Neural crest induction by paraxial mesoderm in *Xenopus* embryos requires FGF signals

Anne-Hélène Monsoro-Burq*, Russell B. Fletcher and Richard M. Harland

Department of Molecular and Cellular Biology, University of California at Berkeley, CA 94720, USA

*Author for correspondence (e-mail: monsoro@uclink.berkeley.edu)

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SUMMARY

At the border of the neural plate, the induction of the neural crest can be achieved by interactions with the epidermis, or with the underlying mesoderm. Wnt signals are required for the inducing activity of the epidermis in chick and amphibian embryos. Here, we analyze the molecular mechanisms of neural crest induction by the mesoderm in *Xenopus* embryos. Using a recombination assay, we show that prospective paraxial mesoderm induces a panel of neural crest markers (*Slug, FoxD3, Zic5* and *Sox9*), whereas the future axial mesoderm only induces a subset of these genes. This induction is blocked by a dominant negative (dn) form of FGFR1. However, neither dnFGFR4a nor inhibition of Wnt signaling prevents neural crest induction in this system. Among the FGFs, *FGF8* is strongly expressed by the paraxial mesoderm. *FGF8* is sufficient to induce the neural crest markers *FoxD3, Sox9* and *Zic5* transiently in the animal cap assay. In vivo, *FGF8* injections also expand the *Slug* expression domain. This suggests that *FGF8* can initiate neural crest formation and cooperates with other DLMZ-derived factors to maintain and complete neural crest induction. In contrast to Wnts, eFGF or bFGF, *FGF8* elicits neural crest induction in the absence of mesoderm induction and without a requirement for BMP antagonists. In vivo, it is difficult to dissociate the roles of FGF and WNT factors in mesoderm induction and neural patterning. We show that, in most cases, effects on neural crest formation were parallel to altered mesoderm or neural development. However, neural and neural crest patterning can be dissociated experimentally using different dominant-negative manipulations: while Nfz8 blocks both posterior neural plate formation and neural crest formation, dnFGFR4a blocks neural patterning without blocking neural crest formation. These results suggest that different signal transduction mechanisms may be used in neural crest induction, and anteroposterior neural patterning.

Key words: FGF, WNT, FGF8, Paraxial mesoderm, *Xenopus* embryo, Neural crest, Neural patterning

INTRODUCTION

The neural crest, a transitory population of cells that is characteristic of vertebrate embryos, forms at the border of the neural plate, posteriorly to the diencephalon. After induction, neural crest cells undergo an epithelial-to-mesenchymal transition and migrate into several locations to give rise to a large variety of derivatives (for a review, see Le Douarin and Kalcheim, 1999). Experimental manipulations in chick, fish and amphibian embryos have shown that both the ectoderm and the neural plate can give rise to neural crest cells when they are juxtaposed (Moury and Jacobson, 1989; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Woo and Fraser, 1998). However, in vivo, the neural crest forms adjacent to three different tissues, the non neural ectoderm, the neural plate and the underlying paraxial mesoderm, all of which thus constitute potential sources of neural crest inducers (Schroeder, 1970). Although many studies have focused on neural crest induction by the ectoderm in the chick embryo (Dickinson et al., 1995; Basch et al., 2000; Knecht and Bronner-Fraser, 2002), a pioneering study by Raven and Kloos (Raven and Kloos, 1945) showed that the paraxial mesoderm can induce neural crest formation in the ectoderm of amphibians. More recent studies also show that recombining the paraxial mesoderm with naive ectoderm in *Xenopus laevis* embryos results in potent neural crest induction in the ectodermal part of the explant and that exciting the paraxial mesoderm results in lack of neural crest formation in vivo (Mancilla and Mayor, 1996; Bonstein et al., 1998; Marchant et al., 1998). In chick embryos, some data also indicate that the melanocytes, which are neural crest derivatives, are induced after neural plate-paraxial mesoderm recombination (Selleck and Bronner-Fraser, 1995). Although tested separately in these experimental assays, the possibility that the inducing activities from the ectoderm and the mesoderm might act in concert during normal development remains to be explored.

In the amphibian embryo, the current analysis of the molecular basis of ectoderm- neural tissue interactions results in a two-step model of neural crest induction detailed below (reviewed by Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002). *Slug* was generally used in these studies as a
specific marker gene for neural crest development (Nieto et al., 1994; Mayor et al., 1995). In the first step of the model, in parallel to what happens during amphibian neural induction (Harland, 2000), BMP activity in the ectoderm must be attenuated by BMP antagonists. Neural crest forms after moderate BMP inhibition while neural tissue induction requires higher levels of inhibition (Marchant et al., 1998). However, the levels of Slug induction in these assays, using BMP antagonists alone, are very low compared with endogenous levels (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998). This suggests that in the embryo, additional factors are required for normal levels of Slug expression and neural crest induction/maintenance.

Co-injection of BMP antagonists with molecules such as Wnts (Wnt7b or Wnt8), fibroblast growth factors (eFGF or bFGF) or retinoic acid (RA) results in strong neural crest formation in ectodermal explants (animal caps) (Mayor et al., 1995; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). Although these molecules do not induce neural crest by themselves in vitro, the in vivo overexpression of positive regulators of the Wnt, FGF or RA pathways expands neural crest-forming domains, whereas blocking these pathways prevents normal neural crest induction in both embryo and explant assays (Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). Together, these data suggest a second phase of induction where partially neuralized ectoderm is specified to become neural crest either by Wnts, FGF, RA or a combination. However, this model does not specifically address the mechanism by which paraxial mesoderm might induce the neural crest. Furthermore, both FGF and Wnt proteins play important roles in mesoderm induction and paraxial mesoderm development (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Fisher et al., 2002; Monica and Gumbiner, 2002) and reagents that affect neural crest induction might do so indirectly by their effects on the mesoderm (Mayor et al., 1995; Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Finally, all three classes of molecules implicated in neural crest induction are also important neural posteriorizing agents (Lamb and Harland, 1995; Bang et al., 1997; Bang et al., 1999; Kiecker and Niehrs, 2001; Kudoh et al., 2002). BMP antagonism results in the formation of anterior neural tissue that is not expected to form neural crest (Lamb et al., 1993; Knecht and Harland, 1997). This raises the possibility that posteriorization of this area into a neural crest-producing tissue might do so indirectly by their effects on the mesoderm (Mayor et al., 1995; Mayor et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). However, the levels of Slug induction in these assays, using BMP antagonists alone, are very low compared with endogenous levels (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998). This suggests that in the embryo, additional factors are required for normal levels of Slug expression and neural crest induction/maintenance.

