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BMP antagonism is required in both the node and lateral plate mesoderm for mammalian left-right axis establishment

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In mouse, left-right (L-R) patterning depends on asymmetric expression of *Nodal* around the node, leading to *Nodal* expression specifically in the left lateral plate mesoderm (LPM). Bone morphogenetic protein (BMP) signaling is also involved, but the mechanistic relationship with *Nodal* expression remains unclear. We find that BMP signal transduction is higher in the right LPM, although *Bmp4*, which is required for L-R patterning, is expressed symmetrically. By contrast, the BMP antagonists noggin (Nog) and chordin (Chrd) are expressed at higher levels in the left LPM. In *Chrd;Nog* double mutants, BMP signaling is elevated on both sides, whereas *Nodal* expression is absent. Ectopic expression of *Nog* in the left LPM of double mutants restores *Nodal* expression. Ectopic *Bmp4* expression in the left LPM of wild-type embryos represses *Nodal* transcription, whereas ectopic *Nog* in the right LPM leads to inappropriate *Nodal* expression. These data indicate that chordin and noggin function to limit BMP signaling in the left LPM, thereby derepressing *Nodal* expression. In the node, they promote peripheral *Nodal* expression and proper node morphology, potentially in concert with Notch signaling. These results indicate that BMP antagonism is required in both the node and LPM to facilitate L-R axis establishment in the mammalian embryo.

KEY WORDS: Noggin, Chordin, Nodal, BMP, Left-right asymmetry, Mouse

INTRODUCTION

The first morphological indication of mammalian left-right (L-R) asymmetry occurs during somitogenesis stages, in the direction of heart looping. Later, heart, lungs, liver, spleen, intestines and the vascular system all display asymmetry about the L-R axis. Morphological abnormalities and misalignments of organs resulting from laterality defects can cause disease or death. In humans, clinically significant laterality defects occur in at least 1 in 10,000 births (Peeters and Devriendt, 2006).

This L-R asymmetric organ morphogenesis depends on earlier L-R axis formation. In mouse, this is established around the node, the location of the Spemann organizer at the end of gastrulation, and is propagated from the midline to the lateral plate mesoderm (LPM) (Shiratori and Hamada, 2006). Nodal, a member of the transforming growth factor beta (TGFβ) superfamily of secreted ligands, is a crucial left-side determinant. *Nodal* expression occurs peripheral to the node and then in the left LPM (Collignon et al., 1996). Perinodal *Nodal* expression is required for *Nodal* expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003). An initially low level of Nodal in the LPM can induce *Nodal* expression itself, via a positive-feedback mechanism (Saijoh et al., 2000). Nodal also activates downstream target genes in the left LPM, such as *Lefty2*, an inhibitor of Nodal itself (Saijoh et al., 2000), and *Pitx2*, which regulates left-side-specific morphogenesis (Logan et al., 1998; Yoshioka et al., 1998). Inappropriate *Nodal* expression in the LPM thus leads to severe laterality defects, underscoring the importance of determining how the asymmetric expression of *Nodal* is regulated.

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Bone morphogenetic proteins (BMPs), another class of the TGFβ superfamily, play an important role in regulating *Nodal* expression in the LPM. In chick embryos, a Cerberus-like factor, *Caronte*, is expressed in the left paraxial mesoderm and left LPM, where it promotes *Nodal* expression; it appears to do so by inhibiting Bmp2, Bmp4 and Bmp7 in the LPM, suggesting a negative role for BMP in regulating *Nodal* (Yokouchi et al., 1999; Rodriguez-Esteban et al., 1999). However, beads coated with BMP in the right LPM activated Nodal, whereas beads coated with the BMP antagonist noggin placed in the left LPM blocked *Nodal* expression (Piedra and Ros, 2002). Thus, data from the chick system have suggested both positive and negative roles for BMP in regulating asymmetric *Nodal* expression.

How these results from the chick model relate to the roles of BMP in mammalian L-R formation is unclear. Molecular regulation of L-R axis formation may differ between chick and mouse (Meyers and Martin, 1999), and there does not appear to be a mammalian *Caronte* ortholog. In mouse, *Bmp2* and *Bmp4* are expressed symmetrically in the LPM (Fujiwara et al., 2002) and are present at the right time and place to influence LPM *Nodal* expression. Experiments in mouse embryos have also suggested either positive or negative roles for BMP signals in regulating *Nodal*. Without embryonic *Bmp4*, *Nodal* is not expressed, and embryos cultured with the BMP antagonist noggin (Nog) do not express *Nodal* in the LPM (Fujiwara et al., 2002). These results suggest a positive role. Nevertheless, embryos lacking the BMP signaling component genes *Smad5* or *Alk2* (*Acvr1* – Mouse Genome Informatics) show bilateral *Nodal* expression in the LPM (Chang et al., 2000; Kishigami et al., 2004), suggesting a negative role in the mouse. This conclusion is consistent with data from other vertebrates; for example, exogenous introduction of a constitutively active BMP receptor causes diminished *Nodal* expression in the *Xenopus* embryo (Ramsdell and Yost, 1999). Similarly, ectopic expression of *bmp2b* throughout the zebrafish embryo causes diminished *Nodal* expression (Chocron et al., 2007). Overall, it is clear that BMP signaling plays an important role in regulating asymmetric *Nodal* expression during mammalian development, but both its function in doing so and the manner in which it is regulated remain unresolved.

The organizer BMP antagonists Nog and chordin (Chrd) regulate BMP signaling prior to ligand-receptor binding (Balemans and Van Hul, 2002). *Chrd;Nog* double-mutant mouse embryos exhibit defects in the formation of all three embryonic axes (Bachiller et al., 2000), but there has been no analysis of the role of these factors in establishing the L-R axis. Here we use genetics and molecular embryology to study the roles of BMP signaling and BMP antagonism in regulating *Nodal* expression during mammalian L-R axis establishment.

MATERIALS AND METHODS

Mouse strains and embryos

Wild-type embryos were generated from random outbred ICR stock (Harlan). Nog^{9e} and $Chrd^{tm1Emdr}$ mutations were maintained in an outbred, ICR background as described (Anderson et al., 2002). *Chrd* is homozygous viable in this genetic background, without the phenotypes of the specific backgrounds previously reported (Bachiller et al., 2003). $Nodal^{tm1Rob}$ (Collignon et al., 1996) and $Bmp4^{tm2Blh}$ (Lawson et al., 1999) heterozygotes were maintained in an ICR genetic background. Triple and quadruple mutants were generated by crossing $Chrd^{-/-};Nog^{+/-};Bmp4^{+/-}$ with $Chrd^{-/-};Nodal^{+/-}$.

Embryos were collected at E8.0-8.5 or E9.0-9.5, or after culture, fixed at 4°C overnight in 4% paraformaldehyde in PBS, washed in PBS and stored at –20°C in methanol. After gene expression analysis, genomic DNA was prepared from each embryo and genotyped by PCR as described: *Nog* and *Chrd* (Anderson et al., 2002); *Bmp4* (Stottmann et al., 2006); *Nodal* (Collignon et al., 1996).

