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BMP2 is a positive regulator of Nodal signaling during left-right axis formation in the chicken embryo

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SUMMARY

A model of left-right axis formation in the chick involves inhibition of bone morphogenetic proteins by the antagonist *Car* as a mechanism of upregulating *Nodal* in the left lateral plate mesoderm. By contrast, expression of CFC, a competence factor, which is absolutely required for Nodal signaling in the lateral plate mesoderm is dependent on a functional BMP signaling pathway. We have therefore investigated the relationship between BMP and Nodal in further detail. We implanted BMP2 and Noggin-expressing cells into the left lateral plate and paraxial mesoderm and observed a strong upregulation of *Nodal* and its target genes *Pitx2* and *Nkx3.2*. In addition *Cfc*, the Nodal type II receptor *ActrIIa* and *Snr* were found to depend on BMP signaling for their expression. Comparison of the expression domains of *Nodal*, *Bmp2*, *Car* and *Cfc* revealed co-expression of *Nodal*, *Cfc* and *Bmp2*, while *Car* and *Nodal* only partially overlapped. Ectopic application of BMP2, Nodal, and Car as well as combinations of this signaling molecules to the right lateral plate mesoderm revealed that BMP2 and Car need to synergize in order to specify left identity. We propose a novel model of left-right axis formation, which involves BMP as a positive regulator of Nodal signaling in the chick embryo.

Key words: BMP, Nodal, CFC, Pitx2, NKX3.2, Left-right asymmetry, Chick

INTRODUCTION

A model of left-right (LR) axis determination during vertebrate embryogenesis has been established in recent years (Burdine and Schier, 2000; Capdevilla et al., 2000). According to this model, the entire process can be divided into three different phases (Mercola and Levin, 2001). The first phase that is mechanistically poorly understood concerns breaking the initial bilateral symmetry of the embryo. During the second phase, LR axis information is transferred to the node and subsequently to lateral plate mesoderm (LPM) where side-specific domains of gene expression are established (Pagán-Westphal and Tabin, 1998). During the third phase LR axis information is translated into organ-specific asymmetric morphogenesis. In the chick embryo several signaling pathways within Hensen's node have been identified that establish polarity of the node (Boettger et al., 1999; Garcia-Castro et al., 2000; Levin et al., 1995; Monsoro-Burq and Le Douarin, 2001; Rodriguez-Esteban et al., 2001). An ActivinβB signal on the right side of Hensen's node upregulates expression of *ActrIIa* and *Bmp4*. *Shh* that displays symmetric expression in Hensen's node at HH stage 4 becomes antagonized on the right side by BMP4 and is therefore asymmetrically expressed on the left side of Hensen's node at HH stage 5. BMP4 also induces *Fgf8* that upregulates expression of the repressor *Snr* in the right LPM (Boettger et al., 1999). Several independent signaling pathways control the upregulation of *Nodal* in a small domain adjacent to the left side of Hensen's node (Rodriguez-Esteban et al., 2001).

Transfer of LR polarity to the lateral plate mesoderm is necessary, because most organs that display LR asymmetry are derivatives of the LPM mesoderm. A second expression domain of Nodal in the lateral plate mesoderm becomes apparent at HH stage 7 and extends rapidly along the anteroposterior (AP) axis. At HH stage 8, *Nodal* is expressed in the entire left LPM. The homeobox genes *Pitx2* and *Nkx3.2* are believed to be genetically downstream of *Nodal*, and are postulated to be involved in the morphogenetic execution of LR asymmetry (Liu et al., 2001; Logan et al., 1998; Nielsen et al., 2001; Schneider et al., 1999). Asymmetric expression of *Nodal* and *Pitx2* indeed correlates with normal development of the LR axis (Burdine and Schier, 2000; Capdevilla et al., 2000). Establishing stable *Nodal* expression in lateral plate mesoderm involves the *Nodal* domain adjacent to Hensen's node and in addition requires a factor expressed in paraxial mesoderm (Pagán-Westphal and Tabin, 1998). Caronte (Car), a member of the Dan family of BMP antagonists, was identified as a candidate that is asymmetrically expressed in the left paraxial mesoderm shortly before *Nodal* becomes upregulated in LPM (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Biochemical analysis has demonstrated that Car is able to bind to both, Nodal and BMP. Based on the ability of Car to induce *Nodal* upon ectopic expression on the right side and the fact that the BMP antagonist Noggin can mimic this

activity, it has been suggested that induction of *Nodal* in the left LPM involves local interference with BMP signaling, possibly by Car (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999).

Both in mouse and zebrafish embryos, Nodal and its ortholog squint are long-range signaling molecules (Chen and Schier, 2001; Meno et al., 2001). LR axis formation therefore depends also on a midline barrier in order to prevent left-sided signals (Nodal) from acting on the right LPM. The Nodal antagonists *Lefty1* in mouse (*Ebaf* – Mouse Genome Informatics) and *Lefty* in chick are expressed on the left side of the embryonic midline and loss-of-function experiments result in left isomerization, demonstrating the importance of Lefty factors for maintaining LR identity (Meno et al., 1998; Schlange et al., 2001). In mouse, *Lefty2* (*Leftb* – Mouse Genome Informatics) is also expressed in left LPM together with *Nodal* and is required to prevent diffusion of Nodal from left to right (Meno et al., 2001).

