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Molecular identification of distinct neurogenic and melanogenic neural crest sublineages

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SUMMARY

Clonal and lineage analyses have demonstrated that although some neural crest cells have the ability to generate multiple cell types and display self-renewal ability, other crest cells generate a single or limited repertoire of cell types. However, it is not yet clear when, and in what order, crest cells become specified to adopt a particular fate. We report that the receptor tyrosine kinases TrkC and C-Kit are expressed by distinct neural crest subpopulations in vitro. We then analyzed the lineages of individual receptor-expressing crest cells and found that TrkC-expressing cells that have just emerged from the neural tube give rise to clones containing neurons or glial cells, or both, but never produce melanocytes. A short time later, TrkC-expressing cells only generate pure neuronal clones. By contrast, from their earliest appearance in neural tube outgrowths, C-Kit-expressing cells invariably give rise to clones containing only melanocytes. Our results directly demonstrate that distinct neurogenic and melanogenic sublineages diverge before or soon after crest cells emerge from the neural tube, that fate-restricted precursors are present in nascent neural crest populations and that these sublineages can be distinguished by their cell type-specific expression of receptor tyrosine kinases.

Key words: Neural crest, Clonal analysis, Lineage, TrkC, C-Kit, Neurogenic, Melanogenic, Quail

INTRODUCTION

Many populations of undifferentiated embryonic cells ultimately generate a wide array of differentiated cell types. Studies of the vertebrate neural crest have been useful in elucidating the developmental mechanisms that regulate cell diversification (LeDouarin and Kalcheim, 1999). However, it is not yet known precisely when neural crest cells are specified to adopt particular fates. Such information will be required before the mechanisms by which cell fates are specified at the molecular level can be elucidated.

Clonal and lineage analyses have demonstrated that individual neural crest cells differ with respect to cell fate. A large number of studies have unequivocally demonstrated the ability of some neural crest cells to generate multiple differentiated cell types (for reviews, see LeDouarin et al., 1993; Selleck et al., 1993; Stemple and Anderson, 1993; Anderson, 1997). Importantly, it has also been demonstrated that some cells behave as stem cells, displaying both self-renewal and multipotent behavior both in vivo and in vitro (Stemple and Anderson, 1992; Shah et al., 1996; Morrison et al., 1999). The ability to isolate enriched populations of these cells on the basis of antigen expression has provided a wealth of information about how specific environmental cues direct their cell fate decisions and differentiation. Likewise, the ability to ectopically express different relevant molecules in specific cells and in precise locations has helped resolve the temporal actions of instructive factors as well as identify the tissues that produce them (Reissman et al., 1996; Schneider et al., 1999).

By contrast, immunoablation, clonal analysis and lineage studies in vivo and in vitro have also documented the presence of early neural crest cells that produce only a single type or class of derivative (Vogel and Weston, 1988; Frank and Sanes, 1991; Schilling and Kimmel, 1994; Raible and Eisen, 1994; Henion and Weston, 1997). In addition, the examination of genes selectively expressed in a specific derivative during earlier stages of development and the affects of misexpression of these genes have also implied the existence of fate-restricted precursors (Perez et al., 1999; Greenwood et al., 1999). However, just as gene expression is not always a faithful indicator of cell lineage at the level of individual cells, it has also been argued that even when cell lineage is analyzed at the single cell level, the interpretation of fate-specification may reflect only a bias in the differentiative behavior of a multipotent population.

Taken together, the current evidence suggests that the nascent neural crest comprises both multipotent stem cells and fate-restricted precursors, as well as partially restricted precursors. However, the identity of precursor cells with limited or restricted developmental potential has as yet only been inferred from clonal and lineage analysis of randomly

selected cells or by inference from gene expression patterns and the manipulation of gene expression. The direct correlation of gene expression by individual neural crest cells and their ultimate fate has remained elusive.

We have now accomplished this correlation by performing clonal analysis of individual receptor tyrosine kinase-expressing cells present in nascent neural crest populations in vitro. The receptors TrkC and C-Kit are expressed only by neurons or melanocytes, respectively, in differentiated cultures and in vivo (Tessarollo et al., 1993; Kahane and Kalcheim, 1994; Henion and Weston, 1995; Wehrle-Haller and Weston, 1997). We found that TrkC and C-Kit are expressed by non-overlapping subpopulations of early neural crest cells in culture. The expression patterns of these receptors raised the possibility that TrkC-expressing cells may represent a subset of the fate-restricted neuronal precursors previously identified by clonal analysis of randomly labeled neural crest cells (Henion and Weston, 1997), whereas C-Kit-expressing cells may represent fate-restricted melanocyte precursors. To test this idea, we first identified TrkC and C-Kit-expressing neural crest cells in live cultures and then performed clonal analysis of expressing cells by microinjection of lineage dye into individual cells and subsequently determining the phenotype(s) of their clonal descendants in differentiated cultures.

MATERIALS AND METHODS

Neural crest cultures

Embryonic neural tubes isolated from the level of the last seven somites of stage 11-12 quail embryos (Zacchei, 1961) were explanted as previously described (Henion and Weston, 1997). Briefly, dissected neural tube pieces were treated pancreatin (Gibco) and triturated to remove attached tissue. The neural tubes were then washed in medium, cut into two or three segments with a tungsten needle, and five to seven segments were explanted into 5 mm Sylgard (Dow Corning) tissue culture well inserts on tissue culture plastic (Corning). The cultures were grown for a total period of 108 hours after neural crest cells first emerged from the neural tube. In most cases, neural tube explants were removed from the cultures with a tungsten needle 24 hours after the initial observation of the emergence of neural crest cells from neural tube segments.