Gene expression assays and immunostaining

Whole-mount in situ hybridization (WMISH) was performed according to standard procedures (Yamamoto et al., 2004) with the following probes: *eGFP*, *Lefty2*, *Lefty1* (Nakamura et al., 2006); *Lfng* (Kume et al., 2001); *Cryptic* (Shen et al., 1997); *Nodal* (Collignon et al., 1996); *Pitx2c* (Liu et al., 2001); *Nog* (McMahon et al., 1998); and *Chrd* (Klingensmith et al., 1999). *lacZ* staining was performed as described (Stottmann et al., 2001). Quantitative (q) RT-PCR used total RNA prepared from 20 pieces of left or right 4-5s LPM using Trizol (Invitrogen) and glycogen carrier (Ambion), with reverse transcription employing a Taqman RT-PCR Kit (Applied Biosystems) after DNase I treatment (Ambion). qPCR was performed using the MyiQ Real-Time PCR System and SYBR Green mix (Bio-Rad) with the following primers (forward and reverse): *Nog*, 5′-TTTTGGCCACGCTACGTGAA-3′ and 5′-CTAGCAGGAACACTTACACT-3′; *Chrd*, 5′-TTCCCAGAGAATCAGAGCTG-3′ and 5′-TCTGGAAGGGTTCTAGTCTC-3′; *Nodal*, 5′-ACTTTGCTTTGGGAAGCTGA-3′ and 5′-ACCTGGAACTTGACCCTCCT-3′; *Bmp4*, 5′-AGACCCTAGTCAACTCTGTT-3′ and 5′-CTCTACCACCATCTCCTGAT-3′; β-actin, 5′-AAGAGCTATGAGCTGCCTGA-3′ and 5′-CACAGGATTCCATACCCAAG-3′. Whole-mount immunostaining was performed as described with anti-phospho-Smad1/5/8 antibody (Cell Signaling) (Yang and Klingensmith, 2006) or anti-acetylated tubulin (Sigma) (Nakaya et al., 2005). Node areas were calculated by NIH Image software and statistical treatment used the χ^2 test.

Whole embryo and explant culture

For whole embryo culture, headfold-stage embryos were isolated and parietal endoderm removed. Liposomes composed of expression vectors and Lipofectamine 2000 (Invitrogen) were injected between the endoderm and LPM (see Fig. S3 in the supplementary material). Expression vectors were *eGFP*, with the *eGFP* gene in the pCAGGS vector (Okabe et al., 1997), and *Nog*, *Bmp4* or *Nodal*, in which each coding region was inserted into pEF-BOS (Mizushima and Nagata, 1990). For control embryos, *GFP* vector was injected alone. For experimental embryos, equal concentrations of eGFP and secreted product vectors were used. Liposome solution was prepared as follows: 12.5 μl OPTI-MEM (Invitrogen) was mixed with 1 μl Lipofectamine 2000 or with 1 μg expression vector(s). These solutions were combined and incubated for 5 minutes at room temperature; 0.1 μl solution was injected. Embryos were cultured with rotation for 14 hours in a humidified atmosphere of 5% CO_2/95% air at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with 50% rat serum. Embryos with direct eGFP fluorescence in the desired regions were selected for WMISH with probes for *GFP* and a relevant marker. For Nodal signaling experiments, 3s ICR embryos were cultured with DMSO (0.1% in DMEM) or SB431542 (Sigma) in 0.1% DMSO (100 μg/ml) for 3 hours until 5s.

Node explants (including peripheral tissues) were isolated from 1-3s ICR embryos and cultured for 4 hours at 37°C and 5% CO_2 in DMEM containing 10% FCS. Control culture was performed with BSA (200 ng/ml). Experimental culture was performed with recombinant human BMP2 (200 ng/ml) (R&D Systems). Twenty explants from either treatment were used to isolate total mRNA. qRT-PCR was performed using the following primers (forward and reverse): *Lfng*, 5′-CACCATTGGCTACATTGTAG-3′ and 5′-CAAACATGCCATAGCTTCAGG-3′; *Dll1*, 5′-AAGTGCCAGTCACAGAGCTC-3′ and 5′-TGCAGACAGAACATACACCG-3′; *Notch1*, 5′-AGTCAGGCAGATGTACAACC-3′ and 5′-AGGAACTGGGTAGTGGTCAT-3′; *Rbpjk*, 5′-ACCTTCACCTACACACCAGA-3′ and 5′-GACGATGTGACACTGGTAGA-3′.

RESULTS

Reciprocal elevation of asymmetric BMP signaling activity and BMP antagonist expression in LPM

Given the ambiguity concerning the roles of BMP signaling in regulating *Nodal* expression during mouse L-R axis formation, we wanted to know where BMP signaling was active in the relevant spatiotemporal context. *Nodal* is expressed in the left LPM at the 3- to 5-somite stages (3-5s) (Collignon et al., 1996; Nakamura et al., 2006). Accordingly, we assayed the spatiotemporal distribution of BMP signaling activity during early somite stages. We used immunohistochemistry to visualize binding of antibodies to phosphorylated Smads 1, 5 and 8 (p-Smad1/5/8; Smad 8 is also known as Smad9 – Mouse Genome Informatics) in mouse embryos. Activation of the BMP receptor complex by BMP ligand binding causes Smad1/5/8 phosphorylation and subsequent signal transduction (Kishigami and Mishina, 2005). As previously shown (Yang and Klingensmith, 2006), the distribution of active BMP signaling during gastrulation and neurulation largely coincides with expression of *Bmp4* and other BMP genes (Furuta et al., 1997; Solloway and Robertson, 1999). At 2s, p-Smad1/5/8 expression was observed bilaterally in all embryos. At 3s, 75% of embryos (9/12) still expressed bilaterally, but 25% (3/12) showed weaker expression in left LPM. Strikingly asymmetric p-Smad1/5/8 distribution was observed in all embryos at 4-5s, with elevated levels in the right LPM relative to the left (Fig. 1A-C). By contrast, *Bmp4* is expressed bilaterally in the LPM at 4-5s (Fig. 1D,E) (Fujiwara et al., 2002). Expression of *Bmp2* is bilateral like *Bmp4* (Fujiwara et al., 2002), and *Bmp7* is in the node and midline (Solloway and Robertson, 1999). In summary, we observed higher levels of BMP signaling activity in the right LPM than in the left, although none of the known BMP ligands active at this time shows a similarly biased expression pattern. This left-sided decrease in BMP signaling occurs at or just before the time when *Nodal* begins to be expressed in the left LPM.

Our results suggest that asymmetric activation of BMP signaling in the mammalian LPM does not depend on BMP gene expression patterns. It might instead be created by asymmetric BMP antagonist activity. We therefore examined expression of *Chrd* and *Nog* in this context. At embryonic day 8 (E8.0), *Nog* is expressed robustly in the node, notochord and lateral neural folds (McMahon et al., 1998), and at lower levels in heart, allantois and LPM. At 4-5s, elevated expression was observed in the left LPM relative to the right (Fig. 1F-H). *Chrd* was expressed broadly at low levels, with higher levels in node and notochord; however, we also detected increased *Chrd*

