Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm

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Two independent signals are necessary for neural crest (NC) induction in Xenopus: a Bmp signal, which must be partially attenuated by Bmp antagonists, and a separate signal mediated by either a canonical Wnt or an Fgf. The mesoderm underlying the NC-forming region has been proposed as a source of this second signal. Wnt8 and Fgf8a are expressed in this tissue around the time of NC induction and are therefore good candidate NC inducers. Loss-of-function studies indicate that both of these ligands are necessary to specify the NC; however, it is unclear whether these signaling molecules are operating in the same or in parallel pathways to generate the NC. Here, we describe experiments addressing this outstanding question. We show that although Wnt8 expression can restore NC progenitors in Fgf8a-deficient embryos, Fgf8a is unable to rescue NC formation in Wnt8-depleted embryos. Moreover, the NC-inducing activity of Fgf8a in neuralized explants is strongly repressed by co-injection of a Wnt8 or a β-catenin morpholino, suggesting that the activity of these two signaling molecules is linked. Consistent with these observations, Fgf8a is a potent inducer of Wnt8 in both whole embryos and animal explants, and Fgf8a knockdown results in a dramatic loss of Wnt8 expression in the mesoderm. We propose that Fgf8a induces NC indirectly through the activation of Wnt8 in the paraxial mesoderm, which in turn promotes NC formation in the overlying ectoderm primed by Bmp antagonists.

KEY WORDS: Fgf8, Wnt8, Bmp, Neural crest, Induction, Xenopus

INTRODUCTION

The neural crest (NC) is a population of cells unique to the vertebrate embryo. NC progenitors originate from the neural plate border, and as the neural tube closes undergo an epithelial-to-mesenchymal transition that allows them to migrate into the periphery and to contribute to multiple lineages, including the developing heart, the peripheral nervous system and much of the craniofacial skeleton (LeDouarin et al., 2004). At the time of its induction, the NC-forming region is flanked by the neural plate on one side and the non-neural ectoderm on the other, and sits on top of the underlying paraxial mesoderm. Because of their position relative to the NC, each one of these tissues has been proposed as a source of NC inducer(s). The relative contribution of these tissues to NC induction appears to vary greatly from one species to another (reviewed by Knecht and Bronner-Fraser, 2002; Huang and Saint-Jeannet, 2004).

At least three major signaling pathways have been implicated in NC induction (reviewed by Jones and Trainor, 2005). Studies in frog and fish have shown that NC forms in regions of the ectoderm where Bone Morphogenetic Protein (Bmp) signaling is partially attenuated by Bmp antagonists, such as Chordin, Noggin and Follistatin, which are derived from the axial mesoderm (Marchant et al., 1998; Nguyen et al., 1998; Tribulo et al., 2003). However, it is also true that changes in Bmp signaling levels in the ectoderm are not sufficient for NC induction and that other signaling pathways are involved (LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002). A large body of work indicates that signaling through the canonical Wnt pathway is crucial to specify the NC in fish, frog and chick (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Bang et al., 1999; Deardorff et al., 2001; Garcia-Castro et al., 2002; Lewis et al., 2004) (reviewed by Wu et al., 2003; Heeg-Truedsell and LaBonne, 2007). The source of this Wnt signal has been proposed to reside in the paraxial mesoderm of frog and fish (Bang et al., 1999; Lewis et al., 2004), and in the ectoderm of birds (Garcia-Castro et al., 2002). In the mouse, the situation is not as clearly defined. Genetic analyses suggest that Wnt signaling may have a role in NC lineage specification, rather than in induction (Ikeya et al., 1997; Hari et al., 2002). However, because of functional redundancy, an earlier role of Wnt in NC formation cannot be completely excluded.