Genetic and biochemical studies have shown that Nodal and its orthologs require EGF-CFC factors for biological activity (Saijoh et al., 2000; Shen and Schier, 2000; Yeo and Whitman, 2001). EGF-CFC factors are attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchors (Minchiotti et al., 2000) and contain a modified EGF-like domain and a cysteine-rich CFC domain (Shen and Schier, 2000). Both domains are required for physical interaction of EGF-CFC factors with Nodal and its signal-transducing receptor complex consisting of type I receptors, ALK4 or ALK7 and the type II receptors, ActRIIA or ActRIIB (Reissmann et al., 2001; Yeo and Whitman, 2001). In contrast to other members of the TGFβ super family, receptor complex formation and signal transduction of Nodal is absolutely dependent on the presence of EGF-CFC factors (Reissmann et al., 2001; Yeo and Whitman, 2001). During LR axis formation in the chick embryo, *Cfc* is expressed in Hensen's node, the forming notochord, and symmetrically in the right and left LPM (Schlange et al., 2001). Similar to the other vertebrate orthologs, *Cfc* is also important for maintaining *Nodal* expression in LPM and *Lefty* expression in the embryonic midline (Schlange et al., 2001). Expression of *Cfc* in the midline is controlled by an activin-like signal, whereas expression in the LPM depends on BMP. These findings create an apparent paradox: while induction of *Nodal* in left LPM involves Car-mediated inhibition of BMP, its maintenance in LPM requires the presence of CFC that is dependent on BMP signaling. A possible solution to this paradox might lie in a tight spatiotemporal control of BMP signaling within LPM that would allow for both, *Cfc* and *Nodal* expression.

In order to study this problem in greater detail, we have re-examined the role of BMP signaling during the process of lateralization of the left LPM. We here demonstrate that *Bmp2*, *Nodal* and *Cfc* display largely overlapping expression domains in the lateral plate. By contrast, expression domains of *Car* and *Nodal* are adjacent to each other with little overlap. Moreover, *Car* and *Bmp2* expression domains do not overlap. In contrast to previous reports (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), we find that implantation of BMP2 in left LPM enhances the expression of left-sided marker genes, such as, *Nodal*, *Pitx2* and *Nkx3.2*. In addition, expression of both *ActRIIA* and *Cfc* was upregulated by BMP2 implants. We also investigated whether Car, Nodal or BMP2 could ectopically induce *Nodal* or *Pitx2* expression on the right side and we found that Car needed to synergize with BMP2 in order to upregulate *Pitx2*. Taken together, our observations suggest that BMP orchestrates the competence of LPM to respond to Nodal signals from Hensen's node. Based on these findings, we propose a new model for how LR asymmetry might be established in the chick embryo.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Whole-mount in situ hybridization and double color whole-mount in situ hybridization were carried out as described (Andrée et al., 1998; Stern, 1998). For expression analysis of marker genes, the probes used hybridized to: *Cfc*, 1 kb (Schlange et al., 2001); *Nodal*, 500 bp (Levin et al., 1995); *Shh*, 1.6 kb (Riddle et al., 1993); *Car*, 600 bp (Rodriguez Esteban et al., 1999); *Pitx2*, 1 kb (St Amand et al., 1998); *Snr*, 1800 bp (Isaac et al., 1997); *Lefty1*, 1.1 kb (Ishimaru et al., 2000); *Nkx3.2*, 1.2 kb (Schneider et al., 1999); *ActrIIa*, 2.2 kb (Stern et al., 1995).

Implantation of cell aggregates

CHO.B3.A4 cells expressing *Xenopus* Noggin and CHO control cells, were cultured as previously described (Schlange et al., 2000). Q2bn cells producing BMP2 and control cells were cultured as previously described (Andrée et al., 1998). Chick embryonic fibroblasts (CEF) were transfected with RCAS-BP(A) constructs encoding Car (Rodriguez Esteban et al., 1999b), mature chick Nodal fused with the BMP4 pro-region (Levin et al., 1997) or alkaline phosphatase (AP) (Fekete and Cepko, 1993). Cell aggregates for implantation were produced by trypsinizing confluent culture dishes and subsequently culturing the cells in bacteriological Petri dishes. After 1 or 2 days the cells formed cell aggregates that were suitable for implantation.

RESULTS

A recent model of LR axis formation proposed that induction of *Nodal* in the left LPM involves local interference with BMP signaling (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). Autoregulation of *Nodal* in the lateral plate is dependent on the presence of CFC (Yan et al., 1999). We have shown previously that expression of *Cfc* depends on the presence of a functional BMP signaling pathway (Schlange et al., 2001). Thus, a tight spatiotemporal control of BMP signaling must be postulated in order to allow for *Nodal* induction and maintenance to occur. In this study, we therefore evaluated in further detail the role of BMP in establishing LR identity.

In the chick, Nodal is first expressed in a small domain adjacent to left side of the node at HH stage 6 (Fig. 1A) (Rodriguez Esteban et al., 1999). At HH stage 7, Nodal became also expressed in the left LPM and subsequently this domain enlarged along the AP axis and also dorsolaterally (Fig. 1B-E). In many stained specimens, a weakly stained tongue-like expression domain (arrows in Fig. 1D,E) was observed that appeared to connect both expression domains. Expression of *Nodal* touched the lateral border of paraxial mesoderm in the midst of the LPM expression domain, while it narrowed down at the anterior and posterior ends (Fig. 1D). Aggregates of BMP2-expressing cells were implanted in the left LPM of chicken embryos at HH stages 4 (Fig. 1F) and 5 (Fig. 1G), and cultures were terminated when the embryos had reached the