All cultures were grown in QMED medium and half the volume of medium was replaced every day. QMED is a Ham's F12-based medium (Gibco) supplemented with 15% fetal bovine serum (Hyclone) and 4% E10 chicken embryo extract (see Henion et al., 1995). Several batches of serum and extract were tested for their optimal ability to support the robust survival and differentiation of neurons, glia and melanocytes. The batch of each selected was used in all experiments.

For the experiments described, we have defined four time points in culture (Henion and Weston, 1997). The 1-6 hour time point corresponds to the first 1 to 6 hours after the first neural crest cell was observed to have segregated from a neural tube explant. The next interval was 13 to 16 hours after the initial emergence of cells from explants, and the last interval was 30 to 36 hours after initial emergence of cells from explants. Cultures were analyzed 108 hours after explantation of neural tubes, which corresponds to a time in culture when more than 95% of the cells in populations can be unambiguously identified as neurons, glia or melanocytes (Henion et al., 2000) (see below).

Immunocytochemistry

A monoclonal antibody generated against a fusion protein corresponding to a region of the extracellular domain of the chicken c-kit receptor (see below) and an antiserum that recognizes the extracellular domain of the chicken trkC receptor (gift from Dr Francis Lefcort) (Lefcort et al., 1996) were used to identify cells expressing these proteins in live neural crest cultures. The monoclonal antibody against chicken C-Kit was obtained by immunization of a fusion protein consisting of the first three N-terminal IgG-like domains of the extracellular domain of the chicken C-Kit receptor linked to the Fc region of human IgG1. The 870 bp N-terminal fragment of a chicken c-kit cDNA (obtained from Dr Gary Ciment) (Sasaki et al., 1993) was blunt end ligated at a unique *Bst*EII site and cloned in-frame into a CDM8 expression vector containing the sequence for the Fc part of human IgG1 (obtained from Dr S. Nishikawa) (Zettlmeissl et al., 1990). The construct was transiently expressed in COS-7 cells using lipofectamin (Gibco) and serum-free conditioned medium was collected from day 2 onwards. The cC-Kit-IgG1 fusion proteins were purified by Protein A affinity chromatography using standard protocols. Obtained hybridomas were negatively selected for their reactivity for a fusion protein consisting of the signal peptide of chicken C-Kit fused to the same IgG1 sequence. The signal peptide of chicken c-kit was amplified by PCR with an upstream T7 primer and a reverse primer: ATAGGATCCTCATGAGGCACTGAACCAC containing an in-frame *Bam*HI site for integration into the IgG1 vector. This fusion protein was produced as indicated above. Antibodies reacting with the cC-Kit-IgG1 fusion protein but not with the SP-IgG1protein were further tested by immunofluorescence of pigmented quail neural crest cultures and immunofluorescence of COS-7 cells transiently transfected with the full length chicken *c-kit* cDNA (Sasaki et al., 1993).

To identify live, receptor-expressing neural crest cells, cultures grown for the desired amount of time (see above) were removed from the incubator, and culture medium was removed from the wells and replaced with medium containing primary antibody. The cultures were incubated at room temperature for 30 minutes. After extensive washes in medium, the cultures were incubated in medium containing the appropriate secondary antiserum and returned to the incubator for 30-45 minutes. The cultures were then rinsed extensively in medium and viewed using a Zeiss Axiovert fluorescence microscope to reveal immunoreactive cells in preparation for microinjection and clonal analysis.

Interestingly, we were able to detect C-Kit expression in neural crest cells at an earlier time during development than previously reported in avian embryos and neural crest cultures (Lahav et al., 1994; Lecoin et al., 1995). Although it is possible that our culture conditions permit higher expression levels of C-Kit, we attribute our ability to detect C-Kit on the experimental procedure of immunolabeling live cells. Through interactions between antigen and primary and secondary antisera, it appears that antigen-antibody complexes become clustered, rendering them detectable. If cultures are fixed first and then labeled, virtually no recognizably immunoreactive cells are observed. This same phenomenon was observed using 3T3 cells expressing avian TrkC (not shown). Therefore, we think that the clustering of otherwise rare and evenly distributed receptors is critical for detection of expressing cells.

To determine the phenotype(s) of the clonal progeny of labeled receptor-expressing cells at the end of the culture period, we used the neuron-specific antibody 16A11 that recognizes Hu proteins (Marusich et al., 1994), monoclonal antibody 7B3 that specifically recognizes glial cells in neural crest cultures (Henion et al., 2000), and the presence of melanin granules to identify melanocytes [for a more detailed protocol see Henion and Weston (Henion and Weston, 1997)]. Importantly, at the end of these experiments (108 hours after initial segregation of the first neural crest cells from neural tube explants) virtually all of the cells in these cultures are identifiable as either differentiated neurons, glia or melanocytes (Henion et al., 2000). Clonal progeny of injected receptor-expressing progenitors

were identified by rhodamine fluorescence, melanocytes (melanin) by visible light and neurons and glia by either fluorescein or AMCA fluorescence.

Cell counts of TrkC or C-Kit-expressing neural crest cells at different times during development in vitro and determination of the proportions of neurons, glia and melanocytes in control and experimental cultures were performed as previously described (Vogel and Weston, 1988; Henion and Weston, 1994). Briefly, randomly selected fields of cultures were examined and cells counted by a random sampling method (Chalkley, 1943) using an ocular counting reticule (Curtis, 1960). This procedure provides an estimate of the fraction of an identified cell type based on the area occupied by their nuclei relative to the area occupied by the nuclei of all cells in the field. At least 10 fields were counted for each culture.