Studies in Xenopus have shown that members of the Fibroblast Growth Factor (Fgf) family are also involved in NC induction (Kengaku and Okamoto, 1993; Mayor et al., 1995; Mayor et al., 1997; Villanueva et al., 2002; Monsoro-Burq et al., 2003). Expression of a dominant-negative Fgf receptor blocks NC formation in the whole embryo (Mayor et al., 1997) and in animal explants recombined with paraxial mesoderm (Monsoro-Burq et al., 2003). Fgf8 is expressed in the paraxial mesoderm and is a likely candidate to mediate this activity (Monsoro-Burq et al., 2003). So far, Xenopus is the only model organism in which Fgf signaling has been implicated in NC induction. Therefore, in Xenopus, NC induction depends on a Bmp signal, which must be partially attenuated by Bmp antagonists, and on a separate signal mediated by either a canonical Wnt or an Fgf. However, it is unclear how Wnt and Fgf interact at the neural plate border to generate the NC. While there are suggestions that these pathways might be linked (LaBonne and Bronner-Fraser, 1998), there is also evidence that they may act independently (Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005). In this study, we present a comparative analysis of the NC-inducing activity of Wnt8 and Fgf8a, two candidate NC inducers in Xenopus. Loss- and gain-of-function studies indicate that these ligands share very similar properties. Individually, Fgf8a and Wnt8 are both necessary to specify the NC. By using a number of assays in the whole embryo...
and in animal explants, we also show that Fgf8a requires active canonical Wnt signaling to mediate its activity. Moreover, Fgf8a is a potent inducer of Wnt8 and is required for Wnt8 expression in the paraxial mesoderm. These results indicate that Fgf8a induces NC indirectly through Wnt8 activation, which suggests that these factors function in the same pathway to specify the NC.

**MATERIALS AND METHODS**

**Xenopus embryo injections, morpholinos and explants culture**

Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Wnt8 (25 pg) (Wolda et al., 1993), Fgf8a (5 pg) (Christen and Slack, 1997), and XFD (2 ng) (Amaya et al., 1991) mRNA was synthesized in vitro using the Message Machine Kit (Ambion). Wnt8 (W8MO; AAAGTGGTGTTTGTGATGGAAG; 25-50 ng) (Park and Saint-Jeannet, 2008), β-catenin (BCatMO; 25-50 ng) (Heasman et al., 2000) and Fgf8a (F8MO; 50 ng) (Fletcher et al., 2006) morpholino antisense oligonucleotides were purchased from Gene-Tools LLC (Philomath, OR). In whole embryo experiments, synthetic mRNAs and antisense oligonucleotides were injected unilaterally into two-cell-stage embryos. For Wnt8 and β-catenin, plasmid DNA was injected to avoid axis duplication (100 pg and 200 pg, respectively). Injected embryos were cultured in 0.1 mM EDTA, to eliminate possible contamination by genomic DNA. Immunolabeling experiments were treated with DNase I, to eliminate possible contamination by genomic DNA. The amount of RNA was quantified by measuring the optical density using a spectrophotometer (Beckman). Real-time RT-PCR was performed as previously described, using specific primer sets (Hong and Saint-Jeannet, 2007). In each case, EF1α expression. The histograms in each figure are presented as mean±s.e.m. of three independent experiments. A Student’s t-test was used to define statistically significant values in each group.

**RESULTS**

**Fgf8a and Wnt8 are both required for NC induction**

Fgf and Wnt signaling have been both implicated in NC induction in Xenopus. To better understand their relative contribution to this inductive process, we compared the activity of Fgf8a and Wnt8, two ligands expressed in the paraxial mesoderm around the time of NC induction (Christen and Slack, 1997; Monsoro-Burq et al., 2003; Smith and Harland, 1991; Bang et al., 1999). Morpholino-mediated knockdown of Fgf8a or Wnt8 resulted in a similar loss of NC progenitors at the neurula stage, as determined by the expression of four NC-specific genes: Pax3, Snail2, Sox8 and Sox10 (Fig. 1A). Often this loss of the NC tissue was associated with an expansion of the neural plate (Sox2) on the injected side (Fig. 1A). In these embryos lacking Fgf8a or Wnt8 function, mesoderm appeared to form normally, as determined by the expression at the gastrula stage of the general mesoderm marker Xbra (Fig. 1B). In both knockdowns, the loss of early NC progenitors resulted into a severe reduction of migrating NC cells in the branchial arches at the tailbud stage (Fig. 1C), due to increased cell death (Fig. 1D). These results suggest that Fgf8a and Wnt8 are both required for NC formation.

