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# Timing and pattern of cell fate restrictions in the neural crest lineage

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## SUMMARY

**The trunk neural crest of vertebrate embryos is a transient collection of precursor cells present along the dorsal aspect of the neural tube. These cells migrate on two distinct pathways and give rise to specific derivatives in precise embryonic locations. One group of crest cells migrates early on a ventral pathway and generates neurons and glial cells. A later-dispersing group migrates laterally and gives rise to melanocytes in the skin. These observations raise the possibility that the appearance of distinct derivatives in different embryonic locations is a consequence of lineage restrictions specified before or soon after the onset of neural crest cell migration.**

**To test this notion, we have assessed when and in what order distinct cell fates are specified during neural crest development. We determined the proportions of different types of precursor cells in cultured neural crest populations immediately after emergence from the neural tube and at intervals as development proceeds. We found that the initial neural crest population was a heterogeneous mixture of precursors almost half of which generated single-phenotype clones. Distinct neurogenic and melanogenic sublineages were also present in the outgrowth population almost immediately, but melanogenic precursors dispersed from the neural tube only after many neurogenic precursors had already done so. A discrete fate-restricted neuronal precursor population was distinguished before entirely separate fate-restricted melanocyte and glial precursor populations were present, and well before initial neuronal differentiation. Taken together, our results demonstrate that lineage-restricted subpopulations constitute a major portion of the initial neural crest population and that neural crest diversification occurs well before overt differentiation by the asynchronous restriction of distinct cell fates. Thus, the different morphogenetic and differentiative behavior of neural crest subsets in vivo may result from earlier cell fate-specification events that generate developmentally distinct subpopulations that respond differentially to environmental cues.**

Key words: neural crest, cell fate, lineage, quail, cell migration, melanocyte

## INTRODUCTION

The regulation of cell diversification is a central issue in developmental biology. Embryonic cells become different from one another by a process called specification (Davidson, 1990; Kimmel et al., 1991). As cells are specified they become fate restricted, generating only a subset of possible descendants of the lineage. Although it is important to emphasize that fate restriction does not necessarily imply irreversible commitment, it is generally thought that embryonic precursor cells undergo cell fate restrictions before they overtly express distinct cellular phenotypes. In lineages in which multiple cell types are derived from a seemingly homogeneous precursor population, understanding the mechanisms that regulate cell fate specification requires that both the identity of cells with restricted developmental fates and the timing of their appearance be determined. In vertebrate embryos, the neural crest is one of the more tractable of several embryonic populations that has been studied to understand lineage diversification (Le Douarin et al., 1993; Selleck et al., 1993; Stemple and Anderson, 1993; Weston, 1991).

The neural crest in the trunk region of vertebrate embryos is formed when cells derived from the dorsal portion of the neural tube collect along its dorsal-lateral surface. During embryogenesis, neural crest cells migrate along two spatially discrete pathways and give rise to peripheral neurons, glial cells and melanocytes in precise locations (Le Douarin, 1982). Neural-crest-derived cells do not undergo overt differentiation until after they disperse. Consequently, it has been unclear whether the appearance of differentiated cells in precise locations is the result of the differential localization of developmentally distinct precursors or whether factors in these locations instruct developmentally ambiguous precursors to adopt specific fates. A major unresolved question then is when the fates of individual neural crest cells become different from one another.

Clonal analysis has been used extensively to examine the differentiative behavior of individual neural crest cells. In vitro clonal analysis, in which single crest cells were isolated by limit dilution, has demonstrated that the initial neural crest population contains totipotent precursors, as well as precursors that generate a limited range of derivatives and fate-restricted

precursors that give rise to a single cell type (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988, 1991; Sieber-Blum, 1989; Dupin et al., 1990; Ito and Sieber-Blum, 1991; Deville et al., 1992, Stemple and Anderson, 1992). However, previous in vitro clonal analysis experiments involve secondary cultures of individual cells derived from primary neural tube outgrowth populations. In such culture experiments, it was not possible to establish that the clonal progenitors characterized in secondary culture were representative of the initial primary outgrowth population. Consequently, the proportion of the initial neural crest population represented by specific classes of precursors could not be determined.

In addition to uncertainty as to the precursor composition of the initial neural crest population, very little is known about the timing and order of appearance of developmentally distinct precursors prior to overt differentiation (Vogel and Weston, 1988; Stemple and Anderson, 1992; Raible and Eisen, 1994). In the absence of methods to determine the proportions of different types of precursors in neural crest populations at multiple times during development, the precise way by which interactions between environmental cues and a heterogenous neural crest cell population result in neural crest diversification has remained unresolved.

To learn when and how neural crest cell fates change during development, we have determined the proportions of different types of precursors present in cultured populations, both immediately after crest cells leave the neural epithelium and at intervals prior to overt differentiation. To do so, we performed clonal analysis by intracellularly labeling individual neural crest cells in primary neural crest cell cultures with lineage dye and growing the cells under enriched culture conditions that permitted the differentiation of all three classes of trunk crest derivatives – neurons, glial cells and melanocytes. Further, by showing that the proportions of different cell types generated by labeled clonal progenitors is equivalent to the proportion of these derivatives produced in the population as a whole, we have confirmed that our sampling procedure provides a valid estimate of the precursor composition in crest populations at various times.

