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Wnt signaling and PKA control *Nodal* expression and left-right determination in the chick embryo

Concepción Rodríguez-Esteban*, Javier Capdevila*, Yasuhiko Kawakami and Juan Carlos Izpisúa Belmonte‡

The Salk Institute for Biological Studies, Gene Expression Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
*Equal first authors
‡Author for correspondence (e-mail belmonte@salk.edu)

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SUMMARY

Expression of the *Nodal* gene, which encodes a member of the TGFβ superfamily of secreted factors, localizes to the left side of the developing embryo in all vertebrates examined so far. This asymmetric pattern correlates with normal development of the left-right axis. We now show that the Wnt and PKA signaling pathways control left-right determination in the chick embryo through *Nodal*. A Wnt/β-catenin pathway controls *Nodal* expression in and around Hensen's node, without affecting the upstream regulators *Sonic hedgehog*, *Car* and *Fibroblast Growth Factor 8*. Transcription of *Nodal* is also positively regulated by a protein kinase A-dependent pathway. Both the adhesion protein N-cadherin and PKI (an endogenous protein kinase A inhibitor) are localized to the right side of the node and may contribute to restrict *Nodal* activation by Wnt signaling and PKA to the left side of the node.

Key words: β-Catenin, Left-right, *Nodal*, PKA, Sonic hedgehog, TGFβ, Wnt, Chick

INTRODUCTION

In recent years, a model of left-right determination in the vertebrate embryo has been proposed that involves complex regulatory interactions between signaling pathways (reviewed by Capdevila et al., 2000). Members of the Transforming Growth Factor β (TGFβ), Hedgehog (Hh) and Fibroblast Growth Factor (FGF) superfamilies of secreted factors have all been shown to engage in regulatory loops that initiate and stabilize asymmetric gene expression in the early embryo. Among the genes known to play instructive roles during the development of the left-right (LR) axis, the gene *Nodal* occupies a central position. *Nodal* encodes a member of the TGFβ superfamily of secreted factors, and its expression becomes restricted to the left side of the embryonic node and the lateral plate mesoderm at a time critical for the determination of the LR axis in a variety of vertebrates. Thus, and despite the fact that the cellular and molecular mechanisms that break the initial embryonic symmetry appear not to be conserved among vertebrates, left-specific expression of *Nodal* and its target, *Pitx2*, has been observed in all vertebrates examined so far, and has been shown to correlate with normal development of the LR axis. This observation underscores the importance of understanding the mechanisms that regulate *Nodal* expression in the early vertebrate embryo.

Together with TGFβs, Hh and FGFs, the Wnt family of secreted factors is involved in many developmental decisions in a variety of organisms (Wodarz and Nusse, 1998; Peifer and Polakis, 2000). Although in *Xenopus* Wnt signaling appears to be involved in orienting the LR embryonic axis (Danos and Yost, 1995; Nascone and Mercola, 1997; Yost, 1998), so far no clear role in LR development has been described for any specific Wnt member in either chick or mouse. With the aim of extending earlier observations in *Xenopus* and improving our understanding of the role of Wnt factors in LR development, we have analyzed in detail the expression patterns of several *Wnt* genes during early development of the chick embryo. We show that a *Wnt* gene that operates through β-catenin, *Wnt-8c*, is expressed on the right side of Hensen's node, although it actually acts as a left determinant in the chick embryo. We also show that the activity of β-catenin is both necessary and sufficient to regulate *Nodal* expression. Additionally, protein kinase A (PKA) also acts as a positive regulator of *Nodal*, in a pathway independent of *Wnt-8c*. Antagonism of Wnt/β-catenin signaling (by N-cadherin) and of PKA activity (by the endogenous PKA inhibitor PKI) may also contribute to further restrict and localize *Nodal* expression to the left side of the embryonic node.

MATERIALS AND METHODS

Cell implants and virus production

The entire open reading frame of chick *Wnt-8c* (Hume and Dodd, 1993) was cloned into an *RCAS* retroviral construct that was used to infect chick embryonic fibroblasts, which were then pelleted and used for implants. Production of *RCAS* viruses was as described (Rodríguez-Esteban et al., 1999). *Wnt-8c* cell pellets were implanted to the right or the left side of Hensen's node of HH stage 4 chick embryos grown in vitro as described (New, 1955). As controls, chick embryonic fibroblasts infected with empty *RCAS* constructs or with

RCAS-alkaline phosphatase virus were used, and no phenotypic alterations or changes in gene expression were observed. As judged by whole-mount in situ hybridization using a *Wnt-8c* riboprobe, the cell pellets expressed *Wnt-8c* at a level comparable with endogenous expression. *RCAS-β-catenin^{ACT}* has been previously described (Capdevila et al., 1998). Embryos were infected by right or left side blastoderm injection of the virus at HH stage 4. Control infections with *RCAS* alone or other irrelevant viruses (such as *RCAS-alkaline phosphatase*), produced no phenotypic alterations or changes in gene expression. A full-length mouse *Axin* clone was kindly provided by Dr Frank Costantini (Columbia University, NY). In the chick, the *Axin* gene appears to be widely expressed in the early embryo (not shown). The entire *Axin* ORF was cloned into an adenoviral construct, and viral particles were produced and isolated as described (Rodríguez-Esteban et al., 1999). Control infections with adenovirus alone produced no phenotypic alterations or changes in gene expression.