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In this study, we address two questions. First, what is the nature of the mesodermal signal(s) inducing neural crest in the ectoderm? Second, how is neural crest induction related to early anteroposterior (AP) patterning of the neural plate? To study the molecular mechanisms of neural crest induction by the paraxial mesoderm in the Xenopus laevis embryo, we focused on the neural crest-inducing properties of the dorsolateral marginal zone (DLMZ) on animal cap explants. Using various neural crest markers, we show that the DLMZ and the dorsal marginal zone (DMZ) exhibit qualitative differences in their inducing properties. In order to study the role of specific growth factor signaling in neural crest induction, we then took advantage of previously characterized molecular tools, consisting of broad range or more specific inhibitors of the Wnt and FGF pathways. These reagents include Nfz8, GSK3, dnTCF3 and a truncated form of Dishevelled (Xdd1) for Wnt signaling, and SU5402, XFD and dnFGFR4a for FGF signaling (Amaya et al., 1993; Sokol, 1996; Mohammadi et al., 1997; Deardorff et al., 1998; Hongo et al., 1999; Deardorff et al., 2001), for reviews see (Galzi et al., 1999; Brantjes et al., 2002; Moon et al., 2002). We have also used these reagents in vivo to address whether neural crest formation can be uncoupled from repatterning of the mesoderm or changes in AP patterning of the neural plate.

**MATERIALS AND METHODS**

**RNA injections**

* X. laevis embryos were staged according to Nieuwkoop and Faber (1956) and analyzed according standard procedures described by Sive et al. (2000). Nuclear targeted β-galactosidase (pCS2-Nis-NlacZ, 200 pg) mRNA was co-injected with the test mRNA for lineage tracing. Capped messenger RNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion).

To block the response of the ectoderm to endogenous Wnt molecules, we injected mRNAs encoding either xNFz8, Glycogen Synthase Kinase 3 (GSK3), dnTCF3 or Xdd1. The pCS2-xNFz8 encodes a wide spectrum dominant-negative Wnt receptor (Deardorff et al., 1998), Xdd1 is a truncated form of Dishevelled, which acts as a dominant-negative in both the canonical and the non canonical planar cell polarity (PCP) pathways (Sokol, 1996; Wallingford and Harland, 2002). The pCS2-xGSK3, pT7Tsv-dnTCF3, p64T-XWnt8 and pCS2-dnXWnt8 plasmids have been described previously (Christian et al., 1991; Molenaar et al., 1996; Pierce and Kimelman, 1996; Hoppler and Moon, 1998). We blocked FGF signaling in the responding ectoderm using either a dominant-negative form of xFGFR1, constructed by S. Dougan (pCS2-XFGF-DN) similar to the XFD construct published by Amaya et al. (Amaya et al., 1991), or a truncated FGFR4a (p64T-dnXFGFR-4a) (Hongo et al., 1999), subcloned into pCS108. XFGF8 (Christen and Slack, 1997) was subcloned into pCS107.

**Tissue recombination, SU5402 treatment of the recombinants**

Stage 10-15 DLMZ or DMZ were recombined with stage 8-9 animal caps (Fig. 1A) (Bonstein et al., 1998). Dissections and culture were performed in 3/4 Normal Amphibian Medium (NAM) containing gentamycin (100 μg/ml). The recombinants were harvested when Stage 18. For inhibition of FGF signaling by the SU5402 (Calbiochem) (Mohammadi et al., 1997), the recombinants were cultivated in 50 μM SU5402 diluted into 3/4 NAM (Shinya et al., 2001; Maroon et al., 2002). Controls were grown in DMSO diluted in 3/4 NAM.

**In situ hybridization**

The in situ hybridization protocol was simplified by directly prehybridizing embryos younger than stage 20 after rehydration in PBT. The rest of the procedure remained unchanged. This shorter protocol allows a better staining of superficially located tissues (such as the neural crest).

The probes for Slug, Twist, Snail, Krox20, Cpl-1 and Otx2 have...
been described elsewhere (Richter et al., 1988; Hopwood et al., 1989; Bradley et al., 1993; Lamb et al., 1993; Mayor et al., 1993; Grammer et al., 2000). The Sox9 probe was a kind gift of R. Spokony and J-P. Saint-Jeannet (Spokony et al., 2002). Sox9 and FoxD3 probes were derived from a library made by A. Zorn (Khokha et al., 2000). The probe for muscle actin, and recombined with a stage 8-9 animal cap (AC) (Fig. 1B).

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence (upstream and downstream, respectively)</th>
<th>Number of cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slug</td>
<td>5'-TCCGCCTCCGAGGGATGTACTGCGCTC-3'</td>
<td>23-25</td>
<td>Mizuseki et al. (1998)</td>
</tr>
<tr>
<td>Zic5</td>
<td>5'-AGACTGAGATCGCGTCGCGGCAAG-3'</td>
<td>23</td>
<td>Nakata et al. (2000)</td>
</tr>
<tr>
<td>FoxD3</td>
<td>5'-CCAGAAGGAAAGGAGACGACG-3'</td>
<td>23</td>
<td>This work</td>
</tr>
<tr>
<td>Snail</td>
<td>5'-GGGGCTTACTACACCTTGTGCAC-3'</td>
<td>23</td>
<td>This work</td>
</tr>
<tr>
<td>Sox9</td>
<td>5'-AACAGGAGTTCTCATCCTCC-3'</td>
<td>25</td>
<td>This work</td>
</tr>
<tr>
<td>FGFR3</td>
<td>5'-GGCTGAGAGAGAGACCTAAGTA-3'</td>
<td>25</td>
<td>This work</td>
</tr>
<tr>
<td>eFGF/FGF4</td>
<td>5'-CCGGTTCCTCTCCAGAGA-3'</td>
<td>25</td>
<td>Isaacs et al. (1992)</td>
</tr>
<tr>
<td>FGFR1</td>
<td>5'-TGCGGAGACTGTACTACAGTG-3'</td>
<td>25</td>
<td>This work</td>
</tr>
<tr>
<td>FGFR2</td>
<td>5'-ATGCTCTTTGGTCTGCTGTG-3'</td>
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<td>This work</td>
</tr>
<tr>
<td>FGFR3</td>
<td>5'-ACAGGAGGAGACGAGAAGAC-3'</td>
<td>25</td>
<td>This work</td>
</tr>
<tr>
<td>FGFR4a</td>
<td>5'-TCAGTACCTCTAAGGCGAC-3'</td>
<td>25</td>
<td>This work</td>
</tr>
</tbody>
</table>

