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Broad mesodermal and endodermal deletion of *Nodal* at post-gastrulation stages results solely in left right axial defects

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Abstract

Nodal signaling is a critical regulator of multiple aspects of early vertebrate development including asymmetry along the left/right (LR) axis. To study Nodal function occurring specifically in the post-gastrulation embryo, we have used Cre/loxP based conditional mutagenesis. A floxed allele of *Nodal* was generated and shown to have wild type function. This allele was then used in conjunction with the T-Cre line, which expresses Cre recombinase broadly in the mesodermal and definitive endodermal lineages posterior to the cranial region. T-Cre activity leads to complete deletion of *Nodal* prior to its normal transient expression in the early somite stage lateral plate mesoderm, thereby causing severe LR developmental defects. No other abnormalities were found, suggesting that Nodal signaling has no additional essential functions in developmental patterning within the extensive mesodermal and endodermal domains marked by T-Cre activity.

Keywords

left right axis; conditional mutagenesis; *Nodal*; T-Cre; lateral plate mesoderm

Introduction

Nodal, a transforming growth factor (TGF)- β like secreted signaling molecule, plays multiple essential roles in axial patterning and germ layer specification during early vertebrate development (Ang and Constam, 2004; Shen, 2007). These various functions are reflected in the complex and dynamic expression pattern found for the *Nodal* gene. During early mouse development, expression occurs at several sites and at several developmental stages indicative of Nodal signaling being used and reused to progressively pattern the embryo. One of the most striking domains is the strictly left sided expression in the lateral plate mesoderm (LPM), occurring in a narrow developmental window at early somite stages. The ultimate outcome of Nodal signaling from the LPM, which is intimately tied into the action of its antagonist Lefty (reviewed by Tabin, 2006), is the characteristic left/right (LR) asymmetric location and/or structure of the visceral organs (reviewed in Levin, 2005).

Insight into Nodal function has come from the analysis of various mutant alleles. The null mutant arrests prior to gastrulation with defects in anterior-posterior patterning and without forming mesoderm or endoderm (Iannaccone et al., 1992; Conlon et al., 1994). More recently conditional mutagenesis using the Cre/loxP approach (Lewandoski, 2001; Branda

and Dymecki, 2004) has been used to analyze *Nodal* expression specific to the epiblast (Lu and Robertson, 2004). The introduction of loxP sites and selectable markers can often result in hypomorphic alleles that are informative even in the absence of Cre mediated recombination (Meyers et al., 1998). Two alleles with reduced *Nodal* function and with less severe defects than the null mutant have been described (Lowe et al., 2001; Saijoh et al., 2003). Embryos carrying the *Nodal^{fl1}* hypomorphic allele, when compound heterozygous with the null allele, develop past the block at gastrulation seen in *Nodal* null homozygotes, but then display a variety of developmental abnormalities. Analysis of the least severe class of mutants verified the critical role *Nodal* signaling plays in patterning the LR body axis in mouse development (Lowe et al., 2001). Embryos homozygous for the *Nodal^{neo}* allele undergo normal gastrulation but then develop LR defects due to loss of *Nodal* expression from the LPM (Saijoh et al., 2003). To dissect the various temporal and spatial domains of *Nodal* function further, we have generated a new conditional mutant allele, *Nodal^{fl2}*. Here we use this allele in conjunction with the recently described T-Cre transgenic line, which expresses Cre recombinase broadly in the mesodermal and endodermal lineages commencing soon after gastrulation initiates (Perantoni et al., 2005). We find that extensive deletion of *Nodal* affects only LR development, resulting in a suite of defects characterized by randomization and right isomerisms of the thoracic and abdominal viscera.

