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# The genesis of avian neural crest cells: A classic embryonic induction

**(chicken embryo/neural development/peripheral nervous system/inductive interactions)**

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**ABSTRACT Neural crest cells arise from the ectoderm and are first recognizable as discrete cells in the chicken embryo when they emerge from the neural tube. Despite the classical view that neural crest precursors are a distinct population lying between epidermis and neuroepithelium, our results demonstrate that they are not a segregated population. Cell lineage analyses have demonstrated that individual precursor cells within the neural folds can give rise to epidermal, neural crest, and neural tube derivatives. Interactions between the neural plate and epidermis can generate neural crest cells, since juxtaposition of these tissues at early stages results in the formation of neural crest cells at the interface. Inductive interactions between the epidermis and neural plate can also result in "dorsalization" of the neural plate, as assayed by the expression of the *Wnt* transcripts characteristic of the dorsal neural tube. The competence of the neural plate changes with time, however, such that interaction of early neural plate with epidermis generates only neural crest cells, whereas interaction of slightly older neural plate with epidermis generates neural crest cells and *Wnt*-expressing cells. At cranial levels, neuroepithelial cells can regulate to generate neural crest cells when the endogenous neural folds are removed, probably via interaction of the remaining neural tube with the epidermis. Taken together, these experiments demonstrate that: (*i*) progenitor cells in the neural folds are multipotent, having the ability to form multiple ectodermal derivatives, including epidermal, neural crest, and neural tube cells; (*ii*) the neural crest is an induced population that arises by interactions between the neural plate and the epidermis; and (*iii*) the competence of the neural plate to respond to inductive interactions changes as a function of embryonic age.**

The nervous system of vertebrates derives entirely from the most dorsal germ layer of the embryo, the ectoderm. As a result of interactions with adjacent tissues during neural induction (1, 2), medial ectoderm is induced to thicken into a neural plate, which subsequently invaginates into a neural tube and separates from the adjacent ectoderm (3). The surface ectoderm develops into two tissue types: skin epidermis and cranial placodes, which contribute to the peripheral nervous system (4). The neural tube gives rise to the central nervous system (CNS) and to the remainder of the peripheral nervous system via a population of migratory cells, called the neural crest, that arises from the dorsal neural tube. These cells migrate extensively throughout the embryo and differentiate into a diverse range of both neuronal and nonneuronal derivatives. At trunk levels, these include sensory neurons, postganglionic autonomic neurons, melanocytes, adrenal chromaffin cells, and Schwann cells. At cranial levels, neural crest derivatives include melanocytes, cranial sensory ganglia, and most of the skull and facial cartilage (5).

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### Segregation of Ectodermal Lineages

At early stages of gastrulation in the chicken embryo [definitive primitive streak-stage, or stage 4 of Hamburger and Hamilton (6)], the ectoderm shows no overt subdivision into its prospective tissue types. Nevertheless, fate mapping experiments have demonstrated that future neural and epidermal cells occupy separate territories, with prospective neural tissue lying in a semicircular area of ectoderm around the rostral primitive streak (7–10) and prospective epidermis lying lateral to the future neural tissue (however, see ref. 9). Presumptive neural crest cells lie between the neural and epidermal fields (9, 11).

A little later in development, the prospective neural ectoderm thickens to form the neural plate (3). Careful examination of the caudal end of a 7 somite-pair chicken embryo reveals a distinct, open neural plate bordered laterally by elevated neural folds (Fig. 1). The margins of the neural plate have been thought to represent a lineage boundary separating neural cells medially from epidermal cells more laterally. According to this view, the neural crest is a segregated population of cells lying within the lateral neural plate that comes to lie within the dorsal neural tube after the plate has folded.