Whole-mount in situ hybridization

Antisense riboprobes for *Nodal*, *Shh*, *Car*, *Fgf-8*, *cSnR* and *Pitx2*, and in situ hybridization procedures were as described (Rodríguez-Esteban et al., 1999). The *Wnt-8c* antisense riboprobe was derived from the entire ORF of chick *Wnt-8c*, cloned by RT-PCR from HH stages 12-16 chick embryos. Antisense riboprobes for chick *β-catenin* and the gene encoding subunit α of chick PKI were similarly derived form RT-PCR fragments. The entire ORF of chick *β-catenin* was cloned using primers derived from the published sequence (GenBank Accession Number, U82964). In the case of PKI, degenerated primers were designed after comparison of consensus amino acid residues from human and mouse PKIα subunit sequences. The 230 base pairs PCR fragment obtained is identical to part of GenBank sequence U19496.

Bead implants

For treatment with blocking anti-Shh antibody (Pagán-Westphal and Tabin, 1998), Affigel blue beads (BioRad) were soaked in the antibody for several hours before they were implanted in the left or right side of HH stages 4-5 chick embryos. Controls were obtained by implanting beads soaked in PBS, and no phenotypic consequence or changes in gene expression were observed. As previously described, and based on its effects on *Nodal* and *patched* expression (Pagán-Westphal and Tabin, 1998), this antibody is a powerful blocker of Shh signaling in vivo. Implantation of beads soaked in a blocking anti-N-cadherin antibody was exactly as described (Garcia-Castro et al., 2000). We must point out that Garcia-Castro et al. did not describe changes in *Nodal* expression after implantation of the blocking antibody. This may be due to slight differences in the experimental conditions used by these authors and by ourselves. In previous experiments, we have noticed that visualization of ectopic domains of *Nodal* expression may require long periods of development of the in situ detection reaction, which may lead the researcher to score some experimental embryos as false negatives for ectopic *Nodal* expression. Beads soaked in forskolin (an activator of PKA via its effect on adenylyl cyclase) or H89 (a specific inhibitor of PKA), both at concentrations of 150 μM (dissolved in 4% DMSO in embryo medium) were applied into the New cultured blastoderms at HH stage 4. These chemicals appear to reach large areas of the embryo even when applied locally. Control embryos were obtained by similar treatment with 4% DMSO in embryo medium, and we never detected any phenotypic alteration or change in gene expression.

RESULTS AND DISCUSSION

Wnt-8c acts as a left determinant in the chick embryo

We began our analysis by characterizing in detail the pattern of transcription of several *Wnt* genes expressed at or near tissues known to be involved in LR determination in the chick embryo. We found that *Wnt-3a*, *Wnt-5a* and *Wnt-8c* are expressed in the primitive streak and the node area at the stages at which molecular asymmetries are detected in the chick, as previously described (Hume and Dodd, 1993; Hollyday et al., 1995; Levin, 1997; Baranski et al., 2000). However, we focused our attention on *Wnt-8c* (Fig. 1A), as it displays asymmetric expression in the Hensen's node. As shown in Fig. 1B, expression of *Wnt-8c* is restricted to the right side of the node at HH (Hamburger and Hamilton, 1951) stage 5, at around the same time at which *Sonic hedgehog* (*Shh*) becomes restricted to the left side and before the restriction of *Nodal* transcription to the left side of the node (Fig. 1C,D). The right-specific expression of *Wnt-8c* in the node suggested a possible role as a right determinant during LR development. We tested this possibility by implanting pellets of *Wnt-8c*-expressing cells either to the left or to the right side of the node of HH stage 4 embryos (Fig. 1F shows a right-sided implant). We scored for alterations in LR development by looking at the direction of heart looping in the implanted embryos approximately 24 hours after the operation, as looping of the developing heart tube to the right side of the embryo is the first clear morphological sign of asymmetry in the chick and other vertebrates. Unexpectedly, we found that ectopic *Wnt-8c* had no detectable effect when applied to the left side of the node (n=50). In contrast, ectopic expression of *Wnt-8c* to the right of the node resulted in a high incidence of isomeric hearts (in 30/54 embryos) that developed in a middle position in the embryo, and of reversal of heart situs (in 8/54 embryos; Fig. 1G,H). These results are similar to those obtained in comparable experiments using right-sided treatment with left determinants such as *Shh*, *Caronte* (*Car*) or *Nodal*, and they are consistent with Wnt-8c acting as a left determinant in the chick embryo, despite its right-sided expression in the node.

