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BMP signaling positively regulates Nodal expression during left right specification in the chick embryo

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SUMMARY

Exogenous application of BMP to the lateral plate mesoderm (LPM) of chick embryos at the early somite stage had a positive effect on *Nodal* expression. BMP applications into the right LPM were followed by a rapid activation of *Nodal*, while applications into the left LPM resulted in expansion of the normal domain of *Nodal* expression. Conversely, blocking of BMP signaling by Noggin in the left LPM interfered with the activation of *Nodal* expression. These results support a positive role for endogenous BMP on *Nodal* expression in the LPM. We also report that BMP positively regulates the expression of *Caronte*, *Snail* and *Cfc* in both the left and right LPM. BMP-treated embryos had molecular impairment of the midline with downregulation of *Lefty1*, *Brachyury* and *Shh* but we also show that the midline defect was not sufficient to induce ectopic *Nodal* expression. We discuss our findings in the context of the known molecular control of the specification of left-right asymmetry.

Key words: BMP, Left/right, Nodal, CFC, Car, Pitx2

INTRODUCTION

A great deal of research carried out in recent years has shown that the development of the left-right (LR) axis is a complex and highly regulated process (for reviews, see Capdevilla et al., 2000; Burdine and Shier, 2000; Yost, 2001; Mercola and Levin, 2001). Conceptually, the process of LR specification can be considered to evolve in successive phases (Capdevilla et al., 2000). The first phase involves the breaking of the initial bilateral symmetry of the embryo. In the mouse, this is thought to occur in the node (Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999). In the chick and *Xenopus*, it starts in the peripheral tissues and is then transferred to the node (Pagán-Westphall and Tabin, 1998; Hyatt and Yost, 1998; Levin and Mercola, 1998; Levin and Mercola, 1999). From the node, the information passes into the lateral plate mesoderm (LPM) where side-specific domains of gene expression are established and later translated into the actual asymmetric morphogenesis of the developing organs.

In the chick, several important signaling molecules exhibit small asymmetric domains of expression in the node (Levin et al., 1995; Levin et al., 1997; Boettger et al., 1999; Shamim and Masson, 1999; Garcia-Castro et al., 2000; Monsoro-Burq and Le Douarin, 2000; Monsoro-Burq and Le Douarin, 2001; Kawakami and Nakanishi, 2001; Rodriguez-Esteban et al., 2001). Multiple regulatory relationships between these molecules have been shown to control LR asymmetries. ActivinβB and Bmp4 signaling excludes *sonic hedgehog* (*Shh)* from and induces *Fgf8* expression in the right side of the node (Levin et al., 1995; Boettger et al., 1999; Monsoro-Burq and Le Douarin, 2001). Asymmetric *Shh* expression in the left side of Hensen's node causes asymmetric *Nodal* expression in the left LPM, an effect mediated by the induction of Caronte (Car) (Pagán-Westphal and Tabin, 1998; Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Car is a member of the Cerberus/DAN family of bone morphogenetic protein (BMP) antagonists and is expressed in the left LPM. Considering that Car acts upstream of *Nodal* and that several BMPs are symmetrically expressed in the LPM, the current model postulates that *Nodal* expression results from the abolition of the repressor effect of BMP signaling by Car (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999).

Nodal is a member of the TGFβ superfamily that is expressed in the left LPM and is considered to be a left determinant because it is sufficient to control the laterality of the heart and other organs (Levin et al., 1997; Lowe et al., 2001). Identified target genes of Nodal are the transcription factors *Pitx2* and *Nkx3.2*, both implicated in the control of organ laterality (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St Amand et al., 1998; Yoshioka et al., 1998; Campione et al., 1999; Schneider et al., 1999; Rodriguez-Esteban et al., 1999; Nielsen et al., 2001).

Several observations have implicated members of the TGFβ superfamily in LR specification in several vertebrates. For example in *Xenopus*, BMP signaling establishes right-sided identity in mutual antagonism with the Vg1-dependent left-sided pathway (Ramsdell and Yost, 1999; Burdine and Shier, 2000; Yost, 2001). In zebrafish, asymmetric expression of *Bmp4* is involved in the control of heart development (Cheng

et al., 1997). Members of the Lefty subfamily have also been identified in several vertebrate species and implicated in LR development (Meno et al., 1996; Meno et al., 1997; Meno et al., 1998; Thisse and Thisse, 1999).

In the present work, we have performed a functional analysis of the role of BMP signaling in the late phase of LR asymmetry specification in the chick embryo. We found that exogenous application of BMP to the right LPM rapidly and consistently induced *Nodal* expression and subsequently that of *Pitx2*. Increased BMP signaling in the left LPM also upregulated the normal *Nodal* and *Pitx2* expression. *Car*, *Snail* and *Cfc* expression in the LPM were also positively regulated by BMP signaling. Conversely blocking of BMP signaling by Noggin interfered with the expression of all the above-mentioned genes. Our experiments also showed that the right sided BMP-dependent induction of *Nodal* was not mediated by the alteration of the midline. We discuss the implications of our findings in the context of the present knowledge of the control of LR asymmetries in the chick embryo.

MATERIALS AND METHODS

Embryos and experimental manipulations

Fertilized hen eggs were obtained from local sources, routinely incubated, opened and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). For the experiments the embryos were explanted in New culture (New, 1955) with the modifications described (Yuan et al., 1995; Chapman et al., 2001).

For the placement of a midline barrier a longitudinal incision (immediately to the right or left of the neural tube) was made in the embryo using a tungsten needle. The barrier was introduced through the incision and inserted into the agar-albumen substrate of the New culture so that it remained vertical. As barriers we used metal foil (aluminum or platinum) or pieces of the internal eggshell membrane (Le Douarin and Fontaine, 1970; Fernandez-Teran et al., 1997). For the removal of the left LPM, the profile of a rectangle spanning the desired area was cut with a tungsten needle and discarded.

In situ hybridization in wholemounts

The embryos were fixed and processed for whole-mount in situ hybridization as in Nieto et al. (Nieto et al., 1996). Chick antisense riboprobes for *Nodal*, *Shh*, *Snail*, *Pitx2*, *Brachyury*, *Noggin* and *Cfc*, which have been described previously, were kindly provided by Thomas Brand, Angela Nieto, Gary Schwoenwolf and Cliff Tabin. The chicken *Car* and *Lefty1* probes were isolated by RT-PCR and their identity confirmed by sequencing. When required, hybridized embryos were routinely embedded in paraffin wax, sectioned and analyzed.