Single cell labeling of receptor-expressing cells

In order to determine the differentiative behavior of neural crest cells expressing either TrkC or C-Kit we intracellularly injected a single immunoreactive cells per culture well iontophoretically using a glass microelectrode containing a solution of 6% lyseinated rhodamine dextran (Fluoro-Ruby; Molecular Probes) in 0.2 M KCl. Briefly, the microelectrode was lowered into the culture medium and then the microscope was focussed on the adherent neural crest cells. Using fluorescence, immunoreactive cells were identified. The electrode was lowered to the level of the cells and a single immunoreactive cell was injected with lineage dye. Cultures were returned to the incubator for 2 hours and then re-examined to determine whether the injected cell survived the procedure. Slightly less than half the cells survived with no obvious damage and these were subsequently used for clonal analysis and grown for the remainder of the experimental period. Virtually all of the cells (>90%) that survived the injection produced clones, similar to random injections of individual neural crest cells (Henion and Weston, 1997). At the end of experiments, cultures were fixed and processed for immunocytochemistry with cell type-specific markers (see above).

In some experiments, we determined the differentiative behavior of cells that did not express TrkC or C-Kit. In these experiments, the experimental procedures were identical except individual non-immunoreactive cells were injected and cultured for clonal analysis.

RESULTS

Development of neural crest cultures

When the region of the neural tube of stage 11-12 quail embryos between the five most recently formed somites is dissected and placed in explant culture, most neural crest progenitors have yet to undergo an epithelial-mesenchymal transition and emerge from the neural tube. However, shortly thereafter neural crest cells do begin to segregate from neural tube explants. Because neural crest cells emerge from the dorsal region of the neural tube and the orientation of neural tube explants is random, the time after explantation when neural crest cells are first observed is variable. We have defined the time at which neural crest cells are first observed as time zero (0 hours). The time periods designated for populations present in cultures that were analyzed were defined from this time point. For example, the 1-6 hour period corresponds to neural crest cell populations present between 1 and 6 hours after observation of the first neural crest cells that had segregated from neural tube explants. The time periods at which the fate of individual receptor-expressing cells (see Fig. 1) were analyzed were 1-6 hours, 13-16 hours and 30-36 hours. As previously reported (Henion and Weston, 1997),

the initiation of the overt differentiation of melanocytes (melanization) occurs at 54 hours in our cultures and the overt differentiation of neurons (expression of Hu proteins) is first observed at approximately 72 hours. The cultures were fixed after 108 hours in vitro, at which time more than 95% of the cells can be unambiguously identified as neurons, glial cells or melanocytes (Fig. 2) (Henion et al., 2000).

The receptor tyrosine kinases TrkC and C-Kit are expressed in separate early neural crest cell subpopulations

Using an antiserum that specifically recognizes the extracellular domain of TrkC (Lefcort et al., 1996), we found that TrkC was expressed by a subset of neural crest cells in populations present within the first 6 hours of segregation from neural tube explants (Table 1; Fig. 1A,B). Likewise, a subpopulation of TrkC-expressing neural crest cells was also present in populations present after 13-16 hours of dispersal (Table 1). In differentiated neural crest cultures, TrkC was selectively expressed by a subpopulation of neurons (Fig. 1G,H).

By contrast, using a monoclonal antibody raised against a peptide within the extracellular domain of C-Kit and specific for C-Kit (see Materials and Methods), we found that the initial neural crest population (1-6 hours, see Materials and Methods) lacked C-Kit-expressing cells (Table 1). However, a significant number of C-Kit-expressing cells were present in neural crest populations at periods 13-16 hours (Fig. 1C,D) and 30-36 hours after the initiation of segregation of neural crest cells from neural tube explants (Table 1). In differentiated cultures, all melanocytes and only melanocytes expressed C-Kit (Fig. 1I,J). Double labeling of neural crest cultures during the 13-16 hour period revealed that TrkC and C-Kit are expressed by entirely distinct subpopulations of early neural crest cells (Fig. 1E,F).

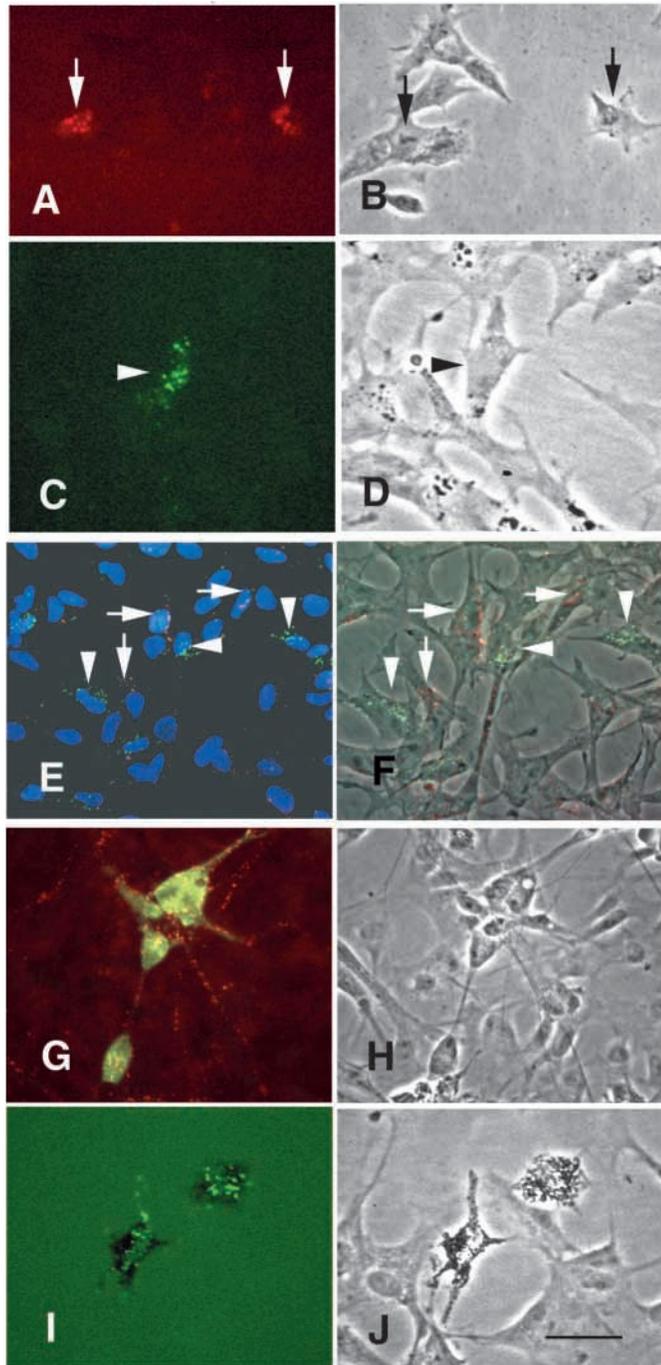### Detection of live TrkC and C-Kit-expressing neural crest cells and clonal analysis

