Isolation of a mammalian neural crest stem cell and environmental control of cell fate choices

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Abstract

One question central to the study of developmental biology is: How does phenotypic diversity arise? The neural crest provides an excellent model system for investigations into the nature of cell-fate decisions and generation of lineage diversity. In the studies described here, I have examined two aspects of this question in cell culture. The first aspect concerns the cellular dynamics underlying the cell-fate decisions. A necessary prerequisite to the study lineage diversification is the reliable production of differentiated cells from undifferentiated precursors in a controllable environment. I have developed a system for the growth of rat neural crest cells. The system allows the serial propagation of a defined sub-population of neural crest cells at clonal density. In the system neural crest cells can differentiate into at least two identifiable cell types; peripheral neurons and Schwann cells. By sub-cloning I have been able to address specific predictions made by a stem cell model of neural crest development, and found neural crest cells to possess multipotency, self-renewal and the capacity to divide asymmetrically.

The second aspect of the question addressed in this study concerns the ability of the neural crest cell environment to control the choice of cell fate. I have examined various culture conditions for their ability to affect the choice of cell fate both in clonal cultures and in mass cultures. In the clonal cultures I found that the fate of neural crest cell clones can be altered in an instructive fashion by the composition of the substrate, or by the presence of fetal bovine serum. In mass cultures, we have examined the effect of medium composition on the expression of adrenergic traits and on the expression of a transcription factor, MASH-1, thought to participate in neural determination. Finally, in mass cultures of neo-natal adrenal chromaffin cells, we have examined the effects of basic fibroblast growth factor on neuronal differentiation, mitosis and acquisition of trophic dependence.

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CHAPTER 1. Introduction

The neural crest is an unusual entity. A uniquely vertebrate structure, its appearance during evolution correlates roughly with the transition from filter-feeding to active predation. The neural crest provides a number of characteristics that make active predation possible. For fish it provides the gill structure to the pharynx allowing gas exchange. The neural crest provides the gnathal animal with teeth and facial bone with which the animal can capture and chew its prey. In addition the crest provides the enteric and autonomic nervous system with which digestion can be controlled (Gans and Northcutt, 1983; Northcutt and Gans, 1983). The neural crest fails to fall conveniently into a classical germ layer. It shares properties with both ectoderm and mesoderm. The neural crest, like ectoderm, produces neuronal, glial, and dermal elements. Like mesoderm, however, it produces bone, muscle, vascular structures and cartilage. Mesenchymal neural crest cells are unique in that they are not restricted to a germ layer during gastrulation. To distinguish neural crest mesenchyme from true mesoderm or ectoderm, investigators have named the tissue ectomesenchyme or mesectoderm (Le Douarin, 1982). The neural crest comprises a set of migratory precursor cells that originate from virtually every axial level of the neural folds. The neural crest cells individualize, emigrate from the dorsal neural tube at about the time of neural tube formation, and migrate throughout the developing embryo. Problems posed by neural crest development are common to much of developmental biology. How are neural crest cells induced to migrate and what pilots them to appropriate locations in the embryo? How do neural crest cells interact with other embryonic tissue to produce mature tissues? And, how are individual neural crest cell fates controlled? The focus of this discussion will be the latter of these questions. A necessary prerequisite for the examination of cell fate choices is to map the fate and to establish the developmental potential of the tissue.

Mapping of neural crest cell fate

Early investigations into the development of the neural crest concerned the mapping of its contribution to mature structures in the animal. In these early studies, performed on amphibia and avians, the fate of neural crest cells was mapped chiefly using histological marking and surgical techniques (Horstadius, 1950). In some studies, investigators marked regions of the neural crest by focal applications of vital dyes. The fate of the marked regions could be followed until the concentration of the dye in the marked cells became undetectably low. Using [^{3}H]thymidine to label donor tissue, Weston was able to map neural crest fates by grafting tissue between animals of the same species (Weston, 1963). In other studies, by removal of the neural crest or dorsal neural tube, the contribution of the neural crest to mature structures was inferred by the failure of those structures to form (Harrison, 1924). Interspecific grafts between closely related animals have also been used. In such studies grafted cells are easily distinguishable by cytological characteristics. These characteristics are stable and allow contributions to mature structures to be mapped at any point during development. Perhaps the most widely employed marker system of this type is the quail-chick chimera system of Le Douarin (Le Douarin, 1982). In the quail-chick system tissue can be transplanted from a quail embryo to a chicken embryo and allowed to develop. Tissue from the two sources can be easily distinguished because the nucleoli of the two species possess distinct structures that can be easily discriminated by histological staining. One of the most striking features of the neural crest to emerge from these studies is the diversity of derivative cell types produced. The neural crest produces virtually all the peripheral nervous system, all the melanocytes of the skin, the bone, cartilage, and some muscle of the face. The neural crest produces important structural elements of the cardiac outflow tract. The neural crest also produces the endocrine cell types such as epinephrine producing chromaffin cells of the adrenal medulla and the *calcitonin* producing C-cells of the thyroid gland.

The studies described above provide important information concerning the fate of populations of neural crest cells. It is important, however, to realize the limitations of each type of marking study. The most extensive mapping of avian neural crest cell fate has been carried out using the quail-chick marker system. The primary concern with this mapping technique is that it is an interspecific grafting technique. Quail embryos are smaller and develop more quickly than chicken embryos. When the neural crest begins its migration, however, embryos are remarkably similar in size and at a given temperature develop to the same stage within a few hours of each other. Chimeric structures that form, however, are apparently normal in terms of the cell types that form and the timing of development (Le Douarin, 1982). Another problem arises in the post-hatching chimera. In attempts to construct brain chimera to study species-specific behaviors, investigators found that the chimeras became sick a few weeks after hatching (Kinutani et al., 1986; Kinutani et al., 1989). At that time the host immune system becomes fully developed and does not recognize that grafted quail tissue as "self," thus an immune response is mounted against the grafted tissue. In the case of spinal cord grafts the graft rejection begins in the form of central nervous system demyelination in the grafted region that then spreads to the host tissue. A solution to this problem was eventually found by grafting a piece of quail thymic rudiment into the host chicken thymus (Ohki et al., 1987; Balaban et al., 1988; Ohki et al., 1988). More relevant to embryological studies is another potential problem. It has been suggested that quail neural crest cells may be more invasive than the corresponding chicken neural crest cells (Bellairs et al., 1981; Serbedzija et al., 1991). If this is true then a transplantation study may actually reveal a latent potential of the quail neural crest cells, rather than the fate normally expressed by the chicken cells. This possibility motivates the use of marking techniques wherein either no transplantation is used or the transplanted tissue is from the same species.

Problems associated with interspecific grafting are avoided if the fate mapping is performed by labeling cells with a vital marker. These techniques have the advantage that the same species of animal may be used. In general, fate mapping results obtained from labeling experiments support the results generated by chimeric studies. In addition vital labeling techniques can make it possible to study neural crest cell fate in animals that lack an established interspecific grafting technique. By injecting the vital fluorescent dye DiI into the lumen of the neural tube of embryonic mice, Serbedzija et al., were able to define pathways of neural crest cell migration in the mouse (Serbedzija et al., 1990). In addition, they were able to determine the sacral neural crest contribution to the enteric nervous system in both the chick and the mouse (Serbedzija, et al., 1991). Prior to this result there was some controversy concerning the sacral neural crest contribution to the enteric nervous system. The neural crest cell labeling allowed Serbedzija et al., to demonstrate directly the neural crest cell contribution to the enteric nervous system without the worry that quail cells might be more invasive and infiltrate the gut in an inappropriate fashion. A drawback of these techniques, however, is that the label will be diluted as the marked cells divide. This opens the possibility that some of the progeny of the originally marked tissue will not be seen at the time of analysis. In effect, this limits the amount of time that development can proceed before the embryos must be analyzed. Other caveats associated with this technique will be explored further below.

Bronner-Fraser and Fraser have extended the labeling technique to the level of single neural crest cells (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). Single pre-migratory or migrating avian truncal neural crest cells were injected with lysinated rhodamine dextran (LRD). Embryos were allowed to develop for 2 days then were fixed, sectioned and analyzed. The most striking result of these studies is that some single neural crest cells were found to produce diverse progeny in virtually every neural crest derived structure at that

axial level. Furthermore, those clones apparently comprised neurons, melanocytes, Schwann cells and satellite cells. The conclusion that the clones are multipotent, however, should be viewed with some caveats. Specifically, the only molecular marker of differentiation that was used was neurofilament (NF), which will recognize only neuronal cells. The phenotype of non-neuronal cells was inferred from their location and morphology. It remains possible that non-neuronal cells present in the ganglia represent neuronal precursors that have not yet undergone neuronal differentiation, or cells that have migrated inappropriately and are destined to die. Without a molecular marker for glia, it may be incorrect to conclude that non-neuronal cells in a ganglion are glia. The same caveat holds for the cells found in the dorsolateral pathway of migration. Without the observation of melanocyte differentiation it may be incorrect to conclude that those cells are melanocyte precursors. They could be cells of another phenotype that simply have migrated to an inappropriate location.

Another caveat concerns the timing of the analysis. The embryos were injected at ~stage 11-13, corresponding to ~2 days of incubation, and analyzed 2 days later, which corresponds to ~stage 22-24 (Hamburger and Hamilton, 1951). Given the constraint that the label will be present through only a limited number of cell divisions, it is understandable that the injected animals were only allowed to develop for 2 additional days. The timing of neuron and glial birth, however, is such that 2 days may not be enough time to see all of the clonal progeny divide that normally would. Neurons are born until ~stage 30 and glia are produced until at least ~stage 36, corresponding roughly to day 7 and day 10 respectively (see description by Frank and Sanes, 1991). Finally, concerning timing, it remains possible that clones producing neurons in both dorsal root ganglia (DRG) and sympathetic ganglia (SG) may actually be producing neurons of only one phenotype in two different locations, and that given enough time the neurons inappropriate for their locations may be eliminated by

selection. The first timing caveat is addressed somewhat by lineage tracing studies of Frank and Sanes (Frank and Sanes, 1991).

Frank and Sanes injected dilute solutions containing replication deficient retroviruses into the lumen of ~stage 11 neural tube. Viruses infected neural tube cells including pre-migratory neural crest cells. The viruses were dilute enough that < 4 infection events occurred per neural tube injection. They employed a mixture of two types of virus; one virus containing a gene encoding normal β-galactosidase and another virus containing a modified β-galactosidase with a nuclear localization signal. Cells infected with the two viruses can be easily distinguished by the localization of the marker gene product. By using the mixture of viruses they were able to argue that cohorts of cells labeled in the same way represented clones, that is, they were progeny of the same founder cell. Because the retroviruses integrate into the host cell genome, the β-galactosidase expression is a heritable trait whose expression is not necessarily lost through rounds of cell division. Hence, the marker can be detected at any point in development. Frank and Sanes were able to allow the injected embryos to develop beyond the time of significant neuron birth, i.e., to ~stage 31-34. They considered only the DRG and found that a large fraction of the clones contained only neurons, as marked by the expression of long processes. The most common clone, however, contained a mixture of neurons and non-neuronal cells. The most phenotypically diverse clones contained the highest numbers of cells. The morphological criterion for neuronal differentiation precludes the need for a molecular marker of differentiation for neurons (although different types of neurons may not be distinguished), but the morphology of non-neuronal cells found in the DRG and nerve roots is not sufficient evidence to conclude that those cells have undergone glial differentiation. The fact that the embryos were fixed after most neuronal differentiation has taken place supports the notion that non-neuronal cells, if they are not actually glia, may eventually produce glia. It is possible, however, that the non-neuronal cells are undifferentiated progenitors.

Fate mapping studies using populations of neural crest cells reveal that the neural crest produces a diverse array of differentiated phenotypes. Fate mapping of single neural crest cells reveals, with a few caveats, that at least some multipotent neural crest cells exist. These studies do not necessarily reveal the developmental potential of the neural crest at the level of either single cells or populations of cells, because it is possible that some multipotent cells in such an experiment, will not experience the embryonic environment necessary to reveal their total developmental potential. The growth of neural crest cells in clonal culture has allowed a more complete examination of the multipotency of individual cells.

The development of single cells from the neural crest and some of its derivative structures has also been studied in tissue culture. Several groups have grown clones of neural crest cells. In an early study of Sieber-Blum and Cohen, neural crest cells taken from 18 hour old neural tube outgrowths of 2-day old quail embryos produced large clones of 3 measurable phenotypes (Sieber-Blum and Cohen, 1980). Some clones contained only melanocytes, another group was composed only of non-pigmented cells and a third group contained mixtures of both melanocytes and non-pigmented cells. In addition some of the non-pigmented cells expressed formaldehyde-induced fluorescence (FIF), a characteristic of adrenergic cells. In more recent studies, Baroffio et al., cultured single primary cephalic neural crest cells onto feeder layers of growth arrested 3T3 cells (Baroffio et al., 1988). These investigators, using several antigenic markers to analyze clone composition, found a variety of phenotypes among clones. For example, they found some clones that contained only Schwann cells, as defined by expression of Schwann cell myelin protein (SMP) (Dupin et al., 1990). They also found clones that expressed virtually all of the markers that they tested including: substance-P (SP), which is expressed by some sensory neurons *in vivo*; tyrosine hydroxylase (TH), marking adrenergic differentiation; melanin, indicating melanocyte formation; and toluidine blue staining, which when done at pH 4.0 indicates cartilage

formation (Baroffio et al., 1991). There are two key results that one can draw from these studies. First, there exist some cells in the neural crest that are able to produce cells of virtually every measurable neural crest derivative, i.e., multipotent cells. Thus, the multipotency of the neural crest as a whole can be explained by the multipotency of many individual neural crest cells, consistent with the *in vivo* studies. Moreover, since a number of molecular marker of different neural crest lineages were examined, the fact of multipotency of neural crest cell is more firmly established in these studies than in the *in vivo* studies. Second, under conditions that allow multipotent cells to express their multipotency, some crest cells appear to be committed to form only a single phenotype.

Multipotency is manifest in the clonal cultures as clones comprising mixtures of differentiated cells and other unidentified cells. In addition some clones appeared to be the products of restricted or committed progenitors. Similar results were obtained with *in vivo* lineage tracing. How might these results be explained in terms of the pattern of cell division and cell fate choices that are made? Two extreme views that remain consistent with these results can be imagined. On one hand, it may be that the earliest neural crest cells to emerge are totipotent for neural crest lineages at a given axial level, but that every cell division of that cell is a restrictive cell division. That is the products are more restricted in developmental potential than the progenitor. No progeny of a clone would possess the potency of the founder cell. (Although multipotent intermediate cell types that possess a proper subset of the developmental potential of the founder may be found.) Eventually, symmetrically dividing progenitors that have a finite capacity for cell division would be produced. Mitosis is a feature that may be controlled by environmentally derived signals. Ultimately those cells would differentiate and survive in the appropriate environment or simply die in an inappropriate environment. On the other hand, it may be that the totipotent neural crest cell is a self-renewing cell, that can produce committed progenitor cells when given the

appropriate intrinsic or environmentally derived signals. Under either mechanism the outcome is the same; a group of differentiated progeny develop from a single progenitor cell. The key difference between the two mechanisms is that in the later mechanism, a stem cell mechanism, the multipotent progenitor will self-renew. The property of self-renewal can only be detected if individual progeny cells are assayed for the multipotency, i.e., sub-cloned to test whether they produce the same range of phenotypes as the original founder cell. There is a rich precedent for the existence of stem cells in certain mature vertebrate tissues, but a role in developmental systems is less clear. In the next section I will address this issue.

Stem cells

During the development of vertebrate organisms, a series of patterning events establish the major axes of the embryo and the germ layers (Spemann, 1938). The rudiments of the major organ systems are established through a series of interactions between the germ layer tissues. Within a developing organ, large numbers of relatively few cell types are produced. The mechanisms governing the production of mature cells within organs are generally unknown in detail, but for a few cases the pattern of cell proliferation and differentiation has been described (Hall and Watt, 1989; Potten and Loeffler, 1990; Ikuta et al., 1992). Two important features that emerge from analyses of progenitor cells in developing vertebrate organs are the diversity and size of the differentiated cell populations they generate. To produce sufficiently large numbers of differentiated progeny cells, many vertebrate progenitors have substantial proliferative capacity. The diversity of cell types generated from a single multipotential progenitor often reflects progressive mechanisms, in which progenitor cells gradually undergo changes in their developmental potential (Dexter et al., 1990). The identification and characterization of progenitor cells at different stages of development has been a major focus of research in cellular immunology. Similar work has been performed for skin

and intestine that, like the immune system, are required to replenish the differentiated cells throughout the life of the organism (Hall and Watt, 1989; Potten and Loeffler, 1990). Such studies have led to the concept that progenitor cells may be divided into two broad categories: "blast" cells, or "transit amplifying" cells that are limited both in their proliferative capacity and in their developmental repertoire; and more developmentally-primitive "stem" cells that have an extensive capacity for self-maintenance and are often (but not always) multipotent (Hall and Watt, 1989; Potten and Loeffler, 1990). While these studies concern with the maintenance of adult tissues, the "stem" cell and "blast" cell concepts may be useful when applied to developmental situations.

The major task during organogenesis, that of producing a large number of a limited array of different cell types, is very similar to what is required in some adult tissues. There are situations encountered in the adult when an entire structure or complement of cells needs to be replaced, for example, during bone marrow transplantation or intestinal epithelial regeneration after irradiation. The regeneration of a structure has many similarities to the original formation of that structure, although the details may differ somewhat. Finally, the signaling systems and transcription machinery employed during the initial construction of the organ may be preserved and re-utilized in a regeneration or maintenance paradigm.

For some tissues in the adult mammal there is an ongoing requirement for new cells. As stated above, a relatively simple example is the epithelium of the intestine (Potten and Loeffler, 1990). The cells of the intestinal epithelium are specialized transport cells that are exposed to topologically external surfaces of the animal and thus are keenly subject to abuse. These cells are constantly sloughed and replenished by stem cells that exist at the base of the intestinal villi. There are two basic properties that these stem cells exhibit. They renew themselves and they produce differentiated intestinal epithelial cells. They produce cells at a relatively constant rate. The intestinal

epithelial stem-cell has an enormous capacity for the production of differentiated progeny, but the range of cell types produced is small. For the development of most organs the progenitor is multipotent. A well studied example of a multipotent stem cell is the hematopoietic stem cell.

All blood cells derive from one type of cell called the hematopoietic stem cell (HSC) (Spangrude et al., 1988; Ikuta, et al., 1992). The composition of blood, in terms of the relative proportion of cell types is fairly constant within an individual but can rapidly respond to requirements for specific cell types, such as lymphocytes and neutrophils when a pathogen infects the individual. Hematopoiesis is a process that begins in the embryo and continues throughout the life of the individual. In mice, HSCs can be detected in the embryonic yolk-sac, at a stage preceding major organogenesis. Later in development, HSC come to reside in the liver and ultimately in the bone-marrow of mature animals. The fetal HSC is capable of long-term repopulation of the blood of a lethally irradiated adults and in this sense gives rise to the adult form of the cell (Ikuta et al., 1990; Jordan et al., 1990). There are, however, some subtle differences that distinguish it from the adult HSC. For example, only fetal, but not adult, HSCs can produce $V\gamma3^+$ and $V\gamma4^+$ T cells in the fetal thymic microenvironment (Ikuta, et al., 1992). In the example of hematopoiesis we find a situation wherein the initial development of the organ, blood, is mimicked by the maintenance process in the adult. A similar transition from an embryonic to an adult form of the progenitor may be found in the formation of the myelinating glia of the central nervous system (CNS).

Oligodendrocytes, the myelinating glia of the CNS, can be generated by the O-2A progenitor, which can renew itself under certain conditions and can produce differentiated glia under other conditions (Raff et al., 1983). The extent of self renewal was at first thought to be limited to a few cell divisions after which O-2A progenitors differentiated into oligodendrocytes. It was found, however, that under the control of

certain combinations of growth factors the O-2A progenitor has an apparently unlimited capacity for self-renewal and at any point the progeny of an O-2A progenitor can be forced to differentiate into one of two glial fates (Bogler et al., 1990). Further, it was found that the embryonic O-2A progenitor could be caused to produce an adult form of the cell that persists *in vivo* through the life of the organism (Wren et al., 1992). The O-2A progenitor can also exhibit multipotency, at least *in vitro*, as it can be caused to differentiate to the GFAP expressing type-2 astrocyte phenotype by appropriate growth factor treatment.

Recently multipotent progenitor cells capable of self-renewal have been isolated from the striatum of adult mouse (Reynolds and Weiss, 1992). Initially these cells express nestin, a marker that is characteristic of neuroepithelial progenitors cells (Lendahl et al., 1990). These cells can be induced *in vitro* to form astrocytes and neurons when attachment to a substrate is promoted. They will self-renew in suspension culture and are stimulated to divide by epidermal growth factor (EGF). These multipotent cells exist in the adult striatum as a relatively small percentage population. They also exhibit an extensive capacity for self-renewal under EGF stimulation. These cells are apparently stem cells for the striatum, but it is not clear what specific neuronal and glia potentials are available to them, nor is it is clear what role these cells play in the maintenance of adult brain tissue or in the regeneration of damaged regions. In a similar study, Cattaneo and McKay found that embryonic striatal neuron progenitors (nestin expressing cells) were stimulated to divide by fibroblast growth factor (FGF) and by nerve growth factor (NGF) in combination with FGF (Cattaneo and McKay, 1990). When the growth factors were withdrawn some of the cells underwent neuronal differentiation. A clonal culture of rat embryonic forebrain reveals that some cells are capable of producing both neurons and glia, but does not address the issue of self-maintenance (Temple, 1989).

Using the deficient retrovirus lineage tracing methodology described above, several groups report that a variety of cell types may arise from multipotent progenitors within the mammalian neocortex (Price and Thurlow, 1988; Walsh and Cepko, 1988; Walsh and Cepko, 1992). Further, they find that single progenitors can contribute neurons to several cortical layers. Some have termed these cells stem cells (Lendahl, et al., 1990), however, in none of these studies is self-renewal demonstrated. Further, these studies reveal only the fate of labeled cells and not the developmental potential of the progenitor. In the normal development of the mammalian neocortex, a laminar structure, the first neurons to be born populate the deepest layers starting with the cortical plate (for review see McConnell, 1991). Subsequent cell divisions produce neurons destined to populate more superficial layers. Neurons are all born in the ventricular zone and migrate toward the pial surface. In the ferret, neurons destined to populate the cortical plate and layer 6 are born at embryonic day 29 (E29). The neurons of the superficial layer 2/3 are born postnatally. Earlier work of McConnell suggested that the primary factor controlling the commitment of cortical neurons to a particular layer is the environment in which it goes through its final cell division (McConnell, 1988; McConnell, 1989). Neurons born in E29 ventricular zone migrated to and took on traits characteristic of the deep layers even when they were transplanted to the postnatal ventricular zone. McConnell and Kaznowski have investigated the environmental control cortical neuron fate and its relation to the cell cycle (McConnell and Kaznowski, 1991). S-phase neuronal precursors are labeled with [^{3}H]thymidine in the ventricular zone of E29 ferrets, then transplanted to neonatal brains after a time interval from 0 hours (i.e., cells removed and labeled *in vitro*) to 24 hours (i.e., label injected into the donor brain and cells are allowed to be labeled for 24 hours before being transplanted). They find that cells that are allowed to undergo their last division in the donor ventricular zone (the 4, 8 and 24 hour timepoints) populate the deep layers when transplanted (donor behavior). By contrast, if the cells undergo their last division

in the host (the 0 hour timepoint), the labeled cells populate the superficial layers (host behavior). In addition, if they allowed the late transplants (i.e., 4, 8 and 24 hour timepoints) to develop for an additional round of cell division before analysis, then *lightly* labeled cells were found only to populate the superficial layers. They conclude that each progenitor is a dividing multipotent cell that responds to a changing environment. The results do not exclude an alternative hypothesis that the set of progenitors is heterogeneous with respect to developmental potential and that the differences observed are due simply to selective migration of labeled cells (van der Kooy, 1992). Indeed, McConnell and Kaznowski found that only 20% of the labeled cell migrated out of the injection site. They respond to the criticism by arguing that if a heterogeneous population of progenitors existed, the exact interval between labeling and transplantation would be irrelevant to the laminar fates of the progeny. They found, however, that the population of labeled cells behaved homogeneously, i.e., in the 0 hour transplant > 90% of the migrating cells populated to the superficial layers; in the 4 hour transplant > 90% of the migrating cells populated the deep layer; and the lightly labeled migrating cells always populated only the superficial layers.

It seems reasonable to conclude that the precursor cells are multipotent cells responding to a changing environment. They may not be stem cells however. A progressive restriction model of development may apply. The transplants that have been done, however, take cells from a young animal and move them to an older animal. Hence, the multipotent cell would not necessarily undergo self-renewal, but instead could take on the more restricted state specified by the older environment. To address the possibility of self-renewal, one would have to label cells of the older animal, allow them to divide in the younger environment, and then test them for the ability to choose the deep layer fate. In such an experiment if the cells choose the deep layer fate then the precursor probably undergo self-renewal. If they choose the superficial fate, however, they have become restricted.

Do neural crest derivatives develop via a stem cell mechanism?

If neural crest-derived tissues develop by way of a stem cell mode there are several properties that the neural crest cells will exhibit. The first property is multipotency. While multipotency is not a property of all stem cell systems, it is a property of many and would be expected for neural crest cells given the range of derivatives that arise from the neural crest. The second property, which may be the most defining property of stem cells, is self-renewal. Strictly speaking, self-renewal refers to the ability of a given stem cell to replace itself at every cell division. Self-renewal may arise through either symmetric or asymmetric cell divisions. An asymmetric cell division being one in which one daughter of the pair is a relatively restricted cell, i.e., a cell whose developmental potential is less than the parent, and the other daughter is, like the parent, a stem cell. A third important property of stem cells is the ability to produce differentiated progeny either by way of a blast cell population or by direct conversion.

To study the problem at this level, it is necessary to determine the fates of single progenitors either *in situ* or in novel environments where latent potentials may be revealed. As discussed earlier, *in situ* clonal analysis of pre-migratory and migrating neural crest cells has been done (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). These studies suggest that single pre-migratory neural crest cells are multipotent and capable of forming all of the derivatives available to the trunk neural crest. Earlier the two extreme models of neural crest development were discussed. It is important to distinguish between these two models because it affects the way one thinks about the normal development of neural crest-derived structures. If self-renewing multipotent neural crest cells are present in a developing structure the choices made by the precursor cells would very different from the choices

that would be made by cells already committed to particular differentiated fates. For example, consider the development of a sensory ganglion. The ganglion contains two cell types, neurons and glia. If the stem cell model were true, then the choices made by a stem cell in the ganglion would be to produce a neuron or to produce a glial cell. In addition the stem cell may choose to self-renew. If, however, the restrictive model were true, committed progenitors of many lineages would form, then either they cells would migrate in a directed fashion to their appropriate location or a mixture of committed progenitors would populate the ganglion. Once in the ganglion the choices available to those cells would be to survive and divide, if they were neuronal or glial progenitors; or to die, if they were progenitors of any other cell type.

In order to determine the developmental potential of mammalian neural crest cells and further to distinguish between the two models of neural crest development I developed a system in which rat neural crest cells can be grown in clonal culture. These investigations are detailed in chapter 2.

Environmental control of neural crest cell lineage specification

Mapping of neural crest developmental potential

The first evidence that the fate of neural crest cells may be controlled by the embryonic environment in which they develop came from studies designed to test the developmental potential of neural crest derived from different axial levels. Using the chick-quail marker system, Le Douarin and her colleagues found that the neural crest of any given axial level possesses a larger developmental potential than is normally realized (Le Douarin, 1980; Le Douarin, 1982). Regions of the neural crest that do not normally produce a given cell type possess the capacity to do so when challenged with the appropriate environment. Here are examples of transplantation studies that reveal the environmental effect on neural crest cell fate:

The axial level corresponding to somites 18-24 in the chick is the only part of the neural crest that normally produces adrenal chromaffin cells. However, when neural crest from any other axial level is transplanted to that region, adrenal chromaffin cells arise from the transplanted tissues (Le Douarin and Teillet, 1974). Hence, while every level of neural crest tested possesses the ability to form chromaffin cells, only neural crest from the level of somites 18-24 actually does. It is at that level of the organism that the adrenal gland forms and, as will be discussed later, the environment of the adrenal gland directs the formation of chromaffin cells (Anderson, 1993).

The trunk of the neural crest does not normally generate ectomesenchymal derivatives. In a study of Nakamura and Ayer-Le Lievre, however, when adrenomedullary neural crest of quail was transplanted to the level of the rhombencepahlon in host chick embryos, the transplanted neural crest differentiated into mesectodermal derivatives, including connective cells in the walls of blood vessels and dermis of the neck (Nakamura and Ayer-Le Lievre, 1982). The quail cells, however, were not found to form cartilage or bone . Thus the truncal crest, which never contributes ectomesenchyme can be caused or allowed to do so in the environment of the rhombencephalon.

The fact that cartilage and bone do not form from truncal neural crest indicates that profound differences in developmental potential may exist between different axial levels of the neural crest. Within the cephalic crest there may exist differences in developmental potential at different axial levels. There are certainly differences in the expression of homeobox containing genes that may be in part responsible for the axial level-specific structural differences found during craniofacial development (Noden, 1991). The mesencephalic neural crest expresses an engrailed homolog before and during its migration, whereas adjacent regions of the neural crest apparently do not (Gardner et al., 1988; Gardner and Barald, 1991). There are indications that the vagal neural crest, which normally will produce enteric ganglia, is more invasive than trunk

neural crest. Vagal neural crest, when transplanted to the level of adrenomedullary neural crest, will invade the gut and form much of the enteric ganglia posterior to that axial level (Le Douarin and Teillet, 1974). Adrenomedullary neural crest can not normally invade the gut, but when it is transplanted to the vagal level is capable of populating the pre-umbilical gut and forming enteric ganglia (Le Douarin and Teillet, 1974; Le Douarin et al., 1975). The differences in developmental potential that exist between axial levels of the neural crest are important to note and may reflect intrinsic differences between classes of neural crest cells. Those differences may be crucial for the normal formation of complex structure in the head and neck. Much of the developmental potential of different axial levels, however, is overlapping and similar mechanisms of control over the generation of differentiated fates may operate even though the range of possible fates may vary somewhat.

Undetermined neural crest cells can be found in developing neural crest-derived structures

When tissue is transplanted from a neural crest derivative structure of a donor embryo to the neural crest of a younger host embryo, it has been found that often the donor tissue possesses a developmental potential greater than would be normally realized in the source tissue. In a series of experiments carried out by Le Douarin and her co-workers, pieces of E4.5 to E6 quail sympathetic and dorsal root ganglia were back-transplanted into neural crest migration pathway at the adrenomedullary level of 2-day old chick embryos (Le Lievre et al., 1980). Sympathetic ganglion grafts were found to contribute neurons and satellite cells to the sympathetic ganglia, chromaffin cells to the adrenomedullary cords and Schwann cells to the ventral roots, but did not contribute neurons to DRGs. On the other hand, grafted DRGs were found to contribute the same array of cells as the sympathetic ganglia, but, in addition, were able to contribute neurons to the DRGs. For the most part DRGs do not express adrenergic

traits. Price and Mudge reported, however, that in 3-4 week old rats, some DRGs contained a small number of tyrosine hydroxylase (TH) expressing neurons. They found that the DRGs never expressed dopamine-β-hydroxylase (DβH), nor were TH expressing cells ever found in embryonic or perinatal DRGs of rats or chickens (Price and Mudge, 1983). This observation is supported by Katz in his studies of cranial sensory ganglia, such as the petrosal ganglion, where there are many adrenergic cells. He found that the neural crest derived neurons express TH only transiently, whereas TH is stably expressed by placode derived neurons. In addition, he does not find adrenergic neurons in embryonic or perinatal DRGs (Katz, 1991). Mercer et al., using a DβH promoter to drive β-galactosidase expression in transgenic mice found at most 2 β-galactosidase expressing cells in any DRG (Mercer et al., 1991). Hence, even though adrenergic properties are rarely expressed in the DRG, and never expressed in the embryonic DRG, the developing ganglion possesses the capacity to produce sympathetic neurons and chromaffin cells. Similarly, Ciment et al., found that early DRGs, from embryos up to 5 days old, possess the capacity to produce melanocytes even though melanocytes are never found in the DRG (Ciment et al., 1986). These examples point up the fact that although certain phenotypes are never manifest in a given derivative structure the potential for their formation exists.

These transplantation studies provide suggestive evidence that the environment serves to control cell fate decisions in the development of neural crest derived structures. By transplantation to a novel environment, one can test the developmental potential of a given tissue. (Although the total developmental potential of a transplanted tissue will not necessarily be revealed, specific developmental potentials can be tested.) In addition, the fact that a lineage decision has been made can be measured. That is, transplantation studies show that a choice between lineages, e.g., sympathetic neuron vs. sensory neuron, can be made by cells within the transplanted tissue. The problem is that transplantation studies cannot show what the nature of the decision is. For

example, one cannot distinguish between the induction of a novel phenotype from an undifferentiated progenitor, and the selective growth (or selective loss) and differentiation of pre-committed progenitors. This type of ambiguity is primarily due to the fact that in these transplant studies only populations of cells are examined, and selection can always explain the outcome of the experiment.

Sympathoadrenal lineage

As stated above, the sympathoadrenal (SA) lineage comprises the neurons of the sympathetic ganglia and the chromaffin cells of the adrenal medulla. These two cell types share several characteristics, perhaps most importantly they are both adrenergic cells and thus express a common neurotransmitter biosynthetic pathway. The two cells, however, have dramatically different morphologies and serve different physiological roles for the organism. Although both cell types synthesize catecholamines, most chromaffin cells express an additional enzyme, phenylethanolamine-N-methyl transferase (PNMT), whose function is to convert norepinepherine to epinepherine. The environment in which these cells develops appears to control the differences between them (for review see Anderson, 1993). The adrenal gland produces large amounts of glucocorticoid steroid hormone (Roos, 1967). The glucocorticoids appear to affect the development of chromaffin cells from SA progenitors in two ways. Initially, glucocorticoids can inhibit the neuronal differentiation of embryonic SA progenitors taken from E14.5 rat adrenal glands. Later, glucocorticoids promote the maturation of chromaffin cells, as marked by the induced expression of PNMT (Michelsohn and Anderson, 1992). On the other hand adrenal SA progenitors can undergo neuronal differentiation in the absence of glucocorticoids (Anderson and Axel, 1986). SA progenitors derived from developing sympathetic ganglia appear to behave in the same way (Carnahan and Patterson, 1991).