BMP and Noggin misexpression

Human recombinant BMP2, BMP4 and BMP7 proteins (obtained from Genetics Institute and from R&D Systems) were loaded into heparin acrylic beads at the indicated concentration, mainly 0.1 μg/μl. Noggin protein (R&D Systems) was also used loaded in beads at a concentration of 1 μg/μl. The beads were loaded by soaking for at least 1 hour at room temperature and then implanted under the hypoblast or endoderm in the appropriate location in the New-cultured embryos. Noggin was also applied as pellets of Noggin-expressing cells. Chick embryonic fibroblast (CEF) were transfected with RCAS-*Noggin* construct (Pizette and Niswander, 1999).

Beads loaded with PBS were used as control. We also used normal embryos or cultured but untreated embryos as a control for comparisons of patterns of expression.

RESULTS

BMP signaling positively regulates *Nodal* expression in the LPM

Nodal expression is transiently detected in the left LPM from stage 7 to stage 11 HH (Levin et al., 1995) (Fig. 1A). Its pattern of expression is very dynamic with downregulation progressing in an anterior-to-posterior wave. To analyze the effect of BMP signaling on *Nodal* expression, we exogenously

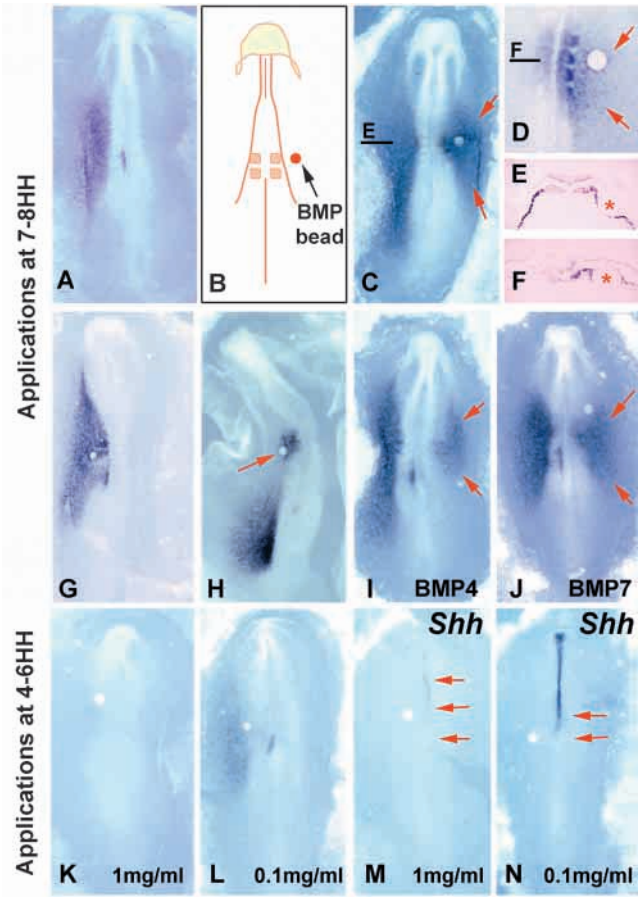

Fig. 1. BMPs positively regulates *Nodal* expression. (A) Normal pattern of *Nodal* expression in a stage 8 embryo. (B) Schematic drawing delineating the experiment of bead placement (red circle) into the stage 7-8 lateral plate mesoderm (LPM). Only the placement on the right is depicted, but for each experiment left and right placements were made. (C) A BMP2-soaked bead induces ectopic expression of *Nodal* in the right LPM. (D) Detail of ectopic right-side expression of *Nodal* in another embryo with the same treatment. (E,F) Transverse sections of the embryos shown in C,D, respectively, at the level indicated. (G) A BMP2-soaked bead in the left LPM expands the normal domain of *Nodal* expression. (H) BMP2 maintains *Nodal* expression, as indicated by the arrow, when the normal domain of expression has moved posteriorly. Treatment with BMP4 (I) and BMP7 (J) had the same effect as treatment with BMP2. (K,L) Repression of Nodal expression after the application of a BMP2-soaked bead (1 mg/ml, K; 0.1 mg/ml, L) at earlier stages (4-6 HH). (M,N) *Shh* expression is downregulated (arrows) by BMP2 (1 mg/ml, M; 0.1 mg/ml, N) application at earlier stages (4-6 HH). The red asterisk marks the position of the bead in the sections. The bead of the embryo in I was dislodged during hybridization.

applied BMP to the LPM. This was performed by inserting heparin acrylic beads soaked in BMP2 human recombinant protein (Genetics Institute; 0.1 μg/μl) into the LPM of stage 7-8 chick embryos in New culture as schematically illustrated in Fig. 1B. Very surprisingly, the implantation of a BMP2-soaked bead in the right LPM was followed by a clear activation of *Nodal* expression (37 out of 40, 92.5%) (Fig. 1C). *Nodal* activation preferentially occurred around the bead (Fig. 1C-D) but in quite a broad domain that occasionally was a mirror image of the normal left pattern (see, for example, Fig. 1J). The ectopic right-side activation of *Nodal* expression by BMP2 was rapid and clearly perceptible from 2 hours after the placement of the bead. Interestingly, any tissue in the vicinity of the bead, including the somites and the neural tube, activated *Nodal* expression (Fig. 1D). This occurred disregarding the side, left or right, of placement of the bead and is shown, for the right side, in Fig. 1E,F, that correspond to transverse sections of the embryos in Fig. 1C and Fig. 1D, respectively. When the BMP2-loaded bead was implanted in the left LPM, the normal expression of *Nodal* was upregulated (Fig. 1G). Owing to the normally high level of *Nodal* expression, increases in the level of expression were difficult to evaluate but the domain of expression of *Nodal* was expanded. Interestingly, the ectopic BMP2 protein resulted in *Nodal* expression being maintained longer than normal, so that when the normal domain of *Nodal* expression moved posteriorly, transcripts were maintained in the proximity of the bead (Fig. 1H).