To test this hypothesis and to investigate the lineage history of the neural crest, we have used iontophoretic injection of lysinated-rhodamine-dextran to follow the fate of single cells of the ectoderm and ectodermal derivatives (12–15). Lineage analysis of cells within the dorsal (closed) neural tube has revealed that some single cells can contribute to both CNS and neural crest derivatives (12–14), indicating that the neural crest is not a segregated population even after tube closure. It is reasonable to conclude that divergence of the neural tube and neural crest lineages occurs later, as neural crest cells are emigrating from the neuroepithelium. Using the same experimental approach, we have also investigated the early lineage decisions of ectodermal cells at the open and closing neural plate stages (15). To our surprise, some single cells within lateral neural plate contributed to CNS, neural crest, and epidermis (Fig. 2), indicating that the visible margin of the neural plate is not a barrier separating future nervous system cells from epidermal cells. Since we found such "tripotent" cells in the neural folds before, but not after, neural tube closure, we may conclude that the CNS/neural crest lineage

Abbreviation: CNS, central nervous system.

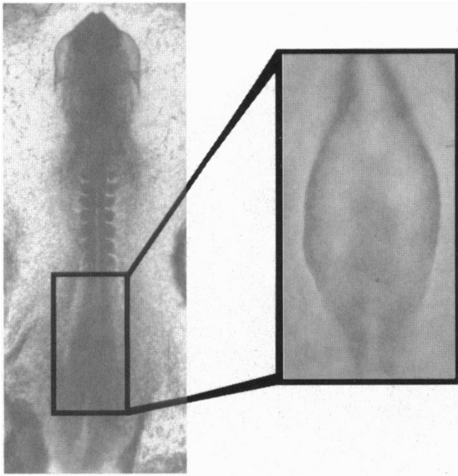

FIG. 1. At early stages (stages 7–10) of chicken development, multiple stages of neural tube formation can be found within a single embryo. (*Left*) In this whole mount preparation of a 7 somite stage embryo observed from the dorsal side, the neural tube is closed in rostral regions (toward the top), whereas the neural plate remains open in more caudal regions (outlined at the bottom). (*Right*) At higher magnification, the open neural plate region remains tear-shaped, with Hensen's node lying at its center. Lateral to Hensen's node, the borders of the neural plate are well-defined; more caudally, the edges of the neural plate are less distinct.

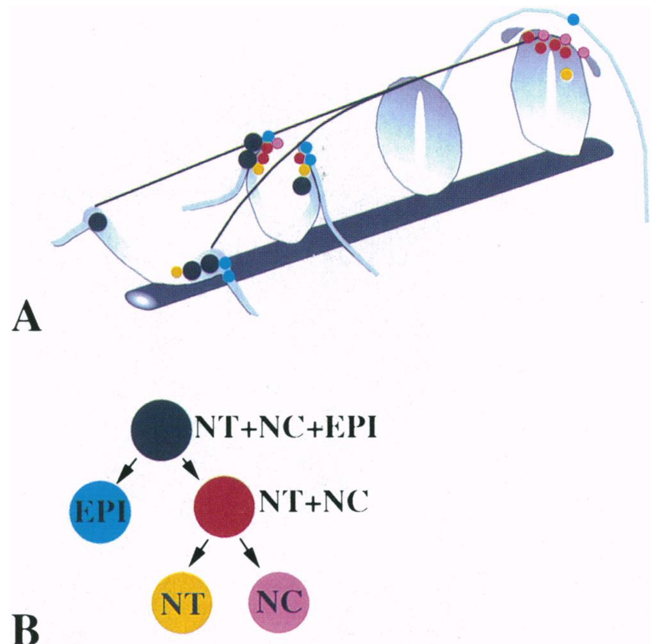


FIG. 2. (*A*) A schematic representation of the results of single cell lineage analysis of the open and closing neural plate. After injection of a single cell (black) in the neural folds, the progeny are located within all three ectoderm derivatives, neural tube (NT), neural crest (NC), and epidermis (EPI). Only after neural tube closure are "tripotent" black cells no longer observed. Some precursors (red) within the dorsal neural tube form both neural tube and neural crest cells. (*B*) A summary of deduced ectodermal lineages.

separates from the epidermal lineage around the time of neural tube closure.