We next explored the pathway by which the Wnt-8c signal might influence LR development in the chick. β-catenin has been shown to act as an intracellular mediator of Wnt signals (including Wnt-8c) in a variety of systems, by translocating to the nucleus in response to Wnt and interacting with TCF/LEF factors to generate transcriptionally active complexes that regulate expression of Wnt targets (reviewed by Wodarz and Nusse, 1998; Peifer and Polakis, 2000). The chick *β-catenin* gene is transcribed in the whole embryo at the early stages examined, although it is more strongly expressed throughout the primitive streak, with no detectable asymmetry in the node (Fig. 1E). After infection of the right side of HH stage 4 embryos with a retroviral construct expressing an activated form of β-catenin (*RCAS-β-catenin^{ACT}*; Capdevila et al., 1998), which activates Wnt targets independently of the presence of Wnts, we observed a high incidence of reversal of heart situs and of hearts located in a middle position that failed to loop towards either side of the embryo. This is similar to what was observed with right-sided *Wnt-8c* implants, and infections of the left side had no detectable effect (data not shown). This result, together with the fact that Wnt-8c has been shown to operate through β-catenin in other organisms, led us to conclude that β-catenin is likely to mediate the effects of Wnt-8c on LR development in the chick embryo. Moreover, and consistent with a requirement for β-catenin in normal LR development, overexpression of Axin (a well-characterized

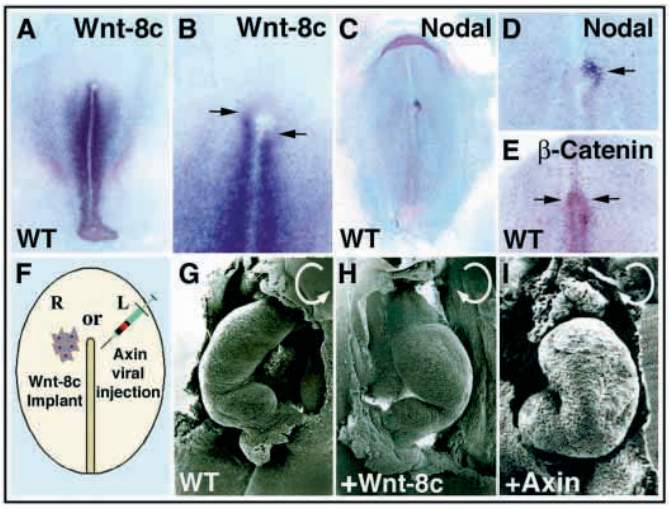

Fig. 1. Wnt-8c signaling regulates heart looping in the chick embryo. (A) *Wnt-8c* expression in a HH stage 5 chick embryo. Transcripts are observed throughout the entire length of the primitive streak and symmetrically in the presumptive mesoderm cells lateral to the streak. (B) Detail of the same embryo, where *Wnt-8c* expression in the node is restricted to the right side (indicated by top arrow). (C) At HH stages 5+ to 6, *Nodal* becomes restricted laterally and anteriorly to the left side of Hensen's node. (D) A close-up of the Hensen's node area of the embryo in C, showing left-specific expression of *Nodal* mRNA in the node (arrow), a pattern complementary to that of *Wnt-8c* in the node (compare B with D). (E) At the same stage, *β-catenin* transcripts appear to be symmetrically expressed (arrows). (F) Scheme showing the implantation of *Wnt-8c* cell pellets to the right side of Hensen's node of a HH stage 4 chick embryo. The syringe indicates the area to the left of the node where the *Axin* adenovirus is injected at the same stage. (G,H) 24-36 hours after implantation of *Wnt-8c* cells to the right side of the node, specimens were processed for scanning electron microscopy. Normal heart looping (G) is altered after implantation of *Wnt-8c* cells. Like right-sided ectopic expression of *Nodal* (Levin et al., 1995; Levin et al., 1997), right-sided *Wnt-8c* cell implants induce bilaterally symmetric hearts (not shown) or reversed heart looping (H). (I) Injection of an adenovirus expressing the Axin protein to the left of the node of a HH stage 4 chick embryo (see F) also results in reversed heart looping (I). White semicircular arrows in (G-I) indicate direction of heart looping. In this and the following figures, WT indicates wild-type or normal embryos, which are the controls mentioned in the Materials and Methods section.

negative regulator of β-catenin) (Wodarz and Nusse, 1998; Peifer and Polakis, 2000) in the left side of HH stage 4 embryos (schematized in Fig. 1F), results in heart alterations that reveal disruption of the LR axis (in 9/26 embryos; Fig.1I, compare with control heart in Fig. 1G). Right-sided overexpression of Axin had no detectable effect on LR development (*n*=15).

