Mechanisms of cell fate determination and differentiation in the mammalian neural crest

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Dedicated to my parents and Ramnikmama

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ABSTRACT

The neural crest provides a model system to study the generation of cellular diversity during development. Crest cells derive from the vertebrate neural tube and migrate to many locations in the embryo where they give rise to different lineages. Cell types differentiating from the neural crest include neurons and glia of the peripheral nervous system, neuroendocrine cells, melanocytes of the skin and ectomesenchymal lineages such as smooth muscle and bone. Multipotential crest cells have been demonstrated to exist in vivo and in vitro. How do multipotential crest cells make lineage decisions? In one scenario, neural crest cells may differentiate using only cell autonomous mechanisms. Another scenario is that differentiative signals present in the environment direct the lineage choices of multipotential crest cells. These two models represent the extremes of possible mechanisms of cell fate specification.

I have tried to determine the mechanisms involved in lineage specification of rat neural crest stem cells (NCSCs). NCSCs form clones containing neurons, glia and smooth muscle in vitro. I have identified three growth factors that can direct differentiation of NCSCs to each of these fates in culture. Neuregulin-2 (GGF2), BMP2 and TGFß1 promote predominantly glial, autonomic neuronal and smooth muscle differentiation from these cells, respectively. My experiments suggest that these polypeptides may exert an instructive rather than a selective influence on NCSC lineage specification. Furthermore, each of these growth factors is present at a time and place in the embryo consistent with it being able to influence crest differentiation. Although NCSCs possess functional receptors for all three polypeptides, glial differentiation in GGF2 in vitro may not occur as fast as neuronal and smooth muscle differentiation in BMP2 and TGFß1, respectively. NCSCs also exhibit differential dosage sensitivity in their differentiation response to these growth factors. Such differences in the response

to environmental signals can affect the outcome of situations in which NCSCs encounter competing instructive cues. Taken together, my results suggest that instructive environmental signals in conjunction with cell intrinsic mechanisms may play an important role in neural crest cell fate specification.

TABLE OF CONTENTS

Acknowledgments......iii

Abstract......v

Table of contents......vii

Chapter 1

Introduction......1

Section A: Differentiation of the neural crest......3

References......42

Section B: Regulatory mechanisms in stem cell biology......66

Chapter 2

Glial growth factor restricts mammalian neural crest stem cells to a glial fate......79

Chapter 3

Alternative neural crest cell fates are instructively promoted by TGFß superfamily members......92

Chapter 4

Integration of multiple instructive signals reveals differences in growth factor responsiveness of multipotential rat neural crest cells......106

Abstract......107

Results and discussion......108

Materials and methods......117

References......118

Figure 1......123

Figure 2......127

Figure 3......130

Figure 4......133

Chapter 5

Summary and future directions....136

References....148

Appendix 1

The cellular function of MASH1 in autonomic neurogenesis....154

Chapter 1

Introduction

I have reviewed the mechanisms of lineage diversification in this chapter. In Section A, I focus on the development of the vertebrate peripheral nervous system from the neural crest. Here, I highlight some of the outstanding issues concerning neural crest differentiation. Since much is now known about this subject, Section A is necessarily long and detailed. However, I feel this is necessary to get a global picture of neural crest development. In Section B, the mechanisms regulating stem cell differentiation are explored. This latter section is reproduced from a published review.

Section A

Differentiation of the neural crest

A major focus in the study of development is to understand the mechanisms involved in generating the tremendous diversity of cell types found in the adult organism. The available evidence strongly suggests that this occurs through a series of steps in which cells undergo progressive differentiation to achieve the mature phenotype. What is the basis for such a progressive differentiation of cells? Furthermore, how is it that adjacent cells, often separated by only a few micrometers in the embryo, can differentiate into dramatically different tissues? An intuitive answer is that different cells possess different genomic material which limits their developmental options. However, the preponderance of evidence suggests that, with certain notable exceptions, different cell types in an organism have identical genomes. Thus, differentiation cannot be explained, in most cases, by postulating the existence of unique genomes in different cells.