Taken together, these results raise the tantalizing possibility that ectodermal lineages segregate as a consequence of mechanical separation of the ectodermal derivatives: neural tube closure prevents cells of the neural tube from becoming epidermis, and epithelial-to-mesenchymal conversions prevent neural crest precursors from contributing to the CNS. If this is the case, the columnar epithelial morphology of the neural plate cells may be of mechanical importance only, facilitating the rolling of ectoderm into a neural tube (3, 16, 17). This model stands in contrast to more traditional ones that hold that the ectodermal lineages segregate when the neural plate is formed during the process of neural induction. Some recent experiments in *Xenopus* have indicated that the generation of neural crest and the formation of neural plate are indeed separate events (18), possibly occurring at different times in development [compare Kengaku and Okamoto (19) with Zhang and Jacobson (20)].

## Inductive Interactions Generate Neural Crest Cells

Since neural crest precursors in the dorsal neural tube share a lineage with CNS derivatives, such as commissural neurons and roofplate cells, the question of how neural crest cells are generated from the ectoderm becomes one of how the neural tube becomes polarized dorsoventrally such that motor neurons and floor plate cells develop ventrally and neural crest cells and commissural neurons arise dorsally. Many lines of evidence suggest that the notochord can induce adjacent neural tube cells to develop into ventral derivatives such as motor neurons and floor plate (21–28). This polarizing ability of the notochord has been attributed to its production of *Sonic hedgehog* (29–31). One possibility, therefore, is that the entire neural tube is polarized by such a signal from the notochord: high levels of signal cause neuroepithelial cells to differentiate into ventral derivatives, while those neural tube cells furthest from the notochord see less (or no) signal and consequently develop into neural crest cells. Studies in our laboratory, however, show that notochord is not sufficient to polarize the entire neural tube. By performing notochord grafting experiments, Artinger and Bronner-Fraser (32) confirmed the findings of others (24, 27) that an implanted notochord is able to induce ventral cell types after grafting adjacent to dorsal neural tube. However, they also found that an implanted notochord is unable to suppress the formation of neural crest cells, suggesting that some degree of commitment to a neural crest fate has already occurred by the time of grafting. Notochord grafts also failed to suppress formation of commissural neurons, a cell type that first differentiates in the dorsal neural tube (32). Furthermore, cell lineage analysis demonstrates that single dorsal neuroepithelial cells can form neural crest cells, dorsal neural tube cells, motor neurons, and floor plate cells (33). Thus, single cells or their progeny can respond to multiple signals, resulting in the formation of dorsal and ventral cells types within the same clone.

What tissue might play a role in inducing dorsal neural tube cell types? Throughout neurulation, prospective neural crest cells are located at the boundary between neural plate/neural tube and epidermis. Therefore, one possibility is that interactions between these two cell populations are responsible for the formation of neural crest. Indeed, experiments in amphibian embryos (34–36) indicate that neural crest cells are generated wherever these tissues approximate. To test whether neural plate–epidermal interactions lead to the formation of neural crest cells in the avian embryo, we have performed experiments in which prospective neural plates from definitive streak stage chicken embryos (stage 4; ref. 6) were grafted adjacent to prospective epidermis in host embryos of the same stage (ref. 15; Fig. 3). Using an antibody that recognizes HNK-1, an epitope associated with neural crest cells (37, 38), we found that HNK-1 immunoreactive cells (putative neural crest) were generated *de novo* at the site where these tissues lie apposed (Fig. 3 *B* and *C*). Similar results were obtained when prospective (stage 4) neural plate and epidermis were combined in a collagen gel explant system, grown in serum-free medium (39). To test whether such interactions can generate neural crest derivatives, the same tissues were grown on

