Isolation of a Stem Cell for Neurons and Glia from the Mammalian Neural Crest

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Summary

We have isolated mammalian neural crest cells using a monoclonal antibody to the low affinity NGF receptor, and established conditions for the serial propagation of these cells in clonal culture to assess their developmental potential. This analysis indicates that, first, single mammalian neural crest cells are multipotent, able to generate at least neurons and Schwann cells like their avian counterparts. Second, multipotent neural crest cells generate multipotent progeny, indicating that they are capable of self-renewal and therefore are stem cells. Third, multipotent neural crest cells also generate some clonal progeny that form only neurons or glia, suggesting the production of committed neuroblasts and glioblasts. Manipulation of the substrate alters the fate of the multipotent cells. These findings have implications for models of neural crest development in vivo, and establish a system for studying the generation of cellular diversity by a multipotent stem cell in vitro.

Introduction

The neural crest is an attractive system for investigating the mechanisms underlying cell lineage diversification in higher vertebrates. Neural crest cells detach from the dorsal neural tube and migrate to diverse locations in the developing embryo, where they generate a number of different cell types. These include the neurons, glia, and secretory cells of the peripheral neuroendocrine system, as well as nonneural cells such as melanocytes, chondrocytes, and smooth myocytes (LeDouarin, 1982). Transplantation experiments performed in avian embryo chimeras have suggested that the fate of neural crest cells is determined, at least in part, by their local environment (LeDouarin, 1980). This idea has been supported by studies of cultured neural crest cells and their derivatives (for reviews see Anderson, 1989; Patterson, 1990). Taken together, these observations pose a general question: how does an apparently homogeneous population of nondifferentiated cells become channeled into different developmental pathways?

An important first step toward answering this question is the elucidation of neural crest cell lineages. This information is essential because it constrains the possibilities for when and how cell fate decisions are made. Lineage analysis of avian neural crest cells in vivo (Bronner-Fraser and Fraser, 1988, 1990; Frank and Sanes, 1991) and clonal analysis of such cells in vitro (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Duff et al., 1991) have revealed that at least some of these cells are multipotent. These observations, however, do not explain how the different lineages become segregated from their multipotent parental cells. At one extreme, a neural crest cell newly generated from the neural tube could divide to generate two different daughter cells, each already committed to distinct lineages or subsets of lineages; these cells would then undergo further divisions and restrictions before differentiating. At the other extreme, such a newly generated neural crest cell could undergo a series of self-renewing divisions to produce a clone of multipotent cells, which would migrate and generate different cell types in different environments. While intermediate models are also possible, any mechanism involving the self-renewal of multipotent neural crest cells implies that these cells are stem cells.

A resolution of this issue is important because it affects the timing and mechanisms of cell fate decisions in the neural crest. In developing peripheral ganglia, for example, both neurons and glia must differentiate. If such ganglia are formed primarily by multipotent cells able to generate either neurons or glia, the mechanisms determining cell fate must be very different than if the forming ganglia are populated mainly by blast cells already committed to distinct neuronal or glial lineages. While single-cell lineage tracing of migrating neural crest cells in vivo has indicated that at least some of these cells are still multipotent (Fraser and Bronner-Fraser, 1991), it is not clear whether these cells divided between the time of emigration and the time of marking.

We have isolated mammalian neural crest cells using an antibody to a cell surface antigen and have used subcloning to examine the developmental potential of these cells and their clonal progeny. Three main findings emerge from this analysis. First, single mammalian neural crest cells are multipotent. Second, multipotent neural crest cells generate multipotent progeny, indicating that they are capable of self-renewal and therefore are stem cells. Third, multipotent neural crest cells also generate some clonal progeny that form only neurons or glia, implying that the stem cells may eventually produce committed neuroblasts and glioblasts. These experiments suggest that in vivo neural crest cells may maintain their multipotency as they migrate and proliferate, and that initial lineage decisions may occur within developing ganglia via the generation of committed blast cells. They also demonstrate the isolation and clonal propagation of a mammalian stem cell for neurons and glia, and establish a system permitting facile experimental manipulation of such cells and their environment. An initial set of such manipulations reveals that the neuronal differentiation of neural crest cells can be instructively controlled by the molecular composition of their substrate.
Results

Isolated Rat Neural Crest Cells Generate Both Peripheral Neurons and Schwann Cells in Clonal Culture

To isolate mammalian neural crest cells, we have exploited the fact that these cells express the low affinity nerve growth factor receptor (LNGFR) (Bernd, 1988; Smith-Thomas and Fawcett, 1999; Heuer et al., 1990), and that several monoclonal antibodies are available that react with extracellular epitopes of the LNGFR in rat (but not mouse) (Table 1) (Peng et al., 1982; Chandler et al., 1984; Stemple and Anderson, 1991). These antibodies provide a nondestructive means to identify and sort rat neural crest cells. Neural crest cells were isolated from 24 hr explants of E10.5 trunk neural tubes on a fibronectin (FN) substrate (Cohen and Konigsberg, 1975); the neural crest cells emigrate from such explants onto the surface of the culture dish (Figure 1A). Live cell labeling of such cultures confirmed that most of the emigrating cells expressed LNGFR (Figure 1B), whereas cells of the neural tube were not labeled (not shown). Fixation and staining of the cultures with monoclonal antibody Rat 401 (Table 1) revealed that all of the LNGFR+ cells also expressed nestin (Figure 1B), an intermediate filament protein characteristic of immature neuroepithelial cells (Lendahl et al., 1990).