To characterize the neural crest induced by the DLMZ in this explant assay in more detail, we analyzed several other genes in addition to Slug: in

**RESULTS**

**The DLMZ and the DMZ induce distinct subsets of neural crest markers in the ectoderm**

Mesoderm explants were dissected at stage 10 to 10.5 (Fig. 1A) and recombined with a stage 8-9 animal cap (AC) (Fig. 1B). Neural crest formation in the recombinants was first assessed by the induction of Slug expression in the recombinants, as reported by Marchant et al. (Marchant et al., 1998) and Bonstein et al. (Bonstein et al., 1998) (Fig. 1C). The DMZ was dissected above the pigment line, indicating the future dorsal lip at stage 10 or along an equivalent width at stage 10.5 (Fig. 1A). When analyzed using RT-PCR at stage 17-18, DMZ explants grown in isolation expressed the notochord marker Xnot but very little muscle actin (MA) (Fig. 1C, lane 6). After recombination with animal caps, the DMZ-AC did not show Slug expression (Fig. 1C, lane 7). By contrast, the paraxial mesoderm (DLMZ) (Fig. 1A) expressed both muscle actin and Xnot (Fig. 1C, lane 4), and DLMZ-AC recombinants showed a strong Slug signal (Fig. 1C, lane 5). Previous studies have shown that the inducing tissue in this system is the DLMZ and that neural crest forms from the animal cap (Bonstein et al., 1998; Marchant et al., 1998). Xnot expression in the DLMZ area corresponds to the lateral extension of the notochord domain at stage 10.5 (Yasuo and Lemaire, 2001).

If slightly larger DMZ explants were cut, extending beyond the stage 10.25 dorsal lip, they variably expressed muscle actin and Slug upon recombination (not shown). Thus, for consistency in the experiments illustrated in this study, we dissected the DMZ as a narrow band of tissue taken at stage 10-10.5, and cut DLMZs that contained robust Slug inducing activity.

To characterize the neural crest induced by the DLMZ in this explant assay in more detail, we analyzed several other genes in addition to Slug, all expressed mainly by the neural crest around stage 18 (Fig. 1C,D). Snail (Essex et al., 1993; Mayor et al., 1993), Twist (Hopwood et al., 1989), Zic5 (Nakata et al., 2000), Sox9 (Spokony et al., 2002) and FoxD3 (Pohl and Knochel, 2001; Sasai et al., 2001) were all upregulated when the DLMZ was recombined with animal caps (Fig. 1C, lane 5). FoxD3 responded in a very similar manner to Slug.
particular, neither was induced in the AC-DMZ recombinants (Fig. 1B, lane 7). Both showed weak expression in the mesoderm, corresponding to what was observed in vivo (Fig. 1C, lanes 4 and 6) (Linker et al., 2000; Sasai et al., 2001). By contrast, Sox9, Zic5 and Snail expression were also upregulated in the AC-DMZ, although at a low level in the case of Sox9. Interestingly, Snail and Zic5 induction was as strong with the DMZ as with the DLMZ, perhaps reflecting the normal expression of these genes in the midline of the anterior neural fold (Fig. 1D) (Linker et al., 2000; Nakata et al., 2000).

This analysis suggests that neural crest induction observed in this recombination assay reproduces the complexity of in vivo mechanisms. Because of their basal expression in the isolated animal caps and/or mesoderm explants, Snail and Twist were not analyzed further in this study. We focused on Slug, FoxD3, Sox9 and Zic5, which were specifically upregulated in the recombinants.

Blocking Wnt signaling does not prevent induction of neural crest by the DLMZ

The canonical Wnt pathway has been shown to be important in neural crest formation in other systems. In addition, the Slug promoter contains LEF-TCF binding sites suggesting a direct regulation by this pathway (Vallin et al., 2001). To test the hypothesis that the DLMZ requires Wnt signals to induce neural crest, we blocked the response of the ectoderm to Wnt signaling using the antagonists NFz8, GSK3 and dnTCF3. The xFz8 receptor has been shown to mediate the activity of Wnt1, Wnt2c, Wnt3a, Wnt5a, Wnt7b, Wnt8 and Wnt11 efficiently (Deardorff et al., 2001). NFz8, a truncated and diffusible form of xFz8, acts on gastrulation movements and neural plate patterning as expected for a Wnt antagonist, but does not prevent dorsal mesoderm specification (Deardorff et al., 1998). In contrast to NFz8, glycogen synthase kinase 3 (GSK3) and dnTCF3 prevent Wnt signaling in a cell autonomous manner (Brantjes et al., 2002; Moon et al., 2002). In this series of experiments, positive controls of Wnt inhibiting activity showed that 400 pg of NFz8 mRNA efficiently blocked XWnt8-induced secondary axis formation (100% reversal of double axis formation, after co-injecting 400 pg of NFz8 and 50 pg p64T-XWnt8 mRNAs, n=31, not shown). Moreover, the injected embryos displayed defects in dorsal neural tube closure, as shown when Wnt signaling is blocked (Wallingford and Harland, 2002). Thus, injections of 400 to 800 pg of NFz8 mRNA per embryo were generally used in the next experiments, although doses above 1 ng were also tested. Moreover, as Wnt antagonists, NFz8 and GSK3 overexpression is expected to anteriorize the neural plate and, later, increase cement gland formation (Deardorff et al., 1998; Kiecker and Niehrs, 2001). After injecting GSK3 or NFz8 (400 to 1600 pg) in the animal hemisphere of two- or four-cell stage embryos, the cement gland was enlarged in more than 96% (n>53) of the embryos (Fig. 2A). This phenotype was used as a routine control, when sibling embryos were analyzed for neural crest formation as described below.