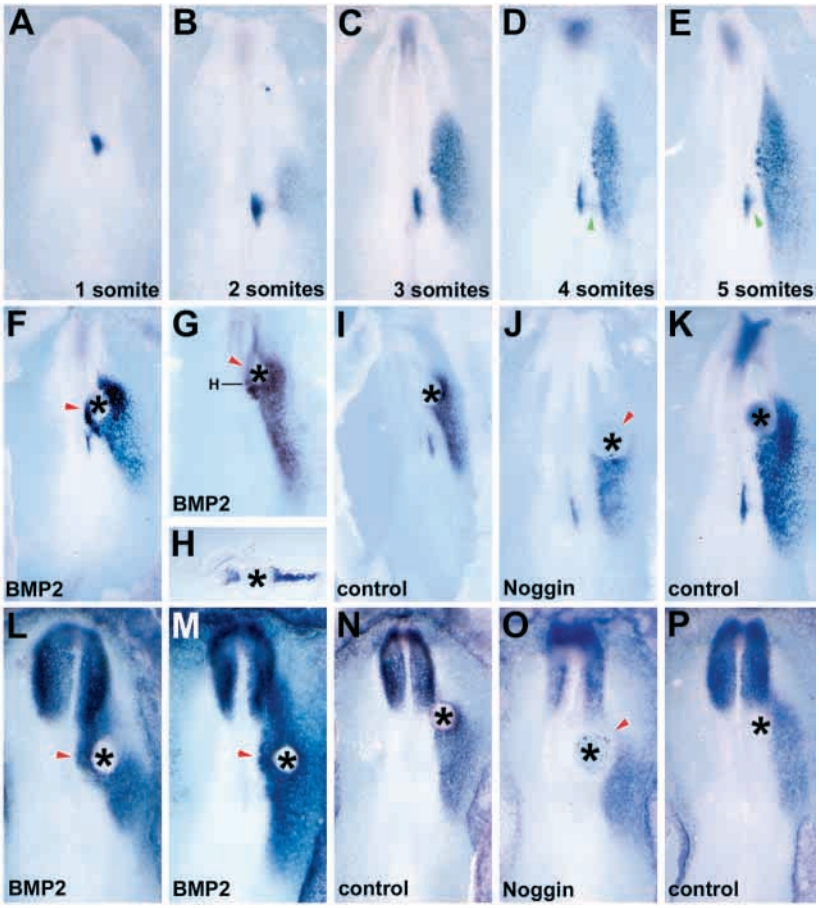

Fig. 1. Expression of *Nodal* and *Pitx2* in the left lateral plate mesoderm is dependent on BMP. Ventral views of stained embryos at HH stage 7 (A-C), or HH stage 8 (D-P). (H) A transverse section through the embryos shown in G. The plane of sectioning is indicated. Normal control embryos in A-E were hybridized with a probe for *Nodal*. The number of somites is given in the bottom right-hand corner in A-E. All other embryos were placed in New culture and implanted with BMP2-expressing cells (F,G,L,M), control cells (I,K,N,P), or with Noggin-expressing cells (J,O). Cell aggregates were placed in the lateral plate mesoderm at HH stages 4 (F), 5 (G,I-L,N-P) or 6 (M). The embryos were cultured until four somites had formed and subjected to whole-mount in situ hybridization with probes for *Nodal* (A-K) and *Pitx2* (L-P). Green arrowheads indicate the tongue-like expression domain of Nodal that connect the paraxial and LPM expression domains in D,E. Red arrowheads indicate ectopic or loss-of expression as a result of the manipulation. Asterisk marks the implanted cell aggregate.

four-somite stage. In contrast to previous reports (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), BMP2-producing implants on the left side strongly enhanced *Nodal* expression in our hands (86%, *n*=14). The induced *Nodal* expression domain completely surrounded the BMP2 cell implant and extended into the paraxial mesoderm. This was especially apparent at the anterior end of the Nodal domain, which was greatly enlarged when compared with embryos that were implanted with control cell aggregates (Fig. 1I) or with untreated normal embryos of the same developmental stage (Fig. 1D). Conversely, implantation of cell aggregates that overexpressed the BMP antagonist Noggin at HH stage 5 strongly reduced *Nodal* expression in the left LPM (80%, *n*=10; Fig. 1J), while control implants had no effect (Fig. 1K). Similar to *Nodal*, expression of *Pitx2*, the direct downstream target of Nodal signaling (Campione et al., 1999) was also strongly enhanced by BMP2 implantation at HH stages 5 and 6 (100%, *n*=5; Fig. 1L,M). Consistent with its effect on *Nodal*, ectopic Noggin in the left LPM downregulated expression of *Pitx2* (83%, *n*=6; Fig. 1O). Control cell implants had no effect on the *Pitx2* expression (Fig. 1N,P). We never observed BMP2-induced inhibition or reduction of *Nodal* or *Pitx2* expression in left LPM.

The homeobox gene *Nkx3.2* is symmetrically expressed in somitic mesoderm but transiently expressed asymmetrically in left LPM (Schneider et al., 1999). Implantation of BMP2 in the lateral plate enhanced expression in left LPM but disrupted expression in somites (100%, *n*=4; Fig. 2A,C). Implanting Noggin-producing cells abolished left-sided expression of *Nkx3.2* in the LPM; however, it did not interfere with the expression in