Given an identical genome in most cells of the embryo, what then is the basis for progressive differentiation? Molecular and genetic analyses strongly suggest that each step in differentiation is probably characterized by the expression of a unique combination, either qualitative or quantitative, of gene products. This differential gene expression is likely to be generated by an asymmetric distribution in the information available to cells. Such asymmetry could arise either cell autonomously or via environmental influences. Cell autonomous mechanisms to generate differentiated cells have been well characterized in early development in several organisms. In many cases, autonomous mechanisms involve the spatially restricted distribution of particular RNAs or proteins within a cell. Environmental signals that lead to differential gene expression may arise from an adjacent tissue with a different developmental capacity or from neighboring cells with identical developmental potential. It is unlikely, however, that differentiation of most lineages is dictated completely by cell autonomous or nonautonomous mechanisms. Instead, the developmental read-out of a cell is probably

dependent on its lineal history (i.e., its intrinsic state due to previous developmental events) as well as any environmental signals it encounters.

What is the molecular identity of such cell autonomous and environmental regulators of differentiation? Furthermore, how do they interact to direct appropriate developmental decisions? To answer these and related issues, I will focus on the differentiation of the neural crest. It is easily accessible to different experimental perturbations and it has been studied for most of the last 100 years. As a result, much is known about the mechanisms that may underlie its differentiation. Since it gives rise to many different cell types, the neural crest also offers the opportunity to analyze multilineage differentiation from a precursor pool. While details of neural crest differentiation are interesting in their own right, the general mechanisms and issues that emerge from these studies may be relevant to other developing tissues as well.

OVERVIEW OF THE NEURAL CREST

The neural crest cell population arises from the dorsal part of the embryonic vertebrate neural tube at virtually all levels of the antero-posterior axis. Neural crest cells migrate into different regions of the embryo using well-defined pathways to differentiate into a variety of cell types. Thus, the majority of neurons and glia of the peripheral nervous system (PNS) derive from the neural crest. These neurons and glia form the sensory ganglia, sympathetic, parasympathetic and enteric ganglia; glial cells are also an integral part of peripheral nerves. The neural crest also differentiates into neuroendocrine cells, melanocytes of the skin and several mesenchymal cell types. Neural crest derivatives play divergent roles in vertebrate physiology. The autonomic nervous system and the neuroendocrine cells subserve many important functions: maintaining normal blood pressure, heart rate and contractility, regulating body temperature and maintenance of

calcium homeostasis. The enteric plexuses of Meissner and Auerbach control the various functions of the digestive tract. Sensory ganglia relay to the central nervous system (CNS) our tactile perceptions as well as proprioceptive and skeletal muscle tone information. Melanocytes protect underlying cells from UV damage and, in many species, may camouflage from predators. Mesenchymal cells provide structural integrity to the structures they form.

Given the diversity of cell types originating from the neural crest, it is not surprising that mutations in genes affecting neural crest differentiation lead to disparate phenotypes. Impaired differentiation of mesodermal derivatives of the crest can lead to a cleft palate, abnormal cardiac morphogenesis with its attendant hemodynamic alterations, abnormal skull architecture and thymic aplasia resulting in immunodeficiency syndromes. Failure of development of the enteric plexus impairs intestinal contractility (aganglionic megacolon or Hirschsprung's disease). Medullary carcinoma of the thyroid, pheochromocytomas and neuroblastomas are not uncommon tumors arising from neuroendocrine and autonomic derivatives of the neural crest. These and other diseases, termed neurocristopathies (Bolande, 1973), resulting from abnormal neural crest development, underscore the importance of this tissue during normal development. Thus, the study of neural crest derivatives is likely to lead to benefits in the medical clinic.

The neural crest is probably a vertebrate innovation and it may be at least partially responsible for the evolutionary success of this subphylum. It is present in both jawed vertebrates (Gnathostomes) and more primitive jawless vertebrates (Agnathostmes). An important difference between vertebrates and protochordates is that the former are active predators while the latter obtain nutrients primarily via a passive filter-feeding mechanism. A major reason that vertebrates may be active predators is the presence of "A New Head" in this subphylum (Gans and Northcutt, 1983). This new

head structure, comprised of the cranial sensory and autonomic apparatus as well as most of the skull, is almost entirely derived from the neural crest and epidermal placodes, both vertebrate advances. The new head structure was also accompanied by phenomenal specialization of the CNS. Such a head structure may provide the specialized feeding and respiratory apparatus required for the predatory life-style. That the neural crest gives rise to most of the cephalic skeleton (including most of the skull and facial bones) has recently been demonstrated elegantly using the quail-chick chimera system (see later) (Couly et al., 1993). Active predation may also have been facilitated by the fact that the muscularized digestive system of vertebrates is innervated by neural crest derivatives. Detailed comparative molecular and embryological analyses of the neural crest should lead to important insights into vertebrate origins.