Several investigations reveal that NGF can function to elicit the conversion of chromaffin cells into sympathetic neurons (Unsicker et al., 1978; Doupe et al., 1985a; Doupe et al., 1985b). This result suggests that NGF may serve a similar function in the normal development of sympathetic neurons. At the time of neuronal differentiation, however, there is no NGF present in the sympathetic ganglia (Davies et al., 1987; Korsching and Thoenen, 1988). This suggests that perhaps some other factor or factors act in the sympathetic ganglion to promote neuronal differentiation. The rat pheochromocytoma line PC12, has been useful for investigations into the roles of specific factors in neuronal differentiation (Greene and Tischler, 1976). PC12 cells have been found to undergo neuronal differentiation in response to NGF (Greene and Tischler, 1976). Several reports indicate that both basic and acidic FGF can mimic NGF and act to induce neuronal differentiation in PC12 cells (Togari et al., 1985; Wagner and D'Amore, 1986; Rydel and Greene, 1987; Schubert et al., 1987). Given the fact that PC12 cells derive from a mature rat chromaffin cell it is reasonable to expect that FGF may also act to induce neuronal differentiation of primary adrenal chromaffin cells. Chapter 3 details investigations into this possibility.

The sympathoadrenal lineage includes also another neuronal phenotype. Patterson and his colleagues found that when cultures of sympathetic neurons were grown in the presence of medium conditioned by embryonic heart cells, the neurons change their neurotransmitter phenotype. Specifically, they down-regulate the production of catecholamines and begin to produce acetylcholine (Patterson and Chun, 1977; Fukada, 1985) (for reviews see Patterson, 1978; and Patterson, 1990). This activity may be important in the ultimate specification of sympathetic neuron phenotype and has been demonstrated to occur *in vivo* (Schotzinger and Landis, 1988; Schotzinger and Landis, 1990). The factor responsible for the activity, as derived from embryonic heart cells, was recently found to be leukemia inhibitory factor (LIF) (Yamamori et al., 1989). In addition, postmitotic neurons can undergo further

phenotypic diversification with respect to the neuropeptide expression (Nawa and Patterson, 1990). This effect is modulated by specific non-LIF components of the heart cell conditioned medium. Thus we see in the sympathoadrenal lineage that not only do environmental factors affect cell fate choices in dividing progenitors, but the environment can re-specify or modulate the ultimate phenotype of post-mitotic sympathetic neurons.

The sympathoadrenal lineage provides a system with fairly well defined progenitors and products that permits detailed investigations into the role of specific factors in cell fate choice. It would be of interest, however, to be able to describe the relationship between the earliest neural crest cells and a specific lineage, like the SA lineage, in terms of the intermediate cell types and the factors required to make the transitions between them. The first step toward this goal, as outlined above is to determine the developmental potential of the earliest neural crest cells and to establish a culture system in which specific environmental factors can be tested. Some investigations into the developmental potential of early neural crest cells are described in chapter 2. Some of the problems associated with attempts to address the role of specific environmental factors are discussed in the next section.

Primary culture of early neural crest cells

Most of the work concerning the effect of environment on neural crest cell differentiation has been performed with large populations of primary neural crest cells, either in primary explants of migrating neural crest cells or in aggregates of neural crest cells formed when neural tubes are cultured on a non-adhesive substrate. The results of such experiments are difficult to interpret for several reasons. First, in such an experiment it is difficult to distinguish inductive, mitogenic and trophic activities. Second, there is reason to believe the target population is heterogeneous. For example, in quail primary neural crest cultures, the neural crest cells are heterogeneous with

respect to expression of both the HNK-1 and R24 epitopes (Maxwell et al., 1988). Barald finds that mesencephalic neural crest cells are heterogeneous with respect to their expression of a choline uptake mechanism antigen (Barald, 1988). Further, this heterogeneity may reflect some commitment to cholinergic differentiation (Barald, 1989). Barbu et al., have described heterogeneity in migrating neural crest cell expression of the antigen recognized by the monoclonal antibody G1N1 (Barbu et al., 1986). Primary, neural crest cells of rat are heterogeneous in their expression of low-affinity nerve growth factor receptor (LNGFR) (Smith-Thomas and Fawcett, 1989; Smith-Thomas et al., 1990). In addition, the Long-Evans strain of rat is heterogeneous for HNK-1 epitope expression (Erickson et al., 1989). Nonetheless, studies of mass cultures of neural crest may be useful for providing a relatively easy culture system to screen factors for activities that affect a large percentage of the neural crest cells. Murphy et al., found that LIF can promote the formation of sensory neurons in cultures of mouse neural crest (Murphy et al., 1991). Forbes and Maxwell found enhanced adrenergic differentiation in the presence of EHS sarcoma extracellular matrix material (Maxwell and Forbes, 1990). Morrison-Graham and Weston found that skin cells promote pigment cell formation in cultures of mouse neural crest (Morrison-Graham et al., 1990). Kalcheim et al., found that FGF in solution promotes the survival of non-neuronal cells in primary neural crest cultures and can promote neuronal differentiation when presented by FGF expressing cells (Kalcheim, 1989; Brill et al., 1992). In addition Kalcheim reported that neurontrophin-3 (NT-3) is a mitogen for primary avian neural crest cells (Kalcheim et al., 1992). Finally, Erickson and Turley, found that epidermal growth factor (EGF) is a mitogen for neural crest cells and can also serve to regulate the production of extracellular matrix molecules, hyaluronic acid, chondroitin sulfate proteoglycan, and cell-surface heparin sulfate proteoglycan (Erickson and Turley, 1987).

Clusters of neural crest cells isolated from neural tubes cultured on a non-adhesive substrate lend some insight into another type of environmental factor. Vogel and Weston picked neural crest cell clusters at various times after formation, dispersed them and cultured them (Vogel and Weston, 1988). The crest cells taken from early clusters, 18-24 hours old contained a majority of cell that expressed a ganglioside antigen recognized by the antibody A2B5. These cell produced many neurons in the dispersed cultures and relatively few melanocyte. On the other hand, if the clusters were left aggregated for an additional 24 hours, with or without the neural tube present, neural crest cells were found to no longer express the ganglioside and differentiated into melanocyte rather than neurons. There are several possibilities to explain these results. There might be distinct populations of neuroblasts and melanoblasts within the cluster and the cluster environment is not supportive of the neuroblasts and favors the proliferation of melanoblasts. Another possibility is that the cluster is comprised of stem cells and derivatives and that the cluster environment favors the production of melanoblasts over neuroblasts. Or, the cluster environment does not allow differentiation to occur but dispersal does. Further, over time the stem cells lose neurogenic potential, so that when the cells are dispersed they differentiate. If they are dispersed early they differentiate into neurons, and if they are dispersed late they differentiate into melanocytes. These possibilities are cannot be distinguished when populations of cells are examined. To resolve the issue one needs to analyze the behavior of single cells. If single cells were marked in 18 hour clusters, one could test whether the progeny of those marked cells (some fraction of which will produce neurons when dispersed at 24 hours and some fraction of which will produce melanocytes when dispersed at 48 hours) are supported by the cluster environment. That is, does the cluster environment select against neuroblasts or does it convert would-be neuroblasts into melanoblasts. The clusters provide an example of an environmental effect that may be mediated by cell-cell contact and not soluble factors.

Without a clonal analysis of their development, however, it is impossible to resolve what the primary activity of the cluster environment is.

There are some published examples of environmental effects on cell fate choices in clones of neural crest cells. Satoh and Ide found that application of αMSH to clonal cultures of quail neural crest had the effect of increasing the number of clones producing pigment cells (Satoh and Ide, 1987). Sieber-Blum and Cohen showed that when clones of quail neural crest cells are grown on a extracellular matrix extract that both the number of adrenergic clones and the number of adrenergic cells within each clone is increased (Sieber-Blum and Cohen, 1980). In a recent study Sieber-Blum shows that the number of SSEA-1 expressing cells within quail neural crest clones is increased when the clones are grown with BDNF (Sieber-Blum, 1991). Kalcheim et al., had previously shown that BDNF could stimulate neuronal differentiation and survival in mass cultures of avian neural crest cells (Kalcheim and Gendreau, 1988). In these studies it is clear that the environmental factor has some effect. It is difficult, however, to distinguish between inductive, trophic or mitogenic activities acting either on the clone founders themselves or on specific progeny cell types.

As discussed above, transplantation studies indicate that the environment can affect the choices of fate made by populations of cells. *In vivo* clonal analyses indicate that many primary neural crest cells are multipotent. *In vitro* clonal analyses indicate that many neural crest cells are multipotent, and may be controlled by environmental factors to express specific differentiated phenotypes. There are important details concerning the development of neural crest derivative that remain to be addressed. What are the intermediate cell types that form in the transition from multipotent (totipotent) stem cells to the ultimate derivative cell types? For example, Baroffio et al., observed several types of clones that contained sub-sets of the possible derivatives (Baroffio, et al., 1988). Were such clones founded by relatively restricted, multipotent but not totipotent intermediate cell types that were the progeny of a stem cell? Are there

unique stem cell types generated at different axial levels? The apparent lack of a mesectodermal developmental potential among trunk neural crest cells, and the multipotency of cephalic neural crest suggest that there are at least two distinct stem cell types. What are the factors that actually control cell fate decisions? And how do those factors act in tissues where distinct cell types, in close contact, derive from the same progenitor? The answers to these questions will lend important insight into problems such as gangliogenesis, where the production of several distinct cell types all arising from the same cell are tightly controlled to produce the mature structure.

In this review, I have outlined some of the problems of neural crest cell biology. The work to be presented will speak to two problems. First, how do neural crest cells achieve the observed diversity of lineage specification in terms properties of precursor cells and their immediate progeny? The work presented in chapter 2, concerning the identification and characterization of a primary neural crest stem cell, addresses a question that distinguishes between the extreme models of neural crest development I have presented. That is, do the earliest multipotent neural crest cells undergo self-renewal, in addition to the production of differentiated progeny? In other word are they stem cells or do they develop by way of continual restriction? The second question is: how does the environment exert its influence over cell fate choices? Chapter 2 describes the effect of an environmental factor on the fate of neural crest cell clones. In addition, chapter 3 describes the activity of basic FGF on chromaffin cells. Finally, Chapter 4 presents evidence that an antibody originally thought to be a Schwann cell marker actually recognizes the rat low-affinity nerve growth factor receptor.

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CHAPTER 2: Isolation and characterization of self-renewing, multipotent mammalian neural crest cells

Abstract

Clonal and lineage analysis of avian neural crest cells has revealed that many of these cells are multipotent. However, it is not clear whether the immediate progeny of these cells are committed to distinct sublineages or whether these progeny are themselves multipotent, as would occur in a stem cell lineage. To address this issue we have isolated mammalian neural crest cells using a cell surface monoclonal antibody, and examined their developmental potential in culture. These neural crest cells are both multipotent, and able to generate clonal progeny that retain such multipotency. Moreover, the composition of the substrate can determine whether these cells differentiate into both neurons and glia, or just glia. These data provide the first evidence that early neural crest cells have stem cell properties, and establish a system in which the roles of environment and lineage restriction in crest development may be investigated at the single cell level.

Introduction

The vertebrate nervous system contains a very large number of different cell types, but the cellular and molecular mechanisms that generate this diversity during development are largely unknown. Lineage tracing studies performed *in vivo* using retroviral genomic markers or injected tracer dyes have revealed that many neural progenitor cells are multipotent; i.e., they give rise to more than one cell type, including in many cases both neurons and glia (for reviews, see Sanes, 1989; McConnell, 1991). While such studies define the fates of individual progenitor cells in the embryo, they do not necessarily reveal the developmental potentials of these cells, or how these potentials change with time. The development of the hematopoietic system, for example, involves progressive restrictions in the developmental capacities of progenitor cells (for review, see Metcalf, 1989). The isolation and characterization of different populations of hematopoietic progenitor cells has been a major focus of research in cellular immunology (Spangrude et al., 1988; Ikuta, et. al., 1992). By contrast, few neurogenic progenitor cell populations have been similarly studied.

Experimental analysis of purified progenitor cell populations isolated from the developing nervous system has been carried out in the optic nerve-derived O2A lineage (for review, see Raff, 1989) and the neural crest-derived sympathoadrenal (SA) lineage (for reviews, see Patterson, 1990; Anderson, 1993). In both of these cases, isolated progenitor cells placed in culture have been shown to have at least two potential fates, the choice among which is controlled by an interplay between instructive environmental signals and intrinsic developmental programming. These progenitors, however, appear to have a limited repertoire of cell fates and derive from relatively late stages in neural cell lineage diversification. Far less is known about the properties of the early neuroepithelial cells that function in the initial stages of neurogenesis (McKay, 1989; McConnell, 1991). Although progenitor cells from such early stages are often referred to as "stem" cells (e.g., see Cattaneo and McKay, 1990) the experimental criteria used

to define stem cells in other systems (Potten and Loeffler, 1990) have not been rigorously applied. These properties include the ability to self-renew, divide asymmetrically and generate differentiated progeny. In addition, many (but not all) stem cells are multipotent (Hall and Watt, 1989).

The neural crest has provided a system for the study of neural cell type diversification that is simpler and experimentally more accessible than the CNS. Neural crest cells disperse from the dorsal margin of the neural tube and undergo extensive migrations before differentiating to generate the neurons and glia of the peripheral nervous system (LeDouarin, 1982). Classical experiments in avian embryos have revealed that the fate of transplanted neural crest cell populations can be strongly influenced by their environment (LeDouarin, 1980). Clonal analysis of avian neural crest cells has been carried out both *in vitro* and *in vivo*, and has revealed that many (but not all) early neural crest cells can generate the full range of crest derivatives in the trunk and mesencephalic regions (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). Taken together, these data are consistent with the idea that the environment acts to control the choice of fate by multipotent neural crest cells.

Arguing against this simple view, however, are data revealing cellular heterogeneity in the neural crest. For example, some neural crest cell clones contain only a single derivative, such as Schwann cells (Bronner-Fraser and Fraser, 1989; Dupin et al., 1990; Frank and Sanes, 1991). A subpopulation of mesencephalic neural crest cells isolated using a cholinergic surface marker appears restricted to a parasympathetic neuronal lineage (Barald, 1988; Barald, 1989). Furthermore, neural crest cell populations with partially-restricted developmental capacities have been identified both *in vivo* (Artinger and Bronner-Fraser, 1992) and *in vitro* (Vogel and Weston, 1988). To reconcile these observations with the existence of multipotent cells, it has been proposed that neural crest cell lineage diversification may occur by a process

analagous to hematopoiesis (Dexter et al., 1990), in which multipotent neural crest cells give rise to their differentiated progeny via the production of "blast," or "transit amplifying" cells, which are limited both in their proliferative capacity and in their developmental repertoire (Anderson, 1989; Sieber-Blum, 1990; LeDouarin et al., 1991). Environmental factors could then influence the choice of fate by multiopotent cells and/or the survival of specific progenitor subpopulations.

An important question raised by such a model is whether multipotent neural crest cells generate immediate progeny that are already developmentally restricted, or whether such multipotent cells undergo at least limited self-renewal analagous to stem cells in other lineages. To address this question, we have isolated mammalian neural crest cells using a cell-surface monoclonal antibody and examined their properties in clonal culture. We show that these cells are not only multipotent, but that such multipotent cells are capable of self-renewal. Moreover, the fate of these multipotent cells, and not simply their attachment or survival, can be controlled by both their culture medium and the composition of their substrate. With time in culture, some developmentally-restricted progeny cells appear to be generated. These data provide evidence for a hematopoietic-like mechanism of neural crest cell lineage diversification, and establish a system in which the molecular control of this diversification process can be further investigated.

Results

Isolation and cloning of multipotent rat neural crest cells

We have chosen to isolate mammalian neural crest cells from rat rather than mouse embryos for two reasons. First, we wish ultimately to relate the developmental properties of these cells to those of neural crest-derived sympathoadrenal progenitors, which we have previously characterized in the rat using a series of antibody markers, many of which are rat-specific (Anderson and Axel, 1986; Anderson et al., 1991;

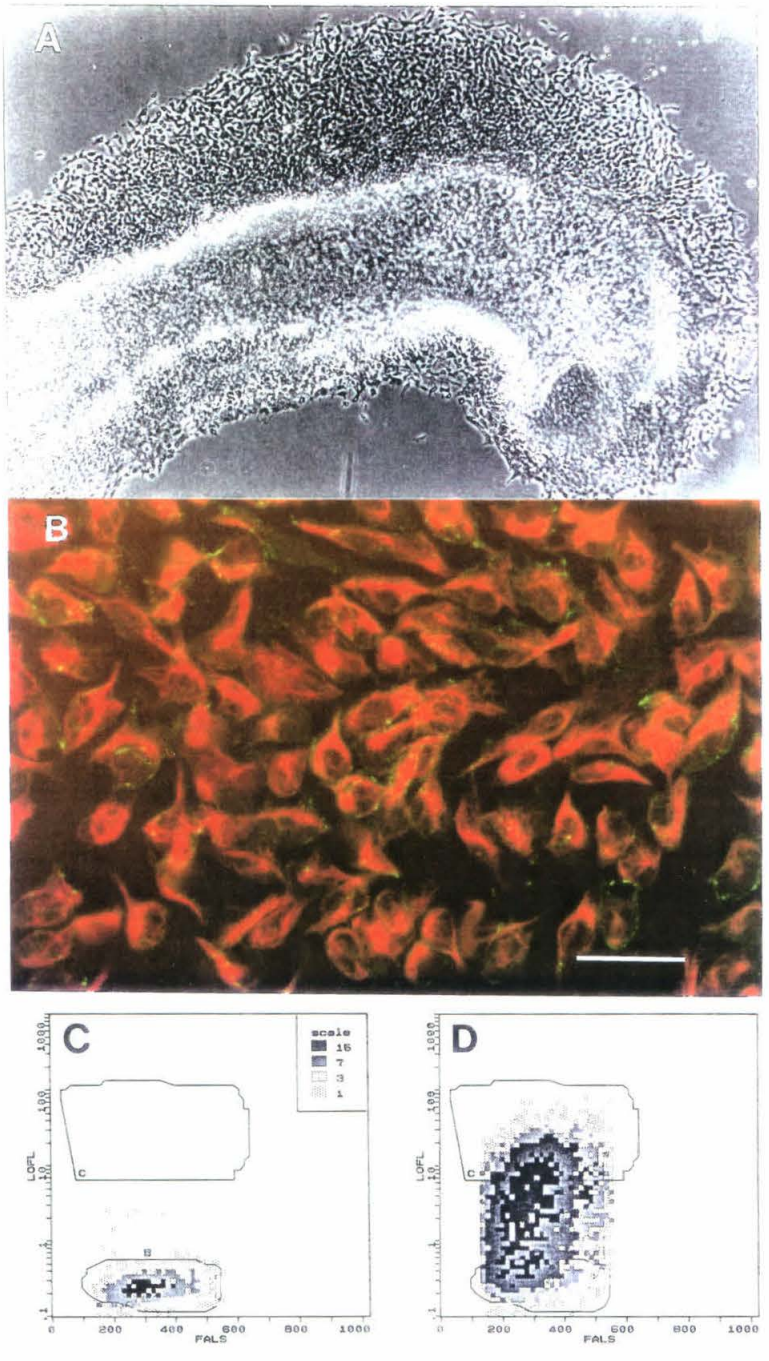
Michelsohn and Anderson, 1992). Second, cell-surface antibody markers were available that react with rat (Smith-Thomas and Fawcett, 1989) but not mouse neural crest cells. These antibodies recognize the mammalian low-affinity NGF receptor (LNGFR) (Chandler et al., 1984; Stemple and Anderson, 1990).

When E10.5 neural tubes are cultured on a fibronectin (FN) substrate, most of the neural crest cells that emigrate from the neural tubes over a 24 hour period (Fig. 1A) express LNGFR, as recognized by the monoclonal antibodies 217c (not shown) and 192IgG (Figure 1B, yellow/green fluorescence). Flow cytometry of 24 hour neural crest explants indicates that > 50% of the neural crest cells are moderately to strongly LNGFR$^+$ (Figure 1D). All of the neural crest cells also express nestin (Fig. 1B, red/orange fluorescence), an intermediate filament protein found in immature neuroepithelial cells (Hockfield and McKay, 1985) and in Schwann cells (Friedman et al., 1990). Double-label immunofluorescence staining at clonal density confirmed the co-expression of LNGFR and nestin by individual neural crest cells (Figs. 1B, 2A-C).

We established conditions that permitted the growth in low-density culture of LNGFR$^+$ cells that had emigrated from the neural tube *in vitro*. In basal L-15 CO_2 medium (Hawrot and Patterson, 1979), the combination of fetal bovine serum (FBS) and chick embryo extract (CEE) allowed the survival and growth of neural crest colonies (data not shown). Under these conditions, however, the neural crest cell clones soon lost LNGFR expression, and subsequent differentiation of neuronal or glial cells was not observed. Since our empirical observations indicate that the ability to differentiate to neurons and glia correlates with the maintenance of early LNGFR expression, we developed a chemically defined medium (see Experimental Procedures) that would permit neural crest cell survival and maintain LNGFR expression. While this medium was sufficient for cell survival at high plating densities it was not able to support colony growth at clonal densities. If the defined medium was supplemented with 10% FBS, colonies were able to grow at clonal density, but LNGFR expression

Figure 1. Neural crest outgrowth from rat E10.5 neural tube at 24 hours in culture.

A neural tube that had been growing for 24 hours in culture is shown in panel 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. In panel B is shown, at higher magnification, a region of neural crest cells after fixation and immunocytochemical staining with a monoclonal antibody to nestin (in red) and a rabbit antiserum to LNGFR (in green). (C) Neural crest cells were labelled with monoclonal anti-LNGFR 192IgG and sorted by a fluorescence activated cell sorter (FACS). A control profile from the FACS shows the background staining for the fluorescent secondary antibody (C). Panel D shows the FACS 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 foward angle light scatter, is plotted on the ordinate. The scale bar in panel B corresponds to a length of 50 μm for panel B and to 500 μm for panel A.


A
B
C
scale
16
7
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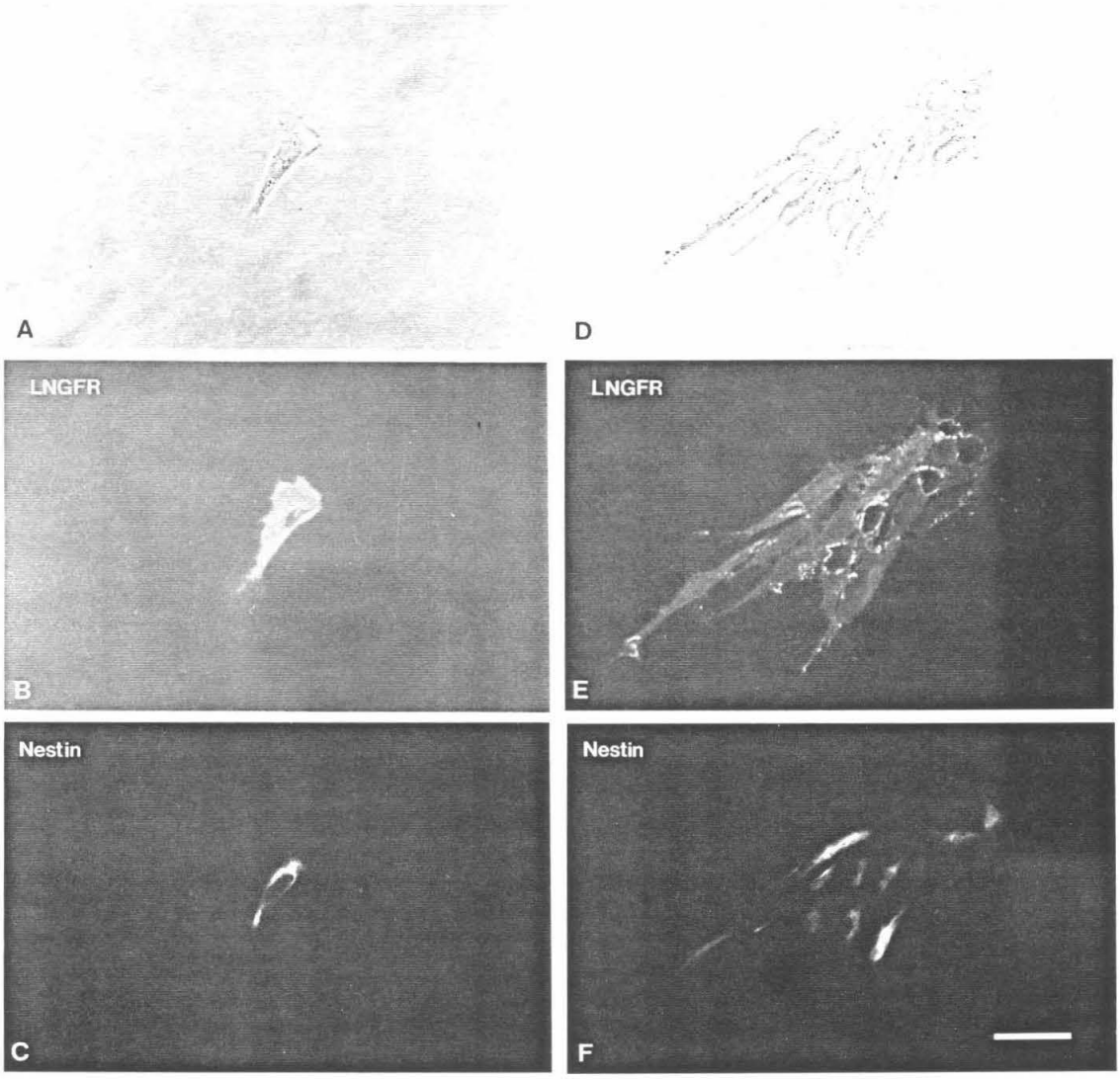
FALS

Figure 2. Clonal expansion of LNGFR+, nestin+ rat neural crest cells.

(A, B, C) a single neural crest founder cell fixed, stained and photographed 24 hours after plating at clonal density. (D, E, F) a clone derived from a similar single founder cell after 3 days in culture. (A, D) phase contrast micrographs. Double-label immunofluorescence was performed with rabbit antiserum to LNGFR (B, E) and monoclonal anti-nestin (C, F). Both founder cell and the cells in the clone co-express LNGFR and nestin. The scale bar in panel F corresponds to a length of 50 μm.


A
D
LNGFR
B
LNGFR
E
Nestin
C
Nestin
F

was lost (not shown). When the defined medium was supplemented with 10% CEE rather than FBS, however, the neural crest cells survived, proliferated at clonal density and continued to express LNGFR and nestin for several cell generations (Figure 2E,F). We term such medium standard medium (SM).

In order to assess the developmental potential of individual neural crest cells, it was necessary to establish that the colonies that developed in low density cultures were actually clones. Neural crest cells taken from 24 hour neural tube explants were plated at a density of ~225 cells / 100 mm dish. After the cells attached, individual LNGFR$^+$ neural crest cells were identified and inscribed with a ~3 mm circle drawn on the bottom of the dish. The circles were generally large enough to permit the unambiguous identification of the progeny of the cell at any time during the experiment. When such single, identified LNGFR$^+$ neural crest cells were grown in SM for 9-14 days, they generated large clones that contained both cells with a neuronal morphology (Figure 3C arrow), as well as non-neuronal cells. Quantification indicated that >60% of the clones contained such neuronal cells (see Fig. 10B). These neuronal cells no longer expressed LNGFR and nestin (Fig. 3D). However, they could be triply-labeled by monoclonal antibodies to pan-neuronal markers such as neurofilament (Fig. 4E) and high-polysialic acid (PSA) NCAM (monoclonal antibody 5A5; Fig. 4D), as well as by a polyclonal antibody to peripherin (Fig. 4F), an intermediate filament protein preferentially expressed by peripheral neurons (Portier et al., 1984; Parysek et al., 1988; Parysek and Goldman, 1988).

The neuron-containing clones also contained non-neuronal cells (Fig. 3A,C). These cells continued to express LNGFR and nestin (Fig. 3B) and displayed an elongated Schwann cell-like morphology (Brockes and Raff, 1979). While immature Schwann cells are known to express both LNGFR and nestin (Friedman et al., 1990; Jessen et al., 1990), these markers are insufficient to identify Schwann cells in this system since they are expressed by the neural crest cell precursor as well. Other

Figure 3. LNGFR and nestin expression in a neuron containing clone.

Two separate fields from a single clone are shown. As in Figure 2, the clone has been double labeled with rabbit antiserum to LNGFR (green fluorescence) and monoclonal anti-nestin (orange fluorescence). Panels A and B show a field containing $LNGFR^+$, $nestin^+$ cells with a Schwann cell-like morphology. Panels C and D show another region containing neurons which express neither LNGFR nor nestin. The scale bar in panel D corresponds to a length of 50 μm.

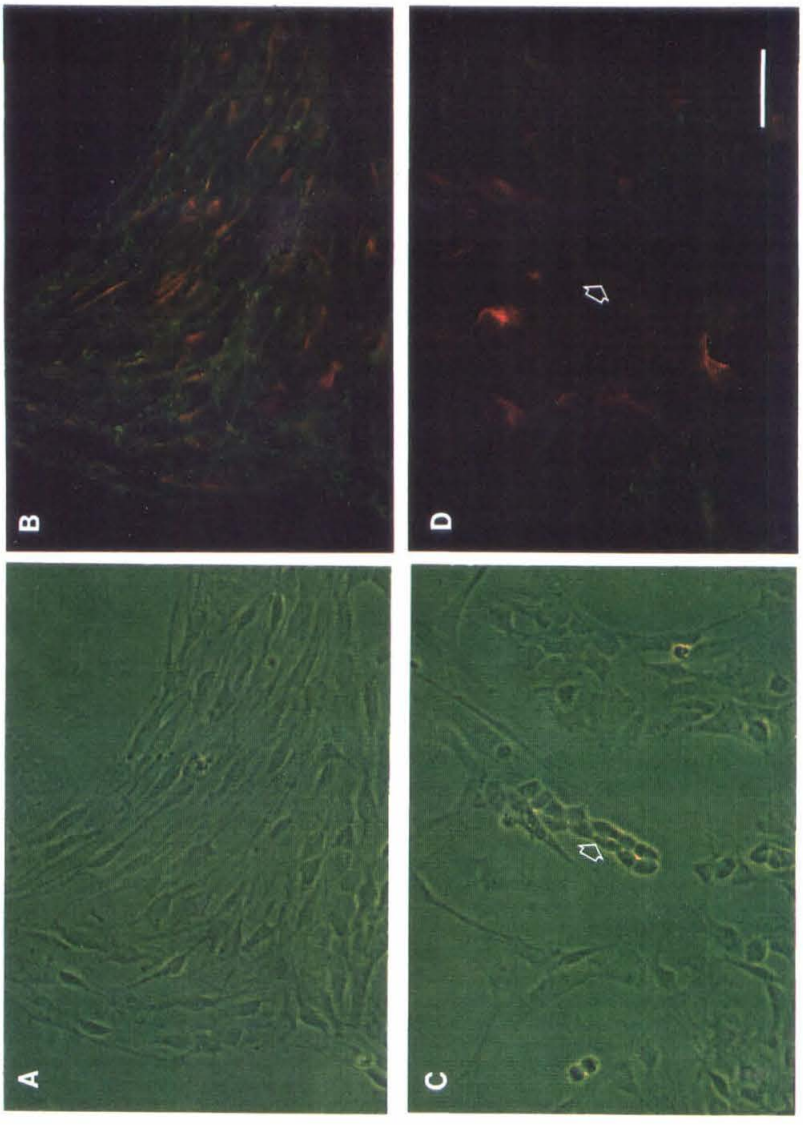
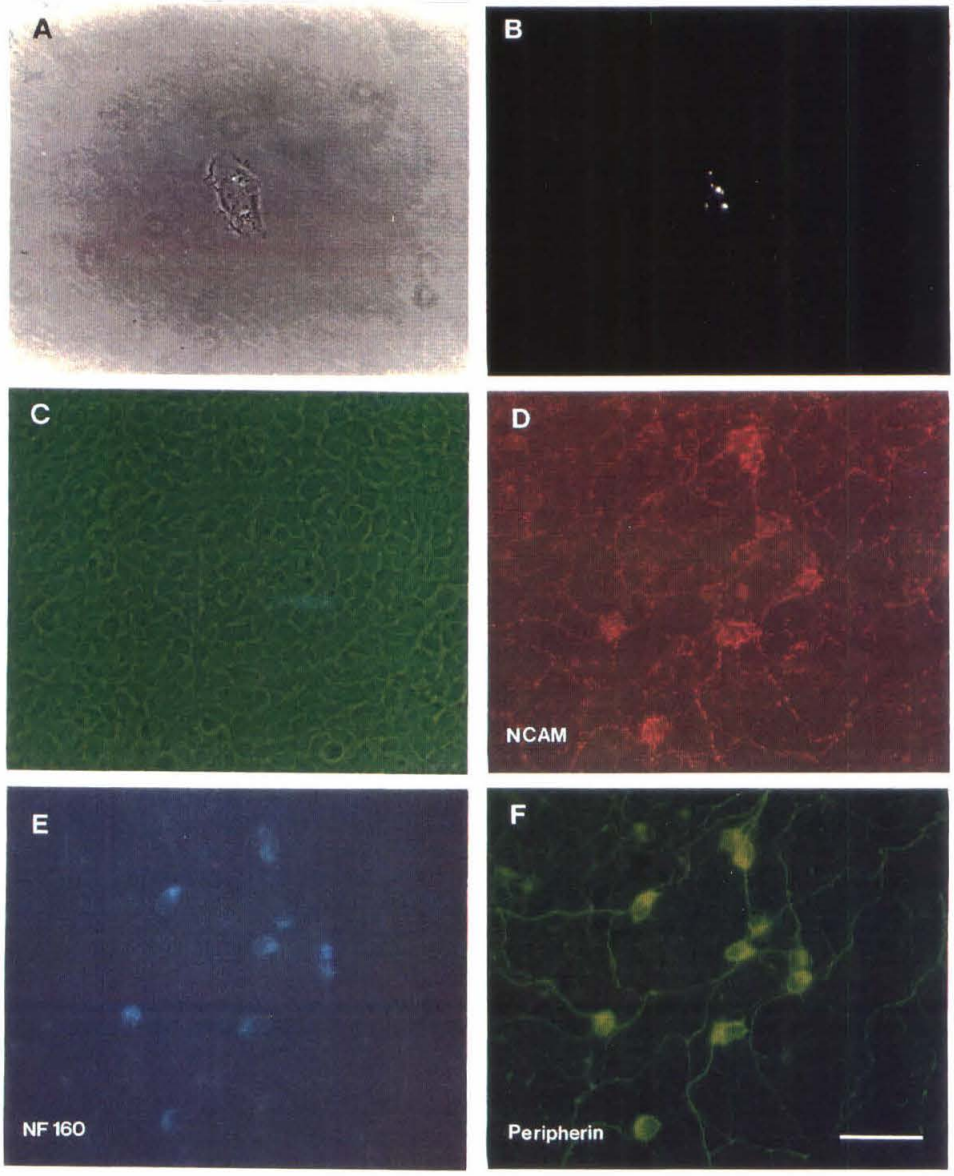

A
B
C
D

Figure 4. Expression of neuronal traits in clones of LNGFR+ founders.