Our results indicate that almost half of the initial neural crest population comprises fate-restricted precursors that generate a single cell type. Further, completely distinct neurogenic and melanogenic sublineages are present almost immediately, but melanogenic precursors emerge from the neural tube only after many neurogenic precursors have done so. In older outgrowth populations, we found that virtually all neurons that subsequently differentiated in the culture were derived from fate-restricted neuronal precursors. However, these precursors became fate restricted well before overt neuronal differentiation and before entirely distinct fate-restricted melanocyte or glial precursor populations arose. Thus, in a presumably homogeneous culture environment that supports the differentiation of all three derivatives, fate-restricted precursors are initially abundant, neurogenic and melanogenic sublineages rapidly segregate, and crest cell fates are asynchronously specified. Taken together, these results support the notion that intrinsic differences between early neural crest cells play an important role in the process of neural crest diversification. Thus, the appearance of distinct neural crest derivatives in different embryonic locations may be a consequence, in large part, of the segregation of developmentally distinct precursor subpopulations prior to migration.

## MATERIALS AND METHODS

### Neural crest cultures

Embryonic neural tubes isolated from the level of the last seven somites of stage 11-12 (Zacchei, 1961) quail embryos were explanted as previously described (Girdlestone and Weston, 1985). Briefly, dissected neural tubes were enzymatically isolated from surrounding tissues by incubation in pancreatin (Gibco) and trituration in a fire-polished Pasteur pipette. The isolated neural tubes were washed with culture medium (see below), cut into 3-5 pieces, and explanted to 5 mm Sylgard (Dow Corning) tissue culture well inserts (5-7 neural tube pieces/well) on tissue culture plastic (Corning), which is permissive for neural crest cell attachment and dispersal (Girdlestone and Weston, 1985; Vogel and Weston 1988). All labeling experiments were performed on cells in the primary neural tube outgrowth. The cultures were grown for a total period of 96 hours after neural crest cells first emerged from the neural tube. In most cases, the neural tube explant was removed with a tungsten needle after 24 hours.

All cultures were grown in our control medium, QMED, and half the volume of medium in every culture was replaced every day. QMED is a Ham's F12-based medium, supplemented with 15% fetal bovine serum (Hyclone) and 4% E10 chicken embryo extract (see Henion and Weston, 1994; Henion et al., 1995). Several different batches of serum and embryo extract were tested for optimal ability to support neural crest cell survival and the differentiation of neurons, glial cells and melanocytes, and the selected batch of both was used for all the experiments reported here.

In one set of experiments, we specifically isolated populations of neural crest cells that dispersed from the neural tube during discreet periods. Thus, 0-6 hour populations were produced by removing neural tube pieces from outgrowth cultures with tungsten needles 6 hours after the first neural crest cells emerged from the neural tubes. To produce 6-16 hour outgrowth populations, the neural tubes removed at 6 hours were replated in separate cultures for an additional 10 hours, then removed with tungsten needles and discarded. Control (0-24 hour) neural crest populations were produced by removing neural tubes with tungsten needles from cultures 24 hours after the initial emergence of neural crest cells from the neural tubes. The resulting populations (0-6 hour, 6-16 hour and 0-24 hour) were then allowed to differentiate (96 hours) and the percentages of melanocytes (cells that contained melanin granules) in the resulting populations were determined by random cell counts as previously described (Vogel and Weston, 1988).

### Single cell labeling

In order to determine the differentiative behavior of individual neural crest cells in cultured populations at intervals after emergence from the neural tube, we intracellularly labeled cells in the primary culture population using glass microelectrodes containing a solution of 6% lyseinated rhodamine dextran (Fluoro-Ruby; Molecular Probes) in 0.2 M KCl. Single, randomly chosen neural crest cells were labeled at one of three time periods as follows: a culture was placed on an inverted fluorescence microscope and the electrode was lowered with a micromanipulator until it just punctured the surface of the medium in the culture well. The tip of the electrode was brought into focus, which was well above the focal plane of the cells below. The electrode and focal plane were lowered to the level of the cells. The cell located just below the electrode tip, or when a cell was not immediately below the electrode tip, the nearest cell to the tip, was then impaled and iontophoretically injected with dye. The electrode was then extracted from the injected cell. To insure that only one cell was labeled, each injected culture was viewed within 3 hours of injection using phase-contrast optics and low intensity fluorescence (Rhodamine filter) with the aid of an image intensification system.

Single cells in more than 420 cultures were injected. About 60% of these cells failed to survive the injection procedure. Such cells were seen to have died within the first 3 hours after injection, presumably

because of irreparable disruption of the plasma membrane by the electrode, or because excess dye was accidentally injected. However, virtually all (ca. 98%) of the cells that survived the injection procedure produced clones that could be readily identified and phenotypically characterized at the end of the 4-day culture period. It should be emphasized that the injected cells were not subcultured and the injection procedure was not selectively lethal, since the proportion of phenotypes exhibited by labeled cells corresponded to that of the population as a whole (see Results; Table 1). In consequence, the marking method provided an unbiased sampling procedure for assessing the proportion of different progenitors within the crest cell outgrowth population at intervals after emergence from the neural tube explant.

Cells of the primary outgrowth of explanted neural tubes were labeled at one of three time periods. In early experiments, cells were injected at the beginning of (1-6 hours) and after (30-36 hours) the period of outgrowth from the explanted neural tube. As reported below, we learned that about half of the cells at the 1-6 hour labeling interval were already fate restricted, whereas the vast majority of labeled cells were fate restricted by 30-36 hours. Therefore, to assess efficiently the time course of cell fate restriction, we selected an intermediate sampling period (13-16 hours) that was roughly the duration of one cell cycle (Maxwell, 1976) after the first sampling period, rather than the midpoint between the two previous sampling periods.

