Genetic network during neural crest induction: From cell specification to cell survival

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Abstract

The concerted action of extracellular signals such as BMP, Wnt, FGF, RA and Notch activate a genetic program required to transform a naïve ectodermal cell into a neural crest cell. In this review we will analyze the extracellular signals and the network of transcription factors that are required for this transformation. We will propose the division of this complex network of factors in two main steps: an initial cell specification step followed by a maintenance or cell survival step.

Keywords: Neural crest; Genetic cascade; Cell survival; Wnt; FGF; BMP; Retinoic acid

Contents

1. Introduction

The neural crest is a unique and highly specialized population of cells found in all vertebrate embryos that has fascinated generations of developmental and evolutionary biologists. The neural crest develops at the border between the neural plate and the epidermis, and following closure of the neural tube these cells delaminate from the dorsal neural tube to migrate along different pathways. On reaching their destination in the embryo, they differentiate into a wide variety of cell types.

In this review, we will analyse recent advances on the signals that induce neural crest and the genes activated by these signals that are required for the early steps of neural crest specification. Induction of neural crest is a multi-step process, and we will propose here some of the steps and the genes that control it.

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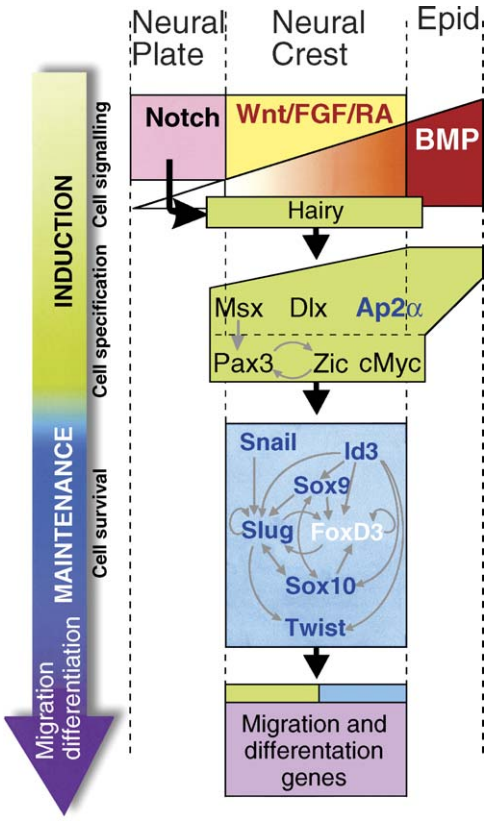

Fig. 1. Neural crest genetic network. Genetic network based on expression patterns and gain- and loss- of function experiments. Green square: Cell Specification genes; Blue square: Cell Survival genes. Genes written in blue indicate antiapoptotic activity. See text for more details.

Complete induction of neural crest cells requires the transformation of a naïve ectoderm into neural crest. This is a complex process that involves the activity of extracellular factors produced by the inductive tissues (*Cell Signalling* in Fig. 1), which activate a genetic program in the ectodermal cells (*Cell Specification*, green arrow in Fig. 1). Once this genetic program is activated in the neural crest cells a second set of genes, the proper neural crest genes (blue box in Fig. 1), are transcribed. One of the important functions of this second set of genes is to allow survival of the recently induced neural crest cells (maintenance or *Cell Survival* in Fig. 1). Finally, the joint action of the Cell Specification and Cell Survival genes work together to control delamination, migration and differentiation of the neural crest cells. In this review we will analyse the factors and genes involved in Cell Signalling, Specification and Survival, but we will not include the analysis of delamination, migration and differentiation as they have been discussed in another review of this issue [1].

The genetic cascade proposed in this review is an updated version of previous proposals [2,3] and it will be based in the expression pattern and gain- and loss- of function experiments performed in different animal models. Thus, although useful as a proposal is far of being definitive as not many cys-regulatory analysis has been carried out. A further limitation of this proposal is that the interaction between the genes is not linear, and many of the genes involved in the early steps of neural crest specification are also involved in later steps of crest development.

