Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction

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The neural crest is induced by a combination of secreted signals. Although previous models of neural crest induction have proposed a step-wise activation of these signals, the actual spatial and temporal requirement has not been analysed. Through analysing the role of the mesoderm we show for the first time that specification of neural crest requires two temporally and chemically different steps: first, an induction at the gastrula stage dependent on signals arising from the dorsolateral mesoderm; and second, a maintenance step at the neurula stage dependent on signals from tissues adjacent to the neural crest. By performing tissue recombination experiments and using specific inhibitors of different inductive signals, we show that the first inductive step requires Wnt activation and BMP inhibition, whereas the later maintenance step requires activation of both pathways. This change in BMP necessity from BMP inhibition at gastrula to BMP activation at neurula stages is further supported by the dynamic expression of BMP4 and its antagonists, and is confirmed by direct measurements of BMP activity in the neural crest cells. The differential requirements of BMP activity allow us to propose an explanation for apparently discrepant results between chick and frog experiments. The demonstration that Wnt signals are required for neural crest induction by mesoderm solves an additional long-standing controversy. Finally, our results emphasise the importance of considering the order of exposure to signals during an inductive event.

KEY WORDS: Neural crest induction, Mesoderm, Wnt, BMP, Slug, Sox2

INTRODUCTION

The neural crest (NC) is a cell population characteristic of vertebrates that gives rise to a variety of cell types, including neurons and glia in the peripheral nervous system, connective tissues of the craniofacial structures and pigment cells of the skin (LeDouarin and Kalcheim, 1999). This population is induced at the neural plate border by interactions between the neural plate and nearby tissues (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Mayor et al., 1997). From studies in chick, amphibian and zebrafish embryos, some of the signals involved in the induction of the NC have been identified, including BMPs, Wnts, FGF, Notch and RA (reviewed by Basch et al., 2004; Steventon et al., 2005).

Although the role of Wnt as a NC inducer has been clearly demonstrated in different animal models (reviewed by Wu et al., 2003; Heeg-Truedell and LaBonne, 2006), the participation of BMPs as an inducer has been more controversial. Experiments in Xenopus and zebrafish show that an inhibition of BMP is required for NC induction, whereas experiments in chick indicate that activation of BMP is sufficient to induce NC (Liem et al., 1995; Marchant et al., 1998; Nguyen et al., 1998; LaBonne and Bronner-Fraser, 1998; Endo et al., 2002). The multitude of signalling molecules involved in NC induction has generated the idea that NC induction is a multi-step process, with the different signals acting at different steps during the inductive process; however, the precise temporal requirement for these signals has not yet been determined.

NC induction is thought to occur through the complex movements of gastrulation and neurulation, and hence the prospective NC is likely to encounter signals from a variety of sources. Several studies have shown that mesoderm is able to induce NC (Raven and Kloos, 1945; Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003), but the exact nature of the signals produced by the mesoderm is unknown. The role of Wnt signalling during NC induction by mesoderm has been controversial. It has been shown that Wnt signals are required for NC induction and that some Wnt ligands are expressed in the mesoderm, but specific inhibition of Wnt signals produced by the mesoderm does not affect NC induction, suggesting that NC induction by mesoderm is Wnt independent (Monsoro-Burq et al., 2003). Moreover, a recent report has clearly shown that FGF acts in a Wnt-dependent manner during the early stages of NC induction towards the end of gastrulation (Hong et al., 2008). It is of importance now to reconcile these results directly with those of Monsoro-Burq et al. (Monsoro-Burq et al., 2003).

To understand better the spatial relation of the mesoderm to the prospective NC, we performed the first fate map of this tissue at gastrula stages. We found that a specific region of the prospective mesoderm (dorsolateral marginal zone, DLMZ) is adjacent to the NC during its induction at the gastrula stage. As gastrulation and neurulation proceed, the DLMZ differentiates into primarily intermediate mesoderm (IM) and moves to become directly underneath the NC at the neurula stage. We show for the first time that induction of NC requires two steps: first, signals from the DLMZ participate in its early induction during gastrulation, and then signals from the IM underlying the NC and adjacent ectodermal tissue are involved in maintenance of the NC identity during neurulation. We demonstrate that Wnt activity is needed for both steps, whereas BMP activity is differentially required between the early and late step of NC induction. The first inductive step requires...
BMP inhibition, but the second maintenance step requires BMP activation. These results allow us to propose a new two step model for NC induction and to explain the discrepancies in the BMP requirement between chick and Xenopus embryos.

