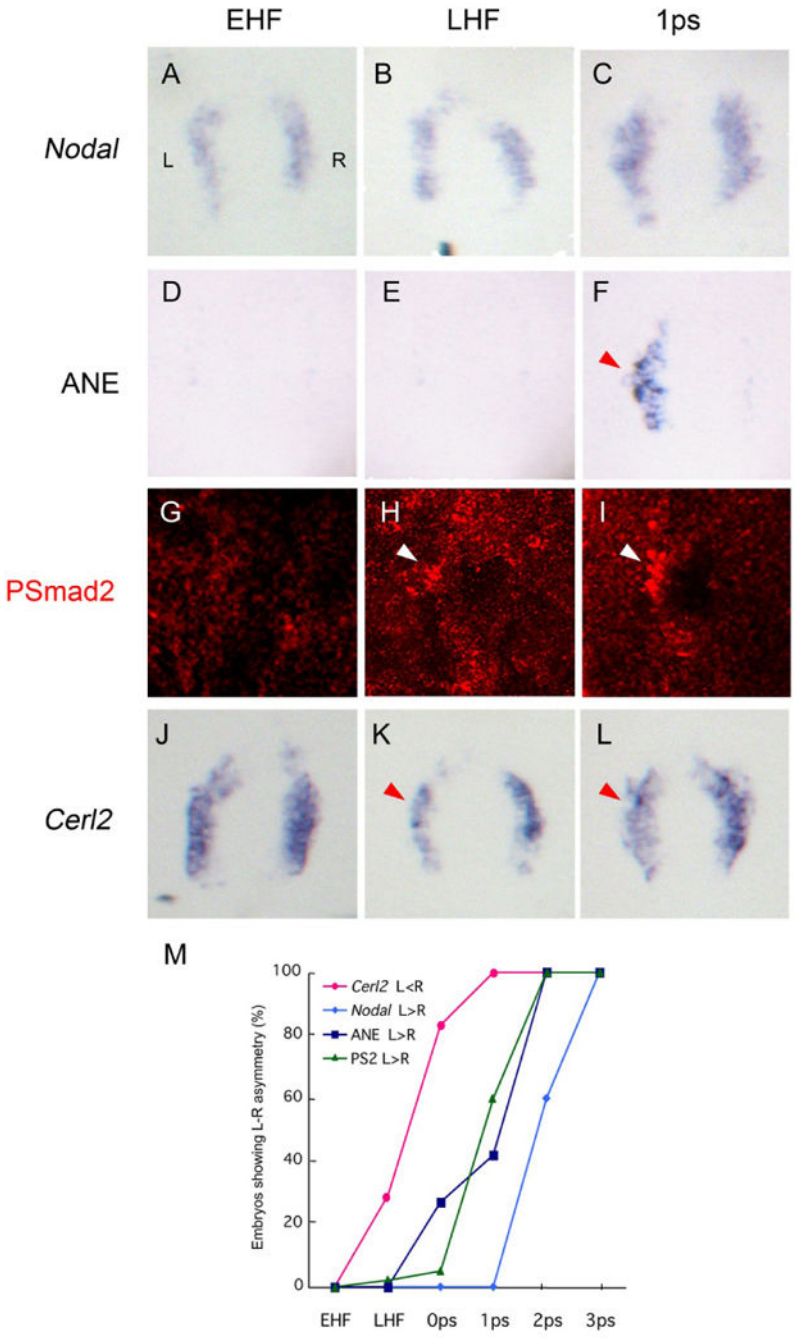

Figure 5. The first L-R asymmetry in perinodal cells is *Cerl2* expression

Nodal expression (**A**–**C**), hNPE7.5-*lacZ* expression (ANE activity) (**D**–**F**), Smad2/3 phosphorylation (**G**–**I**), and *Cerl2* expression (**J**–**L**) were examined in perinodal cells of mouse embryos at the EHF, LHF, and one-somite stages, respectively. Arrowheads indicate the changes in ANE activity (F), Smad2/3 phosphorylation (H, I) and Cerl2 expression (K, L). (**M**) Summary of the frequency of embryos that showed L-R asymmetric patterns for each of the four markers examined at the indicated developmental stages. The number of embryos examined for each marker at each stage ranged from 10 to 17. PS2, pSmad2.