In order to analyze the molecular changes associated with the observed LR defects, we repeated the experiments harvesting the embryos 5-10 hours after implantation of *Wnt-8c* cells. Whole-mount in situ hybridization was performed using riboprobes to detect expression of the *Shh*, *Fgf-8*, *Car*, *Nodal*, *cSnR* and *Pitx2* genes, all known to be involved in LR determination. As indicated in Fig. 2A-G, ectopic *Wnt-8c* to the right side of the node (schematized in Fig. 2A) has no effect on the left determinants *Shh* (*n*=12) and *Car* (*n*=16), and the right determinant *Fgf-8* (*n*=20). However, it strongly activates

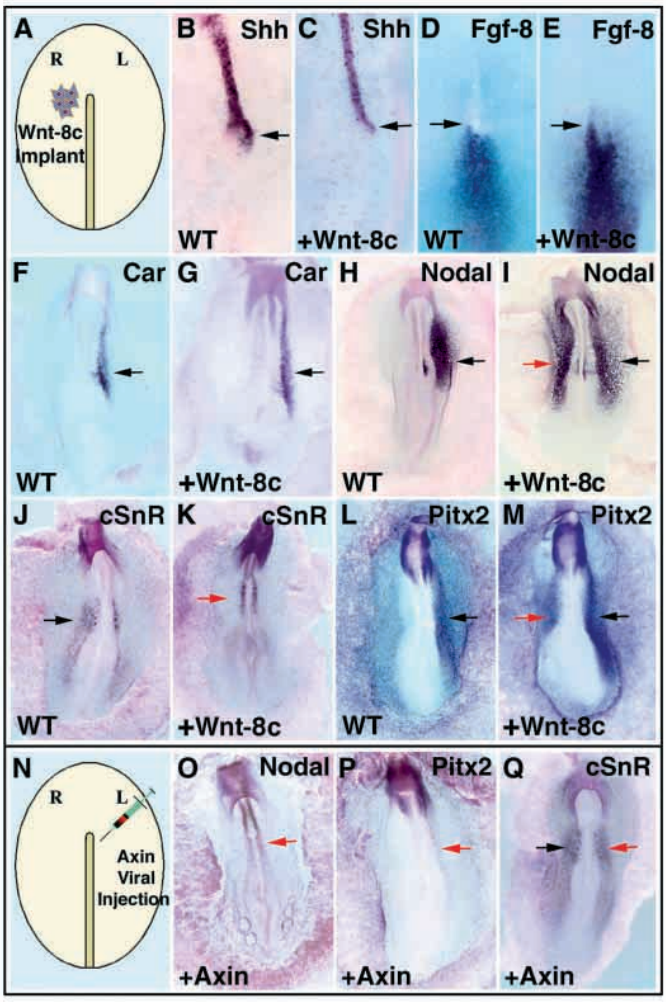

Fig. 2. Wnt-8c signaling regulates *Nodal* expression. (A) Scheme showing the implantation of *Wnt-8c* cell pellets to the right side of Hensen's node of a HH stage 4 chick embryo. (B) *Shh* expression on the left side (arrow) of the node in a HH stage 6 wild-type chick embryo. (C) *Shh* expression was not altered after implantation of *Wnt-8c* cell pellets to the right side of the node. (D,E) *Wnt-8c* cells to the right side of the node do not alter the asymmetric expression pattern of *Fgf-8* on the node (arrows point to stronger expression on the right side). (F,G) Expression of *Caronte* (*Car*; arrows), is not altered after implantation of *Wnt-8c* cells to the right side of the node. (H-M) However, expression of *Nodal* (H,I), *cSnR* (J,K) and *Pitx2* (L,M) is altered by grafting *Wnt-8c* cells to the right side of Hensen's node (red arrows in I,M indicate ectopic expression in the right LPM of *Nodal* and *Pitx2*, respectively, and red arrow in K indicates repression of *cSnR* expression in the right LPM, where it is normally strongly expressed, as shown in J). (N) Injection of an adenovirus expressing Axin to the left side of Hensen's node of a HH stage 4 chick embryo. Both *Nodal* (O) and *Pitx2* (P) are strongly repressed in their normal domains (red arrows). Conversely, the left domain of *cSnR*, which is very weak outside the somites in normal embryos (see J), is strongly activated (red arrow in Q; compare with the normal strong expression of *cSnR* in the right side of the embryo, indicated by the black arrow).