### Immunocytochemistry

In all experiments, cultures were grown for 96 hours after the first neural crest cells emerged from the neural tube explants (0 hour). Cultures were fixed in 4% paraformaldehyde and cell phenotypes determined with visible markers (melanin granules) or cell-type-specific antibodies (Fig. 2). All antibodies were diluted in a buffer containing 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3, 0.1% sodium azide, 2% BSA, and 0.3% Triton X-100 (Henion and Landis, 1992). Neurons were identified by immunoreactivity (IR) with the 16A11 monoclonal antibody (mAb), which recognizes neuron-specific vertebrate ELAV-related proteins (Yao et al., 1993; Marusich et al., 1994) and is an early pan-neuronal marker of neural-crest-derived neurons (Marusich et al., 1994). Glial cells were identified by p75-immunoreactivity (Tanaka et al., 1989) in non-pigmented cells that did not co-express 16A11-IR, or by the unique expression of mAb 7B3. In embryo sections, mAb 7B3 recognizes satellite glia and Schwann cells, but not neurons or melanocytes, and recognizes virtually all non-neuronal (>98%) cells in differentiated neural crest cultures that are not melanocytes. A detailed description of mAb 7B3 will be presented elsewhere (G. Blyss, P. D. Henion and J. A. Weston, in preparation). All antibodies were detected with fluorescein isothiocyanate and aminomethyl coumaric acid-conjugated secondary antibodies.

It is important to emphasize, that the cell-type-specific markers that we used to classify the clonal progenitors were ‘generic’. Therefore, although we were able to designate cells as fate-restricted precursors, such cells can still differentiate along a number of pathways. Thus, neuronal populations derived from fate-restricted neuronal precursors could ultimately contain a number of different neuronal phenotypes including different sensory (e.g. Marusich et al., 1986) and sympathoadrenal (e.g. Hökfelt et al., 1980; Anderson and Axel, 1986; Vogel and Weston, 1990) cells. Likewise, it should be emphasized that glial progenitors ultimately differentiate as either satellite glia and Schwann cells.

## RESULTS

### Clonal analysis strategy and verification of the sampling procedure

To determine the identities and proportions of different types of precursors in cultured neural crest populations, an individual cell within a population of neural crest cells was labeled at random by intracellular injection of lineage dye (Fig. 1A,B; see Materials and Methods). This allowed descendants of clonal progenitors to be unambiguously identified (Fig. 1C,D), while also allowing extensive cell-cell interactions between both clonally related and unrelated crest cells. Neural crest cell populations were cultured in a nutrient environment that was known to permit the differentiation of all three major derivatives (neurons, glial cells and melanocytes) on a time course that approximates that in the embryo (Vogel and Weston, 1988; Marusich et al., 1994). The differentiated phenotype(s) of the labeled clonal descendants at the end of experiments were identified with cell-type-specific markers (Fig. 1E-G). These phenotype(s) were used to classify clonal progenitors (Fig. 2). Finally, crest cell populations were sampled at intervals, so that changes in the precursor composition of the population could be determined (Fig. 1H).

The sequential clonal analysis procedure that we have developed fulfills the requirements for quantitatively assessing changes in the precursor composition of neural crest populations during in vitro development. Dilution of dye did not appear to limit detection of clonal progeny since labeled precursors produced readily detectable clones ranging in size from 1 to 114 cells after 4 days of culture. Importantly, the percentages of the different cell types derived from precursors labeled in later (13-16 hour and 30-36 hour) outgrowth populations were not significantly different from the percentages of derivatives generated by unlabeled populations in control cultures (Table 1). Therefore, since the dye-injection procedure itself was not selectively lethal and did not alter the differentiative behavior of labeled cells, this method appears to provide an unbiased assessment of the composition of the crest cell population, in which the labeling frequency of specific progenitors accurately reflects the proportion of such progenitors in the population. Moreover, sampling the outgrowth population at intervals provided accurate information about changes in the proportions of developmentally distinct precursors that occurred in the population.

### Heterogeneity of the neural crest

The initial neural crest cell population was defined as the population of neural crest cells that had emerged during the first 6 hours of primary outgrowth from the neural tube explant (Fig. 1). We found that this population (1-6 hours; Table 2) is already a heterogeneous mixture of cell types. Importantly, the percentages of different precursor types present were unequal. Although a slight majority of clonal progenitors in the initial population gave rise to more than one cell type, a significant proportion of cells (44.5%) behaved as fate-restricted precursors, generating a single cell type, either neurons, glial cells or melanocytes. Most of these were fate-restricted glial cell precursors (31.5%), but a significant proportion of fate-restricted neuronal precursors (11.1%) was also present.

