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Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse

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SUMMARY

In the mouse and chick embryo, the node plays a central role in generating left-right (LR) positional information. Using several different strategies, we provide evidence in the mouse that bone morphogenetic protein 4 (Bmp4) is required independently in two different sites for node morphogenesis and for LR patterning. *Bmp4* expression in the trophoblast-derived extra-embryonic ectoderm is essential for the normal formation of the node and primitive streak. However, tetraploid chimera analysis demonstrates that Bmp4 made in epiblast-derived tissues is required for robust LR patterning, even when normal node morphology is restored. In the absence of embryonic Bmp4, the expression of left-side determinants such as *Nodal* and *Lefty2* is absent in the left lateral plate mesoderm (LPM). Noggin-mediated inhibition of Bmp activity in cultured wild-type embryos results in suppression of *Nodal* expression in the LPM. Thus, unlike previous models proposed in the chick embryo in which Bmp4 suppresses left-sided gene expression, our results suggest that Bmp acts as a positive facilitator of the left-sided molecular cascade and is required for *Nodal* induction and maintenance in the left LPM.

Key words: Bone morphogenetic protein 4, Mouse, Embryo, LR asymmetry, Node

INTRODUCTION

During vertebrate embryogenesis, the morphogenesis and positioning of internal organs depends upon the establishment of the three primary body axes: anteroposterior (AP), dorsoventral (DV) and left-right (LR). Over the past few years, significant progress has been made towards defining the mechanisms that initiate LR asymmetry in the early embryo and regulate the downstream molecular pathways that reinforce the asymmetry and lead to specific anatomical differences (for reviews, see Wright, 2001; Hamada et al., 2002). However, the precise function of some of the signaling components in LR patterning remains unclear, or contradictory, depending on the model organism and experimental techniques used. Among these components is bone morphogenetic protein 4 (Bmp4), which, from experimental studies in the chick embryo, has been assigned a key role as a suppressor of the L-sided molecular cascade (Monsoro-Burq and le Douarin, 2000). However, until now there has been no genetic evidence for the role of endogenous *Bmp4* in vertebrate LR patterning.

The initiation of LR asymmetry in the vertebrate embryo is thought to involve the unidirectional flow of molecules across the body axis to establish asymmetric gene expression in the node (Wright, 2001; Hamada et al., 2002). In the chick embryo, ion flow in the blastoderm at the streak stage may induce *Shh* on the left side of Hensen's node and this, in turn, activates the left-sided program in the embryo (Pagan-Westphal and Tabin, 1998; Levin and Mercola 1999). In the mouse, monocilia in the ventral node cells rotate unidirectionally and may generate a left-specific flow of morphogens within the node to establish the program for LR asymmetry (Wright, 2001; Hamada et al., 2002). In both cases, the node, influenced by its surrounding environment, serves as a center for establishing LR asymmetry.

The mechanisms for reinforcing the LR molecular cascade within and from the node are different in the chick and the mouse (Wright, 2001; Hamada et al., 2002). In the chick embryo, it is thought that *Shh* induces *Nodal* to reinforce the left-side pathway while on the right Bmp4 induces *Fgf8* that, in turn, locally represses *Shh* (Levin et al., 1995; Monsoro-Burq and le Douarin, 2001). The left-side signaling cascade in the node is subsequently extended to the lateral plate mesoderm (LPM), possibly by the diffusion of Nodal from the node. Significantly, caronte, a secreted Bmp antagonist induced by Shh, is expressed in the left paraxial mesoderm and left

LPM and inhibits the activity of Bmp2, Bmp4 and Bmp7 in the LPM that would otherwise repress *Nodal*. Caronte thereby permits *Nodal* expression in the left LPM (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). Nodal in turn induces a transcription factor, *Pitx2*, in the left LPM to complete the establishment of the molecular left-side patterning (Logan et al., 1998).

In the mouse, as described earlier, the first asymmetric expression of *Nodal* is thought to be driven by asymmetric physical flow leading to higher levels of transcripts on the left side of the node compared with the right (Collignon et al., 1996). However, unlike in the chick, *Shh* and *Fgf8* are not asymmetrically expressed in the mouse node, at least at the RNA level: *Shh* is transcribed throughout the node (Collignon et al., 1996), while *Fgf8* is exclusively expressed in the primitive streak but not in the node (Crossley and Martin, 1995). Genetic studies have provided evidence that these genes have functions distinct from those proposed in the chick. Specifically, *Shh* is required to maintain the midline and so prevent left-side identity 'invading' into the right side (Meyers and Martin, 1999), while *Fgf8* positively facilitates left-side patterning (Meyers and Martin, 1999). How molecular asymmetries at the node are transferred to the left LPM in the mouse embryo remains obscure because, among other things, no caronte homolog has been identified. However, cryptic, which encodes a membrane-bound protein of the EGF-CFC family that is a co-factor of Nodal signaling, is a known player in LR patterning in the mouse. It is expressed in the node, midline and bilaterally in the LPM; analysis of cryptic-null embryos suggests that the protein is required in the left LPM to enable the expression of *Nodal* and other left-side determinants (Shen et al., 1997; Yan et al., 1999; Shen and Schier, 2000; Yeo and Whitman, 2001). Once *Nodal* is induced in the LPM, the molecular left-side signaling pathway seems to be conserved between most vertebrates (Wright, 2001; Hamada et al., 2002). Nodal induces *Lefty2* (*Leftb* – Mouse Genome Informatics), which acts as a feedback repressor of *Nodal* expression (Meno et al., 1999; Whitman, 2001), and *Pitx2*, which maintains the left-sided environment (Meno et al., 2001; Shiratori et al., 2001).

In the early mouse embryo, *Bmp4* is expressed in a dynamic pattern, first in the extra-embryonic ectoderm (ExE) and then in epiblast-derived tissues, including extra-embryonic mesoderm (ExM), posterior primitive streak and bilaterally in the LPM. Moreover, genetic loss of function and chimeric embryo analyses have demonstrated distinct functions for Bmp4 in these different tissues (Winnier et al., 1995; Lawson et al., 1999; Fujiwara et al., 2001). *Bmp4* expressed in the ExE is required for patterning the epiblast along the proximodistal axis that is later transformed during gastrulation into the anteroposterior axis. Consequently, epiblast cells closest to the source of Bmp4 in the ExE give rise to the most posterior cell types, the allantois, ExM, and primordial germ cells (PGCs) (Lawson et al., 1999). *Bmp4* expressed in the ExM regulates the subsequent migration and survival of PGCs, and the development of the allantois and its blood vessels (Fujiwara et al., 2001). We employ a variety of strategies to further investigate the function of *Bmp4* in the early patterning of the mouse embryo. Our data indicate that *Bmp4* expression in the ExE is essential for the normal morphological development of the node and primitive streak. At the same time, *Bmp4* made in epiblast-derived tissues is required for the propagation of the left-side molecular cascade. Taken together, our results highlight the dynamic interplay that must occur between different tissues, signaling pathways and anatomical structures for establishing and maintaining LR axial patterning.

MATERIALS AND METHODS

Mouse strains and tetraploid chimeras

For generating $Bmp4^{tm1blh}$ null mutants, the mutation was maintained on a (129/SvEvTacfBR×Black Swiss) background by intercrossing. $Bmp4^{lacZ/+}$ heterozygotes were maintained on the Black Swiss outbred background and $Bmp4^{lacZ/lacZ}$ embryos were obtained by intercrossing. $Bmp4^{lacZ/tm1}\leftrightarrow$wild-type tetraploid chimeras were generated by aggregating ES cells that were targeted in both *Bmp4* alleles and wild-type ICR embryos, as previously described (Fujiwara et al., 2001).

Whole-mount in situ hybridization and immunostaining

Whole-mount in situ hybridization was performed essentially as previously described (Hogan et al., 1994). For double whole-mount in situ hybridization, RNA probes for *Nodal* and *Foxf1* (*Foxf1a* – Mouse Genome Informatics) were labeled with digoxigenin and fluorescein, respectively. BM-purple and BCIP were used for the color reactions. BCIP Reaction buffer is NTM [100 mM Tris-HCl (pH 9.5), 100 mM $MgCl_2$, 150 mM NaCl]. For sectioning after in situ hybridization, embryos were fixed in 4% paraformaldehyde in PBS, dehydrated into 100% isopropanol, washed with 1:1 isopropanol/paraffin wax, embedded in wax and sectioned at 7 μm. Whole-mount immunostaining with anti VCAM1 antibody (Pharmingen) was performed as previously described (Fujiwara et al., 2001).