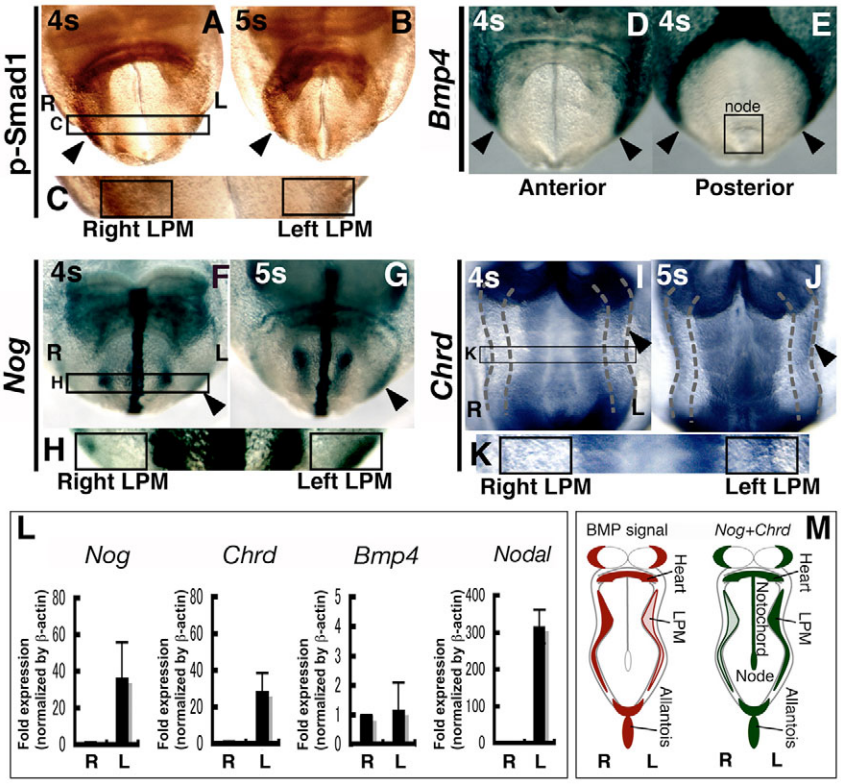

Fig. 1. L-R asymmetric Smad1/5/8 activation and BMP antagonist expression in mouse embryo lateral plate mesoderm. (**A-C**) Whole-mount immunohistochemistry for phosphorylated (p-) Smad1/5/8 in 4s (A) (n=7/10) and 5s (B) (n=8/8) embryos (anterior view). Anti p-Smad1/5/8 staining in lateral plate mesoderm (LPM) is stronger on the right side than on the left (arrowhead). (C) High magnification of the boxed region from A. Rectangles demark left and right LPM. (**D**,**E**) Whole-mount *lacZ* staining of *Bmp4*$^{lacZ/+}$ embryo at 4s (n>10). *Bmp4* expression is observed bilaterally at the edge of the LPM (arrowhead). Anterior (D) and posterior (E) views. The node is boxed. (**F-H**) Whole-mount *lacZ* staining of *Nog*$^{lacZ/+}$ embryo at 4s (F) (n=6/6) and 5s (G) (n=6/6). *Nog* expression in LPM is stronger on the left side than right (arrowhead). (F,G) Anterior view. (H) High magnification of the boxed region from F. (**I-K**) Whole-mount in situ hybridization (WMISH) for *Chrd* at 4s (I) (n=6/12) and 5s (J) (n=10/16). *Chrd* expression is stronger in left LPM than right (arrowhead). Ventral views. The dashed line indicates the extent of LPM on each side. (K) High magnification of the boxed region from I. (**L**) Quantitative RT-PCR results for *Nog*, *Chrd*, *Bmp4* and *Nodal* in right versus left LPM at 4s. R, right; L, left. (**M**) Summary of BMP signaling activity and BMP antagonist expression at 4-5s. BMP signaling activity is represented in red (left), *Nog* and *Chrd* expression in green (right), with higher levels in a darker hue.

hybridization in left LPM relative to the right (Fig. 1I-K). Embryo sections confirmed that these expression domains are in the LPM itself, rather than in adjacent germ layers (see Fig. S1 in the supplementary material). We independently assayed BMP antagonist expression by quantitative RT-PCR (qPCR) using RNA isolated from left or right LPM at 4-5s, finding that both *Nog* and *Chrd* are expressed at higher levels in the left LPM (Fig. 1L). Together, these results indicate that the BMP antagonists *Nog* and *Chrd* are expressed at elevated levels in left LPM (Fig. 1M), implicating them in producing asymmetric BMP signaling activity during early L-R axis formation.

Noggin and chordin are required for normal L-R morphogenesis and asymmetric expression of left-side determinants in LPM

Consistent with the hypothesis that Chrd and Nog are involved in mammalian L-R axis formation, the double-null mutant (*Chrd*$^{-/-}$;*Nog*$^{-/-}$) has defects in directionality of heart looping (Bachiller et al., 2000) (Fig. 2A-D; see Table S1 in the supplementary material). By contrast, *Chrd*$^{-/-}$;*Nog*$^{+/-}$ and *Chrd*$^{-/-}$;*Nog*$^{+/+}$ embryos all exhibited normal heart looping (Fig. 2D). These results suggest that the BMP antagonists Nog and Chrd function redundantly in L-R axis formation. We therefore investigated the expression of *Nodal* and other asymmetrically transcribed genes in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos. *Nodal* expression was greatly diminished or absent in 82% of embryos (n=11) (Fig. 2E,F). A few embryos (9%) expressed *Nodal* in right LPM (Fig. 2I). Expression of *Lefty2* and *Pitx2*, targets of *Nodal* in the left LPM (Hamada et al., 2002), was also diminished or absent in most *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos (Fig. 2F,H,I; see Fig. S2 in the supplementary material). Expression of *Cryptic* (*Cfc1* – Mouse Genome Informatics), a Nodal signaling component, was unchanged (see Fig. S2 in the supplementary material). These results suggest that *Nog* and *Chrd* are required for mammalian L-R axis establishment by promoting *Nodal* expression in the left LPM.

Noggin and chordin are required for *Nodal* expression around the node and for node morphology

A key factor in initiating *Nodal* expression in the left LPM is *Nodal* expression around the node (Brennan et al., 2002; Saijoh et al., 2003). Given that *Chrd* and *Nog* are expressed in the node (Klingensmith et al., 1999), and that LPM expression of *Nodal* is absent in most *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, we also examined perinodal expression of *Nodal*. This assay revealed three populations: ~29% of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants (7/24) expressed perinodal *Nodal* at essentially the same level as in wild type (denoted ++); ~58% of mutants (14/24) showed greatly reduced expression (+); and 12% showed no detectable *Nodal* expression around the node (–) (Fig. 3A-E). These data reveal that *Chrd* and *Nog* play an important role in promoting perinodal *Nodal* expression, but are not necessarily essential for its expression.

We correlated the level of *Nodal* expression around the node with the status of *Nodal* expression in the LPM in the *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants, revealing two populations. Mutants showing essentially normal levels of *Nodal* around the node (the '++' class) showed weak but detectable expression of *Nodal* in the LPM (3/7). By contrast, embryos showing weak (+) or absent (–) *Nodal* expression around the node (17/17) showed no expression of *Nodal* in the LPM (Fig. 3F). Thus, *Chrd* and *Nog* promote *Nodal* expression around the node and subsequent expression in LPM.

These results imply that ectopic BMP activity around the node would downregulate local *Nodal* expression. We observed ectopic anti-p-Smad1/5/8 staining around the nodes of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants relative to wild-type embryos at the stages we assayed,