Nodal (in 6/20 embryos; Fig. 2H,I) and the *Nodal* target *Pitx2* (in 5/13 embryos; Fig. 2L,M), and represses *cSnR* (in 4/12 embryos; Fig. 2J,K), normally strongly expressed on the right side of the embryo (Isaac et al., 1997). Again, very similar

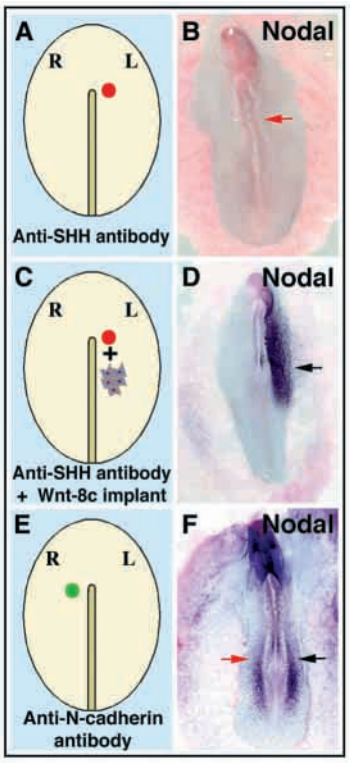

Fig. 3. Involvement of Shh, Wnt-8c and N-cadherin in the control of *Nodal* expression. (A) Blocking of Shh signaling with a bead (in red) soaked in anti-Shh antibody implanted on the left side of the node at HH stage 4. (B) This treatment abolishes normal *Nodal* expression in the left LPM (Pagán-Westphal et al., 1998; red arrow). (C,D) *Nodal* expression in the left LPM is maintained (black arrow in D) if *Wnt-8c* cells are implanted together with the bead soaked in the anti-Shh antibody (as shown in C). (E) Strategy to block N-cadherin activity with a bead (in green) soaked in anti-N-cadherin antibody (Garcia-Castro et al., 2000) implanted on the right side of the node at HH stage 4. (F) This results in ectopic activation of *Nodal* in the right side of the embryo (red arrow; compare with normal domain indicated by the black arrow).

results were obtained after ectopic expression of *RCAS-β-catenin^{ACT}* on the right side (data not shown). Moreover, antagonism of β-catenin in the left side of the embryo by overexpression of Axin (schematized in Fig. 2N) resulted in strong repression of both *Nodal* (in 8/20 embryos) and *Pitx2* (in 7/20 embryos; Fig. 2O,P, respectively) and strong activation of *cSnR* (in 8/22 embryos; Fig. 2Q). *Shh* and *Car* were both unaffected (data not shown). These results confirm that Wnt-8c acts as a left determinant in the chick embryo and that β-catenin is a very likely transducer of the Wnt-8c signal that induces *Nodal*. Functional β-catenin appears to be necessary for normal *Nodal* expression in the left side of the embryo. Moreover, we believe that the induction of *Nodal* by ectopic Wnt-8c or activated β-catenin observed on the right side of the embryo strongly suggests that this induction also operates inside and around the node, especially considering that expression of *Wnt-8c* is mostly restricted to the node and primitive streak at the stages at which the experiments were performed.

Because Shh has been shown to be both necessary and sufficient for *Nodal* expression in the chick embryo (Pagán-Westphal and Tabin, 1998), we decided to further investigate a possible interaction between the Wnt-8c and Shh pathways regarding induction of *Nodal*. Interestingly, while left application of a blocking anti-Shh antibody (Fig. 3A) has been shown to repress *Nodal* expression (as we observed in 9/10 embryos; red arrow in Fig. 3B), a graft of *Wnt-8c* cells implanted together with the blocking anti-Shh antibody (Fig. 3C) is able to counteract this repression, thus maintaining *Nodal* transcription (in 8/10 embryos; Fig. 3D). Application of the blocking anti-Shh antibody alone does not appear to alter the pattern or the level of expression of *Wnt-8c*, whereas expression of the Shh target *patched* is repressed, further confirming the blocking activity of the antibody in our experimental setting (data not shown). These results, together with the expression patterns of *Wnt-8c* and *Shh* in the node, and the fact that *Wnt-8c* misexpression (or Wnt/β-catenin antagonism by Axin) do not affect transcription of *Shh* or *Car*, indicate that specific levels of *Wnt-8c* may activate *Nodal* in a pathway independent of Shh.