Scanning electron microscopy

Dissected embryos were immediately washed three times in Sorenson's phosphate buffer (Sulik et al., 1994). After fixation with 2.5% glutaraldehyde in Sorenson's phosphate buffer at 4°C for 48 hours, embryos were rinsed in Sorenson's phosphate buffer, then post-fixed in 2% osmium tetroxide for 2 hours. After dehydration in a graded series of ethanols, the specimens were critical-point dried, mounted on metal stubs and sputter-coated with gold palladium. Electron microscopy was performed on a JOEL microscope.

Whole embryo culture

Headfold-stage wild-type embryos were isolated and Reichert's membrane removed mechanically. Three to four embryos were cultured with rotation in a silicon-coated vial in 500 μl culture medium with/without 1 μg/ml noggin for 20 hours at 37°C, 5% CO_2/95% air. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 50% rat serum (Harlan Bioproducts), 2 mM L-Glutamine (GIBCO) and 50 μg streptomycin/penicillin (GIBCO). Recombinant mouse noggin (R&D Systems) was dissolved at 100 μg/ml in PBS containing 0.1% bovine serum albumin (BSA) (BSA/PBS). The BSA/PBS solution was used as a negative control. After culture, the embryos were washed in BSA/PBS three times and fixed with 4% PFA in PBS for 75 minutes at 4°C, and subsequently analyzed by whole-mount in situ hybridization.

RESULTS

Reduced Nodal expression in the node and abnormal node and posterior morphology in Bmp4-null mutants

Based on previous findings that the severity of the $Bmp4^{tm1}$-

null mutant phenotype varies with genetic background, the (129/SvEvTacfBR×Black Swiss) background, on which homozygotes develop up to around the 20 somite stage, was chosen for this study. Mutant embryos can be unambiguously identified by the absence of an allantois (Lawson et al., 1999). To analyze LR patterning in mutant embryos, we first examined the expression of *Nodal* by whole-mount in situ hybridization. *Nodal* expression normally begins symmetrically in the crown cells of the node from the late-streak stage, and becomes enriched on the left side at the two- to eight-somite stage, accompanied by transient expression in the left LPM (Collignon et al., 1996). As expected, in all three- to five-somite-stage wild-type embryos examined (n=7), *Nodal* was expressed in the periphery of the node, with enrichment on the left side, and in the left LPM (Fig. 1A, part a). By contrast, in more than 75% (n=14/18) of *Bmp4*^{tm1} mutants examined at the same somite stage as wild type, *Nodal* expression in the node was patchy (Fig. 1A, part b) or significantly reduced (Fig. 1A, part c), and no expression was observed in the left LPM. All *Bmp4*$^{-/-}$ mutants with patchy or reduced *Nodal* expression in the node (n=14/14) had no LPM *Nodal* expression. The remainder (n=4/18) appeared relatively normal at this level of analysis (data not shown). To exclude the possibility that the absence of *Nodal* expression in the LPM was due to abnormal loss of this tissue, we examined mutant embryos by double in situ hybridization with *Nodal* and *Foxf1*, which encodes a forkhead transcription factor bilaterally expressed in the LPM (Mahlapuu et al., 2001). We found that all mutants lacking *Nodal* expression in the LPM nevertheless expressed *Foxf1* at a comparable level to the wild type (Fig. 1A, parts d-f; n=3/3).

Defects in the function of monocilia in the node are associated with LR abnormalities in the mouse (reviewed by Wright, 2001; Hamada et al., 2002). Therefore, the morphology of the node in *Bmp4*^{tm1} mutants was analyzed using scanning electron microscopy (SEM). Although not all mutants have abnormal LR asymmetry, whole-mount in situ hybridization for *Nodal* allowed normal and abnormal embryos to be sorted out prior to SEM. The wild-type embryos had a prominent hindgut pocket, well-formed primitive streak, and a concave node (Fig. 1A, parts g,j). However, in the two *Bmp4*^{tm1} mutants examined, the posterior region had an abnormal bulge that projected ventrally (Fig. 1A, parts h,i). At high magnification, the node was flat or slightly convex and its periphery was irregular (Fig. 1A, parts k,l).

It was not possible in the above study to compare the detailed morphology (Sulik et al., 1994) of ventral node cells of mutant and wild-type embryos because the cell boundaries and architecture of the monocilia were disrupted by the in situ hybridization protocol. We therefore analyzed by SEM the gross morphology of five additional, untreated, *Bmp4*^{tm1} mutants from the headfold to the six-somite stage. Wild-type embryos (n=5) showed a well-formed primitive streak and a concave and tear-drop shaped node with monocilia in almost every cell. The extended notochord had well demarcated borders with the endoderm (Fig. 1B, parts a,b). By contrast, a ventrally projected bulge was present in the posterior region of all *Bmp4*^{tm1} mutants examined (n=5). The shape of the node was also abnormal and was either flat or slightly convex (Fig. 1B, parts, e,f). Most cells had monocilia, but one embryo at the headfold stage showed large and irregularly shaped unciliated cells, resembling endodermal cells, scattered within the node (Fig. 2B, part i). The notochord was formed in all mutants examined, although the boundary of the notochord and the adjacent endoderm was less regular than in the wild-type embryos (Fig. 1B, part e).

The morphology of the posterior region of the embryos was analyzed by cutting through them in a dorsoventral plane and viewing posteriorly. In the wild-type embryos, the amnion extends over the embryo and demarcates a well-expanded amniotic cavity (Fig. 1B, parts c,d,j). However, in *Bmp4*^{tm1} mutants, much of the posterior amniotic cavity is filled up by a mass of mesodermal cells that extends to the apical surface of the ectoderm (Fig. 1B, parts g,h). This phenotype closely resembles that of *Fgf8*-null mutants, in which mesodermal cells that have failed to move out of the streak fill up the amniotic cavity (Sun et al., 1999). In situ hybridization with an *Evx1* probe (Dush and Martin, 1992) confirms that the *Bmp4*-null cells are embryonic in origin, rather than extra-embryonic (Fig. 1B, parts j,k).

Reduced Lefty2 and cryptic expression in Bmp4-null mutants

To further examine the L-sided molecular cascade in *Bmp4*^{tm1} mutants, we analyzed expression of *Lefty2* and cryptic. *Lefty2* is normally transiently expressed in the left LPM between the two- and five-somite stage (Fig. 1C, part a) (Meno et al., 1996), while cryptic is expressed bilaterally in the LPM, node, and the midline from the headfold to the six- to eight-somite stage (Fig. 1C, parts d,g) (Shen et al., 1997). In about 65% (n=6/9) of *Bmp4*^{tm1} mutants at the 3-4 somite stage, *Lefty2* expression was not detected (Fig. 1C, part b). The remainder (n=3/9) had relatively normal left-side expression (Fig. 1C, part c). In the case of cryptic, three out of three *Bmp4* mutants at the two- to four-somite stage and four out of four at the five- to six-somite stage had no expression of the gene in the LPM, node and midline (Fig. 2C, parts e,f). However, in all earlier (headfold to one somite-stage) mutants examined (n=4/4), cryptic was expressed bilaterally in the LPM, but was greatly reduced in the node (Fig. 2C, part h). This suggests that Bmp4 signaling is required for the maintenance of cryptic expression in the LPM.

The absence of *Lefty2* and cryptic transcripts in the LPM again raised the issue of whether this tissue fails to develop or degenerates in *Bmp4*^{tm1} mutants. To address this possibility, we made use of the fact that in *Bmp4*$^{lacZ/+}$ embryos the expression of *Bmp4* in the LPM is marked by *lacZ* activity (Fig. 1C, parts i,j) (Lawson et al., 1999). Staining for β-galactosidase revealed abundant *Bmp4*^{lacZ}-positive cells with a normal morphology in the LPM of *Bmp4*$^{lacZ/lacz}$ null mutants at the four- to six-somite stage (Fig. 1C, parts l,m). Moreover, analysis of *Foxf1* expression as described above (Fig. 1A, parts d-f) also confirmed that the LPM is formed in all *Bmp4*^{tm1} mutants examined at the two- to six-somite stage (n=8/8) (Fig. 1C, parts k,n).