MATERIALS AND METHODS

**Xenopus embryos, micromanipulation and whole-mount in situ hybridisation**

*Xenopus* embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dissections and grafts were performed as described by Mancilla and Mayor (Mancilla and Mayor, 1996). In situ hybridisation was performed as described by Harland (Harland, 1991). The genes analysed were Snail2 (formerly Slug) (Mayor et al., 1995), Foxd3 (Sasai et al., 2001), Wnt8 (Christian and Moon, 1993), Sox2 (Kishi et al., 2000), Chordin (Sasai et al., 1994) and BMP4 (Wardle et al., 1999).

**Dil injections and construction of fate map**

Injections of Dil (Molecular Probes) were performed at stage 10 as described by Linker et al. (Linker et al., 2000). Photos were taken immediately, at stages 11.5, 13, 17, and at stage 28. Embryos were sectioned at stage 28 and based on their locations and previous positions, the fate of each label was assigned. Each label was then mapped onto a representative stage 10 embryo by counting cells from the blastopore lip.

**RNA synthesis in vitro and microinjection of mRNAs or morpholino oligonucleotides**

All plasmids were linearised and RNA transcribed as described by Harland and Weintraub (Harland and Weintraub, 1985). RNA was co-injected with FLDx (Molecular Probes) as described by Aybar et al. (Aybar et al., 2003); or with 200-600 pg of nuclear lacZ mRNA (kind gift from Ali H. Brivanlou). The constructs used were: dd2 (Sokol, 1996); dnTCF3 (Molenaar et al., 1998); GSK3-pCS2 (Shimizu et al., 2000). A 1 mM mixture of two different chordin morpholino oligonucleotides (Oelgeschlager et al., 2003) was injected into the dorsolateral equatorial region of a two-cell embryo. Treatment with dexamethasone was performed as described previously (Tribulo et al., 2003).

**Protein and chemical inhibitor treatment**

For proteins, heparin acrylic beads (Sigma) were soaked overnight in 40 μg/ml Dkk1 (Calbiochem), 50 μg/ml Noggin (R&D Systems) or 20 μg/ml BMP4 (R&D Systems) all suspended in 0.1% BSA. Beads were grafted into explants/whole embryos for entire culture period prior to fixation.

**Luciferase assay**

Embryos were injected four times at the two-cell stage with 25 pg TOPflash or Vent1-luciferase DNA. Explants or whole embryos were then taken and homogenised as described below. Whole embryo readings were taken with each explant experiment as a further positive control. For each sample, 15-20 explants were taken and homogenised immediately in 25 μl 50 mM Tris-HCl (pH 7.5), centrifuged and a further 25 μl Tris was added to the supernatant. The volume was brought up to 250 μl with the reporter lysis buffer provided with a luciferase assay kit (Promega). The samples were then freeze-thawed and the luciferase activity measured as per manufacturers instructions on a single-tube luminometer (Turner BioSystems). Each reading was standardised by protein concentration as determined by absorbance at 280 nm. Readings were further normalised to control injections of FOPflash that contains a mutated version of the Wnt responsive promoter.