We also compared the ability of Wnt8 and Fgf8a to induce NC markers in blastula-stage animal pole explants neutralized by Bmp attenuation (Chordin injection). The neutralization of these explants was assessed by the expression of the pan-neural gene Sox2. In this assay, Fgf8a had the ability to enhance the neuralization mediated by Chordin (Fig. 1E), as had been previously reported (Lamb and Harland, 1995). We observed that Fgf8a and Wnt8 were very similar in their ability to activate NC markers (Pax3, Snail2 and Sox8) in these explants (Fig. 1E). Importantly, the induction of these NC-specific genes occurred independently of mesoderm formation. Marker genes for skeletal muscle (m-Actin) and notochord (Col2a1) were not significantly increased in these explants, suggesting that Wnt8 and Fgf8a directly convert these cells from a neural (Sox2) to an NC (Pax3, Snail2 and Sox8) fate. Taken together, these results indicate that, individually, Fgf8a and Wnt8 are both necessary to generate NC progenitors in Xenopus. However, it is unclear whether this dual requirement reflects the fact that these two signaling molecules operate in the same or in parallel pathways (Fig. 1F).

**NC induction by Fgf8a requires active canonical Wnt signaling**

To determine whether Fgf8a and Wnt8 are functioning independently, we first compared the ability of Fgf8a and Wnt8 to restore NC progenitors in Wnt8- or Fgf8a-depleted embryos, respectively. Although injection of Fgf8a mRNA expands Snail2 and Sox8 expression domains (Fig. 2A), as previously reported (Monsoro-Burq et al., 2003; Hong and Saint-Jeannet, 2007), Fgf8a expression was unable to restore the expression of these NC markers in embryos injected with Wnt8 or β-catenin morpholino (Fig. 2A). Conversely, injection of Wnt8 or β-catenin plasmid DNA was very efficient at restoring NC progenitors in Fgf8a-depleted embryos (Fig. 2B). These results indicate that NC induction by Fgf8a requires active Wnt signaling in the embryo, whereas Wnt8 NC-inducing activity can occur independently of Fgf8a function.

We also evaluated the relationship between Fgf8a and Wnt8 in animal explants. We found that the NC-inducing activity of Wnt8 and Fgf8a in neutralized explants was dramatically inhibited by co-
injection of a Wnt8 or a β-catenin morpholino, as visualized by real-time RT-PCR (Fig. 3A). The loss of Snail2 expression in these explants co-injected with Wnt8 morpholino was also evaluated by whole-mount in situ hybridization (Fig. 3B). Manipulating Wnt signaling in Fgf8a-injected explants did not significantly change the levels of expression of the neural plate marker Sox2 (Fig. 3A). Whereas in Wnt8-injected explants, the inhibition of Wnt signaling restored Sox2 expression to levels similar to those observed in neuralized explants (Chordin injected; not shown). These results support the view that Fgf8a requires a functional canonical Wnt pathway to mediate its NC-inducing activity, suggesting that Fgf8a may act upstream of Wnt8 during NC induction.

**Developmental expression of Fgf8 and Wnt8**

Although Wnt8 and Fgf8a are good candidate NC inducers, a detailed analysis of their expression pattern as it relates to NC induction has not been reported. At the mid-gastrula stage (stage 11.5), Fgf8 and Wnt8 are expressed around the blastopore in a complementary pattern in the dorsolateral and ventrolateral mesoderm, respectively (Fig. 4A). Their expression overlaps in the lateral region of the mesoderm (Fig. 4A). At stage 12, while Fgf8 remains confined to the posterior mesoderm, the Wnt8 expression domain extends anteriorly as the mesoderm involutes (Fig. 4A,B). It is around stage 12 that early NC markers, such as Sox8 (O’Donnell et al., 2006), are first activated in the prospective NC tissue (Fig. 4B).

