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Extracellular signals, cell interactions and transcription factors involved in the induction of the neural crest cells

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ABSTRACT

The neural crest is induced at the border between the neural plate and the epidermis. A complex set of signals is required for the specification of the crest cells between the epidermis and the neural plate. Here we discuss evidence supporting a model for neural crest induction in which different signals contribute in a sequential order. First, a gradient of bone morphogenic proteins (BMPs) is established in the ectoderm that results in segreggation into neural plate, neural folds and epidermis at increasing levels of BMP activity. Thus, the neural folds are induced at a precise threshold concentration of BMP, but this neural fold has an anterior character. In a second step, these anterior neural folds are transformed into prospective neural crest by posteriorizing signals due to fibroblast growth factor, Wnts and retinoic acid. Finally, the induced cells interact to complete neural crest induction by a process that requires Notch/Delta signaling. Once neural crest formation has been induced by this combination of extracellular and intracellular signals, a cascade of transcription factors is activated in these cells that culminates in the ultimate steps of neural crest differentiation.

Key terms: neural crest, induction, BMPs, Wnts, FGF, retinoic acid, Notch, delta

INTRODUCTION

The neural crest was discovered by His in 1868 and has since remained a focus of intense research in Developmental Biology. The neural crest is comprised of a very particular set of cells, which segregate from the dorsal portion of the neural tube and migrate through the embryo to generate a prodigious array of cell types, including neurons, support cells for the peripheral nervous system, pigment cells, smooth muscle, cranofacial cartilage, as well as bone and fin in amphibian and fishes (for a review on neural crest cells see: Le Douarin and Kalcheim, 1999; La Bonne and Bronner-Fraser, 2000; Aybar and Mayor, 2002)

Much of the work discussed here focuses on neural crest development in *Xenopus laevis* embryos, where detailed knowledge of cellular and molecular aspects of the early development process is available. Results from work on *Xenopus* embryos has contributed substantially to our current understanding of the inductive process, both in terms of the cellular and molecular interactions involved. More recently, an increasing number of genes expressed in the neural crest of this species have been identified. In this review, we will analyze certain aspects concerning the induction of the neural crest in *Xenopus,* chicken and zebrafish. Also, we will discuss the role of different diffusible

and membrane bound molecules in activation of the genetic program required for neural crest specification.

A double gradient Model of neural crest induction

Experiments in *Xenopus* and zebrafish support a BMP gradient model for neural crest induction (LaBonne and Bronner-Fraser, 1998; Nguyen et al., 1998; Morgan and Sargent, 1997; Marchant et al., 1998; Barth et al., 1999, Mayor et al., 2001). However, recent reports in *Xenopus* indicated that induction of neural crest markers could not be explained as the simple consequence of lowering BMP activity in the ectoderm, thereby pointing to the need to identify additional signals (LaBonne and Bronner-Fraser, 1998; Wilson et al., 1997). Some of these have now been identified and include fibroblast growth factor (FGF), Wnts, and retinoic acid (RA) (LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002). In *Xenopus,* these molecules function, at least in part, as posteriorizing signals that pattern the anterior-posterior axis of the neural fold (Villanueva et al., 2002).

We propose the following model for neural crest induction. Initial neural crest induction is dependent on a gradient of BMP activity established in the ectoderm. The neural plate border, induced at a precise location within the medio-lateral axis of the ectoderm, has anterior character. At a later stage, but still during the early neural crest induction phase, posteriorizing signals originating from the posterior part of the embryo transform a region of the previously induced anterior neural plate border into prospective neural crest cells. These signals could correspond to Wnts, FGFs and RA, some of which are expressed at the correct time and place to act as posteriorizing agents in *Xenopus* and zebrafish (Lekven et al., 2001; Erter et al., 2001; Kiecker and Niehr, 2001). The anterior neural plate border either does not receive such signals or these are inhibited by other agents produced by the anterior regions of the embryo, such as *cerberus*, *Dkk1* or *Dan* (Bowmeester et al., 1996; Kasanskaya et al., 2000; Hashimoto et al., 2000; Ogita et al., 2001), three known Wnts inhibitors (for a discussion of Wnt signaling pathways, see Hendriks and Reichmann, 2002). As a consequence, this region does not develop into neural crest. As neurulation proceeds and the neural plate folds into a tube, molecules such as BMPs, Wnts and possibly FGF and RA are expressed in the epidermis and/or in the dorsal part of the neural tube, including the neural crest, where they are required for late induction (Liem et al., 1995; Villanueva et al., 2002; Lekven et al., 2001; Wilson et al., 2001; Jing et al., 2001; Dorsky et al., 1998; Chang et al., 1998; Ikeya et al., 1997). New elements of the Wnt pathway, such as *Xfz3* and *Kermit*, have been shown to be important in neural crest specification and differentiation (Deardorff et al., 2001; Tan et al., 2001). Strong evidence in favor of such a late induction step is available in the chick model, where neural tube cells analyzed at later stages can be transformed into neural crest tissue by the addition of BMPs (Liem et al., 1995; Selleck et al., 1998).