A single founder cell (A, B) and its resulting clone (C-F) after 10 days growth are shown. Phase contrast micrographs of the founder (A) and the clone (C) are shown. The coresponding fluorescence micrographs depict the LNGFR expression of the founder (B) and the expression of several neuronal markers in the clone. Triple labeling with mouse IgM anti-NCAM (D), mouse IgG anti-NF160 (E), and rabbit anti-peripherin (F) reveals the coincidence of these markers in a subset of the cells in the clone. The scale bar in panel F corresponds to a length of 50 μm.


A
B
C
D
NCAM
E
NF 160
F
Peripherin

Schwann cell markers, such as glial fibrillary acidic protein (GFAP) (Jessen et al., 1990), and the sulfatide antigen recognized by antibody O4 (Mirsky et al., 1990) were not expressed by these LNGFR$^+$ cells (not shown). We therefore sought to promote Schwann cell differentiation by transferring the clones into a medium known to maintain differentiation of postnatal Schwann cells isolated from peripheral nerve (Mirsky et al., 1990). This medium consisted of SM with the addition of both 10% FBS and 5 μM forskolin, an activator of adenylate cyclase.

Following 5-10 days in such Schwann cell differentiation (SCD) medium, most of the non-neuronal cells in the clones expressed GFAP (Figure 5E), the sulfatide O4 antigen (Fig. 5B), and the peripheral myelin protein P_0 (Fig. 5C,F). Although GFAP is expressed by astrocytes and O4 by oligodendrocytes in the CNS, the co-expression of these two markers in the same cell is unique to peripheral glial cells (Jessen et al., 1990; Mirsky et al., 1990; Morgan et al., 1991). Moreover, P_0 is uniquely expressed by myelinating peripheral glia (Lemke, 1988). These data therefore indicate that the LNGFR$^+$, nestin$^+$ neural crest cells are able to differentiate into Schwann cells, under appropriate culture conditions.

The addition of serum and forskolin at this late stage of culture did not appear to be toxic to neurons that had differentiated prior to transfer into SCD medium (Fig. 6). In fact the number of neurons appeared to increase after such medium transfer (not shown). Triple-label immunofluorescence staining using rabbit polyclonal anti-peripherin, mouse monoclonal IgM O4, and mouse monoclonal IgG anti-GFAP revealed that sulfatide$^+$ and GFAP$^+$ Schwann cells were present in the same clones as peripherin$^+$ neurons (Fig. 6C,D).

Primary neural crest clones containing identifiable neurons and glia usually also contained a small number of cells, that displayed a flattened, undifferentiated morphology. These cells did not express any of the neuronal or glial lineage markers used (Fig. 5E,F), and did not express LNGFR (data not shown). Low levels of nestin

Figure 5. 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 (SCD) medium and allowed to grow for another 1-2 weeks prior to fixation and immunocytochemistry. Two separate double labeled clones are shown here in phase contrast (A, D). The clones were stained with O_4 (B) and rabbit anti-P_0 (C), or with monoclonal anti-GFAP (E) and rabbit anti-P_0. The same clones also contained neurons (not shown). The scale bar in panel F corresponds to a length of 50 μm.

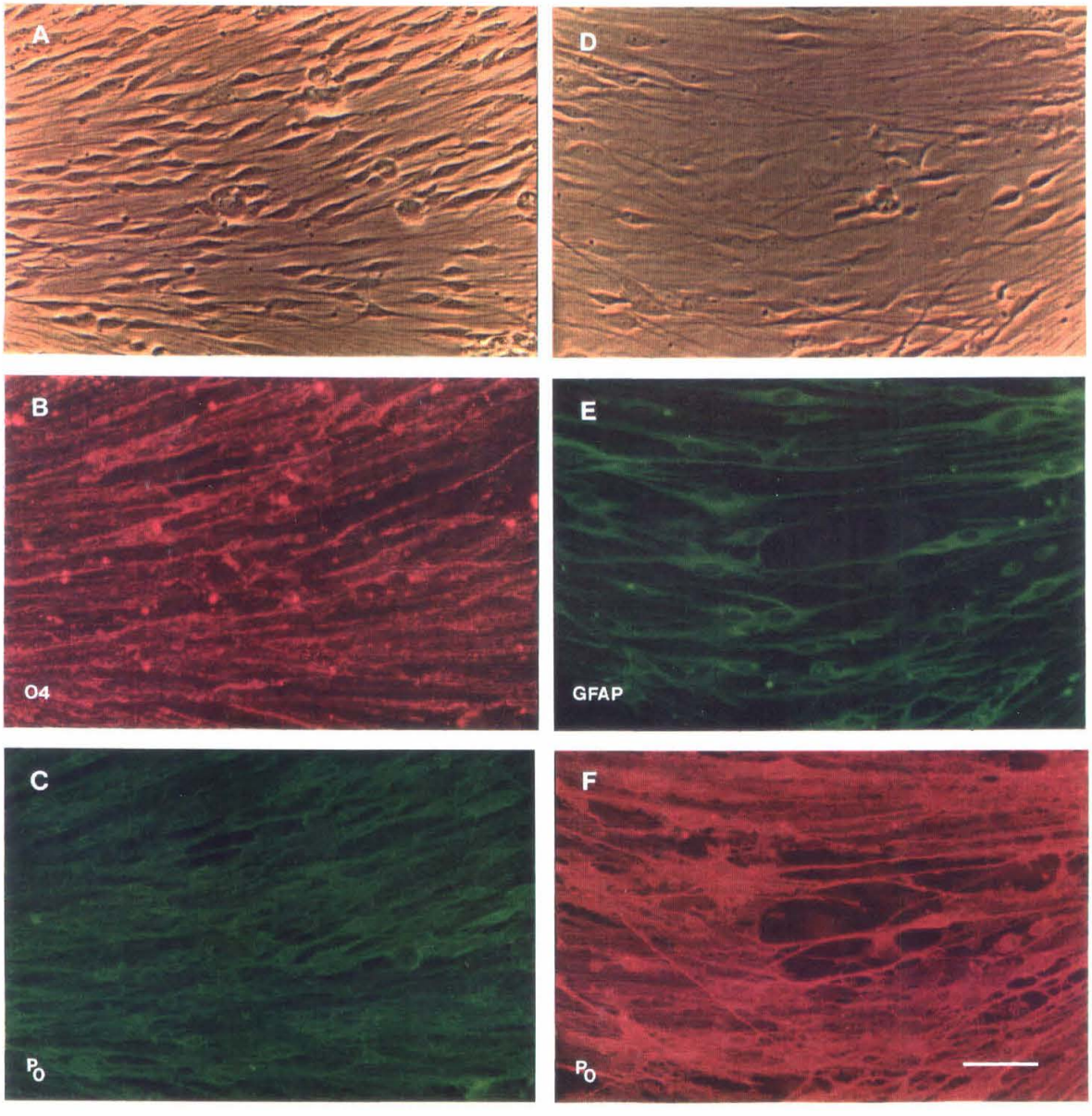
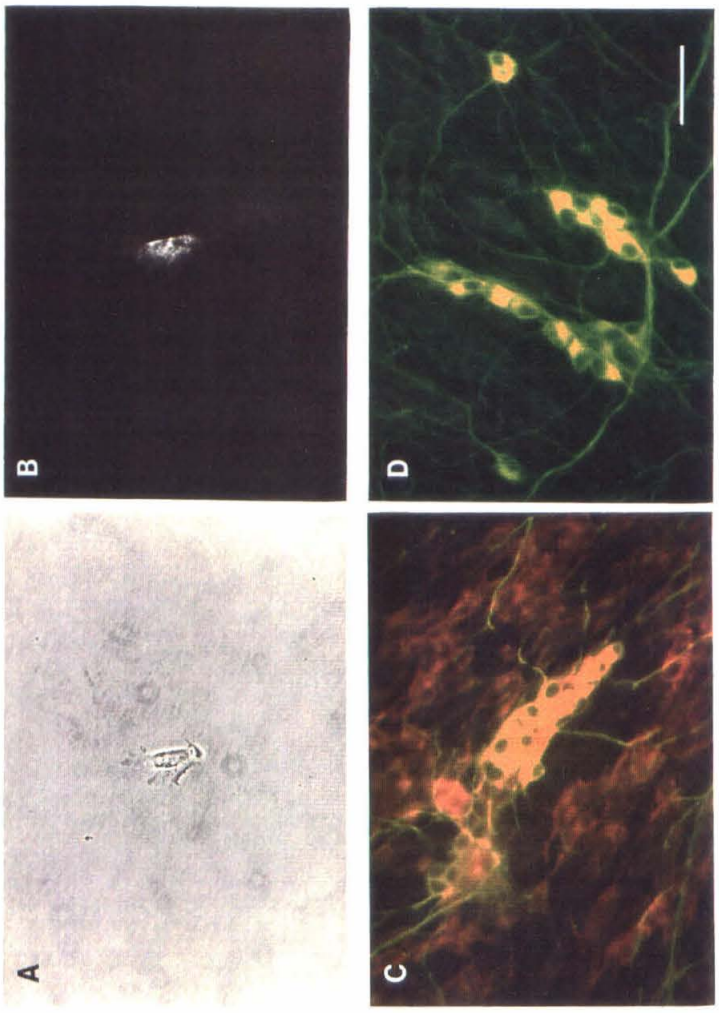

A
D
B
O4
E
GFAP
C
P_0
F
P_0

Figure 6. Multipotency of neural crest cells.

A clone from a single founder cell shown in phase contrast (A) and expressing LNGFR (B) was allowed to proliferate and differentiate in standard medium (containing CEE and lacking serum) and then transferred into SCD medium (containing serum and forskolin). After approximately 10 days, the culture was fixed and triple-labeled with rabbit anti-peripherin (C and D, in green/yellow), anti-GFAP (IgG) (C, in red) and O_4 (IgM) (D, blue). Panels C and D are 2 separate fields from the same clone. The scale bar in panel D corresponds to a length of 50 μm.


A
B
C
D

immunoreactivity could occasionally be detected in these cells. Due to our inability to identify these cells with available markers, we have termed them "other" (O) cells. A small percentage (< 10%) of neural crest cells that were LNGFR$^+$ at 24 hrs after plating generated clones that consisted only of "O" cells (see below, Figs. 10 and 11). Inclusion of 10% FBS in the medium greatly increased the proportion of such purely "O" clones (D.L. Stemple, unpublished observations). This suggests that the "O" phenotype may represent a third, presently unidentified, non-neuronal fate of the neural crest cells which is also sensitive to environmental factors.

Taken together, the foregoing data indicate that single neural crest cells (Fig. 6A,B) expressing nestin and LNGFR are able to give rise to clones of differentiated cells containing both peripheral neurons and glia, as well as a third unidentified cell type. Differentiation to the neuronal phenotype involves the loss of nestin and LNGFR expression, and the acquisition of neuronal markers such as neurofilament, high-PSA NCAM and peripherin. By contrast, in the glial lineage LNGFR and nestin expression are retained, and under appropriate differentiation-inducing conditions additional glial markers (GFAP, O4 and P_0) are acquired. Taken together, these data establish the multipotency of the LNGFR$^+$, nestin$^+$ rat neural crest cells.

Self-renewal of multipotent neural crest cells *in vitro*

The observation that a single neural crest cell gives rise to multiple differentiated derivatives does not distinguish whether the immediate progeny of a multipotent cell are themselves multipotent, or whether they are already restricted to different sublineages. To distinguish these possibilities, we performed a serial subcloning experiment. Primary clones founded by LNGFR$^+$ progenitor cells (Figure 7A) were allowed to grow for 6 days on a pDL/FN substrate. Clones were then picked and replated at clonal density under the same conditions as their founder cells. Individual secondary founder cells were identified visually to ensure that all secondary colonies in fact

derived from single cells (Figure 7B,B'). Although on average 50% of these identified founder cells died (Table I), after 10 days many of the surviving subclones were observed to contain neurons, identifiable by their rounded cell bodies and network of neurites (Figure 7C,C'). These subclones were then transferred to SCD medium and allowed to mature for another week, at which point they were fixed and double-labeled with antibodies to GFAP and neurofilament. This analysis confirmed that, like primary clones, these secondary subclones contained not only peripheral neurons, but $GFAP^+$ (Fig. 8B) and P_0^+ (not shown) glial cells as well.

From a total of 16 different primary founder clones analyzed in two separate experiments, 75% gave rise to at least one secondary clone containing neurons, glia and other cells (Table I, N+G+O). Moreover, in the second experiment (Table I, clones 5.2-8.5), 100% of the founder clones gave rise to at least one N+G+O subclone. These data indicate that a high proportion of primary neural crest cells are capable of self-maintenance. Furthermore, 50% of the primary clones evaluated gave rise to multiple multipotent secondary subclones; for example, one founder cell gave rise to 15 multipotent subclones (Table I, clone 1.1). On average, 54 ±11% of the surviving secondary clones derived from each primary founder were multipotent, i.e., contained both neurons, glia and "other" cells. These data show not only that multipotent neural crest cells are capable of self-renewal, but also indicate that self-renewal does not occur exclusively via asymmetric cell divisions, otherwise only one multipotent clone would be found among each set of secondary clones.

In several cases secondary clones containing several neurons but no other cell type were found (Table I, N only). The number of cells in these N only clones was small (< 10), and such clones were rarely observed in the primary clone population. In addition, many secondary clones contained glia and other cells but no neurons (Table I, G+O). Some of these non-neurogenic clones, moreover, contained only $LNGFR^+$ glial cells and no $LNGFR^-$ "other" cells, suggesting the eventual generation of

Figure 7. Self-renewal of multipotent neural crest cells.

(A), primary clone derived from a single LNGFR$^+$ founder cell, photographed live after 7 days in culture. This same clone was dissociated by trypsinization and replated at clonal density. Two of the progeny subclone founder cells are shown in (B) and (B'). These two cells were allowed to form clones and photographed live after 17 days (C, C'). Both non-neuronal and neurite bearing cells are visible in the clones. Some of the clones were then transferred into SCD medium, fixed and stained after an additional period of culture (see Figure 7.). The scale bar in panel B' corresponds to a length of 50 μm for panels B and B', 60 μm for panels C and C', and 100 μm for panel A.

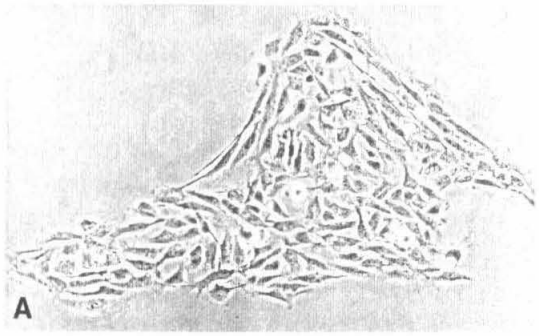

1° Clone
Day 7
A
2° Founders
B
B´
2° Clones
Day 17
C
C´

Table I. Quantification of self-renewal in clonal neural crest cultures.

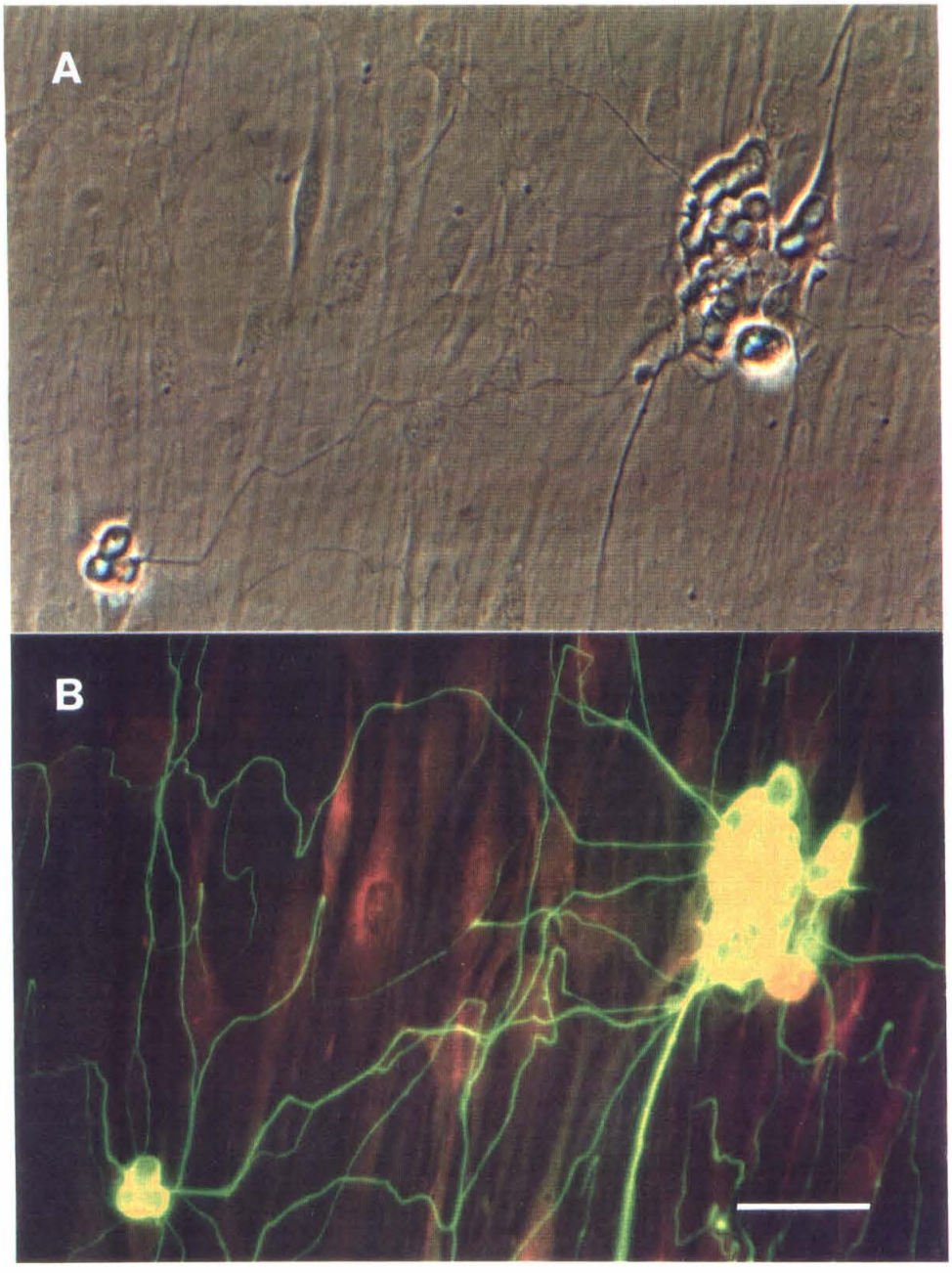
Primary neural crest clones were subcloned and the phenotypic composition of their progeny secondary clones recorded as "N only" (multiple neurons but no glia), "N+G+O" (neurons, glia and other); "G+O" (glia and other), or "No clone found" (i.e., the secondary founder cell failed to form a surviving clone). For both primary and secondary clones, the clonal nature of the colonies was verified by identifying and circling individual LNGFR$^+$ founder cells on the plate. For each primary clone (plate number.clone number), the total number of secondary founder cells identified is given in column two. The total number of subclones of each phenotype is indicated in the subsequent columns. The percentage of total subclones represented by each phenotype is given in parentheses. The mean numbers are given either as the percentage of total subclone founder cells identified, or as the percentage of clonogenic founders; i.e., surviving secondary clones. These means were calculated separately for each primary clone and the average of this number ±SEM was calculated. Note that the predominant clone type found among the surviving subclones is of the N+G+O type, i.e., multipotent. Clones in the "G+O" category included some containing only LNGFR$^+$ cells. The data are derived from two independent experiments (plates 1-4, and 5-8). A total of 16 primary founder cells and 151 secondary founder cells were analyzed.

Table I. Quantification of self-renewal in clonal neural crest cultures

		Sub-Clone Phenotype total# (%)				
Primary Clone ID	# of 2° Founders	N only	N+G+O	G + O	O	No clone found
1.1	21	0	15 (71)	0	0	6 (29)
1.18	6	0	1 (17)	1 (17)	2 (33)	2 (33)
1.24	5	1 (20)	0	1 (20)	2 (40)	1 (20)
2.6	7	0	0	1 (14)	1 (14)	5 (72)
2.18	7	0	0	1 (14)	0	6 (86)
3.14	20	0	2 (10)	4 (20)	0	14 (70)
3.18	4	0	1 (25)	0	0	3 (75)
4.5	1	0	1 (100)	0	0	0
4.8	9	0	0	1 (11)	2 (22)	6 (67)
4.14	10	0	2 (20)	3 (30)	1 (10)	4 (40)
5.2	15	1 (7)	8 (53)	0	0	6 (40)
6.1	13	0	2 (15)	2 (15)	0	9 (70)
6.2	17	1 (6)	2 (12)	4 (24)	0	10 (58)
6.17	2	0	1 (50)	0	0	1 (50)
8.2	5	0	4 (80)	0	0	1 (20)
8.5	9	0	4 (44)	0	0	5 (56)
Mean±s.e.m.						
% of total founders		2.1 ± 1.3	31 ± 7.9	10 ± 2.6	7.4 ± 3.3	49 ± 6
% of clonogenic founders		3.1 ± 1.8	54 ± 11	29 ± 8	15 ± 6	

Figure 8. Multipotency of secondary founder cells.

A clone derived from secondary founder cells such as that shown in Figure 6 was transferred into SCD 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 panel B corresponds to a length of 50 μm.


A
B

Schwann cell "blast" cells. In support of this idea, in separate experiments we have found that subcloning of clones initially containing LNGFR$^+$ and LNGFR$^-$ cells produces both purely LNGFR$^+$ and purely LNGFR$^-$ subclones (D.L. Stemple, unpublished observations). Taken together, these data suggest that although mammalian neural crest cells are capable of self-renewal, under some conditions they may eventually generate progeny with more restricted developmental potential.

The fate of multipotent neural crest cells is influenced by their substrate

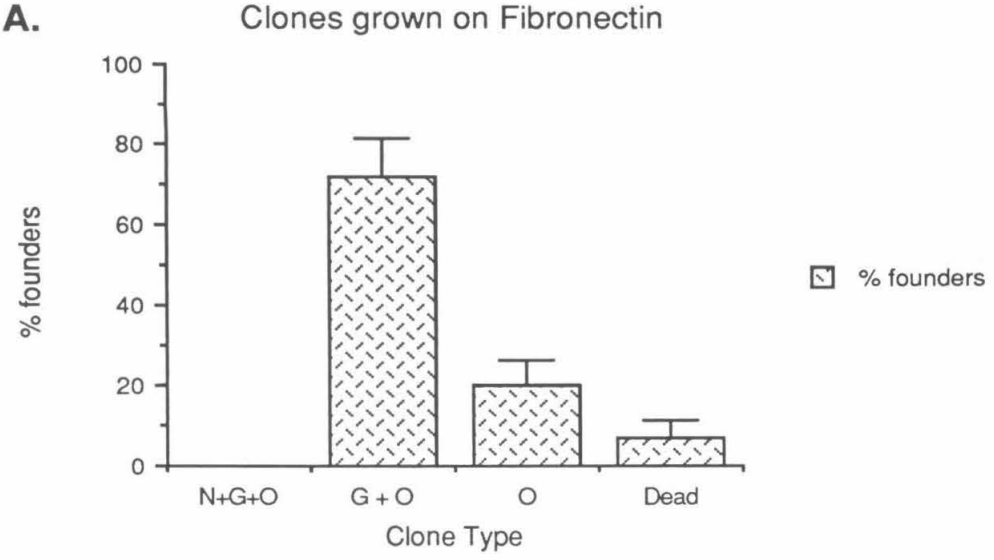
The foregoing experiments indicate that neural crest cells grown on a pDL/FN substrate generate clones containing both peripheral neurons and glia. When the same cell population is grown at clonal density on a substrate containing FN only, the resulting clones contain LNGFR$^+$ non-neuronal cells and O cells, but no neurons (Figure 9, compare C vs. F). By contrast, on pDL/FN the clones contain both LNGFR$^+$, 5A5$^-$ non-neuronal cells and LNGFR$^-$, 5A5$^+$ neurons (Figure 9B, C). Quantification indicated that on FN alone, 70-80% of the clones are of the G+O phenotype and none of the N+G+O phenotype (Figure 10A), whereas on pDL/FN 60% of the clones are of the N+G+O phenotype and only 20% of the G+O phenotype (Figure 10B). These data indicate that the composition of the substrate affects the cellular phenotype of neural crest clones that develop in culture.

Since in these experiments a population of dissociated neural crest cells is plated on either of two different substrates, the preceding results could be explained simply by the failure of neurogenic crest cells to adhere and survive on a FN substrate. To address this possibility, a different experiment was performed in which all of the crest cells were initially plated on a FN substrate. Thus differences in initial attachment and/or survival would not account for differences in eventual clone composition. After either 24 hrs or 4 days of culture, clones containing LNGFR$^+$ crest cells were identified by live-labeling and circled. Purely "O" clones were not further analyzed

Figure 9. Neuronal differentiation of mutipotent 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, E), and monoclonal anti-high PSA NCAM (IgM) (C, F). Labeling was distinguished using class specific secondary antibodies. Neurite-bearing cells (A) expressing NCAM (C) are obtained on pDL/FN but not on FN (D, F). Clones on both substrates contain LNGFR$^+$ cells (B, E). However the intensity of LNGFR labeling is lower on pDL/FN, possibily reflecting the known ability of axonal contact to down-regulate LNGFR expression on Schwann cell. The scale bar in panel F corresponds to a length of 50 μm.

A.


Clones grown on Fibronectin
100
80
60
40
20
0
% founders
N+G+O
G + O
O
Dead
Clone Type
% founders

B.

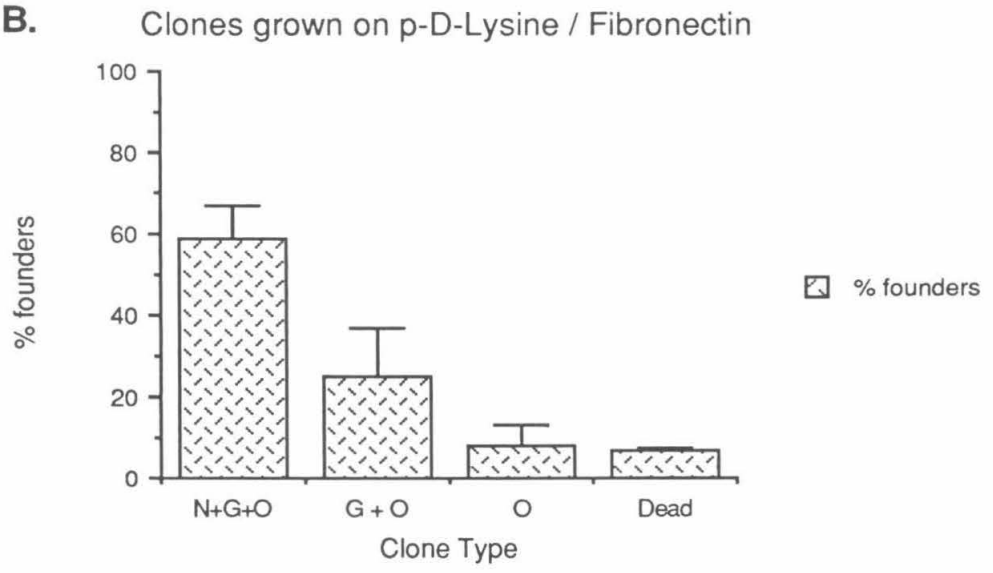
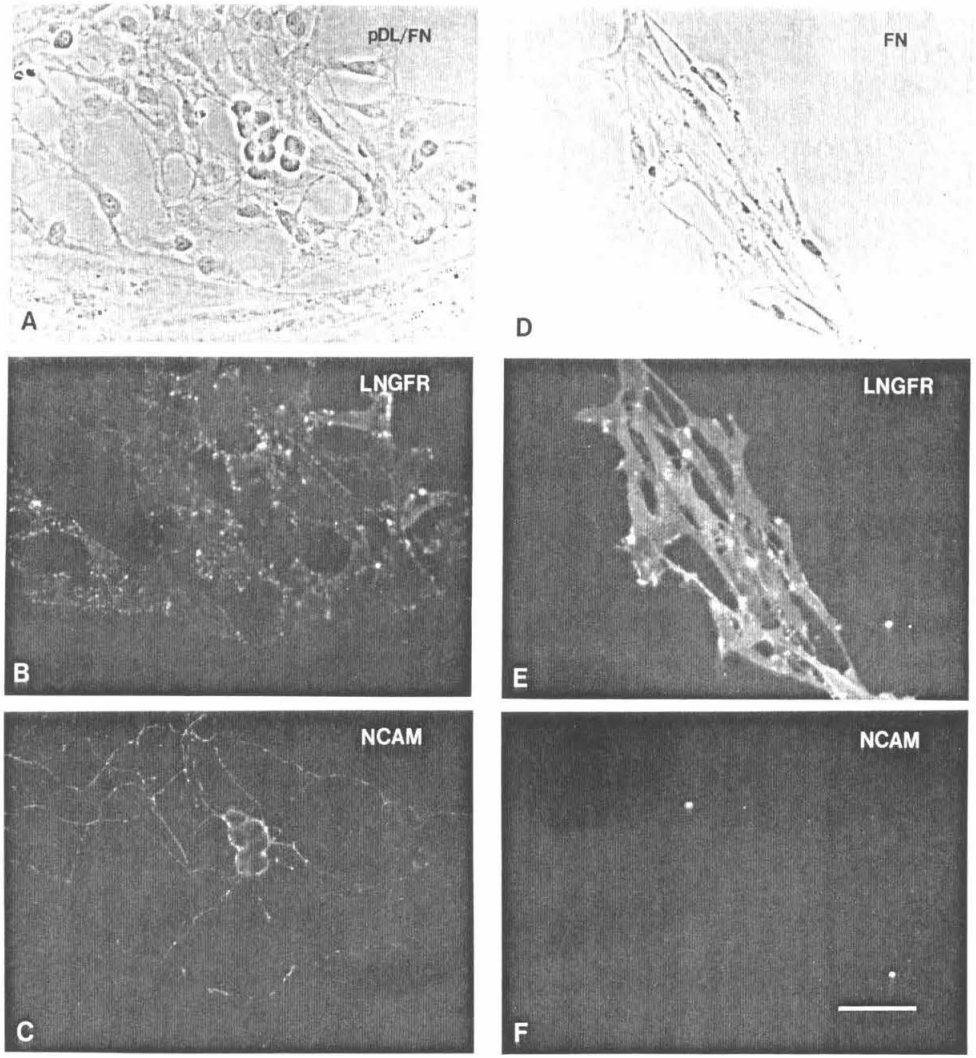

Clones grown on p-D-Lysine / Fibronectin
100
80
60
40
20
0
% founders
N+G+O
G + O
O
Dead
Clone Type
% founders

Figure 10. 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 determined by labeling with anti-LNGFR and anti-high PSA NCAM (see Fig. 9). The proportion of clones containing neurons, glia and other (N+G+O) cells, glia and other cells but no neurons (G+O), other cells only (O) or dead (those founder cells which failed to form clones) was calculated. The values plotted represent the mean ±SEM from over 100 clones in 4 separate experiments. Note that no neuron-containing clones are found on FN (A), whereas 60% of the clones contain neurons on pDL/FN (B).

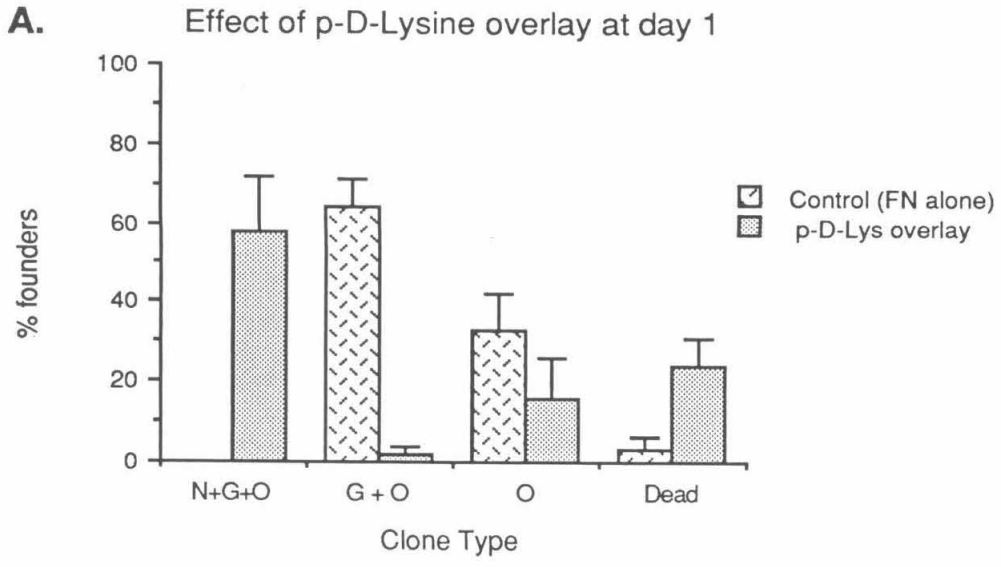

pDL/FN
A
FN
D
LNGFR
B
LNGFR
E
NCAM
C
NCAM
F

since these clones generate neither neurons nor glia under any conditions so far examined (see above). Following LNGFR staining, one group of clones was exposed to pDL as an overlay, while a set of sister clones was maintained on FN alone as a control. After 10 days the cultures were transferred to SCD medium for an additional 2 weeks of culture, and the clone phenotypes then scored as previously described.