RESULTS

**NC fate map**

To locate the prospective NC region, we applied small injections of the lipophilic dye Dil to deep ectodermal cells of stage 10 embryos (Fig. 1A) to mark groups of 10-20 cells; the fate of these labelled cells was analysed at successive time-points until stage 28 (for examples see Fig. 1B-E). On the basis of these data, a stage 10 fate map was constructed (Fig. 1. NC fate map. (A) Stage 10 embryos were injected with small quantities of the lipophilic marker Dil to label prospective neural crest cells and the surrounding tissues. (B-E) Representative examples of the labelled cells contributing to the cephalic NC (B), trunk NC (C), midbrain (D) and posterior intermediate mesoderm (E). Broken lines outline the neural tube (white), notochord (yellow) and somites (green). (F,G) DLMZ becomes IM and underlies the NC. (F) Dil was used to label the DLMZ of a stage 10 embryo. At the neurula stage (stage 16), the embryos were fixed and in situ hybridisation against Snail2 was performed. (G) Section showing fluorescence in the IM. (G') Same section as in G showing Snail2 expression. (G'') Merge of G and G'. (H) The position of each labelled cell was mapped onto a photo of a stage 10 embryo. Each colour corresponds to the key shown in A. (I) A summary of fate map at stage gastrula stage. (J) Summary of the position of NC in relation to IM at the neurula stage.
map showing the position of the prospective NC cells and mesoderm was constructed (Fig. 1H,I). It can be clearly seen from this fate map that at stage 10 the prospective NC population lies adjacent to the DLMZ. Labels within the DLMZ itself were fated to become predominantly intermediate mesoderm, which lies beneath the NC at neurula stage (IM, an example is shown in Fig. 1F-G). In situ hybridisation against Sox3 in these embryos shows that intermediate mesoderm labelled with Dil underlies the NC cells (Fig. 1G-G'). A summary of the gastrula and neurula fate map is shown in Fig. 1I, which shows that DLMZ at the gastrula stage and IM at the neurula stage are adjacent to the NC. The location of the DLMZ supports its involvement in early NC induction and our fate map opens the possibility that IM could play a role in later NC development.

**Two steps of NC specification: induction by DLMZ and maintenance by IM**

To test the inductive ability of DLMZ and IM, tissue recombination assays were performed. Although the inductive abilities of the DLMZ have been already reported (Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003), we include here the DLMZ assay to compare it with the induction by IM. The entire marginal zone of stage 10.25 embryos was dissected and divided into dorsal (DMZ, the region above the blastopore lip), dorsolateral (DLMZ, the region next to the blastopore lip) and lateral (LMZ, the next region that is not adjacent to the blastopore lip); these explants were conjugated with stage 9 animal caps, and the expression of the NC markers Snail2 and FoxD3 was analysed at the equivalent of stage 15 (Fig. 2A). Both genes are expressed in a similar manner; thus, for simplicity, only the expression of Snail2 is shown. Only DLMZ, but not DMZ or LMZ, was able to induce NC markers (Fig. 2A, parts a-c; Snail2: 85%, n=69; FoxD3: 80%, n=44). These results show that DLMZ is involved in NC induction at the gastrula stage, confirming previous reports (Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003).

The role of IM was tested in two different assays. First, explants of different regions of underlying mesoderm were taken from a stage 16 neurula and grafted into the blastocoel cavity of stage 10 embryos (Fig. 2B). Region 1 represents the axial most mesoderm, the notochord. Regions 2, 3 and 4 correspond to predominantly paraxial, intermediate and lateral mesoderm, respectively. Embryos were fixed at stage 16 and analysed for the expression of NC (Snail2) and neural plate markers (Sox3), and for the lineage tracer (FDX) to identify the grafted tissue. Mesodermal explants do not express neural plate or NC markers (Fig. 2B, parts a-h, n=7-10 with 0% expressing markers in all cases). Regions 1, 2 and 3 were able to induce NC markers in ventral epidermis (Fig. 2B, parts i-k), whereas region 4 was not inductive (Fig. 2B, parts p, t, x). However, regions 1 and 2 induced NC at a distance (Fig. 2B, parts m, n, q, r), whereas region 3 induced Snail2 in the adjacent cells (Fig. 2B, parts o, s), suggesting that IM was a direct NC inducer.