Results

Development and characterization of the *Nodal^{fl2}* line

We previously reported that a floxed *Nodal* allele, *Nodal^{fl1}*, originally developed for conditional mutagenesis, was hypomorphic. The overall reduced level of function of this allele in the absence of Cre-mediated recombination provided insight into several aspects of *Nodal* function in early mouse development (Lowe et al., 2001). To generate loss of *Nodal* function in specific tissues and developmental stages in an otherwise normal embryo, we created a new conditional allele, *Nodal^{fl2}*. This new allele differs from wild type only by the presence of loxP sites in the first intron and 3' untranslated region (UTR; Fig. 1). Homozygous *Nodal^{fl2}* animals were derived at the expected frequency and were found to be normal and fertile. However, to determine whether *Nodal^{fl2}* is truly a wild type allele, we examined embryos compound heterozygous for *Nodal^{fl2}* and the null allele, *Nodal^{Δ1}* (Lowe et al., 2001). Using this approach in our previous study of *Nodal^{fl1}*, we found numerous defects indicating it was hypomorphic (Lowe et al., 2001). Even in the small percentage of *Nodal^{fl1/Δ1}* embryos that appeared completely normal at embryonic day (e)14.5, we found transpositions of the aorta and pulmonary trunk and right isomerisms of the lung. Here, a similar analysis of *Nodal^{fl2/Δ1}* embryos revealed none with transpositions of the great vessels or right lung isomerisms, or with any other overt defect (Table 1). Examination of embryos at earlier stages, beginning at e7.5, also revealed no abnormalities with only three embryos found resorbed (Table 2). While the latter could not be genotyped, the frequency is not significantly different from that seen in normal matings. In total, we examined almost 200 embryos derived from crosses of *Nodal^{fl2/fl2}* homozygotes and *Nodal^{+/Δ1}* heterozygotes, of which 52 were genotyped. Of these, 50% were *Nodal^{fl2/Δ1}* (the expected Mendelian frequency). Together, these data provide strong evidence that the *Nodal^{fl2}* allele is fully functional.

Characterization of early T-Cre activity in relation to *Nodal* expression

The recently described T-Cre transgenic line, in which Cre recombinase is driven by regulatory elements of the T/Brachyury gene, has been shown to be activated in mesoderm at the primitive streak stage (Perantoni et al., 2005). This suggested that it would be an ideal genetic tool to examine post-gastrulation *Nodal* function. To confirm that there is no earlier T-Cre activity, which might impact *Nodal* expression required at or prior to mesoderm

formation, we evaluated the exact time of onset using the conditional reporter lines R26R (Soriano, 1999) and R26R-EYFP (Srinivas et al., 2001). To complement this analysis we used the *Nodal*^{f1} line as a reporter. In embryos from this line, which carries an IRES β geo cassette inserted just after the stop codon, β -galactosidase expression mirrors that of *Nodal* mRNA (Lowe et al., 2001). Cre mediated deletion of *Nodal*^{f1} eliminates β geo. Thus, domains of Cre activity that overlap *Nodal* expression can be identified as regions undergoing loss of X-gal staining (Kumar et al., 2007).

The earliest activity detected in embryos derived from T-Cre $\times$ R26R matings was indeed at e6.5, in nascent mesoderm migrating extra-embryonically (Fig. 2A). This was confirmed by confocal analysis of EYFP fluorescence in embryos derived from T-Cre $\times$ R26R-EYFP matings (Fig. 2B). Evidence of recombination was then seen in the lateral mesodermal wings migrating out from the primitive streak (Fig. 2C,D). By the early headfold stage, T-Cre activity was apparent along the entire primitive streak (Fig. 2E-G). At primitive streak stages, *Nodal* is expressed in posterior embryonic ectoderm migrating into the primitive streak but is rapidly downregulated as cells differentiate into mesoderm. X-Gal staining of late streak stage embryos derived from T-Cre $\times$ *Nodal*^{f1} matings did not reveal any difference between T-Cre negative and T-Cre positive embryos (Fig. 3A,B), indicating there is minimal overlap in expression either spatially or temporally between *Nodal* and T-Cre at these stages.

As the primitive streak elongates, *Nodal* expression localizes to the very anterior of the streak and by headfold stages is found further anterior, in cells surrounding the indentation at the distal tip of the embryo. This region has been called the node, but a recent analysis (Blum et al., 2007) suggests it is more properly considered the posterior notochord (PNC) with the term node reserved for the dynamic population of cells at the anterior end of the primitive streak containing the midgastrula organizer (MGO) (Robb and Tam, 2004). Beginning at approximately the 2 to 3 somite stage (2-3 ss), *Nodal* expression around the PNC becomes stronger on the left side. At approximately the same stage, *Nodal* expression commences in the left LPM, persisting until the 8-9 ss (Lowe et al., 1996). Over these same stages, T-Cre activity detected using the R26R reporter was found throughout the lateral and somitic mesoderm (Fig. 2H-K). X-gal staining also began to be evident in the PNC and notochord by the 3-4 ss (Fig. 2K). Given that Cre is driven by primitive streak specific regulatory elements, this result probably reflects the relatively late onset of T-Cre activity in the node/anterior streak/MGO, from which the PNC and notochord are derived, rather than *de novo* expression. Analysis of T-Cre activity over these same stages using the *Nodal*^{f1} line as a reporter revealed a progressive loss of X-gal positive cells around the PNC from late headfold to early somite stages (Fig. 3D,F,H) and complete absence of X-gal staining in the LPM (Fig. 3H). Together these results show that T-Cre should completely delete the *Nodal*^{f12} allele within the mesoderm prior to its normal expression in the LPM, but only partially delete at the PNC well after expression in this domain commences. We also confirmed the broad T-Cre activity in mesoderm and posterior gut endoderm reported for later stages (Perantoni et al., 2005). Although activity does not extend fully into the most anterior cranial region (Fig. 2L), T-Cre should lead to extensive deletion of *Nodal* throughout the majority of the embryo.