Both the antiserum directed against TrkC and the monoclonal antibody directed against C-Kit recognize epitopes within the extracellular domains of the respective receptors (Lefcort et al., 1996) (see Materials and Methods). Therefore, it was possible to immunolabel live TrkC and C-Kit-expressing cells in neural crest cultures and detect them using appropriate fluorescent secondary antisera (Fig. 1A-D). This provided the opportunity to perform clonal analysis of molecularly identified neural crest progenitors by microinjection of individual receptor-expressing cells with fluorescent lineage dye (Fig. 2). In

Table 1. Abundance of TrkC- and C-Kit-expressing neural crest cells in vitro

	Time period		
	1-6 hours	13-16 hours	30-36 hours
TrkC	7.4±2.2% (162/2185)	5.5±1.7% (103/1850)	ND
C-Kit	–*	22.7±6.2% (408/1799)	13.9%±4.0 (227/1633)

*Only very rarely (fewer than 1% of cells) were C-Kit+ cells observed.
Cells in random fields of three cultures per experiment were counted (see Materials and Methods) and three experiments were analyzed for each data point. Data are expressed as mean±s.d.

Table 2. Antibody detection of TrkC- and C-Kit-expressing cells does not affect development

| Treatment*	Fate	Number of cells	Percentage of total
Control[†]	N	406	19.1±4.5
	G	1214	57.2±7.9
	M	502	23.7±8.7
Anti-C-Kit	N	416	19.3±3.9
	G	1188	55.2±6.4
	M	544	25.5±6.9
Anti-trkC	N	431	19.8±2.7
	G	1222	56.1±3.0
	M	525	24.1±3.7

Data are mean±s.d. Statistics for each type of derivative in each treatment category were compared using t tests and found not to be significantly different ($P>0.05$). N, neuron; G, glial cell; M, melanocyte.

*Cultures were labeled with antibodies as described during the 13-16 hour time period and allowed to develop for 108 hours. Cultures were then fixed and cell types analyzed (see Materials and Methods). Three cultures from each of three experiments were analyzed for each condition.

[†]The control treatment was the same as the antibody labeling (incubations, washes) but without added antibodies (see Materials and Methods).

addition, as neural crest cells in culture are accessible throughout development, we were able to perform clonal analysis of identified cells at different times to determine whether the fate of receptor-expressing cells changes with development. Because the lineage dye is inherited by all of the progeny of an injected clonal progenitor, we could detect clones in differentiated cultures and determine the phenotype(s) of cells comprising clones using cell type-specific markers (Henion and Weston, 1997) (see Materials and Methods).

Clonal analysis of neural crest cells by microinjection of lineage dye has been shown to reflect accurately the diversification of neural crest cell populations (Henion and Weston, 1997). One concern with the immuno-detection of receptor-expressing cells and subsequent clonal analysis is the possibility that antibody binding to receptors may alter the development of the labeled cells. To attempt to address this concern, we compared the development of cultures in which TrkC or C-Kit expressing cells were immunolabeled early during development to mock-labeled cultures in which all incubations and washes were performed but without antibodies (see Materials and Methods). As shown in Table 2, the proportions of neurons, glia and melanocytes in differentiated cultures derived from previously immunolabeled or control cultures were equivalent. This suggests that detection of receptor-expressing cells by immunolabeling does not affect the development of these cells or non-expressing cells. In addition, we observed that the persistence of the antibody-receptor complex at the cell surface appears to be brief, as immunoreactive cells are undetectable ~1 hour after

Fig. 2. Experimental design for clonal analysis of molecularly identified cells. (Top) Because we were able to molecularly identify distinct neural crest populations in live cultures (see Fig. 1), we were able to perform clonal analysis of these cells by microinjection of lineage dye (photomicrograph). The lineage dye is inherited by all clonal descendants of the labeled precursor, which permits them to be distinguished in differentiated cultures and phenotypically analyzed using cell type-specific markers. (Bottom) The time periods at which we immunolabeled and injected individual receptor-expressing cells are expressed relative to the initiation of segregation of neural crest cells from neural tube explants (see Results, and Materials and Methods). We labeled individual TrkC-expressing cells in 1-6 and 13-16 hour populations. We labeled C-Kit-expressing cells in 13-16 and 30-36 hour populations. The initial overt differentiation of melanocytes