**Fig. 2. NC induction by DLMZ and IM.** (A) Early NC induction. The prospective mesoderm was dissected out from stage 10.25 embryos and divided in dorsal marginal zone (DMZ), dorsolateral marginal zone (DLMZ) and lateral marginal zone (LMZ). This tissue was then conjugated with an animal cap taken from a stage 9 embryo and cultured until the equivalent of stage 15 when the expression of the NC marker Snail2 was analysed. (a) Conjugates with DMZ. (b) Conjugates with DLMZ. (c) Conjugates with LMZ. (B) Early NC induction by late mesoderm. Different regions of mesoderm (named 1-4) were dissected from a stage 16 neurula embryo previously injected with FDX and grafted into the blastocoel cavity of a stage 10 gastrula embryo. The grafted embryos were cultured until stage 16 when the expression of Snail2 and Sox3 was analysed. (a-h) Control explants of mesoderm were fixed immediately to analyse the expression of Snail2 and Sox3; no expression of these markers was observed. Purple, in situ hybridisation; blue, grafted tissue. (i-l) Lateral view of grafted embryos showing expression of Snail2. Dorsal is towards the top; anterior towards the right. Only embryos in which the grafted tissue was located in ventral epidermis are analysed. (m-p) Higher magnification of the graft showing expression of Snail2. (q-x) Section through the graft showing expression of Sox3. (C) Late NC maintenance. NC explants were dissected from a stage 16 neurula embryo; NC was fixed immediately or cultured alone or together with the underlying IM until the equivalent of stage 23. The expression of Snail2 was analysed. (a) NC dissected from a stage 16 embryo and fixed immediately. (b) NC cultured until the equivalent of stage 23. (c) NC cultured with the underlying mesoderm (IM).
inducer. This idea was supported by the observation that whereas the two most axial regions (1 and 2) of mesoderm induced NC and neural plate markers (Fig. 2B, parts u, v), region 3 induced NC without neural plate (Fig. 2B, part w). This is the region of mesoderm that underlies the NC, is the main derivative of the DLMZ (Fig. 1F,G) and corresponds to the intermediate mesoderm (IM), as judged in sections (Fig. 1E). Although this experiment indicates that the IM has the ability to induce NC directly in gastrula ectoderm, it does not prove that it plays a similar inductive role in normal development. To test the requirement of IM in NC specification, NC from 16 neurula embryos were dissected and cultured in isolation, in the presence or absence of Wnt and BMP inhibition.

To test whether the DLMZ uses Wnt signals to induce the NC, we first blocked this pathway in the ectoderm in a cell-autonomous manner (Fig. 3A). Animal caps were injected with the specific Dishevelled (Dsh) mutant (dd2) (Sokol, 1996), or the dominant-negative form of the TCF3 protein (Hamilton et al., 2001), both potent intracellular Wnt inhibitors. No Snail2 expression was observed in either control animal caps or animal caps injected with either of these constructs. Control conjugates of animal caps and DLMZ for induction of NC cells, a specific inhibition in the maintenance of the induced NC at the neurula stage. Do these two steps of NC specification require the same set of signals?

**Activation of Wnt and inhibition of BMP are required for NC induction by the DLMZ**

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Taken together, these results show that DLMZ is able to induce NC in the ectoderm of the early gastrula and that IM is required for the maintenance of the induced NC at the neurula stage. Do these two steps of NC specification require the same set of signals?
Are these molecules expressed in the DLMZ at the early gastrula stages? In situ hybridisation against Wnt8 and chordin in whole embryos or in dissected DLMZ shows a clear co-expression in the DLMZ (Fig. 3M-P). Taken together, these results indicate that Wnt signalling and anti-BMP molecules, such as chordin, are required for NC induction by DLMZ.

