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Neural induction in *Xenopus* requires inhibition of Wnt-β-catenin signaling

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Abstract

Canonical Wnt signals have been implicated in multiple events during early embryogenesis, including primary axis formation, neural crest induction, and A–P patterning of the neural plate. The mechanisms by which Wnt signals can direct distinct fates in cell types that are closely linked both temporally and spatially remains poorly understood. However, recent work has suggested that the downstream transcriptional mediators of this pathway, Lef/Tcf family DNA binding proteins, may confer distinct outcomes on these signals in some cellular contexts. In this study, we first examined whether inhibitory mutants of XTcf3 and XLef1 might block distinct Wnt-dependent signaling events during the diversification of cell fates in the early embryonic ectoderm. We found that a Wnt-unresponsive mutant of XTcf3 potently blocks neural crest formation, whereas an analogous mutant of XLef1 does not, and that the difference in activity mapped to the C-terminus of the proteins. Significantly, the inhibitory XTcf3 mutant also blocked expression of markers of anterior-most cell types, including cement gland and sensory placodes, indicating that Wnt signals are required for rostral as well as caudal ectodermal fates. Unexpectedly, we also found that blocking canonical Wnt signals in the ectoderm, using the inhibitory XTcf3 mutant or by other means, dramatically expanded the size of the neural plate, as evidenced by the increased expression of early pan-neural markers such as *Sox3* and *Nrp1*. Conversely, we find that upregulation of canonical Wnt signals interferes with the induction of the neural plate, and this activity can be separated experimentally from Wnt-mediated neural crest induction. Together these findings provide important and novel insights into the role of canonical Wnt signals during the patterning of vertebrate ectoderm and indicate that Wnt inhibition plays a central role in the process of neural induction.

Keywords: Neural induction; Wnt; BMP; XTcf3; XLef1; Neural crest; Placode

Introduction

During vertebrate embryogenesis, distinct regions of the ectoderm become specified to form epidermis, CNS progenitors, neural crest, and placodes. During this process information with respect to position along the anterior–posterior (A–P) and dorso-ventral (D–V) axes is provided to each of these cell types (De Robertis and Kuroda, 2004; Gamse and Sive, 2000; Sasai and De Robertis, 1997; Wilson and Maden, 2005). In a model first proposed by Nieuwkoop (1952), an "activator" molecule was thought to impart anterior neural character on cells that would otherwise form epidermis, while "transformer" signals subsequently generated more posterior neural fates (Nieuwkoop, 1999). Later, the model of neural induction, referred to as the 'default model' proposed that BMP antagonists secreted from the organizer function as Nieuwkoop's "activator" signals and induce anterior neural fates, whereas the high level BMP signaling characteristic of ectoderm further from the organizer instructs these cells to form epidermis (Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997; Wilson et al., 1997). While there is significant experimental evidence for the default model in *Xenopus*, recent work in a number of model organisms indicates that other signals in addition to BMP inhibition play important roles in neural induction (Stern, 2005).

Based on the observation that they can confer more caudal identity to neural tissue induced by BMP antagonists such as chordin or noggin, it has been suggested that canonical Wnt signals may act as "transforming" signals during CNS patterning (Erter et al., 2001; Fekany-Lee et al., 2000; Kazanskaya

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et al., 2000; Kelly and Melton, 1995; Kiecker and Niehrs, 2001; Kudoh et al., 2002; McGrew et al., 1995). Wnt signaling is also required for induction of the neural crest (Deardorff et al., 2001; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997; Wu et al., 2005), and cranial placodes (Bang et al., 1999; Honore et al., 2003; Saint-Germain et al., 2004; Stark et al., 2000), two groups of cells that arise at the juncture of the neural ectoderm and presumptive epidermis. Wnts are secreted glycoproteins that bind to seven-pass transmembrane receptors, termed Frizzleds, in cooperation with LDL family coreceptors (He, 2003; Yanfeng et al., 2003). Canonical Wnt signaling centers on the stability of the transcriptional coactivator β-catenin (Pandur et al., 2002). In the absence of a Wnt signal, the serine/threonine kinase GSK-3 phosphorylates β-catenin, targeting it for ubiquitin-mediated proteosomal degradation. Receptor activation results in the inactivation of a degradation complex containing GSK-3, APC, Axin and other factors, resulting in an increase in β-catenin stability. Stabilized β-catenin enters the nucleus and interacts with Lef/Tcf family DNA binding proteins to activate transcription of Wnt target genes. In the absence of an interaction with β-catenin, Lef/Tcf factors remain bound to the DNA and are thought to function as transcriptional repressors (Hurlstone and Clevers, 2002). The mechanisms whereby canonical Wnt signals can direct such diverse responses in cell types such as the neural crest, placodes, and neural plate, when their induction is so closely linked both temporally and spatially, remain poorly understood. However, recent work has suggested that functional diversity amongst the different Lef/Tcf family proteins may confer distinct outcomes on canonical Wnt signals in some cellular contexts.