(melanization) does not begin until 54 hours and initial overt neuronal differentiation begins at 72 hours. 'Complete' differentiation (>95% of all cells) of the cultures occurs by 108 hours.
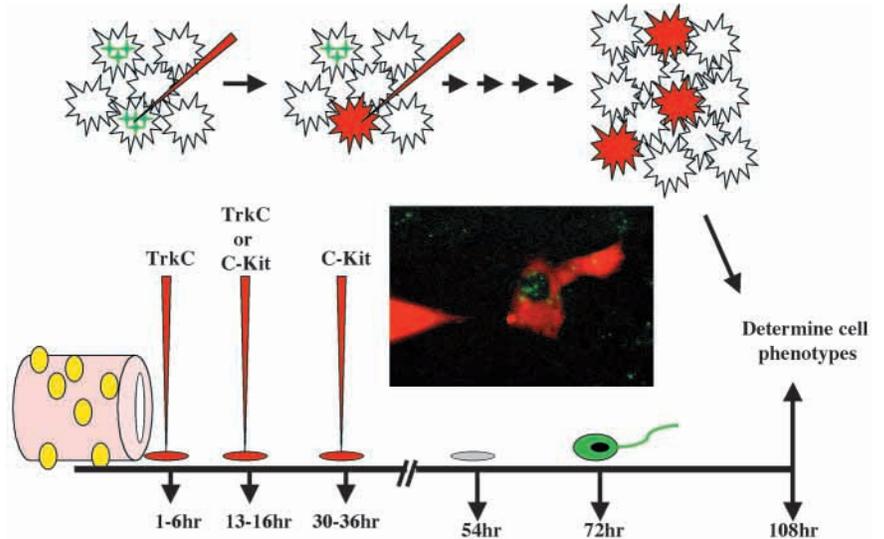
immunolabeling. In addition, although it is not known if or how the C-Kit antibody affects C-Kit function, its continuous presence in the medium does not noticeably affect differentiation in cultures and nor does the continuous presence of the TrkC antiserum (not shown). The TrkC antiserum is known to activate TrkC signaling and can replace the TrkC ligand neurotrophin 3 (NT3) to support cultured neurons (Lefcort et al., 1996). However, our medium is known to contain NT3 and continuous exposure of cultures under our conditions to NT3 does not affect differentiation (Henion et al., 1995), Thus, the apparent lack of any agonistic affect of brief exposure to the TrkC antiserum is not surprising. Taken together, it appears that immunolabeling of receptor-expressing cells is benign and that clonal analysis of immunolabeled receptor-expressing cells faithfully reflects the normal differentiative behavior of these cells.

TrkC expression defines neurogenic and fate-restricted neuronal precursor populations during neural crest development

We performed clonal analysis of TrkC-expressing neural crest cells present at two time periods after the initial emergence of neural crest cells from neural tube explants. TrkC-expressing cells were visualized with an inverted fluorescence microscope after immunolabeling and then injected and with lyseinated rhodamine dextran lineage dye. About half of the clones derived from TrkC-expressing progenitors present during the 1-6 hour period were composed entirely of neurons (Table 3). Importantly, 60% of these precursors were mitotically active and generated multicell clones and thus did not represent postmitotic neuroblasts. The remaining clones derived from TrkC-expressing precursors during the 1-6 hour time period generated clones comprised of neurons and glia, or glia alone. No labeled precursors generated clones containing melanocytes. Therefore, the TrkC-expressing cells present in the initial neural crest population represent a neurogenic sublineage. By contrast, these cells entirely lack melanogenic ability.
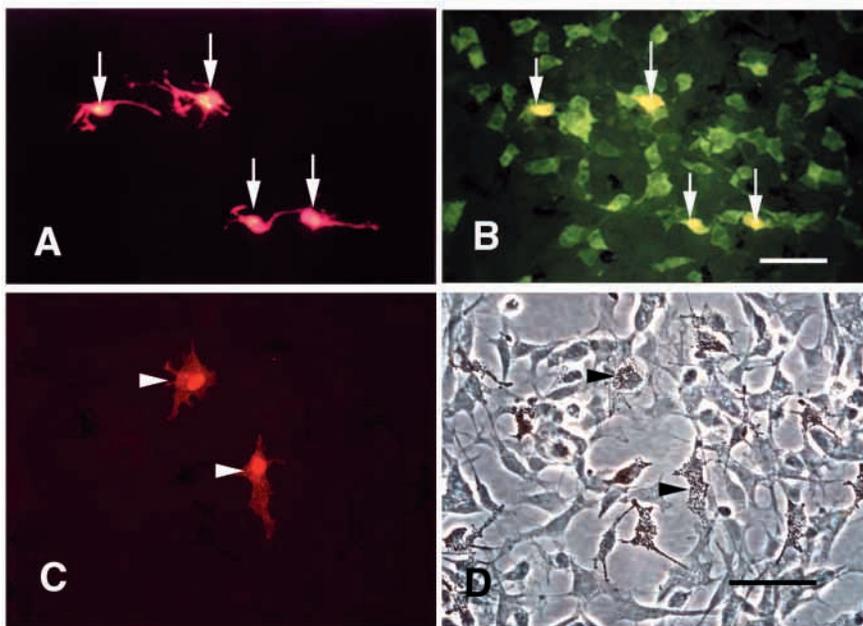
Fig. 3. Examples of clones generated by fate-restricted neural crest cells. (A,B) Images of the same field of cells in a differentiated (108 hour) culture. This clone was derived from a single TrkC-expressing neural crest cell labeled during the 13-16 hour time period. The four members of the clone were identified by fluorescent lineage dye (arrows, A) and were found to be neurons by 16A11 immunoreactivity (arrows, B). (C,D) A two-cell clone in a differentiated culture derived from a C-Kit-expressing neural crest cell labeled during the 13-16 hour period. The two members of the clone (arrowheads, C) were melanocytes, as they contained melanin granules (arrowheads, D, phase-contrast). Scale bars: 25 μm in B; 12 μm in D.

Table 3. Clonal analysis of TrkC-expressing neural crest cells

Precursor phenotype	Time labeled	Clone composition	Average clone size	Range	% of total clones	% single cell clones	% multi-cell clones
TrkC$^+$	1-6 hours	Total=23	2.7	1-7	100.0	21.7	78.3
		N=10	2.7	1-7	43.5	40.0	60.0
		NG=6	3.0	2-5	26.1	0.0	100.0
		G=7	2.6	1-4	30.4	14.3	85.7
TrkC$^+$	13-16 hours	Total=17	1.8	1-4	100.0	58.8	41.2
		N=17	1.8	1-4	100.0	58.8	41.2

N, neuron; G, glial; NG, mixed neuron/glial.

