Genetic dissection of *nodal* function in patterning the mouse embryo

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接受 23 February; published on WWW 19 April 2001

**SUMMARY**

Loss-of-function analysis has shown that the transforming growth factor-like signaling molecule *nodal* is essential for mouse mesoderm development. However, definitive proof of *nodal* function in other developmental processes in the mouse embryo has been lacking because the null mutation blocks gastrulation. We describe the generation and analysis of a hypomorphic *nodal* allele. Mouse embryos heterozygous for the hypomorphic allele and a null allele undergo gastrulation but then display abnormalities that fall into three distinct mutant phenotypic classes, which may result from expression levels falling below critical thresholds in one or more domains of *nodal* expression. Our analysis of each of these classes provides conclusive evidence for *nodal*-mediated regulation of several developmental processes in the mouse embryo, beyond its role in mesoderm formation. We find that *nodal* signaling is required for correct positioning of the anteroposterior axis, normal anterior and midline patterning, and the left-right asymmetric development of the heart, vasculature, lungs and stomach.

Key words: *nodal*, Anteroposterior axis, Left-right asymmetry, Node, Lateral plate mesoderm, Mouse

**INTRODUCTION**

Gastrulation in the vertebrate embryo results in the formation of the three germ layers and the establishment of the primary body axes, the anteroposterior (AP), dorsoventral (DV) and left-right (LR) axes. In the mouse, gastrulation initiates with the formation of mesoderm at the primitive streak, a transient structure at the posterior of the embryo. Mutational studies have revealed an essential role for *nodal*, a transforming growth factor β-related signaling molecule, in the early steps of mouse gastrulation (Conlon et al., 1991; Conlon et al., 1994; Iannaccone et al., 1992; Zhou et al., 1993). Embryos homozygous for the retroviral insertional mutation that originally identified the *nodal* gene fail to form a primitive streak and lack morphological evidence of embryonic mesoderm (Conlon et al., 1994; Iannaccone et al., 1992; Pfendler et al., 2000). The early expression pattern of *nodal* in the normal mouse embryo is consistent with a role in gastrulation. Expression is found prior to gastrulation in the proximal epiblast, then becomes restricted to the posterior embryonic ectoderm as the streak forms and elongates (Varlet et al., 1997). *nodal*-related genes have been identified in other vertebrates, including chick, *Xenopus* and zebrafish (Feldman et al., 1998; Jones et al., 1995; Levin et al., 1995; Rebagliati et al., 1998b; Sampath et al., 1998; Smith et al., 1995). Zebrafish embryos with combined mutations in two *nodal*-related genes, *cyclops* and *squint*, also fail to form mesoderm (Feldman et al., 1998). Ectopic expression studies in *Xenopus* and zebrafish embryos have shown that nodal and related factors can induce the formation of mesoderm (Jones et al., 1995; Joseph and Melton, 1997; Toyama et al., 1995). Additionally, approaches to specifically inhibit nodal signaling in frog embryos lead to a block in mesoderm formation (Agius et al., 2000; Osada and Wright, 1999). Taken together these studies provide strong evidence for a role for the nodal signaling pathway in vertebrate mesoderm induction at the start of gastrulation.

In addition to a role in gastrulation, nodal has been implicated in LR development, in part owing to asymmetric domains of expression found at later stages. In head-fold and early somite stage mouse embryos, *nodal* is expressed in mesendodermal cells around the node (Conlon et al., 1994; Zhou et al., 1993). This transient embryonic structure and its derivatives, the notochord and prechordal plate, are key organizing centers for embryonic pattern formation. During early somite stages *nodal* expression around the node becomes progressively asymmetric, with stronger expression on the left side (Collignon et al., 1996; Lowe et al., 1996). At the same stages, *nodal* is also expressed in the lateral plate mesoderm (LPM) but only on the left side. Asymmetric expression is evolutionarily conserved, being found in every vertebrate examined (Collignon et al., 1996; Fujinaga et al., 2000; Levin et al., 1995; Lowe et al., 1996; Lustig et al., 1996; Rebagliati et al., 1998a; Sampath et al., 1998). It has been proposed that *nodal*, and other asymmetrically expressed genes since identified, act in a molecular pathway directing normal LR asymmetric development of the heart, and other visceral organs (reviewed in Burdine and Schier, 2000; Capdevila et al., 2000). Although misexpression of nodal on the right side of chick and...
**MATERIALS AND METHODS**

**Gene targeting.** The *nodal* cDNA was used to isolate an 18 kilobase (kb) genomic clone from a 129Sv lambda library. An kb *BanHI* fragment covering the upstream region, the first exon, the first intron and 165 basepairs of the second exon, and the 3′ contiguous 4.5 kb *BanHI* fragment covering the rest of the gene and 2 kb downstream were subcloned. These fragments were modified by PCR to introduce a loxP site into the first intron and another loxP site after the 3′ untranslated region. They were then combined with a fragment of the *nodal* cDNA that contained the remainder of the second exon and the third exon up to the stop codon, as well as a fragment that contained the IRES beta geo cassette (Mountford et al., 1994). Details of the construction are available upon request. The final arrangement is shown in Fig. 1A. The loxP sites were tested by transformation of 294-Cre bacteria (Buchholz et al., 1996). For gene targeting the vector was linearized and transfected by electroporation into W9.5 embryonic stem (ES) cells (Buzin et al., 1994). ES cell culture and blastocyst injections were carried out by standard methods.