Once the neural plate border has been specifed as prospective neural crest by the signals mentioned, additional intercellular signals are required to complete the induction of the neural crest. Some of these signals depend on the interaction of Notch/Delta.

Notch/Delta signaling pathway

Notch/Delta signaling pathway has been conserved through evolution from nematodes to human. This pathway is involved in local cell-cell inductive processes that specify

embryonic cell fates, in particular in the neurogenic region of the blastoderm, in the developing nervous system, and in the compartment boundaries of the *Drosophila* imaginal discs (Coffman et al., 1990). Also a role during vertebrate development, in processes such as somitogenesis, selection of neuronal precursors in the central nervous system and glial to neuronal transition in the developing retina is well documented (Christ et al., 2000; Baker, 2000; Scheer et al., 2001). Notch and Delta genes code for single membrane-spanning proteins, Notch being the receptor and Delta the ligand of this signaling pathway. It should be mentioned that another ligand for Notch called Serrate exists, which seems to elicit similar responses (Kiyota et al., 2001). Both Notch ligand proteins have extracellular EGF repeats domain which mediate the interaction. In order to be effective, the interaction between Notch and Delta proteins must occur by contact between adjacent cells. If Notch and Delta are expressed on the same cell, an interaction is possible but does not lead to Notch activation. Additionally, Notch requires a co-receptor, called Fringe (or its vertebrate homologue Lunatic Fringe). This protein is suggested to be a glycosyltransferase that modifies the extracellular domain of Notch and thereby permits correct binding of Delta. In addition to these components, Notch undergoes at least three proteolytic events during maturation and signal transduction. First, the protease Furin cleaves Notch at a site in the extracellular domain (site 1) during transport of the receptor to the cell surface; the resulting amino-terminal domain remains associated with a carboxy-terminal transmembrane domain. Second, ligand binding triggers an additional cleavage of the extracellular region of the carboxy terminal domain (site 2), probably by a protease of the ADAM family. This second cleavage step shortens the extracellular region to 12 amino acids. Third, proteolytic cleavage within the transmembrane domain (site 3) releases the intracellular domain (ICD) so that it can translocate to the nucleus and activate transcription of target genes (Struhl and Adachi, 2000; Struhl and Greenwald, 2001). The intracellular domain of Notch poses ankyrin repeats, which behave as transcriptional activators upon interaction with the transcription factor Suppressor of Hairless which is normally found associated with the transcriptional repressor Groucho. The complex Groucho-Suppressor of Hairless represses the activity of several target genes, among them the genes of the *Enhancer of Split* complex (*E(spl)*, Jennings et al., 1994). Translocation to the nucleus of the intracellular domain of Notch displaces Groucho and transforms the repressor complex to an activator complex, thereby promoting expression of the *E(spl)* complex genes. As mentioned before, components of this cell-cell signaling pathway have been described in vertebrates and their roles have been characterized in mouse, chicken, zebrafish and *Xenopus*. Although, the main function attributed to this signaling pathway in vertebrate models has been selection of neuronal precursors in the central nervous system, more recent studies have additionally unraveled a role for the Notch pathway in gliogenesis of the retina (Scheer et al., 2001) and acquisition of glial fate by neural crest stem cells upon transient activation of this pathway (Morrison et al., 2000). These studies suggest that Notch activity maintains cells in an uncommitted state and view Notch as also being part of the gliogenic instructive program. In addition, studies in zebrafish and chicken have implicated the Notch signaling pathway in neural crest development.