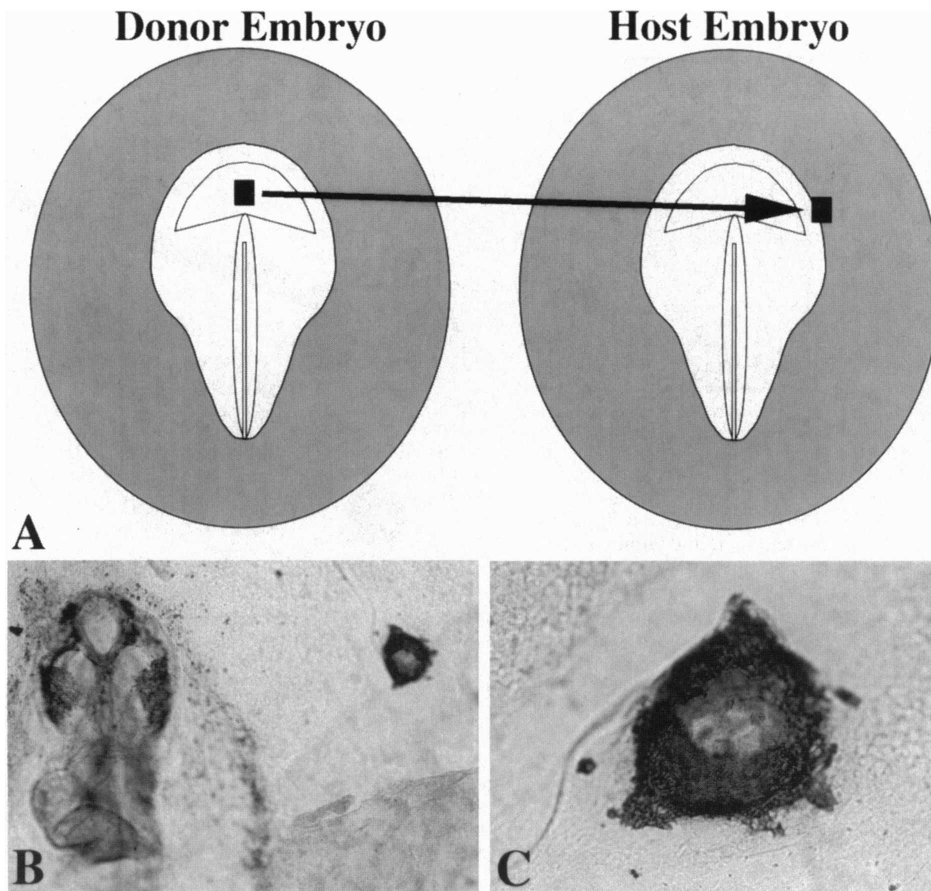


FIG. 3. (*A*) Diagrams illustrating the sites from which tissues were isolated and at which tissues were grafted. Neural plate (black) lying rostral to Hensen's node was isolated from stage 4 donor embryos and grafted adjacent to nonneural ectoderm in the host embryo. (*B* and *C*) Low and high magnifications of host embryos and grafts in which the tissue has been labeled with the HNK-1 antibody, which recognizes neural crest cells. Immunoreactive cells are observed in the host embryo (*B Left*) as well as surrounding the graft. At higher magnification (*C*), the HNK-1 immunoreactive cells are clearly visible around the periphery of the graft.

fibronectin-coated dishes for 10 days in serum-containing medium. We found both melanocytes and adrenergic cells (expressing tyrosine hydroxylase) when these tissues were cocultured. In contrast, neural crest derivatives were not generated when either prospective neural plate or prospective epidermis were cultured alone (15). Although embryos at the definitive streak stage have no visible neural plate, we infer from our results that some differences must already be present between medial and lateral ectoderm, otherwise they could not interact to generate neural crest cells.

By repeating these experiments with older neural plate taken from stage 8–10 embryos, we obtained essentially the same results. Some of our experiments, however, indicate a difference in the competence of young and old neural tissue in terms of their potential to form neural crest cells. The finding that epidermis can induce neural crest cells from neural tissue in the chicken has since been confirmed by others (40).

## The Neural Crest Inducer

To determine whether the neural plate–epidermal interactions that generate neural crest cells are mediated by cell–cell contact or whether diffusible factors are responsible for the induction, we have employed a transfilter micromass assay system previously described by Schramm *et al.* (41). Our results suggest that the neural plate–epidermal interaction is mediated by a diffusible molecule (or molecules; M.A.J.S., unpublished work).