**Mouse breeding and genotyping.** Male germline chimeras were bred with C57Bl6/J females to obtain F1 animals carrying the floxed *nodal* allele (*nodalfl1*), and with 129/Sv females for inbreeding. To derive animals carrying a deleted *nodal* allele (*nodalnb*), we bred *nodalfl1* animals with E1a-Cre mice. Embryos derived from E1a-Cre transgenic females express Cre in all cells of the early embryo (Lakso et al., 1996). Offspring from this cross with the deleted allele were identified by Southern blotting of EcoRI digests (probe shown in Fig. 1A). Subsequent genotyping was by PCR. The following PCR genotyping primers were used: for the wild-type allele, F114/N12 CCCAGCAAAATGCGAATCGACC (specific for *nodal* intron 2) and R403/3′U CCCAGCAAAATGCGAATCGACC (specific for the 3′UTR of *nodal*) for the floxed allele, F580/neo CCGAATATCATGTGAGAATGCGC (specific for the neo gene) and R403/3′U; for the *nodalnb* allele, R878 AGTTCCTCCCACCTCACATACC (specific for *nodal* intron 2); and F654 AGAACCAGAAGGGAATTGGG (specific for the 3′UTR of *nodal*).

**Phenotypic and molecular analysis.** Analysis of cardiac and vasculature malformations was by colored latex injections into right and left ventricles (Oh and Li, 1997). Whole-mount in situ hybridization was carried out as described (Lowe and Kuehn, 2000). Whole-mount X-gal staining to check *lacZ* expression was performed by a modification of existing protocols. Briefly, embryos were fixed in 0.2% Glutaraldehyde, 5 mM EDTA, 2 mM MgCl2, 1× PBS at room temperature for 30 minutes to 1 hour, then immediately placed in 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 1× PBS, 2 mM MgCl2, 2 mg/ml X-gal in DMSO and kept at room temperature overnight. Selected embryos were embedded in JB-4 plastic and sectioned as described (Lowe and Kuehn, 2000).

**RESULTS**

**Generating a floxed allele of *nodal***

We used a promoterless targeting vector to introduce loxP sites around the *nodal*-coding region by homologous recombination in ES cells (Fig. 1A). An IRES-βgeo cassette (Mountford et al., 1994) was placed immediately after the *nodal* stop codon.
This results in a bicistronic message encoding *nodal* as well as providing G418 selection of transfectants and expression of β-galactosidase (β-gal) with a pattern identical to *nodal*. One loxP site was placed within the first intron and another immediately 3’ to the IRES-βgeo cassette. We assumed that loss of this part of the coding sequence, following Cre-mediated recombination, would eliminate function because it encodes the secreted, mature region of *nodal*. The 3’ loxP site was positioned after the IRES-βgeo to allow the extent of Cre-mediated recombination to be assessed in situ, by monitoring colorimetrically the loss of β-gal activity.

We expected to enrich for homologous recombinants using the promoterless targeting strategy. Remarkably, in two separate experiments, greater than 95% of G418-resistant colonies were homologous recombinants. Two pools of five to six homologously recombined ES cell clones were used to establish two lines of *nodal* fl1 mice. Breeding of *nodal* fl1 heterozygotes on a mixed genetic background (129/Sv and C57Bl/6J) produced the expected number of homozygotes (Fig. 1B,C). Further breeding showed that homozygotes were viable and fertile. Whole-mount X-gal staining of embryonic day (E) 6.5, E7.5 and E8.5 embryos gave a pattern that was essentially the same as that of wild-type *nodal* mRNA expression (Lowe et al., 1996; Fig. 1D). To confirm that the *nodal* fl1 allele is capable of undergoing Cre-mediated recombination, we crossed *nodal* fl1 males with EIIa-Cre females (Lakso et al., 1996). As expected for this Cre transgenic strain, in which all cells of the early embryo express Cre, PCR analysis of offspring revealed the specific product resulting from deletion between the loxP sites (Fig. 1C). In addition, embryos collected from these matings revealed almost complete loss of X-gal staining (Fig. 1E), indicating that the *nodal* fl1 allele can be efficiently recombined by Cre to produce the deleted allele (*nodal* fl1).