Abnormal expression of Fgfs and *Dte* but maintenance of midline tissues in *Bmp4*-null mutants

Fgf8 has been implicated as a positive facilitator of left-sided patterning in the mouse embryo, and as a right-sided determinant in the chick (Meyers and Martin, 1999; Monsoro-

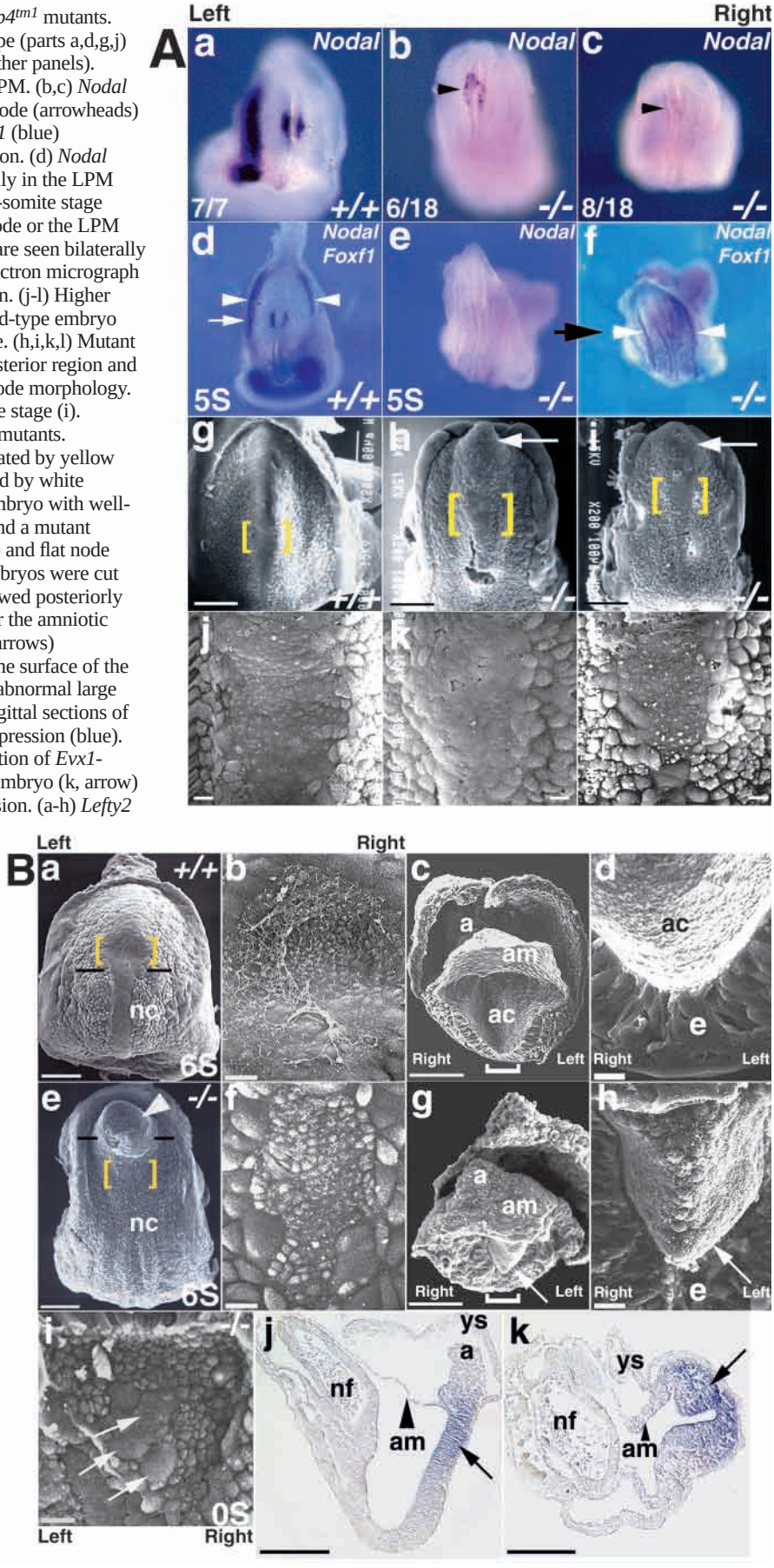
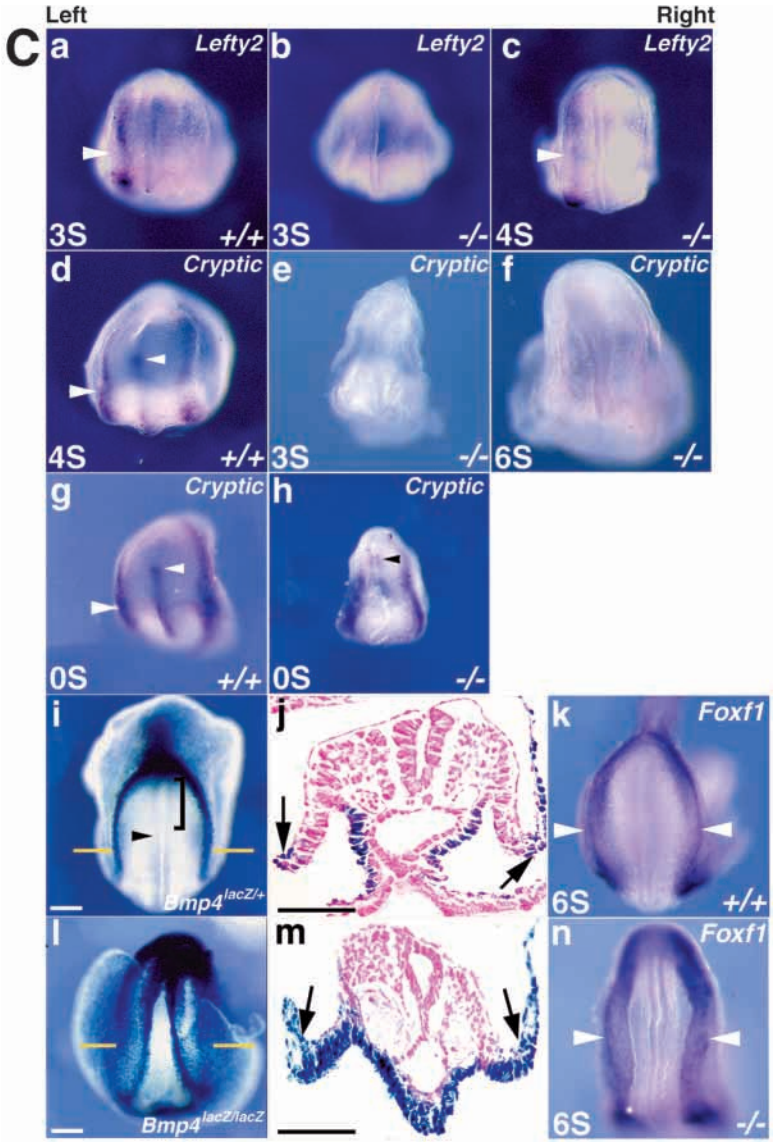