The clonal descendants of labeled precursors in the initial neural crest population (1-6 hour) included significantly fewer melanocytes than in control (unlabeled) populations (Table 1). This apparent discrepancy does not appear to reflect a non-random or inadequate sampling of the population since identical sampling methods and numbers of clonal progenitors labeled at two subsequent time intervals gave rise to proportions of melanocytes that correspond well to unlabeled crest cell populations in control cultures (Table 1). Nor does it reflect defi-

ciencies in the culture environment since differentiated melanocytes were present in the cultures. Rather, the clonal analysis result suggests that the first neural crest population to emerge from the neural tube generally lacks melanogenic precursors. If this inference were valid, outgrowth populations that emerge from explanted neural tubes only during the first 6 hours should generate few melanocytes, whereas the crest cell outgrowth population that emerged from the neural tube later (e.g. after 6 hours) should produce many melanocytes. To test these predictions, early outgrowth populations were isolated by removing the neural tube explants 6 hours, instead of the normal 24 hours after the initial emergence of neural crest cells. We found that very few melanocytes were present in cultures derived from populations that lacked the contribution of later dispersing cells (Table 3). We then determined the proportion of melanocytes that was derived from populations of neural crest cells that dispersed from the neural tube between 6 hours and 16 hours. To do so, neural tubes removed from explant cultures 6 hours after the initial emergence of neural crest cells were re-explanted to separate cultures for an additional 10 hours. The neural tube explants were then removed, and the resulting neural crest populations (6-16 hours) were allowed to differentiate. In contrast to 0-6 hour populations, 6-16 hour populations generated disproportionately large numbers of melanocytes (Table 3). We conclude, therefore, that most melanogenic precursors leave the neural tube after a delay of at least 6 hours relative to the emergence of the first neurogenic precursors from the neural tube.

## Segregation of neurogenic and melanogenic sublineages

Distinct neurogenic and melanogenic sublineages were present soon after neural crest cells leave the neural tube. Thus, virtually all neurons present in the 4-day culture were derived from neuron-glial (NG) or fate-restricted neuronal (N) precursors. Likewise, the vast majority of melanocytes were derived from glial-melanocyte (GM) or fate-restricted melanocyte (M) precursors. We found no clonal progenitors that gave rise to both neurons and melanocytes when individual neural crest cells were labeled 13-16 hours or 30-36 hours after emergence from the neural tube (Table 2). In fact, based on the sampling results, the initial (1-6 hour) outgrowth population contained only a small proportion (<2%) of progenitors that gave rise to both neurons and melanocytes. In this case, the clone generated by a single labeled precursor also contained glial cells (NGM; Table 2). Thus, the segregation of