NFz8 or GSK3 mRNAs were injected into the animal hemisphere of two- to four-cell stage embryos, sometimes with NlacZ mRNA for lineage tracing. Animal caps were cut at
stage 8-9 and recombined with uninjected DLMZ explants. The recombinants were grown up to stage 17-18, fixed and processed for in situ hybridization with a Slug antisense probe or for RT-PCR analysis. In some explants, β-galactosidase activity was revealed before the in situ procedure. After an equivalent treatment, isolated DLMZs or animal caps did not express Slug (Fig. 2B, C). In control recombinants, the animal cap-derived tissues exhibited Slug staining, either as a strong domain of expression or as individual dispersed cells (Fig. 2D). Weaker and more internal Slug expression was detected in the DLMZ-derived tissues (Fig. 2D), consistent with Slug being expressed in the mesoderm (Mayor et al., 2000).

We first blocked signaling by putative endogenous Wnt molecules using NFz8. After injections of 800 pg of NFz8 mRNA, a similar proportion of the recombinants exhibited Slug staining, being virtually identical to controls (Fig. 2E). This observation was confirmed by RT-PCR analysis. After recombination, control explants strongly expressed Slug and other neural crest markers (n=151, on average, 8-10 recombinants were used for each lane; Fig. 2F, lane 7). Moderate to high doses of NFz8, which are fully active in the biological tests described above, did not prevent the induction of any of the neural crest markers tested (400-800 pg injections do not block response to DLMZ signals. Lanes 1 and 2, controls (see Materials and Methods); n.i., non-injected. (G) GSK3 (800 pg) or dnTCF3 (1 ng) mRNA injections do not prevent the ectoderm to form neural crest in response to the DLMZ.

(H) Xdd1 (1 ng) injections do not prevent neural crest marker induction either. (I) The AC+DLMZ recombinants elongate in the same way as controls even in presence of 400 pg of NFz8 (middle) but their elongation is abolished by injections of 1600 pg of NFz8 in the ectoderm (bottom).
We thus focused on 400-800 pg NFz8 doses (lanes 8 and 9): the decrease in Slug and Sox9 neural crest markers expression, in lanes 9, could either reflect the requirement for a Wnt signal acting directly on the ectodermal cells or a change in the DLMZ-inducing properties. To avoid Wnt-dependent changes in the signaling properties of the DLMZ, we blocked the response to the canonical and non canonical Wnt pathways intracellularly in the ectoderm, by injecting either GSK3 (300–400 pg/n=38 and 800–1000 pg/n=40), dnTCF3 (1 ng/n=10) or Xdd1 (1 ng/n=10) (Fig. 2G–H and not shown). None of these blocked the induction of neural crest markers by the DLMZ (Fig. 2G, lanes 6–8 and Fig. 2H). However, the injection of NFz8 or GSK3 did modulate the expression of other genes, such as Krox20 or Otx2, but not Pax3 (not shown). We conclude that neither canonical nor PCP Wnt-dependent pathways are required directly for the ectoderm to respond to the DLMZ neural crest-inducing activity. Blocking Wnt signaling by diffusable antagonists perturbs DLMZ development and most probably its signaling properties. However, if Wnt signaling is not perturbed in the mesoderm, the DLMZ can induce neural crest in the ectoderm, suggesting alternative or redundant pathways for neural crest induction.

FGF signaling is required for neural crest induction by the DLMZ

FGFs bind to one of four tyrosine-kinase receptors, FGFR1-FGFR4, which lead to activation of MAP kinase or phosphatidyl inositol pathways, eventually modulating target gene expression (for a review, see Galzie et al., 1997). Blocking signaling by FGFRs, in vivo or in vitro, has employed either a truncated dominant-negative form of FGFR1, XFD (Amaya et al., 1993) or a synthetic inhibitor (SU5402) that binds to the kinase domain of FGFRs (Mohammadi et al., 1997).

In the first approach, we blocked FGF signaling in the explants by growing them in presence of 50 μM SU5402. Two DLMZs were dissected out of each stage 10 embryo and used to make two recombinants, one was cultivated in the SU5402 solution, the other in the control DMSO medium. RT-PCR analysis (Fig. 3A) showed that the SU5402 treatment completely suppressed Slug induction (Fig. 3A-lane 4, n=19). However, it also prevented normal development of the paraxial mesoderm from the DLMZ as shown by the lack of muscle actin expression. Under these conditions, the lack of Slug induction could be a secondary effect caused by abnormal DLMZ development.

To avoid perturbing FGF signaling in the DLMZ part of the recombinant, we injected XFD into the embryos used for animal cap explants (500 pg). The XFD-injected caps did not express Slug (Fig. 3D, XFD-AC) or the other neural crest markers (Fig. 3B, lane 3). When they were recombined with wild-type DLMZs, in situ analysis showed that most of Slug expression was lost (Fig. 3A-lane 4, n=19). However, it also prevented normal development of the paraxial mesoderm from the DLMZ as shown by the lack of muscle actin expression. Under these conditions, the lack of Slug induction could be a secondary effect caused by abnormal DLMZ development.

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neural crest if the ectodermal part of the explant is unable to respond to FGF signals. In addition, this experiment suggests that different mechanisms control Sox9 and Zic5 induction or maintenance. Another dominant-negative FGFR construct, \(dnFGFR4a\), was tested to address potential specific roles of the distinct FGFRs. This mRNA perturbed several aspects of in vivo development (see below) but interestingly, \(dnFGFR4a\) injections resulted in normal expression of all the neural crest markers tested, both by in situ and RT-PCR analysis (Fig. 3C,D; n=36). Thus, different FGFRs are not equivalent and induction of crest by DLMZ may involve FGFR1 rather than FGFR4a activity.

**FGFs and FGFRs are expressed in the recombinants**

We analyzed the expression of FGF3, FGF4 (\(eFGF\)) and FGF8 in explants during the period of neural crest induction, i.e. stages 10.25-14, using semi-quantitative RT-PCR (Aybar and Mayor, 2002). FGF3, FGF4 and FGF8 were detected in the isolated DLMZ but not in the isolated animal caps at all stages analyzed (Fig. 4A,C). In the DLMZ, the expression of FGF genes preceded that of myotome markers such as MyoD and muscle actin, which appeared around stage 12 (Fig. 4A, lanes 5 and 6), similar to Slug in the ectoderm (Linker et al., 2000). Thus, FGF genes and FGF8 in particular are expressed in the DLMZ during gastrulation and early neurulation, and this expression is maintained without the need for external signals.