It has been published, in apparently similar experiments, that NC induction by DLMZ requires FGF while Wnt signalling is dispensable (Monsoro-Burq et al., 2003). In order to understand this apparent discrepancy, we proceeded to repeat some of these experiments and compare them with our experiments. It is known that inhibition of Wnt signalling can anteriorise the neural plate and later increase cement gland formation (Dear dorff et al., 1998; Kiecker and Niehrs, 2001). In the Monsoro-Burq study (Monsoro-Burq et al., 2003), the expansion of the cement gland was used as a positive control to check the Wnt inhibitor treatments without a direct analysis of the effect on NC markers in whole embryos. This leaves open the possibility that expansion of cement gland and inhibition of NC have different sensitivities to the Wnt inhibitor treatments and could explain the differences in these results. To test this possibility, we compared the capacity of the Wnt inhibitor GSK3 (used by Monsoro-Burq et al.), to expand cement gland and to inhibit Snail2. We observed that injection of 0.5 ng of GSK3 mRNA produced a strong expansion in cement gland (Fig. 4F,F'); black bar in Fig. 4H) but did not inhibit Snail2 expression (Fig. 4D,H); only extremely high concentrations of GSK3 mRNA, higher than those used by Monsoro-Burq et al., were able to block NC induction efficiently (Fig. 4E,H) and expand the cement gland (Fig. 4G,G',H). These results show that expansion of cement gland occurs under a mild Wnt inhibition, whereas a more complete inhibition of Wnt signalling is required to abrogate induction of NC. We next showed that the Wnt inhibitors dd2 and dnTCF3 are able to inhibit NC markers in whole embryo under the conditions used in our conjugates experiments (Fig. 4A-C,H). These results indicate that the conditions used by Monsoro-Burq et al. (Monsoro-Burq et al., 2003) were not sufficient to inhibit NC induction.

**Activation of Wnt and BMP signalling is required for NC maintenance**

We then examined the signals involved in the NC maintenance step (Fig. 2C). Explants of NC and underlying mesoderm (NC/M) dissected at stage 16, were cultured in the presence of Wnt and BMP inhibitors until the equivalent of stage 23 (Fig. 5A). Inhibitors were immobilised into beads that are embedded into the explant. The beads are about one-third of the size of the explant, and thus we assume that most, if not all, of the cells receive the signal. Control conjugates express the NC marker Snail2 (Fig. 5B), whereas its expression was blocked by treatment with the Wnt inhibitor dkk1 (Fig. 5C), suggesting Wnt signaling is also required for NC maintenance. Further support for this possibility comes from the finding that β-catenin activation in isolated NC explants is able to maintain Snail2 expression (Fig. 5H), which is otherwise lost (Fig. 5F,G). Treatment with Noggin, an endogenous BMP inhibitor, resulted in an inhibition of Snail2 expression (Fig. 5D), suggesting that BMP is required for NC maintenance. Accordingly, culturing the NC/M explants in the presence of BMP4 did not inhibit Snail2 expression (Fig. 5E). In addition, isolated NC explants cultured (Fig. 5F) in the presence of BMP4 resulted in maintenance of Snail2 expression (Fig. 5I). Overall, these results show that during the maintenance step at the neurula stage activation of both Wnt and BMP signals is required. Subsequently, we found that at the neurula stage Wnt8 and BMP4 are expressed in tissues surrounding the NC (Fig. 5L-N). Specifically, we found that IM express Wnt8, whereas BMP4 is expressed in the ectoderm adjacent and in the NC (Fig. 5O-Q). That the IM expresses Wnt8 but not BMP4 was further confirmed by direct analysis of these genes in isolated mesoderm (Fig. 5J,K).

We tested the observed change in BMP necessity in whole embryos by analysing the expression of NC markers after performing the same activation or inhibition of BMP activity at the gastrula or neurula stage (Fig. 6A,B). In order to inhibit BMP signal in the whole embryo, we injected an inducible Smad7 construct (Wawersik et al., 2005) into blastomeres fated to differentiate as ventral epidermis. Inhibition of BMP signaling in the ventral epidermis can induce Snail2 expression during gastrulation (Fig. 6C,E) but not thereafter (Fig. 6D,E). No induction was observed in the absence of dexamethosone (Fig. 6E). The amount of Smad7 used in this experiment was able to induce specifically NC with little or no neural plate induction (Fig. 6L). It is known that the epidermis loses competence for NC induction from stage 13 onwards (Mancilla and Mayor, 1996), and hence the observed changes in response to Smad7GR might be due to a change in competence. We next performed the opposite experiment by adding BMP4 to the embryos. Animal cap cells that had been previously injected with high levels of BMP4 mRNA were grafted adjacent to
the NC and the expression of NC markers was analysed. As a control to ensure that the injected animal cap cells are expressing BMP4 at high levels, they were conjugated with explants of DLMZ and animal cap (Fig. 6I-K). When conjugated with cells injected with FDX only, an induction of Snail2 is observed (Fig. 6I), whereas when conjugated with cells injected with FDX/BMP4, an inhibition of Snail2 induction is observed (Fig. 6K), indicating that the injected cells produce enough BMP4 to inhibit NC induction.