Three candidate inducing molecules are dorsalin-1 (42), BMP-4, and BMP-7 (40), all of which are members of the transforming growth factor $\beta$ superfamily. Liem *et al.* (40) have recently shown by *in situ* hybridization that both BMP-4 and BMP-7 transcripts are expressed in prospective epidermis lying lateral to the open neural plate, and subsequently in some midline epidermal/neural structures. More importantly, they have shown that recombinant BMP-4 and BMP-7 can substitute for epidermis in inducing neural crest markers (*Pax3*, *Dsl1*, *Msx*, and *Slug*; see below) in neural plate explants. Taken together, the expression patterns and functional experiments using recombinant protein provide evidence that BMP-4 and/or BMP-7 are sufficient to induce neural crest cells from the neuroepithelium. However, it has yet to be determined whether these molecules are necessary for induction of neural crest cells, whether they cause differentiation into a complete range of neural crest derivatives and whether the induced cells behave like endogenous neural crest cells.

## The Molecular Consequences of Neural Plate–Epidermal Interactions

One might expect that genes directly involved in the formation of neural crest cells would be (*i*) expressed within the dorsal neural tube, and (*ii*) regulated by neural plate–epidermal interactions. A number of genes have been cloned whose transcripts are, at some stage of neural development, restricted to the dorsal neural tube. Some of these genes, such as *Pax3* (40, 43, 44), *Msx-1/2* (40, 45), and *np-1* (M. Barembaum and M.B.-F., unpublished work), are initially expressed in both medial and lateral regions of the neural plate and subsequently become restricted to dorsal neural tube. Other genes are

restricted to the dorsal region of the neural tube; these include *dorsalin-1* (*dsl1*; refs. 40 and 42), *Wnt1* and *Wnt3a* (46–48), and *Slug* (40, 49).

We have investigated whether neural plate–epidermal interactions influence the expression of three genes (*Slug*, *Wnt1*, and *Wnt3a*) that are expressed in dorsal neural tube (39). Tissue recombinations were performed both *in ovo* and in collagen gels maintained in serum-free medium (Fig. 4). When cultured alone, epidermis, stage 4 prospective neural plates, and stage 8–10 neural plates did not express any of the assayed genes. In contrast, *Slug* transcripts were detected by *in situ* hybridization 24 hr after juxtaposing stage 4 prospective neural plates and epidermis: neither *Wnt1* nor *Wnt3a* transcripts were expressed in these recombinants. Since combinations of stage 4 neural plates and epidermis have been shown to produce neural crest cells (15), we conclude that *Wnt1* and *Wnt3a* are not required for the genesis of (all) neural crest cells. When older (stages 8–10) neural plates were combined with epidermis, expression of all three transcripts was induced. Therefore, expression of these mRNAs in dorsal neural tube cells (or cells of the lateral neural plate) is likely to be due to their proximity to nonneural (epidermal) ectoderm. Similar experiments conducted in the laboratory of Jessell (40) have confirmed our results showing that epidermis can induce *Slug* expression in neural plate explants. Furthermore, these workers have extended the analysis to *Pax3*, *Msx-1/2*, and *dsl1*, finding that neural plate–epidermal interactions can induce expression of all of these genes.

In addition to the inductive influences of the epidermis, dorsal neural tube genes appear to be regulated by inhibitory signals from the notochord. For example, the expression of transcripts for *Pax3* (40, 44) *Msx* (40), *Dsl1* (40, 42), *Slug* (40), and *Wnt1* (39) is inhibited by the notochord. Inhibition of expression of some transcripts by the notochord appears to be