To isolate and clone neural crest cells, neural tubes were discarded after 24 hr and the remaining cells were subcultured at clonal density (225 cells per 100 mm dish). To enrich for LNGFR+ cells, 1 of 2 procedures was used: either the cells were surface labeled and fractionated by fluorescence-activated cell sorting (Figure 1D), or the cells were first subcultured and then surface labeled with anti-LNGFR and the positions of individual antibody-positive cells marked by inscribing a circle on the bottom of the dish (for details see Experimental Procedures). Both methods yielded similar results.

The clonal growth and differentiation of isolated neural crest cells required us to establish empirically culture conditions that supported the proliferation of undifferentiated cells expressing the LNGFR+, nestin+ antigenic phenotype (for details see Experimental Procedures). Not all culture media supporting cell growth maintained this phenotype: for example, addition of fetal bovine serum (FBS) resulted in the extinction of LNGFR expression (data not shown). Under optimal conditions, clones containing several hundred LNGFR+, nestin+ cells could be routinely generated. From serial observations of individual clones, we estimate a doubling time of 18–24 hr for the cells. When such clones were cultured for 9–14 days, cells with a neuronal morphology began to appear. To confirm their identity as peripheral neurons, we performed triple label immunofluorescence staining using antibodies to NF160 and high PSA NCAM, two panneuronal markers (Table 1), and peripherin, an intermediate filament protein preferentially expressed by neural crest–derived peripheral neurons (Table 1) (Portier et al., 1984; Parysek et al., 1988; Parysek and Goldman, 1988). The neurons in these clones coexpressed all three markers (Figures 2D, 2E, and 2F). These neurons expressed neither LNGFR nor nestin (data not shown), indicating that they had extinguished the markers characteristic of the founder cell.

In addition to neurons, the 9–14 day clones contained numerous nonneuronal cells unlabeled by the neuronal markers (compare Figures 2C and 2F). Most of these nonneuronal cells expressed LNGFR and nestin (not shown). These cells may represent immature Schwann cells, which also express LNGFR and nestin (Hockfield and McKay, 1985; Johnson et al., 1988). Indeed, the nonneuronal cells often displayed an elongated, Schwann-like morphology. However, since none of the cells expressed late Schwann cell markers, such as glial fibrillary acidic protein (GFAP) (not shown), they could not be distinguished from undifferentiated neural crest cells. We therefore sought culture conditions that could elicit or permit Schwann cell differentiation.

Many features of Schwann cell differentiation and maturation require intimate association with axonal membranes (Jessen et al., 1987, 1990), and the need for such contact can be mimicked by agents that elevate intracellular cAMP (Lehnke and Chao, 1988; Mirsky et al., 1990). We therefore transferred neuron-containing clones into medium containing 5 μM forskolin, an activator of adenylate cyclase, and 10% FBS. Under these conditions, the nonneuronal cells acquired a highly elongated, Schwann-like morphology (Figures 3A and 3D) and expressed three independent Schwann cell markers (Table 1): GFAP (Figure 3E), sulfatide–O, (Figure 3B), and P0 (Figures 3C and

Table 1. Markers Used in the Identification of Neural Crest Stem Cells and Their Derivatives

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Antigen Recognized</th>
<th>Subcellular Localization of Antigen</th>
<th>Cell Type Recognized</th>
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<td>LNGFR</td>
<td>Cell surface</td>
<td>Stem, Schwann</td>
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<td>Anti-P0</td>
<td>Myelin protein 0</td>
<td>Plasma membrane</td>
<td>Schwann</td>
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References for the markers listed are as follows: 192 Ig, Chandler et al., 1984; 217c, Stamp and Anderson, 1981; Rat 401, Friedman et al., 1990; Lendahl et al., 1990; 5A5, Dodd et al., 1988; NF160, Coehard and Paulin, 1984; peripherin, Parysek and Goldman, 1988; GFAP, Jessen et al., 1990; O4, Mirsky et al., 1990; P0, Mirsky, 1982.
Figure 1. Neural Crest Outgrowth from Rat E10.5 Neural Tube at 24 hr in Culture