**The *nodal* fl1 allele is a hypomorph**

Analysis of embryos with a *nodal* Δfl1 Δgeo genotype showed a pre-gastrulation block identical to the original *nodal* insertional mutant, confirming that the *nodal* fl1 allele is a null. Unexpectedly, *nodal* fl1 Δgeo embryos were found that had undergone gastrulation but were clearly abnormal. These embryos did not carry Cre. This result indicated that a single copy of the floxed allele is insufficient for normal development. Analysis of additional embryos (n=133) obtained from crossing *nodal* fl1 Δgeo mice with *nodal* fl1 homozygotes, none carrying Cre, revealed 41% with overt developmental defects at E7.5. A sample set was genotyped and all phenotypically abnormal embryos were *nodal* fl1 Δgeo. Of the abnormal embryos, 6% were arrested at the pre-gastrulation stage and the remaining 35% had clearly undergone gastrulation. Of the embryos examined at E8.5 (n=542), 46% were abnormal with 6% arrested prior to gastrulation and the remainder showing post-gastrulation defects. Although a small percentage of *nodal* fl1 Δgeo embryos appeared normal at these early stages, those found alive at E14.5 were abnormal and PCR genotyping of four litters (n=21) showed that no *nodal* fl1 Δgeo pups survived to birth. These findings confirm that the floxed *nodal* allele has reduced function, i.e. is hypomorphic. As described in the sections below, detailed analysis of the defects caused by the hypomorphic allele has provided new insight into nodal function not revealed by the *nodal* null mutant, identifying several developmental processes for which nodal signaling is crucial.

**Positioning of the AP axis is abnormal in *nodal* hypomorphic mutants**

Examination of E7.5 *nodal* fl1 Δgeo embryos showed morphological abnormalities as well as alterations in the pattern of *nodal* expression, as detected by X-gal staining for...
β-gal expressed from the nodal[\^]{\delta} allele. Normally at the primitive streak/early head-fold stage, nodal is expressed in the posterior embryonic ectoderm, extending the length of the primitive streak (Fig. 2A). Expression in nodal[\^]{\delta}/+[\^]{\delta} embryos did not extend as far distally from the embryonic/extra-embryonic boundary (Fig. 2B), suggesting truncation of the primitive streak. We also found a constriction at the boundary between the embryonic and extra-embryonic regions in mutants (Fig. 2B). In normal embryos, the anterior visceral endoderm (AVE) is expressed anteriorly, both in the AVE and in the overlying mesoderm. In nodal[\^]{\delta}/+[\^]{\delta} embryos, the morphology of the visceral endoderm at the embryonic/extra-embryonic boundary was different from that in a normal AVE (Fig. 2F). Normally, there is direct contact between the AVE and the underlying anterior embryonic ectoderm (Fig. 2E). In nodal[\^]{\delta}/+[\^]{\delta} embryos, the lateral edges of the mesodermal wings encroached on the anterior midline at this position (Fig. 2F). Interestingly, visceral endoderm cells with a distinct cuboidal shape were found in a distal location (Fig. 2J), where visceral endoderm is normally squamous (Fig. 2I). This might represent an abnormally located AVE, but there was also mesoderm between these cells and the embryonic ectoderm (Fig. 2J). In the normal embryo, primitive streak expression ceases by the neural plate stage. nodal is then expressed in mesendodermal cells of the node forming at the anterior end of the primitive streak, which is at the distal tip of the embryo (Fig. 2C,G). In mutants at this stage, localized expression was found only half way down the posterior side (Fig. 2D). This position coincided with the distal-most limit of primitive streak expression found in embryos examined at earlier stages (Fig. 2B). These nodal-expressing cells appeared to be mesodermal or mesendodermal as in a normal node (Fig. 2H), suggesting node development in a more proximal location. It has been proposed that the AP axis arises from a 90° rotation of the pre-existing proximal-distal axis of the pregastrulation embryo (Beddington and Robertson, 1998; Beddington and Robertson, 1999). The altered position of primitive streak and node expression, together with the apparently misplaced AVE, suggests that axial rotation does not occur properly in nodal[\^]{\delta}/+[\^]{\delta} embryos.

