Developmental Biology 365 (2012) 196–207

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Early neural crest induction requires an initial inhibition of Wnt signals

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ARTICLE INFO

Article history:
Received for publication 23 August 2011
Revised 31 January 2012
Accepted 20 February 2012
Available online 25 February 2012

Keywords:
Neural crest
Neural plate
Neural plate border
Slug
Snail
hairy2a
dlx5
Zic3
Msx1
Wnt
BMP

ABSTRACT

Neural crest (NC) induction is a long process that continues through gastrula and neurula stages. In order to reveal additional stages of NC induction we performed a series of explants where different known inducing tissues were taken along with the prospective NC. Interestingly the dorso-lateral marginal zone (DLMZ) is only able to promote the expression of a subset of neural plate border (NPB) makers without the presence of specific NC markers. We then analysed the temporal requirement for BMP and Wnt signals for the NPB genes *Hairy2a* and *Dlx5*, compared to the expression of neural plate (NP) and NC genes. Although the NP is sensitive to BMP levels at early gastrula stages, *Hairy2a*/*Dlx5* expression is unaffected. Later, the NP becomes insensitive to BMP levels at late gastrulation when NC markers require an inhibition. The NP requires an inhibition of Wnt signals prior to gastrulation, but becomes insensitive during early gastrula stages when *Hairy2a*/*Dlx5* requires an inhibition of Wnt signalling. An increase in Wnt signalling is then important for the switch from NPB to NC at late gastrula stages. In addition to revealing an additional distinct signalling event in NC induction, this work emphasizes the importance of integrating both timing and levels of signalling activity during the patterning of complex tissues such as the vertebrate ectoderm.

Introduction

Induction is a process by which an inducing tissue releases a signal that results in a change in the direction of differentiation of the responding tissue (Gurdon, 1987). Recent molecular explanations for many inductive interactions have revealed increasing complexity, with responding tissues receiving multiple signals from a variety of tissues. One example of this is the induction of the neural crest (NC), an embryonic cell population that arises at the neural plate border that later migrates to numerous sites in the embryo. In *Xenopus* embryos, two separate tissue interactions are thought to induce NC cells. The first involves signals from the dorso-lateral marginal zone (DLMZ) (Bonstein et al., 1998; Marchant et al., 1998; Raven and Kloos, 1945). The second involves an interaction between the neural plate (NP) and epidermis (EP) (Mancilla and Mayor, 1996; Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995). Several signalling cascades are required for NC induction, including BMP (Glavic et al., 2004b; Marchant et al., 1998; Mayor et al., 1995; Neave et al., 1997; Nguyen et al., 1998; Wilson et al., 1997), Wnt (Bastidas et al., 2004; Deardorff et al., 2001; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Lewis et al., 2004; Saint-Jeannet et al., 1997; Tribulo et al., 2003), FGF (LaBonne and Bronner-Fraser, 1998; Mayor et al., 1995; Mayor et al., 1997; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Stuhlmiller and García-Castro, 2012), retinoic acid (Begemann et al., 2001; Villanueva et al., 2002) and Notch (Cornell and Eisen, 2000; 2002; 2005; Endo et al., 2002; 2003; Glavic et al., 2004a).

BMP, Wnt, FGF, RA and Notch signals feed into a complex transcriptional network that include neural plate border (NPB) specifiers such as *Hairy2*, *Msx*, *Ap2*, *Dlx*, *Pax*, *Zic* and *c-Myc* gene families (Bang et al., 1997; 1999; Holzschuh et al., 2003; Knight et al., 2003; Luo et al., 2001; Luo et al., 2003; Meulemans and Bronner-Fraser, 2002; Nakata et al., 1997; 1998; 2000; Papalopulu and Kintner, 1993; Sato et al., 2005; Suzuki et al., 1997; Wettstein et al., 1997; Woda et al., 2003). This is followed by a second group termed NC specifiers that include *Snail2*, *Snail*, *Sox9*, *Sox10*, *FoxD3*, *Twist* and *Id3* (Aybar et al., 2003; Honoré et al., 2003; Kee and Bronner-Fraser, 2005; Lee et al., 2004; Light et al., 2005; Linker et al., 2000; Pohl and Knochel, 2001; Sasai et al., 2001).

It remains an open question as to how prospective NC cells interpret multiple extracellular signals such as BMP and Wnt. As a starting point, it is important to ask when each of these pathways is required during NC induction. Interestingly, NC cells change in their requirement for BMP signals (Patthey et al., 2008; Steventon et al., 2009). In *Xenopus* embryos, NC cells require Wnt signals together with intermediate levels of BMP at late gastrula stages for the onset of *Snail2* expression. However, the same cells then require high levels of BMP and

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doi:10.1016/j.ydbio.2012.02.029

Wnt signals during neurulation for maintenance of cell fate (Steventon et al., 2009).

To discover additional steps in the NC induction process, we started by isolating the distinct tissue interactions that are known to be required for NC induction. Based on our previous stage 10 fate map for the neural crest (Steventon et al., 2009) we dissected the prospective NC with different known inducing tissues. We find that in the absence of EP, DLMZ and prospective NC conjugates express the NPB markers *Hairy2a*, *Dlx5*, *Msx1 and Zic3* but not *Pax3* or the NC marker *Snail2*. We next developed a stage 11.5 fate map for the NC from which prospective NC could be taken and cultured in vitro. Interestingly, we find that the NPB markers *Hairy2a* and *Dlx5* are specified at this stage. With these assays we were then able to compare the response of *Hairy2a* and *Dlx5* to NP and NC after modulation of BMP and Wnt signals in distinct time windows. Finally all our in vitro conclusions were confirmed in vivo. Together we present a dynamical model of NC induction, wherein the levels of both BMP and Wnt signalling pathways need to be modulated in three successive steps.