BMP4-expressing cells grafted into gastrula embryos inhibit Snail2 expression (Fig. 6F,H). However, Snail2 expression is not affected if the same graft is performed at neurula stages during the maintenance step (Fig. 6G,H). Graft of control cells did not affect the NC at the gastrula or neural stages (Fig. 6H). Together, these results confirm our previous results obtained in explants and show that a change in BMP activity is required between the induction and the maintenance steps.

To examine dynamic changes in levels of signaling, we measured levels of Wnt or BMP activity in the NC using the TOPFlash luciferase reporter or BMP reporter construct (Fig. 7A). Embryos were injected in the animal hemisphere with the reporter constructs and the luciferase activity we measured at the gastrula (from stage 10 until 12) or neurula (stage 16-18) stages. As our previous results predicted, the level of Wnt activity is kept low in the prospective NC during the early part of gastrulation (stage 10, not shown), with a rise in activity seen at mid gastrula that corresponds to NC induction step (Fig. 7B, gastrula bar). Furthermore, we see a continual rise in Wnt activity during neurulation, consistent with its role in NC maintenance (Fig. 7B, neurula bar). For BMP signaling, we made use of a Vent1-luciferase reporter. As predicted, levels of BMP signaling are lower in the NC than in the epidermis during gastrulation (Fig. 7C, gastrula bar). Interestingly, we observe a sharp rise in the relative levels of BMP signaling during neurulation (Fig. 7C, neurula bar). This correlates both with the requirement of high levels of BMP for maintenance and the observation that BMP4 is expressed at high levels in the neural folds from stage 16 onwards (Fig. 5N,Q).

DISCUSSION

We propose a novel, two-step model for NC induction and identify the signals required for each step (Fig. 8A,B). First, the induction step at gastrula stage when Wnt activation and BMP inhibition are required. The level of both pathways is regulated by signals secreted by the DLMZ which is adjacent to the prospective NC. Second, the maintenance step at neurula stages, when activation of both Wnt and BMP signalling are required and provided by signals secreted from the IM and adjacent ectoderm. This model emphasises the importance of the timing at which ectoderm is exposed to different signals during an inductive event.