The expression of the different FGF receptors has been described in animal caps grown in isolation (Friesel and Dawid, 1991; Golub et al., 2000). Interestingly, this study showed that FGFR1 and FGFR4a genes are expressed when animal caps are dissected but their expression is maintained only in the presence of ongoing FGF signaling (Friesel and Dawid, 1991). We therefore analyzed FGFR1-FGFR4 expression in the different kinds of explants used in this study. FGFR2 expression was maintained in the isolated animal caps, consistent with previous results (Fig. 4B, lane 3) (Friesel and Brown, 1992). This expression was also present in all the recombinants containing ectoderm or DMZ (Fig. 4B, lanes 5-7). FGF3 was more specifically found in the DMZ-containing explants (Fig. 4B, lanes 6 and 7) but was expressed at much lower levels in DLMZ or AC-DLMZ recombinants (Fig. 4B, lanes 3-4). Most interestingly, FGFR1 and FGFR4a expression was hardly detected in the isolated AC or DLMZ (Fig. 4B, lanes 3 and 4) but was present if both tissues were recombined (Fig. 4B, lane 5). This suggests that when the ectoderm and the DLMZ are in contact, interactions between the two parts of the recombinant sustain FGFR1 and FGFR4a expression. To test whether this was due to an active FGF signaling in the recombinants, we cultivated them either in DMSO or in SU5402 as described above. Although this prevented Slug and muscle actin expression (Fig. 3), both FGFR1 and FGFR4a were normally expressed in these explants (Fig. 4D). We conclude that, non-FGF signals act in the AC-DLMZ recombinants to maintain FGFR expression when FGF signaling is blocked.

**FGF8 enhances neural crest formation in embryos and is sufficient to induce neural crest markers in explants**

As FGF8 has recently been shown to be involved in neurogenesis without inducing mesoderm (Hardcastle et al., 2000), we decided to focus on this member of the family and analyze its potential activity in neural crest formation. We examined FGF8 gene expression at gastrula and early neurula stages. FGF8 appears initially as a ring around the blastopore and is reinforced dorsally by stage 11-11.5, when neural crest induction is thought to begin (Fig. 5E) (Christen and Slack, 1997). FGF8 expression level is then enhanced in the dorsolateral mesoderm at stage 13 and onwards, whereas it is downregulated in the dorsal midline (Fig. 5E). FGF8 is thus a good candidate to mediate the FGF-dependent DLMZ activity on neural crest induction. To test this hypothesis in whole embryos, we analyzed Slug expression after FGF8 mRNA injections. Compared with control sibling embryos (Fig. 5A), 50 pg of FGF8 mRNA injections were followed by a strong increase in Slug expression (Fig. 5B, yellow arrows indicate the injected side). This upregulation was not correlated to an expansion of the MyoD domain (Fig. 5C,D, small red arrow). Interestingly, when the injected cells (lacZ staining) were located in the anterior part of the neural plate, this region expressed Slug, suggesting that these injections transformed the anterior neural fold into a more posterior structure (Fig. 5B, red arrow) (Christen and Slack, 1997). However, in the...
embryo, co-factors from the surrounding tissues, such as the mesoderm or the ectoderm, could also be recruited for FGF8 activity on the neural crest.

To test FGF8 activity in a more defined assay, we injected animal caps with 100 pg of FGF8 mRNA at the two- to four-cell stage into the animal pole, cut animal caps at stage 9 and grew them in isolation up to stage 17-18. This did not result in the induction of muscle actin expression, confirming that these injections do not induce paraxial mesoderm (Fig. 5D, lanes 3-4). Nonetheless, the neural crest markers Zic5 and FoxD3 were clearly induced by FGF8 injections. Slug expression was only slightly upregulated in the FGF8-injected animal caps, at much lower levels than in the recombination assay, and Sox9 was not consistently present (Fig. 5F). This first result indicates that FGF8 alone is sufficient to induce the expression of some genes characteristic of the neural crest, although not as efficiently as the DLMZ. To understand FGF8 activity better, we then increased the injected doses and saw that 500 pg of FGF8 consistently induced Sox9 expression when observed at stage 17-18 (data not shown). In addition, we found that, when the animal caps were analyzed at an earlier stage (stage 15), a very strong induction of FoxD3, Sox9 and Zic5 was obtained (Fig. 2G). However, when sibling animal caps from the same injection series were fixed a few hours later, at stage 19, the expression of all markers, except for Sox9, had vanished (Fig. 2G). This demonstrates that FGF8 is able to elicit a strong but transient induction of FoxD3, Sox9 and Zic5. This is obtained in the absence of mesoderm induction and without need for additional downregulation of BMP signaling. By contrast, we did not obtain Slug induction above the background levels shown in Fig. 2F, and thus we conclude that FGF8 does not significantly induce this gene under our experimental conditions.

In conclusion, these data suggest that FGF8 alone is sufficient to mediate both the DLMZ-specific induction of FoxD3 and the common DMZ/DLMZ induction of Zic5 and Sox9. Second, because, in vivo, FGF8 injections show a potent Slug upregulation, we conclude that this aspect of FGF8 activity requires interactions with other DLMZ-specific factors. Moreover, in the AC-DLMZ or AC-DMZ recombinants, the expression of neural crest markers is induced and maintained, indicating that other molecules must reinforce and sustain FGF8 inductive activity.

**Fig. 5.** FGF8 induces neural crest in vivo and in vitro. (A-D) In vivo injections of FGF8 mRNA in one of two-cell stage embryos, analyzed by in situ hybridization for Slug (A,B) or MyoD (C,D) at stage 18-20. (A) Control embryos. (B) FGF8 mRNA unilateral injections result in a strong overexpression of Slug on the injected side (yellow arrows) and sometimes in the contralateral side and the anterior neural fold (red arrowheads). (C) Control embryos. (D) FGF8 mRNA injections (injected side indicated by yellow arrowheads) do not expand paraxial mesoderm, they even reduce it in some embryos (embryo on the right) (red arrowhead). (E) FGF8 mRNA is expressed as a ring around the blastopore at stage 11 (top), reinforced dorsally (red arrow). Later on, FGF8 is expressed in the DLMZ and downregulated in the midline (bottom, red arrow). (F) FGF8 mRNA injections induce neural crest markers in animal caps. RT-PCR analysis shows the induction of FoxD3 and Zic5 by 100 pg of FGF8 mRNA, but not of paraxial mesoderm formation. (G) When the caps are analyzed earlier (stage 15), increased doses of FGF8 induce strongly FoxD3, Sox9 and Zic5. By stage 19, FoxD3 and Zic5 expression was not maintained.
Wnt signals with a dominant-negative form of XWnt8 resulted in reduction of both Slug and Krox20 (Fig. 6E,F), suggesting again that neural crest modifications observed previously could be interpreted in terms of general neural patterning (Villanueva et al., 2002).

