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 (Rodríguez 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 among most vertebrates (Wright, 2001; Hamada et al., 2002). Nodal induces Lefty2 (Left – 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 Bmp4tm1h null mutants, the mutation was maintained on a (129/SvEvTacBRxBlack Swiss) background by intercrossing. Bmp4lacZ/lacZ heterozygotes were maintained on the Black Swiss outbred background and Bmp4lacZ/lacZ embryos were obtained by intercrossing. Bmp4lacZ/lacZ mouse-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 MgCl2, 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% CO2/95% air. The culture medium was Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% 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 Bmp4tm1-
null mutant phenotype varies with genetic background, the (129/SvEvTacFBRxBlack 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. 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 Bmp4tm1 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 Bmp4tm1 mutants was analyzed using scanning electron microscopy (SEM). Although not all mutants have abnormal LR asymmetry, whole-mint 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.i). However, in the two Bmp4tm1 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, Bmp4tm1 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 Bmp4tm1 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 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 Bmp4tm1 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 Fgfl8-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 Bmp4tm1 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 Bmp4tm1 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 Bmp4tm1 mutants. To address this possibility, we made use of the fact that in Bmp4lacZ/+ embryos the expression of Bmp4 in the LPM is marked by lacZ activity (Fig. 1C, parts i,j) (Lawson et al., 1999). Staining for ß-galactosidase revealed abundant Bmp4lacZ-positive cells with a normal morphology in the LPM of Bmp4lacZ/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 Bmp4tm1 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

Fgfl8 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<sup>tm1</sup> mutants. (A) Posteroventral views of five-somite stage wild-type (parts a,d,g,j) and four- to five-somite stage Bmp4<sup>tm1</sup> 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-i) 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<sup>tm1</sup> 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 e,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,m) β-galactosidase staining to detect Bmp4<sup>lacZ</sup> reporter expression in six-somite stage Bmp4<sup>lacZ</sup> heterozygous (i,j) and seven-somite stage Bmp4<sup>lacZ</sup> homozygous-null (m) embryos. (j,m) Transverse sections in the plane indicated (yellow bar) in i and m. Arrows indicate the Bmp4<sup>lacZ</sup>-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,i,k and 10 µm for parts b,d,f,h,i; in C, 100 µm for parts j,l,m.
Bmp4 and LR asymmetry

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 Bmp4tm1 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 Bmp4tm1 mutants (Fig. 2B,C). One six-somite stage Bmp4tm1 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 Bmp4tm1 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 Bmp4tm1 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 Bmp4tm1 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 Bmp4tm1 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 on the left at the four- to six-somite stage (Fig. 2K) (Pearce et al., 1999). In 80% (n=4/5) of Bmp4tm1 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**

Homzygous null Bmp4tm1 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 Bmp4tm1 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 Bmp4tm1 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 Bmp4lacZ/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

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**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,L,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 (arrowhead; \(n=1/5\)) (M), 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 \(\mu\)m for B,C,E,F.
somite-stage tetraploid chimeras \((n=3/3)\) generated using Bmp4lacZ/\(+\) 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 Bmp4tm1 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). 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
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,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/antero-posterior/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. 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.

**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 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).
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)]. 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).

Bmp4 and LR asymmetry

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

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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).
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
anomalies 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/SvEvTagfBR 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 Fgfl8 mutants proposed that Fgfl8 produced
in the primitive streak positively regulates Nodal expression in
the node (Meyers and Martin, 1999). Fgfl8 expression in our
tetraploid chimeras was moderately reduced, but still present
(Fig. 5H). However, whether the level of Fgfl8 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 Fgfl8 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 facilitated during mammalian embryonic development. Two papers have been published that discuss the role of Bmps in the establishment of LR asymmetry.

REFERENCES


identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development 124, 429-442.