To explore whether BMP4 and BMP7 had the same effect as BMP2, we repeated the experiments using beads loaded in human recombinant BMP4 and BMP7 (0.1 μg/μl). The results obtained were similar to those described above for BMP2 and are shown in Fig. 1I,J. We checked BMP concentrations ranging from 0.05 μg/μl to 1 μg/μl and found a dose-dependent effect, the induction being minimal, if it existed, at 0.05 μg/μl and increasing as the concentration was raised (not shown).

All these results strongly indicate a positive effect of BMP signaling on *Nodal* expression. This was a striking result considering that BMP2-soaked beads implanted in the left LPM at stage 6 (Yokouchi et al., 1999), or BMP4-soaked beads implanted in the left side of the node at stage 4 (Rodriguez-Esteban et al., 1999) were shown to downregulate *Nodal* expression. We reasoned that the discrepancy in results might reside in the earlier stage of application used in these experiments compared with ours. To check this point we repeated their experiments implanting BMP2-soaked beads (1 mg/ml) to the left LPM of stage 4-6 embryos and reproduced their results obtaining a repression in *Nodal* expression that was complete in 70% of the cases (seven out of 10; Fig. 1K). When the concentration used was 0.1 mg/ml, the downregulation in *Nodal* expression was dramatic but not total (80%, eight out of 10; Fig. 1L). Thus, BMP2 could have a negative effect on *Nodal* at earlier stages and a positive effect at later stages. However, as it was recently demonstrated the BMP signaling on the left-side of the node repressed *Shh* expression (Monsoro-Burq and Le Douarin, 2001), we analyzed the status of *Shh* expression in these early treated embryos. We found that *Shh* expression in the node of embryos treated with the high concentration was abolished (1 mg/ml; 75%, six out of eight; Fig. 1M) and clearly downregulated in embryos treated with the lower concentration (0.1 mg/ml; 66%, four out of six; Fig. 1N). Thus, we concluded that the downregulation on *Nodal* expression observed after early BMP treatments was mediated by a downregulation on *Shh* expression in Hensen's node.

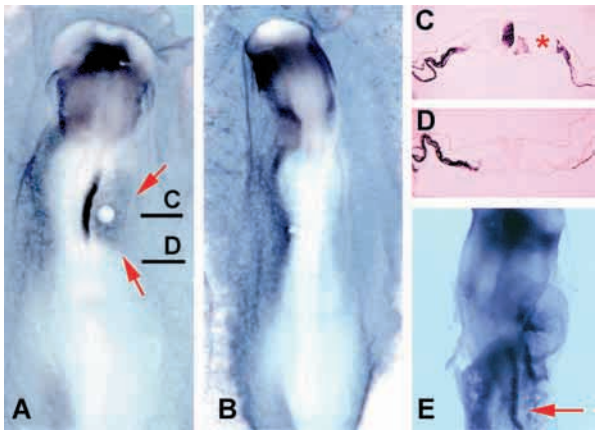

Fig. 2. BMPs positively regulates *Pitx2* expression. (A) Ectopic *Pitx2* expression in the right LPM after BMP treatment. (B) A BMP-soaked bead applied to the left LPM (arrows) enhances *Pitx2* expression. (C) A transverse section through the embryo shown in A at the level of ectopic *Pitx2* expression. The red asterisk marks the bead (arrow) (D) A section through the same embryo at a level of normal *Pitx2* expression. Note that the ectopic activation of *Pitx2* by BMP also affects the neural tube. (E) A BMP-treated embryo 19 hours after the treatment, showing a right-looped heart and maintenance of ectopic right sided *Pitx2* expression. . All the pictures are dorsal views.

Pitx2 is a *paired*-like homeobox gene that is a downstream target of *Nodal* (Harvey, 1998; Shiratori et al., 2001). As expected for a target of Nodal, *Pitx2* was also expressed ectopically following BMP applications into the right LPM (13 out of 16, 81%; Fig. 2A) while applications to the left LPM upregulated the normal pattern of *Pitx2* expression (three out of three; 100%; Fig. 2B). As is true for *Nodal*, *Pitx2* expression was activated by the tissue in the vicinity of the BMP bead, including the somites and the neural tube (Fig. 2A and corresponding section in Fig. 2C). For comparison the normal pattern of expression of *Pitx2* is shown in Fig. 2D.

Finally, to analyze whether BMP-dependent *Nodal* and *Pitx2* ectopic induction resulted in morphological alterations of laterality, some of the treated embryos were allowed to develop in order to assess the direction of cardiac looping. Most of the BMP-treated embryos (16 out of 20, 80%) showed a normal right-sided cardiac loop and maintained bilateral expression of *Pitx2* (Fig. 2E). In spite of the majority of right-looped hearts, malformations of the caudal heart pole were frequent (not shown). Alterations of the morphology of the gut could not be examined because the New culture does not allow development of the embryo up to the required stages.

Blocking of BMP signaling interferes with *Nodal* expression

The positive effect of BMP signaling on *Nodal* expression detected by our experiments, together with normal domains of BMP expression and signaling in the LPM (Streit et al., 1998; Schultheiss et al., 1997; Faure et al., 2002), suggested that endogenous BMP signaling may be involved in normal *Nodal* expression. To check this hypothesis, we blocked endogenous

BMP signaling by the exogenous application of Noggin (Zimmerman et al., 1996). Implantation of Noggin-soaked beads (1 μg/μl) or CEFs transfected with RCAS-*Noggin* into the left LPM at HH stages 5-6 interfered with *Nodal* expression in the LPM (20 out of 25, 80%). In some cases *Nodal* expression was dramatically blocked (28%, seven out of 25; Fig. 3A), while in others it was locally affected around the bead (52%, 13 out of 25; Fig. 3B). Applications of Noggin after *Nodal* initiation of expression (stages 7-8 HH) had a very little effect, if any, on *Nodal* expression (*n*=15, Fig. 3C). When we applied Noggin to the right LPM, the pattern of *Nodal* expression remained unperturbed (*n*=30, 100%; Fig. 3D for Noggin recombinant protein and Fig. 3E for Noggin-expressing cells). These results reinforced the idea that endogenous BMP signaling is required for normal activation of *Nodal* expression. However, the function of BMP in maintaining *Nodal* expression appears to be less significant.