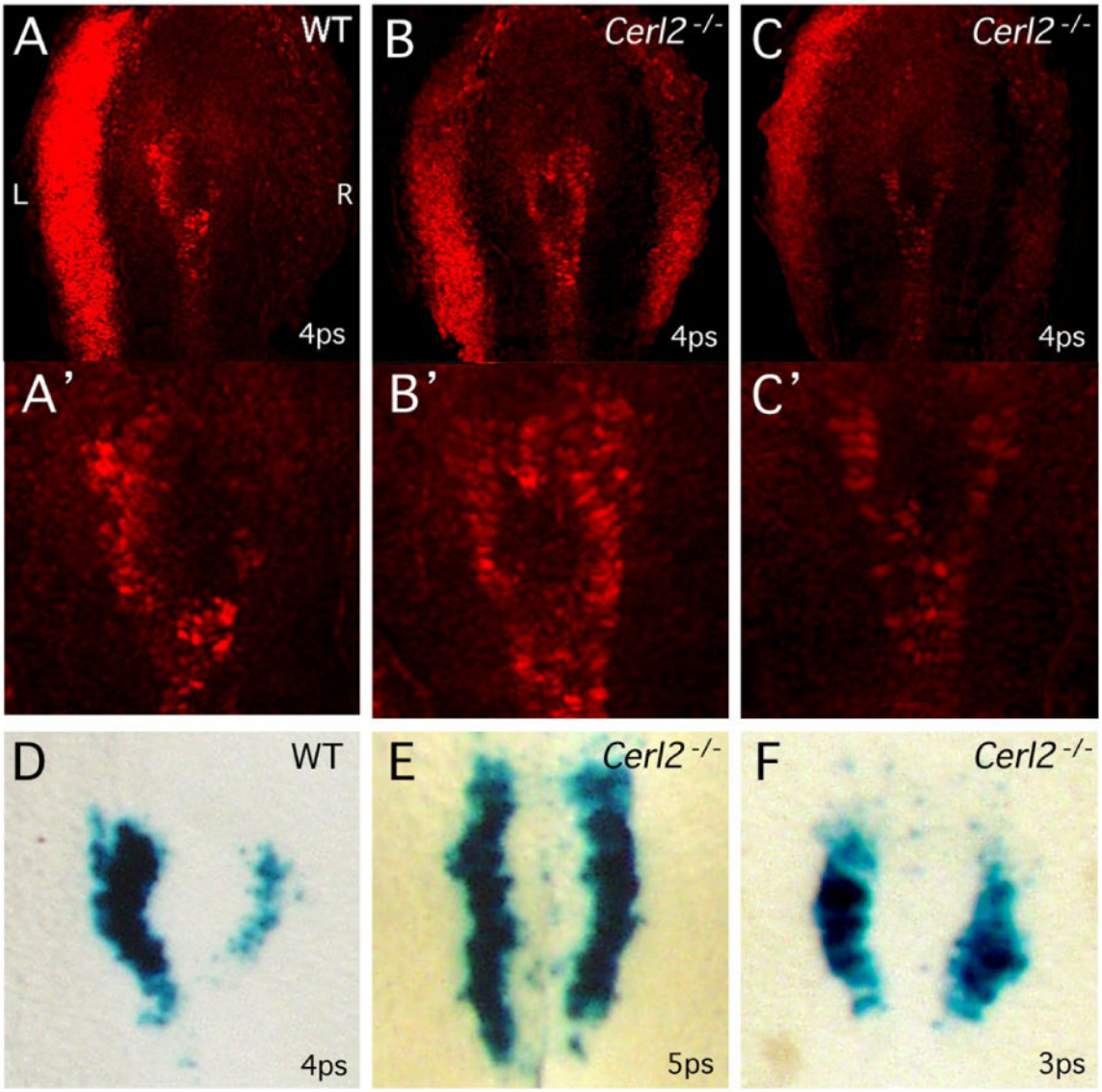

Figure 6. L<R asymmetric *Cerl2* expression is responsible for the asymmetric pSmad2 distribution among perinodal cells

(**A**-**C**) Immunostaining for pSmad2 in wild-type (WT) and *Cerl2*$^{-/-}$ embryos, respectively, at the four-somite stage. (**A′**-**C′**) Higher magnification images of the node region of the embryos shown in (**A**), (**B**) and (**C**), respectively. The distribution of pSmad2 is either bilateral or bilateral with subtle L>R asymmetry in both the node and LPM of the *Cerl2* mutant. (**D**-**F**) Expression of the hNPE7.5-*lacZ* transgene in wild-type and *Cerl2*$^{-/-}$ embryos, respectively, at the indicated developmental stages.