A role for N-cadherin in the control of *Nodal*

The presence of *Wnt-8c* on the right side of the node would seem to argue against its role as a left determinant, and yet our results show that *Wnt-8c* is an upstream regulator of *Nodal* (a key left determinant itself). As other Wnts are also expressed in the node (i.e. Wnt-3a, which also signals through β-catenin (Shimizu et al., 1997), and Wnt-11), it is possible that a combination of Wnt activities, rather than a single Wnt, actually acts as the left determinant identified by our experiments. However, neither *Wnt-3a* nor *Wnt-11* show a left-specific expression pattern that could counteract the right bias of *Wnt-8c* expression in the node. And even if that is actually the case, why isn't *Nodal* also transcribed on the right side of the node in response to Wnt-8c? One way to resolve this apparent discrepancy would be to assume the presence of a repressor or repressors of *Nodal* transcription exclusively on the right side of the node, which could antagonize the activation of *Nodal* by Wnts on the right but not on the left side of the node (where Wnt-8c and other Wnt proteins, and also β-catenin, are presumably present). Accordingly, Wnts would act normally as regulators of *Nodal* on the left side of the node (where the putative antagonist or antagonists are not present).

A good candidate to act negatively on the transcription of *Nodal* on the right side of Hensen's node is the adhesion molecule N-cadherin. Recently, it has been described that expression of N-cadherin in the node of the chick embryo is restricted to the right side, and that blocking its activity results in LR alterations (Garcia-Castro et al., 2000). Moreover, it is known that high levels of cadherin expression negatively correlate with the activation of Wnt targets by β-catenin, because high levels of cadherin may reduce the pool of cytoplasmic β-catenin available for activating nuclear targets of Wnt signals (Fagotto et al., 1996). Thus, we reasoned that high levels of N-cadherin on the right side of the node could potentially interfere with the activation of Wnt targets (such as *Nodal*) by β-catenin. To test this hypothesis, we blocked N-cadherin activity by implanting beads soaked in a blocking anti-N-cadherin antibody to the right side of the node of HH stage 4 embryos (Fig. 3E), and analyzed *Nodal* expression 6-12 hours after implantation. As indicated in Fig. 3F, blocking N-cadherin activity on the right side of the node results in

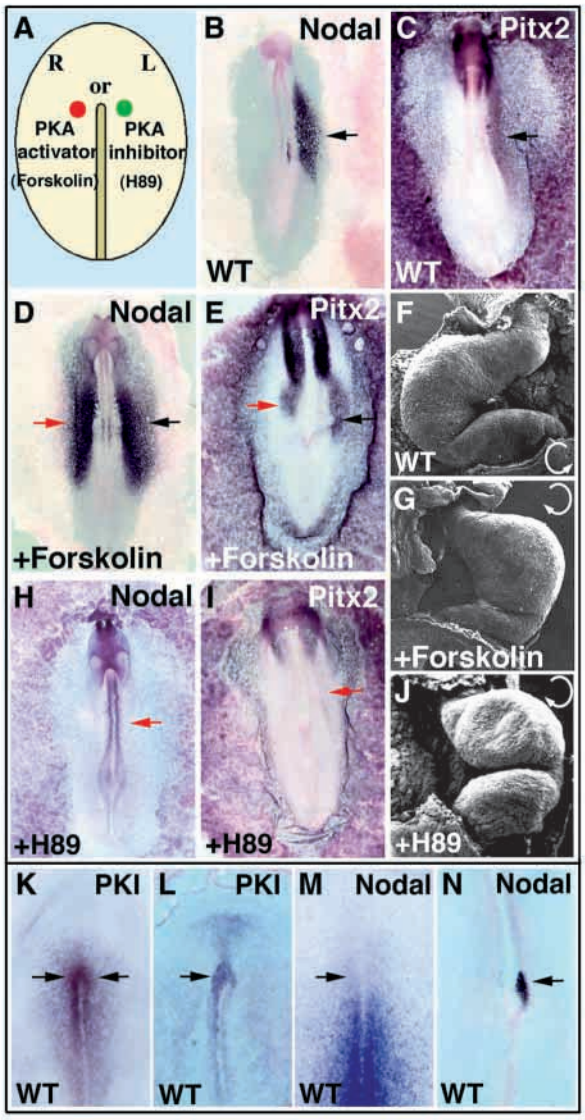

Fig. 4. Alteration of PKA activity induces changes in heart looping that are preceded by changes in both *Nodal* and *Pitx2* expression. (A-E) A bead (red in A) soaked in the PKA activator Forskolin applied to the right side of the node of HH stage 4 chick embryos, results in ectopic induction of *Nodal* (D, compare with B) and *Pitx2* (E, compare with C) in the right LPM, indicated by red arrows (black arrows indicate normal domains). (G) Treated embryos show reversals of heart looping (compare with normal heart in F). (H,I) Application of a bead (green in A) soaked in the PKA inhibitor H89 to the left side of the node of HH stage 4 chick embryos, represses *Nodal* (H) and *Pitx2* (I), indicated by red arrows. (J) H89-treated embryos also develop reversals of heart looping (compare with normal heart in F). Although we applied beads to specific locations, as indicated, these chemicals most likely reach both sides of the embryo. (K,L) The gene encoding PKI, an inhibitor of PKA activity, is expressed symmetrically in Hensen's node (arrows in K) and the adjacent portion of the primitive streak at HH stage 4. At HH stages 5-6, PKI expression becomes much stronger on the right side of the node (arrow in L). At these stages, PKI transcripts appear to be excluded from cells expressing *Nodal* mRNA. (M,N) At HH stage 4, *Nodal* transcripts are present throughout most of the primitive streak, but they are clearly absent from the streak adjacent to the node and from the node itself (indicated by the arrow in M), both regions where PKI is transcribed at the same stage (compare with K). Later on, at HH stage 6, *Nodal* transcripts are detected on the left side of the node (arrow in N), but they are absent from the right side of the node, where PKI is strongly expressed at the same stage (compare with L). We have overexpressed chick PKI in the early embryo using a retroviral vector (data not shown), but so far we have failed to detect any alteration of LR development. This may be due to inefficient production of PKI protein by the retroviral construct, a phenomenon we have previously observed when trying to retrovirally express small proteins (the chick PKI protein comprises only 76 amino acid residues).