A neural tube that had been growing for 24 hr in culture is shown in (A). The upper third of the micrograph contains the neural crest cell outgrowth from the dorsal side of the explanted neural tube. The neural crest cells are in a dispersed monolayer and the neural tube retains a multilayered epithelial structure. A region of neural crest cells is shown at higher magnification after fixation and double labeling with a monoclonal antibody to nestin (B) and a rabbit antiserum to LNGFR (B'). Note that most, but not all, nestin+ cells (B') are also LNGFR+ (B). Neural crest cells were labeled with monoclonal anti-LNGFR 192 (IgG) and sorted by a fluorescence-activated cell sorter (C). A control profile from the fluorescence-activated cell sorter shows the background staining for the fluorescent secondary antibody (C). (D) shows the fluorescence-activated cell sorter profile from anti-LNGFR stained cells. Greater than 70% of the neural crest cells show some LNGFR immunoreactivity; 25% expressed high levels and were collected for subsequent analysis. The log of fluorescence intensity is plotted on the abscissa. The cell number is represented in the plot by the density of the pixels. The scale of pixel densities is displayed in the inset. The cell size, as reported by the forward angle light scatter, is plotted on the ordinate. The scale bar in (B) corresponds to a length of 50 μm for (B) and 500 μm for (A).
Figure 2. Expression of Neuronal Markers in Clones Derived from LNGFR⁺ Founders
A single founder cell (A) and (B) and its resulting clone (C-F) after 10 days of growth are shown. Phase-contrast micrographs of the founder (A) and the clone (C) are shown. The corresponding fluorescence micrographs depict the LNGFR expression of the founder (B) and the expression of several neuronal markers in the clone. Triple labeling with mouse anti-N-CAM (IgM) (D), mouse anti-NF160 (IgG) (E), and rabbit anti-peripherin (F) reveals the coincidence of these markers in a subset of the cells in the clone. Cells expressing the neuronal markers did not express LNGFR or nestin, whereas the nonneuronal cells in the same clone did express these markers (data not shown). The scale bar in (F) corresponds to a length of 50 μm.

Double label immunofluorescence staining confirmed the coexpression of these markers by the Schwann cells (compare Figure 3B versus 3C and Figure 3E versus 3F). These data indicate that the neural crest cells isolated by virtue of LNGFR expression have the capacity to generate not only peripheral neurons but also Schwann cells. Double and triple labeling with neuronal and glial-specific markers confirmed the coexistence of neurons and Schwann cells within the same clones (see Figure 5), demonstrating unequivocally that individual founder cells are multipotent. Quantification indicated that at least 60% of the clones were able to generate both neurons and Schwann cells (see Figure 7B); the majority of the remaining clones contained glia but not neurons.

In addition to neurons and Schwann cells, neural crest clones also contained some flat cells that did not express any of the markers tested, including LNGFR. We call these cells O cells (*other* cells). Approximately 10% of the LNGFR⁺ neural crest founder cells gave rise to clones consisting purely of O cells (see Figure 7B). In serum-containing medium, multipotent LNGFR⁺ founder cells produced clones consisting purely of O cells (not shown). In such serum-containing medium, O cells generated neither neurons nor Schwann cells, and reexpression of LNGFR was never observed. This suggests that the O cell may represent a third, nonneuronal fate of the neural crest cells that is sensitive to environmental factors (see Discussion).

**Self-Renewal of Multipotent Neural Crest Cells In Vitro**
To characterize the progeny of multipotent neural crest cells, we performed a second round of cloning. Six-day-old primary clones founded by LNGFR⁺ progenitor cells (Figure 4A) were dispersed and replated as single cells (Figures 4B and 4B'). These cells formed subclones (Figures 4C and 4C') with an efficiency of approximately 60% (Table 2). Many of these subclones gave rise to both peripherin-positive neurons (Figure 5B, yellow fluorescence) and GFAP-positive Schwann cells (Figure 5B, orange fluorescence). Out of 16 different primary founder clones analyzed in two separate experiments, 75% gave rise to at least one secondary clone containing neurons, glia, and O cells (Table 2, N+G+O); in the second experiment (Table 2, clones 5.2-6.5) 100% of the founder clones gave rise to at least one N+G+O subclone. Furthermore, 50% of the primary clones evaluated generated multiple multipotent secondary subclones; for example, one founder cell gave...
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Figure 3. Expression of Schwann Cell Phenotype by Neural Crest-Derived Glia
Clones were allowed to develop on pDL–FN for a week in standard medium, then transferred into Schwann cell differentiation medium and allowed to grow for another 1–2 weeks prior to fixation and immunocytochemistry. Two separate double labeled clones are shown here by phase-contrast microscopy (A) and (D). The clones were stained with O4 (B) and rabbit anti-PO (C), or with monoclonal anti-GFAP (E) and rabbit anti-PO (F). The same clones also contained neurons (not shown). The scale bar in (F) corresponds to a length of 50 μm.

rise to 15 multipotent subclones (Table 2, clone 1.1). On average, 54% ± 11% of the surviving secondary clones derived from each primary founder were multipotent, i.e., contained neurons, glia, and O cells (Table 2). These data indicate that the secondary founder cells, like their primary progenitors, are multipotent, and that such multipotent cells are therefore capable of self-renewal. They also suggest that self-renewal does not occur exclusively via asymmetric cell divisions; otherwise, only one multipotent subclone would be obtained from each primary clone. We can estimate the extent of self-renewal thus far achieved with these cells in two ways. Serial subcloning of multipotent cells has been performed successfully as late as day 10, by which time some primary clones contain at least 500–1000 cells. Approximately 10% of the secondary subclones obtained are multipotent; assuming no bias in secondary cloning efficiency, this would lead to an estimate of about 50–100 multipotent cells in the primary founder clone. This number would require at least 6–7 symmetric cell divisions of a multipotent cell, and more divisions if some of them are asymmetric (which they must be, since glial and neuronal precursors are also generated). Alternately, based on our upper estimate of a 24 hr generation time, a 10 day culture time is sufficient for approximately 10 cell generations. That at least some multipotent cells are dividing continuously throughout this period is suggested by the ability of these cells to regenerate colonies when replated at clonal density. Both of these estimates, therefore, suggest that multipotent neural crest cells have substantial self-renewal capacity. Carrying serial subcloning experiments beyond two generations is presently difficult: since multipotent cells cannot be distinguished a priori from unipotent cells, an exponentially increasing number of subclones would have to be carried at each recloning step.