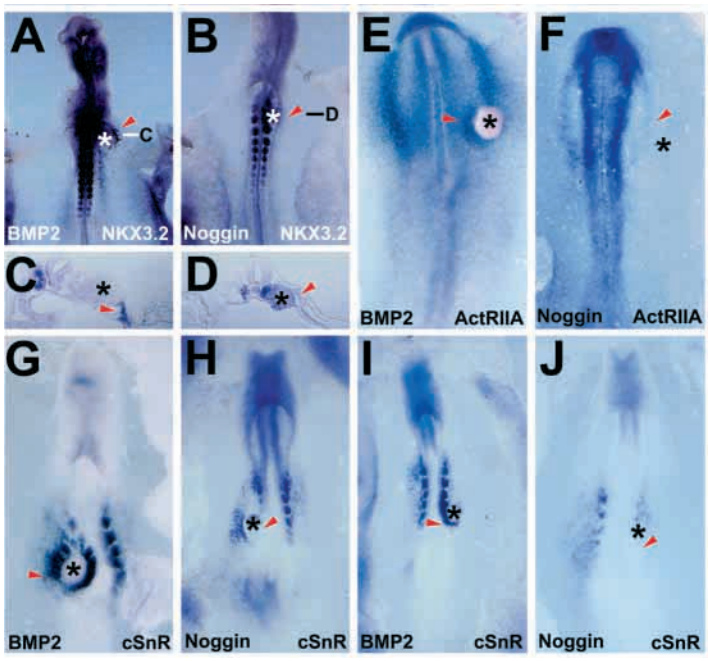

Fig. 2. Expression *Nkx3.2*, *ActrIIa* and *Snr* is dependent on BMP signaling. Ventral views of stained embryos at HH stage 11 (A,B), HH stage 7 (E,F) or HH stage 8 (G-J). (C,D) Transverse section through the embryos shown in A,B. The plane of sectioning is indicated. Embryos were placed in New culture and implanted with BMP2-expressing cells (A,E,G,I) or with Noggin-expressing cells (B,F,H,J) in the left (A,B,E,F,I,J), or right (G,H) LPM at HH stage 5. The embryos were subjected to whole-mount in situ hybridization with probes for *Nkx3.2* (A,B), *ActrII2a* (E,F), or Snr (G-J). Red arrows indicate ectopic or loss-of expression as a result of the manipulation. Asterisk marks the implanted cell aggregate.

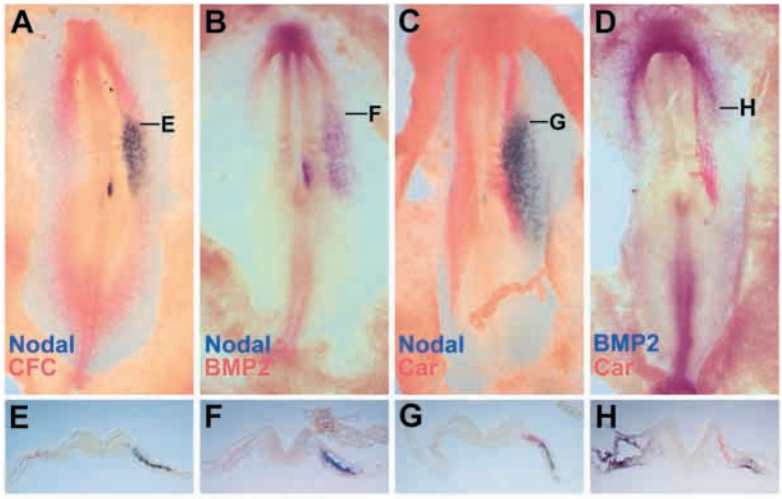

Fig. 3. Analysis of expression domains of determinants of left-right asymmetry. Expression domains of Nodal, CFC, BMP2 and Car were analyzed by double color whole-mount in situ hybridization of HH stage 7 chick embryos. Expression of (A) *Nodal* (blue) and *Cfc* (red), (B) *Nodal* (blue) and *Bmp2* (red), (C) *Nodal* (blue) and *Car* (red), and (D) *BMP2* (blue) and *Car* (red) are shown. (E-H) Transverse sections through the embryos shown in A-D as indicated.

somites (100%, *n*=4; Fig. 2B,D). The presumptive Nodal signal transducing type II receptor *ActrIIa* is expressed in the anterior LPM. We tested whether *ActrIIa* expression was dependent on BMP signaling. BMP2-expressing cell implants enhanced *ActrIIa* expression in LPM (100%, *n*=6; Fig. 2E), while Noggin cell implants resulted in its downregulation (100%, *n*=4; Fig. 2F). These findings suggest that expression of *ActrIIa* depends on a functional BMP signaling pathway in LPM. The zinc finger repressor *Snr*, which mediates suppression of *Pitx2* in the right LPM, has two expression domains in early chick embryos: a symmetric one in somites and a unilateral one in the right LPM (Isaac et al., 1997; Patel et al., 1999). Implantation of BMP2 on the right side resulted in an enhanced expression of Snr in the right LPM and in addition the somitic expression also appears to be enhanced (Fig. 2G). Implantation of Noggin affected both expression domains and resulted in a down-regulation of Snr (100%, *n*=4; Fig. 2H). On the contralateral side, BMP2 enhanced expression of *Snr* in the paraxial mesoderm; however it did not upregulate Snr in the left LPM (80% *n*=5; Fig. 2I). We have previously reported that BMP2 interferes with somite formation and paraxial marker gene expression (Andrée et al., 1998), thus the observed enhanced expression of Snr suggests that BMP probably alters the fate of paraxial mesoderm to a more lateral phenotype. Consistent with previous observations, segmentation of paraxial mesoderm was completely lost in the presence of BMP2 (Andrée et al., 1998). Noggin implantation on the left side resulted in loss of Snr expression in paraxial mesoderm (Fig. 2J). These data suggest that BMP2 positively regulates expression of *Snr* in both, paraxial mesoderm and LPM on the right side of the embryo.

In order to compare the expression domains of *Cfc*, *Nodal*, *Bmp2* and *Car*, double-label whole-mount in situ hybridization was performed. In left LPM at HH stage 7, *Nodal* and *Cfc* have overlapping expression domains (Fig. 3A,E). The expression domain of *Cfc* however, extended more laterally than that of *Nodal*. Likewise, *Bmp2* and *Nodal* expression domains were overlapping. While *Nodal* was present only in LPM, *Bmp2* was expressed in mesoderm and in the underlying pharyngeal endoderm (Fig. 3B,F). *Nodal* and *Car* expression domains were only partially overlapping in the