induction of *Nodal* expression (in 6/15 embryos). This effect is independent of *Car* (which is negatively regulated by *Fgf-8* on the right side of the embryo (Rodríguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999), since *Car* expression is normal in the treated embryos (data not shown). These results indicate that N-cadherin normally represses *Nodal*, suggesting that the presence of N-cadherin on the right side of the node may serve as a mechanism that ensures that the Wnt pathway is unable to activate *Nodal* transcription in that region of the node, despite the presence of Wnt proteins and β-catenin.

Control of *Nodal* by PKA

Given that both Shh and Wnt-8c control *Nodal* transcription, we reasoned that the analysis of modulators of the Shh pathway could reveal additional interactions between these two signaling pathways. As PKA has been shown to antagonize Hedgehog signaling pathways in a variety of organisms, we decided to explore a possible role for PKA as a repressor of *Nodal*, which is a target of Shh during LR determination in the chick. We first implanted a bead soaked in the PKA activator forskolin to the left side of the node of HH stage 4 embryos, expecting a repressive effect on *Nodal* transcription. Surprisingly, *Nodal* and its target *Pitx2* were unaffected on the left side (data not shown), but they were strongly activated on the right side (in 9/20 and 4/20 embryos, respectively), and exactly the same effect was obtained when implanting the bead on the right side (as shown in red in Fig. 4A; ectopic domains of gene expression are indicated by red arrows in Fig. 4D,E). This suggests that PKA, contrary to our expectations, acts as an activator of *Nodal*. *Shh*, *Car* and *Wnt-8c* were unaffected (data not shown), indicating that the activation of *Nodal* was not mediated by an ectopic maintenance of *Car* expression on the right side of the embryo, or by an alteration in the distribution of *Wnt-8c* expression. Alterations of heart looping and gene expression are shown in Fig. 4G (14/32 embryos had either isomeric or left-looped hearts; compare with normal heart in Fig. 4F). The fact that we observe the same effect independently of the location of the bead suggests that forskolin most probably reaches a large area of the embryo, unlike secreted proteins or antibodies also applied using beads, both of which appear to have a more localized effect. When embryos were treated in a similar way with the specific PKA inhibitor H89 (left-sided implant is schematized in green in Fig. 4A), expression of both *Nodal* (in 7/20 embryos) and *Pitx2* (in 4/10 embryos) was completely repressed (red arrows in Fig. 4H,I), and alterations of heart looping occurred in 8/20 embryos (Fig. 4J), which suggests that PKA activity is necessary for normal expression of left-specific genes in the

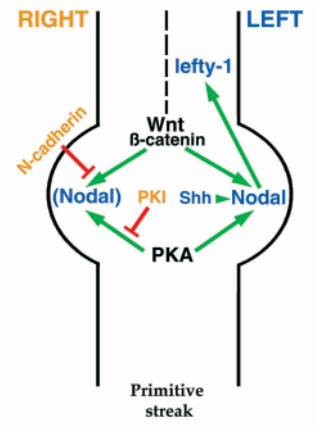

Fig. 5. A model for the role of Wnts and PKA in LR determination in the chick embryo. At the time at which *Nodal* becomes restricted to the left side of Hensen's node (HH stage 5+), Wnt/β-catenin and PKA act as positive regulators of *Nodal* transcription through *Shh*-independent mechanisms. Activation (or maintenance) of *Nodal* on the right side of the node might be prevented by at least two mechanisms: first, the presence of N-cadherin on the right side, which could inhibit activation of *Nodal* transcription by β-catenin; second, the presence of high levels of PKI, which could interfere with the activation of *Nodal* by PKA. Expression of N-cadherin and PKI is biased towards the right side of the node at this stage (indicated in brown); expression of *Shh*, *Nodal* and *lefty-1* is left-specific (indicated in blue). At this stage, several Wnts that are known to signal through β-catenin are expressed in or around the node, and thus in this model we consider a sum of Wnt activities mediated by β-catenin. In the mouse, *Wnt-8c* is expressed in a pattern very similar to that of its chick counterpart, but its role in LR development has not been described yet. Ectopic expression of *Wnt-8c* in a transgenic line induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm (Popperl et al., 1997), but effects of more localized expression of *Wnt-8c* had not been analyzed. Also, mice deficient in β-catenin have been shown to display severe defects of the anterior-posterior axis (Huelsken et al., 2000), which prevents an analysis of possible defects in LR determination. This is a simplified model; only the factors mentioned in the text are indicated.