Several cases of secondary clones containing neurons but no other cell type were found (Table 2, N only). Although the number of cells in these N only clones was small (<10), it is striking that such clones were observed in the secondary but not the primary clone population. In addition, many secondary clones contained glia but no neurons (Table 2, G+O). Some of these nonneurogenic clones contained only glial cells and no O cells, suggesting the production of Schwann cell "blast" cells. In support of this idea, serial subcloning of founder clones containing both LNGFR* stem cells and O cells yielded some purely LNGFR* subclones (data not shown); as mentioned earlier, O cells never were seen to generate neurons or glia. Taken together, these data suggest that although mammalian neural crest cells are capable of self-renewal, under some conditions they eventually generate progeny with more restricted developmental potentials.

Substrate Molecules Can Control Neural Crest Cell Fate
In the foregoing experiments, neuronal and Schwann cell differentiation were obtained from neural crest clones cultured on a substrate containing FN and poly-D-lysine (pDL). (pDL was initially included together with laminin to promote neurite outgrowth; subsequently, it was found
that the laminin could be omitted. By contrast, if the neural crest clones are maintained on a substrate containing only FN, Schwann cells develop but neurons do not (compare Figures 6A–6C with 6D–6F; Figure 7A). Although substrate effects on the phenotype of avian neural crest cells have been previously demonstrated (Loring et al., 1982; Maxwell and Forbes, 1990a), these studies have been performed on mass cultures containing demonstrably heterogeneous cell populations. Therefore, they do not address the question of whether neural crest cell fate is altered by different substrates, or whether these substrates simply support the attachment or survival of different subpopulations of committed cells. We sought to resolve this issue using two different experimental approaches.

In one set of experiments, neural crest cell clones were established on FN and then overlayed at various times with a solution of pDL. This overlay procedure resulted in the appearance of neurons in 60% of the clones, at the expense of glia-only clones, suggesting that pDL promotes a conversion of clone phenotype (data not shown). In a more direct demonstration of such a conversion, a cohort of clones was initially established on FN, and after 5 days each clone was picked and replated at clonal density onto both FN and pDL–FN substrates. Five out of seven primary clones transferred from FN to pDL–FN gave rise to colonies that contained neurons (Table 3). By contrast, their sister cells subcloned onto FN failed to generate any neuron-containing clones. On average, 57% ± 17% of the subclones obtained from each FN-derived founder generated neurons, on pDL–FN. In the complementary experiment, clones founded on pDL–FN and subcultured onto FN failed to generate any neurons; however, all sister subclones generated neurons when replated onto pDL–FN (Table 3).

Transfer of clones from FN to pDL–FN therefore allows overt neuronal differentiation; conversely, transfer of clones from pDL–FN to FN precludes overt neuronal differentiation. Thus, although neural crest cells cultured on FN do not generate neurons, they retain a neurogenic potential that can be revealed by exposure to a different substrate. These data indicate that the composition of the substrate does not simply affect the attachment or survival of different neural crest cell subpopulations, but rather affects the overt differentiation of a multipotent cell.

Discussion

Using an antibody to the LNGFR, we have isolated rat neural crest cells and determined the developmental potential of their progeny by serial cloning experiments. Several basic observations have emerged from this analysis. First, like their avian counterparts, mammalian neural
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The multipotent neural crest stem cells we have isolated are able to self-renew through at least six to ten generations, under our culture conditions. We would argue that the criteria for a stem cell do not require the demonstration of unlimited self-renewal. While many stem cells exhibit extensive self-maintenance capacity (Hall and Watt, 1989), it is also true that the extent of stem cell self-maintenance may be subject to environmental regulation (Potten and Loeffler, 1990). Self-maintenance in a fixed stem cell lineage is unlimited if all cell divisions are obligatorily asymmetric, yielding one stem cell and a different sister (blast) cell. However, in some lineages such asymmetry may be a property of the cell population rather than of individual cells; i.e., all cell divisions are symmetric, but approximately half the time produce two stem cells and half the time two blast cells (Potten and Loeffler, 1990). If the probability of these two kinds of symmetric divisions is environmentally controlled, then under conditions where that probability is greater than 0.5, the stem cell population will increase in size; if it is less than 0.5, the stem cells will eventually be exhausted. Stem cells populations may therefore exhibit finite self-renewal under some conditions, but contain stem cells nevertheless.

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Mammalian Neural Crest Cells Are Stem Cells

The properties of stem cells have been characterized primarily in adult tissues capable of self-renewal, such as skin, intestine, and blood. These properties include the ability to self-renew, divide asymmetrically, and generate one or more irreversibly differentiated progeny cell types (Hall and Watt, 1989; Potten and Loeffler, 1990). It has been widely assumed that the neuroepithelial precursor cells of the vertebrate central nervous system and peripheral nervous system are stem cells (McKay, 1989; McConnell, 1991). However, there has been only indirect evidence in support of this idea (Temple, 1989; Cattaneo and McKay, 1990). Our experiments demonstrate the successful isolation of a mammalian stem cell for neurons and glia and the establishment of conditions for the clonal propagation and differentiation of such cells.
Figure 5. Multipotency of Secondary Founder Cells
A clone derived from secondary founder cells such as that shown in Figure 4 was transferred into Schwann cell differentiation medium to allow the expression of Schwann cell markers. After approximately 10 days the subclone was fixed and double labeled (B) for peripherin (yellow/green) and GFAP (red). The scale bar in (B) corresponds to a length of 50 μm.