To analyze AP axis rotation further, we examined brachyury, Hesx1/Rpx and Otx2 expression. Each of these genes is expressed asymmetrically along the nascent AP axis (Hermes et al., 1996; Herrmann, 1991; Simeone et al., 1993). brachyury is found first in pre-streak stage normal embryos in the proximal embryonic ectoderm (Thomas et al., 1998). Expression then shifts posteriorly, marking in succession the primitive streak (Fig. 3A), node and notochord (Fig. 3C,E,G). In contrast to nodal-null mutants, which express brachyury only transiently or not at all (Conlon et al., 1994; Pfendler et al., 2000), nodal hypomorphs do show primitive streak brachyury expression. However, the domain was foreshortened and located more proximally than in normal embryos (Fig. 3B,D,H). Some nodal hypomorphs had a distinct node showing brachyury expression. However, the node was found in a more proximal location than normal (Fig. 3C,D,E,F). Further anteriorly, brachyury expression was reduced and discontinuous in the midline in some nodal[\^]{\delta}/+[\^]{\delta} embryos (Fig. 3G,H,J,L). In normal embryos, Otx2 is expressed throughout the embryonic ectoderm and visceral endoderm before and during early gastrulation, and then becomes restricted to the anterior ectoderm and visceral endoderm (Fig. 3K,M). In nodal[\^]{\delta}/+[\^]{\delta} mutants, Otx2 expression was consistently found more distally than normal (Fig. 3L,N). Hesx1/Rpx normally is expressed anteriorly, both in the AVE and in the overlying
Fig. 3. Analysis of markers of AP positioning at E7.5. All embryos are shown laterally with anterior toward the left, except in I and J where embryos are viewed ventrally with anterior toward the top. (E,F) Transverse sections through the node of the embryos in C,D, respectively, at the positions indicated by the arrowheads. (A) Wild type (WT) mid-primitive streak embryo with brachyury expression throughout the streak (bracketed). (B) Mid-primitive streak nodalfl1/Δ (fl/Δ) embryo with proximal brachyury expression (bracketed). (C) Mid-head-fold stage WT embryo expressing brachyury in the primitive streak, node (arrowhead) and anterior axial mesendoderm. (D) Mid-head-fold stage nodalfl1/Δ embryo with truncated primitive streak brachyury expression and a more proximally located node (arrowhead). (E) Cross section of the embryo in C showing Brachyury expression in the ventral node (n). At this angle of sectioning, the node lies in a plane with the amnion (a). Embryonic ectoderm, ee; mesoderm, m. (F) Cross section of the embryo in D, showing brachyury expression in the ventral node. Owing to altered AP positioning, the node lies in a plane with the head folds (hf). (G) Late head-fold stage WT embryo, with primitive streak, node and notochord brachyury expression. (H) Late head-fold stage nodalfl1/Δ embryo with truncated primitive streak brachyury expression. (I) Ventral view of the embryo in G. (J) Ventral view of the embryo in H, showing reduced midline brachyury expression. (K) WT mid-primitive streak embryo with anterior Otx2 expression (bracketed). (L) Mid-primitive stage nodalfl1/Δ embryo showing distal Otx2 expression (bracketed). (M) Early head-fold stage WT embryo showing anterior Otx2 expression (bracketed). (N) Early head-fold stage nodalfl1/Δ embryo with more distal Otx2 expression (bracketed). (O) Early head-fold stage WT embryo with anterior Hesx1/Rpx expression (bracketed). (P) A nodalfl1/Δ embryo showing distal Hesx1/Rpx expression.

Defective anterior patterning in nodal hypomorphic mutants correlates with anterior midline mesendodermal defects

One feature shared by all three mutant classes was a severe defect in anterior patterning. Compared with normal embryos (Fig. 4A), Type II and Type III embryos had an abnormal ventral orientation of the head without any obvious demarcation into distinct fore-, mid- and hindbrain regions (Fig. 4B,C). Type I embryos consistently lacked the normal ventral midline separation of the anterior neural folds at E8.5 (Fig. 4D,E,H,I), and displayed holoprosencephaly at E14.5 (Fig. 4F,G). To examine patterning of the brain further in all three phenotypic classes we again analyzed Otx2 expression. In normal E8.5 embryos, Otx2 is restricted to the anterior neur ectoderm where the fore- and mid-brain regions develop (Fig. 4H). This pattern was undisturbed in Type I mutants (Fig.
Fig. 4. Anterior patterning defects in nodal^{fl1/-} embryos. E8.5 embryos in A-C are viewed with anterior towards the left and ventral towards the bottom. E8.5 embryos in D,E,H-J are viewed ventrally. E14.5 embryos in F,G are viewed from the left side. (A) WT embryo enclosed in the extra-embryonic membranes. Type I nodal^{fl1/-} embryos showed the same arrangement. (B) Type II nodal^{fl1/-} embryo (fl/-II) showing the anterior region developing outside the yolk sac. (C) Type III nodal^{fl1/-} embryo (fl/-III) developing completely outside the yolk sac. (D) WT embryo with cranial neural folds showing a clear midline demarcation. (E) Type I nodal^{fl1/-} embryo (fl/-I). The head is small and there is no midline separation in the forebrain (arrowhead). (F) WT embryo. (G) Type I embryo showing an abnormally shaped cranium and snout, and lacking eyes. (H) WT embryo showing Otx2 expression in the midbrain (mb) and forebrain (fb). (I) Type I mutant embryo showing apparently normal Otx2 expression. (J) Type II mutant embryo showing reduced Otx2 expression in the head (arrowhead). (K) Lateral view of an E8.5 Type III mutant embryo with Otx2 expression restricted to the extreme anterior (arrowhead).

