Genetic dissection of nodal function in patterning the mouse embryo

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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 insertion 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
Xenopus embryos is sufficient to alter organ asymmetry (Levin et al., 1997; Sampath et al., 1997), a requirement for nodal signaling in mouse LR development has not been unequivocally shown, owing to the early arrest of the nodal null mutant. Indirect evidence for such a role has come from genetic studies. In mouse and zebrafish mutants with LR defects, asymmetric expression of nodal or the zebrafish nodal-related cyclops is perturbed (Collignon et al., 1996; Heymer et al., 1997; Izraeli et al., 1999; Lowe et al., 1996; Melloy et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998). Mutations in mouse cryptic or zebrafish one eyed pinhead cause LR defects (Yan et al., 1999). These genes encode related EGF/CFC extracellular proteins that are required for nodal signaling (Gritsman et al., 1999; Kumar et al., 2001; Saijoh et al., 2000). Mouse embryos with a mutation in Smad2, which functions to transduce nodal signals (Kumar et al., 2001), show LR abnormalities when rescued beyond early gastrulation (Heymer et al., 1999). Embryos heterozygous for both a nodal null mutation and mutation in Smad2 also show LR defects (Nomura and Li, 1998). However, chimeras with a high percentage of Smad2 mutant cells undergo normal LR development (Tremblay et al., 2000), complicating the interpretation of the compound heterozygotes. In addition, certain mutant alleles of cyclops, presumed to be nulls, do not alter LR patterning of the zebrafish heart (Chen et al., 1997). Furthermore, no LR defects were reported in mouse chimeras made with a large contribution of nodal mutant cells (Varlet et al., 1997). Therefore it is still an open question whether nodal is necessary for patterning the LR axis.

Nodal signaling also has been implicated in anterior neural development. Anterior defects were found in mouse chimeras made with normal and nodal mutant cells, in which the visceral endoderm was composed exclusively of nodal mutant cells (Varlet et al., 1997). Mouse embryos heterozygous for mutations in both nodal and Smad2 also show anterior defects including cyclopia (Nomura and Li, 1998). Zebrafish mutants in either of the nodal-related genes, cyclops or squint, result in anterior patterning defects (Feldman et al., 1998; Hatta et al., 1991; Heisenberg and Nusslein-Volhard, 1997). However, the cyclops and squint mutations both cause midline defects that may interfere with proper anterior patterning, suggesting that nodal may be involved only indirectly in anterior patterning.

A loss-of-function analysis of nodal in LR and anterior development is impossible with the original nodal mutant, owing to its early developmental arrest. Therefore, we have used a Cre/loxP strategy (reviewed by Marth, 1996) to make a conditional mutant allele flanked by loxP site-specific recombination signals (floxed). This floxed allele can undergo Cre-mediated deletion, leading to loss of function. Homozygotes are viable and fertile, and so can be used in conjunction with stage- and tissue-specific Cre transgenic lines to carry out conditional mutational analysis of nodal function. In the course of characterizing the floxed allele, we discovered that although floxed homozygotes were normal, embryos that were compound heterozygotes for the floxed and a nodal null allele were not. These results were found in the absence of any Cre-mediated recombination, indicating that the floxed allele is hypomorphic. Embryos with reduced nodal function undergo gastrulation but then display abnormalities, which fall into distinct phenotypic classes, at later stages. We have carried out a comprehensive analysis of these phenotypes and provide conclusive evidence for nodal regulation of multiple developmental processes in the mouse embryo, beyond its role in mesoderm formation.

**MATERIALS AND METHODS**

**Gene targeting**

The nodal cDNA was used to isolate an 18 kilobase (kb) genomic clone from a 129Sv lambda library. An 8 kb BamHI 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 BamHI 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-galactosidase 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 (nodalfl), we bred nodalfl1 animals with Ella-Cre mice. Embryos derived from Ella-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 wildtype allele, F114/N12 CCCAGCAAAATGCAAATCTGACC (specific for nodal intron 2) and R403/n3U ACGTTTCTGTCCTCGGATAGAC (specific for the 3′UTR of nodal) for the floxed allele, F580/neo CCGAATATCATGTTGAAAAATGCCC (specific for the neo gene) and R403/n3U; for the nodalfl allele, R878 AGTTCCCAACACCTCAATAACC (specific for nodal intron 2); and F654 AGAACGAGAGCGTTTGGG (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 K4Fe(CN)6, 5 mM K3Fe(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-beta-galactosidase 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 5’ 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 nodalβ/allele. Breeding of nodalβ/ mice. Breeding of nodalβ/+ 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 nodalβ/ allele is capable of undergoing Cre-mediated recombination, we crossed nodalβ/ 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 nodalβ/ allele can be efficiently recombined by Cre to produce the deleted allele (nodalβ/).