(Wolswijk and Noble, 1989) and apparently bipotential progenitors have been identified in the adult mouse striatum (Reynolds and Weiss, 1992). Thus, the regenerative capacity of the mammalian nervous system may be more extensive than previously thought. It will be of future interest to determine whether neural crest cells contribute to this regenerative capacity.

The observation that multipotent neural crest cells are capable of self-renewal in vitro has implications for the control of neural crest differentiation in vivo. The migration of neural crest cells occurs in the embryo over a period of several days (Serbedzija et al., 1990). The self-renewal observed in culture suggests that at least some neural crest cells may maintain their multipotency as they migrate and colonize various structures throughout the embryo. Consistent with this notion, some migrating neural crest cells in the somitic mesenchyme have been shown to be multipotent in vivo (Fraser and Bronner-Fraser, 1991), and multipotent cells have been identified in early avian sensory and sympathetic ganglia by in vitro clonal analysis (Duff et al., 1991; LeDouarin et al., 1991). If multipotent cells aggregate to form sensory and sympathetic ganglia, then mechanisms must exist to ensure that these precursors generate adequate numbers of at least two different cell types, neurons and glia, within the same local environment. If, in addition, the production of these cell types occurs through the generation of separate blast cells for neurons and glia, then mechanisms probably exist for controlling blast cell generation, proliferation, survival, and differentiation. The availability of an in vitro system should make it possible to elucidate these mechanisms as well as the factors that control the timing of differentiation (Raff et al., 1986).

Developmental Potential of Neural Crest Stem Cells
Like their avian counterparts (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988), the rat neural crest cells we have isolated are multipotent. Under our present culture conditions, these cells are able to generate Schwann cells, peripheral neurons, and at least one other nonneural cell type (Figure 8). The precise identity of the neurons that develop in this system is unclear. They do not express sympathoadrenal markers, such as tyrosine hydroxylase.
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Figure 6. Neuronal Differentiation of Multipotent Neural Crest Cells Is Affected by Their Substrate

Primary clones established from single LNGFR+ founder cells plated on either pDL-FN (A-C) or FN alone (D-F) were double labeled with monoclonal anti-LNGFR (IgG) (B and E) and monoclonal anti-high PSA N-CAM (IgM) (C and F). Labeling was distinguished using class-specific secondary antibodies. Neurite-bearing cells (A) expressing N-CAM (C) are obtained on pDL-FN but not on FN (D and F). Clones on both substrates contain LNGFR+ cells (B and E). However, the intensity of LNGFR labeling is lower on pDL-FN, possibly reflecting the known ability of axonal contact to down-regulate LNGFR expression in Schwann cells. The scale bar in (F) corresponds to a length of 50 μm.

However, the neurotransmitter phenotype of sympathetic neurons is known to be plastic (Patterson and Chun, 1977; Landis and Keefe, 1983), so we cannot exclude the possibility that these neurons are in fact sympathetic. On the other hand, the neurons could represent one or more noncatecholaminergic lineages such as sensory, parasympathetic, and enteric. Unfortunately, definitive markers for these sublineages have not yet been described in mammals.

O cells are unable to generate either neurons or Schwann cells in our in vitro system, and could therefore be related to other nonneural derivatives of the crest, which include melanocytes, chondrocytes, and smooth myocytes. It seems less likely that these cells are related to bone-forming lineages, since transplantation experiments in chick suggest that the trunk neural crest (which we have used here) lacks osteogenic potential (LeDouarin, 1980). Our ability to detect melanocytes has been precluded by the use of albino rats in these experiments. Preliminary experiments using a strain of pigmented rat have thus far failed to reveal overt melanogenesis in clonal cultures. The culture conditions we have established may not permit melanogenesis at clonal density; alternatively, the stem cells we have isolated may lack melanogenic potential. Establishing the repertoire of fates for the rat neural crest cell is currently limited by the availability of appropriate culture conditions and/or markers, not necessarily by the capacity of the cells. In vivo transplantation experiments may (Huszar et al., 1991) resolve this problem.

Environmental Control of Neural Crest Cell Fate

The ability to propagate neural crest cells through several rounds of cloning provides a system in which the influence of environmental factors can be examined at the single-cell level. We have documented a substrate effect on neuronal differentiation and used serial subcloning to rule out the possibility that this effect simply reflects a selective attachment or survival of different subpopulations of neural crest cells. While previous studies have shown that substrate factors can influence neural crest cell differentiation in vitro (Loring et al., 1982; Maxwell and Forbes, 1990b; Rogers et al., 1990), these experiments were performed on mass cultures, where the potential for cellular heterogeneity precludes a clear interpretation of the results. Our results show that substrate composition can control the differentiation of multipotent cells. Whether this control influences a decision between neuronal and glial fates or, rather, a decision of whether to execute a neuronal differentiation program or remain undifferentiated, remains an open and interesting question.