Conditional mutagenesis of *Nodal* using T-Cre

To determine the phenotypic and molecular consequences of T-Cre mediated deletion of *Nodal*, we first produced animals heterozygous for both T-Cre and the null allele, *Nodal* ^{Δ 1}. T-Cre;*Nodal*^{+/ Δ 1} animals were then bred with homozygous *Nodal*^{f12/f12} animals, and embryos were collected at various stages for analysis and genotyping. We expected approximately 25% to be T-Cre;*Nodal*^{f12/ Δ 1}, the appropriate genotype for conditional mutagenesis resulting from Cre deletion of *Nodal*^{f12}. To confirm that T-Cre indeed deletes

the *Nodal*^{f12} allele and determine the extent of recombination, we carried out PCR reactions that specifically amplify across the deletion site (Fig. 1D). We also assessed any reduction in the level of the normal *Nodal* transcript by performing whole mount in situ hybridization (WMISH) with a probe specific for *Nodal* exon 2, which is removed in the deleted allele, *Nodal*^{Δ2} (Fig. 1C,D). PCR genotyping of e7.5 embryos showed the expected *Nodal*^{Δ2} product in T-Cre positive embryos indicating successful recombination (not shown). As expected, WMISH with the *Nodal* exon 2 probe showed no significant difference between *Nodal*^{f12} control and T-Cre;*Nodal*^{f12/Δ1} embryos at the late streak stage (Fig. 3I,J). However, by the late headfold stage there was reduced expression around the PNC (Fig. 3K,L), providing the first indication of recombination in *Nodal* expressing cells. By the 1-2 ss, the reduction in *Nodal* expression was even more apparent (Fig. 3M,N). In addition, although *Nodal* expression in the left LPM begins by the 3 ss in control embryos (Fig. 3O) it was undetectable in T-Cre;*Nodal*^{f12/Δ1} embryos at this and all later somite stages (Fig. 3P), indicating complete recombination in this lineage. These results indicate that T-Cre activity occurs early enough in the mesodermal lineage to cause complete deletion of *Nodal* from the LPM prior to its normal onset of expression. In contrast, a significant level of *Nodal* expression occurs around the PNC prior to its eventual elimination.

T-Cre mediated deletion of *Nodal* leads to LR phenotypes only

To determine the morphological consequences of T-Cre mediated deletion of *Nodal*, we examined embryos from T-Cre;*Nodal*^{+/-Δ1} × *Nodal*^{f12/f12} matings for any overt defects. We found no abnormalities at e7.5, indicating that T-Cre activity does not interfere with critical *Nodal* functions in the pre- and early gastrula stage embryo. However, there were morphological abnormalities seen at late e8.5 and e9.5, specifically in the direction of heart looping. Normally the heart tube loops to the embryo's right side, but 15% (n=97) of embryos derived from T-Cre;*Nodal*^{+/-Δ1} × *Nodal*^{f12/f12} matings had reversed heart looping (Fig. 4A). Genotyping of a representative subset showed that all of these carried T-Cre and the *Nodal*^{Δ1} allele. The 15% frequency corresponds to approximately half of the 25% of embryos hypothetically expected to have the appropriate genotype for conditional mutagenesis, indicating that in embryos deleted for *Nodal* the direction of heart looping is randomized (half normal, half reversed). At later stages, hearts from embryos genotyped as *Nodal*^{Δ1} and positive for T-Cre showed ventricular septal defects as revealed by mixing of different colored dyes injected separately into the left or right ventricle (Fig. 4B,C). In addition, 26 of 63 embryos examined at e13.5d and later showed right lung isomerism (Fig. 4D,E) as well as transpositions of the great vessels and small or absent spleens (not shown). Seven of these embryos also had stomachs placed on the right side. All of these phenotypically abnormal embryos, but none of the apparently normal ones, genotyped as *Nodal*^{Δ1} and positive for T-Cre (Table 3).