By contrast to clones maintained on FN, where no neurons developed, many of the clones exposed to a pDL overlay at 24 hrs contained neurons at the end of the culture period (Figure 11A, shaded bars, N+G+O). Furthermore, virtually none of the clones were of the G+O phenotype after the pDL overlay (Fig. 11A, shaded bars, G+O). These data suggest that at least some of the N+G+O clones were derived by conversion of founder cells that would have produced G+O clones if maintained on FN. But since application of pDL at day 1 yielded significant cytotoxicity, we could not rule out the possibility that many cells that would have produced G+O clones, even in pDL, simply died. However, when the pDL overlay was performed on LNGFR$^+$ clones at day 4 most of the clones survived the overlay, probably because there were more cells in each clone at the time pDL was applied (compare shaded bars, "Dead," in Fig. 11A,B). Under these conditions, 60% of the starting clones generated neurons and glia (Fig. 11B, shaded bar, N+G+O), whereas 35% generated glia but not neurons (Figure 11B, shaded bar, G+O). By contrast, virtually all of the control clones identified at day 4 and maintained on FN developed to a G+O phenotype (Fig. 11B, hatched bars). Since essentially all clones in the day 4 cohort were recovered live following the overlay, these data imply that pDL must have converted a high proportion of presumptive G+O clones into N+G+O clones. The fact that 35% of the clones still became G+O following pDL overlay at day 4, whereas virtually none did so when the overlay was applied at 24 hours (Figure 11, compare G+O, shaded bars, in A and B), suggests that either some clones become resistant to the effect of pDL between 24 hours

Figure 11. Development of neurons in clones plated on FN and subsequently overlayed with pDL.

Neural crest clones were established on FN and overlayed with pDL after either 24 hours of secondary culture (A), or 4 days of secondary culture (B). After a further incubation clone composition was scored as in Figure 10. Shaded bars represent clones overlayed with pDL; hatched bars represent control sister clones maintained on FN. The percentage of final G+O clones (90%) from the day 4 cohort is higher than that obtained from the day 1 cohort (65%), because 35% of the single LNGFR$^+$ cells identified at day 1 subsequently lost LNGFR and developed into O clones (Panel A, hatched bars, O). In contrast, by day 4 all LNGFR$^+$ clones contained multiple LNGFR$^+$ cells so that purely O clones rarely developed from this cohort (Panel B, hatched bars, O).


A.
Effect of p-D-Lysine overlay at day 1
100
80
60
40
20
0
% founders
Control (FN alone)
p-D-Lys overlay
N+G+O
G + O
O
Dead
Clone Type

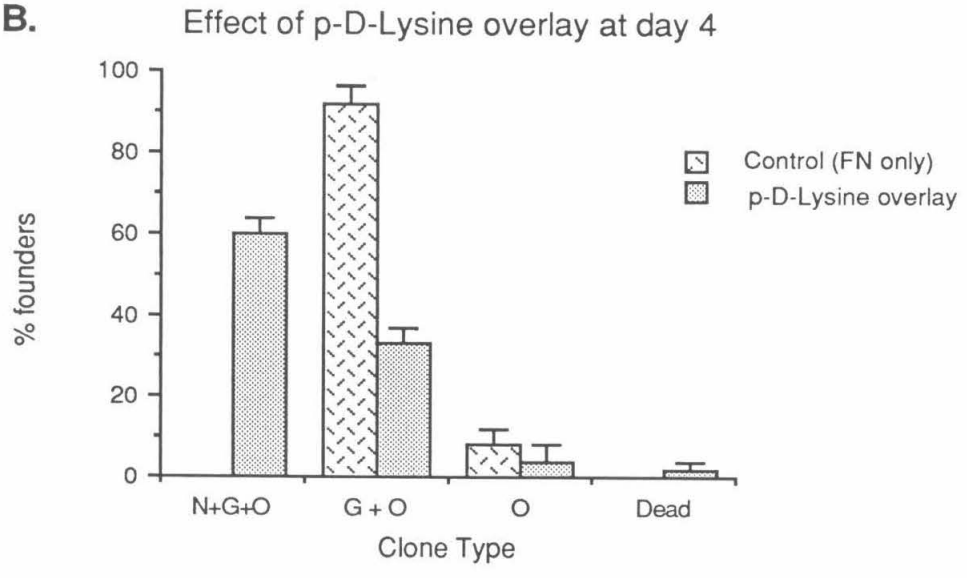

B.
Effect of p-D-Lysine overlay at day 4
100
80
60
40
20
0
% founders
Control (FN only)
p-D-Lysine overlay
N+G+O
G + O
O
Dead
Clone Type

Table II. Alteration of neural crest cell clone fate by subculture onto different substrates

Neural crest clones were established on either FN or pDL/FN ("Primary Substrate"), and then sub-cultured after 5 days. Cells from each primary clone were replated at clonal density onto either FN or pDL/FN ("Secondary substrate"). The phenotypic composition of resulting sub-colonies was analyzed ten days later. "+" indicates that one or more sub-colonies of a primary clone contained neurons under that condition. "-" indicates that no neurons were found in any sub-colonies of the primary clone. The fractions represent the proportion of total primary clones analyzed that generated any neuron-containing sub-colonies.

Table II. Alteration of neural crest cell clone fate by subculture onto different substrates

	Primary Substrate	
Secondary Substrate	**Fibronectin**	**pDL/Fibronectin**
Fibronectin	- (0/7)	- (0/5)
pDL/Fibronectin	+ (5/7)	+ (5/5)

and 4 days of culture, or that a greater number of presumptive G+O clones survived following the pDL overlay at day 4.

Clonal culture on fibronectin precludes overt neuronal differentiation but permits the maintenance of neurogenic potential

The preceeding experiment provides statistical evidence that neural crest clones grown on FN are unable to differentiate into neurons, but retain the capacity to do so when subsequently exposed to pDL. A more direct test of this idea would be to plate clones on FN and then to sub-culture them onto pDL/FN; such cells should generate neuron-containing sub-colonies, sister cells sub-cultured onto FN should not. To this end, 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. The replated cells were allowed to grow and mature in culture, and the resulting colonies were scored for the presence of cells bearing a neuronal morphology. In striking contrast to their sister cells subcloned onto FN, which failed to generate any neuron-containing clones, five out of seven primary clones transferred from FN to pDL/FN at day 5 gave rise to colonies that contained neurons (Table II). Among these sub-colonies transferred to pDL/FN, on average, 57 $\pm$ 17 % contained neurons. Conversely, 0/5 primary clones founded on pDL/FN generated neurons when subcultured onto FN , although these clones all generated neuron-containing subclones when replated onto pDL/FN (Table II). Thus, sub-culture on pDL/FN permits overt neuronal differentiation by neural crest cells initially plated on FN; conversely sub-culture on FN precludes overt neuronal differentiation by cells initially plated on pDL/FN. These data indicate that the composition of the substrate is able to control the overt neuronal differentiation of neural crest cells. However, since the capacity to produce neurons is retained for some time on FN, overt neuronal differentiation can apparently be controlled independently of neurogenic potential.

Discussion

The properties of stem cells have been characterized primarily in adult tissues capable of self-renewal or regeneration in response to injury, such as skin, intestine and blood. These properties include the ability to self-renew, divide asymmetrically and to generate one or more irreversibly differentiated progeny cell types (Hall and Watt, 1989; Potten and Loeffler, 1990). Progress in the characterization of different mammalian stem cell populations has been hampered by the difficulty of obtaining adequate cell markers, and of establishing functional assay systems. It has been widely assumed that the neuroepithelial precursor cells of the vertebrate CNS and PNS are stem cells (McKay, 1989). However, there has been only indirect evidence in support of this idea (Temple, 1989). Here we describe the properties of isolated mammalian neural crest cells *in vitro*. We show that these cells are multipotent, generate differentiated progeny, divide asymmetrically and are capable of self renewal. By these criteria, neural crest cells exhibit the properties of stem cells.

Mammalian neural crest cells are multipotent

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 glia, neurons and at least one other unidentifiable cell type. Using a panel of antibody markers, we have identified the glial cells as Schwann cells. Similarly, we have characterized the neurons using a battery of markers that include NCAM, neurofilament and peripherin, an intermediate filament protein preferentially expressed in the PNS (Parysek and Goldman, 1988). Although these markers identify the neurons as peripheral, their precise identity is unclear. They do not express markers characteristic of noradrenergic sympathetic neurons, such as tyrosine hydroxylase (TH). 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 non-catecholaminergic lineages such as sensory, parasympathetic and enteric. Unfortunately, definitive markers for these sub-lineages have not yet been described in mammals.

Other non-neuronal cell types known to derive from the neural crest include melanocytes and mesectodermal cells such as chondrocytes. *In vivo*, mesectodermal lineages normally develop only from the cephalic neural crest (LeDouarin, 1982), and transplantation experiments in chick suggest that the trunk region lacks mesectodermal potential (LeDouarin, 1980). While melanocytes originate from all regions of the crest, for various reasons albino rats proved a superior source of crest cells for these experiments. It is conceivable that the O cells represent melanocytes or their progenitors.

Mammalian neural crest cells undergo self-renewal

By performing serial subcloning experiments we have been able to demonstrate that multipotent neural crest cells, expressing an LNGFR$^+$, nestin$^+$, lineage$^-$ antigenic phenotype, give rise to clonal progeny which are themselves multipotent and express the same markers. Thus, by both antigenic and functional criteria multipotent neural crest cells are capable of self-renewal. This observation suggests that *in vivo*, the progeny of proliferating neural crest cells are not immediately restricted to different sublineages. Rather, these cells may maintain their multipotency as they migrate and colonize various structures throughout the embryo. Consistent with this notion, migrating neural crest cells in the somitic mesenchyme have been shown to be multipotent *in vivo* (Fraser and Bronner-Fraser, 1991), and clonal analysis of cells from early avian sensory and sympathetic ganglia has revealed multipotency *in vitro* (Duff et al., 1991; LeDouarin et al., 1991).

Experiments to assess further the extent of neural crest cell self-renewal capacity are currently in progress. We have performed serial subcloning experiments as late as

day 11 when clones appear to contain >500 cells (9 cell divisions), and found that > 50% of the sub-clones exhibit multipotency (not shown). While the accepted definition of a stem cell usually includes extensive or "unlimited" self-maintenance capacity (Hall and Watt, 1989), it is also accepted that the extent of self-maintenance may be subject to environmental regulation (Potten and Loeffler, 1990). Self-maintenance in a stem cell lineage may be achieved if individual cell divisions are asymmetric, producing 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 self-renewing divisions is environmentally-controlled, then under conditions where that probability is >0.5 the stem cell population will increase in size; if it is < 0.5 the stem cells will eventually be exhausted. Stem cells populations may therefore exhibit finite self-renewal under some conditions.

Unlimited self-maintenance capacity is generally a property of stem cells found in regenerating tissues, such as skin, intestine and the hematopoietic system. The mammalian nervous system has traditionally been thought to lack extensive regenerative capacity. On the other hand, evidence has recently been presented for an adult glial progenitor cell in the optic nerve (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 contibute to this regenerative capacity.

The composition of the substrate can control the fate of neural crest cells

The fate of neural crest cell populations can be altered by their local environment *in vivo* (LeDouarin, 1980; LeDouarin, 1982). However, little is known about the identity of the environmental signals that influence neural crest cell fate, or how these signals act on individual neural crest cells. We have shown that multipotent neural crest cells generate different derivatives when cultured on different substrates: both neuronal and non-neuronal cells are generated on pDL/FN whereas on FN only non-neuronal cells are generated. Both the pDL overlay and cross-subcloning experiments rule out the possibility that the substrate acts selectively to promote the differential attachment of neurogenic and non-neurogenic neural crest cells present in the starting cell population. Furthermore, continuous observation of clones indicates that neurons do not appear transiently on a FN substrate. It thus appears unlikely that the pDL/FN substrate acts selectively to permit the survival of neurons that are initially generated at equal frequency on both substrates. Whether the substrate conditions we have defined control a choice between neuronal and non-neuronal directions of differentiation, or rather influence the overt expression of a latent neuronal differentiation program, remains an open and interesting question. The notion that different environments can influence the fate of multipotent neural crest cells fits well with data suggesting that the progeny of a single neural crest cell express different phenotypes in different locations *in vivo* (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991).

The observation that neural crest clones founded on a pDL/FN substrate fail to produce neurons when subcultured on FN suggests that FN is either non-permissive for, or inhibitory to, neuronal differentiation. Consistent with this idea, preliminary observations indicate that multipotent neural crest cells plated at clonal density on untreated tissue culture plastic are able to generate neurons, by contrast to cells plated

on FN-coated dishes. Clones grown on fibronectin appear more disperse than those grown on untreated plastic or on pDL/FN. This suggests that culture on a FN substrate may preclude neuronal differentiation by inhibiting cell-cell interactions. *In vivo*, FN has been demonstrated to be 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 neuronal differentiation by promoting cell dispersal. Consistent with this view, neuronal differentiation *in vivo* is associated with the aggregation of neural crest cells to form ganglion primordia. Such aggregation is correlated with the expression of cell adhesion molecules such as N-cadherin (S. Fraser, personal communication), which may counteract the effects of FN. Similarly, in our culture system poly-D-lysine may act to override the dispersal promoting activity of FN; in this respect it may mimic the action of a natural molecule *in vivo*.

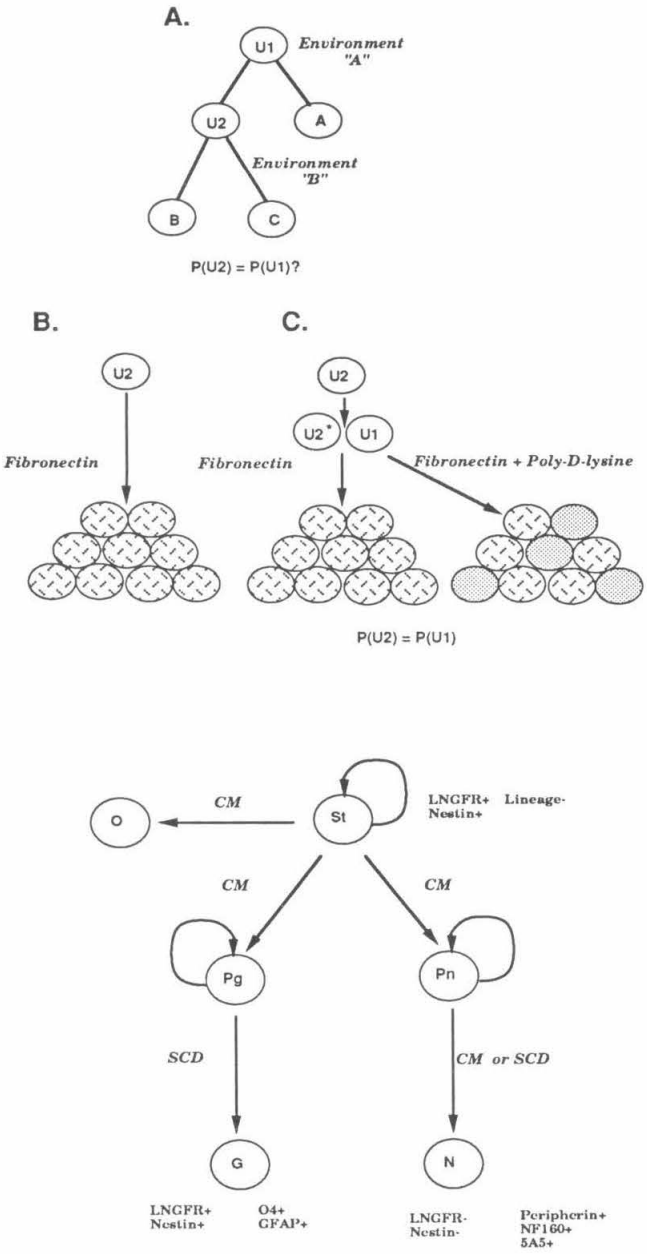
Developmental fate versus developmental potential

When neural crest cells are subcloned from FN onto pDL/FN within the first few days of culture, they are able to generate clones containing both neurons and Schwann cells. This result indicates that although the fate of cells on FN would have been to generate only glia, such cells nevertheless have neurogenic potential (Fig. 12 upper, B and C). This suggests that *in vivo*, some neural crest cells that generate clones containing only glia (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; LeDouarin et al., 1991) may in fact be multipotent, but may manifest only one of their developmental capacities due to their local environment. A more general restatement of this notion is that although the progeny of a single neural crest cell may generate different derivatives at different times or different places *in vivo*, the developmental potentials of these progeny cells may be equivalent (Figure 12 upper, A). Such a mechanism would be consistent with the observation that multipotent crest cells are capable of self-renewal.

Figure 12. Schematic illustration of neural crest cell fate vs. developmental potential.

Upper: (A) Hypothetical *in vivo* crest lineage in which a dividing crest cell gives rise to different cell types in different environments. It cannot be determined whether the developmental potentials of the undifferentiated cell (U1) and its daughter (U2) are equivalent. (B) The fate of an undifferentiated crest cell (U2) grown on fibronectin *in vitro* is to generate glia but not neurons; however the neurogenic potential of its daughter is revealed by serial subcloning onto pDL/FN (C). Therefore undifferentiated cells U2 and U1 have the same developmental potential.

Lower: Summary diagram indicating the *in vitro* behavior of neural crest stem cells defined in this study. St, stem cell; Pg, glial progenitor; Pn, neuronal progenitor; G differentiated glial cell; N differentiated neuron; O, "other" unidentifiable cell.


A.
U1
Environment "A"
U2
A
Environment "B"
B
C
P(U2) = P(U1)?
B.
C.
U2
Fibronectin
U2
U2*
U1
Fibronectin
Fibronectin + Poly-D-lysine
P(U2) = P(U1)
CM
O
St
LNGFR+ Lineage- Nestin+
CM
CM
Pg
Pn
SCD
CM or SCD
G
N
LNGFR+ Nestin+
O4+ GFAP+
LNGFR- Nestin-
Peripherin+ NF160+ 5A5+

On the other hand, our serial subclonal analysis has also revealed that some secondary subclones contain only neurons (Table II), a clone composition never observed among primary clones. Moreover, in preliminary experiments (D. Stemple and D. Anderson, unpublished observations) we have found that when neural crest cells are subcloned from FN to pDL/FN after a more extended culture period, many subclones fail to generate neurons although glial differentiation capacity is retained. This suggests that under some conditions, multipotent neural crest cells may generate progeny that do not have neurogenic potential (Fig. 12, lower). The generation of such lineally-restricted progeny from multipotent progenitor cells is consistent with a progressive model of neural crest development. The timing, mechanism and environmental control of such developmental restrictions remain to be investigated further.

In summary, our data support the idea that the early stages of neural crest cell lineage segregation involve the self-renewal of multipotent stem-like cells, analagous to the early stages of hematopoiesis (Spangrude et al., 1988; Ikuta et al., 1992). In the immune system, substantial progress has been made in identifying factors controlling the development of hematopoietic progenitor cells (Metcalf, 1989; Dexter et al., 1990). Interestingly, an increasing number of hematopoietic growth factors have been shown to have biological effects on neurons and neuronal precursor cells (Nawa et al., 1990; Ip et al., 1992), suggesting that the relationships between hematopoiesis and "neuropoiesis" (Anderson, 1989) may extend beyond mere analogy (Bazan, 1991) The isolation of neural crest stem cells now provides an assay system to investigate the roles of these and other growth factors in peripheral nervous system development, at the single-cell level. In addition, our culture system provides an assay for neural crest stem cells in tissues at later stages of development. Such 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 neurologic and neurodegenerative disease.

Experimental procedures

Neural crest cell preparation

For a given preparation 5-10 timed pregnant female Sprague-Dawley rats (Simonson Laboratories, Gilroy, CA) were killed by CO_2 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 Hank's Balanced Salt Solution (HBSS) (GIBCO, Grand Island, NY) at 4°C for 2-4 hours. Under a dissecting microscope, at room temperature, a block of tissue from a region corresponding approximately to the caudal most 10 somites was dissected from each embryo using an L-shaped electrolytically sharpened tungsten needle. Trunk sections were transferred in HBSS into one well of a 3 well depression slide that had been chilled to 4°C. Trunk sections were treated with collagenase (152 units/mg)(Worthington Biochemical, Freehold, NJ) made to a concentration of 0.75 mg/ml in Howard's Ringer's solution (per 1 liter of dH_2O: NaCl 7.2g; CaCl2 0.17g; KCl 0.37g) and sterilized by passage through a 0.22 μm filter prior to use. The collagenase solution was exchanged at least 3 times and with each exchange the trunk sections were vigorously triturated. After incubation at 37°C for 20 minutes in humidified CO_2 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 quenched by repeated exchanges with cold standard medium (SM) (medium composition described below). The neural tubes were plated onto fibronectin-coated (substrate preparation is described below) 60 mm tissue culture dishes (Corning, Corning, NY) that had been rinsed with SM. After a 30 minute incubation to allow the neural tubes to attach, dishes were flooded with 5 ml of SM. After a 24 hour culture

period, using an L-shaped electrolytically sharpened tungsten needle and an inverted phase contrast microscope equipped with a 4X objective lens, each neural tube was carefully scraped away from the neural crest cells that had migrated onto the substrate. Neural crest cells were removed by a 3 minute 37^{o}C treatment with 0.05% Trypsin solution (GIBCO). The cells were centrifuged for 4 minutes at 2000 r.p.m. and the pellet was resuspended into 1 ml of fresh SM. Typically the cells were plated at a density of 225 cells / 100mm dish.

Media and additives

A. Chemically defined medium

The basal medium used in all experiments is a chemically defined medium that we developed based on the recipes of several other defined media. The medium consists of L-15 CO_2 formulated as described by Hawrot and Patterson (Hawrot and Patterson, 1979), supplemented with additives described by Bottenstein and Sato (Bottenstein and Sato, 1979; Wolinsky et al., 1985), and further supplemented with the additives described by Sieber-Blum and Chokshi (Sieber-Blum and Chokshi, 1985). The recipe is given here: to L-15 CO_2 add, 100 μg/ml transferrin (Calbiochem, San Diego, CA), 5 μg/ml insulin (Sigma, St. Louis, MO), 16 μg/ml putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM selenious acid (Sigma), 1 mg/ml bovine serum albumin, crystallized (GIBCO), 39 pg/ml dexamethasone (Sigma), 35 ng/ml retinoic acid (Sigma), 5 μg/ml α-d, 1-tocopherol (Sigma), 63 μg/ml β-hydroxybutyrate (Sigma), 25 ng/ml cobalt chloride (Sigma), 1 μg/ml biotin (Sigma), 10 ng/ml oleic acid (Sigma), 3.6 mg/ml glycerol, 100 ng/ml α-melanocyte stimulating hormone (Sigma), 10 ng/ml prostaglandin E1 (Sigma), 67.5 ng/ml triiodothyronine (Aldrich Chemical Company, Milwaukee, WI), 100 ng/ml epidermal growth factor (Upstate Biotechnology, Inc., Lake Placid, NY), 4 ng/ml bFGF (UBI), and 20 ng/ml 2.5S NGF (UBI). Each concentration given is the final concentration in the medium. In general each additive was dissolved to a crude stock concentration and then diluted further to a 1000X stock concentration in L-15 CO_2 and then stored at -80°C. Most additives were dissolved in dH_2O with the exception of the following: Biotin was dissolved to a crude stock concentration of 10 mg/ml in DMSO; Prostaglandin was dissolved in 95% ethanol to a crude stock concentration of 1 mg/ml; Triiodothyronine was dissolved in DMSO to a crude stock concentration of 10 mg/ml; Retinoic acid was dissolved in DMSO to a crude stock concentration of 17.5 mg/ml and then diluted to a 1000X concentration in a 1:1 mixture of 95% ethanol and L-15 CO_2; and α-d, 1-

tocopherol was dissolved to a 1000X stock concentration of 5 mg/ml in DMSO. The final solvent concentrations are 0.11% DMSO (v/v) and 0.07% ethanol (v/v). Transferrin, insulin, selenious acid, putrescine, and progesterone were prepared as described by Wolinsky and Patterson (Wolinsky et al., 1985).

B. Chick embryo extract

For most experiments standard medium (SM) was used. SM was made by further supplementing the chemically defined medium with 10% chick embryo extract (CEE). To prepare CEE, white leghorn chicken eggs were incubated for 11 days at 38°C in a humidified atmosphere. Eggs were washed and the embryos were removed, and placed into a petri dish containing sterile Minimal Essential Medium (MEM with Glutamine and Earle's salts) (GIBCO) at 4°C. Approximately 10 embryos each were macerated by passage through a 30 ml syringe into a 50 ml test tube (Corning). This typically produced ~25 ml of homogenate to which was added 25 ml of MEM. The tubes were rocked at 4°C for 1 hour. Sterile hyaluronidase (1 mg / 25 g of embryo) (Sigma) was added and the mixture was centrifuged for 6 hours at 30,000 g. The supernatant was collected, passed first through a 0.45 μm filter, then through a 0.22 μm filter and stored at -80°C until used.

C. FBS and forskolin

For some experiments the medium was further supplemented with fetal bovine serum (FBS) and forskolin. The FBS was obtained from JR scientific and was heat inactivated by treatment at 55°C for 30 minutes. The FBS was stored at -20°C and passed through a 0.22 μm filter prior to use. Forskolin was obtained from Sigma as a dry powder and resuspended in 95% ethanol to a concentration of 20 mM.

Substrate preparation

A. Fibronectin

Tissue culture dishes were coated with human plasma fibronectin (New York Blood Center, New York, NY) in the following way. Lyophilized fibronectin was resuspended in sterile distilled water (dH_2O) to a concentration of 10 mg/ml and stored at -80°C until use. The fibronectin stock was diluted to a concentration of 250 µg/ml in Dulbecco's phosphate buffered saline (D-PBS) (GIBCO). The fibronectin solution was then applied to tissue culture dishes and immediately withdrawn.

B. poly-D-lysine

Sterile poly-D-lysine (pDL) (MW 30-70 kDa) (Biomedical Technologies, Inc., Stoughton, MA) was dissolved in dH_2O to as 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 dH_2O and allowed to dry again. Fibronectin was then applied, as described above, over the pDL.

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

Cloning procedure

Cloning of cells was accomplished by first plating cells at a low density of ~225 cells/100mm dish, allowing the cells to attach and then identifying single cells with an inverted phase contrast microscope equipped with a 4X objective lens. The single founder cells were marked by inscribing a circle around them with a grease pencil on the bottom of the dish. The circles were made to a diameter of 3-4 mm, which was generally large enough to allow the unambiguous identification of the progeny of the founder at any time during an experiment. The dishes were stained for expression of LNGFR using the monoclonal antibody 192IgG (Chandler et al., 1984). The 192IgG antibody was produced by hybridoma cells grown in the chemically defined medium described above. The staining procedure used is described below.

Serial sub-cloning

For serial sub-cloning experiments clones were harvested and re-plated 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 i.d.) 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) for 3 minutes at 37°C in a humidified 5% CO_2 incubator. At room temperature 70 µl of the trypsin solution was removed and replaced with 70 µl of SM. The cells were resuspended into the 100 µl volume by vigorous trituration and the whole volume was diluted into 5 ml of SM. The 5 ml was then plated onto one or two 60 mm dishes which were placed in a humidified 5% CO_2 incubator for 2 hours. The medium was then exchanged for fresh SM and single founders cells were identified as described above.

Immunohistochemistry

A. 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 minutes at room temperature. The cultures were washed twice with L-15 medium (GIBCO) supplemented with 1:1:2, fresh vitamin mix (FVM) (Hawrot and Patterson, 1979), and 1 mg/ml bovine serum albumin (L-15 Air). The cultures were then incubated for 20 minutes at room temperature with Phycoerythrin-R conjugated secondary antibody (TAGO) 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-LNGFR antiserum was a kind gift of Dr. Gisela Weskamp, University of California, San Francisco and was used at a 1:1000 dilution. Monoclonal anti-NCAM 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 O4 were the gift of Dr. Monique DuBois-Dalc (NIH) and were grown in a medium consisting of RPMI-1640 (GIBCO), 10% FBS, and 1% penicillin-streptomycin solution (5000u penicillin; 5000 μg streptomycin) (GIBCO).

B. Fixation

i. Formaldehyde

For most of the immunocytochemistry formaldehyde fixation was done. Formaldehyde solution 37% was diluted 1:10 into S-MEM with 1mM HEPES buffer (GIBCO). Cultures were treated for 10 minutes at room temperature with the 3.7% Formaldehyde solution and then rinsed 3 times with D-PBS (GIBCO).

ii. Acid/ethanol

For some intermediate filament proteins (NF and GFAP) formaldehyde fixation was not possible. Cultures were fixed by treating with a solution of 95% ethanol and 5% glacial acetic acid at -20^{o}C for 20 minutes.

C. Cytoplasmic antigens

For the staining of cytoplasmic atigens, fixed cells were first treated with a blocking solution consisting of D-PBS, 0.1% Tween-20 (Bio-Rad Laboratories, Richmond, CA), and 10% heat inactivated normal goat serum (NGS) for 15 minutes at room temperature. Primary antibodies were diluted into a solution of D-PBS, 0.1% Tween-20, and 5% NGS. The fixed cells were incubated overnight at 4^{o}C in primary antibody solution then rinsed twice with D-PBS, 0.05% Tween-20. Fluorescent secondary antibodies were diluted into D-PBS, 1% NGS and applied to the cells for 1 hour at room temperature. The cells were rinsed twice with D-PBS, 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-glial fibrillary acidic protein (GFAP) antibody G-A-5 (IgG) and mouse monoclonal anti-NF160 antibody NN18 (IgG) were purchased from Sigma and used at a 1:100 dilution. Mouse monoclonal anti-neurofilament heavy chain antibody SMI 39 (IgM) was purchased from Sternberger Monoclonals Inc., Baltimore, MD, and used at a dilution of 1:500. Purified rabbit antibodies to peripherin (preparation 199-6) was the kind gift of Dr. Linda Parysek, University of Cincinatti, OH, and was used at a dilution of 1:300. Rabbit antisera generated against P_0 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; Trapp et al., 1981). (used at a dilution of 1:500).

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CHAPTER 3. Basic FGF Induces Neuronal Differentiation, Cell Division, And NGF Dependence In Chromaffin Cells: A Sequence of Events In Sympathetic Development

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Basic FGF Induces Neuronal Differentiation, Cell Division, and NGF Dependence in Chromaffin Cells: A Sequence of Events in Sympathetic Development

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Summary

To define further the molecules that control sympathoadrenal differentiation, we have investigated the effects of FGF, NGF, and glucocorticoid on cultured neonatal rat adrenal chromaffin cells. Basic FGF (bFGF), like NGF, induces cell division and neurite outgrowth from these cells. Dexamethasone inhibits neuronal differentiation but not proliferation induced by bFGF. Unlike NGF, bFGF will not support the survival of chromaffin cell–derived sympathetic neurons. However, bFGF induces a dependence on NGF. The overlapping but distinct responses to NGF and bFGF may underlie a sequence of events in sympathetic differentiation. bFGF (or another factor) may act locally in developing ganglia to stimulate mitotic expansion and initial axon outgrowth. Subsequent survival and maturation are then controlled by NGF, which is provided by peripheral targets of innervation. In the adrenal gland, glucocorticoids may permit bFGF to amplify the chromaffin population, while preventing neuronal differentiation.

Introduction

The neural crest is a transient neurectodermal structure of the vertebrate embryo that gives rise to a variety of cell types including those of the sympathoadrenal lineage: the neurons of the sympathetic ganglia and the chromaffin cells of the adrenal medulla (Le Douarin, 1982). In vitro studies have shown that chromaffin cells and sympathetic neurons can be derived from a common precursor (Doupe et al., 1985b; Anderson and Axel, 1986). Interestingly, mature chromaffin cells can be induced to transdifferentiate into sympathetic neurons by addition of nerve growth factor (NGF) and a factor in heart cell–conditioned medium (Unsicker et al., 1978; Doupe et al., 1985a). This effect is blocked by glucocorticoid, suggesting that the high local concentration of steroids in the adrenal medulla may be important for the maintenance of an endocrine phenotype. In support of this, glucocorticoids are required for the survival of rat chromaffin cells in vitro (Doupe et al., 1985a). In addition to inducing neuronal differentiation, NGF can simultaneously serve as a mitogen for these cells (Lillien and Claude, 1985). A cell line of adrenal medullary origin, PC12, is also capable of adopting a neuron-like phenotype in the presence of NGF (Greene and Tischler, 1976). These findings, in conjunction with the original characterization of the role of NGF in the embryo (Levi-Montalcini, 1976), have established NGF as a mitogen and differentiation agent, as well as a survival factor.

Acidic and basic fibroblast growth factors (aFGF and bFGF), though originally characterized as potent mitogens for a variety of cell types of mesodermal origin (for review, see Gospodarowicz et al., 1986b), have recently been demonstrated to support the survival of a variety of vertebrate neurons. Embryonic rat hippocampal neurons, cerebellar granule cells, and Purkinje cells, as well as cortical neuronal precursor cells, can all be sustained by bFGF in vitro (Walicke et al., 1986; Morrison et al., 1986; Gensburger et al., 1987; Hatten et al., 1988). In addition, FGF supports the survival of chick parasympathetic ciliary ganglion neurons (Unsicker et al., 1987). The response of PC12 cells to aFGF and bFGF is very similar to their response to NGF (Togari et al., 1985; Wagner and D'Amore, 1986; Neufeld et al., 1987; Rydel and Greene, 1987; Schubert et al., 1987). Thus far, FGF and NGF are the only proteins known to be capable of inducing neuron-specific mRNAs in PC12 cells (Leonard et al., 1987; Stein et al., 1988a).