Neural crest clones grown on FN do not generate neurons but retain neurogenic potential. Clones grown on FN appear more disperse than those grown on pDL–FN, suggesting that culture on an FN substrate may suppress neuronal differentiation by inhibiting cell contact. In vivo, FN is present on the routes followed by migrating, undifferentiated crest cells (Newgreen and Thiery, 1980). Our results suggest that this substrate molecule may not only favor cell migration, but may also prevent premature neu-
A. Clones grown on Fibronectin

B. Clones grown on p-D-Lysine / Fibronectin

Figure 7. Phenotypic Composition of Clones Grown on FN and pDL-FN at Day 10

Clones derived from LNGFR+ neural crest founder cells were established on either FN (A) or pDL-FN (B) substrates. After 10 days, the cultures were fixed and the phenotype of the clones was determined by labeling with anti-LNGFR and anti-high PSA N-CAM (see Figure 6). The proportion of clones containing neurons, glia, and O (N+G+O) cells, glia and O cells but no neurons (G+O), O cells only (O), or dead cells (those founder cells that failed to form clones) was calculated. The values plotted represent the mean ± standard error of the mean from over 100 clones in four separate experiments. Note that no neuron-containing clones are found on FN (A), whereas 6Cr% of the clones contain neurons on pDL-FN (B).

Table 3. Alteration of Neural Crest Cell Clone Fate by Subculture onto Different Substrates

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<th>Secondary Substrate</th>
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<tr>
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Neural crest clones were established on either FN or pDL-FN (Primary substrate), and then subcultured after 5 days. Cells from each primary clone were replated at clonal density onto either FN or pDL-FN (Secondary substrate). The phenotype composition of resulting subcolonies was analyzed 10 days later. A plus sign indicates that one or more subcolonies of a primary clone contained neurons under that condition. A minus sign indicates that no neurons were found in any subcolonies of the primary clone. The fractions represent the proportion of total primary clones analyzed that generated any neuron-containing subcolonies.

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Neural crest clones were established on either FN or pDL-FN (Primary substrate), and then subcultured after 5 days. Cells from each primary clone were replated at clonal density onto either FN or pDL-FN (Secondary substrate). The phenotype composition of resulting subcolonies was analyzed 10 days later. A plus sign indicates that one or more subcolonies of a primary clone contained neurons under that condition. A minus sign indicates that no neurons were found in any subcolonies of the primary clone. The fractions represent the proportion of total primary clones analyzed that generated any neuron-containing subcolonies.

1990). Similar advances in our understanding of late stages in neural crest development have emerged from in vitro studies of isolated sympathoadrenal progenitors (for reviews see Patterson, 1990; Anderson, 1993). In both of these cases, however, the progenitors appear to have a relatively limited repertoire of cell fates. The isolation of progenitor cells representing the earliest stages of neural crest development should lead to a better understanding of the logic underlying the progressive narrowing of the differentiation choices available to a multipotent cell. Such neural differentiation by promoting cell dispersal, an idea consistent with conclusions drawn from previous mass culture experiments in avian systems (Rogers et al., 1990).

Consistent with this view, the aggregation of neural crest cells to form ganglion primordia is associated with a loss of FN expression in vivo (Rogers et al., 1986).

The experiments reported here establish a system in which the cellular mechanisms controlling neural crest lineage diversification can be further dissected. In the central nervous system, the establishment of a culture system for the OPA progenitor cell has yielded important insights into the control of proliferation, differentiation, and survival in a glial lineage (for reviews see Raff, 1989; Lillien and Raff, 1990). Similar advances in our understanding of late stages in neural crest development have emerged from in vitro studies of isolated sympathoadrenal progenitors (for reviews see Patterson, 1990; Anderson, 1993). In both of these cases, however, the progenitors appear to have a relatively limited repertoire of cell fates. The isolation of progenitor cells representing the earliest stages of neural crest development should lead to a better understanding of the logic underlying the progressive narrowing of the differentiation choices available to a multipotent cell. Such

Figure 8. Summary of the Behavior of Isolated Neural Crest Cells

Neural crest stem cells (NCSC) are capable of giving rise to at least three cell types in vitro, as indicated. These multipotential cells are capable of self-renewal, indicated by the circular arrow. Pertinent antigenic markers used to identify the cells are indicated in the boxes. "cAMP" indicates that elevated intracellular levels of this compound are necessary for overt Schwann cell differentiation. "Serum" indicates that in the presence of serum, O cells predominate. Schwann and O cells can be obtained either on FN alone or on FN plus PDL; by contrast, neurons are obtained at clonal density on FN plus pDL or on uncoated plates, but not on FN alone.
studies should not only contribute to our understanding of basic stem cell biology and neural development, but may also have therapeutic applications in the treatment of neurololgic and neurodegenerative diseases.