Notch/Delta signaling in neural crest induction

In zebrafish, attention has centered on the function of Notch signaling in differentiation of the neural crest cells. In a *Delta A* mutant fish, Cornell and Eisen (2002) have shown that the population of Rohon-Beard neurons, a group of cells that arise in the most dorsal region of the neural tube, expands at expense of the neural crest cells. Therefore, the activation of Notch favors neural crest cell development and is required

to restrict the Rohon-Beard population (Cornell and Eisen, 2002). This study was extended by using specific morpholinos, which block the transcription of down stream genes, to demonstrate that the proneural gene *Neurogenin* is an essential element required for Rohon-Beard development and inhibition of neural crest fate. In summary, Notch signaling represses *Neurogenin* that in turn represses neural crest fate (Cornell and Eisen, 2002). Alternatively, inhibition of Notch activity (in a *Delta A* minus fish) promotes *Neurogenin* expression and Rohon-Beard development.

In chicken, neural crest cells are induced at the junction of the neural plate and the epidermal ectoderm. The current model of chicken neural crest induction involves interaction between the neural plate and the epidermis. This event induces *Bmp-4* transcription at the border of the neural plate, thereby inducing neural crest in this region. In a recent paper, Endo and colleagues have shown that at the time when neural crest cells are induced *Delta1* (a Notch ligand) is expressed in the epidermis and prevents *Notch1* expression in the neural folds. The contact produced by the cells expressing *Notch* and *Delta1* is able to induce the expression of *Bmp-4* in a row of cells in the epidermis and represses *Slug* expression in the same cells. In this manner, *Notch* activation promotes the formation of neural crest cells through *BMP-4* induction and thereby specifies sites at which neural crest cells are induced by suppression of *Slug* transcription (Endo et al., 2002). However, this model does not account of all the experimental data. First, previous results in chicken have shown that overexpression of Lunatic Fringe, the Notch co-receptor, leads to excessive proliferation of neural crest cells (Nellemann et al., 2001). Second, and more importantly, overexpression of the intracellular domain of Notch, enhances Notch activity, and represses *Bmp-4* transcription (Endo et al., 2002).

As in other vertebrates, activation of the Notch signaling pathway in *Xenopus* promotes the selection of neuronal precursors within the central nervous system (Coffman et al., 1990) and participates in somitogenesis. By the time the neural crest cells are induced, *Notch1* is expressed homogeneously in the neural plate and probably in part of the neural folds. On the other hand, *Delta1* and *Serrate* are expressed in a dynamic pattern surrounding the anterior part of the prospective domain where the neural crest is induced. Interestingly, *Hairy 2A*, one of the genes that belongs to the vertebrate *Enhancer of Split* complex and lies downstream of Notch, is expressed in the neural folds including the territory where the neural crest develop. This expression profile strongly suggests a role for Notch signaling in *Xenopus* neural crest development. Our experiments demonstrate that Notch signaling activates and is necessary for *Xslug*, as well as *Xmsx1* expression, a target gene of the BMP4 signaling pathway. In addition, chicken Notch activity is required for *Bmp-4* expression and, in Zebrafish, the Notch signaling pathway participates in differentiation of Rohon-Beard neurons where it controls *Neurogenin* expression (Silva et al., 2001; Glavic, unpublished results). Thus, in *Xenopus* the Notch signaling pathway is involved in early stages of neural crest development and later on in neural crest differentiation. In this manner, local cell-cell interactions through the Notch/Delta pathway help define the complex genetic program required for formation of neural crest cells.

Transcriptional activity in the neural crest during induction

A number of genes that are expressed at the neural plate border have been identified in the recent years. These genes are expressed at different stages of neural crest development, in the prospective neural crest, in migratory neural crest or during their differentiation. In this section, we will briefly discuss how gene expression territories

are established in a timely manner and then outline a possible sequence of the transcriptional activation events required during induction of the neural crest.