Exogenous Noggin applied at early stages (5-6 HH) also impaired *Pitx2* expression in the LPM (nine out of 10, 90%; Fig. 3F). Applications at later stages also resulted in downregulation of *Pitx2* expression around the source of Noggin (10 out of 17, 58,8%; Fig. 3G,H), an effect that we never observed with control PBS beads (seven out of seven, 100%; Fig. 3I). This was a striking result because, at these stages, Noggin application had no effect on *Nodal* expression and indicated that BMP signaling was essential for maintenance of *Pitx2* expression. The downregulation of *Pitx2* expression was circumscribed to an area of variable extension around the bead (indicated by the red arrows in Fig. 3G,H). Application of Noggin into the right LPM never resulted in activation of *Pitx2* expression (*n*=14, 100%; Fig. 3J).

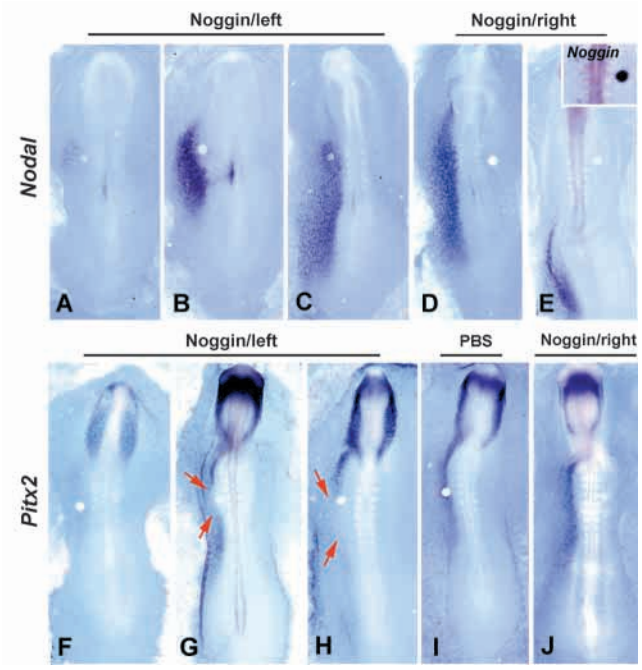

Fig. 3. Noggin interferes with *Nodal* and *Pitx2* expression. (A) A Noggin-bead that has almost completely abolished *Nodal* expression in the LPM. (B) Another example in which Noggin had a partial effect on *Nodal* expression. (C) When the Noggin bead is applied at stage 7-8 HH, the effect is minimal. (D,E) Blocking of BMP signaling by Noggin in the right LPM, either by using recombinant protein (D) or an aggregate of Noggin-expressing cells (E), has no effect on *Nodal* expression. The insert in E shows the high level of *Noggin* expression by the cellular aggregate. (F) Applying Noggin to the left LPM before activation of *Pitx2* expression prevents its normal expression. (G,H) Noggin applied after *Pitx2* activation of expression represses *Pitx2* expression around the bead (G) or in a more ample domain (H), as marked by the arrows. (I) A PBS-loaded bead has no effect on *Pitx2* expression. (J) Noggin does not modify *Pitx2* expression when applied on the right.

BMP positively regulates *Caronte* and *Snail*

Car is transiently expressed in the left LPM in a pattern parallel to *Nodal* (Fig. 4A) (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Owing to its ability to bind BMP and Nodal we decided to explore its pattern of expression after the BMP treatment. BMP application to the left LPM of stage 7-8 embryos resulted in an appreciable expansion of the domain of *Car* expression (eight out of 12, 66.6%; Fig. 4B). BMP application to the right LPM ectopically activated *Car*, resulting in a bilateral pattern of expression (10 out of 12, 83.3%; Fig. 4C). Thus, *Car* also appears to be positively regulated by BMP signaling. As in the case of *Nodal*, Noggin application to the left LPM interfered with *Car* expression but only if applied before its normal initiation of expression (five out of eight, 62%; Fig. 4D). When Noggin was applied at stages 7-8 HH, *Car* expression was only slightly reduced around the bead (indicated by arrows in Fig. 4E; five out of nine, 55%). *Car* expression in a staged-matched control embryo is shown in Fig. 4F for comparison. Thus, the effect of BMP on *Car* expression paralleled that seen for *Nodal*.

Snail is a member of the *Snail* family of transcription factors that is asymmetrically expressed in the right LPM with a clear right-side bias (Isaac et al., 1997; Sefton et al., 1998) (Fig. 4G). It has been proposed that expression of *Snail* and *Nodal* are mutually exclusive (Isaac et al., 1997; Patel et al., 1999). Thus, we decided to analyze the pattern of expression of *Snail* in our BMP-treated embryos in which *Nodal* expression was enhanced. *Snail* expression appeared upregulated in the left LPM by the application of a BMP-soaked bead (three out of three; Fig. 4H), whereas in the right LPM *Snail* expression remained unmodified or occasionally upregulated (43%, three embryos out of seven showed upregulation; Fig. 4I). As there is some variability in the intensity of the expression among different embryos, it is sometimes difficult to assess this upregulation. Interestingly, Noggin clearly interfered with *Snail* expression regardless of the side of application, as shown in Fig. 4J for right-sided application of Noggin (10 out of 10; 100%). In some cases (two out of 10, 20%) applying Noggin to one side resulted in complete abolition of *Snail* expression on both sides, as shown in the embryo in Fig. 3K (which received a pellet of Noggin-expressing cells in the right LPM at stage 7). Taken together, our results indicate that BMP signaling positively regulates the expressions of all the genes analyzed so far, including the right determinant *Snail*.