To avoid the diffusible effects of NFz8, we also inhibited Wnt signaling cell-autonomously using GSK3 injected either in one half of the embryo or into the prospective neural fold at the 16-cell stage. Control injections did not alter Slug expression (Fig. 7A,D) or paraxial mesoderm formation (Fig. 7A). However, in both types of GSK3 injections, the decrease or a lack in Slug expression was correlated with altered paraxial mesoderm and neural patterning (Fig. 7B-F). Thus, in these in vivo assays, we have not been able to dissociate the effects of Wnt signaling on neural crest formation from those on neural plate and mesoderm patterning.

**Neural plate patterning and neural crest induction can be uncoupled in vivo**

To understand better how Wnt or FGF signaling might affect neural crest formation by changing early neural patterning, we analyzed NFz8 and dnFGFR4a effects on AP neural pattern. We compared the AP neural pattern at stage 11.5 to Slug expression in sibling embryos fixed around stage 18. Blocking of Wnt signaling by NFz8 mRNA injections, or of FGF signaling by XFD injections in vivo, resulted in a reduction of Slug expression together with perturbed gastrulation and neural plate formation when observed at stage 18 (Fig. 8A-C). However, by marked contrast, dnFGFR4a injections resulted in dramatic gastrulation defects without obvious downregulation of Slug expression levels (Fig. 8D). The Slug expressing domain was shifted around the blastopore in the most affected embryos, but we did not observe as strong a decrease in Slug staining as after NFz8 or XFD treatment. To test if this observation was correlated with distinct activities of these molecules on neural AP patterning, we fixed the injected embryos at stage 11.5, i.e. before neural crest induction is established, and when Slug expression is hardly detected (not shown). At this stage, neural AP pattern is already well established and the Otx2 expression domain is restricted to the anterior part of the neural plate (Fig. 8E) (Kiecker and Niehrs, 2001). This domain corresponds to the presumptive forebrain and midbrain and does not give rise to neural crest. After NFz8 dorsal injections at the two-cell
Fig. 8. Neural crest formation can be experimentally uncoupled from neural plate patterning. (A-D) Injected embryos were analyzed around stage 18 and stained for Slug expression. (A) Control embryo. (B) XFD injections result in gastrulation defects and loss of most Slug staining. (C) NFz8 injections most often produce an abnormally shaped neural plate, gastrulation defects and reduced Slug expression. (D) dnFGFR4a-injected embryos show severe gastrulation defects but still present a strong Slug staining (the right hand embryo is shown in side view). (E,F) Similar injections were analyzed at stage 11.5-12 for Otx2 expression. Otx2 labels the area anterior to the neural crest-forming regions. It is found further from the blastopore as development proceeds (blue bars measure the distance between the posterior part of the Otx2 domain and the blastopore; anterior is indicated by the red star). (E) Stage 11.5 (left) and stage 12 (right) control embryos. (F) NFz8-injected embryos show a strongly reduced posterior neural crest-forming domain. (G) dnFGFR4a injections result either in normal sized posterior domain (left) or strongly reduced ones (right). Both types of embryos will show a strong Slug expression at stage 18 (D). Red stars indicate anterior.

stage, we observed a clear anteriorization of the neural plate, assessed by the posterior border of the Otx2 domain (Fig. 8F). The area between the blastopore and the posterior limit of the Otx-positive area was reduced or absent (blue bars). This corresponds to a strong reduction of the neural crest-forming structures, the rhombencephalon and the spinal cord. Similarly, dnFGFR4a injections resulted in a moderate reduction of the posterior structures (Fig. 8G, left embryo) or their complete absence (Fig. 8G, right embryo). When similar dnFGFR4a-injected embryos fixed at stage 18, the Slug-positive domain formed around the blastopore corresponded an area where Krox20 was expressed. However, Krox20 was strongly reduced and abnormal (not shown). This result indicates that dnFGFR4a injections do perturb neural AP patterning deeply without resulting in a deficiency in Slug induction and maintenance. This implies that, in vivo, although we could not separate the roles of dnXWnt8, NFz8 or XFD injections on neural and neural crest patterning, these two phenomena can be uncoupled if we injected dnFGFR4a. We conclude that neural crest formation is not a strict consequence of proper neural plate patterning, although it is closely related to it, as signaling via FGF4a is required for neural patterning but not for neural crest formation.

DISCUSSION

In this study, we show that the paraxial mesoderm of Xenopus embryos induces neural crest by an FGF-dependent mechanism and that FGF8 is sufficient to induce neural crest markers in the naive ectoderm without requiring additional BMP antagonists. Moreover, FGF8 can cooperate with additional factors to modulate Slug expression in vivo. Wnt signaling, via the canonical or the planar cell polarity pathways, is not necessary for the response of the ectoderm to the paraxial mesoderm. In examining the relationships between neural plate patterning and neural crest formation in vivo, we show that blocking Wnt signaling affects both AP neural patterning and neural crest formation; by contrast, a truncated FGF4a acts on neural pattern but does not block neural crest induction and maintenance.