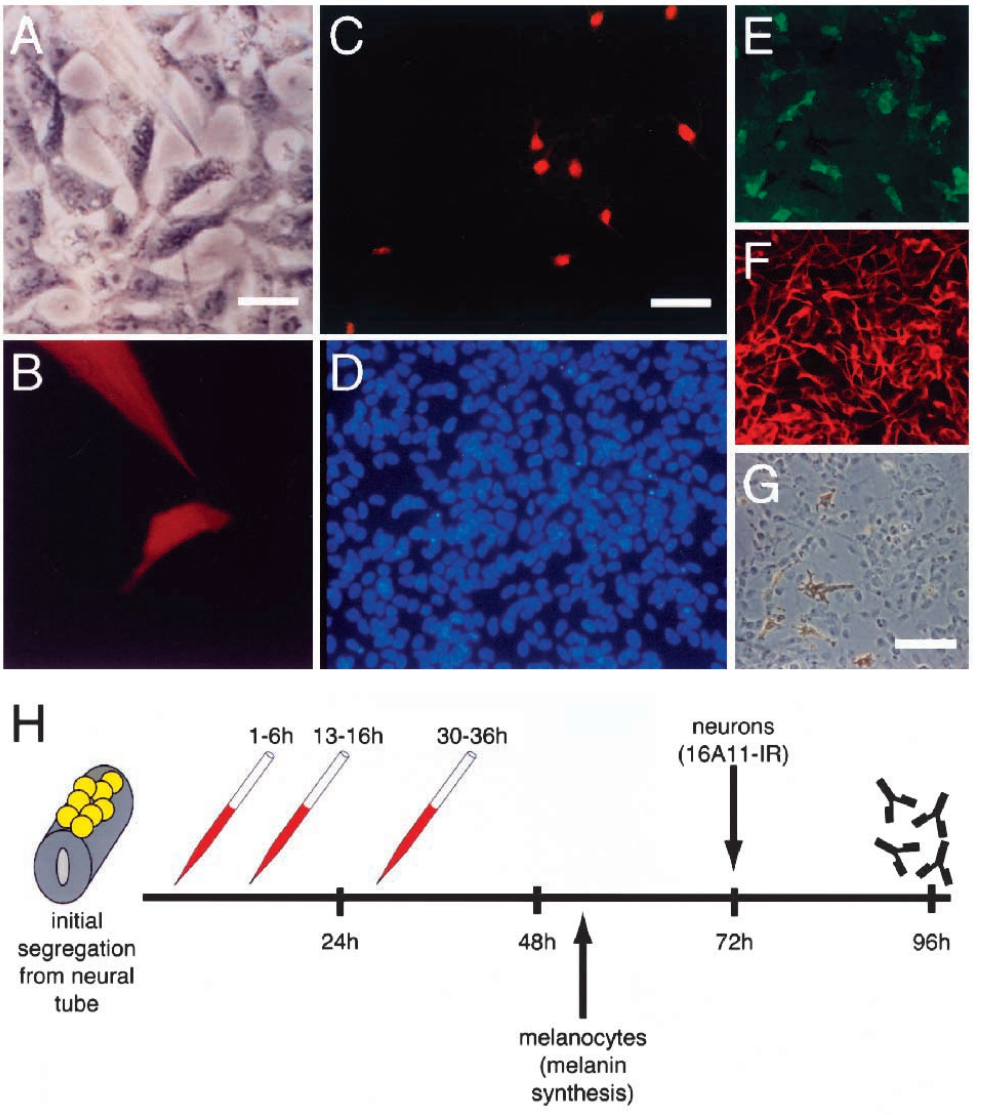


**Fig. 1.** Sequential clonal analysis. To determine the precursor composition of cultured neural crest cell populations, an individual neural crest cell in a population derived from explanted neural tubes (A) was labeled intracellularly at random with lysinated rhodamine dextran (B, see Methods). All cultures were allowed to develop for 96 hours after the first neural crest cells emerged from the neural tube (see Methods), at which time virtually all cells (>98%) had differentiated. At this time, clonal progeny of a labeled cell (C) were readily identifiable (D, same field as C labeled with DAPI). The phenotypes of clonal descendants were determined at the end of experiments (96 hours) by the expression of cell-type-specific markers. (E-G) The same field of a culture at 96 hours shows neurons identified with the monoclonal antibody (mAb) 16A11, (E; Marusich et al., 1994), glial cells labeled with the mAb 7B3 (F, see Methods) and melanocytes were identified by the presence of melanin (G). To assess changes in the differentiative behavior of clonal progenitors during development, cultured populations were surveyed, as described above, at one of three successive times after segregation from the neural tube and well before the initial expression of differentiated properties (H). Scale bars: A, 4.1 μm; C, 24 μm; G, 27.1 μm.

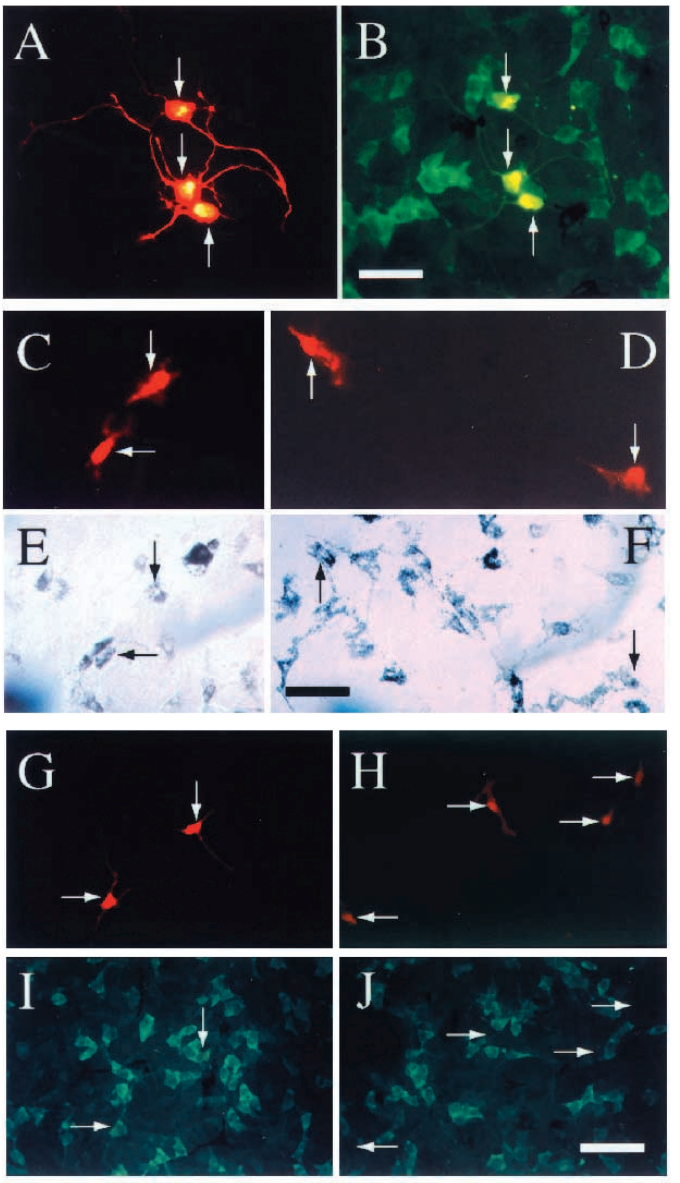


**Fig. 2.** Examples of clones. (A,B) The clonal descendants at 96 hours of a cell labeled 4 hours after the initial emergence of neural crest cells from the neural tube. The three cells containing LRD (A) express neuron-specific 16A11-IR (B). Thus, the precursor cell was classified as a fate-restricted neuronal precursor. (C-F) Two fields of the same culture showing the clonal descendants at 96 hours of a precursor cell labeled at 14 hours. All four labeled cells (C,D) contain melanin granules, thus the precursor was classified as a fate-restricted melanocyte precursor. (G-J) Two fields of the same culture showing the clonal descendants (G,H) at 96 hours of a precursor labeled at 13 hours, and the same fields as in G and H labeled with the neuronal marker mAb 16A11 (I,J). The two cells in G are 16A11-IR, whereas the four cells in H are not, and are in fact glial cells (7B3-IR, not shown). Therefore the precursor of this clone was classified as a neuron-glial precursor. Scale bars: B, 14.2 μm; F, 14.2 μm; J, 26.1 μm.

distinct neurogenic and melanogenic sublineages appears to occur during the earliest stages of neural crest development.