The response of PC12 cells to FGF raises the possibility that this factor may play a previously unanticipated role in the normal development of the sympathoadrenal lineage. To address this question, the actions of bFGF on primary cultures of neonatal rat adrenal chromaffin cells were examined and compared with those of NGF.

Results

bFGF Induces the Neuronal Differentiation of Chromaffin Cells

The effects of bFGF (purified some 140,000-fold from bovine pituitary) on chromaffin cells were initially examined by measuring neurite outgrowth, a morphologic index of neuronal differentiation (Figure 1; Figure 2). bFGF at a concentration of 10 ng/ml rapidly induced process outgrowth from cultured chromaffin cells (Figure 1E). The kinetics of this induction were roughly equivalent to those observed with NGF (Figure 2A, compare NGF and FGF, filled symbols), except that in bFGF the percentage of process-bearing cells reached a plateau of 83% at day 6, whereas in NGF it continued to increase to 99% by day 10. In agreement with observations reported for PC12 cells (Neufeld et al., 1987; Rydel and Greene, 1987), it was found that the dose-response for neurite outgrowth from chromaffin cells was biphasic with respect to bFGF (Figure 2B), although this was less apparent after longer periods of exposure to the factor.

Dexamethasone (dex) inhibits NGF-induced neurite outgrowth from chromaffin cells (Unsicker et al., 1978; Doupe et al., 1985a). It was therefore of interest to determine whether this synthetic glucocorticoid would have a similar effect on process outgrowth induced by bFGF. When cultures were simultaneously exposed to bFGF plus 1 μM dex, the extent of process outgrowth was in-

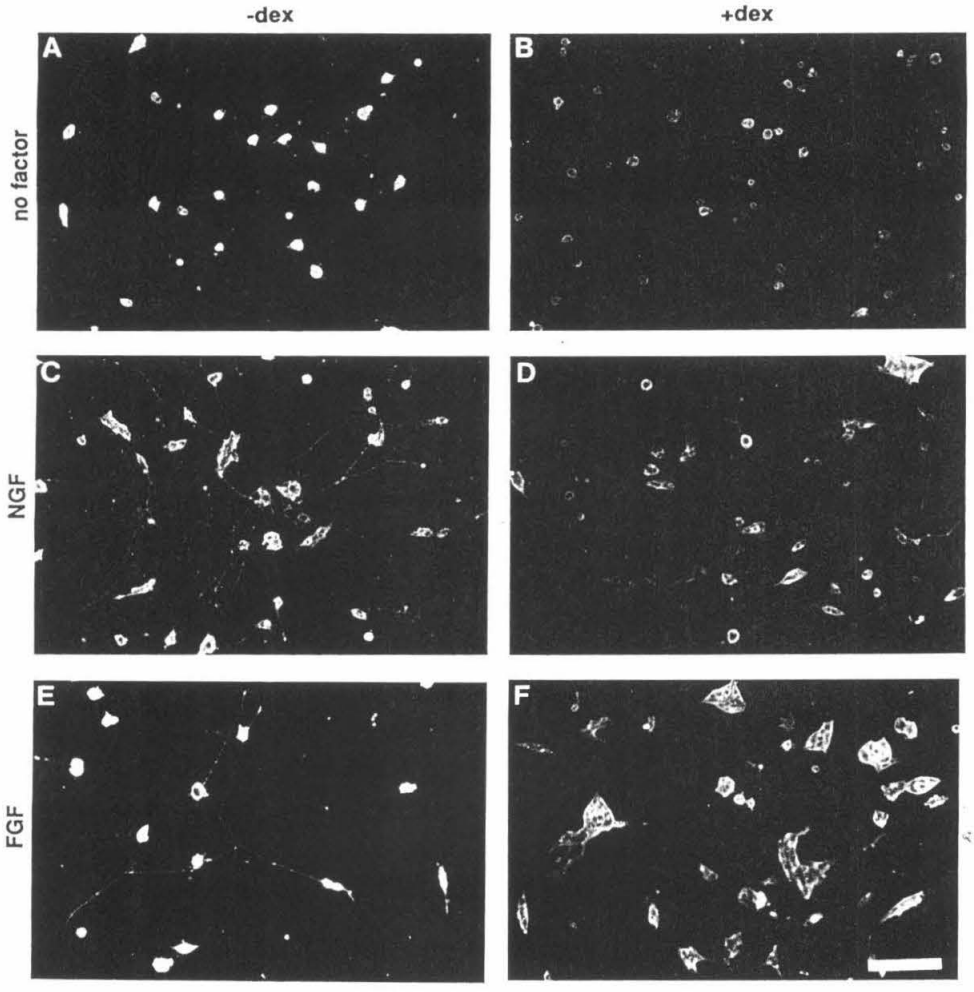

Figure 1. Morphology of Chromaffin Cells Treated with bFGF, NGF, and/or dex

Cells were grown for 6 days in growth medium alone (A) or in growth medium supplemented with the following factors: 1.0 μM dex (B), 1.0 μg/ml 7S NGF (C), NGF plus dex (D), 10.0 ng/ml bFGF (E), and bFGF plus dex (F). Fluorescence micrographs were taken of cultures stained with OX-7 monoclonal anti-Thy-1 antibody. Bar, 50 μm.

hibited by about 50% (Figure 2A, FGF, open symbols). However, the rate of accumulation of process-bearing cells was only slightly diminished by the steroid. In this culture condition, chromaffin cells were often found in large, flattened clusters bearing processes that were thicker, shorter, and less arborized than those observed in bFGF alone (compare Figures 1E and 1F). Furthermore, most process-bearing cells in bFGF plus dex did not express immunohistochemically detectable amounts of the neural-specific marker protein SCG10 (Stein et al., 1988b) (data not shown). The quantitative data may therefore underestimate the inhibition of neuronal differentiation caused by dex. The inhibitory effect of dex on NGF-induced neuronal differentiation was more pronounced than in the case of bFGF, affecting both the rate and the extent of neurite outgrowth (Figure 1D; Figure 2A, NGF, open symbols).

To distinguish whether the neuronal differentiation caused by bFGF reflects the phenotypic conversion of chromaffin cells (as observed for NGF [Doupe et al., 1985a]) or the selection for preexisting process-bearing cells (the adrenal medulla contains a small proportion of ganglionic neurons [Anderson and Axel, 1985; Trojanowski et al., 1986]), the number of cells in each dish was measured as a function of time in bFGF. This experiment revealed that between days 2 and 6, the number of cells

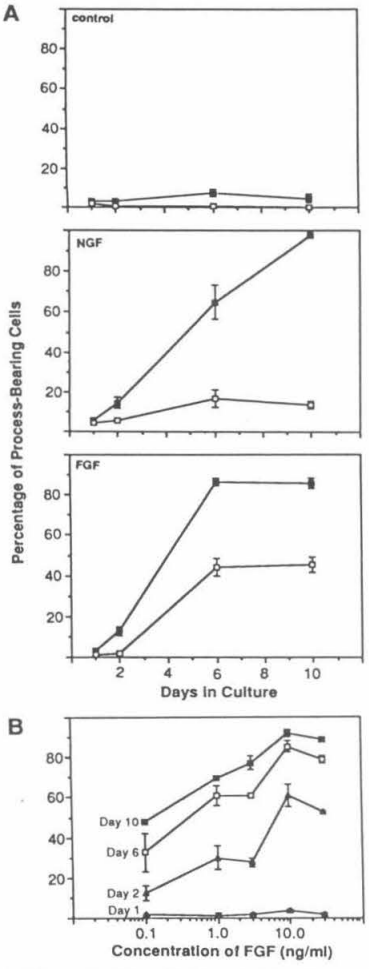

Figure 2. Effects of bFGF and NGF on the Time Course of Neurite Outgrowth

(A) Cells were counted and scored for the presence of neurites. For each of the experimental conditions, the percentage of cells bearing a process is plotted against the number of days in culture. In all panels, filled squares correspond to the presence of the factor indicated in the upper left corner of the panel and open squares correspond to the factor in combination with 1.0 μM dex. Control indicates that cells were grown in growth medium alone; NGF indicates the presence of 1.0 μg/ml 7S NGF; FGF indicates the presence of 10.0 ng/ml bFGF. The values plotted are averages of several dishes for each condition on each day, obtained from two independent experiments (days 1 and 2, n = 10; day 6, n = 8; day 10, n = 4). Error bars indicate the standard error of the mean.

(B) Dose-response characteristics of bFGF-induced neurite outgrowth. Cells were counted and scored as in (A). Each point represents the average value of counts from at least two dishes with the exception of day 10, 0.1 ng/ml and 1.0 ng/ml conditions (n = 1). Error bars indicate the standard error of the mean.

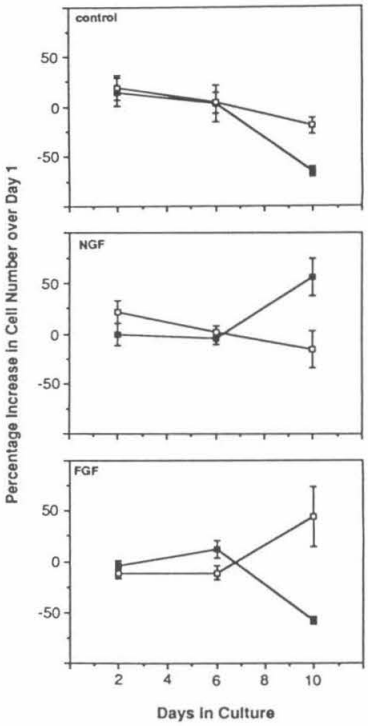

Figure 3. Effects of bFGF and NGF on Chromaffin Cell Number

For each of the experimental conditions, the percent increase in the number of cells over day 1 (to normalize for initial differences in plating efficiencies) is plotted against the number of days in culture. In all panels, filled squares correspond to the presence of the factor indicated in the upper left corner of the panel and open squares correspond to the factor in combination with 1.0 μM dex. Control indicates that cells were grown in growth medium alone; NGF indicates the presence of 1.0 μg/ml 7S NGF; FGF indicates the presence of 10.0 ng/ml bFGF. The values plotted are averages of several dishes for each condition on each day, obtained from two independent experiments (days 1 and 2, n = 10; day 6, n = 8; day 10, n = 4). Error bars indicate the standard error of the mean.

in bFGF increased only slightly (Figure 3, FGF, filled symbols), whereas the percentage of process-bearing cells rose to over 80% during this same interval (Figure 2, FGF, filled symbols). It is therefore likely that bFGF, like NGF (Figure 2; Figure 3; Doupe et al., 1985a), acts by inducing the phenotypic conversion of chromaffin cells to neurons, rather than by selecting for preexisting neurons in the culture. This interpretation is supported by serial observations of identified chromaffin cells (described below).

bFGF Is a Chromaffin Cell Mitogen

In cultures exposed to bFGF, a striking increase in the frequency of cell clusters was observed. This raised the possibility that, in addition to stimulating neuronal differentiation, this factor might also be a mitogen for chro-

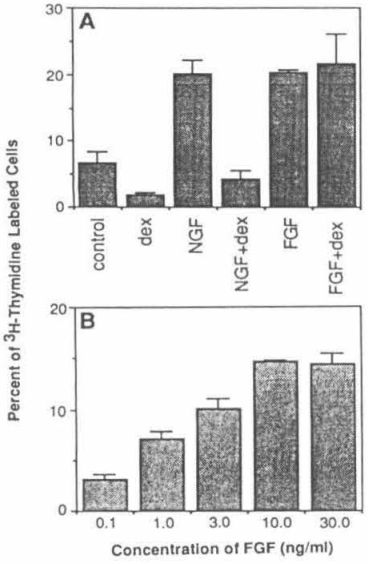

Figure 4. Effects of bFGF and NGF on the Incorporation of [^{3}H]Thymidine in Chromaffin Cells

Cells were grown for 6 days in the various culture conditions and then exposed to [^{3}H]thymidine for 24 hr. Cultures were processed, and OX-7-positive cells were scored for the presence of silver grains over the nuclei. The percentage of chromaffin cells that incorporated thymidine was calculated. (A) Control indicates that cells were grown in growth medium alone; dex indicates the presence of 1.0 μM dex; NGF indicates the presence of 1.0 μg/ml 7S NGF; NGF + dex indicates the both NGF and dex were present; FGF indicates the presence of 10.0 ng/ml bFGF; FGF + dex indicates that both bFGF and dex were present in culture. The average of data from five dishes in each condition is plotted. Error bars indicate the standard error of the mean. (B) Dose-response for thymidine incorporation. Data represent the average from three dishes for each condition. Error bars indicate the standard error of the mean.

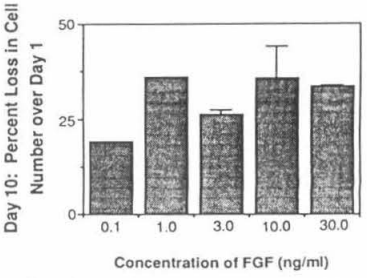

Figure 5. Effect of Varying bFGF Concentration on Chromaffin Cell Survival

The percent decrease in cell number at day 10 relative to day 1 is shown. Data for 3, 10, and 30 ng/ml represent the average from two sister cultures, while data for 0.1 and 1.0 ng/ml are from a single culture; all are based on initial counts of 350–400 cells per culture on day 1. The apparent slight decrease in cell loss at 0.1 ng/ml bFGF may not be statistically significant. However, at this concentration a significantly smaller proportion of the cells were process-bearing (48% compared with 95% for 10 ng/ml bFGF); therefore it is possible that at low concentrations bFGF may improve the viability of those chromaffin cells which have not yet converted to neurons.

maffin cells. This possibility was also suggested by the previous observation that NGF is a chromaffin cell mitogen (Lillien and Claude, 1985). [^{3}H]thymidine labeling of cultures on day 6 confirmed that bFGF, like NGF, stimulated DNA synthesis about 3-fold in chromaffin cells (Figure 4A). The dose-response for mitogenic stimulation confirmed that a concentration of 10 ng/ml was saturating for this effect (Figure 4B). Although dex strongly inhibited the mitogenic action of NGF (as previously reported [Lillien and Claude, 1985]), it did not do so for bFGF (Figure 4A). This difference in the inhibitory effect of dex was further reflected in cell number measurements: in bFGF plus dex, cell number increased significantly by day 10 in culture (Figure 3, FGF, open symbols), whereas dex inhibited the increase in cell number caused by NGF (Figure 3, NGF, open symbols).

bFGF Induces a Dependence on NGF but Does Not Itself Promote Survival

Although bFGF and NGF had similar effects on chromaffin cells between days 2 and 6 in vitro, between days 6 and 10 a significant difference was observed. In bFGF, the number of cells decreased precipitously during this later interval, while the cell number increased in NGF (Figure 3, filled symbols). One possible explanation for this difference is that bFGF can induce the neuronal differentiation of chromaffin cells, but it cannot support their subsequent survival as sympathetic neurons. In support of this interpretation, cultures of neonatal sympathetic neurons (derived from superior cervical ganglia) cannot be maintained by bFGF (data not shown), consistent with the findings of Rydel and Greene (1987). Thus, although bFGF can support the survival of some CNS neuronal populations (Walicke et al., 1986; Morrison et al., 1986; Hatten et al., 1988), and even parasympathetic periperal neurons (Unsicker et al., 1987), it does not do so for neonatal rat sympathetic neurons.

In a separate experiment (Figure 5), it was determined that varying the concentration of bFGF over a wide range (0.1–30 ng/ml) did not significantly improve the viability of cells when measured at day 10, by which time most have converted to a neuronal phenotype. In all concentrations of bFGF tested, a net cell loss occurred, whereas in the experiment of Figure 3, a 60% increase in cell number at day 10 was observed in the presence of NGF. Although under control conditions (no added factors), the extent of cell loss at day 10 was similar to that seen in 10 ng/ml bFGF (Figure 3), the attrition in control cultures reflects the elimination of chromaffin cells, which are glucocorticoid-dependent (Figure 3, control versus dex); in contrast, the loss of cells in bFGF-containing cultures occurs in a population expressing a neuronal phenotype.

The simplest interpretation of the decline in cell number in bFGF after day 6 is that at about this time, the chromaffin-derived neurons have acquired an absolute

Table 1. NGF Rescues Chromaffin Cells Previously Exposed to bFGF

Condition[a]	Mass Culture: % of Cells at Day 6 Present at Day 10[b]	Identified Cell: % of Cells at Day 6 Present at Day 12[c]
NGF → NGF	147 ± 25	100
bFGF → bFGF	37 ± 7	16
bFGF → NGF	108 ± 1	92

[a] Cells were switched to the indicated factor on day 6 of culture.
[b] Cell counts were made as for Figure 3. Values reflect the average cell numbers obtained from at least two dishes for each condition. Cells (200–400 in each dish) were scored on day 6. The number of cells at day 10 after switching from bFGF to NGF on day 6 was 404 ± 59; in cultures maintained in NGF it was 455 ± 115; in those maintained in bFGF it was 172 ± 28.
[c] Numbers based on following individual identified cells as described in the text. The mass culture data and the identified cell data are from two separate experiments.

dependence upon NGF, which cannot be substituted by bFGF. A prediction of this hypothesis is that NGF should rescue cells previously triggered to convert to sympathetic neurons by exposure to bFGF. To test this prediction, cells were exposed to FGF for 6 days and then switched to medium containing NGF. This treatment prevented the steep decline in cell number that ocurred after day 6 in bFGF, maintaining about 73% of the total number of cells maintained in control cultures exposed only to NGF (Table 1, Mass Culture column).

To exclude the possibility that bFGF and NGF may act on different subpopulations of chromaffin cells, in a separate experiment individual cells were identified during exposure to bFGF and then serially observed after switching to medium containing NGF. These experiments confirmed that most cells which had initially extended processes in response to bFGF died after day 6 when maintained in bFGF, as suggested by the cell counting data (Figure 6, FGF → FGF, days 8 and 12). In dramatic contrast, however, process-bearing cells in sister cultures survived when the medium was switched to NGF after day 6 (Figure 6, FGF → NGF, day 12). Of the identified cells, 92% survived when switched to NGF (n = 13), while only 16% survived when maintained in bFGF (n = 19) (Table 1, Identified Cell column). The rescued cells acquired the large cell bodies characteristic of cells maintained continuously in NGF (Figure 6, NGF → NGF). These single-cell tracking data thus strongly support the idea that when chromaffin cells are triggered to undergo neuronal differentiation by bFGF, they acquire a dependence upon NGF for survival that is not satisfied by bFGF.

The effects of the purified bovine pituitary bFGF preparation reported here could be mimicked by a preparation of recombinant human bFGF at similar concentrations (data not shown). As discussed previously elsewhere (Kimelman and Kirschner, 1987), this control makes it highly unlikely that the biological activities observed with the purified bovine preparation are due to any molecule other than bFGF. Also, we observed a similar response to bFGF when chromaffin cells were plated on a simple collagen substrate rather than Matrigel (data not shown), making it unlikely that the effects we observed in the latter case are due to a synergistic interaction (Kimelman and Kirschner, 1987) between bFGF and other growth factors which may be present in Matrigel (Hynda Kleinman, personal communication).

Discussion

aFGF and bFGF have mitogenic and/or differentiation-inducing effects on a variety of precursor cell types, including myoblasts and chondroblasts (Linkhart et al., 1980; Kato and Gospodarowicz, 1985; for review, see Gospodarowicz et al., 1986b). It was found that bFGF exhibits both activities on cultured adrenal chromaffin cells, which function as stably differentiated endocrine cells in the adult organism. (A preliminary report indicates that aFGF has similar effects [Claude et al., 1987, Soc. Neurosci., abstract].) In this case, bFGF induces the transdifferentiation of chromaffin cells into sympathetic neurons, rather than the further differentiation of an endocrine phenotype. This effect, as well as the mitogenic effects, is similar to the action of NGF on chromaffin cells (Doupe et al., 1985a; Lillien and Claude, 1985). Similarly, both NGF and bFGF cause PC12 cells to extend neurites (Togari et al., 1985; Wagner and D'Amore, 1986; Neufeld et al., 1987; Rydel and Greene, 1987; Schubert et al., 1987) and to increase the expression on neuron-specific mRNAs (Leonard et al., 1987; Stein et al., 1988a). The present work also reveals a difference between the actions of bFGF and NGF: bFGF cannot support the long-term survival of chromaffin cell–derived neurons, while NGF can (Table 2). However, bFGF does induce a dependence on NGF for survival, perhaps as part of a program of neuronal differentiation that it triggers in chromaffin cells.

The changes in the size of the chromaffin cell population in response to NGF and bFGF (Figures 1–3) can be analyzed in terms of the contributions of cell division, survival, and death. The increase in total cell number in NGF (Figure 3) reflects the mitotic expansion of the differentiating population, as well as its subsequent survival as neurons. However, the proliferation effect is delayed, as indicated by both [^{3}H]thymidine incorporation on day 2 (data not shown) and single-cell tracking analysis (Figure 5). This delay in the mitogenic effect of NGF yields the flattened early portion of the growth curve (Figure 3). Since many cells extend neurites during this interval, this lag indicates that proliferation is not necessary for neuronal differentiation, consistent with the fact that neurite outgrowth from chromaffin cells can be observed in the presence of anti-mitotic agents (Doupe et al., 1985a). For bFGF, the decline in cell number after day 6 (Figure 3) reflects the fact that as these cells become neurons, they acquire a dependence on NGF for survival that is not satisfied by bFGF. In support of this interpretation, serial observations of identified cells revealed that NGF can rescue cells that have been triggered to undergo neuronal differentiation by bFGF (Table 1; Figure 6). In contrast to the case of bFGF alone,

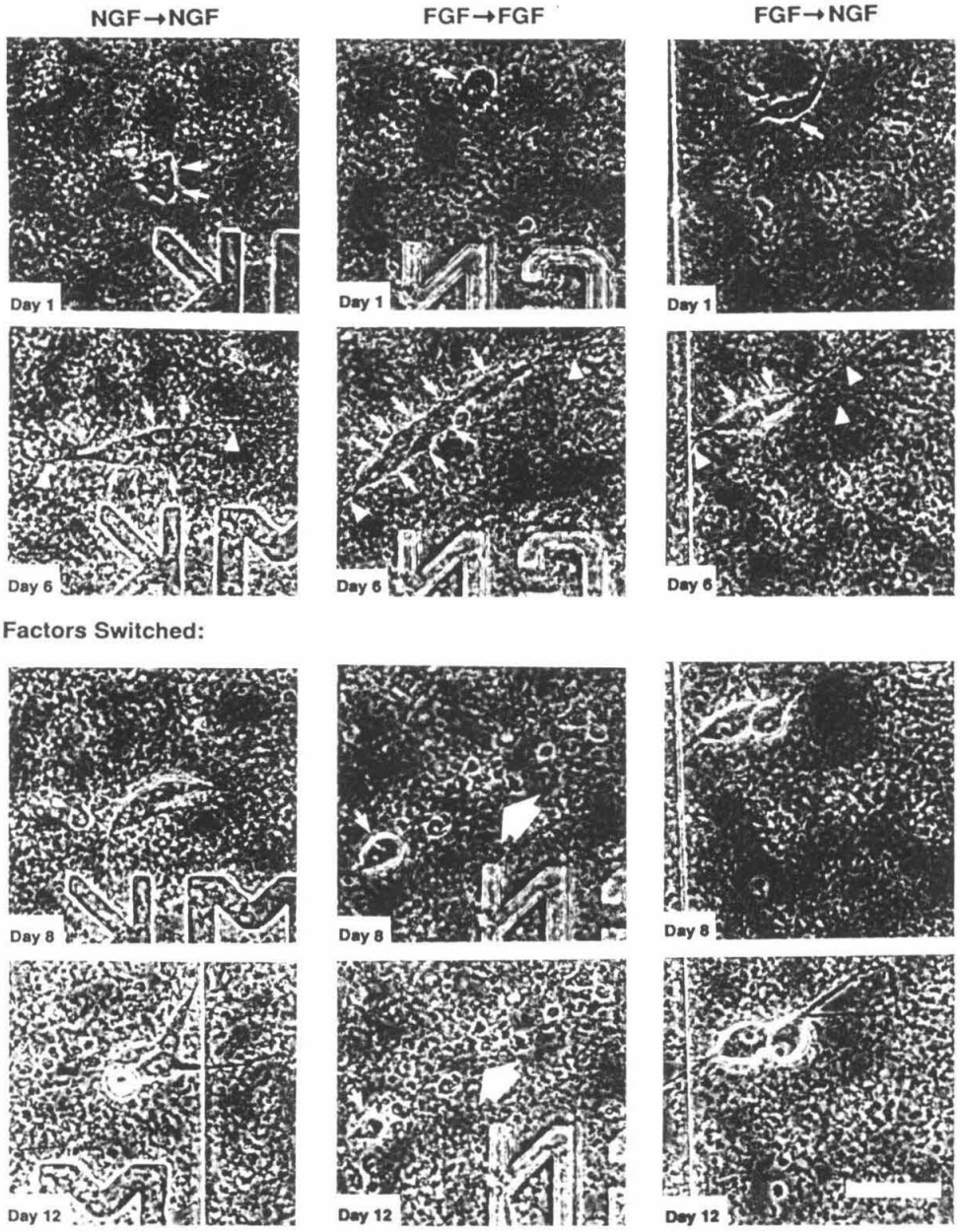

Figure 6. Chromaffin Cells Induced to Become Neurons by bFGF Are Rescued by Treatment with NGF

For each of these conditions, cells were photographed on the first day and on several subsequent days in culture. For the NGF → NGF condition, cells were maintained in 1.0 μg/ml 7S NGF throughout the series. For the FGF → FGF condition, cells were maintained in 10.0 ng/ml bFGF throughout the series. For the FGF → NGF condition, cells were initially treated with FGF and then switched on day 6 to medium containing NGF instead of bFGF. The small triangles indicate neurites emerging from the chromaffin cell clusters. The small arrows indicate the nuclei of some of the cells in the clusters. The large arrows indicate cellular debris left where clusters once existed. Bar, 25 μm.

Table 2. Summary of NGF and bFGF Effects on Neonatal Chromaffin Cells

Factor	Neuronal Differentiation	Mitogenic	Neuronal Survival
NGF	+	+	+
bFGF	+	+	–
NGF + dex	–	–	–
bFGF + dex	–	+	–

the combination of bFGF and dex causes cell number to increase dramatically by day 10 (Figure 3). This difference can be explained by two different effects of the glucocorticoid. In the presence of dex, the neuronal differentiation effect of bFGF is suppressed (Figure 1; Figure 2), while the mitogenic effect is not (Figure 4). Thus, cells remain in the chromaffin state and continue to proliferate. (As in the case for NGF, the mitogenic effect of bFGF is delayed.)

NGF was originally characterized as a survival factor for sympathetic neurons (Levi-Montalcini, 1976). Glucocorticoids, on the other hand, have been shown to function in part as a survival factor for adrenal chromaffin cells (Doupe et al., 1985a; Figure 3, compare control versus dex, day 10). In the absence of glucocorticoids, but in the presence of NGF, chromaffin cells survive and extend neurites. This initially suggested that NGF might substitute for dex as a chromaffin cell survival factor, but that in the absence of the inhibitory influence of glucocorticoid (Unsicker et al., 1978), the endocrine cells would undergo neuronal differentiation by default. The data presented here suggest a more complex mechanism. In the absence of any added factors, chromaffin cells survive for several days but do not undergo spontaneous neuronal differentiation. Therefore, the neuronal differentiation of chromaffin cells is not a default pathway, but requires both the presence of an inducer (bFGF or NGF) and the absence of an inhibitor (dex). The fact that bFGF can induce neuronal differentiation but is not sufficient for subsequent survival suggests that the ability of NGF to induce neuronal differentiation may be distinct from its effects on survival (Doupe et al., 1985b; Patterson, 1987).

Developmental Implications of the Chromaffin Cell Response to FGF

The similar but distinct responses of chromaffin cells to NGF and FGF (Table 2) imply that these two factors may activate different intracellular signaling pathways. The existence in chromaffin cells of such alternative signal transduction cascades, producing overlapping but nonidentical sets of biological responses, could be simply coincidental. Alternatively, if the response properties of chromaffin cells are similar to those of embryonic sympathetic precursors, these properties would fit well with some of the spatial and temporal constraints that recent data have placed on the embryonic development of sympathetic neurons. For example, NGF, which is synthesized by targets of sympathetic innervation (Heumann et al., 1984; Shelton and Reichardt, 1984), is not produced until the time at which embryonic neurons have grown axons to these targets (Davies et al., 1987; Korsching and Thoenen, 1988). Therefore, some other factor(s) may act at or near the forming ganglia to trigger the initial outgrowth of axons from neuroblasts to the periphery. Our results suggest that FGF, or some other molecule exerting identical effects, could serve such a function in vivo. The intracellular signaling pathway activated by this factor would not only initiate axon outgrowth, but would expand the population of ganglionic neuroblasts as well. The adrenal gland is also known to be a source of bFGF (Gospodarowicz et al., 1986a), and thus the established dex insensitivity of the mitotic response would allow the expansion of the developing medullary cell population within the glucocorticoid-rich adrenal gland, while simultaneously preventing neuronal differentiation.

In chromaffin cells, the FGF response pathway also leads to the acquisition of NGF dependence. By extension, in embryonic sympathetic precursors, the timing of this NGF-dependence might coincide with the arrival of sympathetic axons at their NGF-enriched peripheral targets. Simultaneously, the inability of the FGF signaling pathway ultimately to support neuronal survival would ensure the elimination of those neurons that failed to reach an appropriate peripheral source of NGF. Local NGF secretion by the target would then activate an alternative intracellular signaling pathway in sympathetic neurons, which would allow their subsequent survival and further maturation. In this way, a combination of factors, acting at different stages of development and triggering overlapping but distinct response cascades, could control a crucial sequence of steps in the early development of sympathetic neurons.

Preliminary observations suggest that the response properties of chromaffin cells to bFGF and NGF may indeed reflect the response properties of embryonic sympathetic precursor cells. In particular, it has been observed that bFGF stimulates neuronal differentiation in cultures of E14.5 paravertebral and superior cervical sympathetic ganglia (D. L. S. and J. Carnahan, unpublished observations). Moreover, sympathetic neurons at this stage do not require NGF for survival in vitro (Coughlin and Collins, 1985). In addition, bFGF appears to be mitogenic for E14.5 precursors in the presence of dex (A. Michelsohn, unpublished data). Thus, bFGF seems to induce a set of responses in embryonic sympathetic precursors similar to those induced in neonatal chromaffin cells. Confirmation of such a role for bFGF in vivo, however, awaits the development of methods for the sensitive detection of FGF in the mammalian embryo as well as the perturbation of its function.

Experimental Procedures

Cell Preparation

The adrenal medullae of neonatal (1–3 days) Simonsen albino rats (Simonsen Laboratories Inc., Gilroy, CA) were dissected free of cortex in L-15 air medium (Hawrot and Patterson, 1979). The medullae were dissociated by a modification of the method of Unsicker et al. (1978) in Hank's buffered saline solution without Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, NY) containing 1.0 mg/ml collagenase

(Worthington Diagnostics, Freehold, NJ). Cell suspensions were centrifuged twice at 1000 rpm for 10 min and resuspended into growth medium. To remove the more substrate-adherent cortical cell population, the cell suspension was preplated on 100 mm Corning tissue culture plates (Corning Glass Works, Corning, NY) for 6 hr in growth medium in a humidified incubator at 37°C and 5% CO_2. The chromaffin cells were removed from the plates by gentle trituration, centrifuged at 1000 rpm for 10 min, resuspended into growth medium, and plated on previously prepared plates at a density of 400–3000 cells per well.

Culture Media

The growth medium used in this study was prepared as described by Mains and Patterson (1973; Hawrot and Patterson, 1979) and was supplemented with 5% adult rat serum and 5% fetal calf serum (HyClone, Logan, UT). To generate the various experimental media, the growth medium was supplemented with one or more of the following additives: dexamethasone phosphate (Merck, Sharpe & Dohme, Teterboro, NJ) at a final concentration of 1.0 μM, NGF (7S) (Mains and Patterson, 1973) at a final concentration of 1.0 μg/ml, or bovine bFGF, prepared according to Gospodarowicz (1975), or recombinant human bFGF at a final concentration of 10 ng/ml. The bovine bFGF was a gift of Gera Neufeld, University of California, San Francisco, and the recombinant human bFGF was a gift of Forrest Fuller, California Biotechnology, Inc., Mountain View, CA.

Culture Dishes and Substrate

For the experiments in which the time course of process outgrowth was assayed, cells were growing on Aclar (Allied Chemical Corp., Morristown, NJ) coverslips in 35 mm Falcon petri dishes (Becton Dickinson and Company, Oxnard, CA) in which an 8 mm well had been lathed (Hawrot and Patterson, 1979). The dishes were sterilized by UV irradiation. To prepare the substrate, dishes were cooled to 4°C and coated with 50 μl of Matrigel (Collaborative Research, Bedford, MA), an Engelbreth-Holm-Swarm sarcoma extracellular matrix (Hadley et al., 1985), mixed 1:1 with growth medium. After 10 min, the coated dishes were flooded with 2 ml of experimental media and left for 1 hr at 4°C to allow the substrate meniscus to flatten. They were then transferred to a 37°C incubator for at least 1 hr to induce substrate polymerization and equilibration with the 5% CO_2. After 1 day in culture, the cells were overlaid with Matrigel mixture as follows. The medium and dishes were chilled to 4°C. Most of the medium in the dishes was drawn off, and an additional 50 μl of the Matrigel mixture was spread over the cells. The dishes were then flooded with cold medium and returned to the incubator, where they were maintained throughout the experiment. Cultures were fed every 4 days.

For [^{3}H]thymidine incorporation experiments, 15 × 3 mm glass rings (Thomas Scientific, Swedesboro, NJ) were attached to the bottoms of 35 mm Corning tissue culture dishes with high-vacuum silicone grease (Dow Corning, Midland, MI). The bottoms of the wells formed by the glass rings were coated with 100 μl of Matrigel mixture as described above.

For identified cell studies, a BB-Press petri dish press (World Precision Instruments, Inc., New Haven, CT) was used to imprint a grid pattern on the bottoms of 35 mm Corning tissue culture dishes. Glass rings were attached to the dishes outside the grid pattern as described above.