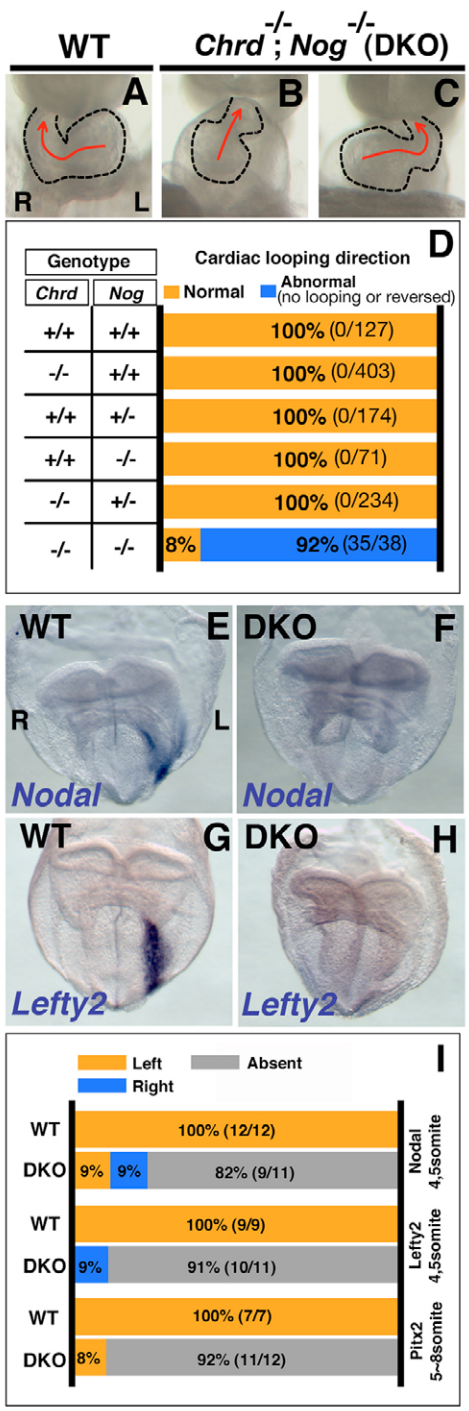

Fig. 2. Chordin and noggin are both required for correct heart looping and expression of left-side determinants. (**A-C**) Arrows indicate laterality of heart looping from left ventricle to outflow tract. Dashed lines outline hearts. (A) E9.0 wild-type (WT) mouse embryo showing normal rightward heart looping. (B) E9.0 *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (DKO) embryo showing no directional looping of the heart. (C) E9.0 *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryo showing reversed, leftward looping. (**D**) Summary of cardiac looping at E9.5 in various genotypes, each investigated in >20 embryos. (**E-H**) Embryos subjected to WMISH at 5s. WMISH for *Nodal* probe in wild type (E) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (F) and for *Lefty2* in wild type (G) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (H). (**I**) Summary of expression patterns of the L-R asymmetric markers *Nodal*, *Lefty2* and *Pitx2* in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos. The percentages and numbers of embryos in each class are shown.

including presomitic bud-stage embryos (data not shown) and 4-5s embryos (see Fig. S3 in the supplementary material). This indicates that BMP signaling is indeed increased perinodally in the absence of Chrd and Nog. To directly test the consequences of ectopic BMP around the node on *Nodal* expression, we cultured explants isolated from 1-3s wild-type embryos with recombinant human BMP2 protein. Whereas the control carrier protein BSA had no affect, ectopic BMP markedly decreased perinodal *Nodal* expression (see Fig. S3 in the supplementary material).

Notch signaling is a positive regulator of perinodal *Nodal* expression (Krebs et al., 2003; Raya et al., 2003). To assess Notch signaling in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos, we assayed expression of lunatic fringe (*Lfng*), a positive transcriptional target in the presomitic paraxial mesoderm (Morales et al., 2002). We observed sharply reduced levels of *Lfng* expression just lateral and anterior to the node at the early somite stage in the mutants (see Fig. S3 in the supplementary material), suggesting that BMP reduces the activity of the Notch pathway. To test this, we cultured explants from the node region of wild-type embryos with BMP2. Expression of the Notch signaling targets Dll1 and Lfng was reduced (see Fig. S3 in the supplementary material). We also observed dysmorphic nodes in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos, of abnormal shape and with fewer cilia (Fig. 3G-I). Along with L-R heart looping defects, similarly dysmorphic nodes and absent perinodal *Nodal* expression have been observed previously in embryos lacking the Notch signaling components Dll1 (Przemeck et al., 2003) and Baf60c (Smarcd3 – Mouse Genome Informatics) (Takeuchi et al., 2007). Collectively, these findings indicate that BMP antagonism in the node via Chrd and Nog is necessary for expression of *Nodal* around the periphery of the node, and suggest that the loss of this expression in *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos results from decreased activity of the Notch pathway.

Ectopic noggin in left LPM rescues local *Nodal* expression in *Chrd;Nog* mutants

Some *Chrd*$^{-/-}$;*Nog*$^{-/-}$ mutants with significant perinodal *Nodal* expression nevertheless lacked *Nodal* expression in the left LPM (Fig. 3B,F). Moreover, in wild-type embryos, both *Chrd* and *Nog* are elevated in left LPM, whereas BMP signal transduction through Smad1/5/8 is reduced there relative to the right LPM. These findings raise the possibility that beyond promoting *Nodal* expression around the node, *Nog* and *Chrd* have an additional direct function to promote *Nodal* expression in the LPM. Accordingly, we investigated BMP signaling activity in the LPM of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos by assaying p-Smad1/5/8 staining. We observed increased staining, with bilaterally equivalent levels in left and right LPM (Fig. 4A,B), indicating that Chrd and Nog are required for the left-sided reduction in BMP signaling.

To directly test whether BMP antagonism in the left LPM per se can promote *Nodal* expression therein, we introduced a *Nog* expression vector into the left LPM of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos at the late headfold stage (schematized in Fig. S4 in the supplementary material). *GFP* was included with *Nog* to mark transfected cells. In control injections, we introduced the *GFP* vector alone. Embryos were then cultured to 5-6s and assayed by in situ hybridization for *Nodal* and *GFP*. The results are summarized in Fig. 4F. Injection of *Nog* and *GFP* vectors into the left LPM of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos resulted in *Nodal* expression in left LPM in 40% of embryos (10/25), whereas introduction of *GFP* vector alone resulted in 4% of mutants (1/23) expressing *Nodal* in the left LPM (Fig. 4Fa), a highly significant difference (P<0.001).