Fig. 1. Gene expression and node morphology in *Bmp4*^{tm1} mutants. (A) Posteroventral views of five-somite stage wild-type (parts a,d,g,j) and four- to five-somite stage *Bmp4*^{tm1} mutants (all other panels). (a) *Nodal* expression in the node periphery and left LPM. (b,c) *Nodal* expression is either patchy or greatly reduced in the node (arrowheads) and absent in the LPM. (d-f) *Nodal* (purple) and *Foxf1* (blue) expression by double whole-mount in situ hybridization. (d) *Nodal* expressed in the left LPM (arrow), and *Foxf1* bilaterally in the LPM (arrowheads) (*n*=5/5). (e,f) These show the same five-somite stage embryo. There is no *Nodal* expression in either the node or the LPM (e), but, after second hybridization, *Foxf1* transcripts are seen bilaterally in the LPM (f, arrowheads; *n*=3/3). (g-l) Scanning electron micrograph (SEM) of the embryos in a-c after in situ hybridization. (j-l) Higher magnification of the node (yellow brackets). (g,j) Wild-type embryo with well-formed primitive streak and a concave node. (h,i,k,l) Mutant embryos show abnormal bulge (h,i; arrows) in the posterior region and flat node morphology with irregular periphery. (B) Node morphology. SEM of embryos at the six somite (a-h) and presomite stage (i). (a-d,j) Wild-type embryos; all other panels, *Bmp4*^{tm1} mutants. (b,f,d,h) Higher magnification of the node (b,f), indicated by yellow brackets in a,e, and the amniotic cavity (d,h), indicated by white brackets in c,g. (a,b,e,f) Ventral views of wild-type embryo with well-formed primitive streak (a) and a concave node (b), and a mutant embryo with abnormal posterior bulge (e, arrowhead) and flat node morphology with irregular periphery (f). (c,d,g,h) Embryos were cut through the plane (black line) indicated in a,e and viewed posteriorly (c) or dorsoposteriorly (g). (c,d) Amnion extends over the amniotic cavity in wild-type embryo; (g,h) mesodermal cells (arrows) abnormally fill up the amniotic cavity and extend to the surface of the ectoderm in mutant embryo; (i) mutant embryo with abnormal large endoderm-like cells inside the node (arrows). (j,k) Sagittal sections of three- to four-somite stage embryos showing *Evx1* expression (blue). Anterior is towards the left. Note abnormal accumulation of *Evx1*-positive mesodermal cells in the posterior of mutant embryo (k, arrow) compared with wild-type (j, arrow). (C) Gene expression. (a-h) *Lefty2* (a-c) and cryptic (d-h) expression by whole-mount in situ hybridization. (a,c) *Lefty2* in the left LPM (arrowheads) of the 3- to 4-somite stage wild type (a, *n*=2/2) and some mutants (c, *n*=3/9). (b) Other mutants show no *Lefty2* expression at the three- to four-somite-stage (*n*=6/9). (d-f) Cryptic is bilaterally expressed in the LPM and node (arrowheads) of wild-type (d, *n*=2/2), but not of mutants at the two- to four- (e; *n*=3/3) and five- to six- (f; *n*=4/4) somite stage. (g,h) Cryptic is expressed in the LPM and node (arrowheads) of wild type (g, *n*=3/3), whereas expression in the node (arrowhead) is reduced in mutants (h, *n*=4/4) at the headfold to one-somite stage. (i,j,l,m) β-galactosidase staining to detect *Bmp4*^{lacZ} reporter expression in six-somite stage *Bmp4*$^{lacZ/+}$ heterozygous (i,j) and seven-somite stage *Bmp4*$^{lacZ/lacZ}$ homozygous-null (l,m) embryos. (j,m) Transverse sections in the plane indicated (yellow bar) in i and l. Arrows indicate the *Bmp4*^{lacZ}-positive LPM. Note that no *Bmp4* expression is detected in either the primitive streak (bracket), or the node (arrowhead) in i. (k,n) *Foxf1* expression by whole-mount in situ hybridization. *Foxf1* is expressed bilaterally in the LPM (arrowheads) in both two- and six-somite stage wild type (k, *n*=4/4) and mutants at the same stage (n, *n*=8/8). a, allantois; ac, amniotic cavity; am, amnion; e, ectoderm; nc, notochord; nf, neural fold; s, somite stage; ys, yolk sac. Scale bars: in A, 10 μm for parts g-i; in B, 100 μm for parts a,c,e,g,j,k and 10 μm for parts b,d,f,h,i; in C, 100 μm for parts i,j,l,m.



Burq and le Douarin, 2001). In the mouse, *Fgf8* is symmetrically expressed in the primitive streak, and expression of *Fgf4* and *Nodal* is dependent on the gene dose of *Fgf8* (Crossley and Martin, 1995; Meyers and Martin, 1999; Sun et al., 1999). We therefore examined the expression of both Fgf genes in one- to six-somite-stage *Bmp4*^{tm1} mutants. In five- to seven-somite stage wild-type embryos, *Fgf8* expression was detected in the primitive streak (Fig. 2A). By contrast, *Fgf8* expression was significantly reduced in around 80% (n=5/6) of five- to six-somite stage *Bmp4*^{tm1} mutants (Fig. 2B,C). One six-somite stage *Bmp4*^{tm1} mutant showed *Fgf8* expression in the primitive streak at a level comparable with the wild type (data not shown). As for *Fgf4*, about 70% (n=3/4) of one- to two-somite stage *Bmp4*^{tm1} mutants showed reduced levels in the primitive streak compared with wild-type embryos (Fig. 2D,E), while one mutant showed comparatively normal expression (Fig. 2F).

Because of the importance of the midline in maintaining LR asymmetry, we confirmed that trunk midline tissues still develop robustly despite the posterior abnormalities in *Bmp4*^{tm1} mutants. In the mouse, *Shh* is normally symmetrically expressed in the node and midline tissues such as the notochord and the prospective floor plate in the headfold to 6-somite stage embryos (Collignon et al., 1996). In 10/11 *Bmp4*^{tm1} mutants examined at the pre- to 6-somite stage, expression of *Shh* was observed in the node and midline tissues at a level comparable with that seen in wild-type embryos (Fig. 2G-J). One headfold-stage *Bmp4*^{tm1} mutant showed scattered *Shh* expression only in the node, possibly reflecting a delay of midline development (data not shown).

Members of the cerberus/*Dan*-related gene family encode proteins that are thought to directly bind to Nodal and Bmps and to inhibit signaling through their receptors (Pearce et al., 1999; Massague and Chen, 2000). One gene, *Dante* (*Dte*), is expressed specifically in the periphery of the node in the mirror image of *Nodal*, with somewhat higher expression on the right than the left at the four- to six-somite stage (Fig. 2K) (Pearce et al., 1999). In 80% (n=4/5) of *Bmp4*^{tm1} mutants examined, *Dte* expression in the node periphery was reduced and patchy but still relatively higher on the right (Fig. 2L), while one mutant was normal (Fig. 2M). Taken together, our results with *Nodal*, cryptic and *Dte* suggest that the normal development of the node requires *Bmp4* expression in the embryo.

Mesocardia in Bmp4 null mutants

Homozygous null *Bmp4*^{tm1} mutants do not survive to the stage when there is clear asymmetry of organs such as the lung and gut, and the only anatomical indicator of early LR patterning that can be scored is heart looping. In about half of the *Bmp4*^{tm1} mutant embryos examined at the nine- to ten-somite stage (n=4/7) the heart tube lay centrally in the midline, and showed no evidence of heart looping, either to the left or right (mesocardia) (Fig. 3A-F). This was seen both in the intact embryo and after sectioning. The remainder of the *Bmp4*^{tm1} mutants (n=3/7) had normal rightward looping, as in the wild-type embryo at the same somite stage.

Extra-embryonic ectoderm Bmp4 signaling restores node and primitive streak morphology

Bmp4 is normally expressed in the extra-embryonic ectoderm (ExE) and extra-embryonic mesoderm (ExM) in pre- and post-gastrulation embryos, respectively (Lawson et al., 1999). Later at the somite stage, it is expressed in the ExM, posterior primitive streak, and the LPM (Fig. 1C, parts h,i). As shown previously (Fujiwara et al., 2001), aggregation of *Bmp4*$^{lacZ/tm1}$-null ES cells and tetraploid (4N) wild-type host embryos generated chimeras (hereafter called tetraploid chimeras) in which *Bmp4* expression is restored in the ExE but not in the embryo (including the ExM). Analysis of such chimeras showed that *Bmp4* activity in the ExM is required for the proper migration and survival of PGCs, and for the development of the allantois, while anterior structures and the primitive streak appear normal (Fujiwara et al., 2001). In this present study we obtained 19 tetraploid chimeras from the late-streak to 10-somite stage. The gross morphology of the node and posterior region was analyzed by SEM in five of them, from the headfold to 3-somite stage. The primitive streak was normal and did not

bulge ventrally, and the concave and tear-drop shape of the node was restored (Fig. 4A-D). Monocilia projected ventrally from the node cells, the overall number of which was about the same as in wild-type embryos. Therefore, Bmp4 produced in the ExE restores at least the morphology of the node and primitive streak. Molecular analysis confirmed that the midline notochord is properly formed in tetraploid chimeras; the level of *Shh* expression in five- to six-somite-stage tetraploid chimeras (n=2/2) was comparable with that seen in wild-type embryos (Fig. 4E,F).