Cell Counting

For the time course of process outgrowth studies, chromaffin cells were distinguished on the basis of their phase-dense granular cytoplasm under phase-contrast optics at a total magnification of 200×. A cell was counted as bearing a process if the process extended >3 cell body diameters from the soma. Where cells were clustered, the number of nuclei was recorded as the total number of cells, and the number of emerging processes was recorded as the number of process-bearing cells. Cell numbers were measured by counting all of the cells in two parallel strips constituting ~30% of the total growth surface area. In this way, from 100 to 600 cells were scored for each dish, depending upon time and condition.

[^{3}H]Thymidine Labeling

At day 6 after plating cultures were exposed to [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) at a final concentration of 2 μCi/ml for 24 hr. Cultures were rinsed with PBS and fixed with 3.7% formaldehyde in PBS. The cultures were then incubated with OX-7, an anti-Thy-1 monoclonal antibody, and strained with an FITC-conjugated goat anti-mouse IgG secondary antibody (TAGO, Burlingame, CA) to label specifically the chromaffin cells in the cultures. The OX-7 hybridoma cell line was a gift of Alan Williams, MRC Cellular Immunology Unit, Oxford University. The labeled cultures were coated with photographic emulsion, Kodak NTB2 (Eastman Kodak Company, Rochester, NY), diluted 1:1 in 0.5 M sodium acetate, and exposed for 7 days at 4°C. The dishes were developed with Kodak D-19 developer and scored. OX-7-positive cells were counted with epifluorescence illumination, and those cells that had incorporated [^{3}H]thymidine were scored under bright-field illumination. All positive cells in all cultures were counted at a final magnification of 200 ×.

Identified Cells

Cells were identified by means of a gridded dish and photographed with 35 mm Plus-X film at ASA-125 under phase-contrast optics at a final magnification of 400×. For several days after initial plating, the same fields were photographed.

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CHAPTER 4. A Schwann cell antigen recognized by monoclonal antibody 217c is the rat low-affinity nerve growth factor receptor

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A Schwann cell antigen recognized by monoclonal antibody 217c is the rat low-affinity nerve growth factor receptor

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Monoclonal antibody 217c was generated against an antigen expressed on the rat glial cell line, C6 glioma. 217c has been shown to recognize Schwann cells in mixed cultures as well as in tissue sections and has been used to identify Schwann cells independent of other markers, such as monoclonal antibody 192-IgG, which recognizes the rat low affinity nerve growth factor (NGF) receptor. Here we show that the antigen recognized by 217c is the rat low-affinity NGF receptor. This indicates that monoclonal antibodies 192-IgG and 217c are not independent markers and therefore that additional criteria need to be used for the identification of Schwann cells early in development.

Antigenic markers that are expressed on the cell surface provide a powerful means to identify and isolate subpopulations of cells in a non-destructive way. With such a marker one may purify populations of cells and, for example, study their developmental potential, or growth factor and hormone responsiveness, in the absence of activities that may be contributed by other cell types. One can also use the marker to identify individual cells in mixed populations and to establish progenitor-progeny relationships, as has been carried out for glia derived from the O2-A progenitor in cultures of optic nerve [13].

With the aim of identifying cell-surface markers useful for the study of early Schwann cell development, we screened tissue sections of early rat embryos and cultures of neural crest with antibodies that had been previously characterized as independent Schwann cell markers. Among the antibodies tested were the monoclonal anti-NGF receptor antibody 192-IgG [3] and the monoclonal antibody 217c. 217c is thought to recognize the RAN-1 antigen [1, 5] and has been utilized as an independent Schwann cell marker [14, 15]. Recently, Jessen et al. [8] have shown that the 217c antigen is selectively expressed by non-myelin-forming Schwann cells in adult rats, but is expressed by essentially all Schwann cells earlier in development. We found that 192-IgG and 217c labeled very similar subsets of cells in the embryo including spinal cord motorneurons, cells of the dorsal root and sympathetic ganglia, and cells associated with the ventral roots of the spinal cord (Fig. 1). The coincidence of NGF receptor and 217c immunoreactivity suggested that 217c also recognized the NGF receptor.

To test whether 217c and 192-IgG recognize the same molecule two experiments were performed. In the first experiment, NGF receptors of PC12 cells were chemically cross-linked with iodinated 2.5S NGF as described [6]. Detergent extracts from these cells were immunoprecipitated with antibodies 217c, 192-IgG and, as a control ASCS4, a monoclonal antibody that recognizes another PC12 surface antigen NILE/L1/NgCAM [16]. Both antibodies 217c and 192-IgG precipitated a labeled protein complex of identical size, which was not brought down by ASCS4 (Fig. 2). The molecular weight of this complex, ~103 kDa, is appropriate for the NGF/low-affinity NGF receptor cross-linked product [7]. These results are similar to those recently obtained independently by Kumar et al. [9] in C6 glioma cells. While these immunochemical results strongly suggest that 217c recognizes the rat NGF receptor, they do not formally exclude the possibility that the antibody recognizes only a complex between NGF and its receptor, or another protein associated in detergent extracts with the NGF-NGF receptor complex which is not itself the receptor.

To prove that both 192-IgG and 217c recognize the low-affinity NGF receptor, an independent genetic experiment was performed. A stably-transfected mouse

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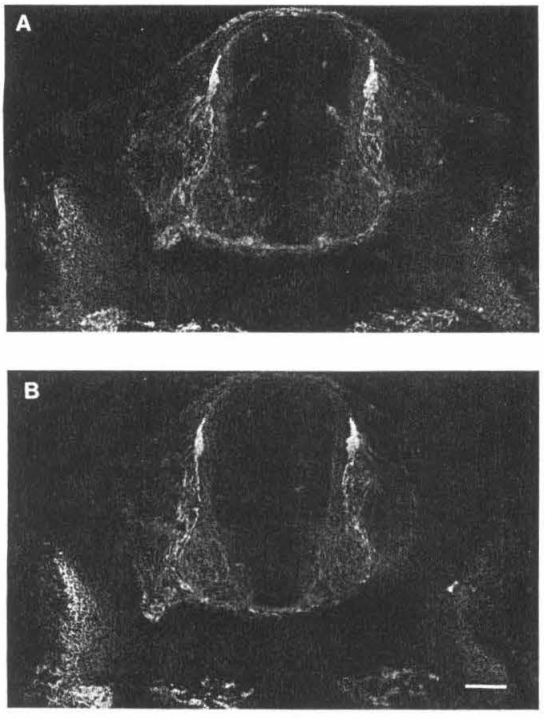


Fig. 1. Adjacent trunk sections of embryonic day 12 rat stained with 192-IgG (A) and 217c (B). Scale bar is 100 μm.

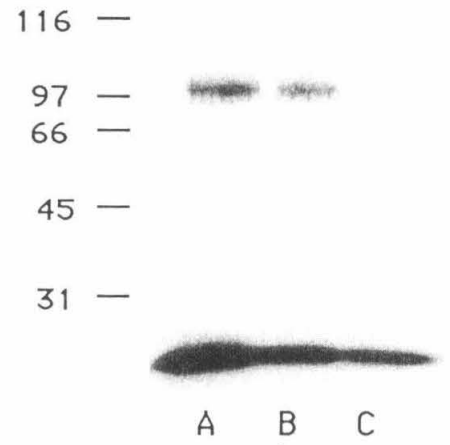


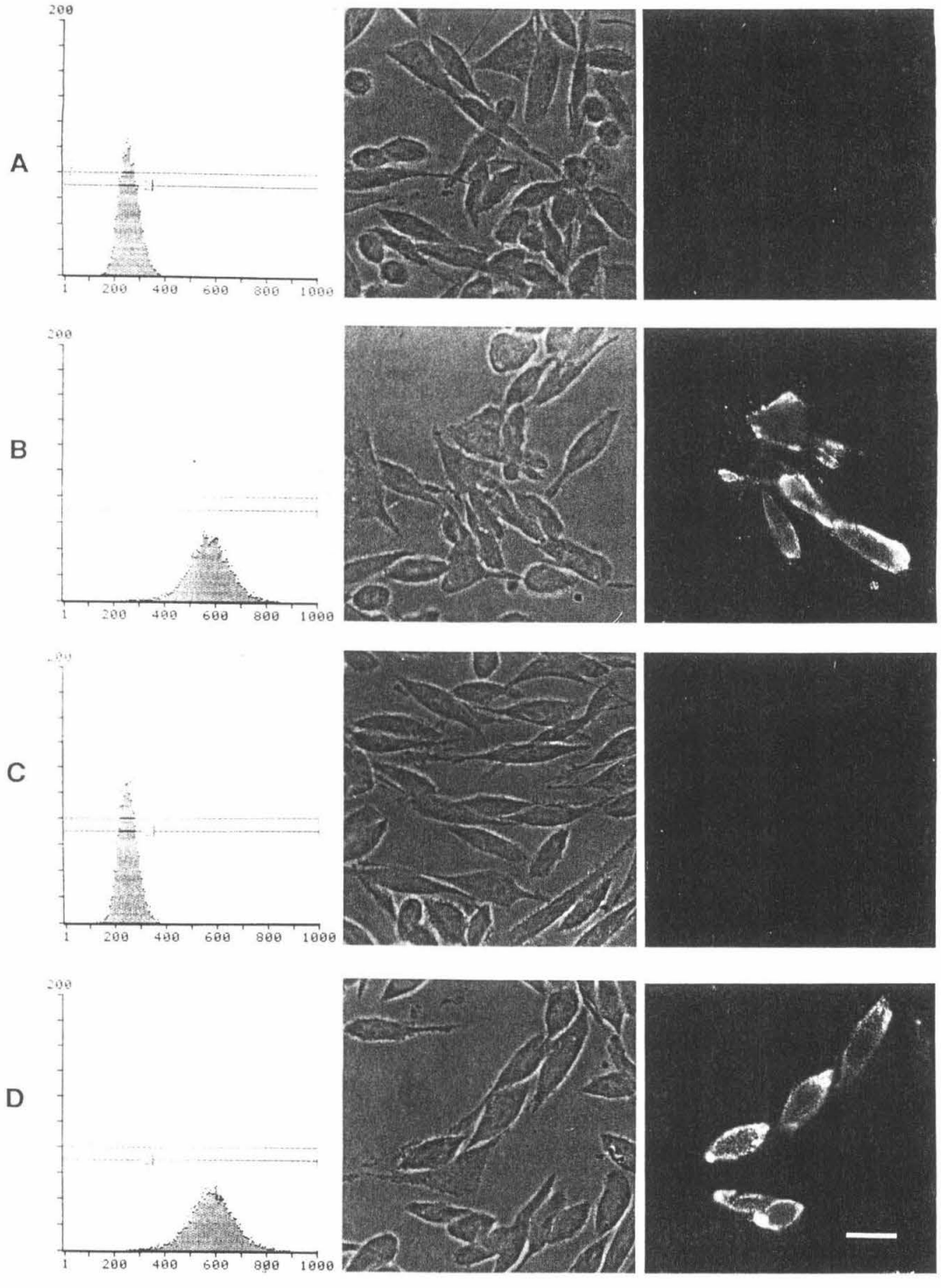
Fig. 2. An autoradiogram of a polyacrylamide gel showing the immunoprecipitation of ^{125}I-NGF cross-linked to its receptor using 192-IgG (lane A), 217c (lane B), and ASCS-4 (lane C). Equal amounts of lysate and antibody were used in each reaction.

L-cell line carrying a rat NGF receptor cDNA [2] was compared with the untransfected parental cell line by immunofluorescence staining and flow cytometry, with both 217c and 192-IgG. The transfected cell line (LpMVR) was stained by both 217c and 192-IgG, but the untransfected line Ltk- was not stained by either antibody (Fig. 3). Because both 217c and 192-IgG are specific to rat NGF receptor (not shown) and Ltk- is a mouse cell line, this result cannot be explained by the induction of the endogenous NGF receptor gene, or indeed of any other associated endogenous mouse product, in the transfected cell line.

We demonstrate here that both 217c and 192-IgG immunoprecipitate an NGF-NGF receptor complex, from PC12 cells, and that a stable mouse cell line transfected with rat NGF receptor cDNA is immunoreactive with both antibodies whereas the parental cell line is not. We conclude, therefore, that both 217c and 192-IgG recognize the low-affinity subunit of the rat NGF receptor.

It is necessary, however, to reconcile the conclusion of this study with previous reports showing that the immunoprecipitable products of 192-IgG and 217c have different molecular weights. Specifically, when 217c is used to immunoprecipitate ^{125}I labeled membrane proteins from C6 glioma, a ~64 kDa product is observed [10]; when 192-IgG is used to precipitate ^{125}I labeled membrane proteins from PC12, an ~83 kDa product is found [12]. The discrepancy between the published molecular weights may be explained in one of several ways. One possibility is that the immunoprecipitated products of 217c and 192-IgG may be glycosylated to different extents. Radeke et al. have suggested that extensive glycosylation may explain the difference between the observed ~83 kDa protein and the ~42 kDa polypeptide predicted from the sequence of NGF receptor cDNA. Another explanation may be that the NGF

Fig. 3. Untransfected mouse Ltk- cells (Panels A and C) and a stable line of Ltk- cells transfected with rat NGF receptor cDNA, LpMVR cells (Panels B and D) stained with antibody 192-IgG (Panels A and B) and 217c (Panels C and D). The left frame in each panel shows the profile from cells analyzed by flow cytometry; cell number is plotted on the ordinate; relative fluorescence in arbitrary units is plotted on the abscissa. The middle and right frames of each panel are phase contrast and fluorescence micrographs, respectively, of representative fields from dishes of stained cells. The heterogeneity of staining as seen by immunofluorescence reflects a broad distribution of levels of expression of the transfected gene in the cell population, as seen by the flow cytometric analysis. Scale bar is 25 μm.


A
B
C
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200
1
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600
800
1000

60

receptor, even as recognized by 192-IgG, exists in multiple molecular weight forms [4]. In addition to the ~83 kDa form, a ~50 kDa form has been detected in the culture medium of a rat schwannoma cell line, and a ~77 kDa form has been detected in plasma during early development. The biochemical basis for the relationship between these different forms is not yet clear.

The work described here was initiated to identify markers for embryonic Schwann cells. In the past 217c and the NGF receptor have been used as independent markers for Schwann cells [14, 15]. This study indicates that they are not independent markers. Thus, additional criteria need to be applied to distinguish Schwann cells from other cell types early in their development.

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SUMMARY

Mammalian neural crest stem cells

The experiments described in chapter 2 were designed to distinguish two possible explanations for both the heterogeneity of primary neural crest cell clones and the observed multipotency in some of those clones. The two extreme models described in the introduction make distinct predictions about the outcome of the sub-cloning of a multipotent neural crest cell clone. For example, under the restrictive model one would expect none of the sub-clones to contain all of the differentiated phenotypes of the original clone. On the other hand under a stem cell model one would expect to find at least one sub-clone containing all of the phenotypes found in the original clone. That is, the key difference between the two models is that, if there is a stem cell, the multipotent cell will self-renew. No published experiments to date have specifically examined self-renewal of multipotency in the neural crest.

In our studies, a majority of primary rat neural crest cell clones, produce a mixture of differentiated cells including peripheral neurons and Schwann cells. We also find that some primary clones appear to be founded by relatively restricted cells and produce clones that contain no neurons. When multipotent clones are sub-cloned, we find that many of the sub-clones are also multipotent and can produce that same range of measurable phenotypes as the original founder clone. In addition, we find some clones that are comprised of a single phenotype as well as some multipotent sub-clones that do not produce the full range of phenotypes displayed by the original clone. Hence, a significant proportion of primary neural crest cells are both multipotent and capable of self-renewal. Furthermore, the fact that some of the sub-clones are comprised of only a single phenotype or a subset of the phenotypes available to the original founder clone indicates that some of the cell divisions of stem cells in the founder clone are asymmetric.

Our data establish the existence of a stem cell capable of producing neurons, glia and probably other neural crest derivatives. The data also establish some of the properties of neural crest stem cells and their derivatives at various stages of development. We also know that cell fate choices are being made within developing clones. Are those choices being made by an internal cellular program or are they controlled by external signals? If by an internal program, is it a deterministic or stochastic program? If by an external signal, do those signals cause the cell to become determined for some particular lineage or do they signal some general information, such as "divide," "survive" or "differentiate"? A few studies have been done that indicate that environmental signals can control the composition of clones derived from multipotent founder cells.

In cultures of rat neural crest cells we have obtained evidence that environmental factors can act directly on founder cells to direct the fate of clones. Specifically, we find that clones grown fibronectin fail to generate neurons. The clones contain only Schwann cells and another unidentified cell type. When clones are grown on poly-D-Lysine and fibronectin, however, they generate peripheral neurons, in addition to Schwann cells and the other cell type. We have carried the analysis further with two other experiments. In the first experiment we start with two sets of identified founder cells on a fibronectin substrate. We then apply pDL to one group of founders as an overlay, and allow both groups to grow and differentiate. We find that, with a high cloning efficiency of ~ 90%, that the pDL treated founders produce clones containing neurons, whereas, the clones grown on fibronectin alone do not. In a second experiment, a group of clones is initially grown on a fibronectin substrate. Each clone is then split into two different dishes, one dish is coated with fibronectin and the other is coated with pDL and fibronectin. Sub-clones are allowed to grow and mature and then analyzed. We find that most of the original founder clones produced neurogenic sub-clones when grown on pDL/FN but not on FN. Hence, even though clones do not

form neurons when grown on FN they retain neurogenic potential and can produce neurons when sub-cloned onto pDL/FN. On FN it is not the case that neurons or neuroblasts appear and then die for lack of trophic support or an adhesive substrate. Instead FN appears to promote dispersion of the cells in a clone. On pDL/FN, close cell-cell contacts are maintained. Furthermore, when neural crest cells are plated at clonal density on tissue culture plastic rather than FN or pDL/FN neurons are produced by the resulting clones. These data rule out selection as a mechanism to explain the effect of the substrate on the fate of clones. These results argue strongly that control over some cell fate choices derives from the environment in which stem cell and their progeny development. In this example, multipotent neural crest cells when grown on pDL/FN produce neurons but their neurogenic potential is not realized on FN alone. Hence, neural crest cells do not appear to develop by some set internal program. Instead they seem to be controlled directly by environmental factors.

We have demonstrated that primary mammalian neural crest cells *can* self-renew, but do they self-renew in the animal. It is possible that the conditions of culture lead to self-renewal, even though it does not happen *in vivo*. Consider the example of the development of neocortical laminae. McConnell and Kaznowski have found that the fate of neurons produced by a multipotent progenitor is specified at a particular point in the cell cycle by the local environment (McConnell and Kaznowski, 1991). Normally as the animal matures the environment of the progenitor cells is changing, and that change is uni-directional with respect to the neuronal fates that can be specified. As I argued in the introduction they have not demonstrated self-renewal of the multipotent cortical progenitor. But if one were to back-transplant postnatal progenitor cells to the E29 environment and self-renewal were observed, that observed self-renewal may be artifactual in the sense that it would never normally occur. Such may be the case for cultures of primary neural crest cells.

There are several approaches that might be taken to address the possibility that self renewal does normally occur during the development of neural crest derived structures. As Fraser and Bronner-Fraser have shown, it is possible to label single migrating neural crest cells (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991). If one were to label single cells as they have (with lysinated rhodamine dextran), allow the cells to divide once, and then with a distinct marker (e.g., lysinated fluorescine dextran) mark one of the two daughters, one may find that the daughters have equivalent and extensive developmental potentials. That is the parent cell has undergone self-renewal, at least with respect to the fates manifest by the progeny of the daughter cells. Such an experiment, however, can only reveal the fate of the cells not necessarily the developmental potential. Another approach is to ask; do neural crest stem cells, as we have defined them in chapter 2, exist in developing neural crest derived structures? For example, can one dissect out an E14.5 rat adrenal gland, dorsal root ganglion, sympathetic ganglion, sciatic nerve or enteric ganglion and find LNGFR$^+$, nestin$^+$ cells that are multipotent (forming neurons, glia and other) and exhibit self-renewal?

While the cell we have described is clearly a stem cell for the production of neurons, glia and the other cell type, it is not clear if that cell is a totipotent neural crest progenitor or some intermediate cell type, restricted to the production of only a proper subset of all possible neural crest derivatives. We never observed the production of melanocytes for example. Other normal neural crest derivatives might be produced by the cell, but we lack either the markers to detect them or the culture conditions to produce them. Before an extensive survey of culture conditions is carried out it may be appropriate to challenge the stem cell to an environment where the appropriate signals are know to exist, i.e., a whole embryo as a transplant host or perhaps a slice of an embryo in culture.

Sympathoadrenal lineage

Our goal was to test whether bFGF could act on primary adrenal chromaffin cells as it had been shown to for PC12 cells. We found that bFGF acts as a neuronal differentiation factor, a mitogen and can induce NGF dependence in neonatal chromaffin cells. FGF cannot act as a trophic factor for sympathetic neurons. During the normal development of the sympathetic ganglion NGF is not present in the ganglion at the time of neuronal differentiation, but is supplied by the peripheral targets of innervation at a later time. We then proposed that perhaps FGF or something like FGF acts in the developing ganglion to control neuronal differentiation. Since the publication of the effects of FGF on chromaffin cells there have been several reports published that have some bearing on our hypothesis.

Birren and Anderson produced a cell line by immortalizing embryonic adrenal derived sympathoadrenal progenitors. They found that the cell is not responsive to NGF but responds dramatically to FGF by extending neurites and undergoing neuronal differentiation, including the induction of low affinity NGF receptor expression. A variety of other purified polypeptide growth factors were tested, and none except FGF caused the cell line to undergo neuronal differentiation (Birren and Anderson, 1990). In addition Birren and Augsburger have found that primary E14.5 HNK-1^+, $B2^-$ adrenal cells respond to FGF by undergoing neuronal differentiation and a small minority of those cells eventually become NGF responsive, forming mature sympathetic neurons (Dr. Susan Birren and Adela Augsburger, unpublished observations). FGF has been detected in developing sympathetic ganglia by immunocytochemistry (Kalcheim and Neufeld, 1990) and by PCR amplification of ganglion derived cDNA (Derek Stemple, unpublished observations).

It seems clear that FGF is present at the right time and place to induce neuronal differentiation but the issue will not be resolved until a direct test of the activity in a

developing ganglion can be performed. Several possible approaches exist for such a test. It may be possible to find a reagent, such a a blocking antibody, or antisense oligodeoxynucleotide, that can disrupt the activity of FGF specifically, and apply the reagent to a developing ganglion *in vivo* or to an *in vitro* embryonic slice preparation. The other possibility is to perturb the activity of the receptor. It may be possible to construct a transgenic mouse that expresses a dominant negative form of the FGF receptor (Amaya et al., 1991) under the control of a sympathoadrenal specific promoter such as the DβH promoter (Mercer et al., 1991). Such a construct would lack FGF receptor activity in the sympathoadrenal progenitor and the simple hypothesis would be that no sympathetic neurons would be produced, even though SA progenitors can form.

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APPENDIX 1: Environmental control of neural crest cell fate *in vitro*

Abstract

Previous clonal studies of neural crest cells revealed that the population is heterogeneous with respect to developmental potential. We have studied the development of mammalian neural crest cell clones *in vitro* and provide evidence for the progressive restriction of neural crest cell developmental potential under certain environmental conditions. We find that multipotent clones can produce progeny that are apparently committed to the production of only a single lineage. These results may serve to explain the observed heterogeneity of primary clones. In addition, the observed heterogeneity may be in part controlled by the environment in which the neural crest cells are grown. We find that the fate of primary neural crest cell clones is affected by the presence of serum in the culture medium. The serum responsiveness of neural crest cells or their progeny differ over time in culture. Clones founded at 24 hours by LNGFR$^+$ neural crest cells respond to FBS by extinguishing LNGFR expression. If neural crest cells are allowed to develop in serum-free medium for a period of time before clonal culture many of the clones are no longer sensitive to FBS and divide to form large LNGFR$^+$ clones. The differential responses of clone founders provide an assay for phenotypic differences between them that are not apparent by morphological or immunocytochemical assays.

Introduction

We have focused our study of neural crest cell development on the capacity of single early neural crest cells to produce differentiated progeny. In previous work the heterogeneity with respect to the ultimate phenotype of neural crest cell clones was highlighted as a key feature of the population of founders (Baroffio et al., 1988; Dupin et al., 1990). That is, although a large percentage of the clones were multipotent, different clones produced different subsets of the available lineages. Relatively few clones produced all of the available phenotypes and some clones appeared to arise from committed progenitors and were comprised of differentiated cells of only one or a few phenotypes. There are several ways that such heterogeneity might arise. One possibility is that the heterogeneity may be programmed into the population of neural crest cells before or as they emerge from the neural tube. Another possibility is that members of a relatively homogeneous population of clone founders are caused to manifest a restricted developmental potential by some extrinsic (e.g., microenvironmental cue) or intrinsic (e.g., stochastic) signal. Finally, it may be possible that emerging crest cells are homogeneous in their developmental potential but that since they are an actively dividing population they are in the process of producing progeny that have been specified to a relatively restricted set of fates. Chapter 2 described sub-cloning experiments in which multipotent primary clones produced a mixture of secondary clone founders. Specifically we found that primary multipotent clones produced progeny that, upon subcloning, were found to be of a variety of phenotypes. Some multipotent subclones contained neuron ("N"), glia ("G") and another unidentified cell type ("O"). Other subclones contained subsets of those differentiated phenotypes, e.g. N-only, G+O and O-only clones. These results, therefore, allow for the possibility that the observed heterogeneity of primary clones arises by the production of relatively restricted progeny of multipotent cells. In this chapter we examine the heterogeneity of neural crest cell clones in a culture condition

that allows the formation of a subset of phenotypes described in chapter 2. Neural crest cell clones grown on fibronectin (FN) contain only glial (G) and other (O) cells. We find that the majority of early (i.e., 24 hour old) LNGFR$^+$ founders produce clones of a mixture of LNGFR$^+$ and LNGFR$^-$ cells; whereas older (i.e., 8 day old) LNGFR$^+$ founders produce a majority of clones comprised entirely of LNGFR$^+$ cells.

The observation that heterogeneity arises within multipotent clones presents another problem. How is the production of relatively restricted and differentiated progeny controlled within the spatially restricted environment of a clone? There is a substantial amount of evidence indicating that the fate of neural crest cells is controlled by the environment in which they develop (Le Douarin, 1982). And a fair number of studies that implicate specific growth factors or culture conditions in the control of neural crest cell fate (Satoh and Ide, 1987; Barald, 1989; Maxwell and Forbes, 1990; Morrison-Graham et al., 1990; Murphy et al., 1991; Sieber-Blum, 1991) (see also review, Sieber-Blum, 1990). In Chapter 2 we presented evidence that the composition of the substrate can substantially alter the fate of neural crest cell clones in an instructive fashion. In this chapter we present evidence that the formation of O cells may be controlled by the presence of serum in the culture medium. The serum seems also to act instructively on LNGFR$^+$ cells leading to the formation of clones comprised only of O cells.

Results

Primary neural crest cell undergo progressive restriction in developmental potential

Colony analysis

We sought to test the notion that over time in culture the developmental potential of neural crest cells becomes restricted. We reasoned that cells taken from early neural crest cultures would be predominately multipotent and that over time in culture they would produce relatively restricted or committed offspring. In these experiments we limited our analysis to neural crest cells expressing low-affinity nerve growth factor receptor (LNGFR), a population we have shown to be capable of producing several identifiable neural crest derivatives (Chapter 2). As shown in Figure 1A colonies of early (24 hour) or late (day 8) founders were examined. Example micrographs of the resulting colony types "LNGFR$^+$" (i.e., all cells in the colony express LNGFR) and "mixed" (i.e., the colony is a mixture of LNGFR$^+$ and LNGFR$^-$ cells) are shown in figure 2. Considering only colonies that contain any LNGFR expressing cells, we found that colonies resulting from cells plated at day 1 were predominately of the "mixed" type (of the total number of colonies 7.5 ± 2.5 % were "mixed" and $< 2\%$ were "LNGFR$^+$")(Figure 1B). In sharp contrast, colonies resulting from cells plated at day 7 (whether analyzed at day 14 or at day 22) were predominately of the "LNGFR$^+$" type (11% "LNGFR$^+$" vs 5% "mixed" for day 14; and 18% "LNGFR$^+$" vs 10% "mixed" for day 21). Over time in mass culture, a population of cells develop that are apparently committed to the production of only LNGFR$^+$ cells. This population of cells develops from a starting population that is apparently not committed and can produce both LNGFR$^+$ and LNGFR$^-$ cells.

Serial subcloning analysis

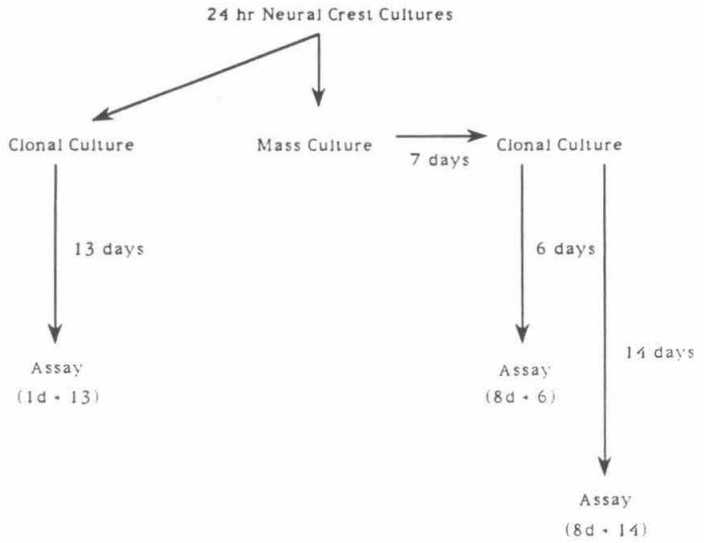
One interpretation of the results of the preceding experiment is that primary neural crest cell founders are multipotent and as they grow, they give rise to progeny

Figure 1. Neural crest cell colony analysis.

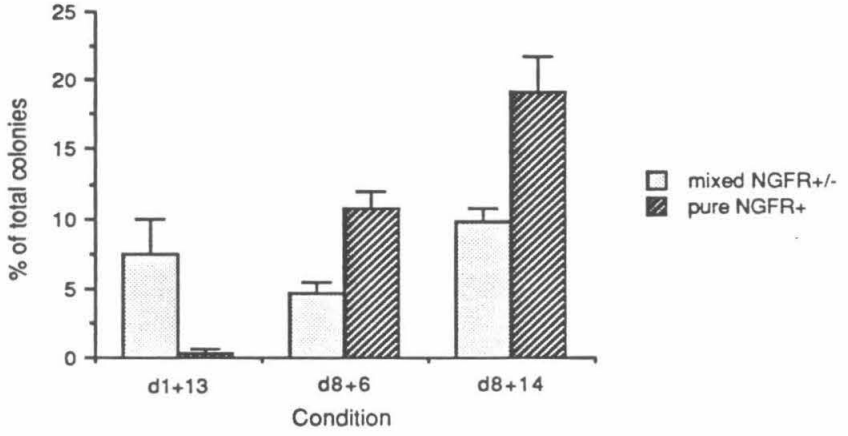
A. This is a schematic outline of the colony analysis. Initially neural crest cell were plated either at clonal density or in mass cultures. Dishes plated at clonal density were allowed to grow in culture for an additional 13 days, at which time they were fixed and analyzed for LNGFR expression. The cells grown in mass culture were replated at clonal density at day 8 and allowed to grow for an additional 6 days, or an additional 14 days, after which the resulting colonies were fixed and analyzed for LNGFR expression. Colonies were assayed by examining them with a fluorescence microscope.

B. $LNGFR^+$ colonies in which either all of the cells expressed LNGFR (striped bars) or only some of the cells expressed LNGFR (stippled bars) are presented as a percentage of the total number (#) of colonies in each 100 mm dish. At least 3 dishes per condition were analyzed. In this analysis, the phenotype of the founder was not determined at the time of plating. In fact the majority of founders are of the $LNGFR^-$ phenotype which in turn produce only $LNGFR^-$ colonies. This accounts for the relatively low percentage of LNGFR expressing colonies.

A. Colony Analysis



B. Schwann Cell Colony Analysis



that are relatively restricted in their developmental potential, perhaps restricted to the extent that they are committed to produce only one differentiated phenotype. In the preceding experiment, founder cells were plated at clonal density, but colony founders were not screened to ensure that they were only single cells. This is a problem for analysis of "mixed" colonies since a "mixed" colony may have been founded by more than one cell and not a single multipotent cell. Furthermore, the phenotype of founders with respect to LNGFR expression was not determined. This is a problem because the relative number of $LNGFR^+$ and $LNGFR^-$ founder cells is variable between preparations of neural crest cells due to the variable amounts of $LNGFR^-$ non-crest cells. To ensure that "mixed" colonies indeed arise from single cells, and to test directly the hypothesis that founders of "mixed" colonies can produce "$LNGFR^+$" clone (i.e., apparently committed) founders, we executed a serial sub-cloning analysis of $LNGFR^+$ primary neural crest cells.

Neural crest cells taken from 24 hour neural tube explants were plated at a density of ~225 cells / 100 mm dish. After the cells attached, individual $LNGFR^+$ neural crest cells were identified and inscribed with a ~3 mm circle drawn on the bottom of the dish. The circles were generally large enough to permit the unambiguous identification of the progeny of the cell at any time during the experiment. After 7 days in clonal culture the primary clone were scored for the continued expression of LNGFR. No $LNGFR^-$ founder was ever found to produce an "$LNGFR^+$" or "mixed" clone. Among the $LNGFR^+$ founders $20 \pm 8\%$ produced "$LNGFR^+$" clones and $50 \pm 8\%$ produced "mixed" clones. A substantial percentage of the $LNGFR^+$ founders, $22 \pm 10\%$, gave rise to clones containing no $LNGFR^+$ cells; and $8 \pm 2\%$ of the $LNGFR^+$ founders did not produce clones (Figure 3A).

Analysis of primary clones at day 7 was done by live-labeling with LNGFR antibody, this allowed us to subclone some of the primary clones to test whether "mixed" primary clones contained precursors apparently committed to produce

Figure 2. Quantification of primary and secondary neural crest cell clones.