In contrast to the initial (1-6 hour) neural crest cell population, numerous melanogenic precursors were present in the population just a few hours later (13-16 hours; Table 2). Most of these behaved as fate-restricted melanocyte precursors and gave rise to smaller clones than glial-melanocyte precursors (Tables 2, 4, 5). Importantly, the relative number of neurogenic cells (including fate-restricted neuronal precursors and neuron-glial precursors) and fate-restricted glial (G) precursors present at 13-16 hours were very similar to those observed at 1-6 hours. The decrease in the frequency of labeling these types of precursors between the 1-6 hour and 13-16 hour sampling periods results from the appearance of numerous melanogenic cells in the population after 6 hours (Tables 2, 3). Thus, although neurogenic and melanogenic sublineages have become distinct by 13-16 hours, segregation of neuronal and glial fates has yet to be completed.

**Table 1. Populations derived from clonal progenitors**

| | Percentage of phenotypes | | | |
|---|---|---|---|---|
| Phenotype* | Control† | 1-6 hours | 13-16 hours | 30-36 hours |
| Neurons | 21±7 | 38 | 15 | 22 |
| Glial cells | 62±11 | 61 | 62 | 67 |
| Melanocytes | 17±7 | 1 | 23 | 11 |
| $\chi^2$‡ | – | 27.013; ($P$<0.001) | 3.428; ($P$>0.100) | 2.569; ($P$>0.250) |

*The phenotypes of the clonal descendants of 54 precursors labeled at each of three time points were determined (total of 1624 cells generated by 162 labeled precursors). The data represent the percentage of all cells (at 96 hours) generated by precursors labeled during the indicated time that were either neurons, glial cells, or melanocytes.

†Control values represent the mean ± range of phenotypes present in unlabeled cultures. 3,345 cells were counted in 5 separate experiments.

‡$\chi^2$ analysis confirmed that there was no significant difference between the proportions of the three phenotypes in control cultures and in the clonal progeny of cells sampled at 13-16 hours and 30-36 hours. In contrast, the proportions of phenotypes in control cultures and the clonal progeny of cells sampled at 1-6 hours are significantly different ($P$<0.001).

## Segregation of neuronal and glial fates

When the neural crest population was sampled 30-36 hours after emergence from the neural tube, virtually all the labeled cells that produced neurons were fate-restricted neuronal precursors (94%, see Table 4) whereas very few neuron-glial precursors were observed (Tables 2, 4). Since the relative numbers of neuron-glial precursors changed little between 1-6 hours and 13-16 hours, the specification of distinct fate-restricted neuronal and glial precursors from neuron-glial progenitors seems to occur between 13-16 hours and 30-36 hours (Tables 2, 4). Since no cells expressing neuronal markers are present in 30-36 hour populations, and significant numbers of neuronal cells can only be detected after 72 hours (not shown; but see Vogel and Weston, 1988; Marusich and Weston, 1992; Henion and Weston, 1994), neuronal and glial fates segregate well before overt neuronal differentiation. Importantly, fate-restricted neuronal precursors were still mitotically active, as illustrated by the fact that the vast majority of neuronal clones contained more than one cell (Table 5).

By 30-36 hours, the neural crest population is composed primarily (87%) of fate-restricted precursors (see Table 4). Although neuron-glial precursors were virtually extinct by this time (Table 2), numerous glial-melanocyte precursors were still present. Therefore, the segregation of melanocyte and glial fates does not occur until after the segregation of neuronal and

**Table 2. Temporally defined precursor-type composition of neural crest cell populations**

| | Percentage of total clones | | |
|---|---|---|---|
| Precursor type | 1-6 hours | 13-16 hours | 30-36 hours |
| N | 11.1 | 9.3 | 24.1 |
| NG | 44.4 | 20.4 | 3.7 |
| NGM | 1.9 | 0 | 0 |
| NM | 0 | 0 | 0 |
| G | 31.5 | 24.1 | 48.1 |
| GM | 9.2 | 7.4 | 9.3 |
| M | 1.9 | 38.8 | 14.8 |

Labeled clonal precursors were classified (Precursor type) according to the phenotype of cells that they generated as determined by the expression of cell-type-specific markers after 96 hours of culture (see Figs 1, 2). These phenotypes identify both the precursor and the resulting clonal descendants, as follows:

N, neuronal; NG, neuron-glial; NGM, neuron-glial-melanocyte; NM, neuron-melanocyte; G, glial; GM, glial-melanocyte; M, melanocyte.

The clonal descendants of 54 individual precursor cells labeled at each time-point were analyzed (total of 162 clones). The data are presented as the percentage of all labeled precursors represented by each precursor type at the indicated time.

glial fates, despite the fact that, in our culture conditions, the initial appearance of differentiated melanocytes precedes neuronal differentiation (see Fig. 1H).

In the melanogenic lineage, the proportion of fate-restricted melanocyte precursors in the outgrowth population decreased between the 13-16-hour and 30-36 hour sampling periods (Tables 2, 4). The decrease in the proportion of fate-restricted melanocyte precursors during this period can be explained by the observation that glial-melanocyte precursors generate larger clones, and therefore divide more rapidly than fate-restricted melanocyte precursors (Table 5). Thus, the abrupt appearance of numerous fate-restricted melanocyte precursors that accounts for a large proportion of both the total number of precursors (Table 2) and the majority of melanogenic precursors (Table 4) at 13-16 hours is subsequently diluted by the more mitotically active glial-melanocyte precursors. We have not yet determined how long glial-melanocyte precursors persist in our cultured neural crest populations.