In normal embryos, both the AVE and the midline mesoderm are thought to participate in head patterning (Camus et al., 2000; Tam and Steiner, 1999). Thus, the observed anterior patterning defects could have their origin in early AVE defects, or in problems in the midline mesoderm, or both. To assess the state of the midline mesoderm we examined the expression of sonic hedgehog (Shh), which is normally found in the node, notochord, prechordal plate and gut endoderm (Fig. 5A,F,I). In Type I embryos, Shh expression in the brain did not extend as far anteriorly as in normal embryos (Fig. 5A,B). Cross section analysis revealed the absence of prechordal plate in the forebrain region of type I embryos, and showed that the foregut also does not extend as far anteriorly as in normal embryos (Fig. 5E,G). However, an apparently normal Shh-expressing notochord was evident in the hindbrain and in the trunk region of Type I embryos (Fig. 5B,G). Type II mutants were found either with Shh expression in the midline (Fig. 5C) or without (Fig. 5D). In those with Shh expression, notochord was found in the trunk, ending abruptly just at the level of the head (Fig. 5H,J). Shh-negative Type II embryos lacked notochord completely (Fig. 5K). Cross section analysis revealed the abnormal ventral orientation of the head in Type II embryos, as well as the disorganization and repeated folding of the head neur ectoderm (Fig. 5H). Type II embryos lacked a recognizable prechordal plate and foregut pocket, as well as a distinct floorplate in the trunk region (Fig. 5J,K). Type III embryos had a closed neural tube with an underlying notochord that expressed Shh (Fig. 5E,L). Notochord, and Shh expression, ended well short of the most anterior region, and no recognizable prechordal plate was found. These results suggest that ventral midline defects contribute to the anterior patterning abnormalities found in all three classes of mutant embryos.

Type II and Type III embryos also showed other phenotypic abnormalities in the midline, as well as in the gut. Type II embryos had abnormally narrow and prematurely closed hindguts (Fig. 5C,D). In Type III embryos, the only Shh expression found was in notochord, suggesting that this mutant class lacks gut endoderm. Indeed there was no morphologically recognizable gut tube in any Type III embryo. All Type II embryos had fusion of somites across the midline (Fig. 5J,K), regardless of the presence or absence of notochord. This was clearly demonstrated by analyzing paraxis (Burgess et al., 1995) gene expression. In normal E8.5 embryos, paraxis is found in the paired somites and presomatic mesoderm (Fig. 5M). Type II embryos showed either single broad somites in the mid-trunk (Fig. 5N) or complicated patterns of splitting and crossing over (Fig. 5O). Most Type III embryos had paired somites as in normal embryos (Fig. 5P), although some were found with fused somites or with only disorganized paraxial mesoderm (not shown).

Altered nodal expression in E8.5 nodal hypomorphic mutants

The midline mesoderm defects described above may result from alterations in the development of the node, perhaps due to reduced nodal expression. Therefore, we examined all three classes of E8.5 hypomorphs both for the structure of the node and for expression from the nodal^{fl1/-} allele by X-gal staining. Normally at this stage, nodal is expressed around the node and is expressed around the node and
of X-gal staining around the node, which also appeared to be normal in structure (Fig. 6K,L). With X-gal staining it was difficult to detect asymmetry of nodal expression around the node in either floxed heterozygotes or Type I mutants (Fig. 6A,C). Analysis of nodal mRNA confirmed normal asymmetry in floxed heterozygotes. However, mRNA levels were too low to assess asymmetry in Type I mutants (not shown). Therefore, β-gal activity exaggerates nodal mRNA levels, and does not reflect the lower level that exists in Type I embryos compared with floxed heterozygotes. For Type II embryos, X-gal staining around the node was clearly reduced, suggesting extremely low nodal mRNA levels. The structure of the node was abnormal as well (Fig. 6E,I,M). Normally, nodal-positive cells lie at the periphery of the node (Fig. 6K). In Type II embryos, nodal-positive cells were found across the midline. In addition, endodermal or mesendodermal cells were found extending ventrally into the hindgut (Fig. 6M). For Type III embryos there was no recognizable node, nor any apparent LPM (Fig. 6J). However, X-gal staining was consistently found in mesodermal or mesendodermal cells in a discrete area in the presumed posterior end that may represent an abnormal node (Fig. 6F,N). These results suggest a function for nodal signaling in the formation of the node.