**Experimental Procedures**

**Neural Crest Cell Preparation**

For a given preparation, five to ten timed pregnant female Sprague-Dawley rats (Bilshorn Laboratories, Bilshorn, CA) were killed by CO2 asphyxiation, as approved by the California Institute of Technology Animal Care Committee in accordance with National Institutes of Health guidelines. Embryos were removed and placed into Hanks’ balanced salt solution (GIBCO/BRL, Grand Island, NY) at 4°C for 2–4 h. Using a dissecting microscope at room temperature, a block of tissue from a region corresponding approximately to the caudalmost 10 somites was dissected from each embryo using an L-shaped electrolytically sharpened tungsten needle. Trunk sections were transferred in Hanks’ balanced salt solution into 1 well of a 3-well depression slide that had been chilled to 4°C. Trunk sections were treated with collagenase, made to a concentration of 0.75 mg/ml in Howard’s Ringer’s solution (per 1 liter of sterile distilled water [dH2O]: 7.2 g of NaCl; 0.17 g of CaC12; 0.37 g of KCl) and sterilized by passage through a 0.22 μm filter prior to use. The collagenase solution was exchanged at least three times and with each exchange the trunk sections were vigorously triturated. After incubation at 37°C for 20 min in humidified CO2 atmosphere, the trunk sections were triturated very gently until most of the neural tubes were clean and free of somites and notochords. The neural tubes were then triturated very gently until most of the tissue from a region corresponding approximately to the caudalmost 10 somites was dissected from each embryo using an L-shaped electrolytically sharpened tungsten needle. Trunk sections were transferred in Hanks’ balanced salt solution into 1 well of a 3-well depression slide that had been chilled to 4°C. Trunk sections were treated with collagenase, made to a concentration of 0.75 mg/ml in Howard’s Ringer’s solution (per 1 liter of sterile distilled water [dH2O]: 7.2 g of NaCl; 0.17 g of CaC12; 0.37 g of KCl) and sterilized by passage through a 0.22 μm filter prior to use. The collagenase solution was exchanged at least three times and with each exchange the trunk sections were vigorously triturated. After incubation at 37°C for 20 min in humidified CO2 atmosphere, the trunk sections were triturated very gently until most of the neural tubes were clean and free of somites and notochords. The collagenase solution was then quenched by repeated exchanges with cold balanced salt solution (GIBCO/BRL, Grand Island, NY) at 4°C for 20 min.

**Substrate Preparation**

**FN**

Tissue culture dishes were coated with human plasma FN (New York Blood Center, New York, NY) in the following way. Lyophilized FN was resuspended in dH2O to a concentration of 10 mg/ml and stored at −80°C until use. The FN stock was diluted to a concentration of 250 μg/ml in Dulbecco’s phosphate-buffered saline (DPBS) (GIBCO/BRL). The FN solution was then applied to tissue culture dishes and immediately withdrawn.

**pDL**

Sterile pDL (30–70 kd) (Biomedical Technologies, Inc., Stoughton, MA) was dissolved in dH2O to a concentration of 0.5 mg/ml. The pDL solution was applied to tissue culture plates and immediately withdrawn. The plates were allowed to dry at room temperature, rinsed with 5 ml of dH2O, and allowed to dry again. FN was then applied, as described above, over the pDL.

For pDL overlay experiments, dishes were rinsed once with L-15 medium (GIBCO/BRL), then treated for 3 min with a solution of 0.05 mg/ml pDL in L-15. The dishes were rinsed twice with L-15 and then reseeded with complete medium.

**Cloning Procedure**

Cloning of cells was accomplished by first plating cells at a low density of 225 cells per 100 mm dish, allowing the cells to attach, and then identifying single cells with an inverted phase-contrast microscope equipped with a 4× objective lens. The single founder cells were marked by inscribing a 3–4 mm circle around them with a grease pencil on the bottom of the dish. The identification and encircling of individual founder cells allowed us to verify that the colonies formed by such cells were indeed clones. At the low plating density achieved (1 cell per 250 mm2), the clones would be contaminated by migrating or floating cells was extremely low. Moreover, we would have easily identified any such contaminating cells in the perimeter of the circumscribed area, and did not observe them. The dishes were stained for expression of LAGFR (see below) using the monoclonal antibody 192 IgG (Cherendel et al., 1994). The 192 IgG antibody was produced by hybridoma cells grown in the chemically defined medium described above.
Serial Subcloning
For serial subcloning experiments, clones were harvested and replated as follows. The clones were examined microscopically to ensure that there were no impinging colonies and that the whole clone fit within the inscribed circle. Using sterile technique throughout the procedure, glass cloning cylinders (3 mm inner diameter) were coated on one end with silicone grease (Dow Corning, Midland, MI) and placed about the clone so that the grease formed a seal through which medium could not pass. The cells were removed from the cylinder by first treating them with 100 μl of 0.05% trypsin solution (GIBCO/BRL) for 3 min at 37°C in a humidified 5% CO2 incubator. At room temperature, 70 μl of the trypsin solution was removed and replaced with 70 μl of complete medium. The cells were resuspended into the 100 μl volume by vigorous trituration and the whole volume was diluted into 5 ml of complete medium. The 5 ml was then plated onto one or two 60 mm dishes that were placed in a humidified 5% CO2 incubator for 2 hr. The medium was then exchanged for fresh complete medium and single founder cells were identified as described above.