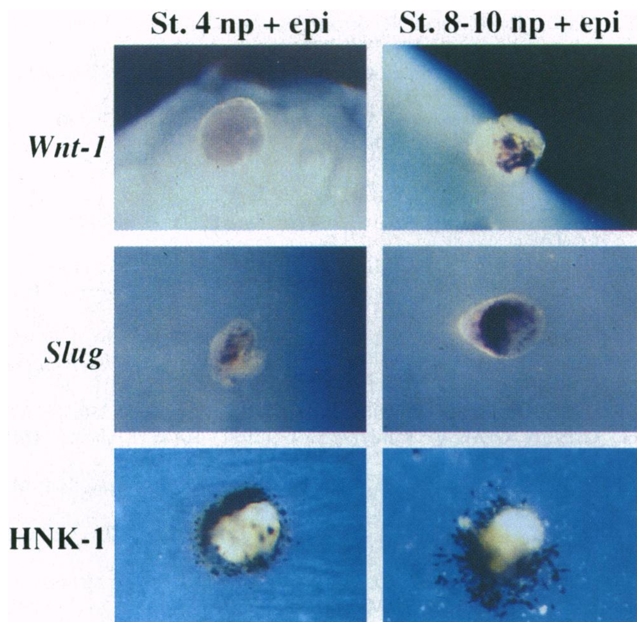


FIG. 4. Neural plate (np) from stage 4 or stage 8–10 embryos were combined with presumptive epidermis (epi) and grown in defined media within collagen matrix gels; after 24–48 hr, they were analyzed for the expression of *Wnt-1*, *Slug*, and HNK-1 epitope expression. Recombinants made from stage 4 neural and epidermis had no *Wnt-1* expression, but did express *Slug* and the HNK-1 epitope, indicating that neural crest cells had been induced. However, *Wnt-1* expression was observed after similar recombinants were made between stage 8–10 intermediate neural plate and epidermis, which also contained neural crest cells, as assessed by *Slug* and HNK-1 reactivity. These experiments suggest that the ability of the neural plate to respond to epidermal interactions changes as a function of time.

direct: for other genes, the notochord may act indirectly by antagonizing the action of the epidermis.

## Regulative Ability of the Neural Tube to Form the Neural Crest

Experiments performed in our laboratory have shown that ventral neural tube cells at some axial levels retain the ability to form neural crest cells after surgical ablation of the dorsal neural tube. We have ablated the cranial neural folds, and by labeling the tissue flanking the rostral, caudal, or ventral margins of the ablated region, we have examined the ability of these adjacent neural tube cells to compensate for the loss of endogenous crest (50). We found that the neural tube cells immediately ventral to the ablation regulate to form a migratory cell population that colonizes neural crest-derived structures. This regulation following ablation is most robust at the level of the caudal midbrain and rostral hindbrain and is temporally regulated, occurring maximally at the 3–4 somite stage and declining after the 5–6 somite stage (51). Interesting, this slightly precedes the onset of neural crest cell emigration, which occurs at the 6–7 somite stage.

In examining molecular markers that are induced after ablation, our results suggest that the zinc finger transcription factor, *Slug*, is up-regulated at the cut edge, probably by interactions with the overlying ectoderm (51). Up-regulation of *Slug* appears to be an early response to ablation, with *Slug* transcripts appearing proximal to the ablated region 5–8 hr after surgery and before emergence of neural crest cells. Our results suggests that neural tube cells normally destined to form CNS derivatives can adjust their prospective fates to form peripheral nervous system and other neural crest derivatives until the time of normal onset of neural crest cell emigration from the neural tube. Because neural crest regeneration occurs only after apposition of the remaining neuroepithelium with the epidermis, the developmental mechanism underlying regeneration of the neural crest appears to recapitulate initial generation of the neural crest.