2. Extracellular Cell Signalling during neural crest induction

Induction of the neural crest involves a complex set of extracellular signals that transform the fate of cells lying along the medio-lateral and anterior-posterior axes of the embryo. The signals that position the neural crest cells along these axes are released from the neural plate, the epidermis and the lateral mesoderm [4–10].

It has been shown in *Xenopus* and zebrafish embryos that prior to gastrulation BMP signalling is active throughout the entire ectoderm [11,12]. At this time, the dorsal mesoderm releases anti-BMP molecules such as noggin, follistatin and chordin, that directly bind to BMPs, inhibiting their activity. In addition, the expression of BMPs is down regulated at the most dorsal ectoderm during gastrulation by a Wnt-dependent mechanism [13]. As a consequence of all these interactions a dorso-ventral gradient of BMP activity is generated. Strong evidence in *Xenopus* and zebrafish embryos indicate that neural plate, neural crest and epidermis are specified at progressively higher levels of BMP activity [9,14–18]. The addition of BMPs to dissected neural crest induces the expression of neural crest markers in chick, supporting a role for this molecule in this animal model as well [19]. However, in this latter work it is likely that BMP is playing a role in a late step of neural crest development as the tissue used in these experiments was taken from relatively older embryos (see [20] for a comparison between the stages of different animal models concerning the timing of neural crest induction). Nevertheless, although intermediate BMP activity is required for neural crest induction in chick, *Xenopus* and zebrafish this molecule is not sufficient to induce neural plate or neural crest cells [21,22].

A second group of signals, Wnts, FGFs, and RA, are required for neural crest induction and have also been implicated in the antero-posterior patterning of the neuroectoderm [23–28]. These molecules come from the involuting endomesoderm located at the posterior part of the embryo. Their expression and inhibition are respectively required to posteriorize and anteriorize the neural tube [29–33], as well as to induce neural crest on the posterior neural folds [34–39].

Recently, the emphasis has shifted in favor of Wnt signalling as one of the key elements in neural crest induction [40]. Gain and loss of function experiments in chick, *Xenopus* and zebrafish show that Wnt signals are essential for neural crest induction. However they are not able to induce neural crest by itself in naïve ectoderm as they require working in combination with anti-BMP signals or in a previously induced neural plate [36,38,41–46]. A possible candidate to be the source of Wnt signalling in the chick embryo is the epidermis as it expresses Wnt6 at the right time to be the inducer

[43]; however, there is no experimental evidence demonstrating that Wnt6 is required for neural crest formation. In *Xenopus* and zebrafish embryos Wnt8 is a good candidate to be a neural crest inducer. It is expressed in the mesoderm that is able to induce neural crest, and gain and loss of function experiment show its role in neural crest induction [36,39,41,45,46].

FGF signalling is also important in neural crest induction as gain and loss of function experiments show an essential role of this molecule, and a direct, though transient, induction of some neural crest markers has been observed in naïve ectoderm treated with FGF8 [10,14,36,37]. Retinoic acid (RA) has also been implicated in neural crest induction in *Xenopus* and zebrafish embryos. Addition of RA to neuralized animal caps or expression of dominant negatives in *Xenopus* embryos, as well as analysis of zebrafish mutant indicate the requirement of this molecule for the correct specification or survival of the neural crest cells [39,47]. Moreover, it has been shown that the induction activity of these three molecules is, at least in part, related to its posteriorization activity [3,20,39]. Recently, it has been possible to dissociate the posteriorizing role of FGF and Wnt signalling from their ability to induce neural crest [48,49]. In conclusion, induction of neural crest requires a specific level of BMP and the concerted action of Wnt, FGF and RA signalling (see Fig. 1). It is likely that Wnt, FGF and RA works in the posteriorization of the neuroectoderm in an early step of neural crest specification and also as direct neural crest inducer at later steps.