Further analysis failed to detect abnormalities in other tissues or developmental processes, including the limbs and tail, or in kidney and urogenital development. Notably, none of the embryos examined in this study displayed midline or anterior/head morphological defects, which arise in the majority of *Nodal*^{f11/Δ1} hypomorphic embryos (Lowe et al., 2001). These negative results indicate that T-Cre mediated conditional mutagenesis of *Nodal* solely targets *Nodal* function in LR development.

Molecular analysis of T-Cre deleted embryos

The observed abnormalities in LR development no doubt arise due to the loss of *Nodal* from the left LPM. To determine the molecular consequences of T-Cre mediated deletion of *Nodal* at early somites stages, we carried out WMISH with probes for genes that have been implicated as downstream targets of *Nodal* signaling, including *Lefty-2*, *Pitx2* and *Nodal* in the left LPM, *Lefty-1* in the left prospective floor plate (PFP) and *Nodal* around the PNC. As

expected given the complete loss of *Nodal* from the LPM in *Nodal^{fl2/Δ1}* embryos positive for T-Cre, the normal expression of *Lefty-2* in the LPM (Fig. 5A) was lost (Fig. 5B). In addition, the normal expression of *Pitx2* in the LPM and heart (Fig. 5C) was not detected in T-Cre;*Nodal^{fl2/Δ1}* embryos (Fig. 5D). Similarly, expression of *Lefty-1* in the PFP (Fig. 3O; Fig. 5A,E) also was not found (Fig. 3P; Fig. 5B,F). Although there is no direct evidence, the most posterior expression of *Lefty-1* in the midline of the PNC (brackets in Fig. 3O and Fig. 5E) may be induced wholly or in part by Nodal signals coming from nearby PNC crown cells. If so, our results suggest that by the 2-3 ss Nodal levels in the PNC of T-Cre deleted embryos are below the threshold critical for *Lefty-1* induction.

To examine expression of the deleted *Nodal* allele, we used a probe specific for the still remaining exon 1 (Fig. 1D). This probe detects the undeleted allele as well, as seen by the normal WMISH pattern in wild type control embryos (Fig. 5E). For T-Cre;*Nodal^{fl2/Δ1}* embryos an estimation of the amount of continued expression of the deleted allele was made by comparing the hybridization signal detected by the exon 1 probe to that from the exon 2 probe. Using the exon 1 probe we were unable to detect any expression in LPM of 3-4 ss T-Cre deleted embryos, but there were near normal levels around the PNC (Fig. 5F), compared to the weak and patchy hybridization signal detected using the exon 2 specific probe (Fig. 3N,P). This result indicates that the deleted allele continues to be transcribed around the PNC of T-Cre;*Nodal^{fl2/Δ1}* positive embryos. Importantly, by the 3-4 ss this expression was stronger on the left side thereby displaying the same asymmetric pattern seen in normal embryos (Fig. 5E,F).

We also examined the expression of *Cerl-2/Dante*, which is asymmetrically expressed around the PNC at early somite stages in a pattern opposite to that of *Nodal* (Fig. 5G) but is not known to be a target of Nodal signaling. Importantly, there was no loss of expression of *Cerl-2/Dante* in T-Cre;*Nodal^{fl2/Δ1}* embryos (Fig. 5H). In addition, right asymmetric expression was apparent at the same stages as in control embryos. As another indicator of PNC function, we asked if there was any alteration in the expression of two genes expressed within the PNC, *Dnahc5* (Fig. 5I) and *Lrd* (not shown), encoding components of the dynein motor complex involved in ciliary beating and nodal flow (Hirokawa et al., 2006). Both were found to be expressed normally in T-Cre deleted embryos (Fig. 5J and not shown). In addition, we found normal expression of the midline markers T/Brachyury, Shh and Foxa2 (not shown). In summary, these data argue that T-Cre mediated conditional mutagenesis of *Nodal* results in the complete loss of asymmetric signals from the LPM while having apparently no impact on gene expression patterns at and around the PNC.

