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

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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-\(\beta\)-catenin\(^{CT}\) 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 \(\beta\)-catenin and the gene encoding subunit \(\alpha\) of chick PKI1 were similarly derived form RT-PCR fragments. The entire ORF of chick \(\beta\)-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 PKI\(\alpha\) 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 \(\mu\)M (dissolved in 4% DMSO in embryo medium) were applied into the New cultured blastoderm 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. \(\beta\)-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 \(\beta\)-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 \(\beta\)-catenin (RCAS-\(\beta\)-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 \(\beta\)-catenin in other organisms, led us to conclude that \(\beta\)-catenin is likely to mediate the effects of Wnt-8c on LR development in the chick embryo. Moreover, and consistent with a requirement for \(\beta\)-catenin in normal LR development, overexpression of Axin (a well-characterized
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, CnR, 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
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...
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
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 (Ohmeyer 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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