Embryonic Bmp4 signaling affects early heart development and LR asymmetric gene expression

An important finding from the tetraploid chimera studies described above is that the morphology of the node is restored. We therefore examined whether heart looping and LR asymmetric gene expression were also normal. All tetraploid chimeras examined at the eight- to ten-somite stage (n=4) had mesocardia similar to that seen in about half of the *Bmp4*^{tm1} mutants (Fig. 4G-L). As previous studies have shown that the technique of generating tetraploid chimeras does not itself affect the LR patterning of the resulting embryos (Dufort et al., 1998), the defects in the chimeras must be due to the genotype of the ES-derived cells. In support of this argument, no morphological or looping defects were seen in equivalent

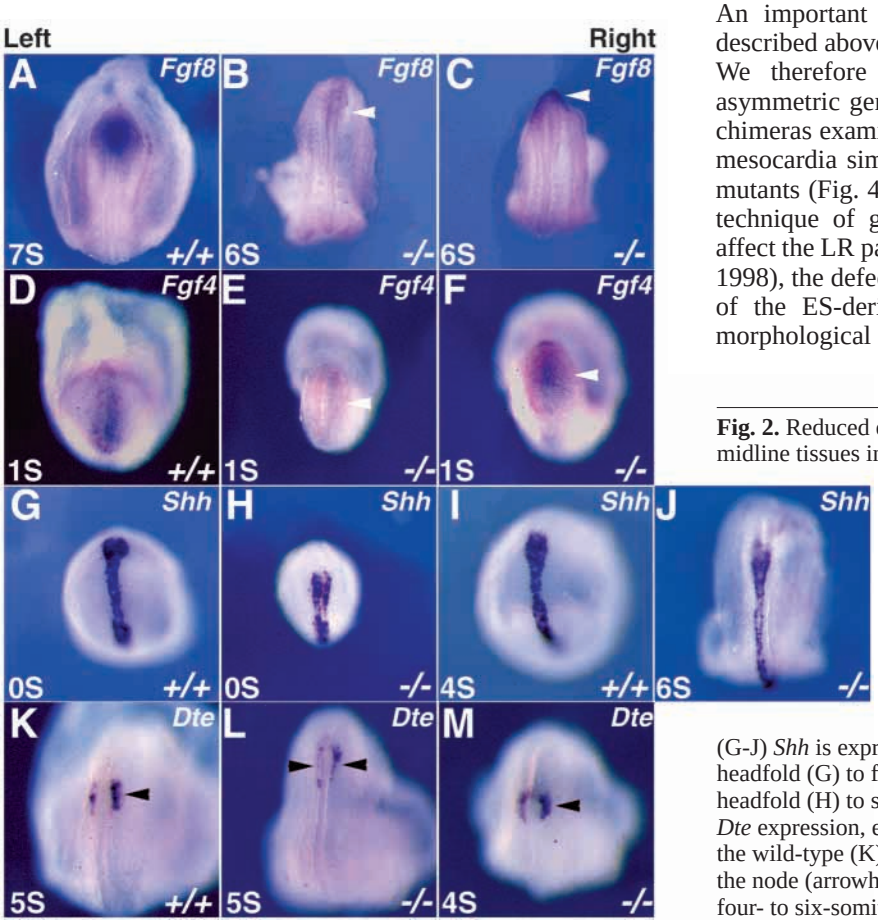


Fig. 2. Reduced expression of *Fgfs* and *Dte* and maintenance of midline tissues in *Bmp4*^{tm1} mutants. Posteroventral views of wild-type embryos (A,D,G,I,K), and mutants (all other panels). (A-C) *Fgf8* is expressed in the primitive streak of wild-type (A), but strongly reduced (n=5/6) in the posterior (arrowheads) of mutant embryos (B,C), at the five- to seven-somite stage. (D-F) *Fgf4* is expressed in the primitive streak of wild-type (D) and one mutant (arrowhead; n=1/4) (F), but strongly reduced (arrowhead; n=3/4) in posterior of mutant embryos (E), at the one- to two-somite stage. (G-J) *Shh* is expressed in the node and midline of wild type at the headfold (G) to four-somite (I) stage, and mutant embryos at the headfold (H) to six-somite (J) stage (n=10/11). (K-M) Asymmetric *Dte* expression, enriched on right side of the node (arrowheads), in the wild-type (K) and one mutant (M; n=1/5), but low and patchy in the node (arrowheads) of other mutant embryos (L; n=4/5), at the four- to six-somite stage.

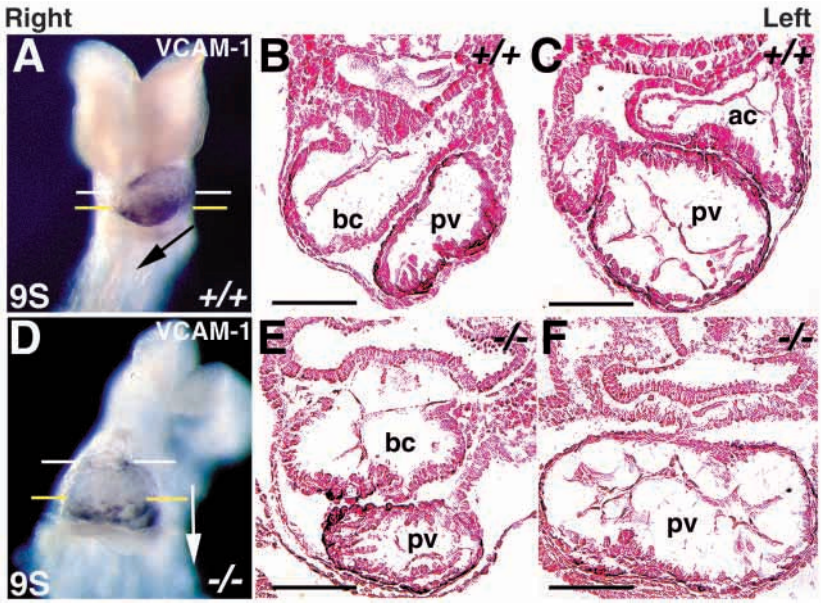


Fig. 3. Abnormal heart development in *Bmp4*^{tm1} mutants. (A,D) Ventral views of nine-somite-stage wild-type (upper panels) and *Bmp4*^{tm1} mutant (bottom panels) stained with anti-VCAM-1 antibody. (B,C,E,F) Transverse section of embryos counterstained with Eosin in planes indicated by white (B,E) and yellow (C,F) bars in A,D. VCAM1 is expressed in the myocardium mostly in the primitive ventricle (purple) of both the wild-type and *Bmp4*^{tm1} mutant, although levels are reduced in the latter. (A) Rightward looping of the heart (arrow). (D) Failure of looping, with midline heart tube (arrow). (B) Anterior heart showing clear boundary of the primitive ventricle to the left of the embryo and the bulbus cordis to the right. (C) Clear distinction between primitive ventricle and atrial chamber in the posterior heart. (E,F) Abnormal positioning of the primitive ventricle and bulbus cordis caused by looping failure. ac, atrial chamber; bc, bulbus cordis; pv, primitive ventricle. Scale bars: 100 μm for B,C,E,F.

somite-stage tetraploid chimeras (n=3/3) generated using $Bmp4^{lacZ/+}$ heterozygous ES cells (data not shown).

In spite of the restoration in node morphology, a reduction in *Nodal* expression was still observed in tetraploid chimeras, at both the late streak (n=2/2) (compare Fig. 5A,D) and the headfold stage (n=2/2) (compare Fig. 5B,E). Later somite-stage tetraploid chimeras (n=2/2) also showed significant reduction of *Nodal* expression in both the node and left LPM (compare Fig. 5C with 5F). These results suggest that *Bmp4* expression in epiblast-derived cells is required for *Nodal* expression in the node and left LPM, independently of node morphogenesis.

In $Bmp4^{tm1}$ null mutants, *Fgf8* expression was strongly reduced in nearly all embryos examined. However, the expression of *Fgf8* in tetraploid chimeras at the six-somite stage (n=2/2) was only moderately less in the primitive streak than in wild-type embryos (Fig. 5H).

Effect of exogenous noggin on LR patterning

Previous studies in the chick embryo suggested that Bmp activity suppresses *Nodal* expression in the left LPM, because caronte inhibits Bmp2, Bmp4 and Bmp7 and permits *Nodal* expression in this tissue (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). In the mouse embryo, *Bmp2* is expressed bilaterally in the LPM but not in the midline (Fig. 6A) but no specific expression of *Bmp6* or *Bmp7* is observed in the LPM, although *Bmp7* expression is seen in the node and midline (data not shown). We therefore addressed the role of Bmps produced in the LPM by culturing embryos in the presence of noggin, an inhibitor of Bmps normally expressed in the node and notochord at the headfold to four-somite stage (Fig. 6B) (Massague and Chen, 2000). This was carried out at the headfold stage when embryos have already established *Nodal* expression in the node. Of the nine control embryos cultured to the two- top five-somite stage, seven showed left-sided and two showed bilateral *Nodal* expression in the LPM (Fig. 6C and data not shown). By contrast, none of the embryos cultured with noggin to the same stage (n=17) showed any *Nodal* expression in the LPM (Fig. 6D). Unlike in tetraploid chimeras, *Nodal* expression in the periphery of the node was still observed in all noggin-treated embryos (Fig. 6D). Moreover, the addition of noggin did not affect cryptic expression in the LPM and midline (Fig. 6E; n=16/16), a result that indirectly demonstrates that the LPM did develop in cultured embryos. Proper midline development was confirmed by *Shh* expression in embryos cultured with exogenous noggin (Fig. 6F; n=7/7).