Another important molecule involved in early crest specification is Notch. It has been proposed that Notch participates in neural crest specification by controlling BMP expression in chick and *Xenopus* embryos, although the exact mechanism could be different in these two species [50–54]. In addition, Notch signalling seems to control neural crest development by repressing neurogenesis [51]. We will not discuss Notch signalling further as it is analyzed elsewhere in this issue [55].

3. Early genetic network involved in Cell Specification of the neural crest

As consequence of the extracellular signalling working on the ectoderm a first set of genes that encode for transcription factors are activated in the prospective neural crest cells. These are the genes that control Cell Specification (see Fig. 1).

Gene expression pattern suggests that *Msx*, *AP2α* and *Dlx* genes are amongst the earliest genes activated in the ectoderm fated to become neural crest. We have grouped these three genes together because they exhibit a similar pattern of expression. They are initially expressed in the non-neural ectoderm, and later they are restricted to the neural fold region in *Xenopus* and chick embryos [56–58]. Interestingly amphioxus, a non vertebrate chordate, shows a similar early AP2 expression pattern suggesting that this molecule also had an important role in neural crest evolution as well as in other vertebrate traits [59,60]. Their expression pattern correlates with the assumed ventro-dorso gradient of BMP activity, and identification of cys-regulatory elements in some of these genes indicate that is very likely to be direct targets of BMP [58,61–63]. Loss of function experiments in mouse, zebrafish and *Xenopus* embryos show that they are required for the early specification of the neural crest [44,58,59,64–67]. *Msx1* seems to play a key role in neural crest development, and epistatic experiments in *Xenopus* suggest that works upstream of the *Snail* genes [44,48,67]. However, once the neural crest is induced *Msx1* expression is switched off from the neural crest, and become to play a role in controlling neural crest apoptosis [48,68]. The zebrafish *AP2a* mutant, *mont blanc*, shows a defect in the early specification of the neural crest, as well as in neural crest derivatives [64,69–71]. It also shows a later role as an inhibitor of apoptosis in the neural crest cells [69]. Morpholino mediated knockdown experiments in *Xenopus* indicates that *AP2a* is essential for the early specification of neural crest cells [66]. Thus, *AP2a* seems to be a gene involved in early as well as late neural crest development.

Dlx genes are important in the positioning of the neural plate border, including neural crest and placodal cells [72–74], although *Dlx5* seems to be more specific for the neural crest cells [75]. It is important to note that *Msx*, *Dlx* and *AP2α* have an earlier role in epidermal development, and in consequence it could be suggested that specification of the neural crest at this early stage involves the dorsalization of epidermis.

The next group of genes, *Zic*, *Pax3* and *c-Myc*, are expressed in a more restricted domain that the previous one, they are not expressed in the entire epidermis at earlier stages but they are present in a wider domain than the premigratory neural crest cells [76–81]. Multiple *Zic* genes have been identified in mouse and *Xenopus* that are expressed in overlapping but distinct patterns [80,82]. Disruption of mouse *Zic* genes leads to multiple defects in neural and neural crest derivatives [82–84]. In *Xenopus*, expression of the Zic genes are a very early response to neural inducing signals, and ectopic expression of these Zic genes leads to neural plate and neural crest formation [67,78–80,85–87].

Pax3 is expressed in the neural folds and loss of function experiments in mouse and *Xenopus* show a key role in neural crest development [48,67,88–90]. Using a combination of overexpression and morpholino mediated knockdown experiments, Monsoro-Burq et al. [48], show that *Msx1* and *Pax3* are required for neural crest formation and that *Msx1* seems to work upstream of *Pax3* (see Fig. 1). Interestingly, it is proposed in their work that Wnt controls neural crest induction through *Pax3* activity, while FGF8 requires *Msx1* and *Pax3* actions; however the molecular mechanism by which the combination of the anti-BMP molecule noggin and *Msx1* is able to induce neural crest in naïve ectoderm is not explained [48]. A similar strategy based on morpholino injections has been recently used to show a cooperative function of *Pax3*

and *Zic1* in neural crest specification and a mutual regulation between these two factors has also been shown (see Fig. 1 and [67]). The exact pathway network connecting *Msx1*, *Pax3* and the *Zic* genes requires further careful consideration.