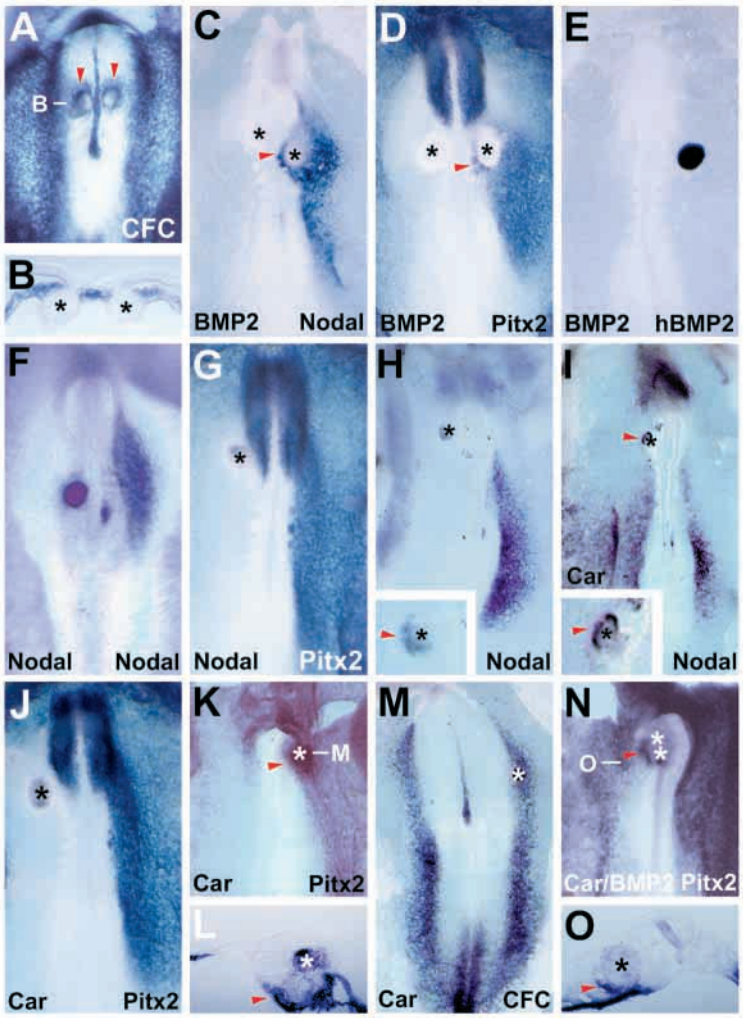

Fig. 4. Car and BMP2 synergize to upregulate *Pitx2* in mesoderm. Ventral views of chicken embryos implanted with cell aggregates expressing BMP2 (A,C-E), Nodal (F,G), Car (H-K,M), and BMP2 and Car (N). Cell implants were placed in left and right LPM (A-D), left LPM (E,K,M) or right LPM (F-J,N). Embryos were cultured in New culture and subsequently subjected to whole-mount in situ hybridization with probes for *Nodal* (C,F,H,I,) *Pitx2* (D,G,J,K,N), *Cfc* (A,M) or human *BMP2* (E). (B,L,O) Transverse sections through the embryos shown in (A,K,N). Insets in H,I show cell implants expressing *Car*. Red arrowheads indicate ectopic expression as a result of the manipulation. Asterisk marks the implanted cell aggregates.

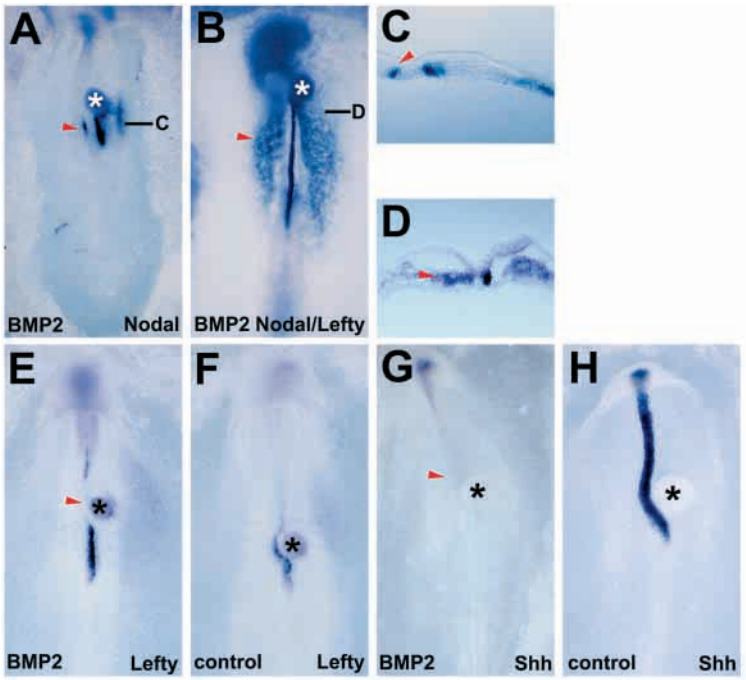

Fig. 5. BMP signaling interferes with a functional midline barrier. Ventral views of stained embryos at HH stage 7 (A) or HH stage 8 (B,E-H). (C,D) Transverse sections through the embryos shown (A,B). Plane of sectioning is indicated in the individual panels. Chicken embryos are shown that were implanted with BMP2-expressing cells (A,B,E,G) or control cells (F,H) adjacent to the midline on the left side and stained for *Nodal* (A), *Nodal* and *Lefty* (B), *Lefty* (E,F), or *Shh* (G,H). Red arrowheads indicate ectopic expression (A,B) or loss-of expression (E-H). Asterisk marks the implanted cell aggregates.

region of forming somites but were clearly separated in more anterior and posterior position (Fig. 3C,G). Regions of *Bmp2* and *Car* expression appeared to be completely separated (Fig. 3D,H).