Discussion

As shown previously (Perantoni et al., 2005) and confirmed here, T-Cre activity in the primitive streak commences early enough and broadly enough to result in recombination throughout the mesodermal lineages of the developing embryo. In addition, activity within cells of the gastrula organizer at the anterior of the primitive streak leads to recombination also occurring in the endoderm and midline mesendoderm, although the timing is such that only the more posterior domains of these lineages are affected. Even so, it is clear that T-Cre activity can cause deletion of floxed alleles throughout an extensive portion of the embryo. Thus, utilization of this line in conjunction with our newly described conditional allele of *Nodal* provided a unique opportunity to assess the consequences of broad deletion of Nodal activity commencing after gastrulation. The fact that we find only LR defects, which presumably derive from loss of Nodal signaling from the e8.5 LPM, suggests that there are no other essential developmental functions for Nodal in mesodermal or endodermal tissues arising at later stages. In fact, there appear to be very few additional domains of *Nodal* expression beyond e8.5. The only well documented expression is that found in the first

branchial arch and forebrain roof starting at e9.5 (Andersson et al., 2006). We do not see evidence of T-Cre activity in either of these regions of the embryo, so an evaluation of Nodal function in these domains awaits development and/or utilization of appropriate Cre lines. Expression of Nodal receptors has been seen in the developing limb bud (Jornvall et al., 2004) although *Nodal* has not been detected, perhaps because levels are too low or transient. However, the lack of any limb defects in T-Cre deleted embryos suggests that if Nodal signaling is active in the limb bud, it is dispensable.

Two previous studies describing targeted deletion or disruption of the *Nodal* enhancer that specifically drives expression in the PNC have shown that this results in almost undetectable levels of *Nodal* expression at the PNC, and is accompanied by absence of expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). These findings have led to the conclusion that the principal role for Nodal signaling originating from the crown cells of the PNC is to initiate expression of *Nodal* in the most proximal part of the LPM, where activity then subsequently amplifies and expands *Nodal* expression throughout the LPM and induces downstream target genes that ultimately control the LR asymmetric development of the viscera. One question not addressed is whether Nodal signaling at the PNC plays any additional role, in LR development or otherwise. Theoretically, this could be addressed using embryos lacking *Nodal* in PNC or LPM, but not both, and comparing phenotypes to embryos lacking *Nodal* in both domains. While it is technically challenging to generate embryos in which *Nodal* is removed from the PNC but retained in the LPM, T-Cre deletion of *Nodal* results in embryos that lack LPM expression but do retain, at the late headfold stage and perhaps beyond, a substantial level of expression at the PNC. The level we see is at least as high, if not higher, than that seen in certain allelic combinations reported by Brennan et al. and Saijoh et al., which were sufficient to initiate expression of *Nodal* in the LPM and thus produced no LR patterning abnormalities (Brennan et al., 2002; Saijoh et al., 2003). As a functional test of the residual levels of *Nodal* remaining in the PNC, we asked whether these could induce expression of the T-Cre deleted *Nodal* allele in the LPM. However, we could not detect any transcription of the residual exon 1 fragment by WMISH, probably due to the absence of the Nodal positive feedback loop that in normal embryos amplifies the level of *Nodal* transcripts in the LPM to robust levels. As an alternative readout of Nodal dependent *Nodal* expression, we examined the PNC and found that although there is partial T-Cre deletion, Nodal activity at the PNC is sufficiently high to allow continued expression of the *Nodal* gene. Importantly, the T-Cre deleted gene still develops the left asymmetric expression pattern seen in wild type. This asymmetry may be dependent on continued Nodal signaling but is clearly independent of antagonism by Lefty-1, perhaps because of the unaltered activity of Cerl-2/Dante, another Nodal antagonist. In addition, this finding shows that *Nodal* asymmetry around the PNC does not require Nodal activity feeding back from the left LPM, although it may augment it in normal embryos. Together these results argue that T-Cre deleted embryos at pre-somite and early somite stages, when symmetry is first broken, have an apparently functionally normal PNC with intact Nodal signaling. The fact that the LR defects we observed are identical to those found in embryos that lack both PNC and LPM Nodal signaling provides strong support for the idea that the sole contribution of *Nodal* expression at the PNC is to initiate LPM expression.

In summary, we find that the consequences of broad deletion of *Nodal* in the developing post-gastrulation embryo can be wholly assigned to the loss of Nodal activity in the left LPM at early somite stages, arguing that essential developmental functions in the extensive mesodermal and endodermal domains marked by T-Cre activity are complete by e8.5. The question of whether Nodal signaling is essential for post-gastrulation cranial development or has a redundant function in organogenesis is still open, as is whether there is any role in homeostasis in the adult or in disease states.