The paraxial (but not the axial) mesoderm, induces a whole range of neural crest-specific genes in the ectoderm

Elegant experiments using albino Xenopus embryos have shown that the ectoderm can form neural crest in response to DLMZ signals and that the DMZ was a less efficient Slug inducer than the DLMZ (Bonstein et al., 1998; Marchant et al., 1998). We show here that the induction of Slug by mesoderm explants is closely correlated to the presence of muscle actin in the inductive tissue, i.e. to the presence of some paraxial tissue (Fig. 1). When DMZs are cut medially, they consistently fail to induce Slug. This suggests that the quantitatively lower activity of the DMZ reported previously might reflect some variability in the width of the explants. We also show that the DLMZ is able to induce a whole range of neural crest markers: Slug, FoxD3, Sox9 and Zic5 (Fig. 1C, lane 5). By contrast, the DMZ does not induce Slug or FoxD3 but upregulates Zic5 and Sox9 expression at various levels (Fig. 1C, lane 7). The distinct inducing activities of the DLMZ and of the DMZ might be due either to a dorsal-to-lateral increasing gradient of neural crest inducing activity or to a different combination of inducing molecules produced by each kind of tissue. According to the first hypothesis, Zic5 and Sox9 genes would be upregulated by low levels of this inducer, whereas Slug and FoxD3 activation would require a higher concentration. According to the second hypothesis, the DMZ would express a molecule able to induce Zic5 and Sox9 whereas the DLMZ would express an additional signal(s) required for inducing either the complete range of neural crest markers or Slug and FoxD3 specifically. We therefore consider Slug and FoxD3 to be most characteristic of
neural crest induction because they are specifically induced by the DLMZ and because their in vivo expression pattern is mostly restricted to the neural crest (Fig. 1D).

**Neural crest induction by the paraxial mesoderm requires functional FGF-FGFR1 signaling**

Previous studies have shown that neural crest formation can be induced by a combination of BMP antagonists plus Wnt/FGF signals in *Xenopus* animal cap assays (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Moreover, the same classes of molecules regulate the expression of Slug and FoxD3. FoxD3 is induced by a combination of either chordin+bFGF or chordin+Wnt3a (Sasai et al., 2001). Similarly, Slug is upregulated in animal caps by combining chordin with either eFGF or XWnt8 (LaBonne and Bronner-Fraser, 1998). The regulation of Zic5 and Sox9 genes has not yet been studied, although these genes are required for neural crest development in vivo (Nakata et al., 2000; Spokony et al., 2002). Both Wnt and FGF signals are expressed in the paraxial mesoderm. They might play a role in mesoderm development itself, as well as mediating mesodermal signaling activities toward the ectoderm. These activities could be redundant and do not exclude the possibility that alternative mechanisms may also be active.

To analyze the mechanisms of action of the DLMZ, we blocked the response of the ectoderm to either endogenous Wnt or FGF signals, in the DLMZ-AC recombination assay (Fig. 2). Many previous studies have used secreted antagonists such as dnWnt8 to block Wnt signals in embryos or in explants: this results in downregulation of Slug in *Xenopus* (LaBonne and Bronner-Fraser, 1998) and blocks Pax3 induction by the chick paraxial mesoderm (Bang et al., 1999). After NFz8 injections in the ectoderm of the recombinants, we only saw a moderate downregulation of Slug, Sox9 and Pax3 at high doses (Fig. 2; data not shown). By blocking the intracellular downstream canonical and PCP Wnt pathways, we show that none of the four neural crest markers analyzed depend directly on Wnt signaling to be induced by the DLMZ. Therefore, the effects of diffusible antagonists observed in these recombination assays might reflect a Wnt-dependent modulation or maintenance of the paraxial mesoderm-inducing activity, or indicate that the Wnt pathway may have an overlapping activity.

By contrast, blocking FGF-FGFR1 signaling, by injecting XFD in the ectoderm, strongly reduced the induction of Slug, FoxD3 and to a lesser extent Sox9 (Fig. 3). The induction of Zic5 was unaffected by the XFD injections. The most affected genes corresponded to those specifically induced by the DLMZ but not by the DMZ. This suggests that the DLMZ-specific aspect of neural crest inducing activity requires functional FGF signaling, probably through FGFR1. FGF signaling is also required in vivo for normal neural crest formation as XFD injections strongly downregulate Slug expression (Mayor, 1997) (this work). Interestingly, we found that dnFGFR4a did not affect neural crest induction. In contrast to this observation, FGFR4a plays a prominent role in neurogenesis (Hardcastle et al., 2000). This raises the attractive possibility that different FGFRs might display different roles in neuronal versus neural crest development.

**FGF8 induces neural crest**

We show that the DLMZ expresses FGF3, FGF4 and FGF8 at gastrula and early neurula stages (Fig. 4). We further show that FGF8 can account for the neural crest induction by the DLMZ, either alone or in cooperation with other DLMZ factors (Fig. 5). First, in vivo FGF8 expression is detected at early gastrula stages as a ring around the blastopore, it is then reinforced in the DLMZ area. Second, FGF8 mRNA injections in vivo are followed by a large increase in Slug expression without expansion of the paraxial mesoderm. Finally, FGF8 injections in the animal cap assay induce the expression of neural crest markers without inducing mesoderm formation. This contrasts with previous studies using FGF4 (eFGF) or bFGF in similar assays, which showed (1) mesoderm induction, (2) absence of neural crest induction by FGF4 or bFGF alone and (3) requirement for co-expression with a BMP antagonist (LaBonne and Bronner-Fraser, 1998; Mizuseki et al., 1998). The unique properties of FGF8 on neural crest can be compared with its ability to induce neurogenesis without mesoderm induction, when it is expressed from blastula stages (Hardcastle et al., 2000). Other FGF molecules can also be direct neural inducers, but only if they are expressed after the period of competence to form mesoderm, and in tissue that has attenuated BMP signaling (Lamb and Harland, 1995). FGF8 is thus a good candidate for mediating FGF neural-specific roles during the period of early neural crest development defined by Aybar and Mayor (Aybar and Mayor, 2002).