Although Lef/Tcf factors are often depicted as equivalent in simplified models of canonical Wnt signaling, these proteins possess distinct structural attributes that are proving to be of functional significance. All Lef/Tcf factors have an HMG-type DNA binding domain and an N-terminal β-catenin binding motif (Brantjes et al., 2002), but outside these regions, they differ significantly. For example, although it is believed that all Lef/Tcf factors can bind Groucho/TLE family corepressors (Brantjes et al., 2001; Cavallo et al., 1998; Daniels and Weis, 2005; Levanon et al., 1998; Roose et al., 1998), only certain Tcfs can recruit the unrelated corepressor, CtBP, through motifs present in their extended C-terminus (Brannon et al., 1999). Such structural variations between Lef/Tcf factors are likely to underlie many of the functional differences that have emerged from studies in *Xenopus*. For example, using constitutively repressing mutants that are unable to bind β-catenin, Roel et al. demonstrated that XTcf3 is required to mediate the Wnt-dependent dorsalization of the embryonic axis, whereas XLef1 is required for subsequent Wnt-mediated ventralization of the mesoderm (Roel et al., 2002). The presence or absence of "LVPQ" and "SXXSS" motifs in the region N-terminal to the HMG domain was shown to underlie the distinct activities displayed by these Lef/Tcf factors during mesodermal patterning (Liu et al., 2005), and these motifs have been proposed to function in transcriptional repression (Gradl et al., 2002).

In this study, we set out to examine whether XLef1 and XTcf3 play distinct roles during the patterning of the embryonic ectoderm. We found that while a Wnt-unresponsive mutant of XTcf3 potently blocks neural crest formation, an analogous mutant of XLef1 did not; however, blocking XLef1 function did interfere with other effects of Wnt signaling. The constitutively repressing XTcf3 mutant also blocked formation of cement gland and anterior placodes, a finding not predicted by the prevailing view of Wnts as posteriorizing factors. Importantly, we found that blocking canonical Wnt signals in the ectoderm, using the inhibitory XTcf3 mutant or by other means, led to a dramatic increase in the size of the neural plate, as evidenced by the increased expression of early pan neural markers such as *Sox3* and *Nrp1*. By contrast, upregulation of canonical Wnt signals was found to inhibit neural plate formation, and this activity could be distinguished experimentally from Wnt-mediated neural crest induction. Our findings demonstrate that not only is activation of Wnt/β-catenin signaling required for neural crest induction, but also that inhibition of canonical Wnt signaling is an essential step in the process that leads to neural plate formation.

Materials and methods

DNA constructs

The wild-type and mutant Lef and Tcf isoforms used in this study (accession numbers AF287148 and X99308 respectively) were generated by low cycle number PCR using a high fidelity polymerase (TGO, Roche) and verified by sequencing. ΔXLef1 (which consists of aa 110–373) and ΔXTcf3 (which consists of aa 88–555) are based on previously described dominant negative mutants (Huber et al., 1996; Molenaar et al., 1996; Roel et al., 2002) and delete the β-catenin binding domain of the respective proteins. Both of these constructs were cloned into a pCS2 expression vector (D. Turner) that incorporates six in frame N-terminal myc tags. β-CateninΔXLef1 and β-cateninΔXTcf3 were constructed by inserting a portion of β-catenin (accession number M77013; aa 737 to 868) in frame between the N-terminal myc tag and the corresponding ΔXLef1 or ΔXTcf3 coding sequence (Hsu et al., 1998; Staal et al., 1999). XLef1HMGEnR (aa 266 to 359) and XTcf3HMGEnR (aa 320–412) were created by placing the HMG domain into a pCS2 expression vector incorporating the engrailed repressor domain and an N-terminal myc tag (D. Turner). The β-catenin binding domain of XTcf3 (aa 2–87) and XLef1 (aa 2–97) was N-terminally myc tagged in pCS2 to create NTCF and NLEF respectively, and includes an NLS engineered in at the C-terminus (PKKKRKV) (Hamilton et al., 2001; Molenaar et al., 1996). For ΔXLef1-Tailswitch, the N-terminal portion of ΔXLef1 (aa 110–344) was fused to the C-terminal portion of XTcf3 (aa 398–554). Δβ-Catenin was constructed by removing the N-terminal region that confers instability (aa 1–167) (Yost et al., 1996) and inserting the remaining reading frame into a pCS2 incorporating six N-terminal myc tags. The dominant inhibitory FGFR1 (dnFGFR), constitutively active BMPR (caBMBR), and Neurogenin expression constructs have been previously described (Amaya et al., 1991; Candia et al., 1997; Ma et al., 1996).

Embryo manipulations and morpholinos

All results shown are representative of at least two independent experiments. Collection, injection, and in situ hybridization of *Xenopus* embryos were as described (Bellmeyer et al., 2003; LaBonne and Bronner-Fraser, 1998). RNA for injection was produced in vitro from linearized plasmid templates using the Message Machine kit (Ambion). β-Catenin morpholino (Heasman et al., 2000) was injected into one cell at the eight-cell stage. Embryos were staged according to Nieuwkoop and Faber (1967). For animal