A. Primary clones founded by LNGFR+ crest cells were analyzed for their expression of LNGFR after 7 days in culture. "LNGFR$^+$" indicates that all of the cells of the clone expressed LNGFR. "Mixed" indicates that both LNGFR$^+$ and LNGFR$^-$ cells were in the clone. "LNGFR$^-$" clones contained no LNGFR expressing cells. "Dead" indicates that no clone was formed. Values are presented as the percentage of the total number of founders ± s.e.m.

B. Secondary clones founded by LNGFR$^+$ cells derived either from "mixed" primary clones (hatched bars) or from primary clones containing only LNGFR$^+$ cells (striped bars) were analyzed for expression of LNGFR after 6 days in secondary clonal culture.

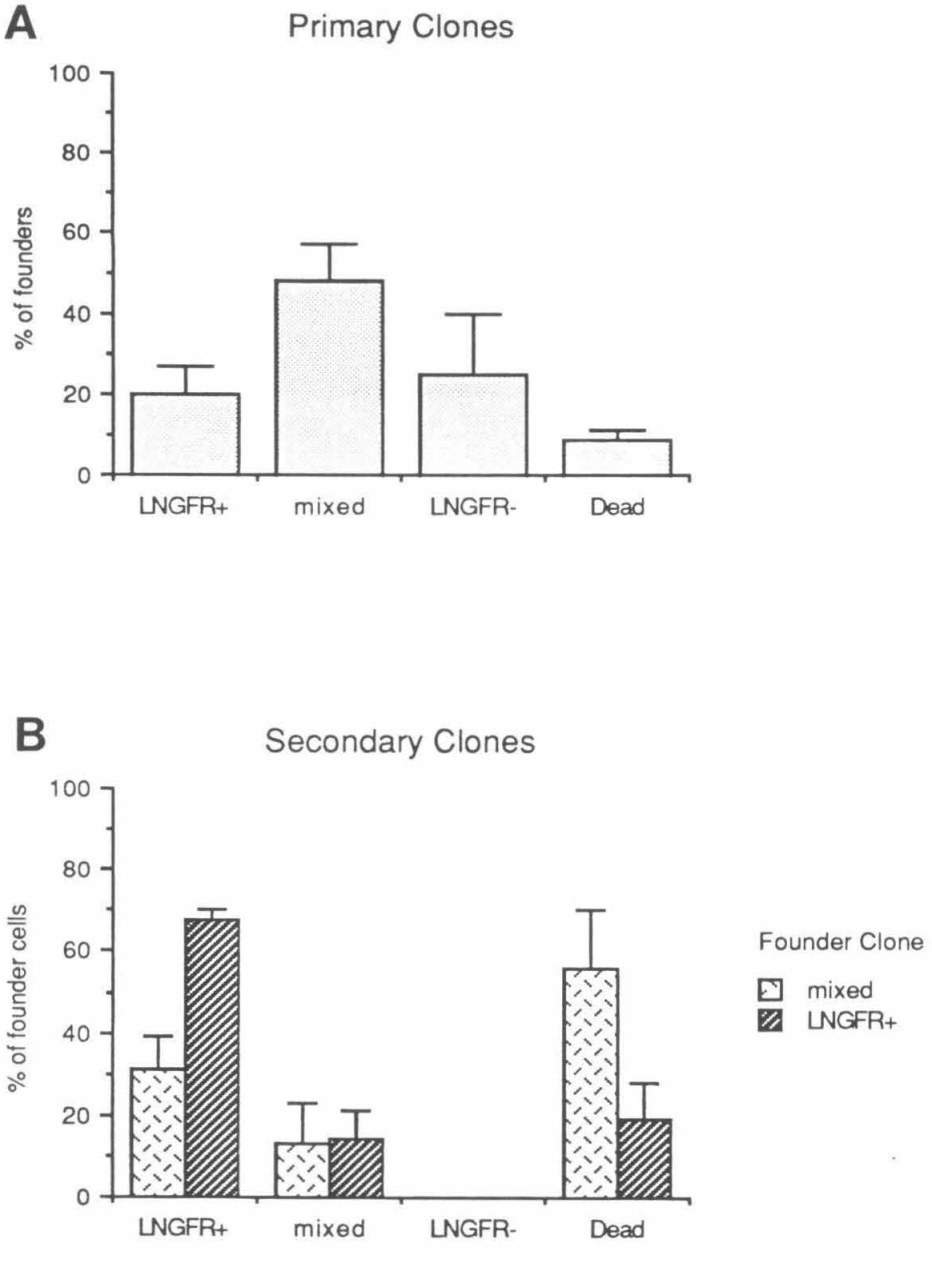
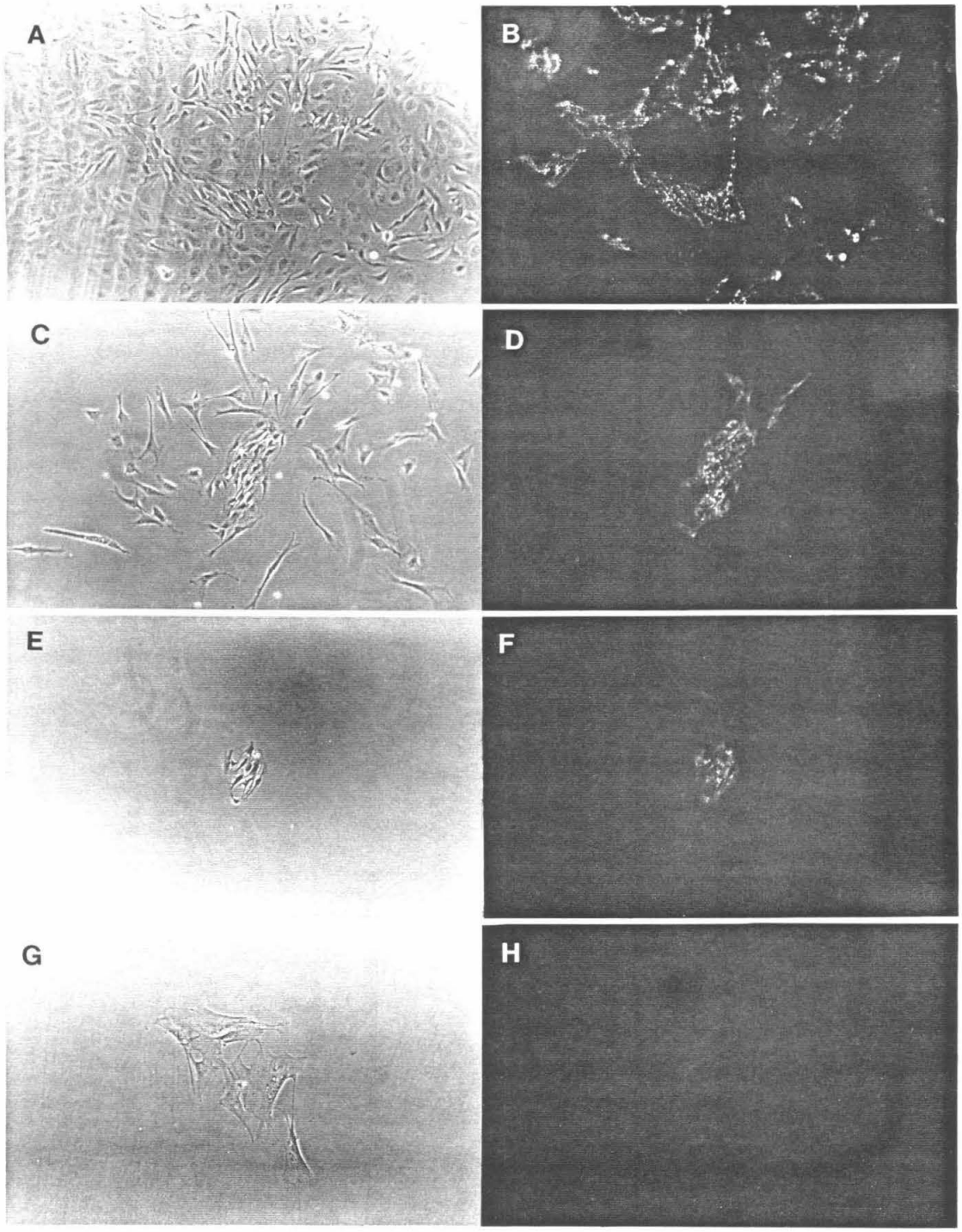

A
Primary Clones
% of founders
100
80
60
40
20
0
LNGFR+
mixed
LNGFR-
Dead
B
Secondary Clones
% of founder cells
100
80
60
40
20
0
LNGFR+
mixed
LNGFR-
Dead
Founder Clone
mixed
LNGFR+

Figure 3. Serial sub-cloning of a "mixed" primary clone.

Micrographs of a primary clone (A, B) and three of its progeny secondary sub-clones are shown. The primary clone produce a "mixed" secondary clone (C,D), an "LNGFR$^+$" (i.e., containing only LNGFR$^+$ cells) secondary clone (E,F) and an "LNGFR$^-$" (i.e., containing only LNGFR$^-$ cells) secondary clone (G,H). LNGFR immunofluorescence is shown in B, D, F and H. The corresponding phase contrast images are shown in A, C, E, and G.


A
B
C
D
E
F
G
H

"LNGFR$^+$" clones. In this experiment "mixed" and "LNGFR$^+$" clones were replated at clonal density on day 7. As for the primary founders, individual LNGFR$^+$ secondary founders were identified and inscribed with a circle for subsequent identification. Secondary clones were allowed to grow for an additional 6 days and then were analyzed for LNGFR expression. As shown in figure 3B both "mixed" and "pure" primary clones produced both "mixed" and "pure" secondary clones. Proportionally more "pure" secondary clones were produced by the "pure" primary clones. Proportionally more "dead" clones resulted from subcloning "mixed" clones than from "pure" clones. LNGFR$^-$ secondary founders from "mixed" primary clones either produced no clone or gave rise only to LNGFR$^-$ cells. Hence some of the phenotypic heterogeneity observed among primary clone may be explained by multipotent founder cells producing offspring apparently committed to the production of a single phenotype.

The fact that some secondary clones of pure LNGFR$^+$ were of the "mixed" phenotype suggest that perhaps the expression of LNGFR is not a stable trait and calls into question whether "mixed" clones give rise to truly "pure" clones. It may be that, given enough time in culture, those "pure" secondary clones would eventually produce LNGFR$^-$ cells. In a preliminary experiment to address this problem we sub-cloned secondary "pure" clones. We found that most of the secondary "pure" clones gave rise only to LNGFR$^+$ cells but that the continued proliferation of those cells required the presence of FBS, forskolin and either glial growth factor (GGF) or basic fibroblast growth factor (bFGF) (not shown). Subsequently those tertiary clones produced mature Schwann cells expressing glial fibrillary acidic protein (GFAP) and the sulfatide antigen recognized by the antibody O4 (not shown).

Primary neural crest cells are sensitive to FBS

Although the LNGFR$^+$ cells taken at different points in the culture period are morphological and immunocytochemically indistinguishable, they may represent several cell types distinguishable by other functional properties. For example, both the primary neural crest cell and mature Schwann or satellite cells express LNGFR and nestin and have a similar morphology, but we found that the primary neural crest cells eventually lose LNGFR expression in the presence of FBS. Schwann cells grow well in FBS and may actually require some FBS components to divide in culture. In addition the observation that "pure" primary clones can produce secondary "mixed" clones coupled with the observation from the colony analysis that the probability of a founder to produce a "pure" clone increases over time in culture, suggests that there may exist more that one LNGFR$^+$ cell type in neural crest cultures.

In our initial attempts to grow LNGFR$^+$ neural crest cells we found that while neural crest cells could grow to produce large clones in the presence of FBS they soon lost expression of LNGFR. Moreover, we found that LNGFR$^-$ clones failed to produce either Schwann cells or neurons. One possibility to explain the loss of LNGR$^+$ cells is that the FBS is somehow toxic to LNGFR$^+$ cells and simply eliminates them. Another possibility is that the FBS leads to the conversion of LNGFR$^+$ crest cells to LNGFR$^-$ cells. To test whether LNGFR$^+$ cells are lost by selection or induction we performed a clonal analysis of LNGFR$^+$ founder cells initially plated under identical conditions and subsequently grown in the presence or absence of FBS. In this experiment individual LNGFR$^+$ neural crest cells in clonal culture were identified and marked as described above. The founders were plated onto several 100 mm dishes. A few hours after plating individual LNGFR$^+$ cells were identified and FBS was added to some of the dishes. Clones were allowed to grow for 7 days and *then* were analyzed for LNGFR expression. Examples of the resulting clones are shown in the micrographs of figure 4 (A-B). The results are graphically presented in

Figure 4. Primary LNGFR+ neural crest cells are sensitive to FBS.

LNGFR$^+$ neural crest cells taken from 1 day neural tube explants (A-D) or from 8 day secondary mass cultures were grown as identified clones either in standard medium (A, B, E, F) or in standard medium supplemented with 10% FBS (C, D, G, H). Founder cells in all conditions were plated under identical conditions, identified as single LNGFR+ cells then switched to the experimental conditions. Shown are clones stained with monoclonal anti-LNGFR and a fluorescent secondary antibody (B, D, F, H) and the corresponding phase-contrast images (A, C, E, G).

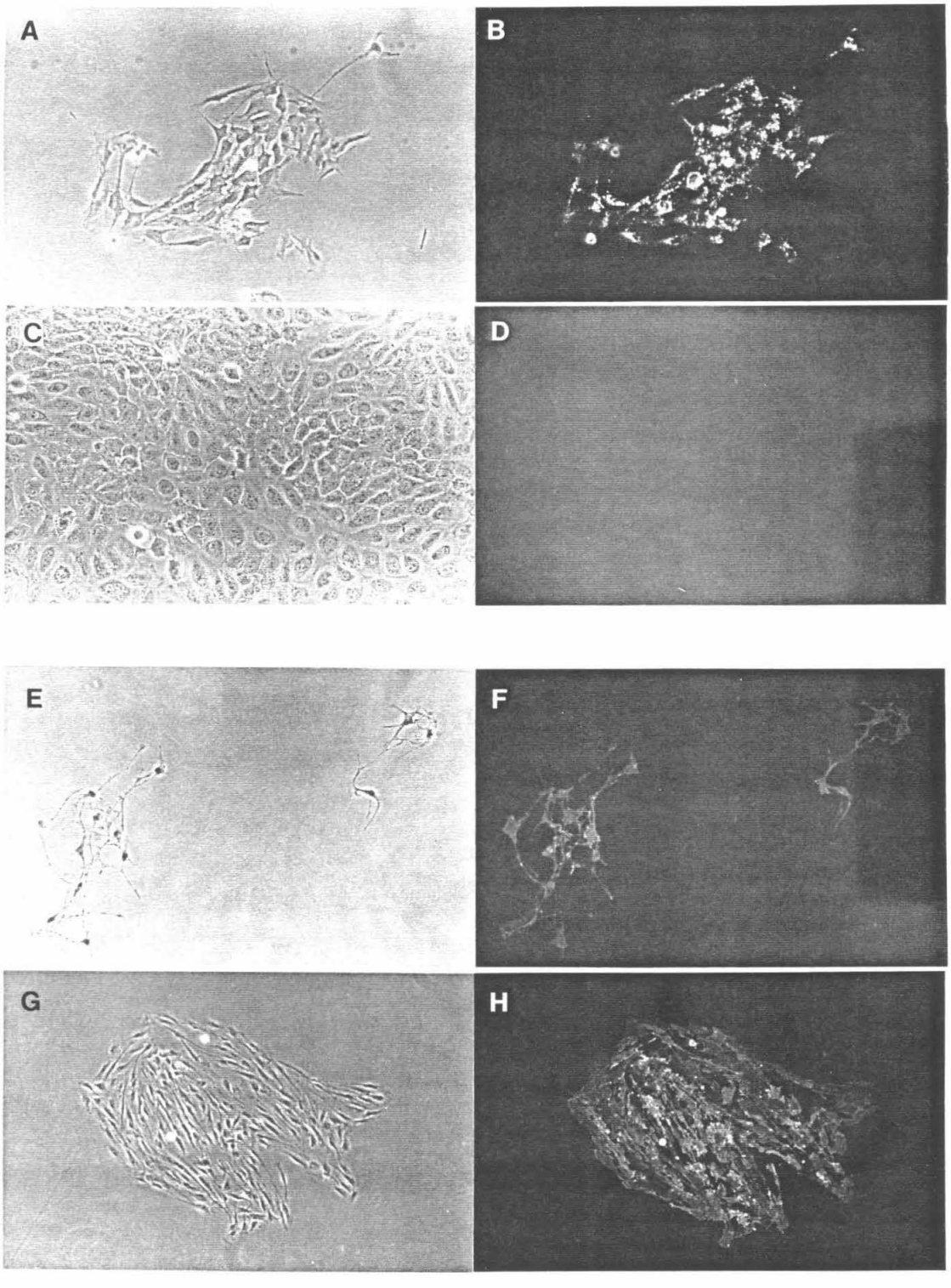
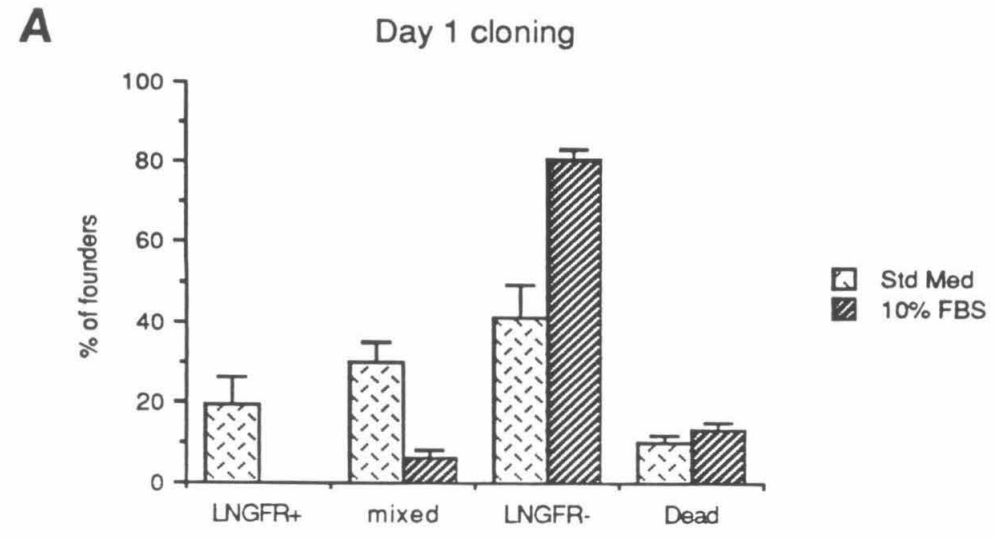

A
B
C
D
E
F
G
H

Figure 5. Quantification of serum sensitivity experiment.

A. Primary LNGFR$^+$ were grown for 7 days in either standard medium (hatched bars) or in standard medium supplemented with 10%FBS. The clones were then analyzed for LNGFR expression as previously described (Figure 2).

B. LNGFR$^+$ cells from secondary mass cultures of neural crest cells were grown as described above.


A
Day 1 cloning
100
80
60
40
20
0
% of founders
LNGFR+
mixed
LNGFR-
Dead
Std Med
10% FBS

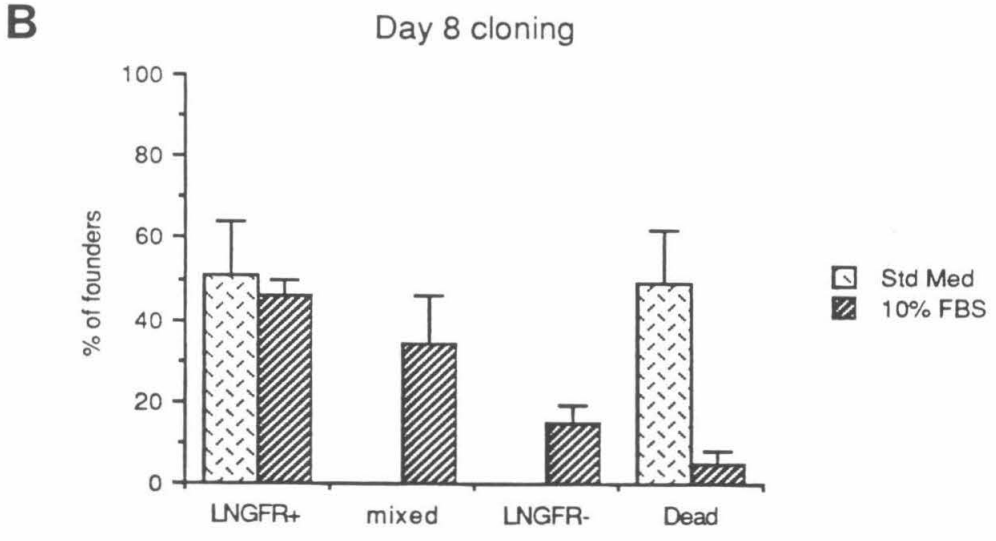

B
Day 8 cloning
100
80
60
40
20
0
% of founders
LNGFR+
mixed
LNGFR-
Dead
Std Med
10% FBS

figure 5A. We found that under both conditions (with and without 10% FBS) the cloning efficiency was high (~90%) but that in the presence of FBS, 80% of LNGFR+ founders produced clones in which no cell expressed LNGFR. By contrast, ~50% of the founders grown without added FBS produced clones containing LNGFR expressing cells (i.e., both "LNGFR$^+$" and "mixed" clones combined; see figure 5A "LNGFR$^+$" and "mixed" columns). Thus, primary LNGFR$^+$ neural crest cells are sensitive to the presence of FBS.

It has been well established that primary Schwann can grow in the presence of FBS and continue to express LNGFR under those conditions. It is possible that some founders producing "pure" clones when plated at day 8 (see the colony analysis described above) are actually committed Schwann cell blasts. If that is the case then we would not expect them to be sensitive to the presence of FBS. To test this notion we cloned neural crest cells late in culture and tested them for sensitivity to FBS. Primary neural crest cells were taken from 1 day old explants and cultured at a moderate density of ~ 5000 cells / 60 mm dish. After 7 days the cells were replated at clonal density onto 100 mm dishes. A few hours after plating individual LNGFR+ cells were identified and inscribed as described above. To some dishes was added 10% FBS and all dishes were allowed to grow for an additional 7 days. The dishes were then analyzed for LNGFR expression. Examples of the resulting clones are shown in the micrographs of figure 4 (C-D). There are two majors results of this experiment. First, in contrast to the behavior of LNGFR founders at day 1, more than 70% (Figure 5B, LNGFR+ and Mixed categories combined) of them produce clones containing LNGFR+ cells in the presence of FBS. Secondly, the FBS appears to have a trophic activity for the LNGFR+ founders, producing a cloning efficiency of ~90% compared to ~ 50% in the absence of FBS.

Discussion

We find that primary LNGFR$^+$ neural crest cells tend to produce colonies comprised of both LNGFR$^+$ and LNGFR$^-$ cells ("mixed" colonies). By contrast primary LNGFR$^-$ neural crest cells produce only "LNGFR$^-$" clones. If, however, neural crest cells are allowed to grow in mass culture for ~1 week before being plated at clonal densities, then the proportion of colonies comprised only of LNGFR$^+$ cells ("LNGFR$^+$" colonies) increased substantially. In a serial sub-cloning analysis we show that single "mixed" clones can produce "mixed" and "LNGFR$^+$" clones, as well as "LNGFR$^-$" clones. Finally, we show that even though LNGFR$^+$ founders of "mixed" clones, and LNGFR$^+$ founders of pure "LNGFR$^+$" clones are antigenically and morphologically indistinguishable, differences between them can be revealed by their differential sensitivity to FBS. "Mixed" founders in the presence of FBS will, propagate and differentiate to form an LNGFR$^-$ clone. By contrast, "LNGFR$^+$" founders, which probably represent Schwann cell blast cells, maintain LNGFR expression in FBS and propagate to form Schwann cells. FBS appears also to act as a trophic agent for these cells. Under Schwann cell differentiation conditions (described in Chapter 2) these cells will express the combination of O4 immunoreactivity and glial fibrillary acidic protein (GFAP) (not shown), i.e., they will differentiate into Schwann cells (Mirsky et al., 1990; Morgan et al., 1991).

The sub-cloning experiments described here (like the sub-cloning experiments described in chapter 2) allow for the possibility that the observed heterogeneity of primary clones may result from the production of relatively restricted or committed cells from multipotent progenitors. An apparent inconsistency arises when one considers the sub-cloning of clones comprised purely of LNGFR$^+$ cells. These clones contained cells that, upon sub-cloning, produced "mixed" secondary clones. A strong possibility is that the primary LNGFR$^+$ founders, which form "mixed" and "LNGFR$^+$" clones,

comprise a cell type distinct from LNGFR$^+$ founders taken from day 8 cultures or primary clones.

The LNGFR$^-$ cells that form under these conditions are most likely the same as the "O" cells described in chapter 2. These cells display a flattened fibroblastic morphology and fail to express neuronal and glial differentiation markers. Currently we cannot relate this cultured cell to any normal neural crest derivative. There are, however, several possibilities. We have observed under Schwann cell differentiation condition that some "O" cell will fuse to form multinucleate cells that twitch (not shown). These are probably striate muscle cells. They form from subclones of LNGFR$^+$ founders that also produce glia, and thus do not represent somitic cell contaminants. The cephalic neural crest is known to produce mesectodermal cell types but the trunk neural crest is thought to have only a limited potential for mesectodermal differentiation. This very preliminary observation has been made on several occasions. No molecular markers of muscle differentiation were used but they may be used in the future and may reveal myogenic potential at early times, i.e., before fusion and twitching. Another possibility is that the "O" cell represent melanocytes or melanoblasts. Since the rats used in these experiments are albino, melanocyte formation is difficult to determine. It may be possible to use the monoclonal antibody TA99 to mark melanogenic cells in these cultures (Vijayasaradhi and Houghton, 1991). The cells may also represent fibroblasts, osteoblasts or chondrocytes. Specific marker for these cell types exist and should be applied to the "O" cells. If the "O" cells do represent a normal neural crest derivative then the fact that FBS is so effective in promoting its formation may provide some clue to the environmental control over the formation of those cells.

Finally the findings presented in this appendix provide a useful assay for measuring the difference between two otherwise indistinguishable cell types, the neural crest stem cell and what may be a glial or glial-and-other blast cell. Current

investigations are underway to measure the transition from a serum-sensitive to a serum-dependent state, relative to the loss of neurogenic activity, as assayed by growth on a poly-D-lysine / fibronectin substrate.

Experimental procedures

Procedures used for this work are described in Chapter 2.

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APPENDIX 2: Adrenergic differentiation and MASH-1 expression in cultures of rat neural crest cells

with Alice Paquette

Introduction

Lineage specification in the development of neural crest derivatives appears to be controlled by environmental factors present in anlage of specific derivative structures (Le Douarin, 1982). It is thought that the set of environments encountered by migrating neural crest cells are responsible for the specification of differentiated traits. This view derives in part from the observation that the founder cells of each class of neural crest derivative structure follow a distinct pathway of migration encountering a unique set of embryonic environments on the way to their ultimate destination. Sympathoadrenal precursors, for example, emerge from the neural crest, pass through the anterior part of the sclerotome, migrate past the ventral neural tube and notochord and ultimately arrive at the dorsal aorta where they undergo adrenergic and neuronal differentiation. The production of sympathoadrenal progenitors appears to be directed in part by the peri-aortic environment into which relatively undifferentiated neural crest cells migrate. In a study of Stern et al. the necessity of notochord or ventral neural tube for the formation of adrenergic cells in the peri-aortic space is established by surgical removal of the notochord or inversion of the neural tube (Stern et al., 1991). *In vitro* studies of the primary neural crest suggest that interaction with the somitic environment may be necessary for sympathoadrenal specification (Fauquet et al., 1981). There are several published cell culture studies in which specific environmental factors enhance adrenergic differentiation of primary neural crest cells. Maxwell et al. found that an extract of EHS sarcoma significantly enhanced the production of adrenergic cells in secondary cultures of quail neural crest cells, apparently at the expense of melanocytes (Maxwell and Forbes, 1990). These authors also found that adrenergic differentiation was enhanced by culture at high cell density. Howard and Bronner-Fraser found that both a low-molecular weight component of chick embryo extract (CEE) and a soluble component of neural tube conditioned medium lead to the production of adrenergic cells in primary neural crest explants (Howard and Bronner-Fraser, 1985). Sieber-Blum

and Cohen found that CEE led to the generation of adrenergic cells in clonal cultures of quail neural crest cells (Sieber-Blum and Cohen, 1980). These authors also found that when the clones were grown on an extracellular matrix material taken from chick embryonic fibroblasts, both the number of adrenergic clones and the number of adrenergic cells per clone were increased. Others have described activities that enhance the production of adrenergic cells from developing neural crest derivatives, such as the DRG. Xue et al. found that insulin and IGF-1 enhance adrenergic differentiation in DRG cultures, but apparently do not have the same effect on primary neural crest cells (Xue et al., 1988). Finally, Fauquet et al. infected primary neural crest cells with a v-myc oncogene containing virus (Fauquet et al., 1990). The expression of v-myc led to enhanced proliferation and adrenergic differentiation in a medium that is not otherwise permissive for adrenergic differentiation. Thus both external and internal factors can result in increased adrenergic differentiation among neural crest cells. The myc proto-oncogene may normally participate in the expression of adrenergic traits, but it is expressed in a wide variety of cell types and may not provide specific information about adrenergic determination.

A gene whose expression is fairly specific and may reflect determination of neural crest cells along an adrenergic pathway is the mammalian achete-scute homolog-1 gene (MASH-1). MASH-1 was identified by its homology with known neural determination genes in Drosophila melanogaster (Johnson et al., 1990). A member of the basic-helix-loop-helix family of transcription factors, MASH-1 may serve an important role in patterning of the nervous system by participation in neuronal cell type specification. MASH-1 was originally isolated from sympathoadrenal cells lines MAH and PC12. MASH-1 is expressed in the sympathetic ganglia at least a day before significant adrenergic differentiation takes place. MASH-1 is not expressed at any time in DRGs (Lo et al., 1991). MASH-1 may be an important transcription factor in the specification of adrenergic cells in the peripheral nervous system.

In order to define some of the factors necessary for the formation of adrenergic cells from neural crest cells, we developed a culture system for rat neural crest in which adrenergic differentiation could take place. In addition, we have studied the expression of MASH-1 in these cultures, both to relate its expression to the acquisition of adrenergic traits and to study environmental factors that may control MASH-1 expression independent of adrenergic differentiation.

Results

Culture conditions that promote expression of TH also promote MASH-1 expression.

To study the formation of sympathoadrenal progenitors from primary neural crest we first needed to establish conditions that would promote the survival, growth and adrenergic differentiation of early neural crest cells. Culture conditions that satisfy these requirements have been described for avian neural crest. Similarly, conditions for the culture of mouse neural crest have also been described (Ito and Takeuchi, 1984; Ito et al., 1988). A screen of a variety of culture conditions revealed the following. In a relatively simple formulation of L-15CO_2, 10% fetal bovine serum (FBS), 10% chick embryo extract (CEE) neural crest cells migrated from the neural tube, proliferated extensively and within 5 days gave rise to some tyrosine hydroxylase (TH) expressing cells (not shown, however, Figure 1A shows an example of the same cell type at a later time in culture, day 10). If either FBS or CEE was not included, neural crest cells migrated from neural tubes, but they did not proliferate extensively, nor did they form TH^+ cells. In addition, cultures underwent extensive death by 5 days. The TH^+ cells seen in the day 5 cultures were large diameter cells with a fibroblastic appearance and were probably not sympathoadrenal progenitors (Birren and Anderson, 1990). In a test of other conditions, at later times in culture, we found that two other TH^+ cell types would form.

Primary neural crest cells were grown at high cell density in the medium described above for 5 days. The medium was then exchanged either with medium supplemented with basic fibroblast growth factor (FGF) and dexamethasone (DEX) or with medium lacking CEE and supplemented with FGF and DEX. Three distinct TH^+ cell types emerged after 10 days in culture. The fibroblastic TH^+ cell type (the same cell type as was seen in day 5 cultures) was found in the regions of the dish with the lowest cell density (Figure 1A, B). In the high density regions two other TH^+ cell

types were found in clusters. Some clusters were comprised of process bearing cells with a relatively low-level of TH (Figure 1C, D). The other cluster type contained cells with short processes expressing high-levels of TH (Figure 1E, F). This cell type resembled the sympathoadrenal progenitor both in its morphology and level of TH expression. If the medium was exchanged at day 5 for medium lacking CEE but supplemented with bFGF and dexamethasone, the fibroblastic TH^+ cell was not observed but the other two TH^+ cell types were generated. TH was never expressed in a high percentage of the cells. TH^+ cells probably represented ~0.1% of the total cell population at day 10 of culture. A few general qualitative observations from these experiments are: 1) Expression of TH in the fibroblastic cell appears to require low cell density and the presence of both CEE and FBS. 2) Expression of TH in both process-bearing cells and sympathoadrenal-like cells appears to require high cell density, FGF, DEX, and FBS, but does not require CEE in the last 5 days of culture. 3) If at day 5 the CEE was removed, by day 10 very few TH^+ cells remained. Those that did remain, however, were found only in the most dense regions of the culture and were all of the low-TH^+ process bearing type.

To establish a relationship between MASH-1 expression and TH expression, cultured neural crest cells were double-labeled with antibodies at various times in TH promoting conditions. After 5 days in culture (in L-15 CO_2, 10%FBS, 10%CEE, FGF and DEX), cells were found to express MASH-1 but very few TH^+ cells were observed (not shown). By day 10 of culture both TH^+ and MASH-1^+ cells were found in the cultures (Figure 2A,B). Some of the TH^+ cells also expressed MASH-1 (Figure 2C). Moreover, TH^+ cells were found clustered with MASH-1^+ cells (Figure 3). Cultures grown without added FGF and DEX appeared to produce fewer MASH-1^+ cells both at day 5 and at day 10. Adrenergic differentiation was very limited under those conditions. These results are summarized in table I.

Figure 1. TH expression in day 10 cultures.