## DISCUSSION

Because we were able to determine the precursor composition of trunk neural crest cell populations both immediately after they emerge from the neural tube and at intervals thereafter, our results allow us to make five main conclusions. (1) Almost half of the neural crest population that first emerges from the explanted neural tube consists of fate-restricted precursors that give rise to a single cell type. (2) Neurogenic (N + NG) and melanogenic (M + GM) sublineages segregate almost immediately. (3) Melanogenic precursors generally emerge from the neural tube after neurogenic precursors. (4) Different neural crest cell fates are specified asynchronously, relative to one another and relative to emergence from the neural tube. (5) All of these sublineages and fate-restricted precursors arise well before overt differentiation of the various classes of crest cell phenotypes in a permissive culture environment.

**Table 3. Delayed dispersal of melanogenic precursors**

| | Percentage of total population | | |
|---|---|---|---|
| | 0-6 hours | 6-16 hours | 0-24 hours |
| % melanocytes | 2.6±1 | 30.4±3 | 17±7 |

The melanogenic ability of neural crest populations that dispersed from neural tubes between 0-6 hours, 6-16 hours, and 0-24 hours were determined (see Results and Methods). Populations derived from neural crest cells that dispersed from the neural tube in neural tube explant cultures between 0-6 hours, 6-16 hours and 0-24 hours were allowed to differentiate (96 hours) and the percentages of melanocytes in the resulting populations were determined. The results are presented as the mean ± range of at least 1,500 cells counted randomly (Vogel and Weston, 1988) in no fewer than 3 sets of experiments with at least 4 wells per experiment.

**Table 4. Percentages of fate-restricted precursors**

| | Percentage of labeled precursors | | |
|---|---|---|---|
| Phenotype | 1-6 hours | 13-16 hours | 30-36 hours |
| N | 12.8 | 24.0 | 94.0 |
| G | 33.9 | 27.0 | 86.0 |
| M | 18.2 | 90.0 | 50.0 |
| Total population | 44.5 | 72.2 | 87.0 |

The data represent the percentage of labeled precursors at one of three time points (1-6 hours; 13-16 hours; 30-36 hours) that gave rise to cells of a single phenotype (N, G, or M). For example, 12.8% of the cells labeled at 1-6 hours that gave rise to neurons were fate-restricted N precursors. For the total population, the numbers indicate the percentage of all precursors at the indicated time that were fate-restricted. N, neuronal; G, glial; M, melanocyte.

### Abundance of fate restricted neural crest cells

Our quantitative analysis revealed that almost half of the nascent neural crest population comprises fate-restricted precursors. The fact that very unequal proportions of different types of precursors were present, even in a presumably uniform cell culture environment in which all three derivatives readily differentiate, argues against the possibility that these precursors arose at random from developmentally unbiased cells. Further, the observed heterogeneity of growth factor receptor expression in the premigratory neural crest in vivo (Tessarallo et al., 1993; Kahane and Kalcheim, 1994; Zhang et al., 1994; Henion et al., 1995; Wehrle-Haller and Weston, 1995) seems to correspond to the presence of large subpopulations of

**Table 5. Average clone sizes**

| | Number of cells/clone | | |
|---|---|---|---|
| Clone phenotype | 1-6 hours | 13-16 hours | 30-36 hours |
| N | 8.5 | 2.4 | 3.5 |
| NG | 30.8 | 16.7 | 5.0 |
| NGM | 29.0 | – | – |
| NM | – | – | – |
| G | 13.0 | 4.4 | 5.0 |
| GM | 4.6 | 5.0 | 5.0 |
| M | 2.0 | 3.6 | 1.5 |

Number of cells/clone for each clone type was determined (at 96 hours) by dividing the sum of all cells in clones of a given phenotype by the number of clones of that phenotype for each sampling interval. In general, precursors that gave rise to more than one derivative generated larger clones than fate-restricted precursors (compare NG to N and GM to M).

N, neuronal; NG, neuron-glial; NGM, neuron-glial-melanocyte; NM, neuron-melanocyte; G, glial; GM, glial-melanocyte; M, melanocyte.

distinct fate-restricted precursors in the initial crest population in vitro. It will be of interest to determine whether growth factor receptor expression correlates with cell fate in a permissive environment when analyzed at the single cell level.

Our results are consistent with the notion that the neural crest is a developmentally heterogeneous population of cells (Sieber-Blum, 1989; Ito and Sieber-Blum, 1991; Weston, 1991; Erickson and Goins, 1995). However, our estimates of the proportion of progenitors in the initial crest population that generate all three classes of derivatives clearly differ from previous inferences achieved by limit-dilution cloning (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988, 1991; Deville et al., 1992; Stemple and Anderson., 1992). There are several possible explanations for this apparent discrepancy. In the limit-dilution experiments (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988, 1991; Sieber-Blum, 1989; Dupin et al., 1990; Deville et al., 1992; Stemple and Anderson, 1992), primary neural tube outgrowth populations are removed from cultures, dissociated and then replated as single cells. It is probably impossible to know what fraction of the primary population survived to be analyzed in secondary cultures. After dilution for secondary culture, individual cells were grown in isolation and in some cases under conditions in which some derivatives were absent (Stemple and Anderson, 1992) or were not identified (Baroffio et al., 1991; Stemple and Anderson, 1992). In addition, in some studies, small clones were not included in the detailed analysis (Baroffio et al., 1988). Since we found that fate-restricted precursors generated smaller clones than unrestricted cells, studies that ignored small clones may have missed the majority of restricted precursors. Taken together, it is possible that these culture methods may have selected for a limited number of precursor types. Most importantly, in the present study, the proportions of the different derivatives generated from labeled clonal progenitors is equivalent to the proportion of these derivatives produced in unlabeled populations. Since this type of controlled analysis was not performed in previous studies, it is impossible to know whether the population sampled by limit-dilution clonal analysis was representative of the population as a whole. Taken together, the methods used in limit-dilution-type clonal analysis studies, while clearly demonstrating that some neural crest cells generate multiple derivatives, were insufficient to determine accurately the types and proportions of precursors present in early neural crest populations.