The proto-oncogene c-Myc has been found to be expressed in the neural plate border of amphibian embryos earlier and in a broader domain than many specific neural crest genes [81]. Morpholino knockdown and overexpression experiments show an essential role of c-Myc in neural crest development that seems to be independent upon cell proliferation or cell death [81]. c-Myc is expressed almost at the same time and in the same cells as *Msx1* and *Pax3*, and it is likely that an interaction between these factors is required for the early step of neural crest specification.

4. Specific neural crest genes involved in Cell Survival

Following the initial specification of neural crest precursors at the neural plate border, a network of genes is set up that function to maintain these precursors (blue arrow in Fig. 1). The genes transcribed at this step of neural crest induction are expressed later that the Cell Specification genes and only in the neural crest territory. They encode for transcriptions factors of the *Snail* and *Sox* family of genes, and for the genes *FoxD3*, *Id3* and *Twist*. Functional experiments are insufficient to predict a cascade of genetic interactions amongst these genes as the up or down regulation of one gene will effect the expression of others. It is also important to note that *Snail*, *Slug* and *FoxD3* all function as transcriptional repressors [91–94]; this means that there are additional transcription factors mediating the activity of these genes. In addition, as many of these genes are involved in cell survival it is not known in many cases whether the observed effect is due to changes in rates of cell death/proliferation or changes in the transcription of neural crest genes. Therefore the grey arrows included in Fig. 1 that connect these genes are tentative.

4.1. Snail/Slug

Two members of the *Snail* family of genes, *Snail* and *Slug*, play key roles in neural crest development. The observed function of these genes appears to be dependent upon the species examined. In *Xenopus snail* is expressed in the prospective neural folds slightly earlier than *Slug*, and based on animal caps and epistatic experiments *Snail* seems to work upstream of *Slug* in neural crest development (see Fig. 1 and [94,95]). In addition, *Slug* can produce an expansion in the *Slug* and *Twist* expression domains in whole embryos [36]. Loss of function experiments in *Xenopus* using different dominant negative constructs have shown that *Slug* is required for full expression of *Slug*, *Sox9*, *Twist*, *Ets-1*, *FoxD3* and *Sox10* [91,92,94,96,97]. Electroporation of *Slug* into the cephalic regions of chick neural tubes produces an expansion in the expression of several neural crest markers including HNK-1, RhoB and *Pax3* [98] but in the trunk additional signals are required [99]. In conclusion, *Snail/Slug* genes are required for the specification of the neural crest cells in chick, mouse and *Xenopus* embryos.

In addition to this early role of the *Snail* genes another important function is to control cell-cycle progression in neural crest cells [100] and to inhibit apoptosis in the crest cells, by controlling the expression of Bcl-x_L and specific caspases [68,100]. Thus, this is the first example of this group of genes expressed specifically in the neural crest that have an anti-apoptotic or cell survival activity.

4.2. Id3

Two recent studies in *Xenopus* have analysed the role of *Id3* in neural crest formation [101,102]. They showed that depletion of *Id3* by morpholino knockdown experiments results in the down-regulation of early neural crest markers (*Slug*, *Sox10*, *FoxD3* and *Twist*) and an increase in apoptosis in the neural crest cells. The down-regulation of neural crest markers in *Id3* depleted embryos occurs very early and could not be rescued by *Slug* expression suggesting that *Id3* functions to maintain the expression of neural crest progenitor genes as well as control cell proliferation and apoptosis [101,102].