Table 4. Clonal analysis of C-Kit-expressing neural crest cells

Precursor phenotype	Time labeled	Clone composition	Average clone size	Range	% of total clones	% single cell clones	% multi-cell clones
C-kit$^+$	13-16 hours	Total=21	2.8	1-13	100.0	33.3	66.7
		M=21	2.8	1-13	100.0	33.3	66.7
C-kit$^+$	30-36 hours	Total=28	1.8	1-5	100.0	53.6	46.4
		M=28	1.8	1-5	100.0	53.6	46.4

M, melanocyte.

We then analyzed the development of TrkC-expressing cells present in 13-16 hour populations. In every case these progenitors generated pure neuronal clones (Table 3; Fig. 3A,B). Interestingly, the TrkC subpopulation in 13-16 hour populations lacked the ability to generate glial cells even though many of the cells in the population as a whole present at this time retain the ability to do so (Henion and Weston, 1997) (Table 5). Using the Usual Hypothesis Test for Probability, we calculated the probability of obtaining this result by chance. That is, we calculated the probability of obtaining our result (0 glia-containing clones out of 17 total clones, which were all pure neuronal) given the frequency of observing a glia-containing clone when the 13-16 hour population was surveyed by random labeling of individual cells (Henion and Weston, 1997). This probability was approximately 0.0002. This low probability supports the interpretation that TrkC-expressing cells in 13-16 hour populations represent fate-restricted neuronal precursors and that neuronal fate-restricted TrkC-expressing precursors segregate between the 1-6 hour and 13-16 hour periods. In addition, over 40% of these were multicell clones, indicating that many of these cells were mitotically active. Overall, the average clone size of clones derived from TrkC-expressing precursors was comparable with that of fate-restricted neuronal precursors labeled at random (Henion and Weston, 1997). As was the case for TrkC-expressing precursors in earlier populations, no TrkC-expressing precursors in 13-16 hour populations generated clones containing melanocytes.

Taken together, TrkC-expressing neural crest cells in initial (1-6 hour) neural crest populations represent a neurogenic, non-melanogenic subpopulation of neuron-glial precursor cells. Just a few hours later, TrkC expression identifies a fate-restricted neuronal precursor subpopulation. Thus, days before the first neurons will differentiate (Fig. 2), fate-restricted, but still mitotic, neuronal precursors are present in cultured crest cell populations, consistent with clonal analysis of randomly labeled cells (Henion and Weston, 1997) Importantly, a

subpopulation of these cells can be identified based on the expression of TrkC.

C-Kit expression identifies fate-restricted melanocyte precursors

Using the same methods that we used to perform clonal analysis of TrkC-expressing neural crest cells, we determined the fate of C-Kit-expressing neural crest cells present in early undifferentiated cultures. As described above, neural crest cell populations present 1-6 hours after the onset of segregation from neural tube explants only rarely included C-Kit-expressing cells. By contrast, 13-16 hour and 30-36 hour populations contained numerous C-Kit-expressing neural crest cells (Table1; Fig. 1C-F).

We analyzed the clonal descendants of 21 C-Kit-expressing neural crest cells labeled in 13-16 hour populations (Table 4). All 21 clones were composed exclusively of melanocytes and two-thirds of the clones contained multiple melanocytes. Likewise, all 28 clones derived from C-Kit-expressing cells present in 30-36 hour cultures were composed entirely of melanocytes and nearly half were multicell clones (Table 4). Based on these results from 49 clones, we conclude that C-Kit-expressing neural crest cells, which are present long before the overt differentiation of melanocytes in our cultures (Fig. 2), represent fate-restricted melanocyte precursors and thus never give rise to neurons or glia.

Fates of TrkC$^-$ and C-Kit$^-$ neural crest cells

Because the neuronal fate-restricted TrkC-expressing neural crest cells present in 13-16 hour populations represent a minority of the population of fate-restricted neuronal precursors present based on random sampling during this period (Table 1) (Henion and Weston, 1997), we asked what the fates were of cells that did not express TrkC (TrkC$^-$). To do so, we labeled TrkC$^-$ neural crest cells present in immunolabeled 13-16 hour populations with lineage dye and determined the phenotype(s) of their clonal descendants in

Table 5. Clonal analysis of TrkC⁻ and C-Kit⁻ neural crest cells

Precursor phenotype	Time labeled	Clone composition	Average clone size	Range	% of total clones	% single cell clones	% multi-cell clones
TrkC⁻	13-16 hours	Total=11	3.4	1-9	100.0	18.1	81.8
		N=3	3.0	1-4	27.3	33.3	66.7
		NG=1	6.0	6-6	9.0	0.0	100.0
		G=4	5.0	1-9	36.4	25.0	75.0
		M=3	2.7	2-4	27.3	0.0	100.0
C-Kit⁻	13-16 hours	Total=18	2.6	1-7	100.0	50.0	50.0
		N=5	1.5	1-2	27.8	60.0	40.0
		NG=5	4.2	2-6	27.8	0.0	100.0
		G=4	1.3	1-2	22.2	75	25
		GM=4	3.5	2-7	22.2	0.0	100.0

N, neuron; G, glial; NG, mixed neuron/glial; M, melanocyte; GM, mixed glial/melanocyte.

differentiated cultures (Table 5). A variety of clones containing different cell types were observed, including neurons, glia and melanocytes, although no clones containing both neurons and melanocytes were observed. Importantly, fate-restricted neuronal precursors were present in the TrkC⁻ population indicating that while TrkC-expressing cells are fate-restricted neuronal precursors, other molecularly distinct fate-restricted neuronal precursors are present in the population.