Recent evidence suggests that neural crest formation may involve inductive interactions between the overlying ectoderm and the developing neural tube. I will not discuss these and related data in this Section {for a review of these data, see (Selleck and Bronner-Fraser, 1996)}. Another hotly pursued area of investigation is the mechanisms directing neural crest migration (Bronner-Fraser, 1993). This subject is also not discussed here except when it pertains to neural crest cell fate determination. I will, for the remainder of this Section, focus on mechanisms that generate lineage diversity from the neural crest. An essential part of such an undertaking is to define the fate map and developmental potential of neural crest cells. These studies set constraints on the various models and mechanisms one can invoke to explain empirical observations on neural crest lineage differentiation. Studies dealing with fate mapping and developmental potential of the neural crest are therefore summarized first. I will then discuss data regarding lineage segregation in the neural crest. Finally, the molecular mechanisms that may effect neural crest lineage differentiation are described.

MAPPING OF NEURAL CREST FATE AND DEVELOPMENTAL POTENTIAL

Before describing the fate map and developmental potential of neural crest cells, it is important to distinguish between two different states of differentiation (Slack, 1991). The first, termed specification, is a state in which a tissue (or alternatively, a cell or a small group of cells) differentiates exactly as it would in the intact embryo when explanted into a neutral culture condition. The second, termed determination, is a state in which the tissue differentiates exactly as it would in the intact embryo irrespective of the culture condition and irrespective of the region of the embryo into which it is transplanted. Thus, a specified tissue is endowed with all the information it needs to differentiate appropriately. A determined tissue has this information, but in addition, it does not respond to any other signal by altering its course of differentiation. These definitions were originally used to describe differentiated states of *X. laevis* tissues which can be explanted into simple salt solutions. Admittedly, in other organisms requiring more complex growth requirements upon explantation, these tests of specification and determination may be difficult to apply. However, as detailed below, studies mapping developmental fate and potential can suggest the specification and determination status of cells.

The fate map

A fate map summarizes what cells will do at a particular time in development. Thus, for the neural crest, the fate map is a description of the delamination of crest cells from the neural tube, their migratory pathway(s) and the different structures to which they contribute. Before I detail some of the results of such studies, it is worthwhile pointing out some of the issues not addressed by a fate mapping experiment (Slack, 1991). A

fate map of neural crest cells does not reveal their full developmental potential. For example, a restricted repertoire of fates in situ may reflect the restrictive nature of the environment rather than the potency of the crest cells. Furthermore, while a fate map shows what neural crest cells become in the embryo, it does not demonstrate that neural crest cells are determined to give rise to a particular structure. As mentioned earlier, determination along a certain path of development should be demonstrated by changing the environment of the cells. In other words, a fate map of the neural crest establishes what the cells do in situ. It does not always reveal what the cells can or cannot do.

Experiments done in the early part of this century exploited varying techniques to analyze the contributions of the neural crest in the embryo {reviewed in (Hörstadius, 1950)}. Most of these studies were done using different species of amphibians. The free living embryos were easy to manipulate, maintain and observe (especially given their semi-transparent nature). Since cells in early ectodermal lineages contain yolk granules that differ from those found in the surrounding mesodermal tissues, the simplest experimental design was to observe the neural crest, a neuroectodermal derivative, based on this property alone. In some studies, regions of the embryo from which the crest was thought to arise were prospectively labeled using vital dyes such as Nile blue sulfate. Contribution from the crest was inferred if a particular structure contained labeled cells at a later time in development. In other cases, prospective neural crest forming regions were ablated. If certain cell types failed to form, they were assumed to have derived from the neural crest. Recent studies have demonstrated a massive, but transient, regulative capacity of the neural tube and the epidermal placodes after ablations (Kirby, 1988; Kirby, 1988; Scherson et al., 1993) of the neural crest. It is, therefore, not surprising that previous ablation studies, many done at different times during development, often led to confusing results. For example, many investigators argued that sympathetic ganglia could not arise from the crest since they often developed

normally even if the crest had been "successfully" ablated. (At the time it was also not appreciated that sympathetic ganglia can receive crest contributions from many rostrocaudal segments.) Another approach was to perform xenoplastic transplantation and observe what the donor crest differentiated into in the host. Donor cells were distinguished from host cells based on cellular and nuclear morphology. In spite of the relatively crude nature of these early manipulations, these pioneering studies reached several conclusions that are still true today. They revealed that neural crest cells give rise to a diverse set of derivatives, including the neuronal and support elements of the PNS, melanocytes of the skin and mesodermal derivatives. Further, neural crest cells were shown to migrate along several distinct pathways in the embryo. Finally, these studies demonstrated that the neural crest can give rise to different derivatives at distinct levels of the rostrocaudal axis.