Adjacent transverse sections of stage 12 and stage 12.5 embryos were hybridized with Sox8 or Wnt8 probes to further evaluate their spatial relationship (Fig. 4C). At stage 12, Wnt8 is detected in the mesoderm immediately contiguous to the NC-forming region where the first Sox8-positive cells are detected (Fig. 4C). At stage 12.5, Sox8 is greatly increased in the ectoderm adjacent to Wnt8 expression in the mesoderm. At this stage, Wnt8 is no longer confined to the mesoderm and is also detected in the ectoderm layer, as previously reported (Bang et al., 1999). The hybridization of adjacent serial sections with Sox8, Wnt8 and Fgf8 probes confirms that Fgf8 is never co-expressed with Wnt8 in the mesoderm underlying the NC-forming region (Fig. 4D). Fgf8 expression is
restricted to the posterior mesoderm at this stage (Fig. 4E). With the understanding that we are looking at the mRNA expression of two secreted factors, and in the absence of appropriate antibodies to further evaluate the localization of the corresponding proteins, these data suggest that compared with Fgf8a the spatiotemporal expression of Wnt8 is more consistent with a role in NC induction.

**Fgf8a is a potent inducer of Wnt8 and is required for Wnt8 expression in the paraxial mesoderm**

Our results so far indicate that Fgf8a requires an intact canonical Wnt pathway to activate NC-specific genes in whole embryos and in animal explants, suggesting that Fgf8a may act upstream of Wnt8 during NC induction. Moreover, the expression pattern of these two factors is consistent with this view. These observations directly imply that Fgf8a must have the ability to activate Wnt8 expression. We tested this possibility in the context of animal explants, and found that expression of Fgf8a alone or in combination with Chordin was a very potent inducer of Wnt8 (Fig. 5A), whereas Wnt8 expression had virtually no effect on Fgf8a expression levels, supporting the idea of a unidirectional relationship between these two ligands. Furthermore, we showed that the induction of Wnt8 by Fgf8a in neuralized explants was mediated through the MAPK pathway, as Wnt8 expression is severely reduced in the presence of the MAPK inhibitor U0126 (see Fig. S1A in the supplementary material).

In the whole embryo, targeted injection of an Fgf8a morpholino or expression of a dominant-negative Fgf receptor (XFD) (Amaya et al., 1991) resulted in a reduction of Wnt8 expression in the paraxial mesoderm of late-gastrula-stage embryos (76%, n=73; and 91%, n=40; respectively; Fig. 5B). Conversely, overexpression of Fgf8a dramatically expanded the Wnt8 expression domain in most injected embryos (98%; n=83) (Fig. 5B). These results indicate that Fgf8a is required for Wnt8 expression in the paraxial mesoderm, which is consistent with the proposal that Fgf8a functions upstream of Wnt8 during NC induction.

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**Fig. 2. Fgf8a and Wnt8 differ in their ability to restore NC progenitors in Wnt8- and Fgf8a-deficient embryos.** (A) Fgf8a mRNA injection fails to rescue Snail2 and Sox8 expression at the neural plate border of embryos injected with Wnt8MO (25 ng) or β-CatMO (25 ng). A single injection of Fgf8a mRNA (2.5 pg) expands Snail2 and Sox8 expression domains. (B) Conversely, Wnt8 (100 pg) or β-catenin (200 pg) plasmid DNA injection restores Snail2 and Sox8 expression in embryos injected with Fgf8aMO (50 ng). Injection of Wnt8 or β-catenin in sibling embryos expanded Snail2 and Sox8 expression domains. In all panels, embryos are viewed from the dorsal side with anterior to the top. The injected side is to the right.