**Is FGF8 a neural crest inducer?**

To be considered a physiologically significant activity, a neural crest inducer must satisfy the following properties. First, it should be expressed by tissue(s) with a neural crest inducing potential, in early neurula stage embryos. *FGF8*, which is expressed in the paraxial mesoderm as early as stage 10, satisfies this first condition (Fig. 5E). By contrast, Wnt1 expression is detected by stage 14, i.e. after Slug induction (Deardorff et al., 2001). Thus, Wnt1 and Wnt 3a, which act via Xfzrizzled-3 and Kermit, are more likely to play later roles in neural crest development, such as maintenance of the induction or fate choice (Dorsky et al., 1998; Basch et al., 2000; Dorsky et al., 2000; Deardorff et al., 2001; Jin et al., 2001; Tan et al., 2001). Thus, in amphibians, the activity of an ectoderm-restricted Wnt, equivalent to the chick Wnt6 gene, remains to be found (Garcia-Castro et al., 2002). The activity of β-catenin on early neural crest formation (LaBonne and Bronner-Fraser, 1998) could rather reflect a role of Wnt7b and Wnt 8, which are present in the early ectoderm/neurectoderm and paraxial mesoderm, respectively (Bang et al., 1999; Wu et al., 2003). Second, the activity of the inducer should be necessary to obtain neural crest formation, although this can be missed if redundant pathways are activated in the same assay. We show here that FGF signaling is required to mediate paraxial mesoderm induction of Slug and FoxD3 (Fig. 3). Active FGFR1 signaling is also necessary in vivo (Mayor et al., 1997). Further analysis by a selective knockdown of FGF8 will determine if FGF8 is specifically required in the DLMZ for neural crest induction or if other FGFs have overlapping activity.

In addition to these two properties, the neural crest-inducing activity could be mediated either by a single factor or a combination of molecules. Tested separately, these molecules might be able to evoke neural crest formation even if the robust induction of neural crest markers and further development of
neural crest cells might require additional inputs. In *Xenopus* animal cap assay, FGF8 induces FoxD3, Sox9 and Zic5 (but Slug is only very slightly upregulated) (Fig. 5,F,G). Moreover, the induction by FGF8 in this assay is transient, showing the requirement for other factors to maintain and complete the induction of the full range of neural crest markers. It has been shown by similar experiments that, although they do not induce neural crest markers by themselves, Wnts, eFGF and bFGF synergize with noggin or chordin to induce neural crest (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Mizuseki et al., 1998). The cooperation of FGF8 with other molecules such as BMP antagonists or Wnts in the maintenance of neural crest induction remains to be explored.

FGF8 activity must be modulated to become a potent crest-inducing activity, as FGF8 is expressed in both dorsal and dorsolateral marginal zones (Fig. 5), and these have qualitatively different neural crest-inducing activity (Fig. 1). FGF8 might account for DMZ-DLMZ common induction of Zic5 and Sox9. However, in the recombinant assay, neither XFD nor dn FGFR4a injections prevented Zic5 or Sox9 induction, supporting the idea that redundant inducing mechanisms are provided by the DLMZ (Fig. 3). In addition, Zic5 and Sox9 are not restricted to the neural crest, but also expressed in the anterior neural fold and the prospective otic placode, respectively. They are thus expected to respond to neural crest specific inducers as well as to other signals (Fig. 1). Our study also revealed distinct regulation for Slug and FoxD3. Both genes were considered specifically induced by the DLMZ (Fig. 1) and this induction requires FGF signaling (Fig. 3). However, FGF8 is sufficient to induce expression of FoxD3 but not of Slug. Cooperation of FGF8 with additional signals could account for the expansion of the Slug domain observed in the embryo (Fig. 5). Alternatively, our in vitro conditions might not induce the right relative levels of FoxD3/Sox9/Zic5: each of these factors is necessary for normal neural crest development and/or Slug expression. In particular, overexpression of FoxD3 can either increase or prevent Slug activation, suggesting that a fine balance is controlled in the embryo (Pohl and Knochel, 2001; Sasai et al., 2001). Finally, we cannot rule out the possibility that the neural crest induction we observed in the isolated ectoderm occurred secondarily to FGF8-induced neural tissue (Hardcastle et al., 2000), secondary to the formation of a border between the ectoderm and induced neural tissue. Further experiments will test if FGF8 is a direct neural crest inducer or if it switches on a developmental program eventually resulting into neural crest induction. However, by its neural crest inducing activity in the animal cap assay, FGF8 stands as an excellent candidate inducer when compared with previously proposed ones such as WNT8 or WNT7b, which do not act alone in this assay (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998).

In the chick embryo, the ectoderm can induce neural crest from early neural tissue and WNT6 signaling seems necessary and sufficient to mediate this activity (Garcia-Castro et al., 2002). In *Xenopus*, blocking Wnt signaling strongly downregulates neural crest formation in vivo, whereas in animal cap assay, Wnt signals require additional downregulation of BMPs to act on Slug induction (LaBonne and Bronner-Fraser, 1998). Combined with our data, this suggests that, in vivo, both the ectoderm and the mesoderm participate in inducing the neural crest and that they have different requirements to achieve neural crest induction. The coordinate activity of both Wnt and FGF pathways may account for the robust neural crest formation observed in normal embryos.

**Neural crest induction and neural plate posteriorization**

Neural crest induction is achieved experimentally by combining the same classes of molecules as those required for neural plate patterning: BMP antagonists, Wnts and FGFs. All three kinds of molecules have been shown to downregulate Bmp4 expression or BMP4 activity, either in *Xenopus* or in chick embryos (Lamb et al., 1993; Lamb and Harland, 1995; Baker et al., 1999; Wilson et al., 2000). In addition, FGF and Wnts also posteriorize the neural plate (Lamb and Harland, 1995; Domingos et al., 2001; Kiecker and Niehrs, 2001). We show here: (1) that Slug expression in vivo strongly correlates to proper neural and mesoderm development (Figs 6, 7), but (2) that blocking FGFR4a signaling strongly affects the AP neural pattern without preventing robust Slug expression (Fig. 8). We conclude that although the AP position of the Slug-positive domain might vary under these conditions, Slug induction can occur independently of AP neural patterning. Thus, we postulate that the loss of Slug expression observed after blocking Wnt or FGFR1 signaling (Mayor et al., 1997; LaBonne and Bronner-Fraser, 1998) (this work) reflects a role of these pathways in neural crest formation, on top of their role on neural patterning (Villanueva et al., 2002).

In conclusion, our study shows that, in the *Xenopus* embryo, (1) normal early development of the neural crest can occur in a context of abnormal AP neural patterning in vivo, (2) the paraxial mesoderm induces neural crest by an FGF-dependent pathway and (3) FGF8 is likely to mediate this activity. Our data still agree with the two-signal model of neural crest induction, and even suggest a multiple-signal model: in this model, the neural crest would arise in a location where a ‘cocktail’ of positive regulators is expressed. We propose that simultaneous moderate downregulation of BMP4 signaling, upregulation of ectodermal-derived factors (Wnt) and mesoderm-produced FGFs provides this suitable environment.

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