Cfc expression in the LPM requires BMP signaling

The signaling pathway of Nodal is highly regulated. It has recently been shown that cells become competent to respond to Nodal by expressing EGF-CFC proteins (Gritsman et al., 1999; Yan et al., 1999; Minchiotti et al., 2000; Shen and Schier, 2000; Minchiotti et al., 2001; Yeo and Whitman, 2001;

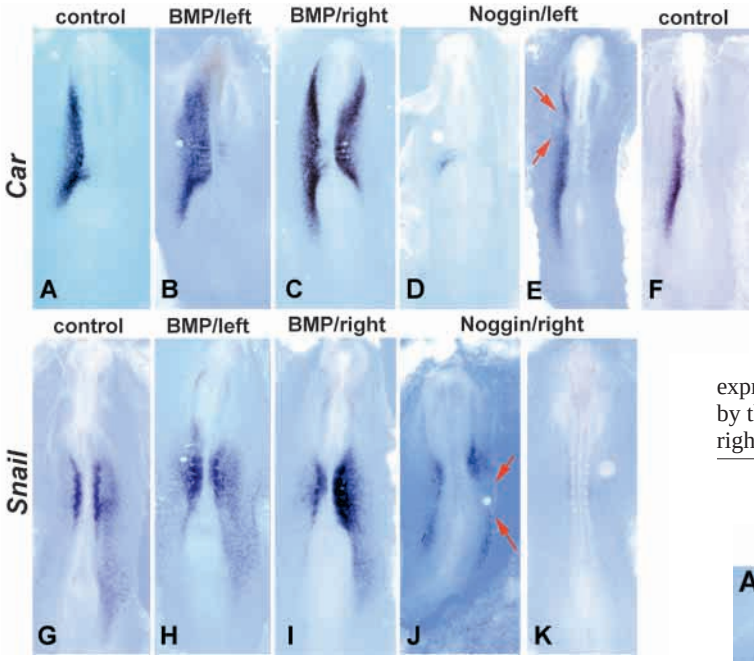

Fig. 4. *Car* and *Snail* expression are positively regulated by BMP signaling. (A) A stage 7 embryo hybridized for *Car*. (B) The normal domain of expression of *Car* is expanded after ectopic BMP signaling to the left LPM. (C) Exogenous BMP signaling in the right LPM results in ectopic *Car* expression. (D) A Noggin-bead that has clearly inhibited the normal expression of *Car*, compare with the stage-matched control in A. (E) Application of Noggin at later stages only minimally downregulated *Car* expression (arrows). (F) Normal expression of *Car* in a stage-matched for comparison. (G) A control stage 8 embryo hybridized for *Snail*. (H) Exogenous BMP signaling in the left LPM results in ectopic activation of *Snail*. (I) BMP application on the right does not modify or upregulate the pattern of *Snail* expression. (J) Inhibition of *Snail* expression by Noggin, indicated by the arrows. (K) A pellet of Noggin-expressing cells applied to the right that completely abolished *Snail* expression.

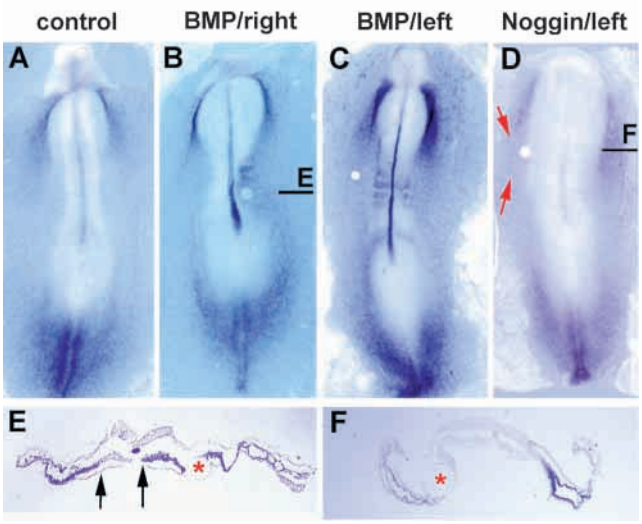

Fig. 5. BMP positively regulates *Cfc* expression. (A) A control stage 8 embryo hybridized for *Cfc*. Exogenous BMP signaling applied to the right (B) or to the left (C) enhances *Cfc* expression. Note that the ectopic expression is stronger in the somites. (D) Noggin application (here shown on the left) inhibits the normal expression of *Cfc*. (E) A transverse section through the embryo shown in B at the indicated level (arrows indicate the medial boundary of each side of *Cfc* expression). (F) A transverse section through the embryo shown in D at the indicated level. The red asterisk marks the position of the bead in the sections.

Whitman, 2001). The EGF-CFC proteins encode extracellular cell-autonomous factors essential for Nodal signaling. Recently a chick member of this family was identified (Colas and Schoenwolf, 2000; Schlange et al., 2001). Following the new nomenclature (Bamford et al., 2000), the chick member has been named *Cfc* and has been shown to be implicated in LR regulation during chick development (Schlange et al., 2001). As it has been shown that at early stages *Cfc* expression depends on BMP signaling (Schlange et al., 2001), we decided to explore the status of *Cfc* expression after our late (stage 7-8) BMP applications that highly upregulated *Nodal* expression. We found that, regardless of which side BMP was applied to, *Cfc* expression was clearly upregulated (*n*=8 100%, Fig. 5B,C; control pattern shown in Fig. 5A). This is clearly seen in the transverse section shown in Fig. 5E. Reciprocally, Noggin applications clearly inhibited *Cfc* expression on the side of application (*n*=8, 100%; Fig. 5D and corresponding section in Fig. 5F). Thus, these results indicate that *Cfc*, a required co-factor for Nodal signaling, is highly dependent on BMP signaling for expression during the stages of our experiments.

Exogenous BMP from the LPM interferes with midline gene expression

The midline is an essential regulator of normal LR development (reviewed by Capdevilla et al., 2000). As it is known that BMP abolishes *Lefty1* expression in the midline (Yokouchi et al., 1999), it was necessary to analyze the midline of our BMP-treated embryos. In addition to *Lefty1*, proposed as the midline molecular barrier (Meno et al., 1998), we also analyzed *Brachyury* (*Bra*) (Knezevic et al., 1997) and *Shh* (Echerlard et al., 1993) as midline markers. We found that ectopic BMP applied to the LPM, either to the left or to the right, rapidly downregulated the expression of *Lefty1* (five out of six, 83%; Fig. 6B,C), *Bra* (six out of eight, 75%; Fig. 6G,H) and *Shh* (four out of six, 66%; Fig. 6L,M) on the midline while PBS-soaked beads had no effect (eight out of eight, 100%; Fig. 6A,F,K). The inhibition of expression predominantly affected the region opposite the bead (between the red arrows in Fig. 6). *Lefty1* expression was completely abolished in the affected area (Fig. 6D). For comparison, the normal pattern of *Lefty1* expression in a non-affected region of the same embryo is shown in Fig. 6E. Similarly, *Bra* expression was dramatically downregulated, as can be appreciated by comparing the sections at an affected (Fig. 6I) and at a non-affected level (Fig. 6J). The analysis of the sections demonstrated that the molecular damage of the midline was not accompanied by gross morphological alteration.