**Fig. 3. NC induction by Fgf8a requires active canonical Wnt signaling in animal explants.** (A) In animal explants the induction of NC markers (Snail2 and Sox8) by the co-expression of Chordin (10 pg) and Wnt8 (25 pg; C+W), or Chordin (10 pg) and Fgf8a (5 pg; C+F), is dramatically reduced in the context of embryos injected with Wnt8MO (W8MO, 50 ng) or β-CatMO (βCMO, 50 ng). Interference with the Wnt signaling pathway did not affect (C+F) or restore (C+W) the neuralization of these explants (Sox2). Values (n=3) are presented as mean±s.e.m.; *P<0.05, versus C+W (upper graphs) or C+F (lower graphs) samples. U, uninjected animal explant. (B) The expression of Snail2 detected by whole-mount in situ hybridization in Chordin and Wnt8 (C+W8), or Chordin and Fgf8a (C+F8a), treated animal explants is abolished by the co-injection of Wnt8MO (W8MO; 50 ng).
Fgf8a promotes NC fate at the anterior neural fold by up-regulating Wnt8

The absence of NC tissue at the anterior edge of the neural plate (Fig. 6A) is believed to depend on the activity of an endogenous Wnt inhibitor, Dkk1, whose function is to prevent Wnt-mediated expansion of the NC tissue in this region of the ectoderm (Carmona-Fontaine et al., 2007). Consistent with this view, inhibition of Dkk1 function expands the NC domain anteriorly (Carmona-Fontaine et al., 2007), and excess Wnt signaling in this region of the embryo results in ectopic NC formation at the anterior neural fold (Wu et al., 2004; Voigt and Papalopulu, 2006; Carmona-Fontaine et al., 2007) (Fig. 6B). Surprisingly, several laboratories have also reported that Fgf misexpression can also induce the expression of NC markers in this NC-free domain (Villanueva et al., 2002; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005) (Fig. 6C), suggesting that a mechanism independent of Dkk1 may preclude NC formation in this region. Our findings placing Fgf8a upstream of Wnt8 may help to resolve this apparent discrepancy. We observed that Fgf8a-mediated induction of Snail2 and Sox8 at the anterior neural fold was associated with a dramatic upregulation of Wnt8 anteriorly (Fig. 6C),
when compared with control embryos (Fig. 6D), suggesting that the activity of the Wnt inhibitor Dkk1 can fully account for the exclusion of the NC from the anterior neural fold.

**DISCUSSION**

In this study we have addressed the outstanding question of the relative contribution of Fgf and Wnt signaling pathways to the induction of the NC in *Xenopus* by comparing the activity of Wnt8 and Fgf8a, two putative NC inducers expressed in the paraxial mesoderm. By using a number of assays in the whole embryo and animal explants, we demonstrate that Fgf8a induces the NC indirectly through the activation of Wnt8 in the paraxial mesoderm, suggesting that signaling through Wnt8 can fully account for the NC-inducing activity of the paraxial mesoderm in *Xenopus*. How can these observations be reconciled with other studies that have implicated an Fgf, rather than a Wnt signal, as the paraxial mesoderm-derived signal required for NC induction (Mayor et al., 1997; Monsoro-Burq et al., 2003)?

The existence of a paraxial mesoderm-derived Wnt signal in NC induction, which was first proposed almost 10 years ago (Bang et al., 1999), has been recently challenged (Monsoro-Burq et al., 2003). In this study, the authors proposed that by interfering with Wnt signaling extracellularly, by using Wnt antagonists, such as dominant-negative Wnt8 (LaBonne and Bronner-Fraser, 1998; Bang et al., 1999) or Nfz8, a truncated and diffusible form of the Wnt receptor Frizzled 8 (Monsoro-Burq et al., 2003), NC formation was impaired not by blocking the activity of a Wnt signal derived from the paraxial mesoderm but rather, indirectly, by altering the character of the mesoderm and therefore changing its signaling properties. In support of this view, these authors reported that interfering with the response of the ectoderm to Wnt signaling by means of intracellular Wnt antagonists, such as Gsk3 and dominant-negative Tcf3, did not prevent the induction of NC markers by the paraxial mesoderm (Monsoro-Burq et al., 2003). However, in these studies we cannot exclude the possibility that these intracellular inhibitors were not fully active at blocking Wnt signaling (Huang and Saint-Jeannet, 2004). Moreover, these findings conflict with other studies that have clearly demonstrated that interfering with the reception of Wnt signaling in the ectoderm, by using dominant-negative forms of Frizzled 3 (Fz3), Frizzled 7 (Fz7) and their co-receptor Lrp6, or by morpholino-mediated knockdown of Fz3, Fz7, Lrp6, Kremen and β-catenin, was sufficient to block NC formation in the whole embryo (Tamai et al., 2000; Deardorff et al., 2001; Wu et al., 2004; Abu-Elmagd et al., 2006; Hassler et al., 2007).