The ectodermal tissue can be divided into three principal domains (neural, neural fold and epidermis) in order to better explain the expression territories and action of the different transcription factors determining neural crest development. The two principal molecules involved in establishing gradients with positional information in the ectoderm at the early gastrula stage are BMP and Shh. In response to these signals, the expression of transcription factors is activated in each ectodermal domain or in superimposed domains. A broader discussion of transcriptional activity in the neural and epidermal domains exceeds the scope of this review, so we will only consider some aspects that influence neural crest formation. In the neural domain, pre-pattern genes of *Gli* and *Zic* families of transcription factors are successively activated in response to Shh and low levels of BMP (for a review on hedgehog/Gli signaling; see Ruiz Altaba , 1999, Brewster et al., 1998; Ruiz iAltaba et al., 2002). The combination of *Gli* and *Zic* genes activates pro-neural genes in the neural domain, which later on, once activated, are able to repress neural fold genes. The expression of *Zic* genes in the neural fold region may be controlled by the *Meis1/Pbx1* genes, as over-expression of *XMeis* is able to upregulate *Zic3* and *Gli3* expression (Maeda et al., 2001; 2002). At the end of gastrulation, *Zic1-3* disappears from the neural plate (Nakata et al., 1997; 1998, Mizuseki et al., 1998) and residual expression of *Zic3* remains at the neural fold and is probably required to induce *Zic5*, which is expressed in a territory more restricted to the neural fold than other members of the *Zic* family. Over-expression of *Zic5* induces neural crest genes in the absence of anterior neural markers (Nakata et al., 2000). In this sense, *Zic5* seems to be downstream of other *Zic* genes but is likely to control more specifically the early specification of neural crest cells.

At the epidermal border of the neural folds, a series of ventral or epidermal genes are expressed, such as *Msx*, *Dlx, Xvent*, *Gata*, etc. Many of these genes are expressed as a result of elevated levels of BMP, and some of them are assumed to be direct targets of this secreted molecule. Recent studies have pointed to *Msx-1* as a major factor in the initial specification of epidermis (Bendall and Abate-Shen, 2000; Maeda et al., 1997; Yamamoto et al., 2000; Takeda et al., 2000). *Dlx3, Dlx5* and *Dlx6* transcription factors are required for the development of the epidermis, and act as repressors of neural crest genes defining the lateral boundary for the cranial neural crest (Luo et al., 2001a, 2001b). By the end of gastrulation, the transcription factor *AP-2* is restricted to prospective epidermis where it controls epidermal development by regulating the transcription of cytokeratin genes (Luo et al., 2002). *AP-2* is later expressed in the pre-migratory neural crest but its function in this tissue remains unknown. The dynamic expression patterns of *Msx1* and *AP-2* genes suggest they could have dual functions, early in epidermal induction and later also in the neural crest induction. Thus, a cascade of different genes is activated at both sides of the neural fold, in the neural plate and the epidermis, which regulates the expression of neural crest genes.

On the other hand, in the neural fold domain a cascade of genes is activated, which positively controls the development of the neural crest. One of the earlier genes expressed in this cascade is *Pax-3*, although its transient expression at the border of the neural plate is not exclusive of the neural fold (Bang et al., 1997; 1999). When over-expressed, *Pax-3* is able to up-regulate *Xslug* expression (Mayor et al. 2000). The best-characterized transcription factors expressed in the neural fold of *Xenopus* are the zinc finger proteins *Xsnail* and *Xslug* (Sargent and Bennett, 1990; Mayor et al., 1995), two representative members of the *Snail* superfamily (for review see Nieto, 2002). The

earliest gene expressed specifically in the prospective neural crest region is *Xsnail* (Essex et al., 1993, Mayor et al., 1993; Linker et al. 2000; Aybar and Mayor, 2001; Aybar et al., 2002). This gene controls the transcription of *Xslug* and other transcription factors expressed in the neural crest such as *Xtwist*, *FoxD3, Zic-5, Ets-1* (Linker et al., 2000; Mayor et al., 2000; Aybar et al., 2002). *Xsnail* upregulates these genes in the absence of expression of neural or mesodermal markers, strongly suggesting that *Xsnail* alone is sufficient to convert presumptive ectodermal cells into neural crest cells. Thus, *Xsnail* is likely to be one of the upstream gene groups in the genetic cascade controlling transcription at the neural fold. A new *Xslug* gene (*Xslugß*) has been identified recently in *Xenopus* (Vallin et al., 2001). Although both *Xslug* genes seem to yield almost identical protein products, they have very different 5´-flanking regions. The biological significance in neural crest development of these two very similar genes controlled by different promoters remains unknown. However, it has been demonstrated that induction of *Xslug* requires intermediate BMP levels and posteriorizing signals such as FGF, Wnt and Retinoic Acid (Villanueva et al., 2002). This indicates that the neural fold tissue could integrate different signaling pathways to define appropriate expression of tissue markers. Experiments employing over-expression, antisense oligonucleotides or dominant negatives of *Xsnail* and *Xslug* showed that both genes play a key role in neural crest development in different animal models (Mayor et al., 2000; LaBonne and Bronner-Fraser, 1998; LaBonne and Bronner-Fraser, 2000; Carl et al., 1999; Nieto et al., 1994; Del Barrio and Nieto, 2002; Aybar et al., 2002; Tribulo and Mayor, 2001).