The nodalβ/ allele is a hypomorph

Analysis of embryos with a nodalβΔ/ genotype showed a pre-gastrulation block identical to the original nodal insertion mutant, confirming that the nodalβ allele is a null. Unexpectedly, nodalβΔ/ 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+/ mice with nodalβ/ homozygotes, none carrying Cre, revealed 41% with overt developmental defects at E7.5. A sample set was genotyped and all phenotypically abnormal embryos were nodalβΔ/. 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 nodalβΔ/+ 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 nodalβΔ/+ 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 nodalβΔ/+ embryos showed morphological abnormalities as well as alterations in the pattern of nodal expression, as detected by X-gal staining for
**Fig. 2.** Abnormalities in E7.5 nodal\(^{fl/\lambda}\) embryos.

(A-D) Embryos X-gal stained for β-gal activity, viewed laterally with anterior towards the left. (E-J) Transverse sections from the embryos directly above them, taken at the positions indicated. (E,F,I,J) Anterior is towards the top. (G) Distal is to the right. (H) Posterior is towards the right. (A) A nodal\(^{fl/\lambda}\) (fl/+\) embryo with a pattern of staining indicating normal nodal expression in the primitive streak (bracketed). (B) A similarly staged nodal\(^{fl/\lambda}\) (fl/\Lambda) embryo. Staining (bracketed) does not extend as far distally and there is a constriction at the embryonic/extra-embryonic boundary (arrow). (C) An older nodal\(^{fl/+}\) embryo showing staining in the node (arrowhead). (D) A similarly staged nodal\(^{fl/\lambda}\) embryo showing abnormally positioned staining (arrowhead) and a constriction. (E) Proximal cross section of the embryo in A revealing the normal direct contact (arrowhead) between the anterior visceral endoderm (ave) and the embryonic ectoderm (ee). Mesoderm, m. (F) Section of the embryo in B revealing the abnormal accumulation of mesoderm (arrowhead) between the embryonic ectoderm and visceral endoderm (ve). (G) Section of the embryo in C. Staining is apparent in the mesendodermal cells (me) around the node (arrowhead). (H) Section of the embryo in D. Staining is also seen in mesendodermal cells (arrowhead). (I) Distal cross section of the embryo in A showing the normal accumulation of mesoderm (arrowhead) between the thin squamous visceral endoderm layer and the embryonic ectoderm. (J) Distal cross section of the embryo in B showing the thick layer of mesoderm (arrowhead) between the embryonic ectoderm and the abnormally cuboidal visceral endoderm.

β-gal expressed from the nodal\(^{fl/1}\) 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\(^{fl/\lambda}\) 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 a morphologically distinct group of cells at the anterior midline close to the embryonic/extra-embryonic boundary (Fig. 2E). In nodal\(^{fl/\lambda}\) 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\(^{fl/\lambda}\) 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. 2F). 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\(^{fl/\lambda}\) 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 (Hermesz 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\(^{fl/\lambda}\) 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\(^{fl/\lambda}\) 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...
nodal patterning function in mouse embryos

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. 4I). In nodal hypomorphic mutants, Hesx1/Rpx expression also was located more distally than normal (Fig. 3P). Together these data indicate an early nodal function in the axial rotation that establishes the normal position of cells along the AP axis.

The AP axial positioning defects seen at E7.5 were more pronounced by E8.5. Analysis of mutant embryos (n=219) revealed three distinct phenotypic classes (Fig. 4A-C). One class, which represented 20% of mutants, developed with the anterior region outside the yolk sac (Fig. 4B). Another 29% of mutants developed completely outside the yolk sac (Fig. 4C). The remaining 51% developed normally within the yolk sac and constituted another class that we hereafter refer to as Type I. Embryos that developed partially and completely outside the yolk sac are designated as Type II and Type III mutants, respectively.