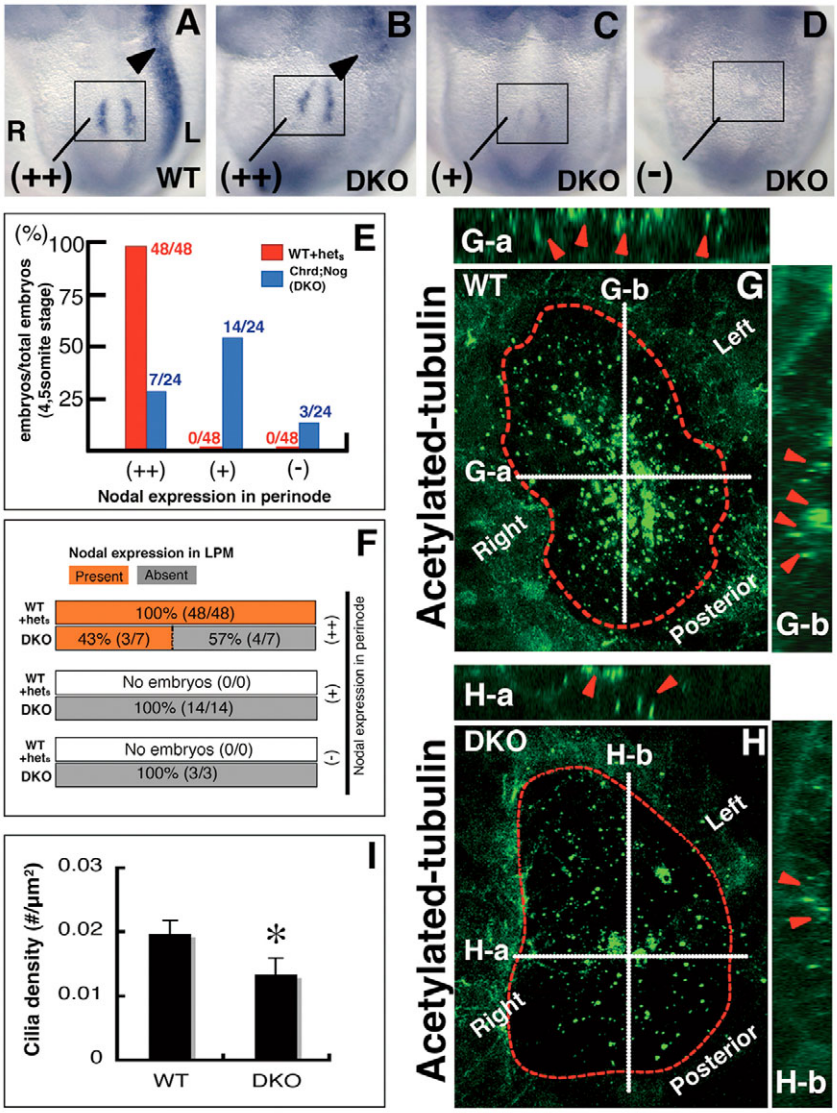

Fig. 3. Noggin and chordin promote *Nodal* expression around the node, the level of which correlates with *Nodal* expression in left LPM. (**A-D**) Ventral views of 4s mouse embryos hybridized with *Nodal* probe. The node area is boxed. The approximate level of *Nodal* expression around the node is judged as normal (++), weak (+) or undetectable (–). Arrowheads indicate *Nodal* expression in LPM. (A) Wild-type embryo. (B-D) *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (DKO) embryos showing variable levels of perinodal *Nodal* expression. (**E**) Summary of perinodal *Nodal* expression in non-mutant control (wild type, *Chrd*$^{-/-}$;*Nog*$^{+/+}$ and *Chrd*$^{-/-}$;*Nog*$^{+/-}$) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos. Numbers above bars indicate embryos of a given result among the total assayed. (**F**) Summary of the relationship between *Nodal* expression around the node and in LPM of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos. (**G**,**H**) Wild-type (G) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (H) nodes at 1s stained for acetylated tubulin, marking cilia. Dashed lines indicate node boundaries. Confocal sections along planes G-a, -b and H-a, -b are shown alongside. Red arrowheads, example cilia. (**I**) Node cilia density at 1s, comparing wild-type (n=3) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (n=3) embryos. *, significant difference (mean <0.05).

We then considered how the expression of *Nodal* in the left LPM related to the expression of *Nodal* around the node in the mutants injected with *Nog* and/or *GFP* vectors (Fig. 4Fb). Among embryos showing robust *Nodal* expression around the node (++), 4/6 also showed *Nodal* expression in the LPM when *Nog* was ectopically expressed there. By contrast, only 1/5 of such embryos expressed *Nodal* in the left LPM when only *GFP* was injected. In *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos with low perinodal *Nodal* expression (+), 6/15 showed *Nodal* in the left LPM when *Nog* was injected into this tissue (Fig. 4E). None (0/14) expressed *Nodal* in the left LPM when only *GFP* was introduced (Fig. 4D). This difference is significant (P=0.007). When the mutant embryos showed no detectable *Nodal* around the node, however, ectopic *Nog* in the left LPM never resulted in *Nodal* expression there (0/4). These data indicate that BMP antagonism in the left LPM promotes *Nodal* expression in this tissue, but BMP antagonism might not be sufficient for *Nodal* expression in the left LPM. Instead, it is likely to depend on synergistic influences from the node region, such as *Nodal* itself. Thus, BMP antagonism appears to create a permissive environment for *Nodal* expression in the left LPM.

BMP activity in the LPM represses local *Nodal* expression

The direct, positive effect of BMP antagonist expression on *Nodal* expression in the left LPM of the *Chrd*$^{-/-}$;*Nog*$^{-/-}$ double-null implies that local BMP activity in the LPM inhibits *Nodal* expression. We tested this by introducing a *Bmp4* expression vector into the left LPM of wild-type embryos. About 80% (16/20) of such embryos showed absent *Nodal* expression in the left LPM, versus 4/18 when the vector contained *GFP* alone (Fig. 4G,H,K), a highly significant increase (P<0.001). This manipulation increased left-side BMP signaling (Fig. 4G,H). We created a similar imbalance by expressing ectopic *Nog* in the right LPM of wild-type embryos. This resulted in ectopic right-sided *Nodal* expression in several embryos: 4/15 showed bilateral *Nodal* expression in the LPM, and one showed only right-sided expression in the LPM (Fig. 4J,L). When the control *GFP* vector alone was transfected into the right LPM, none (0/16) showed right-sided *Nodal* expression (Fig. 4I,L), again a significant difference (P=0.023). These results indicate that BMP signaling in the LPM represses *Nodal* expression there, whereas local BMP antagonism in the LPM derepresses *Nodal* transcription in this tissue.

Chordin and noggin synergize with *Nodal* to promote left-side determination by impeding endogenous *Bmp4*

Nodal promotes its own expression in the left LPM via a positive-feedback loop (Shiratori and Hamada, 2006), and our results indicate that BMP antagonism also promotes *Nodal* expression in the LPM. To genetically assess the relevance of our embryo culture experiments to *Nodal* regulation, we produced embryos mutant for combinations of *Chrd*, *Nog*, *Nodal* and *Bmp4* null alleles, then assayed *Nodal* expression. Embryos of the control genotypes $Nodal^{+/-}$, $Chrd^{-/-}$ and $Chrd^{-/-};Nog^{+/-}$ were indistinguishable from wild type, showing normal robust *Nodal* expression in the node and left LPM (Fig. 5A,B,C,F). By contrast, $Chrd^{-/-};Nodal^{+/-}$ and $Chrd^{-/-};Nog^{+/-};Nodal^{+/-}$ embryos fell into two distinct classes (see Table S2 in the supplementary material). One had robust *Nodal* expression around the node, as in wild-type embryos (denoted ++). Among these, 66% of $Chrd^{-/-};Nodal^{+/-}$ embryos and only 12.5% of $Chrd^{-/-};Nog^{+/-};Nodal^{+/-}$ embryos showed *Nodal* expression in the left LPM (Fig. 5D,G). Embryos in the second class showed markedly reduced perinodal *Nodal* expression (+), of which ~42% of $Chrd^{-/-};Nodal^{+/-}$ embryos expressed *Nodal* in the left LPM but at reduced levels, whereas the rest showed no expression (Fig. 5E).