Experimental Procedures

Gene targeting

To generate the *Nodal*^{f12} targeting vector, we began with intermediate constructs from the *Nodal*^{f11} targeting vector containing only the 5' loxP site (Lowe et al., 2001). Site directed mutagenesis was used to modify four nucleotides within the 3' untranslated region (UTR), and thereby generate two new restriction sites (Fig. 1A). These were used to directionally introduce a floxed PGKneo cassette from the ploxneo vector (Yang et al., 1998). ES cells correctly targeted with this vector were transiently transfected with a Cre containing expression plasmid and desired recombinants were identified using the PCR primer pair B/C (Fig. 1C). Primer B: 5'-ATGATTGTGGAGGAGTGTGGGTGC-3'; Primer C: 5'-TCTCTGGCTTGGCAGGTCTAAG-3'.

Mouse and embryo genotyping

Nodal^{f12} animals were genotyped routinely using primer set B/C (Fig. 1C). To genotype embryos for the *Nodal*^{Δ2} allele, primer set A/C was used (Fig. 1D). Primer A: 5'-AGCACTGGATGCTTGCTCTTCC-3'. Genotyping for the *Nodal*^{f11} and *Nodal*^{Δ1} alleles was described previously (Lowe et al., 2001). For genotyping T-Cre mice and embryos, we utilized primers wholly within the Cre sequence as described (Lowe et al., 2000).

Phenotypic and molecular analysis

Procedures for X-Gal staining of R26R and *Nodal*^{f11} embryos and confocal microscopic analysis of R26R-EYFP were described previously (Kumar et al., 2007). Whole mount in situ hybridization (WMISH) was carried out as described (Lowe and Kuehn, 2000) with modifications (Kumar et al., 2007). To detect the deleted *Nodal* transcript, an exon 1 containing EcoR1/BamH1 fragment from the mouse *Nodal* cDNA was sub-cloned into the EcoR1 and BamH1 sites of pBluescriptSK. DIG labeled antisense RNA probe was made using T3 polymerase following digestion with EcoR1. The *Nodal* exon 2 probe specific for the undeleted wild type transcript has been described (Zhou et al., 1993). Analysis of cardiac and vasculature malformations was done by colored latex injections into right and left ventricles as described previously (Oh and Li, 1997).

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Fig. 1. Generating the *Nodal*^{fl2} allele. **A:** Wild type allele showing primary sequence around the sites where the 5' and 3' loxP sites were inserted. Site directed mutagenesis (red arrows) was used to create the Xba I and Hpa I restriction sites in the *Nodal* 3' untranslated region (UTR). **B:** *Nodal*^{fl2} targeting vector. The 5' end of the 6.4 kb left homology arm is the NotI site in Exon 1. The 3' end of the 2.6 kb right homology arm is the Bam HI site approximately 3 kb downstream of the gene. The loxP sequences are shown in red. The floxed PGKneo cassette was an Xba I/Hpa I fragment from ploxpneo plasmid. **C:** The *Nodal*^{fl2} allele was generated by incomplete Cre recombination and identified by PCR using primer set B/C. **D:** Further Cre recombination generates the deleted allele, *Nodal*^{Δ2}, identified by PCR using the primer set A/C.

Fig. 2.

Analysis of T-Cre mediated recombination using the R26R and R26R-EYFP reporter lines. A, C, E-G, and H-L are images of X-gal stained T-Cre $\times$ R26R embryos. B and D are images of EYFP fluorescence from T-Cre $\times$ R26R-EYFP embryos also stained with Alexafluor 633 Phalloidin to mark cell boundaries. A, anterior; P, posterior; D, dorsal; V, ventral; L, left; R, right. **A:** Lateral view of an early streak (ES) stage embryo showing staining in the nascent extraembryonic mesoderm. EE, embryonic ectoderm; EXE, extraembryonic ectoderm. **B:** Confocal image showing EYFP positive cells (green) in the embryonic and extraembryonic regions of a similarly staged embryo. **C:** A mid-streak (MS) stage embryo showing staining in the lateral mesodermal wings. PS, primitive streak. **D:** Confocal optical cross section showing EYFP positive cells in the mesodermal layer of a late streak (LS) stage embryo. M, mesoderm; EN, endoderm. **E:** Lateral view of early headfold (EHF) stage embryo showing staining along the length of the primitive streak. **F:** Posterior view of the same embryo. **G:** Ventral view of the same embryo showing lack of staining in the forming PNC. **H:** Lateral view of a 2 ss embryo with extensive staining