In order to analyze the interaction of *Bmp2*, *Nodal* and *Car* more directly, we compared the effects of implanting cells that express these ligands in the right LPM. We first compared the ability of BMP2 to induce *Cfc*, *Nodal* and *Pitx2* by implanting BMP2 cell implants in LPM on both sides of the embryo. Consistent with previous reports (Schlange et al., 2001), we found that implantation of BMP cell aggregates at HH stage 5 resulted in ectopic upregulation of *Cfc* in right and left paraxial mesoderm with equal efficacy (Fig. 4A,B). Expression of *Cfc* in the notochord, however, appeared unaffected by this manipulation. In contrast to the bilateral inducibility of CFC, Nodal and Pitx2 were not induced on the right side of the embryo, while enhanced expression of both marker genes was observed on the left side (Fig. 4C,D). In order to prove that the BMP2 cell implants produced sufficient amounts of ligand, manipulated embryos were hybridized with a human BMP2 probe that specifically detected the transcript that is encoded by the cell implant. As short as 10 minutes of staining resulted in an intense labeling of the cell implant, suggesting that the implanted cells produce large amounts of the ligand (Fig. 4E). Nodal implants on the right side were unable to induce *Nodal* (0 out of 9 embryos) and only weakly induced *Pitx2* in direct vicinity to the cell implant (80%, *n*=5; Fig. 4F,G). These data are in accordance with previous reports on *Pitx2* and *Nkx3.2* expression, which showed weak induction adjacent to Nodal producing cell implants (Piedra et al., 1998; Schneider et al., 1999). In one report, injection of RCAS virus-expressing Nodal induced a large ectopic *Pitx2* domain (Ryan et al., 1998). However, this result might simply reflect the virus spread after injection. In contrast to the apparent inability to induce *Nodal* and *Pitx2* ectopically in the right LPM, Nodal randomized heart looping when implanted on the right side at HH stage 5 (data not shown, 37%, *n*=11) (Levin et al., 1995). When Car was implanted on the right side, *Nodal* was induced at the lateral margin of the implant (83%, *n*=6 Fig. 4H). Sometimes (one in six embryos) Car induced ectopic *Nodal* expression along the entire AP axis (Fig. 4I). Car did not affect Pitx2 expression when Car was implanted on the right side (Fig. 4J), while left-sided Car implants enhanced *Nodal* (83%, *n*=6) and *Pitx2* expression (100%, *n*=10; Fig. 4K,L and data not shown) without affecting expression of *Cfc* (100%, *n*=5; Fig. 4M). Significantly, co-implantation of BMP2 together with Car resulted in strong ectopic expression of *Pitx2* in both mesoderm and endoderm (100%, *n*=5; Fig. 4N,O); however, Nodal expression was not affected by this type of implant (data not shown). We also tested whether Noggin was able to upregulate Nodal expression when ectopically applied to the right side (0%, *n*=7; data not shown), despite its profound ability to downregulate Snr and CFC expression in the right LPM (Fig. 2H) (Schlange et al., 2001).

In order to analyze the role of BMP2 in LR axis formation further, we also studied its role in midline development. BMP2 implantation at HH stage 4 on the left side of Hensens's node, enhanced expression of *Nodal* when analyzed at HH stage 7, including an ectopic domain on the right side of the node (Fig. 5A,C). Some embryos implanted with BMP2 at HH stage 5 and cultured until HH stage 8 revealed robust bilateral *Nodal* expression (21%, *n*=14; Fig. 5B,D). Bilateral Nodal expression is often the result of midline barrier defects. We have shown previously that a transient loss of *Lefty* expression is sufficient to induce bilateral Nodal (Schlange et al., 2001). We anticipate that BMP2 implantation might interfere with midline barrier formation. Consistent with this idea, expression of *Lefty* (100%, *n*=7; Fig. 5E) and *Shh* (33%, n=9; Fig. 5G) was repressed after BMP2 implantation adjacent to the midline at HH stage 5. Right sided implantation of BMP2-expressing cells also abolished *Lefty* expression but with lower efficacy (33%; *n*=12, data not shown).

DISCUSSION

Unlike earlier reports (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), we have demonstrated that BMP may positively regulate *Nodal* expression in the left LPM during LR axis formation in the chick embryo. Although previous reports have proposed that BMPs act as repressors of *Nodal* expression, we have demonstrated here that BMP regulates the

expression of an array of genes that are involved in LR axis formation. Specifically, we have demonstrated that induction of *Nodal*, *Pitx2*, *Nkx3.2*, *Cfc*, *ActrIIa* and *Snr* occurs after BMP2 implantation, in agreement with the opposite effect of Noggin-expressing cells implanted in the left LPM. Based on these results we propose that endogenous BMP signaling supports Nodal expression in left LPM. Similar results were independently obtained by another group (Piedra and Ros, 2002).

A repressive role of BMP has been suggested recently based on the observation that Noggin applied to right side appeared to induce *Nodal* (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999). The apparent contradiction of the function of BMP in LR axis formation may indicate: (1) differential response to BMP at different times of embryonic development; (2) concentration-dependent BMP actions as repressor or activator of Nodal expression; or (3) a tightly controlled regional difference of Nodal responsiveness to BMP. The three scenarios are not mutually exclusive.