Molecular impairment of the midline does not mediate BMP-dependent activation of Nodal

The damage of the midline raised the possibility that *Nodal*

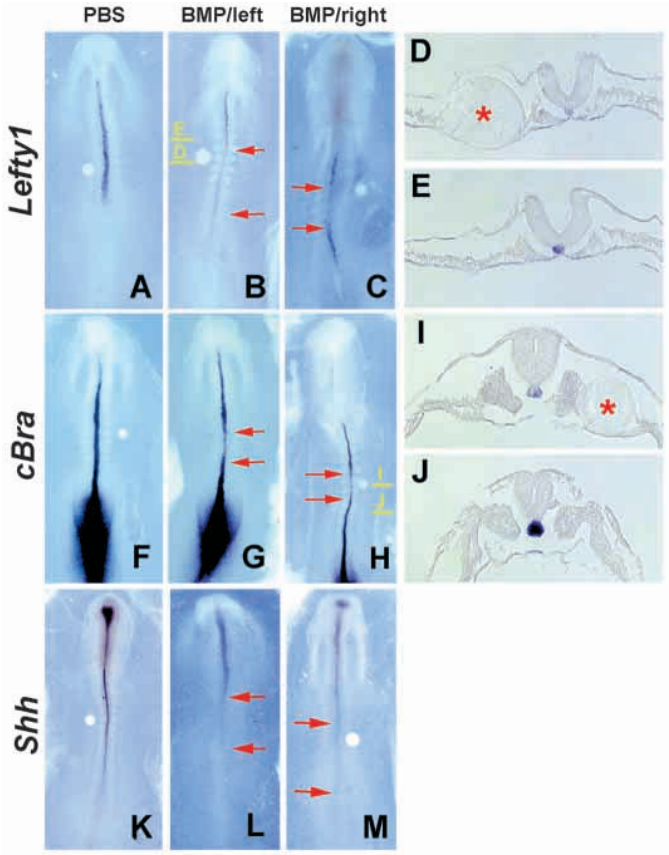

Fig. 6. Exogenous BMP alters normal gene expression in the midline. (A) A PBS-bead in the left LPM does not perturb normal *Lefty1* expression. BMP application, either to the left (B) or right (C) of the LPM represses *Lefty1* (delimited by the red arrows). (D,E) Transverse sections through the embryo shown in B at the indicated levels. (F) A PBS-soaked bead in the left LPM does not perturb normal *Bra* expression. Treatment with BMP either to the left (G) or right (H) dramatically decreases *Bra* expression in the region facing the bead (delimited by the red arrows). (I,J) Transverse sections through the embryo shown in H at the levels indicated. (K) A PBS-soaked bead in the left LPM does not perturb normal *Shh* expression. (L,M) Loss of *Shh* expression in the midline (area between the red arrows) after BMP treatment either to the left (L) or to the right (M). The beads of the embryos in (G,L) were dislodged during hybridization. Red asterisks indicate the bead positions.

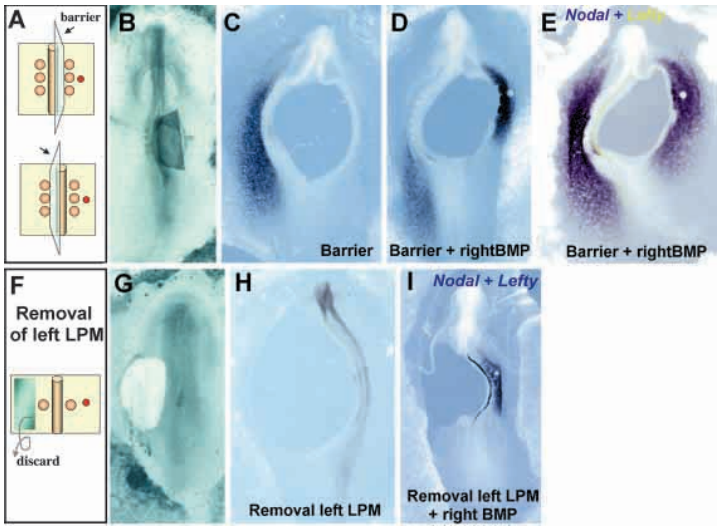

Fig. 7. Barrier and removal experiments. (A) Schematic drawings illustrate the use of a barrier immediately to the right (top) or left (bottom) of the neural tube in a stage 7 embryo. (B) Picture of an embryo immediately after the operation. (C) Normal *Nodal* expression after this experimental manipulation. (D) Ectopic *Nodal* expression induced by a BMP-soaked bead on the right after the placement of the barrier. (E) A double hybridization for *Nodal* (blue) and *Lefty1* (yellow) shows ectopic *Nodal* expression on the right induced by the BMP-bead but *Lefty1* appears unaffected. (F) Schematic drawings illustrate the removal of the left LPM. (G) Stage 6 embryo immediately after the removal of the left LPM. (H) *Nodal* expression in an embryo 5 hours after the operation. (I) An embryo subjected to removal of the left LPM at stage 6 and implanted with a BMP-soaked bead on the right hybridized for *Nodal* and *Lefty1*.

activation on the right could result from diffusion of left-side signaling molecules, rather than being a BMP-specific effect. However, the fact that BMP applications to the left LPM, although hampering the midline (Fig. 6B,G,L), never resulted in ectopic *Nodal* expression in the right LPM (Fig. 1G-H), strongly indicated that the midline defect was not sufficient to activate *Nodal* expression on the right.