Our results raise the possibility that nascent premigratory neural crest populations in vivo also contain significant numbers of fate-restricted precursors. Several in vivo clonal analysis studies have examined the differentiative behavior of clonal descendants of cells in the dorsal neural tube prior to the emergence of the neural crest from the neural epithelium (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Serbedzija et al., 1994). Since cells within the neural epithelium are not formally neural crest cells, and it is unclear whether a single neuroepithelial cell eventually gives rise to more than one neural crest cell, conclusions drawn from these studies about neural crest cell fates might be misleading. In another in vivo study (Fraser and Bronner-Fraser, 1991), individual migrating neural crest cells were labeled and the phenotype(s) of their progeny determined on the basis of location and by neurofilament immunoreactivity. Although the small number of cells analyzed makes it difficult to draw general conclusions about migratory neural crest cell populations, both fate-restricted neuronal precursors and neuron-glial precursors were reported. Thus, these results, if not their interpretation, are consistent with our result that early populations of neural crest cells contain both fate-restricted and partially-restricted cells. In fact, however, clonal analysis of *premigratory* neural crest cells in vivo has only been performed in zebrafish and has revealed that the majority of cells behaved as fate-restricted precursors (Raible and Eisen, 1994; Schilling and Kimmel, 1994) although, in these cases, it is also not clear whether the sampled cells accurately reflected the premigratory population as a whole.

### Delayed dispersal of melanogenic precursors

We found that the majority of melanogenic precursors appeared in the outgrowth only after a significant delay. Nearly identical results at the population level have recently appeared (Reedy et al., 1997; C. Erickson, personal communication). This behavior is strikingly similar to the pattern of neural crest migration in the avian embryo (Erickson and Perris, 1993). Neural crest cells that eventually migrate dorsolaterally and give rise to melanocytes do so only after cells that generate neurons and glia have already dispersed ventrally (Serbedzija et al., 1989; Erickson et al., 1992; Kitamura et al., 1992; Oakley et al., 1994). This late-migrating subpopulation of cells also expresses melanocyte-specific markers in vivo prior to and during migration (Kitamura et al., 1992; Wehrle-Haller and Weston, 1995; C. Erickson, personal communication). Taken together, these results raise the possibility that distinct neurogenic and melanogenic sublineages are specified prior to the onset of selective migration along the two distinct pathways. Our results also raise the interesting possibility that the majority of melanogenic neural crest cells actually segregate from the neural tube after an early, primarily neurogenic population has already done so.

### Asynchronous specification of neural crest cell fates

Our results demonstrate that, even in a uniform culture environment, crest cell fate restrictions occur at different times. It should be emphasized that, within the neurogenic lineage, fate-restricted precursors are generated at two distinct time periods. A significant proportion of fate-restricted neuronal precursors was present in the initial population, and the proportion of these cells remained relatively constant for an extended period. During this same period, the proportion of neuron-glial precursors was also unchanged. Then, within a period corresponding to one or two cell divisions, neuron-glial precursors were no longer detected and virtually all the sampled cells that produced neurons were fate-restricted neuronal precursors. Since, by the last sampling period, the proportion of fate-restricted glial and neuronal precursors has increased and the proportion of neuron-glial progenitors has decreased, we speculate that the former arise from the latter. Thus, fate-restricted neuronal precursors are sequentially specified in the neurogenic sublineage.

The mechanism(s) for such sequential specification is unknown. However, if neural crest diversification occurred by the purely random differentiation of cells from unbiased precursors, no distinct sublineage or precursor would be expected to appear or become extinct in a reproducible order. Since the

permissive nutrient environment of our culture system is unlikely to provide differentially localized cues, such signaling cannot explain this sequential segregation of developmentally distinct precursors. One alternative explanation is that interactions between developmentally distinct neural crest cells play a role in the regulation of cell fate decisions. Importantly, previous in vitro clonal analysis protocols would have abrogated such interactions.

### Implications of early neural crest cell-fate restrictions

The demonstration of initial heterogeneity of the neural crest and the asynchronous diversification of developmentally distinct neural crest populations in cell culture significantly affects the interpretation of the role of environmental factors on the differentiative behavior of neural crest populations. We have suggested that distinct neurogenic and melanogenic sublineages are largely specified prior to the onset of migration, resulting in the temporally and spatially distinct patterns of migration and localization of derivatives in the embryo. We also propose that the abundance of distinct fate-restricted precursors may play an important role in the specification of cell fates of other progenitors while cells reside in close proximity before and during crest cell migration. Fate-restricted precursors could regulate subsequent cell fate decisions by other cells both by cell contact-mediated signaling (Fagotto and Gumbiner, 1996), such as lateral inhibition, and by providing a source of diffusible instructive cues (Shah et al., 1994, 1996; Verdi et al., 1996).

Taken together, we suggest that neural crest diversification is regulated by the combined influences of cell-autonomous differences between individual cells and non-autonomous factors influencing differentially responsive subpopulations. Our results, and the method of sequential clonal analysis used to obtain them, now permit us to assess the potential roles of different factors on the specification, proliferation and survival of identifiable precursors during temporally defined periods of neural crest development.

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