Car and *Bmp2* expression domains do not overlap

In order to distinguish between the three possibilities mentioned above, we carefully re-evaluated the spatial expression of *Nodal*, *Bmp2*, *Car* and *Cfc*. Clearly, the expression domains of *Nodal*, *Cfc* (in mesoderm) and *Bmp2* (in endoderm and mesoderm) overlap, whereas that of *Car* is separated from the others. A small zone of overlap is observed for *Car* and *Nodal*. The distinct regional expression of BMPs and *Car* argue against a neutralizing function of Car for BMP. However agonist and antagonist are not always co-localized. Opposite expression domains of *Chordin* and *Bmp4* in *Xenopus* blastula stage embryos, for example, are important for the formation of a BMP gradient, which is translated into the DV axis. Thus, one possible way of interpretation is that the separate expression domains of *Car* and BMP might generate a BMP gradient in the LPM, which allows for *Nodal* expression at a certain BMP concentration. However, it is also possible that the function of Car is to prevent diffusion of BMP and Nodal to the midline. We have shown that BMP inhibits *Lefty* expression and thereby abolishes the midline barrier that results in bilateral *Nodal* expression. Another argument against an antagonistic relationship between Car and BMP2 relates to the synergistic enhancement of *Pitx2* expression by both molecules. Furthermore, expression of the Nodal competence factor *Cfc* in the LPM has been shown to be dependent on BMP signaling (Schlange et al., 2001), and overexpression of *Car* fails to suppress *Cfc*, suggesting that Car does not antagonize BMP in this setting. There is precedence for molecules that may act as antagonists or agonists, depending on their molecular interaction partners. For example Twisted-gastrulation has been shown to act as both a BMP signaling agonist and antagonist (Chang et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001). Taken together, these observations we like to propose that Car synergizes with BMP2 in activating *Nodal*. This may partly explain why Nodal is expressed unilaterally on the left side despite the bilateral expression of BMPs and *Cfc*. In vitro assays have demonstrated that Car is able to bind both BMP2 and Nodal; however, the full spectrum of Car interaction partners may not be known yet. Loss-of-function experiments are required to define fully the function of *Car* in the setting of LR axis formation in the chick embryo.

Agonist concentrations released from cellular aggregates or beads may elicit different cellular responses

In previous reports (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999), application of BMP to the left side of the LPM resulted in downregulation of Nodal expression, an effect that was never observed in our experiments. Both cited studies applied BMP2/4 at mid-gastrulation between HH stage 4-6, similar to our experiments. We also varied the sites of implantation with no appreciable effect on the outcome of the experiments. The possibility remains that BMP concentrations used in other studies greatly varied from the ones we used. BMP is able to induce apoptosis in various developmental contexts, including lateral plate and paraxial mesoderm (Schmidt et al., 1998) development or limb development (Yokouchi et al., 1996; Zou and Niswander, 1996). We did not see evidence for the induction of apoptosis by expressing BMP2 cells implants. However, it is quite possible that implantation of beads loaded with BMP4 at a concentration of 1 mg/ml (Yokouchi et al., 1999; Rodriguez Esteban et al., 1999) may induce loss of mesodermal cells and thereby yield false negative results. The production of biologically active BMP2 by the implanted quail cells used in this study can be estimated to be in the range of 1 μg/hour/10^6 cells (H. Weich, unpublished). We believe that the cells probably continuously synthesize BMP2 at a constant rate during the entire period of embryo culture. By contrast, bead implantations possibly led to high peak concentrations in the beginning and substantially lower agonist concentrations are released from the bead later on.

It is also striking that, in our hands, application of Car to the right side rarely and Noggin never resulted in activation of *Nodal*. By contrast, Yokouchi et al. (Yokouchi et al., 1999) and Rodriguez-Esteban et al. (Rodriguez-Esteban et al., 1999) reported that both Car and Noggin, when applied individually to the right side, markedly induced bilateral *Nodal* expression. One possible explanation for this discrepancy to our results would be that Noggin was placed close to Hensen's node, rather than into LPM. This then would affect the documented asymmetric expression of BMP4 in Hensen's node (Monsoro-Burq and Le Douarin, 2000; Monsoro-Burq and Le Douarin, 2001). Asymmetric BMP4 controls right-sided activation of *Fgf8* that ultimately effects expression of *Snr* in the right LPM (Boettger et al., 1999). Thus, Noggin and Car might induce *Nodal* on the right side by interfering with *Bmp4* expression in the node rather than by abolishing BMP signaling in the LPM. However, in our hands, implantation of Noggin- and Car-expressing cells adjacent to the node never and rarely, respectively, induced bilateral *Nodal* expression.

A new model of LR axis determination

Recently, a model of LR axis determination has been proposed. In this model, Car plays a central role as a mediator of asymmetric Nodal expression. Car was suggested to act as BMP antagonist in the lateral plate because of its biochemical ability to bind BMP, in line with the observed ability of Noggin, a bona fide BMP antagonist, to upregulate Nodal in the right LPM (Logan et al., 1998; Ryan et al., 1998; Zhu et

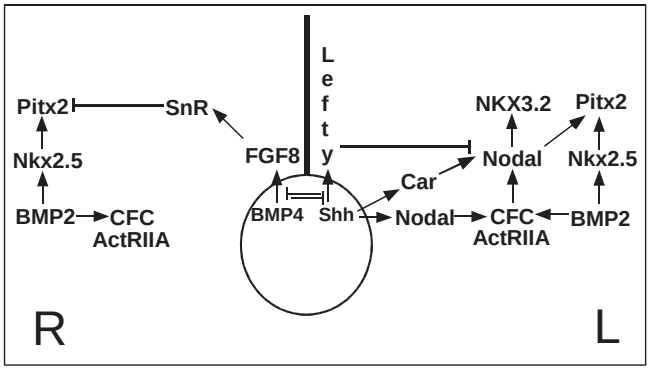

Fig. 6. The proposed interactions among different signaling molecules involved in the lateralization of the left LPM in the chick. Unilateral Shh induces left-sided expression domains of *Nodal* and *Car* adjacent to Hensen's node (Levin et al., 1995; Rodriguez-Esteban et al., 2001). Car probably allows the spread of Nodal to the lateral plate. BMP2, which is present in mesendoderm at stage 4 and in pharyngeal endoderm beginning at HH stage 5 (Andrée et al., 1998) maintains the expression of *Cfc* and *ActrIIa*. Both genes provide competence to respond to the incoming Nodal signal. Nodal upregulates *Pitx2* and *Nkx3.2* (Liu et al., 2001; Logan et al., 1998; Schneider et al., 1999). Expression of Pitx2 at least in mice is maintained by the homeobox gene *Nkx2.5* (Shiratori et al., 2001). Expression of *Nkx2.5* in the mesoderm is dependent on BMP2 signals and thus, BMP2 ochestrates competence for long-range Nodal signals and maintenance of *Pitx2* expression. Expression of *Lefty* in the midline is dependent on CFC (Schlange et al., 2001) and Shh (Tsukui et al., 1999). Ectopic BMP interferes with *Lefty* expression probably by downregulating *Shh* expression. The main function of Lefty is probably to prevent Nodal signals from spreading to the right side. On the right side, FGF8 controls the expression of Snr (Boettger et al., 1999). The proposed function of Snr is to prevent expression of *Nodal* and *Pitx2* on the right side (Patel et al., 1999).