To further analyze the involvement of the midline, we devised two kinds of experiments, one aimed at preventing diffusion across the midline, the second aimed at eradicating the source of the putative left-side diffusing signals. The first experiment consisted in the placement of a longitudinal impermeable barrier immediately to the right or left of the neural tube, as indicated in the scheme in Fig. 7A. An embryo immediately after the operation is shown in Fig. 7B. For barriers we used pieces of metal foil (aluminum or platinum) or the internal eggshell membrane (see Materials and Methods), obtaining similar results independently of the type of barrier. Immediately after the placement of the barrier, a PBS-soaked or BMP-soaked bead was placed in the right LPM. During subsequent development, the incision performed to introduce the barrier opened into a broad aperture, with the embryo taking on the appearance seen in Fig. 7C-E. The embryos that received a PBS-soaked bead or no bead at all after the placement of the barrier showed a normal pattern of *Nodal* expression (n=6, 100%; Fig. 7C). By contrast, the embryos that received a BMP-loaded bead showed a robust activation of *Nodal* expression in the right LPM (n=15, 100%; Fig. 7D). Double color in situ hybridization permitted the analysis of *Nodal* and *Lefty1* expression in the same embryo. The embryo shown in Fig. 7E, implanted with a barrier immediately to the right of the neural tube, showed ectopic *Nodal* expression, while *Lefty1* in the midline was normal (yellow color in Fig. 7E). This indicated that the experiment efficiently prevented BMP from reaching the midline and allowed us to infer that diffusion of putative left-sided signal was also impeded.

In a second series of experiments, we removed the left LPM, aiming to suppress the source of left-side signals. At stage 6-7 HH, we removed a rectangle of left LPM, as indicated in the schematic drawing in Fig. 7F, and immediately afterwards we placed the PBS or BMP bead in the right LPM. Fig. 7G shows an embryo immediately after the operation. The embryos were fixed when they

reached stage 8-9 and were hybridized for *Nodal* or double in situ hybridization for *Nodal* and *Lefty1*. While *Nodal* expression was not detected in the peripheral remnants of the left LPM (*n*=5, 100%; Fig. 7H,I), activation of *Nodal* on the right was consistently observed only if the bead was loaded with BMP (*n*=6, 100%; compare Fig. 7H with 7I). The embryo in Fig. 7I was conjointly hybridized for *Nodal* and *Lefty1*, the signal in the midline corresponding to *Lefty1* transcripts. This outcome shows BMP-dependent activation of *Nodal* expression in the right LPM in conditions of putative complete absence of *Nodal* expression in the left LPM. Taken together, our results indicate that the damage of the midline does not mediate the BMP-dependent activation of *Nodal* in the right LPM.

DISCUSSION

Role of BMP signaling on *Nodal* expression

We demonstrate here that increased BMP signaling in the chick LPM at the early somites stage upregulates *Nodal* expression. Conversely, blocking of endogenous BMP signaling by Noggin in the left LPM, impairs *Nodal* expression. These experiments reveal a positive role for BMP signaling on *Nodal* expression.

Based on the observation that BMP applications to the left of the node or the left LPM at earlier stages (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999) (this study) downregulated *Nodal* expression, BMPs were previously considered to be repressors of *Nodal*. However, we show here that this negative effect was secondary to the repression of *Shh* in the node (Monsoro-Burq and Le Douarin, 2001). These experiments also show that BMP signaling is not sufficient for *Nodal* expression and that the BMP-positive effect on *Nodal* is only effective when *Nodal* expression has been initiated by Shh. Interestingly, the expression of *Cfc*, a co-factor required for Nodal signaling (Gritsman et al., 1999; Yan et al., 1999; Shen and Schier, 2000; Yeo and Whitman, 2001) is highly dependent on BMP signals [see Schlange et al. (Schlange et al., 2001) for earlier stages and this study for later stages].

On the basis of these observations and the present knowledge on Nodal signaling (Whitman, 2001), we propose that the positive action of BMP signaling on *Nodal* is indirect, probably mediated by Cfc. BMPs would primarily induce *Cfc* expression, in this way making the cells competent to respond to Nodal (Fig. 8). The following scenario is conceivable in the chick embryo (Fig. 8): left-sided *Shh* expression in the node would induce the left-sided perinodal domain of *Nodal* (Levin et al., 1995; Pagán-Westphal and Tabin, 1998). The Nodal protein produced would diffuse and autoregulate the *Nodal* gene in competent cells expressing *Cfc* (Meno et al., 2001; Adachi et al., 1999; Saijoh et al., 2000; Norris and Robertson, 1999; Whitman, 2001). As both mouse Nodal and Squint (a zebrafish ortholog of Nodal) have been shown to act as long-range signals (Meno et al., 2001; Chen and Schier, 2001), it can be hypothesized that chick Nodal has the same ability. Diffusion towards the right would be normally prevented by the midline (Meno et al., 1998).

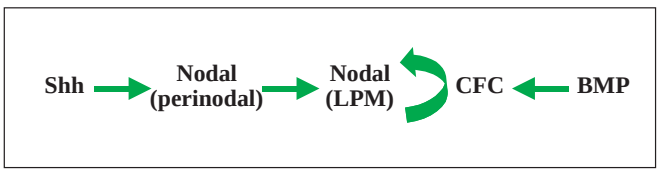

Fig. 8. Role of BMP signals on *Nodal* expression in the LPM. Asymmetric Shh in the left side of Hensen's node induces the medial perinodal domain of *Nodal* expression. BMP endogenous signaling in the LPM induces *Cfc* expression making the cells competent to receive *Nodal* signaling. Nodal is presumed to diffuse from its medial domain of expression up to the area of competent cells where it activates its own expression. For details and references, see text.

In conditions of augmented BMP signaling on the left, *Nodal* expression would result facilitated in the area in which *Cfc* expression is enhanced. A parallelism between the area of ectopic *Cfc* and *Nodal* expression is indeed observed in our experiments (e.g. compare Fig. 1C with 5B). Conversely, blocking of endogenous BMP signaling by Noggin downregulates *Nodal* through the abolition of *Cfc* expression, making the cells refractory to Nodal and thus disabled to activate *Nodal* (Whitman, 2001). *Cfc* expression in the LPM appears to be a prerequisite for *Nodal* expression (Yan et al., 1999; Gaio et al., 1999). It is possible that *Nodal* expression can be initiated in any tissue rendered competent to receive Nodal signaling by the expression of *Cfc*. Thus, the BMP-dependent induction of *Nodal* expression in medial tissues such as the neural tube and the somites, is explained because these tissues were first induced to express *Cfc*. It is worth noting that once *Nodal* expression has been activated in the LPM, it no longer require BMP signaling because Noggin practically has no effect. Neither does it require *Cfc* expression because Noggin blocks *Cfc* expression even when applied at later stages. The control of the highly dynamic and transient pattern of *Nodal* expression in the LPM remains unknown in chick. In mouse it has been shown to be controlled by *Lefty2* (Juan and Hamada, 2001) but a homolog of this gene has not been identified in chick. Similar results and conclusions have been reached independently by Thomas Brand and colleagues (Schlange et al., 2002).