The different requirements for BMP signals at the gastrula or neurula steps resolves a controversy about apparently discrepant results between experiments performed in chick and frog. In chick embryos, only high levels of BMP signalling were reported to be important for NC induction (Liem et al., 1995; Endo et al., 2002), whereas in Xenopus and zebrafish embryos an inhibition of BMP has been reported as a condition for NC induction (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998; Glavic et al., 2004; Villaneuva et al., 2002). However, the chick experiments were performed at neurula stages, much later than the gastrula stages used for the Xenopus and zebrafish experiments. Moreover in chick and Xenopus embryos the levels of phosphorylated Smad1, a read-out of BMP activity, rises in the neural fold only at neurula stages, being lower in the prospective NC
Fig. 6. Distinct temporal requirements for signals during NC development in vivo. (A, C, F) Embryos were manipulated just prior to Snail2 expression at stage 11.5 then analysed at stage 14, to determine the period of NC induction. (B, D, G) Embryos manipulated at stage 15, after Snail2 expression has started, and analysed at stage 18 prior to NC migration, to determine the NC maintenance period. (C, D) Embryos were injected at the 32-cell stage into blastomeres fated to become epidermis. Activation of the construct by the addition of dexamethasone (A, G) with the inducible construct Smad7GR to target injections to injected at the 32-cell stage into blastomeres fated to become epidermis. Activation of the construct by the addition of dexamethasone (A, D) with the inducible construct Smad7GR to target injections to target injections of NC induction. (B) Embryos were manipulated at stage 15, after Snail2 expression has started, and analysed at stage 18 prior to NC migration, to determine the NC maintenance period. (C, D) Embryos were manipulated at stage 15, after Snail2 expression has started, and analysed at stage 18 prior to NC migration, to determine the NC maintenance period. (C, D) Embryos were manipulated at stage 15, after Snail2 expression has started, and analysed at stage 18 prior to NC migration, to determine the NC maintenance period. (C, D) Embryos were manipulated at stage 15, after Snail2 expression has started, and analysed at stage 18 prior to NC migration, to determine the NC maintenance period. During the gastrula stage results in ectopic expression of Snail2 (arrow in C; 25%, n=40). However, when activated during the maintenance phase, no ectopic expression is observed (D; 0%, n=20). No effect is seen in the absence of dexamethasone (not shown; 0%, n=31). (F-G) Small groups of ectodermal cell expressing BMP4 were grafted (arrows) next to the NC. When grafted during gastrulation, a strong inhibition of the NC was observed (F; 100%, n=31). However, when BMP4 cell were grafted during the maintenance phase, no inhibition was observed (G; 0%, n=55). Grafts of cell injected with FDX alone had no effect (not shown; 0%, n=42). The graft was recognised by immunostaining against fluorescein, as FDX was used as a lineage tracer (blue). (E) Summary of Smad7GR injections. (H) Summary of graft of BMP4-expressing cells. (I-K) Positive control to ensure sufficient BMP4 is being released to inhibit NC induction. (I) A small cluster of animal cap cells taken from an embryo injected with FDX or BMP4 mRNA (blue) was conjugated with an explant of stage 10.5 animal cap (AC) and DLMZ, in which NC markers are normally induced. (J) Explants of AC/DLMZ conjugated with cells expressing FDX show strong expression of Snail2 (85%, n=26). (K) Explants of AC/DLMZ conjugated with cells expressing BMP4/FDX show a dramatic inhibition in Snail2 expression (10% expressing Snail2, n=35). (L) Percentage of Sox2 or Snail2 expression after injecting different amounts of Smad7GR into A4 blastomere of a 32-cell stage embryo, as described in C, D. Each experiment was repeated at least three times.

region earlier in development (Faure et al., 2002; Schohl and Fagotto, 2002). We propose that the chick experiments were performed at the maintenance phase of NC induction and thus are consistent with our findings in the frog, that show a necessity of high BMP level at this step. Interestingly, a recent paper demonstrated that NC induction also initiates during gastrulation in chicks (Basch et al., 2006) and it is possible that this early phase requires an inhibition of BMP signalling. Thus, our detailed findings in Xenopus are likely to reflect pathways and phases that are common to all vertebrates.

Mesoderm as NC inducer
The DLMZ has, for a long time, been known to have NC-inducing properties (Raven and Kloos, 1945; Mayor et al., 1995; Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003) (this work). We show here that the DLMZ, which becomes intermediate mesoderm (IM), moves during gastrulation to underlie the NC cells. This mesoderm has the ability to induce NC markers directly, without neural induction, and is essential for NC maintenance.

The expression of Wnt and BMP signal components fit with our model. During gastrulation, Wnt8 is expressed in the lateral and ventral mesoderm, while chordin is expressed in dorsal mesoderm. It is interesting to note that the only tissue co-expressing Wnt8 and chordin is the DLMZ, which is the only mesodermal tissue able to induce NC. At the neurula stage the IM continues to produce the NC inductive signal Wnt, but no longer expresses chordin (or any other BMP antagonist) (Sasai et al., 1994; Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994). The absence of BMP antagonist in the IM and the rise in BMP4 expression in the neural fold lead to the increase in BMP activity necessary for the NC maintenance step.
Furthermore, expression of an expansion of NC cells (LaBonne and Bronner-Fraser, 1998). Overexpression of Wnt (this work) (Christian and Moon, 1993). In zebrafish, Wnt8 (Garcia-Castro et al., 1998). This leaves Wnt8 as the only Wnt candidate to mediate this late step of induction (Garcia-Castro et al., 1998).