The same study proposed that an Fgf rather than a Wnt signal was in fact responsible for the NC-inducing activity of the paraxial mesoderm (Monsoro-Burq et al., 2003). This finding was based on the observation that a piece of dorsolateral marginal zone (DLMZ), which normally induces NC markers in the ectoderm (Bonstein et al., 1998), was unable to induce NC when recombined with animal explants made refractory to Fgf signaling by expression with a dominant-negative Fgf receptor (XFD). However, these experiments do not take into account the fact that intact Fgf signaling is required for neuralization of the ectoderm by Bmp antagonists (Launay et al., 1996; Delaune et al., 2004; Kuroda et al., 2005). Therefore, and because neural and NC induction are tightly linked, an alternative interpretation would be that NC induction was blocked not as a result of the inability of a DLMZ-derived Fgf ligand to signal in the ectoderm, but rather, indirectly, because the neuralization of these explants was impaired by the expression of XFD (Launay et al., 1996). Consistent with this possibility, and as previously described (Kuroda et al., 2005), we observed that the MAPK inhibitor U0126 blocks neuralization by Chordin (see Fig. S1B,C in the supplementary material). Moreover, animal explants co-injected with Chordin and Fgf8a, or Chordin and Wnt8, and cultured in the presence of U0126, showed reduced expression of the NC marker Snail2 (see Fig. S1B,C in the supplementary material). These results confirm previous observations on the active role played by Fgf/MAPK signaling in neuralization of the ectoderm by Bmp antagonists (Launay et al., 1996; Delaune et al., 2004; Kuroda et al., 2005). Furthermore, these observations suggest that the loss of NC in Fgf8a- and Wnt8-injected explants treated with the MAPK inhibitor (see Fig. S1B,C in the supplementary material), or in explants injected with XFD and recombined with DLMZ (Monsoro-Burq et al., 2003), is likely to be secondary to the inability of Bmp antagonists to neuralize the ectoderm in the absence of an active MAPK pathway.

Other evidence suggesting that Wnt and Fgf signaling may function independently during NC induction came from the observation that these factors differ in their ability to regulate the expression of two neural plate border-specifier genes, Pax3 and Msx1 (Monsoro-Burq et al., 2005). However, other studies have shown that Pax3 expression at the neural plate border is not only dependent on a Wnt signal (Monsoro-Burq et al., 2005), but is also tightly regulated by Fgf8a signaling (Sato et al., 2005; Hong and Saint-Jeannet, 2007). Similarly, Msx1 expression in the ectoderm is controlled by either Fgf8 (Monsoro-Burq et al., 2005) or Wnt8 signaling (Bang et al., 1999; Tribulo et al., 2003; Hong and Saint-Jeannet, 2007). The differences in the activity of Wnt8 and Fgf8a reported by different laboratories could be explained by subtle differences in the types of reagent or assay used to evaluate the expression of these genes.

It has been previously shown that the co-expression of Chordin and eFgf induces Snail2 in animal explants, and that this activity is inhibited by the expression of a dominant-negative Wnt8, raising the possibility that the induction of Snail2 by Fgf signaling might be indirect (LaBonne and Bronner-Fraser, 1998). However, because eFgf is also a potent mesoderm inducer (Isaacs et al., 1992), in these
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