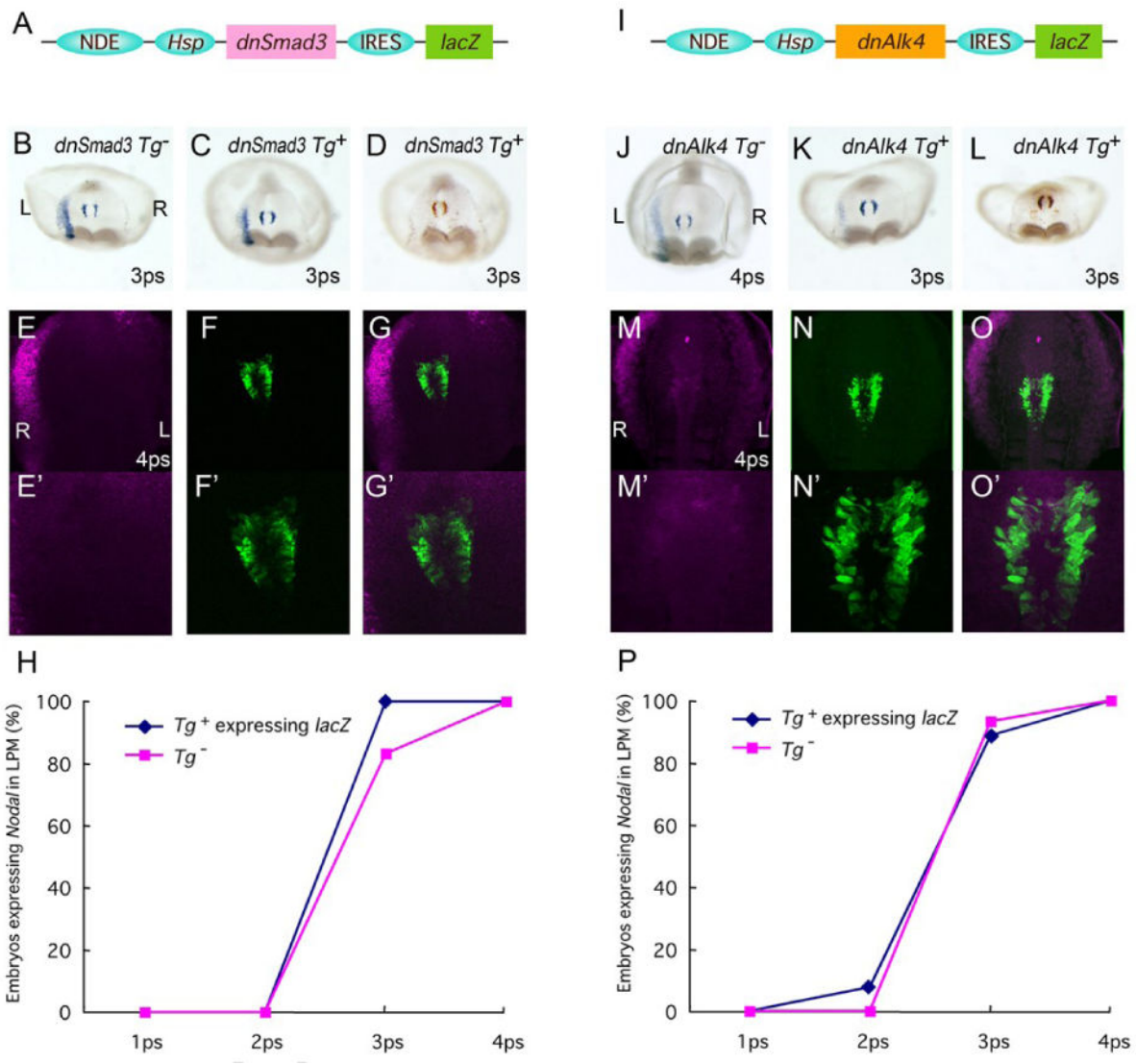

Figure 7. Asymmetric Nodal signaling in perinodal cells is dispensable for L-R patterning of LPM

(**A**) Schematic representation of the transgene encoding dnSmad3. A node-specific enhancer (NDE) is linked to the *Hsp68* promoter, *dnSmad3*, an internal ribosome entry site (IRES), and *lacZ*. (**B**, **C**) In situ hybridization analysis of *Nodal* mRNA in E8.2 nontransgenic (*dnSmad3 Tg*$^{-}$) and *dnSmad3* transgenic (*dnSmad3 Tg*$^{+}$) mouse embryos, respectively. (**D**) Expression of the transgene in the *dnSmad3* transgenic embryo in (**C**) was confirmed by in situ hybridization analysis of *lacZ* mRNA. (**E**–**G**) Immunostaining of pSmad2 (**E**) and beta-galactosidase (**F**) in an E8.2 embryo that expressed the *dnSmad3* transgene. A merged image is shown in (**G**). (**E′**–**G′**) Higher magnification images of the node region of the embryos shown in (**E**) through (**G**). (**H**) Percentage of nontransgenic and *dnSmad3* transgenic embryos positive for *Nodal* expression in LPM at the indicated developmental stages. The number of nontransgenic or transgenic embryos examined at each stage ranged from 9 to 15.

(**I**–**P**) The analyses in (**A**) through (**H**) were repeated with embryos expressing a transgene for dnALK4.