throughout the posterior lateral mesoderm (LM). **I:** Ventral view of the same embryo showing lack of staining in the PNC and notochord (NC). **J:** Lateral view of a 3-4 ss embryo with extensive staining throughout its length. **K:** Ventral view of the same embryo showing limited staining in the PNC and notochord. SM, somitic mesoderm. **L:** Ventral view of a 9-10 ss stage embryo showing strong staining through the LM and SM, and also in the gut endoderm (EN). There is relatively little staining in the heart (HRT) and head (HD).

Fig. 3.

Analysis of T-Cre mediated deletion of *Nodal* using the *Nodal*^{fl1} line as a reporter (left set of panels; A-H) and directly measured in embryos from T-Cre;*Nodal*^{+/Δ1} × *Nodal*^{fl2/fl2} matings using WMISH (right set of panels; I-P). The probes used for WMISH are shown above each set of panels. **A:** Lateral view of a late streak stage control *Nodal*^{fl1} embryo showing X-gal staining predominantly in the posterior embryonic ectoderm. **B:** Similarly staged T-Cre positive embryo with no apparent difference in staining. **C:** Ventral view of late headfold (LHF) stage control *Nodal*^{fl1} embryo showing staining around the PNC. **D:** A similarly staged T-Cre positive embryo with no apparent difference in X-gal staining. **E:** Ventral view of 2-3 ss control *Nodal*^{fl1} embryo showing staining around the PNC. **F:** Similarly staged T-Cre positive embryo with reduced X-gal staining. **G:** Ventral view of 5-6 ss control *Nodal*^{fl1} embryo showing X-gal staining around the PNC and in the LPM. **H:** Similarly staged T-Cre positive embryo showing only patchy staining around the PNC and none in the LPM. **I:** Lateral view of a late streak stage *Nodal*^{fl2} control embryo following WMISH with the *Nodal* exon 2 probe. There is expression in the posterior embryonic ectoderm up to the

primitive streak. **J:** Lateral view of a similarly staged T-Cre;*Nodal*^{fl2/Δ1} embryo with apparently normal *Nodal* expression. **K:** Ventral view of late headfold (LHF) stage control embryo showing *Nodal* expression around the PNC. **L:** Similarly staged T-Cre;*Nodal*^{fl2/Δ1} embryo with a clear reduction in PNC *Nodal* expression. **M:** Ventral view of 1-2 ss *Nodal*^{fl2} control embryo showing strong *Nodal* expression around the PNC. This and following embryos also were hybridized with a probe to *Uncx4.1*, which marks the somites (S). **N:** Similarly staged T-Cre;*Nodal*^{fl2/Δ1} embryo with extensive reduction in PNC *Nodal* expression. **O:** Ventral view of a 3 ss control embryo showing *Nodal* expression around the PNC and left LPM. The embryo also was hybridized with a *Lefty-1* specific probe that marks the midline prospective floor plate (PFP). **P:** Similarly staged T-Cre;*Nodal*^{fl2/Δ1} embryo showing almost complete loss of *Nodal* expression from the PNC and no LPM expression. *Lefty-1* also is not expressed.

Fig. 4.

Phenotypic analysis of LR development. **A:** Frontal, ventral view of e9.5 wild type control embryo at left (WT), and T-Cre;*Nodal*^{fl2/ΔI} conditional mutant (mut) embryo at right, in which the direction of heart looping (arrows) is reversed. **B:** Ventral view of heart isolated from a control wild type e14.5 embryo following injection with blue latex into the left ventricle (LV) and yellow latex into the right ventricle (RV). The aorta (AO) and pulmonary trunk (PT) are outlined by black and white dotted lines, respectively. **C:** Ventral view of similarly injected heart from an e14.5 T-Cre;*Nodal*^{fl2/ΔI} conditional mutant embryo showing extensive mixing of the latex in the ventricular region (V). **D:** Dorsal view of lungs isolated from an e16.5 control embryo, showing the single left lobe (LL) and four right lobes, outlined by white dotted lines. CR, cranial lobe; M, middle lobe; CD, caudal lobe; A, accessory lobe. **E:** Dorsal view of lungs isolated from a similarly staged T-Cre;*Nodal*^{fl2/ΔI} embryo, showing multiple lobes on both right and left, outlined by white dotted lines.