**LR development is defective in Type I nodal hypomorphic mutants**

The lack of LPM expression in mutants allowed us to test the hypothesis that nodal expression in the left LPM is essential for the correct LR asymmetric orientation of the heart. We restricted this analysis to Type I embryos because only this class developed a heart tube. We first analyzed lefty-2 and Pitx2, two other genes also expressed asymmetrically in the left LPM of E8.5 normal embryos, and thought to be regulated by nodal signaling and to play a role in LR patterning. All Type I mutants (n=13) examined lacked lefty-2 expression (Fig. 7A,B). Analysis of Type I embryos (n=5) for Pitx2 revealed no LPM expression, although head and body wall mesoderm showed normal, symmetric levels of expression (Fig. 7C,D). We also found no lefty-1, normally expressed at this stage on the left side of the floorplate (Fig. 7A,B). Thus Type I embryos completely lack asymmetric gene expression in the left LPM and midline. To determine whether this affected cardiac

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**Fig. 5. Midline patterning defects in E8.5 nodalβ1/Δ embryos.** (A-E,M-P) Whole-mount in situ hybridization. (F-L) Transverse sections with dorsal toward the top. Embryos in A-D,N,O are viewed ventrally with anterior toward the top. (A) WT embryo showing normal Shh expression in the brain, midline and hindgut endoderm. (B) Type I embryo showing truncated Shh expression in the brain (arrowhead) but apparently normal trunk midline and hindgut expression. (C) Type II mutant with midline and hindgut Shh expression. (D) Type II mutant lacking midline Shh expression but with expression in the gut. (E) Type III embryo showing Shh expression along the AP axis. (F) Section through the brain of the WT embryo in A showing Shh expression in notochord of the hindbrain (hb) region and prechordal plate (pc) of the forebrain (fb) region. (G) Section through the brain of the Type I embryo in B. Shh is expressed in notochord in the hindbrain region but there is no prechordal plate. (H) Section through the head of the Type II embryo in C, which projects ventrally. Shh is expressed in the notochord. The head neuroectoderm (hne) is extensively folded. hm, head mesenchyme. (I) Section through the WT embryo in A, showing Shh expression in the notochord of the trunk. nf, neural folds; s, somite. (J) Section through the Type II embryo in C, showing Shh expression in the trunk notochord. The neural folds are separated from the notochord by two midline somites. (K) Section through the Type II embryo in D showing the lack of trunk notochord, a single midline somite and flattened neural folds. (L) Section through the Type III mutant in E showing Shh expression in notochord adjacent to the neural tube (nt). (M) WT embryo viewed dorsally showing paraxis expression in the paired somites. (N) Type II embryo with paraxis-expressing somites fused across the midline and a ventrally displaced head. (O) Another Type II embryo showing somite fusions. (P) Type III embryo showing paraxis expression in paired somites.
development, we examined the direction of heart tube looping in E8.5 mutants. Although this process was delayed compared with normal littermates, approximately half of the mutant hearts that had undergone looping were reversed (Fig. 7E,F). This suggested that the loss of nodal expression from the left LPM causes randomization of heart asymmetry.

The continued viability of Type I embryos past E8.5 enabled us to examine later stages of heart development, as well as determine the importance of nodal signaling in the asymmetric patterning of other visceral organs. Examination of organ morphology in E13.5 to E14.5 Type I embryos (n=48) revealed multiple defects (Table 1). We found an equal incidence of hearts in which the apex was pointing rightward or leftward (Fig. 7G,H), and (regardless of orientation) the positions of the aorta and pulmonary trunk were always transposed. Normally the pulmonary trunk lies ventral to the aorta and connects to the right ventricle, whereas the aorta connects to the left ventricle. In latex marker experiments, yellow latex injected into the right ventricle of a normal heart was subsequently found in the pulmonary trunk, while injection of blue latex into the left ventricle labeled the aorta (Fig. 7I). However, in mutants yellow latex injected into the right ventricle was subsequently found in the aorta (Fig. 7J). In addition, sequential injection of yellow and blue latex into the right and left ventricles, respectively, resulted in mixing of the markers, indicating septation defects (Fig. 7J). Normally the right lung has four lobes and the left has one lobe (Fig. 7K). However, mutant lungs consistently showed four lobes on both the right and left sides (Fig. 7L). A fraction of the mutants had stomachs on the right side rather than the normal left-sided location (Fig. 7M,N). In others, rotation of the stomach was incomplete, leaving it close to the midline at the level of the lungs, but on either the left or right side (Fig. 7O,P). This analysis establishes that the nodal signaling pathway is required for proper asymmetric patterning of several visceral organs.

**DISCUSSION**

The goal of this study was to generate a conditional allele to allow an analysis of post-gastrulation nodal function. Fortuitously, the floxed nodal allele we produced is also

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**Table 1. Phenotypic analysis of LR defects in E14.5 nodal<sup>fl1/Δ</sup>/nodal<sup>Δ</sup> embryos**