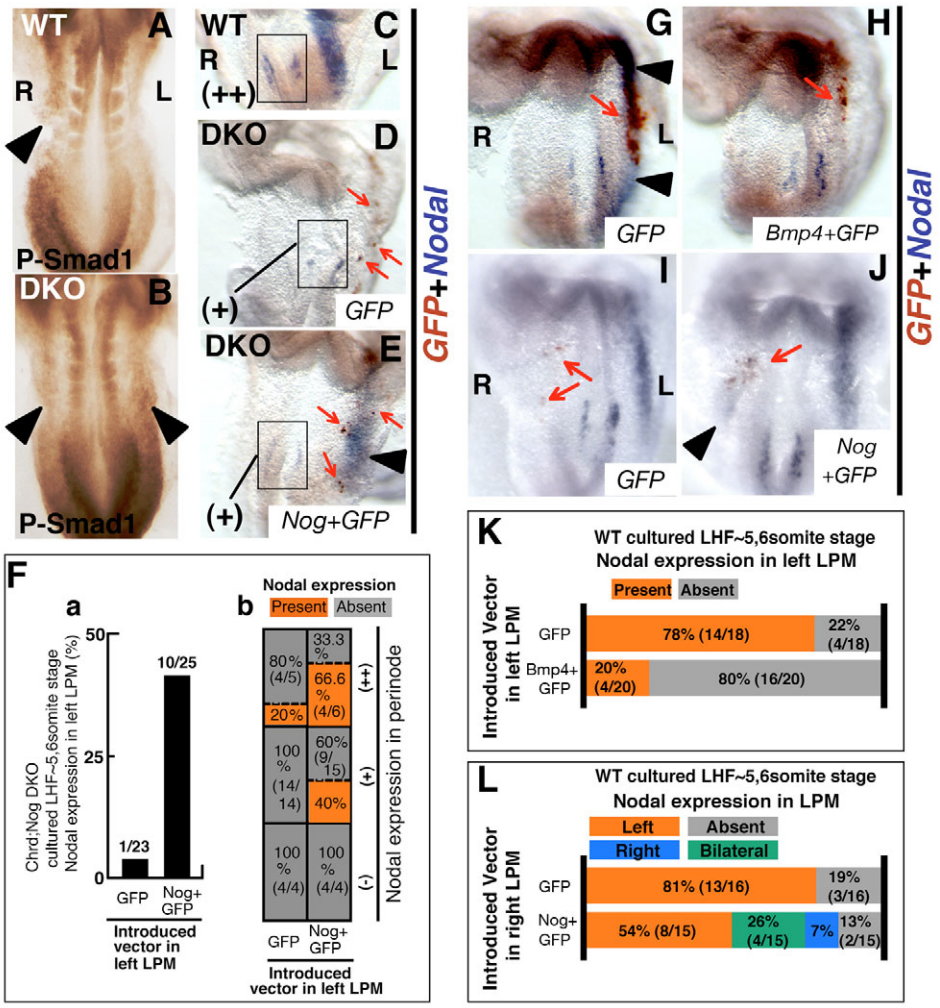

Fig. 4. Endogenous asymmetric BMP antagonism by noggin and chordin regulates *Nodal* expression in LPM. (**A**,**B**) Wild-type (A) and $Chrd^{-/-};Nog^{-/-}$ (B) mouse embryos subjected to immunohistochemistry for p-Smad1/5/8 (p-Smad1). Arrowhead indicates p-Smad1 in LPM. *n*>3 embryos per genotype assayed at 5s. (**C-L**) Cultured embryos assayed for *Nodal* and *GFP* expression by two-color WMISH. Red staining shows *GFP* expression, purple staining *Nodal* expression. (C) Wild-type embryo showing the normal level (++) of perinodal *Nodal* expression (boxed). (D) $Chrd^{-/-};Nog^{-/-}$ embryo injected with *GFP* vector alone into the left LPM, showing *GFP* expression (red arrows) but no *Nodal* expression in LPM. Weak *Nodal* expression (+) occurs in the node (boxed). (E) $Chrd^{-/-};Nog^{-/-}$ embryo injected with both *GFP* and *Nog* vectors into left LPM. *Nodal* expression in the left LPM (arrowhead) occurs in the vicinity of *GFP* (red arrows). (F) Summary of rescued *Nodal* expression in left LPM. (a) Graph of *Nodal* expression in left LPM of manipulated $Chrd^{-/-};Nog^{-/-}$ embryos. Numbers above bars indicate embryos of a given result among total embryos assayed. (b) Subdivision of results as a function of *Nodal* expression around the node [normal (++), weak (+) or absent (–)], comparing $Chrd^{-/-};Nog^{-/-}$ embryos injected with *GFP* vector alone (left column) versus embryos injected with both *GFP* and *Nog* vectors (right column). Orange indicates presence of *Nodal* expression in left LPM, gray indicates its absence (value in each bar is percentage of embryos with absent or present left LPM *Nodal* expression). (G) Wild-type embryo injected with *GFP* vector into left LPM. Arrowheads indicate *Nodal* expression in LPM, red arrows indicate *GFP*. (H) Wild-type embryo injected with *GFP* and *Bmp4* vectors into left LPM. (I) Wild-type embryo injected with *GFP* vector into right LPM. (J) Wild-type embryo injected with *GFP* and *Nog* vectors into right LPM. (K) Summary of *Nodal* expression in embryos injected in left LPM with *GFP* alone or *Bmp4* plus *GFP*. (L) Summary of *Nodal* expression in embryos injected in right LPM with *GFP* alone or *Nog* plus *GFP*.

Strikingly, no *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$ embryos showed any expression of *Nodal* in the LPM (Fig. 5H). Thus *Chrd*, *Nog* and *Nodal* have a positive genetic interaction that promotes robust *Nodal* expression in the left LPM.

Bmp4 is expressed bilaterally in the LPM (Fig. 1D,E) (Fujiwara et al., 2002), raising the possibility that it is an endogenous BMP ligand antagonized by Chrd and Nog in the left LPM. We therefore reduced the gene dosage of *Bmp4* in the triple *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$ mutants to see whether the phenotypic defects were lessened. Indeed, many quadruple mutant *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$;*Bmp4*$^{+/-}$ embryos showed rescue of *Nodal* expression in the left LPM, with 42% expressing robust levels of perinodal *Nodal* (++) and 25% expressing low levels (+) (Fig. 5J,K). By contrast, when *Bmp4* dosage was normal, only 13% of *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$ embryos showed *Nodal* expression in LPM when perinodal expression was robust (Fig. 5G), and none showed such expression when perinodal expression was weak (Fig. 5H). These results suggest that endogenous left-side elevated *Chrd* and *Nog* expression permits activation of a *Nodal* positive-feedback loop in the left LPM by antagonizing local Bmp4 activity.

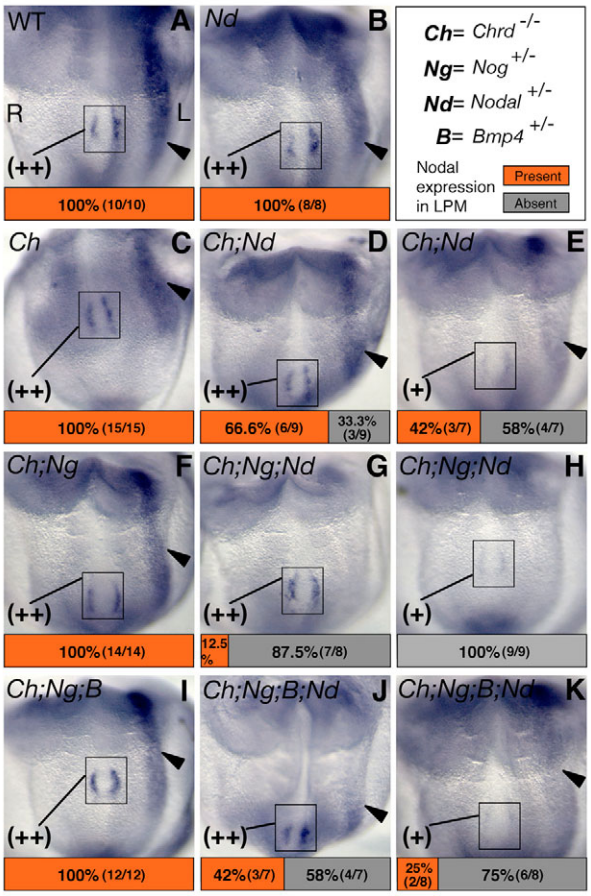

Fig. 5. Genetic reduction of chordin, noggin and *Nodal* causes molecular laterality defects that are rescued by decreasing *Bmp4* dosage. (**A-K**) Ventral views of 5s mouse embryos hybridized with *Nodal* probe. The node region is boxed. Arrowheads indicate *Nodal* expression in LPM. (A) Wild type, (B) *Nodal*$^{+/-}$, (C) *Chrd*$^{-/-}$, (D,E) *Chrd*$^{-/-}$;*Nodal*$^{+/-}$, (F) *Chrd*$^{-/-}$;*Nog*$^{+/-}$, (G,H) *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$, (I) *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Bmp4*$^{+/-}$ and (J,K) *Chrd*$^{-/-}$;*Nog*$^{+/-}$;*Nodal*$^{+/-}$;*Bmp4*$^{+/-}$. At the bottom of each panel is a summary of the *Nodal* expression in the left LPM and the level of perinodal *Nodal* expression [normal (++) or weak (+)]. Orange indicates the presence of *Nodal* expression in left LPM, gray its absence.

Elevated BMP antagonist expression in the left LPM is induced by Nodal

Given the significance of Nodal as a left-side determinant, we considered whether the asymmetry in BMP antagonist expression is initially established by left-side-specific Nodal signaling. *Nog* expression in the left LPM correlates spatially and temporally with *Nodal* expression (see Fig. S6 in the supplementary material); moreover, we never observed asymmetric *Nog* expression in the LPM of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos (Fig. 6B,D), in contrast to wild type (Fig. 1) and *Nog*$^{-/-}$ homozygotes (Fig. 6A,C). Also, *Nodal* is expressed in the left LPM of *Nog*$^{-/-}$ (see Fig. S5 in the supplementary material) but not of *Chrd*$^{-/-}$;*Nog*$^{-/-}$ embryos (Fig. 2F,I). These data suggest that elevated BMP antagonist expression in the left LPM might be a product of left-side-specific Nodal signaling.