be a specific inducer of NC cells (Chang and Hemmati-Brivanlou, 2003). However, the role of Wnt signals from the DLMZ has been challenged (Monsoro-Burq et al., 2003). No inhibition of NC by the intracellular Wnt inhibitors such as GSK3 or dominant-negative TCF3 was found (Monsoro-Burq et al., 2003). However, as the activity of these antagonists was tested only on cement gland expansion, it remains possible that these two intracellular components of the Wnt pathway did not fully block Wnt function. Indeed, we show here that similar conditions to those published (Monsoro-Burq et al., 2003) expand cement gland, without affecting NC. Higher levels of Wnt inhibition are required to impair NC formation. This observation solves a longstanding controversy about the role of Wnt signals on NC induction by mesoderm (see Huang and Saint-Jeannet, 2004) and it is consistent with a recent publication that places Wnt8 as downstream target of β-catenin (LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Garcia-Castro et al., 2002; Tribulo et al., 2003; Lewis et al., 2004; Bastidas et al., 2004; Carmona-Fontaine et al., 2007). Presumably in this context, after the initial induction of NC, the level of Wnt signalling is high enough to compensate for the lower level of BMP signalling in the subsequent maintenance of induction. Alternatively, activated Wnt signalling could interact with the endogenous BMP4 present in the neural fold or in the animal cap, leading to NC maintenance.

In addition to the requirement of BMP and Wnt, it has recently been shown that endothelin 1 plays an important role in the maintenance of the NC (Bonano et al., 2008). It is possible that all three pathways are acting in parallel to maintain the NC, alternatively BMP and Wnt signals might act to maintain the NC by upregulating the endothelin receptor Ednra, although this requires further attention.

This work highlights the importance of looking the spatial relationship of tissues during time. It is likely that all tissues are releasing different signals during development but not all of them are close enough to produce an effective signal. In processes as dynamic as gastrulation and neurulation, this should not be neglected. By taking into account these parameters, we have been able to solve two controversies in NC induction.

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References

Wnt signalling in NC induction by mesoderm

There is compelling evidence indicating that Wnt signalling participates in NC induction in several species (reviewed by Wu et al., 2003; Yanfeng et al., 2003). However, the role of Wnt signals from the DLMZ has been challenged (Monsoro-Burq et al., 2003). No inhibition of NC by the intracellular Wnt inhibitors such as GSK3 or dominant-negative TCF3 was found (Monsoro-Burq et al., 2003). However, as the activity of these antagonists was tested only on cement gland expansion, it remains possible that these two intracellular components of the Wnt pathway did not fully block Wnt function. Indeed, we show here that similar conditions to those published (Monsoro-Burq et al., 2003) expand cement gland, without affecting NC. Higher levels of Wnt inhibition are required to impair NC formation. This observation solves a longstanding controversy about the role of Wnt signals on NC induction by mesoderm (see Huang and Saint-Jeannet, 2004) and it is consistent with a recent publication that places Wnt8 as downstream target of β-catenin (LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Garcia-Castro et al., 2002; Tribulo et al., 2003; Lewis et al., 2004; Bastidas et al., 2004; Carmona-Fontaine et al., 2007). Presumably in this context, after the initial induction of NC, the level of Wnt signalling is high enough to compensate for the lower level of BMP signalling in the subsequent maintenance of induction. Alternatively, activated Wnt signalling could interact with the endogenous BMP4 present in the neural fold or in the animal cap, leading to NC maintenance.

In addition to the requirement of BMP and Wnt, it has recently been shown that endothelin 1 plays an important role in the maintenance of the NC (Bonano et al., 2008). It is possible that all three pathways are acting in parallel to maintain the NC, alternatively BMP and Wnt signals might act to maintain the NC by upregulating the endothelin receptor Ednra, although this requires further attention.

This work highlights the importance of looking the spatial relationship of tissues during time. It is likely that all tissues are releasing different signals during development but not all of them are close enough to produce an effective signal. In processes as dynamic as gastrulation and neurulation, this should not be neglected. By taking into account these parameters, we have been able to solve two controversies in NC induction.

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References

Fig. 8. Model of NC specification in two steps. (A) A diagram summarising the different temporal requirements and activities for Wnt and BMP pathways during NC development. (B) Model of NC induction at the gastrula and maintenance at the neurula stages.