FoxD3, a member of the *Forkhead box*- (*Fox*) or winged-helix class of transcription factors, was also found to play a role in neural crest specification. This gene is expressed in premigratory and migratory neural crest of *Xenopus*, chick and zebrafish. Over-expression of *FoxD3* in *Xenopus* induces different neural crest markers and that expression of *FoxD3* also ocurrs in response to BMP and Wnt signals (Sasai et al., 2001). Studies in chicken showed that *FoxD3* is not able to induce *Slug* or *RhoB* (Dottori et al., 2001) but rather participates in establishing neural crest lineages by repressing melanogenesis (Kos et al., 2001,).

The basic Helix-Loop-Helix (bHLH) class transcription factor *Twist* is also expressed in the pre-migratory and migratory neural crest cells but very little is known about its function in the neural crest (Hopwood et al., 1989; Linker et al., 2000). SRY-like HMG box (*Sox*)-containing genes, such as *Sox9* of zebrafish (Li et al., 2002) and *Xenopus* (Spokony et al., 2002), are expressed in the neural fold. In *Xenopus*, *Sox9* expression persists as development proceeds in migrating cranial crest cells that populate the pharyngeal arches. However, in zebrafish, neural crest cells cease to express *Sox9b* expression during migration (Li et al., 2002). Depletion of Sox9 protein in developing *Xenopus* embryos, using morpholino antisense oligos, causes a dramatic loss of neural crest progenitors and an expansion of the neural plate (Spokony et al., 2002). The transcription factor *Sox10* is required for proper development of various neural crest-derived cell types. Melanocytes, autonomic and enteric neurons, and all subtypes of peripheral glia are missing in mice homozygous for *Sox10* mutations and in zebrafish with the same *Sox10* mutations (Dutton et al., 2001a; 2001b; Kelsh and Eisen, 2000; Kelsh et al., 2000). The available experimental evidence indicates that *Sox10* is required in neural crest stem cells prior to lineage segregation (Paratore et al., 2001).

One other transcription factor expressed in the neural crest of zebrafish, chicken and *Xenopus* is the bHLH factor *Id3* (Dickmeis et al., 2002; Reynaud-Deonauth et al., 2002; Kee and Bronner-Fraser, 2001a). The presence of Su(H) binding sites in both

the enhancer and the promoter of *XId3*, suggest that *Notch*-dependent control of differentiation may involve activation of transcription of *Id* genes (Reynaud-Deonauth et al., 2002). In chick the *Id4* gene is expressed in subsets of migrating neural crest cells (Kee and Bronner-Fraser, 2001b); however the function of this and other members of *Id* gene family in the neural crest is not yet understood.

Lecture overview

In this lecture, results from our laboratory concerning development of the neural crest will be discussed. A new model for induction of the neural crest that involves a gradient of BMP in combination with posteriorizing signals will be presented (Marchant et al., 1998; Mayor and Aybar, 2001; Villanueva et al., 2002; Aybar and Mayor, 2002). This model is based on experiments analyzing the expression of specific markers for the anterior neural fold (*cpl1*, *Xag*) or the neural crest (*Slug*, *Twist*) under different conditions. In addition, gain- and loss- of function experiments were preformed to determine the role of FGF, BMPs and Retinoic Acid. The contribution of the wnt pathway and the role of Notch/Delta signaling were explored using different mutants of these molecules or their respective targets. Finally, we will discuss how transcription factors are activated in crest cells. In particular, we will concentrate on the role the two zinc finger genes *Snail* and *Slug* play during early specification and migration of crest cells.

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