We also determined the fates of cells that were C-Kit⁻ in populations present in 13-16 hour C-Kit immunolabeled cultures (Table 5). As was the case with TrkC⁻ cells, labeled C-Kit⁻ cells generated clones that consisted of a variety of cell types and no clones containing both neurons and melanocytes. Most notable among these results is the presence of clones containing glia and melanocytes and the complete absence of clones comprising exclusively melanocytes. The presence of C-Kit⁻ precursors that generate glia and melanocytes suggests that cells with melanogenic ability do not express C-Kit until they have been specified to exclusively adopt a melanogenic fate. By contrast, the complete absence of fate-restricted melanocyte precursors among the C-Kit⁻ population suggests that the C-Kit-expressing population represents all or nearly all of the fate-restricted melanocyte precursors present.

DISCUSSION

Direct correlation of gene expression and cell fate: molecular identification of fate-restricted subpopulations in the nascent neural crest

Heterogeneity in gene expression among neural crest cell populations has been well documented (Barald, 1988; Maxwell et al., 1988; Sieber-Blum, 1989; Ma et al., 1996; Ma et al., 1999; Greenwood et al., 1999; LeDouarin and Kalcheim, 1999). It has been tempting to speculate that differences in gene expression reflect differences in the ultimate development of unlike cells. However, correlation of gene expression and ultimate cell fate has been problematic, especially at the level of individual cells. At the population level, analysis of HNK-1-immunoreactive and non-immunoreactive neural crest subpopulations isolated by flow cytometry revealed the existence of developmental preferences among neural crest populations (Maxwell et al., 1988). Likewise, the antigenic isolation of neural crest stem cell populations results in enriched but not pure stem cell populations (Morrison et al., 1999).

Temporally overlapping patterns of gene expression in neural crest subpopulations and differentiated neural crest derivatives also suggests developmental heterogeneity in molecularly distinct subpopulations (Ma et al., 1996; Greenwood et al., 1999). These arguments are bolstered by loss- and gain-of-function experiments with relevant genes (Ma et al., 1998; Ma et al., 1999; Parras et al., 2002). However, although gene expression may very well predict lineage decisions in many cases, formally, as gene expression is often dynamic, the expression of gene products cannot be used to infer the fate of individual cells unequivocally unless independent correlations with cell lineages can be established.

The methods we have used here have allowed us to make such correlations because we directly assessed the fate of cells expressing specific gene products at specific times during neural crest development in vitro. We found that the expression TrkC and C-Kit by cells in nascent neural crest populations define distinct fate-restricted subpopulations. Thus, in addition to identifiable neural crest stem cells in rodents, the early avian neural crest contains identifiable subpopulations of fate-restricted cells. The relatively small clone sizes of fate-restricted precursors observed is consistent with the interpretation that fate-restricted precursors are less mitotically active over time that their unrestricted counterparts. Because over 90% of injected cells that survived the labeling procedure (see Materials and Methods) produced detectable clones, we do not believe lineage dye dilution resulted in a large number of, if any, undetected clones. This observation, as well as our control data (Table 2), also argue against the possibility that differential rates of cell death occur between antibody-detected progenitors and unlabeled progenitors. If this were the case, the fact that almost one-third of the population at 13-16 hours expresses either TrkC or C-Kit (Table 1) suggests that differential cell death would almost certainly affect the proportions of different derivatives in differentiated cultures, which was not the case (Table 2). Although the interesting possibility exists that descendants of fate-restricted progenitors are more prone to developmentally regulated cell death than are unrestricted cells, this situation would not fundamentally alter our conclusions concerning the specification and identity of fate-restricted precursors. Our results suggest that the proliferative activity of the population represents an average of precursors with low proliferation rates (fate-restricted precursors) and those with very high proliferation rates (unrestricted precursors), consistent with previous inferences (Henion and Weston, 1997).

It is important to point out that for the purposes of the current study, TrkC and C-Kit were used only as markers for distinct neural crest subpopulations. Although these genes play important roles at least during later development of neural crest-derived cells (see Wehrle-Haller and Weston, 1997; Wehrle-Haller and Weston, 1999; Wehrle-Haller et al., 2001; Conover and Yoncopoulos, 1997; Ernfors, 2001), any gene products identifiable in living cells by our methods could potentially have been used. Indeed, because other fate-restricted and partially-restricted precursors also appear to be present in nascent neural crest populations (Henion and Weston, 1997), it may be possible to identify these subpopulations using similar methods based on differentially expressed gene products.

Restriction of neural crest cell fate: a neurogenic subpopulation that lacks melanogenic ability

When the TrkC-expressing subpopulation of neural crest cells was surveyed during two periods soon after neural crest cells emerged from neural tube explants, none of these 40 clonal progenitors generated melanocytes (Table 3). Although melanogenic cells are rare in 1-6 hour populations (Henion and Weston, 1997) (see below), they are prominent in 13-16 hour populations. Therefore, the fact that TrkC-expressing cells in 13-16 hour populations never generate melanocytes under culture conditions known to support melanogenesis suggests that these cells lack melanogenic ability. These results demonstrate an early segregation of a non-melanogenic, neurogenic sublineage early in neural crest development.

Labeled TrkC-expressing cells present in 1-6 hour populations gave rise to clones consisting of neurons, glia or a combination of neurons and glia (Table 3). The fact that glial cells can be generated by neural crest cells that express TrkC soon after they emerge from the neural tube demonstrates that these early TrkC-expressing cells are not necessarily fate-restricted neuronal precursors. Thus, the initial expression of TrkC in early neural crest cultures is not predictive of cell fate as TrkC is selectively expressed by neurons in differentiated cultures. However, all of the TrkC-expressing cells labeled during the 13-16 hour period generated pure neuronal clones (Table 3, Fig. 3) indicating that these cells have become fate-restricted neuronal precursors. Significantly, many of these TrkC-expressing neural crest cells were still mitotically active neuronal precursors, whereas their counterparts represent mitotically inactive neuronal precursors. Comparison of the proportion of the population present at 13-16 hours that expresses TrkC (Table 1) and the proportion of the same population that behave as fate-restricted precursors as determined by clonal analysis of randomly labeled cells (Henion and Weston, 1997) suggests that the TrkC-expressing cells represent a subpopulation of fate-restricted neuronal precursors. Consistent with this conclusion is the fact that some TrkC⁻ cells present in 13-16 hour populations also display fate-restricted neuronal precursor differentiative behavior (Table 5).