DISCUSSION

Current models for the role of Bmps in early LR patterning are based on experiments with the chick embryo in which *Bmp4* is expressed in Hensen's node, with the highest levels on the right, and bilaterally in the LPM (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). Two roles have been proposed for Bmp4; to positively regulate *Fgf8* expression on the right side of the node (Monsoro-Burq and Le Douarin, 2001) and to prevent initiation of *Nodal* expression in the LPM (Wright, 2001; Hamada et al., 2002). These ideas predict that *Bmp4*-null mouse embryos would show L-side character on both sides of the node and

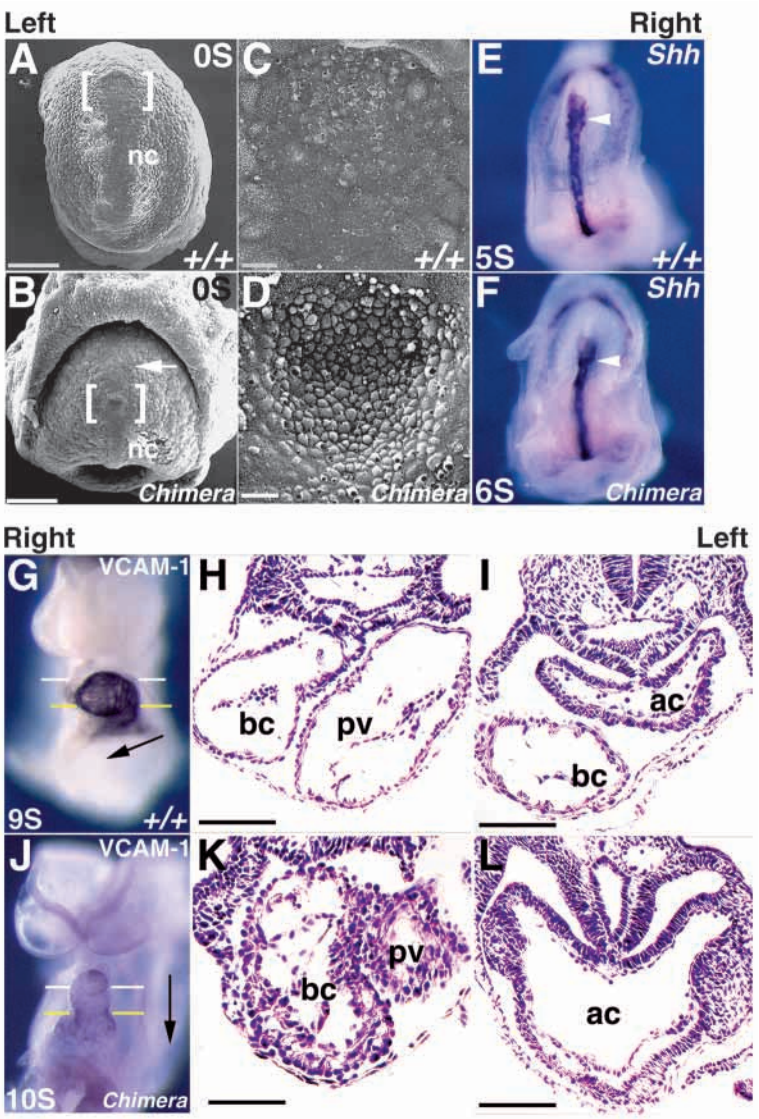


Fig. 4. Development of $Bmp4^{lacZ/tm1}$ ES cell↔wild-type tetraploid chimeras. (A-F) SEM (A-D) and *Shh* expression (E,F) by whole-mount in situ hybridization. Posteroventral views of wild-type embryos (A,E) and tetraploid chimeras (B,F), and higher magnification of the node (C,D) indicated by brackets in A and B. (A-D) Concave node morphology (C,D), well-formed primitive streak (B, arrow), and well-defined notochord in the presomite-stage wild-type embryo (A,C) and tetraploid chimera (B,D). (E,F) *Shh* expressed in the node (arrowheads) and midline of the five- to six-somite-stage wild-type embryo (E) and tetraploid chimera (F). (G,J) Ventral view of the heart after staining with anti-VCAM1 antibody. Nine-somite stage wild-type embryo shows normal rightward looping (G) and ten-somite stage tetraploid chimera showing abnormal midline heart tube (J, n=4/4). (H,I,K,L) Transverse sections stained with Hematoxylin and Eosin taken at levels indicated in white for H,K, and yellow for I,L. ac, atrial chamber; bc, bulbus cordis; nc, notochord; pv, primitive ventricle. Scale bars: 100 μm in A,B,H,I,K,L; 10 μm in C,D.

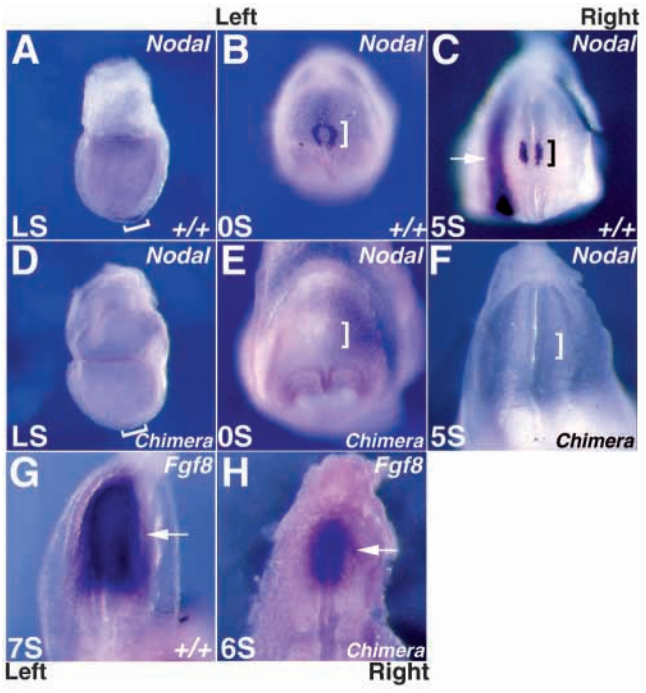


Fig. 5. Gene expression in tetraploid chimeras. (A-C) In wild-type embryos, *Nodal* is expressed in the node at the late-streak stage (A, bracket; lateral view), symmetrically in the node at presomite stage (B, bracket; posteroventral view), and asymmetrically, higher on the left, in the node and left LPM at the five-somite stage (C, bracket and arrow). (D-F) Tetraploid chimeras show no *Nodal* expression in the node (brackets) at all three stages (D-F; *n*=2/2 each) or in the left LPM (*n*=2/2) at the five-somite stage (F). (G,H) *Fgf8* is expressed in the primitive streak (arrow) of the seven-somite stage wild-type embryos (G), and at a moderately lower level (arrow) in the 6-somite stage tetraploid chimeras (H; *n*=2/2). LS, late streak.

bilateral *Nodal* expression in the LPM. By contrast, our data strongly suggest that, in mammals, Bmp4 is in fact required for L-side *Nodal* expression. This reinforces the idea that there are important species differences in the molecular regulation of LR patterning (Meyers and Martin, 1999; Wright, 2001). In addition, our tetraploid chimera studies clearly establish independent functions for Bmp4 produced in two different tissues. Thus, Bmp4 made in the trophoblast-derived ExE regulates the formation of the node and primitive streak, while Bmp4 made in the epiblast-derived ExM, posterior primitive streak, and/or LPM, positively regulates the left molecular pathway of LR patterning. These results highlight the fact that during LR patterning there is a dynamic and continuous interplay between different cell populations and their associated signaling pathways and anatomical structures.

Morphogenesis of the node and primitive streak requires *Bmp4* made in the extra-embryonic ectoderm (ExE)

Comparison of the phenotype of *Bmp4*-null mutants and tetraploid chimeras suggests that Bmp4 signaling from the ExE is a key factor in regulating the development of the posterior mesoderm and the node in the headfold to 6-somite stage mouse embryo (Fig. 1A, parts e,f,h,i; Fig. 2B, parts e,f). This conclusion (summarized in model form in Fig. 7A) is based on the hypothesis that Bmp4 in the ExE patterns the proximodistal axis of the epiblast that is converted, by gastrulation, into the dorsal/anteroposterior/ventral axis of the embryo (Beddington and Robertson, 1999). Fate-mapping studies and previous analysis of *Bmp4*-null embryos, suggest that the epiblast cells closest to the source of Bmp4 are specified as precursors of the posterior ExM, allantois and PGCs, while somewhat more distal cells are specified as precursors of the posterior embryonic mesoderm (Lawson et al., 1991; Lawson et al., 1999). Accordingly, in the absence of ExE *Bmp4* the epiblast is dorsalized, fewer posterior-proximal epiblast cells are specified as prospective ExM, and the development of posterior mesoderm-derived tissues is disrupted. Two kinds of data obtained here support this model. First, posterior proximal epiblast and cells in the posterior primitive streak normally express *Fgf8*, and Fgf8 regulates *Fgf4* expression in the primitive streak (Crossley and Martin, 1995; Sun et al., 1999).