4.3. Sox

The *Sox* genes are a group of transcription factors with considerable importance in neural crest development; their role has been considered in detail elsewhere in this issue [103] and so will not be dealt with in detail here. Briefly, *Sox8*, *Sox9*, *Sox10* and *LSox5* are expressed in the neural crest of mouse, chick, zebrafish and *Xenopus* embryos, at different times during development. The most studied *Sox* genes in the neural crest are *Sox9* and *Sox10*, and multiple interactions of these genes with other members of the Cell Survival group of genes have been described (see Fig. 1 and [96,97,99,104–112]). Sox9-null mice mutants showed massive cell death in the trunk neural crest population prior to or shortly after delamination [99] and in *Sox10* mutant embryos the neural crest undergo apoptosis before they can differentiate [113–115]. Similarly, neural crest of the *Sox10* zebrafish mutant (*colourless*) fails to migrate and undergo cell death [107]. Taken together these results point towards a role of *Sox9* and *Sox10* as a survival factor.

4.4. Twist

Twist is another gene expressed in pre-migratory neural crest cells in *Xenopus* [95] and mice [116,117]. Mice lacking functional *Twist* have defects in neural tube closure and neural crest cell migration [117]. Interestingly, *Twist* has been implicated in controlling cell proliferation and survival during mouse paraxial mesoderm development [118]. The role

of *Twist* in pre-migratory neural crest cells has not yet been examined.

4.5. FoxD3

FoxD3 is one of the earliest neural crest genes to be expressed in mice [119], zebrafish [120], *Xenopus* [92,93] and chicks [121,122]. Functional experiments in *Xenopus* have been somewhat contradictory. Overexpression has been reported to decrease the expression of neural crest markers while causing an expansion of the neural plate [95]. Sasai et al. [92] found that injection of *FoxD3* RNA led to the ectopic induction of *Slug*, *AP-2*, *FoxD3*, *Ets-1*, *Twist* and *Sox2* in embryos and animal caps. It is not known whether *FoxD3* can induce neural crest cells directly or whether the induction is a secondary effect of neural induction. The differences between these two studies are likely to be due to the different doses of RNA used. Indeed, injecting low doses of *FoxD3* RNA results in the expansion of neural crest markers whereas high doses result in the inhibition of neural crest markers (Francisco Romero and R.M. unpublished). Electroporation of *FoxD3* into the chick neural tube can induce HNK-1 expression in neuroepithelial cells as well as an increase in migratory neural crest cells from the dorsal neural tube [99,121,122]. *FoxD3* can also induce changes in the expression of cell adhesion molecules required for delamination, although a dominant negative of SoxE genes can inhibit the effects on HNK-1 induction, it has no effect on the cell adhesion changes [99]. It has not yet been assessed whether FoxD3 is involved in the survival of neural crest cells.

5. Concluding remarks

The combination of an intermediate level of BMP signalling together with the action of Wnt, FGF, RA and Notch activates a genetic program in the ectoderm that defines the prospective neural crest cells. Some of the genes of this early neural crest network are *Msx*, *Dlx*, *Ap2*, *Pax3*, *Zic* and *cMyc* (green box in Fig. 1). The individual and/or concerted action of these Cell Specification genes activates a second group of genes. In this second group the genes are expressed specifically in the neural crest cells and multiple interactions among each gene have been described (blue box in Fig. 1). One of the most interesting outcomes of this analysis is that for almost all the genes in this group an anti-apoptotic or cell survival activity has been described. This is why we propose to call these genes Cell Survival genes and to call this step of neural crest development the Maintenance step (Fig. 1). The only two exceptions to this observation are the *Ap2α* gene (included in the Cell Specification step) which also has an anti-apoptotic activity and the *FoxD3* gene, where no analysis of its role on cell survival has been performed. Interestingly, a recent review proposes the *Ap2α* gene in the same group as *Snail*, *Slug* and *Sox* genes [123].

The need for a genetic program of cell survival is not surprising, as the neural crest are likely to be induced in an hostile environment surrounded by epidermal and neural plate cells, and later they will migrate very far from its original niche. Thus, many of the Cell Specification and Cell Survival genes used during the induction of the neural crest cell will also be used during the last step of the neural crest journey: migration and differentiation.

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