Cultures of neural crest cells were grown for 5 days in L-15CO_2, 10%CEE, 10%FBS then were supplemented with FGF (50ng/ml) and DEX (5μM) and grown for an additional 5 days. Three distinct TH^+ cell types can be found. (A, B) flat, fibroblastic TH^+ cells appear at the margins of the culture. (C, D) TH^+ process bearing cells can be found in dense regions. (E, F) high-level TH expressing cells, apparently sympathoadrenal (SA) progenitors can also be found in dense regions of the culture.

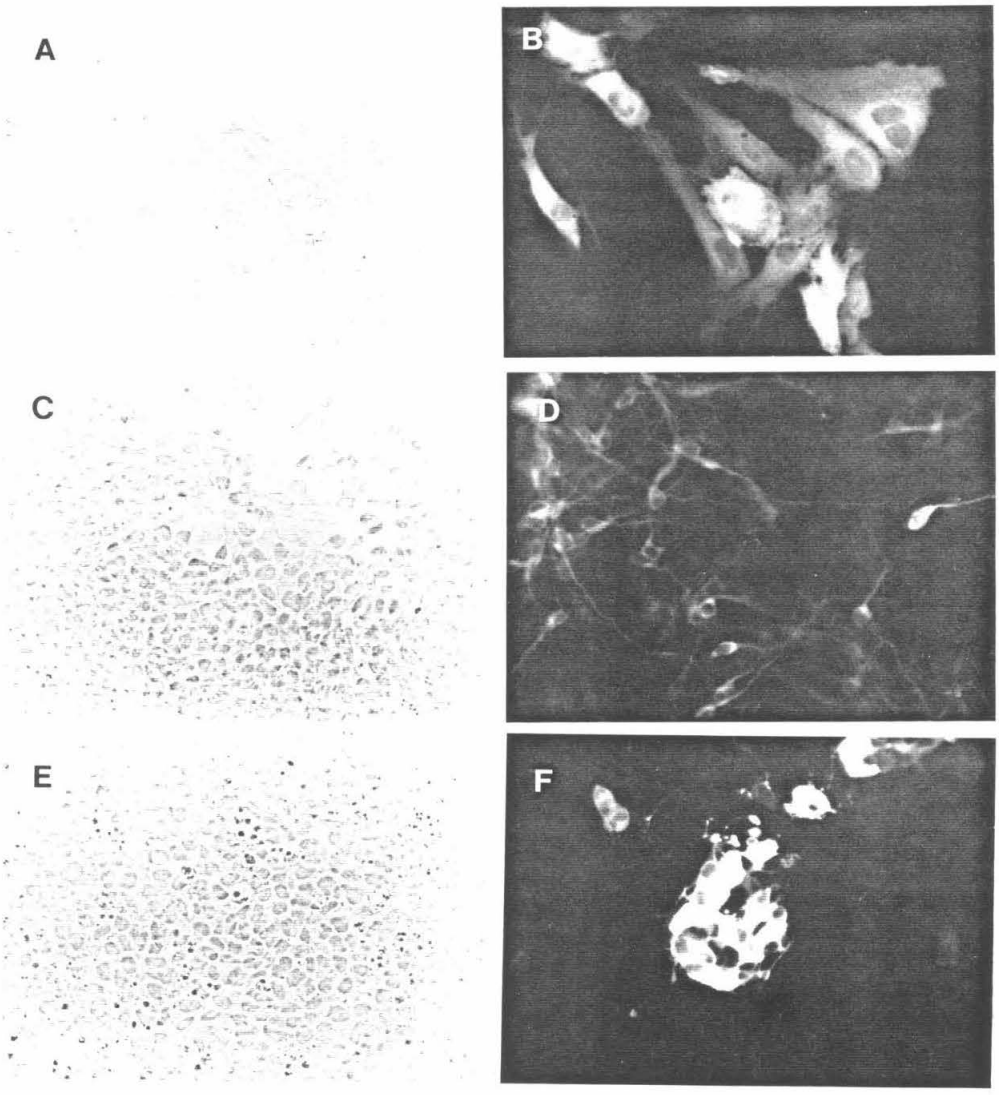
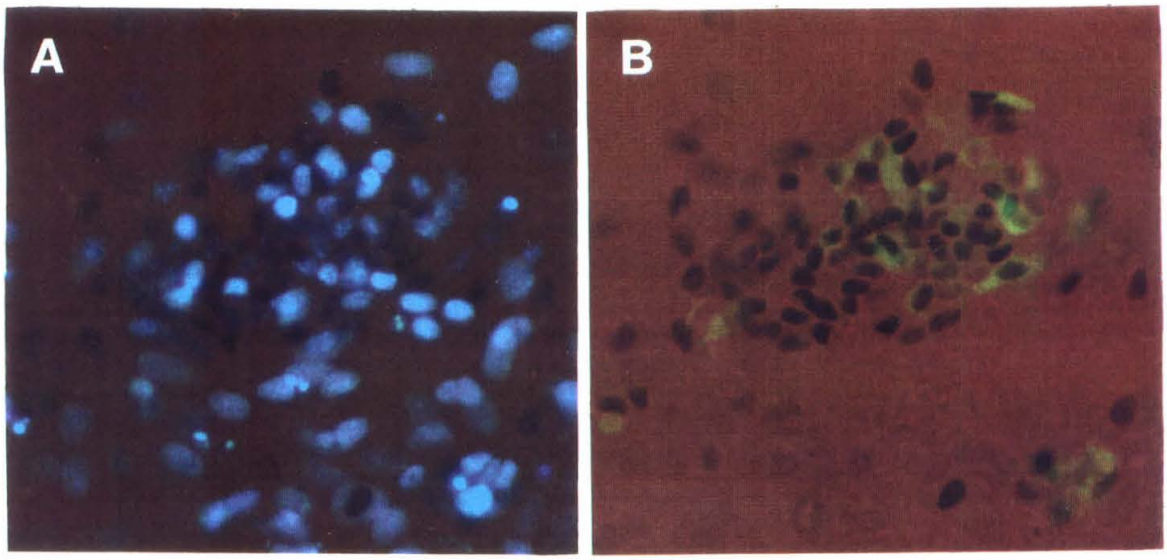

A
B
C
D
E
F

Figure 2. TH and MASH-1 expression in day 10 cultures.

Cultures were grown as described for figure 1, were stained for both TH and MASH-1 expression, and then were counterstained with the DNA dye DAPI to reveal cell nuclei. Panel A shows cell number by nuclear DAPI fluorescence (blue). Panel B, TH (green) and MASH-1 (black) are revealed by immunocytochemistry for the same field shown in panel A. Panel C, another field shows the coincidence of MASH-1 and TH in individual cells.


A
B

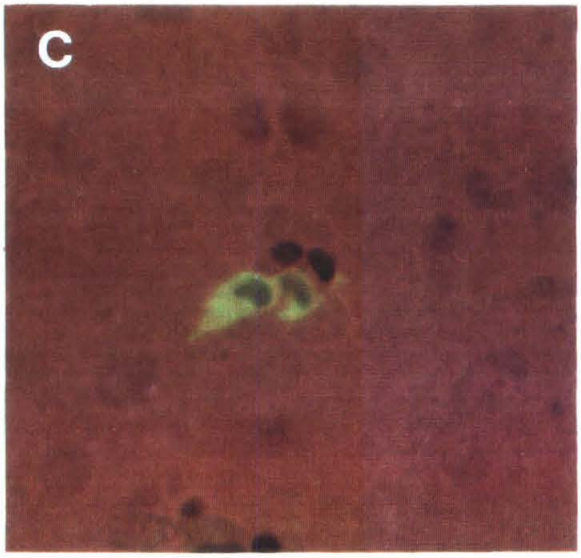

C

Figure 3. TH and MASH-1 expression in day 10 cultures.

Lower magnification images taken from cultures like those described in figure 2 reveal that TH expression and MASH-1 expression is clustered within dense regions of the culture. Panel A shows DAPI nuclear fluorescence. Panel B shows TH (green) and MASH-1 (black) immunoreactivity.

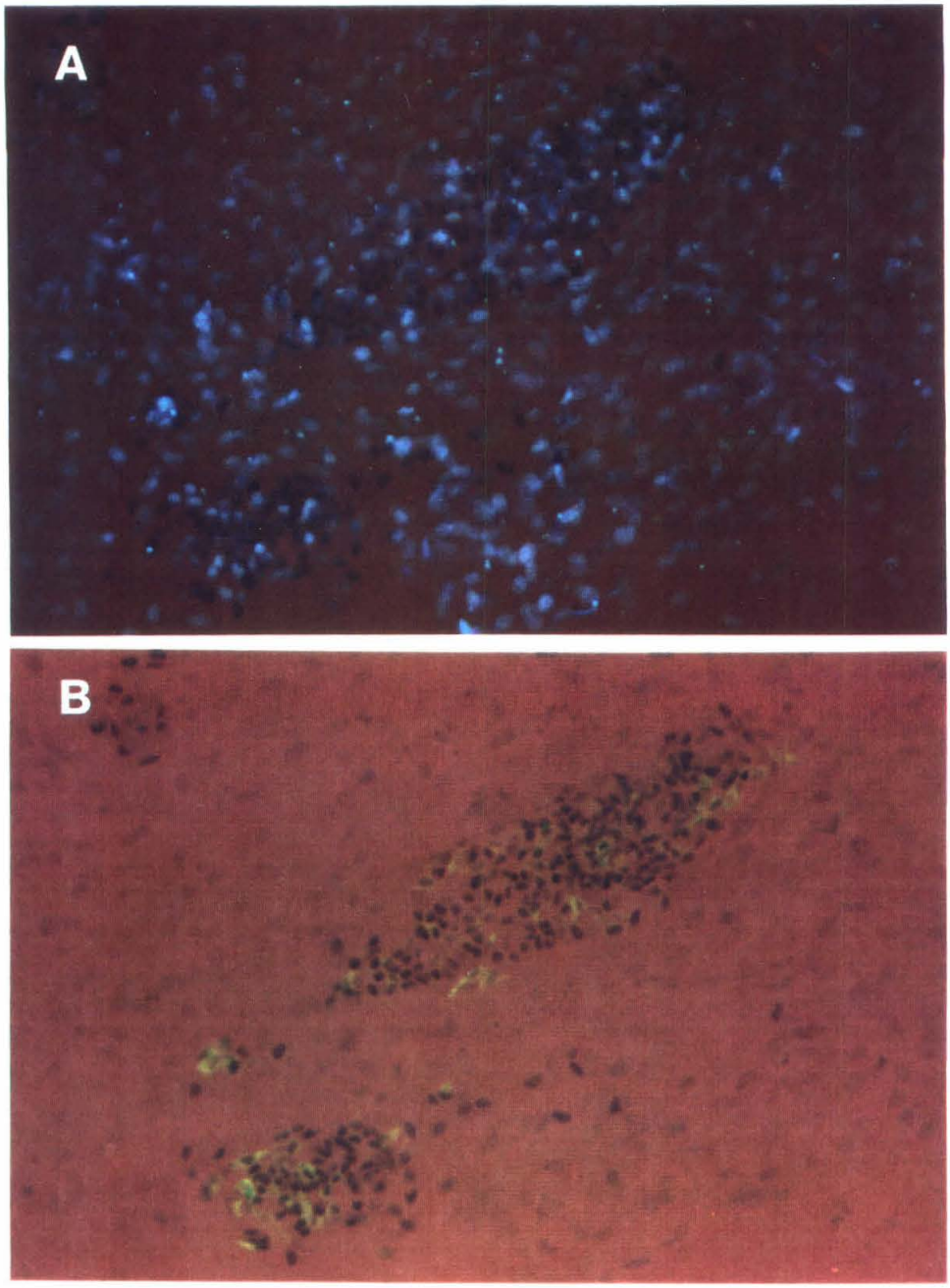

A
B

Table I. TH and MASH-1 expression.

The expression of TH and MASH-1 was tested in day 5 and day 10 cultures of primary neural crest. Cultures were grown in either standard medium, consisting of L-15CO_2, 10% FBS, 10%CEE (Std. Med.) or standard medium supplemented with 50 ng/ml bFGF and 5μM dexamethasone (+F+D). Culture were qualitatively scored on the basis of apparent proportions of stained cells to the total number of cells. Three types of TH^+ cell were observed, a flattened fibroblastic cell (Flat), process bearing low-TH^+ cells (PB), and sympathoadrenal-like cells (SA).

Table I. TH and MASH-1 expression

		Day 5		Day 10	
		Std. Med.	+F+D	Std. Med.	+F+D
MASH-1		+	++	++	+++
	Flat	+	+	++	++
TH	**PB**	-	-	+	++
	SA	-	-	-	+++

MASH-1 expression precedes the expression of adrenergic traits in this culture system. In some cases MASH-1 and TH are expressed in the same cells in day 10 cultures. Furthermore, their coincident expression is also observed as clusters of stained cells separated by unstained cells in high density cultures. Finally, conditions that favor the production of TH^+ cells also enhance the expression of MASH-1. *In vivo,* MASH-1 is expressed in developing sympathetic ganglia by E11.5 and TH first appear at ~ E12 (Lo, et al., 1991). The neural crest that is used in these experiments is taken from E10.5 animals. Thus the expression of the two markers in this culture system is extremely delayed relative to the normal *in vivo* timing. Given the fact that expression of MASH-1 precedes expression of TH in this system, added to the fact that TH and MASH are sometimes co-expressed we sought to ask whether conditions that promote early expression of MASH-1 (e.g., by day 2 in culture) also promote TH expression at an earlier time.

MASH-1 expression in primary neural crest cells can be controlled by several environmental factors.

Several conditions were tested for their ability to enhance MASH-1 expression in day 2 cultures and to produce adrenergic cells at later times. Initially, we found that both CEE and FBS were necessary for survival and growth of neural crest cells. We therefore developed a chemically defined medium (DM) (this chemically defined medium is described in chapter 2) in which neural crest cells could migrate from neural tubes, propagate and survive for a period at least corresponding to the time of maximal adrenergic expression, i.e., at least 10 days. This medium could then be used as a basal medium, to which other factors, e.g. FBS and CEE, could be added to test their effect on the expression of TH and MASH-1. The factors that were tested were FBS, CEE, DEX, FGF, nerve growth factor (NGF), and retinoic acid (RA).

The first experiment was designed to examine the effects of the different culture conditions on the expression of MASH-1 after 2 days of culture. That is we tested the factors for their ability to promote the early expression of MASH-1. In this experiment neural tubes were plated initially in 4 different culture media (Table II). After 24 hours the neural tubes were scraped away from the neural crest cells, and the crest cells were removed from the dish by trypsin treatment and replated into 2 dishes for each condition. After an additional culture period of 24 hours the cells were fixed and stained for MASH-1 and TH. In none of the conditions was TH expressed at that time in culture. In DM alone the neural tubes attached and appeared healthy after 24 hours, but no neural crest cell migrated from the neural tube. In the other three conditions there was robust neural crest cell migration, as measured by the number of cells recovered from an equivalent total length of neural tube (Table II). The MASH-1 expression results were quantified and are presently in Table II. The proportion of MASH-1$^+$ cells was 4-5 times greater in DM with FGF and NGF than in any other condition. It appears that CEE inhibits high level MASH-1 expression in these cells.

It was shown previously that retinoic acid can induce MASH-1 expression in P19 embryonic carcinoma cells (Johnson et al., 1992). Retinoic acid is a component of the DM, so we tested the expression of MASH-1 as a function of RA concentration in secondary cultures of neural crest cells. Neural tubes were initially plated in DM without RA for 24 hours. The crest cells were then replated and grown for 2 days more under a series of RA concentrations. The expression of MASH-1 was the quantified. In no RA, 1.6 ± 0.49 % of the crest cells expressed MASH-1. RA concentrations ranging from 10^{-8} M to 10^{-6} M were tested and all gave about the same level of MASH-1 expression: at 10^{-8} M RA, 19 ± 2.7 % of the neural crest cell expressed MASH-1 (Figure 4A). Micrographs of neural crest cells grown in no RA (Figure 4D,E) and in 10^{-8} M RA (Figure 4B,C) stained for MASH-1 immunoreactivity and counterstained with DAPI are given as examples. The staining in MASH-1$^+$ cells

Table II. Conditions of short term neural crest culture.

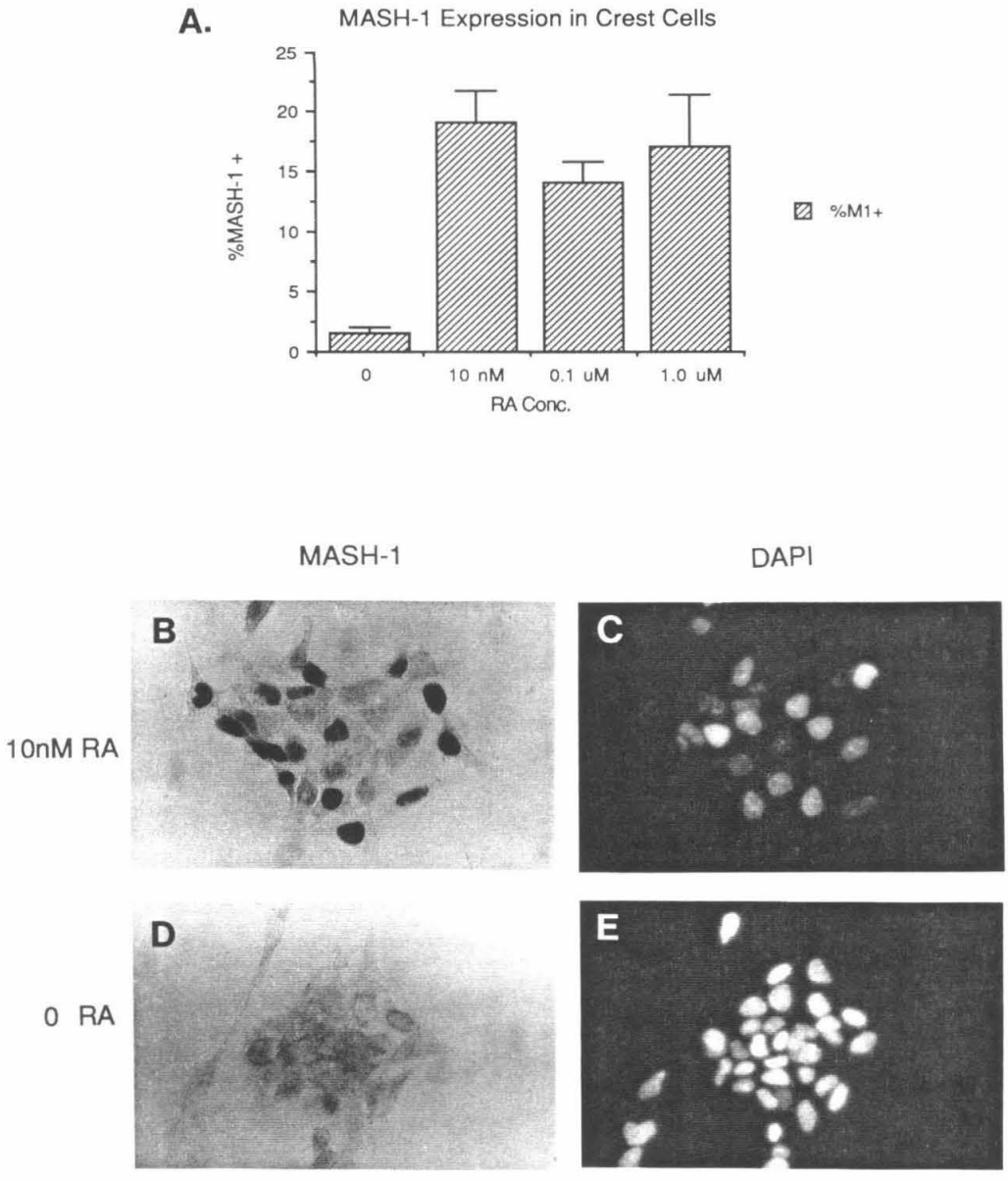
The expression of TH and MASH-1 was tested during short term (48 hour) growth in various compositions of culture medium. Neural tubes were plated in 4 different media, allowed to grow for 24 hours. Neural tubes were scraped away, neural crest cells were harvested, counted (column 2) and replated according to columns 3. "DM" is defined medium lacking FGF and NGF (Chapter 2). "N" is NGF at a concentration of 1 μg/ml. "F" is bFGF at a concentration of 50 ng/ml. "CEE" is 10% chick embryo extract. MASH-1 expression was measured as a proportion of MASH-1$^+$ cells to the total number of cells (Column 4). Errors indicated are the standard error of the mean (s.e.m.) (n=2, except for condition 4, where n=3).

Table II. Conditions of short term neural crest cell growth

	Condition	Cells/µl after 24 hours	µl /plate 2°plating	MASH-1 exprn at day 2 (% total ± s.e.m.)
1.	DM	2	50	4.2 ± 4.2
2.	DM+N+F	156	20	24.1 ± 0.1
3.	DM+CEE	146	50	4.1 ± 0.4
4.	DM+N+F+CEE	158	50	6.2 ± 0.6

Figure 4. Retinoic acid dependence of MASH-1 expression.

Neural tubes were plated in defined medium containing FGF and NGF (DM+N+F) but lacking retinoic acid (RA). After 24 hours the neural tubes were scraped away, neural crest cells were harvested and replated into various concentrations of RA containing DM+N+F. After an additional 48 hours of growth, cultures were fixed, stained for MASH-1 and counterstained with DAPI. Three dishes from each of the conditions were counted. DAPI stained cells were counted for total cell number and MASH-1$^+$ cells were scored by presence of nuclear staining. In panel A, the percent MASH-1$^+$ $\pm$ s.e.m. is graphed for each concentration of RA. In panels B-E example fields from cultures of two different RA concentrations are shown. Panel B shows MASH-1 immunoreactivity in 10nM RA; the corresponding DAPI staining is shown in panel C. Note that cells expressing MASH-1 do not show DAPI fluorescence. Panel D shows MASH-1 immunoreactivity in 0 RA; the corresponding DAPI staining is shown in panel E.


A.
MASH-1 Expression in Crest Cells
%MASH-1 +
25
20
15
10
5
0
0
10 nM
0.1 uM
1.0 uM
RA Conc.
%M1+
MASH-1
DAPI
B
C
10nM RA
D
E
0 RA

was found to obscured the DAPI fluorescence. Total cell number did not significantly vary between cultures grown with different RA concentrations, nor did overt appearance of the cells. TH was not expressed in any cell under these conditions.

Culture conditions were then tested for their effect on TH expression at day 5 and day 10. The conditions used in this experiment are outlined in Table III. Neural tubes were plated in the one medium formulation for the first day (Table III, Column 1), then neural crest cells were replated in a second medium formulation (Table III, Column 2) and allowed to grow for 4 days. At this point, one group of cultures (day 5 cultures) was fixed and stained for MASH-1 expression. The medium in the remaining group (day 10 cultures) of cultures was exchanged (Table III, Column 3). These cultures were allowed to grow for an additional 5 days, then were fixed and stained for MASH-1 and TH expression. MASH-1 was expressed in all day 5 cultures. CEE appeared to decrease the proportion of MASH-1^+ cells. CEE also appeared to inhibit neuron formation, as judged morphologically. In day 10 cultures we found that all conditions produced a large number of MASH-1^+ cells. We found that brightly-TH^+, SA-like cells, were present in large numbers in condition 1, and in all conditions containing CEE for the first 5 days of culture. Under condition 1, where cells were grown with the chemically defined medium supplemented with FBS, SA-like cells were present in high numbers at day 10. When either DEX or FGF or both were added to the medium (Table III, conditions 2, 3 and 4) the number of SA-like cells was dramatically reduced. This result appears to be contrary to results (see Table I) obtained with CEE present either during the first 5 days or throughout the culture period in which FGF and DEX enhance adrenergic differentiation.

Table III. Culture conditions for long term growth of neural crest cells

Neural crest cell secondary cultures were established from neural tube explants grown in the medium described in column 2. During the second 4 days, cultures were grown in the medium described in column 3. The medium was then switched and the cultures were grown for the last 5 day in the medium described in column 5. The medium components are the same as in Table I with the addition: "L-15" is L-15 CO_2 (see Chapter 2) and "FBS"; 10% fetal bovine serum; and "D" is 5 μM dexamethasone. The expression of MASH-1 at day 5 and day 10 of culture, and TH at day 10 of culture was scored qualitatively as the apparent proportion of stained to the total number of cells.

Table III. Culture conditions for growth of neural crest cells

Condition	First Day of Culture	Second 4 days	Final 5 days	MASH-1		TH
				day 5	day 10	day 10
1.	DM+N+F	+FBS	+FBS	++	+++	+++
2.	"	+FBS+F	+FBS+F	++	+++	+
3.	"	+FBS+D	+FBS+D	++	+++	+
4.	"	+FBS+F+D	+FBS+F+D	++	+++	+
5.	"	L15+FBS+CEE+F+D	L15+FBS+F	+	+++	+++
6.	DM+N+F+CEE	L15+FBS+CEE+F+D	L15+FBS+F	+	+++	+++
7.	L15+FBS+CEE	+F+D	L15+FBS+F	++	+++	+++

MASH-1 is expressed by neural crest cell clones under environmental conditions that favor neuronal differentiation.

As reported elsewhere (see chapter 2), the fate of primary neural crest cell clones can be directed by the substrate on which they are grown. Specifically, primary neural crest cell clones grown on poly-D-lysine (pDL) and fibronectin (FN) generate peripheral neurons, glia and other cells. When clones are grown on only FN, however, they do not generate neurons. The expression of MASH-1 was tested under these two conditions. Clones were allowed to grow for 10 days on pDL/FN or on FN in a medium formulation (DM, 10% CEE, FGF, NGF) previously described to allow multipotent neural crest cells to generate neurons and glia in clonal culture. When clones were examined for MASH-1 expression, we found that clones grown on FN produced no MASH-1$^+$ cells (Figure 5D, E, F). On the other hand, every clone grown on pDL/FN produced some MASH-1$^+$ cells (Figure 5A, B, C). Moreover, some MASH-1 process bearing cells were present in this condition (Figure 6). Adrenergic differentiation was not observed in either condition. Hence, a condition that favors neuron production in clonal culture also favors the expression of MASH-1 in neural crest cells.

Figure 5. Substrate dependence of MASH-1 expression in clonal culture.

Neural crest cell clones were grown for 10 days in DM+CEE on either poly-D-lysine / fibronectin (A-C), or on fibronectin only. The clones were fixed, stained for MASH-1 (B, E) and counterstained with DAPI (C, F). Phase contrast images of the clones are shown (A, D). No MASH-1 expressing cells were found on FN in these clonal cultures.

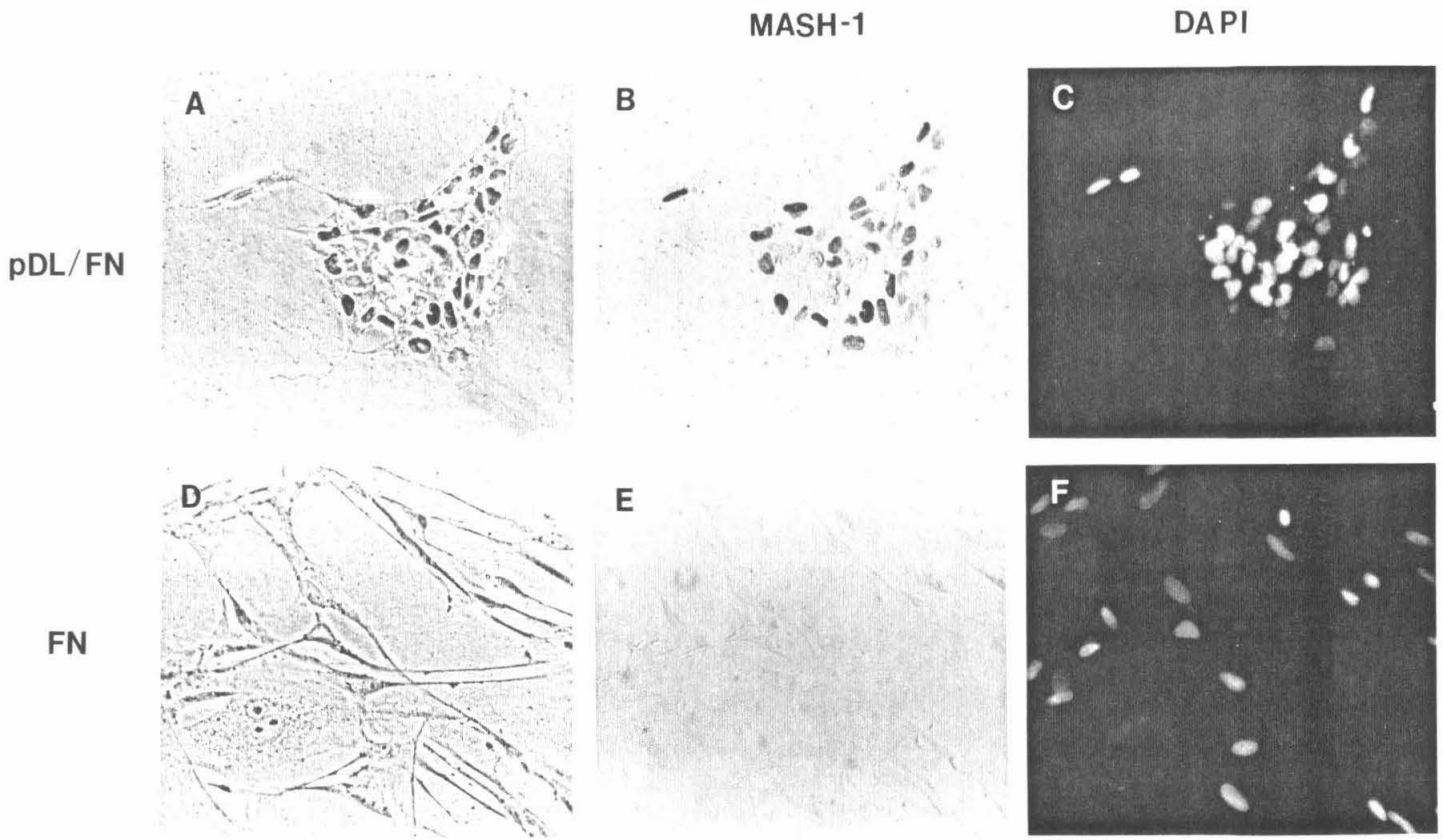
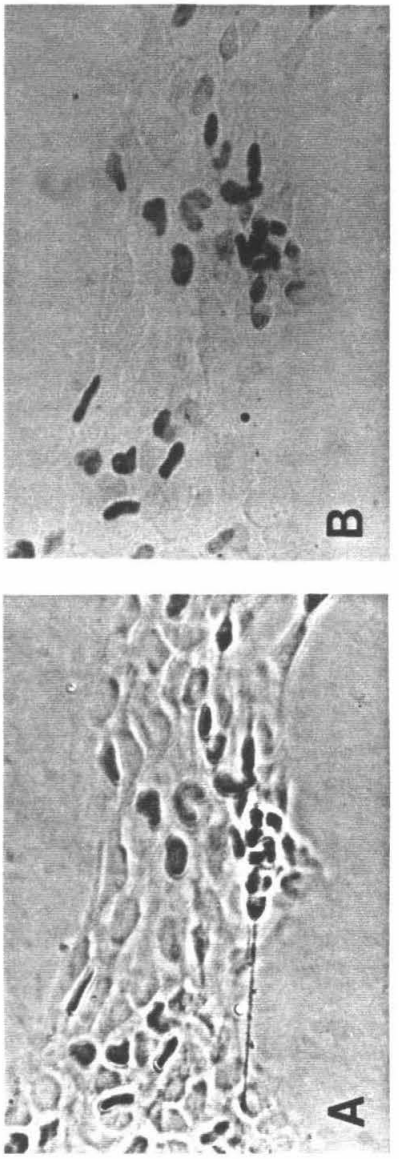

MASH-1
DAPI
pDL/FN
FN
A
B
C
D
E
F

Figure 6. MASH-1 can be expressed by neurons.

Clones grown on pDL/FN as described in figure 6. Were often found to have MASH-1^+ neurons in then. Shown here is a region of one such clone in phase-contrast (A) and in brightfield (B), showing MASH-1 immunoreactivity.


B
A

Discussion

Control of adrenergic differentiation is complex. We can, however, draw some general conclusions about conditions needed for TH expression and about the relationship between MASH-1 expression and TH expression. Under conditions that promote expression of TH in neural crest cells, MASH-1 is always expressed and always expressed *before* TH is expressed. It is not clear whether MASH-1 expression is a necessary prerequisite of TH expression, but TH and MASH-1 are frequently expressed in the same cells. This observation is consistent with the *in vivo* observations of Lo et al. (Lo, et al., 1991). Conditions that prevent or delay neuronal differentiation, i.e., CEE in the culture medium, also severely suppress MASH-1 expression. Conditions that promote strong expression of MASH-1 also promote neuronal differentiation but do not necessarily lead to TH expression. Neural crest cells express high levels of MASH-1 in response to RA, for example, but subsequent expression of TH is not observed. Hence, MASH-1 expression is not sufficient for adrenergic differentiation. A precise understanding of the relationship between MASH-1 expression and adrenergic or neuronal differentiation will have to await the generation of reagents to disrupt the normal function of MASH-1.

The results of Stern et al., indicate that either the floor plate or notochord is necessary, but neither are sufficient for adrenergic differentiation of neural crest cells in the periaortic environment (Stern, et al., 1991). The floor plate has been shown to be sources of retinoids (Wagner et al., 1990). In addition Placzek has shown, in a co-culture experiment, that notochord can induce MASH-1 expression in lateral neural plate (Dr. Marysia Placzek, personal communication). It may be that the adrenergic differentiation of neural crest cells requires a series of specific environmental signals. For example, undifferentiated migrating neural crest cells encounter a MASH-1 inducing signal, e.g., retinoids from the notochord or floor plate, then another signal,

perhaps derived from the dorsal aorta, promotes the later stages of adrenergic differentiation. The nature of such a late signal remains to be determined.

The sequence of embryonic environments encountered by migrating neural crest cells may specify adrenergic differentiation in a sequential fashion. The growth of neural crest cells in a chemically defined medium in co-culture with other embryonic structures, such as notochord, ventral neural tube, dorsal aorta and somite may be useful for testing which structures are necessary for adrenergic differentiation and in what order they must act. The early expression of MASH-1 may be of use to determine which environments must be encountered first to ultimately express TH.

Experimental Procedures

The experimental procedures used in this work are described in Chapter 2.

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