chick embryo. The effect of H89 is also independent of the location of the bead. Whilst both activation and repression of Hedgehog-dependent targets by PKA has been described in *Drosophila* (Ohlmeyer and Kalderon, 1997), it appears that this is the first case described in vertebrates of activation of *Shh* targets by high levels of PKA activity. Complementing the results presented above, we also found that the endogenous PKA inhibitor PKI has an asymmetric pattern of expression, being more strongly expressed on the right side of the node between HH stages 6 and 7 (Fig. 4L). This pattern of expression suggests that PKI could prevent PKA (which is present in all cells) from activating *Nodal* (Fig. 4N) on the right side of the node. Interestingly, PKI and *Nodal* also appear to be mutually exclusive at earlier stages of development (Fig. 4K,M). Ectopic application of *Wnt-8c* to the right side of the embryo failed to alter PKI expression (data not shown).

From all these data, we conclude that a PKA-dependent process is required for left-sided *Nodal* expression in the chick embryo, and that inhibition of PKA activity on the right side of the node by the endogenous inhibitor PKI is a possible mechanism by which *Nodal* is prevented from being transcribed in that location. This PKA-dependent pathway operates as a mechanism that controls *Nodal* expression in parallel to both Shh and Wnt-8c signals.

Multiple pathways contribute to localize *Nodal* expression

Our results describe a situation where multiple regulatory mechanisms control and restrict *Nodal* expression in and around the node. But why is it important to have several independent ways of controlling *Nodal* expression in Hensen's node? Left-specific expression of *Nodal* in the node is likely to play a key role during LR determination, as hinted by the fact that it is the earliest molecular asymmetry known to be conserved among vertebrates (reviewed by Capdevila et al., 2000). Moreover, recent data in mice and zebrafish suggest that the presence of *Nodal* on the left side of the node (and also the normal transduction of the Nodal signal) is absolutely required for the transfer of LR positional information from the node to the lateral plate mesoderm (LPM), including left-specific expression of *lefty-1* (in the presumptive floor plate), and of *lefty-2* and *Nodal* (and hence *Pitx2*) in the LPM (reviewed by Schier and Shen, 2000). The exact reason why left-sided expression of *Nodal* in the node is required for the transfer of LR positional information to the LPM is still unknown. In any event, and complementing the mouse and zebrafish data mentioned above, our results in the chick embryo suggest that at least three signaling mechanisms (triggered by Shh, Wnt/β-catenin and PKA) operate independently to restrict *Nodal* transcription to the left side of Hensen's node.

Taken together, our results allow us to propose a model for the involvement of Wnts and PKA in LR determination in the chick embryo (Fig. 5). We speculate that the sum of Wnt activities in or around Hensen's node results in the activation of *Nodal* transcription on the left side of the node, in a process mediated by β-catenin. The control of *Nodal* by Wnt genes appears to be conserved during evolution, as the promoter of the mouse *Nodal* gene contains LEF-1/TCF sequences, which are known to mediate transactivation by β-catenin (Norris and Robertson, 1999), and in *Xenopus*, the *Nodal*-related genes *Xnr1* and *Xnr3* also have Wnt-responsive regulatory sequences (McKendry et al., 1997; Hyde and Old, 2000). Interestingly, the role of Wnt signals as left determinants may not be limited to Wnts that signal through β-catenin. Wnt-11, which does not signal through β-catenin, has also some activity as a left determinant during LR determination in the chick (C. R.-E., J. C., Y. K. and J. C. I. B, unpublished), further suggesting that a complex network of Wnt signals controls *Nodal* expression. On the right side of the node, the activation of *Nodal* by Wnts may be antagonized by at least two mechanisms: (1) the presence of high levels of N-cadherin, which can presumably inhibit β-catenin-mediated transactivation of Wnt targets; and (2) the presence of the endogenous PKA inhibitor PKI, which may prevent the activation of *Nodal* by PKA. In summary, the results presented here demonstrate a role for Wnt signaling and PKA in LR development of the chick embryo, and underscore the importance of a tight regulation of *Nodal* expression, as the key regulator of LR development in vertebrates.

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