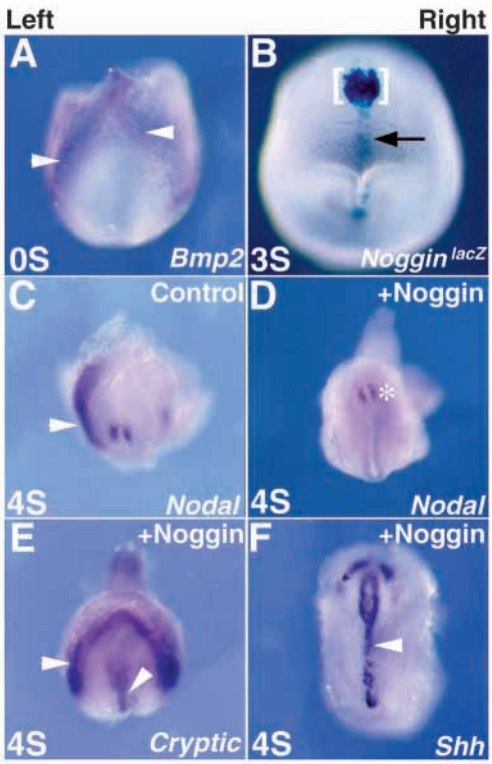


Fig. 6. Effect of exogenous noggin on LR patterning. Whole-mount in situ hybridization of wild-type embryos, except for B (which detects Nog^{lacZ} reporter). (A-F) Posteroventral views. (A) *Bmp2* is expressed bilaterally in the LPM (arrowheads) from the headfold to four-somite stage. (B) Noggin is expressed in the node (bracket) and the notochord (arrow). (C,D) *Nodal* expression in three- to five-somite stage embryos cultured with (D) or without (C) recombinant noggin protein. (C) In control embryos, *Nodal* is expressed in both the left LPM (arrowhead) and node (*n*=7/9). (D) No *Nodal* expression was observed in the LPM when embryos were cultured with noggin (*n*=17/17). However, *Nodal* expression (*) was detected in the node (*n*=17/17). (E,F) Cryptic and *Shh* expression in noggin-treated embryos at the three- to five-somite stage. Cryptic is expressed bilaterally in the LPM and midline (E, arrowheads; *n*=16/16), and *Shh* is expressed in the node and midline (F, arrowhead; *n*=7/7).

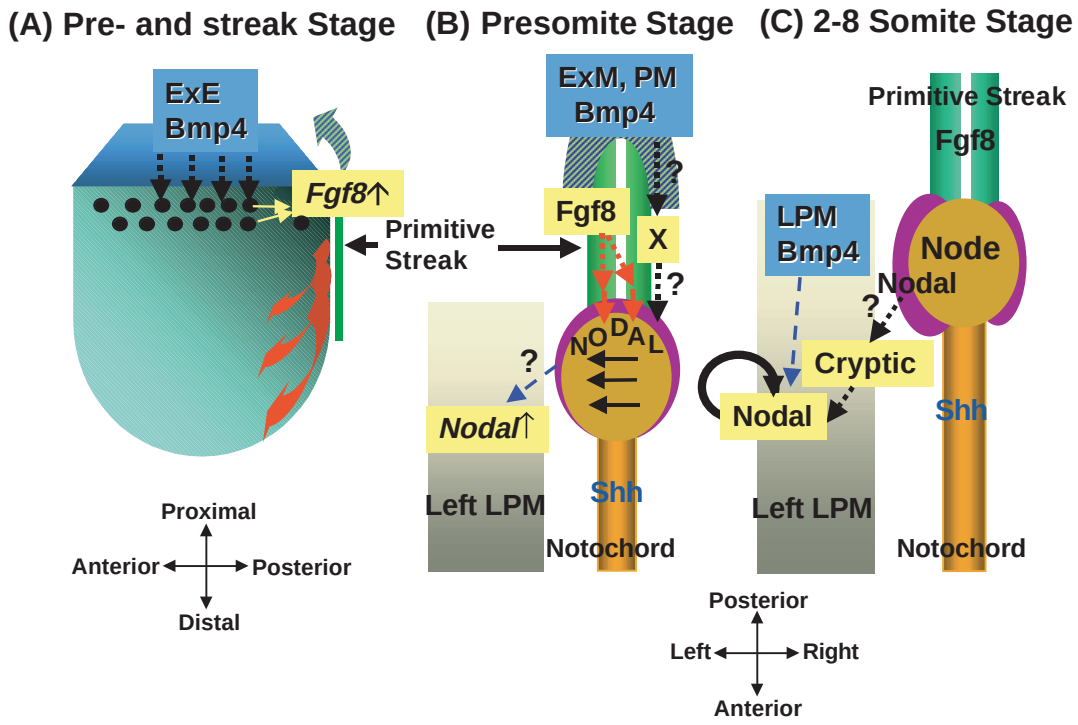


Fig. 7. Model for independent functions of Bmp4 in node and primitive streak morphogenesis, and LR asymmetry. (A) Pre- to early-streak stage; lateral view. Bmp4 produced in the ExE patterns the proximodistal axis of the epiblast and thereby plays a role in specifying cell fates along the anteroposterior axis of the future primitive streak. The most proximal cells (black circle) will give rise (yellow arrows) to the precursors of allantois, PGCs and ExM, which will translocate posteriorly (blue/green striped arrow), while the more distal precursors of embryonic mesoderm move anteriorly and laterally (red arrows). *Fgf8* is expressed in the posteroproximal epiblast. (B) The presomite stage; ventral view. Progenitors of the node are thought to lie in the mid-anterior primitive streak and move anteriorly to be incorporated into the node (red arrows). This morphogenetic process is dependent on Bmp4 expression in the ExE (see A) and Fgf8 in the primitive streak (Sun et al., 1999). We speculate that Bmp4 produced in the ExM and/or posterior mesoderm (PM) positively regulates an unknown gene *X* that initiates the expression of *Nodal* (broken black arrows) in the periphery of the node (purple). Nodal flow (left-pointing black arrows) generates enrichment of left-side *Nodal* expression in the periphery of the node. The future *Nodal* expression in the left LPM is possibly initiated by Nodal protein diffusing from the node (broken blue arrow). (C) The two- to eight-somite stage. Bmp4 in the LPM is required for *Nodal* expression in the LPM (broken blue arrow). Nodal produced in the node may maintain the expression of cryptic that permits the expression of left-side determinant genes such as *Nodal* in the left LPM (broken black arrows).

Both *Fgf8* and *Fgf4* are strongly downregulated in *Bmp4*-null embryos, while *Evx1*-positive embryonic mesodermal cells accumulate in the posterior region (Fig. 2B, part k; Fig. 3B,C,E). Second, the posterior phenotype of *Bmp4*-null embryos analyzed by SEM strongly resembles that of *Fgf8*$^{-/-}$ mutants, with an accumulation of cells in the primitive streak region filling the amniotic cavity and creating a characteristic bulge [compare Fig. 2B, parts g,h with Fig. 1C,D by Sun et al. (Sun et al., 1999)]. This posterior streak defect and the expression of *Fgf8* are rescued in tetraploid chimeras, in which ExE Bmp4 is restored.

Our present study shows that formation not only the posterior streak but also the node is dependent on ExE Bmp4. A recent study has provided insight into the ontogeny of the mouse node (Kinder et al., 2001). At the mid-streak stage, the majority of node precursors appear to be localized in the primitive streak immediately posterior to the cells that colonize the prechordal mesoderm, foregut endoderm, notochord and prospective floor plate of the neural tube. Both *Fgf8* and *Fgf4* are expressed in the prospective node region, and genetic loss of *Fgf8* function results in severe abnormalities in the node (Crossley and Martin, 1995; Sun et al., 1999). In *Bmp4*-null embryos, the abnormal patterning of the epiblast and the reduced expression of *Fgf8* and *Fgf4* (Fig. 3B,C,E) might affect the migration and incorporation of node progenitors into the node. This hypothesis is supported by the rescue of node morphology in the tetraploid chimeras (Fig. 4D).