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<thead>
<tr>
<th>Organ or tissue</th>
<th>Phenotype</th>
<th>Frequency</th>
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<tr>
<td>Great vessels</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Transposed</td>
<td>48 (100%)</td>
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<tr>
<td>Heart direction</td>
<td>Normal</td>
<td>23 (48%)</td>
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<tr>
<td></td>
<td>Reversed</td>
<td>25 (52%)</td>
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<tr>
<td>Lungs</td>
<td>Normal, asymmetric</td>
<td>2 (4%)</td>
</tr>
<tr>
<td></td>
<td>Abnormal, asymmetric</td>
<td>4 (8%)</td>
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<tr>
<td></td>
<td>Right isomeric</td>
<td>42 (88%)</td>
</tr>
<tr>
<td>Stomach</td>
<td>Normal left</td>
<td>37 (82%)</td>
</tr>
<tr>
<td></td>
<td>Normal right</td>
<td>4 (9%)</td>
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<tr>
<td></td>
<td>Midline left</td>
<td>1 (2%)</td>
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<tr>
<td></td>
<td>Midline right</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Liver</td>
<td>Normal appearance</td>
<td>30 (65%)</td>
</tr>
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<td></td>
<td>Reduced size and/or pale</td>
<td>16 (35%)</td>
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nodal patterning function in mouse embryos

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nodal expression is required for AP axial positioning and anterior patterning

It has been proposed that the proximal-distal axis of the pregastrulation embryo is transformed into the AP axis by cell movements that relocate visceral endoderm cells from the distal end of the embryo to the prospective anterior, and proximal embryonic ectoderm to the prospective posterior where the primitive streak then arises (Beddington and Robertson, 1998; Beddington and Robertson, 1999). Our analysis of nodal mutant embryos at E7.5 showed that expression of both nodal and brachyury was more proximally located that normal, closer to their position in pregastrulation embryos. Additionally, anterior markers were found to be in more distal locations than normal. These findings suggest that
the proximal-distal to AP shift initiates in nodal hypomorphs but fails to complete. A more severe example of this phenotype, in which no movement occurs, is seen in mouse embryos with a targeted mutation in the EGF/CFC family member cripto (Ding et al., 1998). Importantly, members of the EGF/CFC family have been shown to be essential for nodal signaling (Gritsman et al., 1999; Kumar et al., 2001; Saijoh et al., 2000). Loss of function of another putative component of the nodal signaling pathway, the type IIA and IIB activin receptors, also leads to mislocalization of anterior and posterior markers, and a very similar overall phenotype to nodal hypomorphic mutants (Song et al., 1999). Finally, the phenotype of zebrafish that lack nodal function, either through mutations in one-eyed pinhead or in cyclops and squint combined, has been interpreted as showing AP positioning defects as well (Schier and Shen, 2000). Our results support a general requirement for early nodal signaling in assuring the proper movement of cells establishing the vertebrate AP axis.

The domain of nodal expression essential for mesoderm formation has been localized to the prestreak posterior embryonic ectoderm (Varlet et al., 1997). Only a small fraction of nodal1/Δ embryos fail to gastrulate, indicating that in most mutants the level of nodal expression in this region is above the threshold required for mesoderm formation. Nodal signaling from the visceral endoderm has been implicated in proper anterior patterning (Varlet et al., 1997). This result has been a key part of a developing hypothesis that anterior patterning in the mouse embryo is regulated, at least in part, by a head organizer located in the AVE and Nodal signaling in the axial midline could have the consequence of interrupting all cell-cell signaling between the AVE and anterior ectoderm, thus improper positioning of cells along the AP axis, is only indirectly responsible for anterior patterning defects.

Nodal signaling is required for normal midline mesendoderm development

Our results indicate a required role for nodal signaling in the formation and function of the node and its derivatives including the notochord and prechordal plate, and the gut endoderm. The midline mesendoderm tissue plays a critical role in anterior development, perhaps by reinforcing and refining an initial unstable neural induction signal provided by the AVE (Bachiller et al., 2000; Camus et al., 2000; Rhinn et al., 1998; Shalwot et al., 1999; Tam and Steiner, 1999). Thus, anterior patterning defects found in nodal hypomorphs may be due to reduced nodal function in the midline mesendoderm as well as in the visceral endoderm. A similar requirement for nodal-related signaling in midline mesendoderm and endoderm formation has been described in zebrafish (Feldman et al., 1998; Gritsman et al., 1999; Sampath et al., 1998). Different levels of nodal signaling have been shown to be necessary for the development of specific cell types in the zebrafish axial midline, with the highest levels required for prechordal plate (Gritsman et al., 2000; Thissie et al., 2000; Thissie and Thissie, 1999). High levels of nodal-related signaling also are required for endoderm development (Schier et al., 1997; Strahle et al., 1997). Our results support a dose-dependent effect for nodal signaling in axial midline and endoderm development in the mouse embryo as well. The least severely affected mutant class, the Type I embryos, lack prechordal plate and the most anterior foregut, and later show forebrain truncations and holoprosencephaly. Type I mutants have a morphologically normal node and, of the three classes of nodal hypomorphs, show the highest nodal expression levels in the node. All Type II embryos lack prechordal plate and have a deficiency of notochord in the head, with some also lacking notochord in the trunk. These embryos lack foregut, have a smaller and precociously closed hindgut, and a morphologically abnormal node. There is an accumulation of endoderm or mesendoderm at the level of the node that projects into the hindgut diverticulum, suggesting abnormal migration of the endoderm that does develop. Expression levels in the nodes of Type II embryos varied but were always well below normal. Type III embryos had nodal-positive cells in what might represent a disorganized node, and consistently developed a notochord while lacking prechordal plate. Type III mutants completely lack gut endoderm. Our results are consistent with a requirement for high nodal levels in the node for correct development of the prechordal plate and foregut, and lower levels required for notochord, node and hindgut formation.