To explore this possibility, we cultured wild-type embryos with a specific inhibitor of Nodal signaling, SB431542 (Inman et al., 2002). It inhibits activation of TGFβ signaling by blocking the phosphorylation ability of the activin type I receptors Alk4, 5 and 7 (Acvr1b, Tgfbr1 and Acvr1c, respectively – Mouse Genome Informatics) without affecting BMP signaling activation (Inman et al., 2002). SB431542 can block both endogenous and exogenous signaling activation via Smad2 phosphorylation in embryos (Ho et al., 2006). We first evaluated expression of the Nodal target gene *Lefty2* in embryos subjected to SB431542 or to the carrier, DMSO. Embryos cultured with DMSO alone showed normal *Lefty2* expression in left LPM (Fig. 6E), whereas embryos cultured with SB431542 lacked *Lefty2* expression (Fig. 6F). Thus, Nodal signaling is inhibited by SB431542 treatment in this assay. The DMSO-treated embryos showed normal elevation of *Nog* expression in the left LPM (Fig. 6G). By contrast, embryos cultured with SB431542 lacked left-side elevation of *Nog* expression, it being reduced to approximately the same basal level as in the right LPM (Fig. 6H). We also assayed BMP antagonist expression by qPCR in left and right LPM of 4-5s embryos treated with DMSO or SB431542. Both *Nog* and *Chrd* expression levels in the left LPM were dramatically decreased by SB431542 treatment (see Fig. S7 in the supplementary material).

Lastly, we investigated the consequences of ectopic *Nodal* in the right LPM on BMP antagonist expression. Control embryos injected with the *GFP* expression vector showed normal *Lefty2* expression in left LPM and left-side elevation of *Nog* expression (Fig. 6I,K). As expected from previous results (Nakamura et al., 2006), embryos injected with the *Nodal* vector into the right LPM showed reversed, right-side expression of *Lefty2* (Fig. 6J). Thus, these conditions created a L-R reversed Nodal signaling context. In such embryos, *Nog* expression was elevated on the right side rather than on the left (Fig. 6L). Altogether, these results reveal that the left-side-specific elevation of *Nog* expression is produced by Nodal-dependent left-side determination, and suggest that increased BMP antagonist expression in the left LPM is induced by Nodal signaling.

DISCUSSION

Here we define for the first time the functions of endogenous BMP antagonism in the establishment of the L-R axis of mammalian embryos. In addition to elucidating crucial roles for BMP signaling attenuation by Chrd and Nog, we demonstrate directly the controversial role of BMP activity in regulating *Nodal* expression in

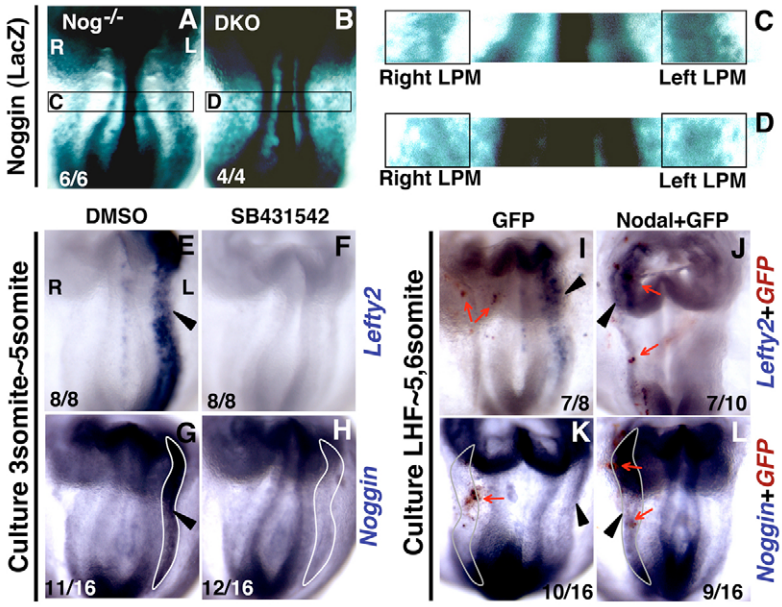

Fig. 6. Nodal induces elevated noggin expression in left LPM. (**A-D**) Ventral views of *lacZ* staining in 5s *Nog*$^{-/-}$ (A,C) and *Chrd*$^{-/-}$;*Nog*$^{-/-}$ (DKO) (B,D) mouse embryos. C and D are high magnification views of the boxed regions from A and B, respectively, showing the left and right LPM regions. (**E-H**) Cultured embryos at 5s hybridized with *Lefty2* (E,F) or *Nog* (G,H) probe. Embryos were cultured with DMSO (E,G) or with the Nodal signaling inhibitor SB-431542 in DMSO (F,H). White lines surround left LPM in G and H. (**I**,**J**) Cultured 5s embryos hybridized with *Lefty2* (purple) and *GFP* (red) probes. (I) Control embryo with *GFP* vector introduced into right LPM. (J) Embryo with *GFP* and *Nodal* vectors introduced into right LPM. Red arrows indicate expression of *GFP* in LPM, arrowheads that of *Lefty2*. (**K**,**L**) Cultured 5s embryos hybridized with *Nog* (purple) and *GFP* (red) probes. (K) Control embryo with *GFP* vector introduced into right LPM. (L) Embryo with *GFP* and *Nodal* vectors introduced into right LPM. Gray line surrounds right LPM. Red arrows indicate expression of *GFP* in LPM, arrowheads that of *Nog*.

the LPM. We found that endogenous levels of BMP signaling activity are higher in the right LPM than in the left; by contrast, *Chrd* and *Nog* are both expressed at higher levels on the left. In vivo, these BMP antagonists are necessary to create this asymmetry of BMP activity in the LPM. In *Chrd;Nog* mutants, *Nodal* expression in the left LPM is reduced or absent. This implies that BMP in the LPM represses *Nodal* expression, and that BMP antagonists function there directly to protect *Nodal* expression. We confirmed these roles by manipulating BMP signaling and antagonism specifically in the LPM of cultured embryos. Further embryo culture experiments showed that Nodal activity is responsible for this left-side-specific upregulation of BMP antagonists. In addition to promoting Nodal activity in the left LPM, Chrd and Nog also promote *Nodal* expression around the node. Consistent with our embryological results, genetic crosses suggested that *Chrd*, *Nog* and *Nodal* act synergistically in L-R patterning, whereas *Bmp4* acts antagonistically to these factors.

Thus, our data indicate that Chrd and Nog function together to facilitate at least two major aspects of L-R axis establishment. First, they are required in the node for normal node morphology and for perinodal expression of *Nodal*. They are then necessary in the left LPM to diminish BMP signaling activity in this domain, and thus inhibit the repressive effect of BMP on the Nodal autoregulatory loop. These findings lead us to propose a model for the mechanisms by which Chrd and Nog function in the establishment of the L-R axis (Fig. 7).

Chordin and noggin promote *Nodal* expression around the node

A crucial leftward flow created by rotating monocilia in the node leads to the asymmetric distribution of extracellular cues, with subsequent transfer of asymmetry cues to the left LPM (Shiratori and Hamada, 2006). Perinodal expression of *Nodal* is required for *Nodal* expression in left LPM (Brennan et al., 2002; Saijoh et al., 2003), and may itself be a component of the laterality signal transferred from the node to the LPM (Oki et al., 2007). *Chrd;Nog* mutant embryos form nodes, but of abnormal morphology and reduced cilia density. They have a variable reduction of *Nodal* expression around the node, ranging from nearly normal to none detectable. These data indicate that BMP antagonists promote perinodal expression of *Nodal* but are not essential for it.