When BMP signaling is augmented on the right, the situation is more complex to explain. As indicated above, the action of the midline, preventing diffusion of Nodal protein from its perinodal domain to the right, would account for the normal absence of *Nodal* expression in the right LPM, even though BMP genes and *Cfc* are normally expressed there. Exogenous BMP application to the right LPM resulted in a rapid and consistent activation of *Nodal* expression, suggesting that BMPs may directly activate *Nodal* transcription. However, as indicated above, several observations suggest an indirect effect. If *Nodal* is not a direct target of BMP but, instead, its induction is mediated by *Cfc*, then our results also suggest that the factor activating *Nodal* expression, possibly Nodal itself, must be present in the right LPM. Although in normal conditions it will not be sufficient to activate *Nodal* expression, it will do so in conditions of enhanced *Cfc* and *Car*.

Function of the midline

Ectopic BMP has been shown to repress *Lefty1* expression in the midline (Yokouchi et al., 1999) and, as expected, we show here that *Bra* and *Shh* expression are also repressed. The fact that our BMP-treated embryos exhibited alterations of the midline regardless of whether BMP was applied to the left or right side, but ectopic *Nodal* in the right LPM was only

observed after right-sided BMP applications, strongly indicated that the midline is not mediating the BMP effect on *Nodal* expression. In addition, the barrier and left LPM removal experiments demonstrated that the right LPM would only activate *Nodal* expression if provided with BMP and that the alteration of the midline was not sufficient to induce *Nodal* in the right LPM.

Although the left LPM removals show that a source of *Nodal* in the left LPM is not required for the BMP mediated induction of *Nodal* on the right, in these experiments the perinodal domain of *Nodal* persisted. In addition, the barrier experiments clearly indicated that the factor inducing *Nodal*, probably Nodal itself (Whitman, 2001), must have diffused to the right LPM before the placement of the barrier. It is worth mentioning that although Nodal diffusion to the right is thought to be normally prevented by *Lefty1*, long-range diffusion of Nodal has been demonstrated to occur with upregulation of *Lefty1* in the absence of *Lefty2* in the LPM (Meno et al., 2001).

The function of BMP in the lateral plate mesoderm

Our experiments demonstrate that BMP signaling, regardless of the side of application, enhances expression not only of *Nodal* and *Cfc* but of all the genes analyzed here: *Pitx2*, *Car* and *Snail*. Conversely, Noggin interferes with the expression of all of them but with unequal intensity.

As *Pitx2* is known to be directly induced by Nodal (Shiratori et al., 2001), the finding that *Pitx2* expression was enhanced by BMP signaling was an expected result. The observation that Noggin blocks *Pitx2* transcription while having little effect on Nodal-mediated maintenance of expression indicates a differential requirement of BMP signaling for maintenance of *Pitx2* and *Nodal* expression. *Nodal* and *Pitx2* expression in the LPM initially overlap, but while *Nodal* expression is transient, *Pitx2* expression remains (Harvey, 1998). Hence, it is conceivable that the mechanisms controlling their transcription are different. It is worth mentioning here that Nkx2.5 has been implicated in maintenance of *Pitx2* expression (Shiratori et al., 2001). As BMP has been shown to regulate *Nkx2.5* positively in different systems (Andree et al., 1998; Schlange et al., 2000; Smith et al., 2000), it is tempting to speculate that BMP maintenance of *Pitx2* expression may be mediated by Nkx2.5.

Of particular interest was the observation that BMP consistently and strongly induced *Car* transcription, in parallel with that of *Nodal*. As in the case of *Nodal*, activation of *Car* expression was impeded by Noggin, while maintenance of expression was little affected. Thus, BMP also appears to be required for activation of Car expression. According to our model, the proposed role for Car in blocking BMP signaling on the left is no longer required, and leaves the biochemical activity of Car unexplained. Car also binds Nodal (Rodriguez-Esteban et al., 1999) and might also bind Wnt as shown for Cerberus. The activity of Car may be complex and will require further investigation.

It is also significant that *Snail*, the only known right determinant in the chick, is also very sensitive to BMP signaling. It is particularly sensitive to Noggin because its expression was consistently and completely abolished around the Noggin-soaked bead. This suggests that *Snail* transcription may primarily depend on BMP signaling.

In summary BMP signals in the chick LPM appear to set up favorable conditions for the expression of all the factors implicated in LR asymmetry and particularly it strongly facilitates *Nodal* expression.

Interspecies conservation of BMP role in LR development

It remains to be determined whether the BMP function identified here in the chick embryo is also conserved in other species. The analysis of mutations in single BMP genes has not given any insight into their role in LR asymmetry. However, BMP genes frequently have overlapping domains of expression, making difficult to obtain information from individual mutations since the function of a particular BMP gene could be replaced by another with a similar pattern of expression (Lyons et al., 1995).

Mutations in several factors involved in the BMP pathway have been reported to exhibit laterality defects. Mice null for *Smad5*, an intracellular factor implicated in transduction of BMP signaling, exhibit bilateral *Nodal* expression (Chang et al., 2000). In addition, mice mutant for Furin or SPC4, members of the family of proprotein convertases implicated in the generation of mature BMP, also present alterations of laterality (Roebroeck et al., 1998; Costam and Robertson, 2000a; Costam and Robertson, 2000b). All these mutations, while somehow interfering with BMP signaling, nevertheless result in upregulation of left-sided markers on the right. In *Xenopus*, a BMP pathway mediated by ALK2 establishes right-sided identity (Ramsdell and Yost, 1999; Yost, 2001). Further work will need to clarify whether the function of BMP in LR asymmetries is conserved interspecies. It is also worth noting that BMP signaling is required at different times during specification of the LR axis in the chick embryo and that blocking of its signaling may have different outcomes depending on the stage at which it is performed. At early stages, BMP4 plays an important role in confining *Shh* expression to the left side of the node (Monsoro-Burq and Le Douarin, 2001). We now report that at early somite stages, BMP signaling positively regulates *Nodal* expression in the LPM. However, the actual mechanisms are complex and we are still missing factors and relationships. Nevertheless, our studies provide new insights into the role of BMPs in the specification of the LR axis in the developing chick embryo.

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