Nodal signaling is required for visceral LR asymmetry

While nodal signaling has been implicated in LR patterning in many vertebrates, it has been difficult to assess in the mouse, owing to the phenotypic severity of the nodal null allele. Our analysis of Type I embryos, which lack left LPM nodal expression, has allowed us to demonstrate the critical role nodal plays in LR development of the heart, as well as of the cardio-vasculature, lungs and stomach. This function has been thought to be mediated, at least in part, by the transcriptional regulator Pitx2, a presumed target of nodal signaling in the left
LPM (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998). Indeed, our analysis of Type I mutants confirms Pitx2 as a downstream target gene. There is no LPM expression of Pitx2 in Type I mutants, and the lungs show a right isomerism phenotype identical to that found in Pitx2 mutants (Gage et al., 1999; Lin et al., 1999; Lu et al., 1999). This indicates a nodal-to-Pitx2 pathway in the proper development of asymmetry in the lungs, which must involve inhibition of branching normally occurring on the left side. However, targeted mutation of Pitx2 has no affect on cardiac asymmetry, whereas reduced nodal function does affect the direction of heart looping. Thus, other downstream targets of nodal signaling, still to be identified, are required for ensuring the proper orientation of the developing heart. Another proposed target of nodal signaling is downstream targets of nodal signaling, still to be identified, are required for ensuring the proper orientation of the developing heart. Another proposed target of nodal signaling is lefty-2, which encodes an antagonist of nodal signaling (Bisgrove et al., 1999; Cheng et al., 2000; Meno et al., 1999; Thissie et al., 2000). Indeed, Type I mutants lack lefty-2 expression in the LPM. Unexpectedly, expression of lefty-1 also is missing, indicating that it is also a target for nodal. The loss of lefty-1 may result because the reduced level of nodal activity in the node falls below a critical threshold for activation or maintenance of lefty-1 expression. It is possible that nodal expression in the node does not become asymmetric in Type I mutants. This might be due to the lack of lefty-1. Therefore, the LR patterning abnormalities we find in Type I mutants may be due not only to the lack of LPM expression of nodal, but also to reduced and symmetric nodal signaling around the node.

In addition to randomized LR cardiac asymmetry, Type I embryos show outflow tract defects. Transpositions of the great vessels were seen in every mutant, regardless of cardiac orientation, and were also found in the small fraction of nodal+/-A embryos that otherwise had no detectable defects. This suggests that the process of conotruncal septation is extremely sensitive to nodal levels. Interventricular septal defects were also found. Thus, it is clear that nodal function in cardiovascular development is more than just to supply cues for sidedness. It also is required for fundamental morphological structuring of the heart and vasculature.

Abdominal LR defects were found in Type I mutants at only a very low frequency. This contrasts with the randomized sidedness of stomachs in cryptic mutants (Yan et al., 1999). Cryptic mutants do not express nodal in the LPM and are presumed to have a block in nodal signaling around the node, whereas Type I mutants retain some level of nodal activity around the node. Therefore we can speculate that nodal signaling from the node plays some role in stomach handedness. This signaling role may be for the proper development of endoderm that later contributes to stomach development, and thus is only indirectly responsible for stomach laterality. In some nodal mutants, the stomach was located dorsal to the lungs, indicating a failure of elongation of the esophagus. These individuals also had underdeveloped lungs, suggesting a connection between esophageal and lung growth. The esophageal growth defect is probably unrelated to the process of stomach rotation, as undescended stomachs were found on either the left or right side of nodal mutants. This phenotype has not been described previously and may indicate another target tissue for nodal signaling, or again reflect an endoderm defect stemming from reduced nodal function at an earlier stage.

In summary, the analysis of the nodal hypomorphic mutation has permitted the dissection of multiple aspects of nodal function in the developing mouse embryo. Our study shows that nodal signaling, in addition to being essential for mesoderm formation, regulates AP positioning and anterior patterning, midline development, endoderm and gut formation, and LR specification. Further elucidation of nodal function in these early developmental processes will benefit from the application of conditional mutagenic approaches, using stage- and tissue-specific Cre transgenic lines as they become available, in conjunction with floxed nodal mice.

We thank Marnie Halpern, Susan Mackem and Shai Izraeli for critical comments on the manuscript.

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