## Other Neural Crest-Generating Interactions

Some early studies on the development of neural crest in the axolotl have indicated that cell fate decisions of the ectoderm are influenced by the underlying archenteron roof. By grafting fragments of archenteron into the blastocoel, Raven and Kloos (52) showed that medial archenteron roof induces the formation of neural tissue and neural crest from competent ectoderm, while the archenteron lying subjacent to the neural folds induces the formation of neural crest only (see also ref. 53). Raven and Kloos (52) proposed that the archenteron roof produces a neural/neural crest "evocator" in a graded manner, such that lateral archenteron produces low levels of evocator that are sufficient to induce neural crest but not brain, while medial archenteron produces more evocator which induces both cell types. Of interest in this regard are some recent experiments by Kengaku and Okamoto (19), who have shown that basic fibroblast growth factor is able to induce both CNS neurons and melanophores (neural crest) from ectoderm of gastrula stage *Xenopus* embryos, acting like the "evocator" proposed by Raven. While there is no evidence concerning a graded secretion of basic fibroblast growth factor by the archenteron roof, Kengaku and Okamoto (19) find that temporal changes in the ectoderm can affect the neuron/melanophore ratio. For instance, basic fibroblast growth factor-treated ectoderm will give rise to CNS neurons and a few melanophores when isolated from stage 9+ to 10 embryos, while "older" ectoderm, taken from stage 10 embryos, will generate mostly (or only) melanophores after similar treatment. Mayor and colleagues (18) have extended previous studies by investigating the neural crest- inducing ability of

mesoderm and some growth factors thought to be important for early development in *Xenopus*. They find that a combination of noggin and FGF are able to induce neural crest in gastrula ectoderm, although mesodermal markers are also induced in such explants.

How do these experiments fit with findings that neural crest cells are generated by neural plate–epidermal interactions? Assuming that neural crest genesis in amphibians is similar to that in avian embryos, one possibility is that the neural crest cells seen after grafting archenteron or mesoderm, or following growth factor treatment, are not induced directly, but as a consequence of induced neural tissue interacting with the surrounding epidermis. Even when neural crest cells are generated in the apparent absence of neural tissue, one cannot eliminate the possibility that all induced neural tissue is subsequently converted to neural crest (54). More importantly, recent experiments by Mancilla and Mayor (55) have shown quite clearly that neural plate–epidermal interactions can induce expression of *Slug* and generate neural crest cells in *Xenopus* embryos, confirming earlier findings (34–36).

**Conclusions and Future Directions**

Good progress has been made toward understanding the genesis of neural crest cells by combining the techniques of experimental embryology with those of molecular biology. Experimentally juxtaposing embryonic tissues can reveal what tissue interactions are important for the expression of gene products thought to play a role in neural crest formation. By perturbating gene expression in whole embryos or in tissue fragments and assaying for the presence or absence of neural crest and specific derivatives, it is possible to determine which genes are involved in the formation of neural crest cells. However, neural crest formation is not likely to be a one-step event, but may involve many different steps, each of which is under the control of distinct sets of genes. Therefore, to understand the precise role of a gene in neural crest formation, we need to know the separate cellular events that occur during neural crest ontogeny. For instance, neural crest formation requires conversion of the epithelial cells of the neural tube to cells with a mesenchymal phenotype, and this process has itself formed the basis of much research (for review, see ref. 56; refs. 57–59). Erickson and Perris (60) identify a number of the processes thought to be critical for initiating neural crest emigration from the dorsal neural tube, including disruption of the basement membrane of the dorsal neural tube and deposition of a migratory substratum dorsal to the neural tube, in addition to the events associated with epithelial-to-mesenchymal conversions.

In addition to the "cell biological" aspects of their formation, genesis of neural crest cells may also involve processes measured in developmental terms, such as cell commitment and developmental potential. One extreme view is that neural crest cells may be no more than migratory neural tube cells, with no change in potential accompanying their emigration from the neural tube. At the other extreme, neural crest formation may involve commitment of migratory cells to a distinct neural crest fate. Experiments are currently in progress to distinguish between these possibilities.

Throughout this discussion, we have assumed that neural crest cells generated from the dorsal neural tube are a homogeneous population with the potential to differentiate into all neural crest derivatives. While our lineage analyses (12–14) and the *in vitro* work of others (61–65) support this view, we cannot eliminate the possibility that some neural crest precursors are more restricted in potential (see refs. 66 and 67). In fact, a number of workers have reported heterogeneity in neural crest precursors (67). If this is so, the formation of neural crest cells might involve a number of different tissue interactions and/or a variety of genes, each of which is important for the formation of a distinct neural crest precursor.

While the genesis of neural crest cells might be exquisitely simple, we must allow for the possibility that this fundamental developmental event is as complicated and involved as other early processes of mesoderm and neural induction.

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