The initial L-R defects in *Chrd;Nog* mutants might be explained by one or more of the following mechanistic possibilities. The sparse cilia or dysmorphic shape in *Chrd;Nog* nodes might be inadequate to generate sufficient levels of essential leftward cues. Or, an unknown BMP-like molecule might directly repress perinodal *Nodal* expression, and the lack of local BMP antagonism in the mutants would leave it unopposed. A third possibility is that *Chrd;Nog* mutants lack sufficient activity of a positive regulator of *Nodal* expression in this domain. Data suggest this might be the Notch pathway, which is known to promote *Nodal* expression around the node (Krebs et al., 2003; Raya et al., 2003). We observed reduced *Lfng* and *Dll1* expression, genes that are positively regulated by Notch signaling, in the vicinity of the node. The phenotypes we observed of heart looping defects, dysmorphic nodes and reduced perinodal *Nodal* expression are similar to those of embryos lacking the Notch signaling components Dll1 (Przemeck et al., 2003) and Baf60c (Takeuchi et al., 2007). These findings imply an antagonistic relationship between BMP and Notch signaling in L-R patterning at the node. A precedent for such a relationship has been documented in the cerebellar rhombic lip (Machold et al., 2007).

BMP signaling represses *Nodal* expression in LPM

We observed higher levels of endogenous BMP signaling activity in the right LPM, i.e. in the side lacking *Nodal* expression. Our site-specific manipulation experiments demonstrated that BMP signaling in the LPM leads to local repression of *Nodal* expression. Introduction of a *Bmp4* expression vector into portions of the left LPM resulted in a coincident reduction in *Nodal* expression, whereas exogenous *Nog* similarly introduced into the right LPM caused ectopic *Nodal* expression in the right LPM. These results strongly support the conclusion that the right-sided elevation of endogenous BMP signal activity functions to repress *Nodal* expression in the right LPM.

Additional evidence from *Xenopus*, zebrafish, chick and mouse studies also supports a negative regulatory role for BMP signaling on *Nodal* expression in the LPM (see Introduction). Nevertheless,

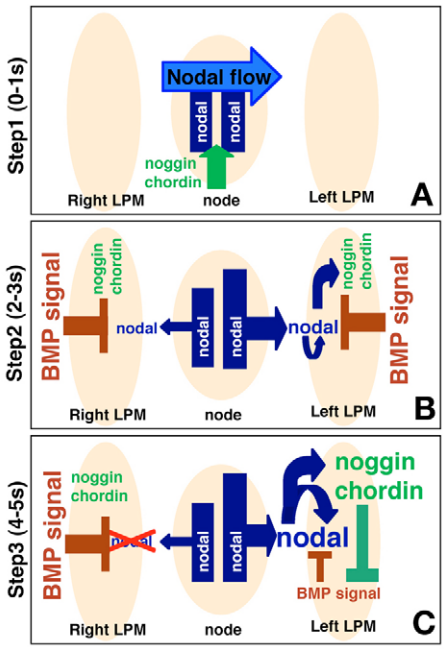

Fig. 7. Three-step model for BMP antagonism by noggin and chordin in promoting *Nodal* expression during left-right axis establishment in mouse. (**A**) Step 1, from presomitic to 1s. Nog and Chrd promote *Nodal* expression around the node. Leftward flow is created by cilia rotation. (**B**) Step 2, 2-3s. *Nodal* expression is higher on the left than right side of the node, but both left and right LPM receive some level of *Nodal*-dependent signals. Bilateral BMP signals in LPM repress Nodal signaling and its positive-feedback loop. Stronger Nodal signals to left LPM induce increased BMP antagonist expression. (**C**) Step 3, 4-5s. Increased *Nog* and *Chrd* expression in left LPM elevates left-side-specific BMP antagonism, inhibiting the repressive action of BMP on *Nodal* expression. As a result, a robust *Nodal* positive-feedback loop is established in left LPM.

some data suggest a positive role for BMP signaling in regulating *Nodal* expression in the LPM. Of greatest relevance is a previous report in mouse that Bmp4 is a positive regulator of *Nodal* expression in the left LPM (Fujiwara et al., 2002), which is the opposite conclusion to ours. Fujiwara et al. found that when *Bmp4* was absent in extraembryonic as well as embryonic tissues, node morphology was abnormal and *Nodal* expression was absent. When only embryonic expression was missing, node morphology was restored but *Nodal* was still absent, in both node and LPM. However, because *Bmp4* is never expressed in the node or its vicinity, it seems very unlikely that Bmp4 has a local role in regulating *Nodal* expression around the node. It is more likely that the node was functionally abnormal owing to defects in primitive streak or mesodermal development, despite looking morphologically intact.

To determine whether there might be a positive role for BMP signaling in the expression of *Nodal* in the LPM, Fujiwara et al. (Fujiwara et al., 2002) cultured wild-type embryos with Nog after *Nodal* expression was established around the node; this resulted in a lack of *Nodal* in the LPM. By contrast, our data demonstrate that BMP activity has a negative role directly in the LPM to downregulate *Nodal*. Moreover, our genetic crosses demonstrate that *Bmp4* per se has an antagonistic relationship with *Chrd*, *Nog* and *Nodal* in expression of *Nodal* in the LPM. We speculate that the discrepancy between the results of these studies might be owing to experimental differences in the timing or specificity of manipulations. For example, because the exposure to Nog recombinant protein in these cultured embryos was ubiquitous rather than specific to the left LPM, we suggest that the lack of *Nodal* expression might have been due to an indirect effect of defective production or transport of the laterality signal from the node to the LPM, rather than to a direct effect on the LPM.

BMP antagonism in the left LPM relieves the repressive effects of BMP on *Nodal* expression

Our analysis revealed asymmetric *Nog* and *Chrd* expression in the LPM. Elevation of expression of these genes on the left side is consistent with the endogenous right-side elevation of BMP signal activity we observed in wild-type embryos. By contrast, *Chrd;Nog* embryos showed bilaterally equivalent BMP signal distribution in the LPM. Introduction of exogenous *Nog* directly into the LPM of *Chrd;Nog* embryos rescued *Nodal* expression in the LPM in some embryos. Thus, *Nog* and *Chrd* promote *Nodal* expression in both the node and LPM, and function synergistically to help establish the L-R axis sequentially in the node and LPM.

The functional significance of this asymmetric BMP antagonist distribution was further supported by the genetic interaction of *Chrd*, *Nog* and *Nodal*. $Chrd^{-/-};Nog^{+/-};Nodal^{+/-}$ embryos showed diminished *Nodal* expression in LPM even in those embryos having robust *Nodal* expression around the node. Removal of one functional allele of *Bmp4* in this compound mutant partially rescued *Nodal* expression in the left LPM, implying that endogenous Nog and Chrd function in the left LPM to protect *Nodal* expression by antagonizing local Bmp4.

Robust *Nodal* expression in the LPM is established by a positive-feedback loop mechanism dependent on Nodal itself (Saijoh et al., 2000). An early step toward *Nodal* expression in the left LPM appears to be the transfer of a small amount of Nodal from the node (Oki et al., 2007). We showed that asymmetric *Nog* expression in LPM is induced by Nodal. Accordingly, the physiological significance of the endogenous asymmetric BMP antagonist expression in the LPM might be to maintain this Nodal positive-feedback loop. Meanwhile, left-side expression of *Nodal* is suggested to suppress *Nodal* expression on the right side through induction of Nodal inhibitors (Nakamura et al., 2006). Our finding of right-side elevated BMP signal distribution and its repressive effect on *Nodal* expression might function synergistically with this mechanism.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/14/2425/DC1

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