Fig. 5. Molecular analysis of LR development. Probes used for WMISH are shown above the panels. **A:** Ventral view of a 4-5 ss wild type control embryo following WMISH with probes for *Lefty-1*, expressed in the PFP and *Lefty-2*, expressed in the left LPM. **B:** Ventral view of a 4-5 ss T-Cre;*Nodal*^{f2/Δ1} conditional mutant embryo hybridized with probes for *Lefty-1* and *Lefty-2*. There is no expression for either. The embryos shown in **A** and **B** were hybridized also to a probe for *Cerl-2/Dante*, which marks the PNC. At this stage, there is no right asymmetry for *Cerl-2/Dante* in wild type or mutant. **C:** Ventral view of a 7-8 ss control embryo following WMISH with probe for *Pitx2*, which is expressed in the LPM and posterior part of the heart (HRT) and in the head (HD). **D:** Ventral view of a 7-8 ss T-Cre;*Nodal*^{f2/Δ1} conditional mutant embryo hybridized with *Pitx2*, showing no expression in the LPM or heart (which shows reversed looping). *Pitx2* expression in the head is unaffected. **E:** Ventral view of a 3-4 ss control embryo hybridized with the probes for *Nodal* exon 1 and *Lefty-1*. *Nodal* expression is seen in the left LPM and around the PNC, stronger on the left side. *Lefty-1* is seen in the PFP **F:** Ventral view of a 3-4 ss T-Cre;*Nodal*^{f2/Δ1} embryo hybridized with the *Nodal* exon 1 and *Lefty-1* probes. *Nodal* expression is

asymmetric around the PNC, stronger on the embryo's left side. No expression is seen for *Nodal* in the LPM or *Lefty-1* in the PFP. **G:** Ventral view of a 2-3 ss control embryo following WMISH for *Cerl-2/Dante*. Right asymmetric expression is seen around the PNC. **H:** Ventral view of a 2-3 ss T-Cre;*Nodal*^{fl2/Δ1} conditional mutant embryo hybridized with the *Cerl-2/Dante* probe. Right asymmetric expression is seen as in the control. **I:** Ventral view of a 2-3 ss control embryo following WMISH with probe for *Dnahc5* marking the pit cells of the PNC. **J:** Ventral view of a 2 ss T-Cre;*Nodal*^{fl2/Δ1} conditional mutant embryo showing an equal level of *Dnahc5* expression.

Table 1LR phenotypic analysis of *Nodal*^{fl2/ΔI} vs. *Nodal*^{fl1/ΔI} embryos

Organ or tissue	Phenotype	<i>Nodal</i> ^{fl2/ΔI} (this study)	<i>Nodal</i> ^{fl1/ΔI} (Lowe et al., 2001)
Great vessels	Normal	19 (100%)	None
	Transposed	None	48 (100%)
Heart Direction	Normal	19 (100%)	23 (48%)
	Reversed	None	25 (52%)
Lungs	Normal, asymmetric	19 (100%)	2 (4%)
	Abnormal, asymmetric	None	4 (8%)
	Right isomeric	None	42 (88%)

Table 2Phenotypic analysis of embryos from $Nodal^{+/ΔI} \times Nodal^{fl2/fl2}$ matings from e7.5 - e16.5

Stage	Number examined	Number resorbed	Number abnormal	Number $Nodal^{fl2/ΔI}$ (total number genotyped)
e7.5	21	0	0	ND
e8.5	70	2	0	ND
e9.5 - e11.5	67	1	0	9 (19)
e13.5 - e16.5	41	0	0	17 (33)
Total	199	3	0	26 (52)

Table 3LR phenotypic analysis of embryos (n=63) from T-Cre;*Nodal*^{+/Δ1} × *Nodal*^{fl2/fl2} matings

Organ or tissue	Phenotype	Controls [*]	Mutants [†]
Great vessels	Normal	37 (100%)	None
	Transposed	None	26 (100%)
Lungs	Normal	37 (100%)	None
	Right isomeric	None	26 (100%)
Spleen	Normal	37 (100%)	None
	Absent, reduced	None	26 (100%)
Stomach	Normal	37 (100%)	19 (73%)
	Right sided	None	7 (27%)

* Controls: T-Cre positive or negative; *Nodal*^{+/fl2}† Mutants: T-Cre positive; *Nodal*^{fl2/Δ1}