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Fig. 4. Anterior patterning defects in nodal∆/∆ 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∆/∆ embryos showed the same arrangement. (B) Type II nodal∆/∆ embryo (fl/∆-II) showing the anterior region developing outside the yolk sac. (C) Type III nodal∆/∆ embryo (fl/∆-III) developing completely outside the yolk sac. (D) WT embryo with cranial neural folds showing a clear midline demarcation. (E) Type I nodal4I/∆ embryo (fl/∆-I). The head is small and there is no midline separation in the forebrain (arrowhead). (F) WT embryo showing Otx2 expression restricted to the extreme anterior (arrowhead). (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 contrast, in both Type II and Type III mutants Otx2 expression was reduced and restricted to a small anterior domain (Fig. 4J,K).

In normal embryos, both the AVE and the midline mesendoderm 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 mesendoderm, or both. To assess the state of the midline mesendoderm 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. 5F,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 mesendoderm 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∆/∆ allele by X-gal staining. Normally at this stage, nodal is expressed around the node and is expressed from the chicken α-globin locus (Camus et al., 2000; Tam and Steiner, 1999). Thus, the midline mesendoderm mutants completely lacked left LPM expression (Fig. 6C-F,H-J). For Type I embryos there was an apparently normal level
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 (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,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
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 nodalfl1/ nodal−/− embryos**

<table>
<thead>
<tr>
<th>Organ or tissue</th>
<th>Phenotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great vessels (n=48)</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Transposed</td>
<td>48 (100%)</td>
</tr>
<tr>
<td>Heart direction (n=48)</td>
<td>Normal</td>
<td>23 (48%)</td>
</tr>
<tr>
<td></td>
<td>Reversed</td>
<td>25 (52%)</td>
</tr>
<tr>
<td>Lungs (n=48)</td>
<td>Normal, asymmetric</td>
<td>2 (4%)</td>
</tr>
<tr>
<td></td>
<td>Abnormal, asymmetric</td>
<td>4 (8%)</td>
</tr>
<tr>
<td></td>
<td>Right isomeric</td>
<td>42 (88%)</td>
</tr>
<tr>
<td>Stomach (n=45)</td>
<td>Normal left</td>
<td>37 (82%)</td>
</tr>
<tr>
<td></td>
<td>Normal right</td>
<td>4 (9%)</td>
</tr>
<tr>
<td></td>
<td>Midline left</td>
<td>1 (2%)</td>
</tr>
<tr>
<td></td>
<td>Midline right</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Liver (n=46)</td>
<td>Normal appearance</td>
<td>30 (65%)</td>
</tr>
<tr>
<td></td>
<td>Reduced size and/or pale</td>
<td>16 (35%)</td>
</tr>
</tbody>
</table>
nodal patterning function in mouse embryos

hypomorphic, independent of Cre-mediated recombination. Expression of the floxed allele may be affected at the level of transcription or mRNA processing, owing to the presence of the IRES-βgeo cassette, which more than doubles the size of the transcript. The floxed allele combined with a nodal-null allele results in three distinct mutant phenotypic classes. These different classes may result from expression levels of the hypomorphic allele falling below critical thresholds in one or more of the multiple domains of nodal expression. Additionally, because nodal may regulate its own transcription and that of components of the nodal pathway (see below), reduced nodal levels at an early stage could have consequences for later domains of expression. Thus, each phenotypic class may reflect a unique conflation of specific temporal and spatial patterns of reduced nodal signaling. Our analysis of these classes provides direct evidence for nodal-mediated regulation of AP axial positioning, and anterior and midline patterning, and confirms a crucial role for this gene in mediating LR patterning of the viscera.

Nodal signaling 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 nodal-fl/fl 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 that anterior patterning in the mouse embryo is regulated, at least in part, by a head organizer located in the AVE

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\frac{\text{nodal}}{\text{normal node}} \quad \text{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 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; Thisse et al., 2000; Thisse and Thisse, 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 lefty-1, which encodes an antagonist of nodal signaling (Bisgrove et al., 1999; Cheng et al., 2000; Meno et al., 1999; Thisse 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 nodalfl1/2A embryos that otherwise had no detectable defects. This suggests that the process of conotruncal septation is extremely sensitive to nodal levels. Intervertricular 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.

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