al., 1999). In contrast to this model, we provide evidence for a positive role of BMP2 for Nodal signaling during LR axis determination. To accommodate our findings, we propose a new working model (Fig. 6). Several antagonistic interactions within Hensen's node, which are not illustrated here but have been described by Rodriguez-Esteban et al. (Rodriguez-Esteban et al., 2001), result in asymmetric Shh expression in Hensen's node. Shh upregulates left-sided expression of *Lefty* within the node and notochord and induces *Nodal* and *Car* left of the node. Nodal is able to act as a long-distance signaling molecule both in zebrafish and mouse embryos (Chen and Schier, 2001; Meno et al., 2001). Target genes of the Nodal signaling pathway in the zebrafish blastula are only activated in cells that express the competence factor *oep*, which is a homolog of *Cfc* (Chen and Schier, 2001). In analogy to this findings in the fish blastula, it can be hypothesized that the presence of the asymmetric *Nodal* domain left of the node can traverse the paraxial mesoderm (possibly with the help of Car) and reach the LPM where competent cells (i.e. cells that do express *Cfc* and *ActrIIa*) are able to respond by upregulating *Nodal* and subsequently *Pitx2* and *Nkx3.2*. Cells in the paraxial mesoderm lack expression of *Cfc* and are therefore presumably unable to respond to Nodal. Placement of BMP2 into the paraxial mesoderm upregulates *Cfc* expression and thereby causing the observed expansion of *Nodal*, *Pitx2* and *Nkx3.2* expression domains. In the mouse, *Pitx2* expression in the left LPM is controlled by a left-side-specific enhancer (ASE) that mediates both the initiation and maintenance of LR asymmetric expression (Shiratori et al., 2001). This element contains three binding sites for the transcription factor FAST that mediates Nodal-dependent initiation of Pitx2 expression. The maintenance of Pitx2 expression requires the Nkx2.5-binding site that is also present within the ASE element. We have previously shown that *Nkx2.5* expression in chick between HH stages 4 and 8 requires the continuous presence of BMP2 (Andrée et al., 1998; Schlange et al., 2000). Thus, BMP2 might not only be important to provide competence to the LPM but may also be important to maintain *Pitx2* expression, as in case of cardiac mesoderm. On the right side, BMP2 is far less effective to induce *Nodal* or *Pitx2*. However, the ability of cells to respond to BMP signaling is not impaired unilaterally on the right side, *Cfc* expression for example is strongly upregulated on both sides by BMP2. However, BMP2 implantation on the right only weakly induced *Pitx2* in endoderm. Despite the fact that CFC is present, and thus the LPM should be competent to respond to Nodal, Nodal was unable to auto-regulate itself and only weakly induced *Pitx2*, or *Nkx3.2* expression on the right (Schneider et al., 1999). Probably Snr interferes with Nodal signaling preventing auto-induction of *Nodal* and any other left-sided gene expression on the right.

Expression of Snr is also modulated by BMP signals

Our data are suggestive of a repressor on the right side that interferes with Nodal upregulation and prevents spreading of Nodal signaling. A candidate for this activity is Snr (Isaac et al., 1997). Treatment of chick embryos with antisense oligonucleotides for *Snr* is sufficient to induce *Pitx2* in right LPM (Patel et al., 1999). It seems that active repression of left-specific gene expression in the right LPM is required to prevent left isomerization. In addition to Snr, it is likely that secreted proteins are also involved in the right-sided inhibition of *Nodal* expression and signal propagation. Interestingly, application of BMP adjacent to the midline is sufficient to downregulate *Lefty* via interference with *Shh* expression. This downregulation is accompanied by a right-sided expression domain of Nodal adjacent to Hensen's node and subsequently bilateral expression of Nodal. Lefty expression in the midline is believed to act as the midline barrier that prevents spreading of Nodal to the right side (Meno et al., 1998). Curiously, it was observed that interference with *Lefty* expression, either by application of BMP to the midline (this study), or antisense CFC treatment (Schlange et al., 2001) is sufficient to induce bilateral *Nodal* expression. By contrast, application of Nodal protein to the right side does not lead to the same result. This may suggest that a repressor that prevents expression of *Nodal* on the right side is either expressed in the midline or dependent on the midline.

Our data provide evidence for the role of BMP2 as positive regulator of LR asymmetry in chick embryos. Whether similar molecular interactions also operate in other vertebrates is yet unclear. The mouse null mutation for *Smad5* causes left isomerism and this has been interpreted as evidence for the role of BMP as a repressor of left identity in mammals (Chang et al., 2000). However, *Lefty1* expression in the midline was also absent in *Smad5* mutants, which equally well explains bilateral expression of *Nodal*. In the *Xenopus* embryo, a BMP/ALK2/

Smad-mediated signaling pathway is active on the right side and antagonizes left-sided Vg1 signaling and both are involved in setting up the LR axis in amphibia (Ramsdell and Yost, 1999). At present, it is unclear at what specific time-point during early development this pathway is active and it is therefore difficult to correlate these data with our observations in the chick embryo. Further work is required to substantiate this new model of LR specification with BMP as a positive regulator.

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