Immunohistochemistry
Live Labeling, Surface Antigens
For cell surface antigens, it was possible to label the living cells in culture. The cultures were incubated with primary antibody solution for 20 min at room temperature and washed twice with L-15 medium (GIBCO/BRL), supplemented with 1:1:2 fresh vitamin mix (Hawrot and Patterson, 1979), and 1 mg/ml bovine serum albumin (L-15 air). The cultures were then incubated for 20 min at room temperature with phycoerythrin R-conjugated secondary antibody at a dilution of 1:200 in L-15 air. The cultures were then rinsed twice with L-15 air and placed back in their original medium and examined with a fluorescence microscope. Rabbit anti-LNFR antiseraum was a kind gift of Dr. Gislea Weekamp, University of California, San Francisco, and was used at a 1:1000 dilution. Monoclonal anti-N-CAM antibody 5A5 was obtained as hybridoma cells from the Developmental Studies Hybridoma Bank and prepared as described by the provider. Hybridoma cells producing the monoclonal anti-sulfatide antibody 04 were the gift of Dr. Monique Dubois-Dalc (National Institutes of Health) and were grown in a medium consisting of RPMI-1640 (GIBCO/BRL), 10% FBS, and 1% penicillin–streptomycin solution (5000 U of penicillin and 5000 μg of streptomycin) (GIBCO/BRL).

Formaldehyde Fixation
For most of the immunocytochemistry, formaldehyde fixation was done. Formaldehyde solution (37%) was diluted 1:10 into S-minimal essential medium with 1 mM HEPES buffer (GIBCO/BRL). Cultures were treated for 10 min at room temperature with the 3.7% formaldehyde solution and then rinsed three times with DPBS (GIBCO/BRL). For most of the immunocytochemistry, formaldehyde fixation was done. Formaldehyde solution (37%) was diluted 1:10 into S-minimal essential medium with 1 mM HEPES buffer (GIBCO/BRL). Cultures were treated for 10 min at room temperature with the 3.7% formaldehyde solution and then rinsed three times with DPBS (GIBCO/BRL). Cultures were then incubated for 1 hr at room temperature with a blocking solution consisting of DPBS, 0.1% Tween 20 (Bio-Rad Laboratories, Richmond, CA), and 10% heat-inactivated normal goat serum for 15 min at room temperature. Primary antibodies were diluted into a solution of DPBS, 0.1% Tween 20, and 5% normal goat serum. The fixed cells were incubated overnight at 4°C in primary antibody solution, then rinsed twice with DPBS, 0.05% Tween 20. Fluorescent secondary antibodies were diluted into DPBS, 1% normal goat serum, and applied to the cells for 1 hr at room temperature. The cells were rinsed twice with DPBS, 0.05% Tween 20. To prevent photobleaching, a solution of 8 mg/ml N-propyl gallate in glycerol was placed over the cells prior to fluorescence microscopy.

Cytoplasmic Antigen Fixation
For the staining of cytoplasmic antigens, fixed cells were first treated with a blocking solution consisting of DPBS, 0.1% Tween 20 (Bio-Rad Laboratories, Richmond, CA), and 10% heat-inactivated normal goat serum for 15 min at room temperature. Primary antibodies were diluted into a solution of DPBS, 0.1% Tween 20, and 5% normal goat serum. The fixed cells were incubated overnight at 4°C in primary antibody solution, then rinsed twice with DPBS, 0.05% Tween 20. Fluorescent secondary antibodies were diluted into DPBS, 1% normal goat serum, and applied to the cells for 1 hr at room temperature. The cells were rinsed twice with DPBS, 0.05% Tween 20. To prevent photobleaching, a solution of 8 mg/ml N-propyl gallate in glycerol was placed over the cells prior to fluorescence microscopy.

Mouse monoclonal anti-GFAP antibody G-A-5 (IgG) and mouse monoclonal anti-NF/180 antibody NN18 (IgG) were purchased from Sigma and used at a 1:100 dilution. Mouse monoclonal anti-neurofilament heavy chain antibody SMB2 (IgM) was purchased from Sternberger Monoclonals Inc., Baltimore, MD, and used at a dilution of 1:100. Purified rabbit antibodies to peripherin preparation 100G-1F) were the kind gift of Dr. Linda Parysek, University of Cincinnati, OH, and were used at a dilution of 1:300. Rabbit antisera generated against P0 were the kind gift of Dr. Jeremy Brockes (Morgan et al., 1991) (used at a dilution of 1:200) or Dr. Greg Lemke (Trapp et al., 1979, 1981) (used at a dilution of 1:500).

Acknowledgments
We thank M. Dubois-Dalc, J. Brockes, L. Parysek, G. Lemke, J. Dodd, S. Hockfield, G. Weskamp, and E. M. Johnson, Jr. for gifts of antibodies. We are grateful to S. Fraser, P. Patterson, and E. Rosenbarg for their constructive suggestions throughout the course of this work. To S. Fraser, M. Reo, S. Birren, B. Wold, and P. Patterson and T. Jessell for their critical comments on various versions of this manuscript, and to R. Axel for a helpful discussion. This work was supported by funds from the National Institutes of Health, the Pew Foundation, and the Howard Hughes Medical Institute. D. J. A. is an Assistant Investigator of the Howard Hughes Medical Institute.

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Received August 24, 1992; revised September 30, 1992.

References
Isolation of a Mammalian Neural Crest Stem Cell