Materials and methods

Xenopus embryos, micromanipulation and whole-mount in situ hybridization

Xenopus embryos were obtained as described previously (Gómez-Skarmeta et al., 1998) and staged according to Nieuwkoop and Faber (1967). Dissections and grafts were performed as described by Mancilla and Mayor (1996). For injection and lineage tracing, β-catenin-GR (Domingos et al., 2001) mRNA was co-injected with FLDx (Molecular Probes) using 8–12 nl needles as described in Aybar et al. (2003). Treatment with dexamethasone was performed as described previously (Tribulo et al., 2003). All plasmids were linearised and RNA transcribed as described by Harland and Weintraub (1985), using SP6 or T7 RNA polymerases, and the GTP cap analogue (New England Biolabs). After DNAse treatment, RNA was purified (BD Biosciences) and resuspended in DEPC-water. For in situ hybridisation, antisense digoxigenin or fluorescein labelled RNA probes were used. Specimens were prepared, hybridized and stained using the method of Harland (1991), and NBT/BCIP or BCIP alone was used as substrates for the alkaline phosphatase. The genes analysed were *Snail2* (formerly *Slug*; Mayor et al., 1995); *Hairy2a* (Wettstein et al., 1997); *Sox2* (Kishi et al., 2000); *Dlx5* (Papalopulu and Kintner, 1993); *Dkk1* (Glinka, et al., 1998); *Zic3* (Nakata et al., 1997); *Msx1* (Maeda et al., 1997) and *Keratin* (Jonas et al., 1989).

DiI injections and construction of fate map

Injections of DiI (Molecular Probes) were performed at stage 10 as described in Linker et al. (2000). Photos were taken immediately and at stages 11.5 and stage 28. Embryos were sectioned at stage 28 and their fate was determined as previously described (Steventon et al., 2009). Each label was then mapped onto a representative stage 11.5 embryo by counting of superficial cell diameters from the blastopore lip.

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 and D systems) or 20 μg/ml BMP4 (R and D systems) all suspended in 0.1% BSA. Beads were grafted into explants/whole-embryos for entire culture period prior to fixation.

Luciferase assay

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 manufacturer instructions on a single-tube luminometer (Turner BioSystems). Each reading was standardised by protein concentration as determined by absorbance at 280 nm. This was important to control for differences in tissues sizes of each explant type.

Results

The DLMZ is able to promote a sub-set of NPB markers from which the epidermis promotes NC

We aim to discover novel steps in the NC induction process. By isolating specific inducing tissues with prospective NC it is possible to ask whether distinct sub-sets of Neural Plate Border (NPB) markers are expressed prior to the induction of the full complement of NC genes. Although the DLMZ is sufficient to induce NC markers in animal caps (Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003; Steventon et al., 2009), it is not known whether this tissue able to promote NC in the absence of prospective epidermis. To test this we explanted the prospective NC with either the DLMZ alone or together with EP. The fate of cells in these regions was known based on our previous stage 10 fate map (Steventon et al., 2009). When explants of the prospective neural crest and neural plate tissue (1A; NC/NP) were cultured in isolation for 30 h, they do not express *Snail2*, *Pax3* (Fig. 1Ai,ii; Table 1), *Sox2* (Fig. 1Aiii; Table 1), *Hairy2a*, *Dlx5* or *Zic3* (Fig. 1Aiv,v,vii; Table 1) markers and are instead reverted to epidermal fate (Fig. 1Aviii, Table 1). A diffuse staining of *Msx1* is observed, which is likely to represent its low level expression in the epidermis (Fig. 1vi; Maeda et al., 1997). When explants of both the prospective NC/NP tissue and DLMZ were cultured for 30 h, specific expression of *Sox2*, *Hairy2a*, *Dlx5*, *Msx1* and *Zic3* markers can be seen (Fig. 1Biii–vii; Table 1) with a reduction in *keratin* expression (Fig. 1Bviii; Table 1). However, these explants still fail to express *Pax3* and *Snail2* (Fig. 1Bi–ii, Table 1) suggesting that further signals might be required for the expression of neural crest markers.

Does the inclusion of EP allow for *Snail2* and *Pax3* expression? Explants of NC/NP together with EP and DLMZ do result in *Snail2* and *Pax3* expression (Fig. 1Ci–ii; Table 1) alongside *Sox2*, *Hairy2a*, *Dlx5*, *Msx1* and *Zic3* (Fig. 1Ciii–vii; Table 1). *Keratin* is also expressed strongly in these explants, confirming the presence of epidermis (Fig. 1Cviii; Table 1). Explants of the DLMZ alone fail to express any of the ectodermal markers examined (Fig. 1D; Table 1). Next we addressed whether the epidermis is releasing signals to induce NC within the NC/NP/DLMZ explants, or whether it is required as additional responding tissue. As expected, neither the NC/NP/DLMZ explants alone (Fig. 1Ei), nor FDX injected-epidermis (Fig. 1Eii) express *Snail2*. When these two tissues are conjugated, a strong expression of *Snail2* in the non-FDX territory is observed, with little or no expression in the epidermis (FDX positive tissue; Fig. 1Eiii). Together, these results suggest that the DLMZ is able to promote the expression of the pan-NPB markers *Hairy2a*, *Dlx5*, *Zic3* and *Msx1* and the NP marker *Sox2*. However, additional signals from the EP are required to induce the posteriorly restricted NPB marker Pax3 and the NC specifier *Snail2*.

Prospective NC cells are specified to express Hairy2a/Dlx5 at mid-gastrula stages

What is the specification state of the prospective NC at mid-gastrula stages? To first determine the position of the NC at