One important character of the mouse node is the formation and function of monocilia. Although these cilia are present in almost all ventral cells in *Bmp4*-null mutants and tetraploid chimeras analyzed, whether leftward nodal flow is present remains to be determined.

Bmp4 made in epiblast-derived tissues is required for molecular LR patterning

A key finding of this study is that in both *Bmp4*-null mutants and tetraploid chimeras, there is reduction of *Nodal* expression in the node and left LPM (Fig. 1A, parts b,c; Fig. 5D-F). This suggests that Bmp4 made in epiblast-derived tissues is an upstream regulator of Nodal signaling and the establishment or maintenance of the left-side molecular pathway.

One unanswered question is whether defects in the molecular LR pathway in *Bmp4* mutants are translated into defect in anatomical structures. Homozygous *Bmp4* mutants

die before asymmetries in gut derivatives develop, and the only anatomical read-out that can be assayed is heart looping. Hypomorphic *Nodal* mutants have randomized heart looping, with ~50% to the right and 50% to the left (Lowe et al., 2001). By contrast, about half of the *Bmp4*-null mutants examined here have mesocardia, in which the heart does not loop at all. We have observed that *Bmp4* is strongly expressed, symmetrically, in the outflow tract of the eight- to ten-somite stage embryo (data not shown). This raises the possibility that abnormalities in the LR patterning of the heart are compounded by a second *Bmp4* function intrinsic to cardiac morphogenesis. Early cardiac specific inactivation of *Bmp4* will be required to test this hypothesis.

The cardiac and LR molecular marker expression defects caused by loss of *Bmp4* function are observed in 50-75% of *Bmp4*-null mutants, but in all tetraploid chimeras. This discrepancy is possibly due to the different genetic backgrounds on which the null mutation is presented. Tetraploid chimeras are completely ES cell-derived (Nagy et al., 1993), so that our chimeras reflect the phenotype of *Bmp4*-null mutants on the 129/SvEvTaqfBR inbred background. As previously shown, homozygous *Bmp4* mutants on an inbred background have more severe defects than on an outbred background (Winnier et al., 1995; Lawson et al., 1999), although the genetic basis for this effect is not yet known.

Role of epiblast-derived Bmp4 in regulating *Nodal* expression in the node

Several epiblast-derived cell populations express *Bmp4*, including the ExM, the most posterior mesoderm of the primitive streak, and, later, the LPM (Lawson et al., 1999). Based on timing of expression, it is likely that Bmp4 expressed in the ExM and/or the most posterior primitive streak affects the initiation of *Nodal* expression in the node, while consolidation and maintenance of this *Nodal* expression is regulated by *Bmp4* expressed in the posterior LPM from the late neural plate stage. Previous genetic loss of function studies using hypomorphic *Fgf8* mutants proposed that Fgf8 produced in the primitive streak positively regulates *Nodal* expression in the node (Meyers and Martin, 1999). *Fgf8* expression in our tetraploid chimeras was moderately reduced, but still present (Fig. 5H). However, whether the level of Fgf8 in these chimeras reaches the threshold necessary to support *Nodal* expression in the node still remains unclear. An attractive alternative possibility is that molecules other than Fgf8 are required in this signaling pathway. Examining the expression of other signaling molecules in *Bmp4*$^{-/-}$ mutants might shed light on the answer to this question in the future.

Role of epiblast-derived Bmp4 in regulating *Nodal* expression in the lateral plate mesoderm

Left side determinant genes, including *Nodal* and *Lefty2*, are first expressed in the left LPM of the embryo at the two- to six-somite stage (Collignon et al., 1996; Meno et al., 1996). By contrast, expression of *Bmp4* in the ExM, posterior primitive streak and LPM starts earlier, at least from the headfold stage. This suggests that Bmp4 positively regulates downstream genes that establish and maintain LR asymmetry, including expression of *Nodal*, *Lefty2* and cryptic. Our genetic loss-of-function studies alone do not address the specific role of LPM-produced Bmp4 in regulating Nodal signaling in the LPM. We therefore took an alternative approach and inhibited the activity of Bmps, including Bmp4, in the LPM by applying noggin in culture to embryos that already express *Nodal* in the node. This resulted in the absence of *Nodal* expression in the LPM, while transcription continued in the node. This finding in the mouse is inconsistent with the model proposed in the chick embryo in which the function of Bmps (Bmp2, Bmp4, Bmp7) is to suppress Nodal signaling in the LPM. An important feature of the chick model is the presence of caronte in the left LPM that inhibits Bmp signaling and consequently permits *Nodal* expression in the left LPM (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). In the mouse, no caronte homolog has been identified, and the Bmp antagonists, noggin and chordin, are not expressed in the LPM (Davidson and Tam, 2000) (Fig. 6A). Therefore, it can be concluded that mouse and chick embryos use different molecular mechanisms in the LPM to establish and maintain LR asymmetry.

Cryptic is a positive co-factor of Nodal signaling (Shen and Schier, 2000; Yeo and Whitman, 2001), and is expressed bilaterally in the LPM, node, and midline axis, from the headfold to six- to eight-somite stage (Shen et al., 1997). Bilateral cryptic expression in the LPM is seen in *Bmp4*-null mutants at the headfold to one somite stage (Fig. 2C, part g), but is undetected in later two- to six-somite-stage mutants (Fig. 2C, parts e,f). This suggests that embryo-derived Bmp4 functions as a maintenance factor for cryptic expression in the LPM. It is of interest that, unlike *Bmp4*-null mutants and tetraploid chimeras, the headfold-stage embryos cultured in vitro with added noggin did express cryptic in the LPM and midline (Fig. 6E). A significant difference between the mutants and the wild-type embryos cultured with noggin (n=17) is that in the latter, *Nodal* expression is still observed in the node (Fig. 6D). This argues that cryptic expression in the LPM and/or midline is initiated and can be maintained by *Nodal* expressed in the node (Fig. 7C). In the future, sorting out the precise contribution of the different tissues expressing *Bmp4* will be best achieved by inactivation of the gene by conditional gene targeting strategies.

Downstream Bmp4 signaling in LR axis formation

Bmps signal through transmembrane receptors and downstream components such as Smad1, Smad5, Smad8 and Smad4 (Massague and Chen, 2000). Recently, it was reported that about half of the *Smad5*-null mutants at the one- to five-somite stage have bilateral *Nodal* expression, while the remainder show no expression in the LPM. Moreover, in *Smad5*-null mutants, *Nodal* expression in the node is normal, with enrichment in the left periphery (Chang et al., 2000). However, Lefty1, which functions as a midline barrier, is absent in all *Smad5*-null mutants examined, suggesting that the bilateral LPM *Nodal* expression is a result of a midline defect (Chang et al., 2000). By contrast, we did not observe any *Bmp4*-null mutants with bilateral *Nodal* expression in the LPM, and the loss of left LPM *Nodal* expression in *Bmp4*-null mutants and tetraploid chimeras was, in all cases, accompanied by strongly reduced *Nodal* expression in the node (mutants, n=14/14; chimeras, n=6/6). The discrepancy in LR patterning between the *Smad5*- and *Bmp4*-null phenotypes may therefore be due to differences in the level of Nodal production in the node, which is required to activate *Nodal* signaling in the left LPM.

Summary: a model for the establishment of LR asymmetry in the mouse embryo

Our genetic loss of *Bmp4* function analyses provide new insight into the signaling cues that initiate *Nodal* expression in the node and left LPM of the mouse embryo. We propose that Bmp4 functions at two stages. As summarized in Fig. 7, Bmp4 made in the ExM and/or posterior primitive streak promotes the initial expression of *Nodal* in the node, possibly by a mechanism independent of *Fgf8* (Fig. 7B). This effect is apparently independent of an earlier role for Bmp4 in proximodistal patterning of the epiblast and the morphogenesis of the node (Fig. 7A). Second, we propose that Bmps, including Bmp2 and Bmp4, function later in the LPM to positively regulate the expression of *Nodal*. Cryptic might ensure the competence of the LPM to respond to the signal that activates the left-side signaling cascade (Fig. 7C). The essential feature of this proposal is that Bmp4 is a left-side-signaling facilitator during mammalian embryonic development.

Note added in proof

Since the submission of this manuscript, two papers have appeared demonstrating that BMP2 is a positive regulator of Nodal signaling in the chick embryo (Schlange et al., 2002; Piedra and Ros, 2002).

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