Of interest is the exact type of precursor or precursors that TrkC identifies in 1-6 hour populations. The most complex possibility is that TrkC is expressed by subsets of fate-restricted neuronal precursors, fate-restricted glial precursors and partially restricted neuron-glial precursors. One argument against this scenario, in addition to its inherent complexity, is the observation that no cells express TrkC in non-neurogenic

populations that result when dispersal of cells from neural tube explants is delayed (not shown) even though these populations retain gliagenic ability (Vogel and Weston, 1988; Henion et al., 1995). Perhaps the simplest explanation would be that TrkC is expressed by neuron-glial (NG) precursors; cells able to generate neurons, glia or both. In this case, the single cell type clones (N or G) result from an early fate restriction of neuron-glial precursors to either a neuronal or glial fate. In addition, as TrkC-expressing cells a short time later (13-16 hours) only generate neurons, cells derived from 1-6 hour TrkC-expressing cells that are specified to adopt a glial fate must have become specified soon after injection of the lineage dye. Presumably, they extinguished expression of TrkC at the same time, but in any case had done so by 13-16 hours. This scenario would suggest that TrkC-expressing neuron-glial precursors undergo a progressive restriction in cell fate by losing gliagenic ability and being specified as fate-restricted neuronal precursors. In addition, it is also possible that some neuronal fate-restricted TrkC-expressing cells present in 13-16 hour cultures represent cells that dispersed from the neural tube after the 1-6 hour period.

Taken together, the most compelling model for the fate of TrkC-expressing neural crest cells present in 1-6 hour populations is that they represent a subset of neuron-glial precursors that become progressively restricted to a neuronal fate. Gliagenic descendants of TrkC-expressing neuron-glial precursors must extinguish expression of TrkC soon after specification, but at least by 13-16 hours. By contrast, neurogenic descendants retain expression and constitute at least a proportion of the fate-restricted neuronal precursor population identified in 13-16 hour populations (Table 3; Fig. 3).

Restriction of neural crest cell fate: melanocyte fate-restricted precursors

All of the C-Kit-expressing neural crest cells analyzed generated pure melanocyte clones (Table 4; Fig. 3). Given the large body of knowledge about the functions of C-Kit in the regulation of melanocyte migration, proliferation and survival during development (see Wehrle-Haller and Weston, 1997) this is not a surprising result. However, it is important to emphasize that it has not previously been demonstrated at the level of individual cells that C-Kit-expressing cells present in early neural crest populations are uniformly restricted to a melanocyte fate. Unlike the case for TrkC, the initial selective expression of C-Kit in early neural crest cultures accurately predicts their fates as C-Kit-expressing melanocytes in differentiated cultures.

In contrast to TrkC-expressing cells, there are essentially no C-Kit-expressing cells present in 1-6 hour populations. The absence of C-Kit-expressing cells could be because C-Kit expression has yet to be induced in the cells present in the population or because C-Kit-expressing neural crest cells have yet to emerge from the neural tube. The prevailing evidence favors the latter interpretation. When neural crest cells are labeled at random during this period, very few behave as fate-restricted melanocyte precursors (Henion and Weston, 1997). In addition, when 1-6 hour populations were isolated by removal of neural tube explants 6 hours after the first crest cells had emerged and cultured, they produced very small numbers of melanocytes (Henion and Weston, 1997; Reedy et al., 1998).

In contrast, when 6-16 hour populations were isolated by re-plating in separate cultures neural tubes removed from the initial cultures at 6 hours, these populations gave rise to disproportionately large numbers of melanocytes compared to control 24 hour outgrowth cultures (Henion and Weston, 1997; Reedy et al., 1998). Thus, 1-6 hour populations generally lack melanogenic precursors. If, as inferred here, C-Kit-expressing cells are fate-restricted melanocyte precursors, it is not surprising that very few such cells are present in the initial 1-6 hour population. Taken together, our results indicate that the specification of fate-restricted melanocyte precursors occurs before, or just as, they emerge from the neural tube.

Importantly, no C-Kit-expressing cells in either 13-16 or 30-36 hour populations generated glial cells, despite the fact that many cells in these populations produce clones containing glial cells as well as melanocytes (Henion and Weston, 1997). The fact that C-Kit⁻ populations contained cells that produced mixed glial-melanocyte clones (Table 5) suggests that melanogenic cells during neural crest development do not express C-Kit until they have been specified as fate-restricted melanocyte precursors.

Neural crest heterogeneity and diversification

Taken together, our results demonstrate that distinct neurogenic and melanogenic sublineages are specified before or soon after neural crest cells emerge from the neural tube. In addition, although it is clear that many neural crest cells are multipotent, it is now equally clear that a significant proportion of early neural crest populations are composed of fate-restricted precursors, some of which we have identified in this study. The existence and molecular identification of fate-restricted precursors provides a means of determining how pluripotent stem cells give rise to multiple derivatives. Furthermore, because the fates of neural crest stem cell populations can be regulated by instructive growth factors (Anderson, 1997; LeDouarin and Kalcheim, 1999) and Notch activation (Morrison et al., 2000; Wakamatsu et al., 2000; Maynard et al., 2000), fate-restricted precursors, in addition to non-neural crest tissues (Reissman et al., 1996; Schneider et al., 1999), present an intriguing potential source of fate-regulating signaling.

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