An important limitation of fate mapping studies is the label used to mark and follow crest cells. A good label should be easy to apply and observe, be specific to the labeled population and be retained in the cells during the entire period of development. Much progress in the precision of crest fate mapping studies is due to the development of better labels. One early innovation was to label the neural tube with [^{3}H]-thymidine and to transplant it in a homotopic and isochronic manner (Weston, 1963). Since crest cells are mitotically active and derive from the neural tube, all crest derivatives should be visualized by [^{3}H]-thymidine labeling. This method generally corroborated the earlier studies described above. A drawback of the technique was that the label would get diluted beyond the level of detection in a highly mitotic population.

A major advance in the analysis of neural crest differentiation has been the development of the quail-chick chimera system (Le Douarin et al., 1996). Quail cells can be easily and reliably distinguished from chick cells because of the association of a large mass(es) of heterochromatin with the nucleolus in the quail nuclei. The chromatin

network in chick nuclei is more homogeneously distributed. This clumpy heterochromatin is visualized using a histochemical reaction and serves as an indelible marker of quail lineages. Using these distinct nuclear profiles, Nicole Le Douarin and other investigators have mapped neural crest fates with exquisite specificity by substituting either early quail or chick neural tubes into a host embryo of the other species in an isochronic and homotopic manner. These experiments have been primarily responsible for demonstrating the various crest derivatives in higher vertebrates (Le Douarin, 1982). Thus, it was shown that the crest gives rise to the dorsal root ganglia (DRG), the sympathetic, parasympathetic and enteric ganglia and neuroendocrine derivatives such as the calcitonin producing parafollicular cells (C cells) of the thyroid, the chromaffin cells of the adrenal medulla and cells in the carotid bodies. It was further demonstrated that the neural crest contributes, along with the ectodermal placodes, to the formation of cranial ganglia. These studies have also been invaluable in demonstrating the various nonneural cells that derive from the crest. These include melanocytes of the skin, the support elements (including smooth muscle) of the cardiac outflow tract and the major branches of the aorta, the dermis of face and neck, stromal elements of the parathyroid, thymus, cornea and adenohypophysis and most of the skeletal elements (bone and cartilage) of the craniofacial region (Kirby et al., 1983; Le Douarin, 1982). It was also established that enteric plexuses of Meissner and Auerbach receive a contribution from neural crest arising from a rostral (vagal crest) as well as a caudal (sacral crest) level of the neuraxis (Le Douarin and Teillet, 1973). Equally important was the demonstration that trunk (thoracolumbar) crest does not normally form enteric ganglia.

Findings of such chimeric studies have been corroborated and extended using lipophilic fluorescent dyes such as DiI. These dyes incorporate into cell membranes and are only passed on to daughter cells with negligible transfer between labeled and

unlabeled cells. These dyes, in combination with whole embryo culture, have advanced our knowledge of neural crest fate maps to mammalian species in which chimeric studies are not yet feasible (Fukiishi and Morriss-Kay, 1992; Serbedzija et al., 1990). Further, lipophilic fluorescent dyes allow the experimenter to observe migrating and differentiating cells at various times after dye application. Of course, this is possible only in species with free living developmental intermediates such as fish and amphibians. In addition to confirming the findings of earlier crest fate mapping work, these studies have raised several important issues. The first concerns the species difference between neural crest contributions. Thus, both the migratory pathways used by the neural crest and the time of entry of crest cells into these paths may differ between amphibians, avians and mammals (Collazo et al., 1993; Serbedzija et al., 1989; Serbedzija et al., 1990). Even the derivatives produced by the neural crest can differ between species. Thus, while avian trunk crest does not form mesenchyme, amphibian trunk crest cells do differentiate into mesenchymal phenotypes (Collazo et al., 1993). Furthermore, amphibian trunk crest cells differentiate into mechanoreceptors and cells of the aorta (Collazo et al., 1993; Collazo et al., 1994). Neither of these derivatives is produced by the avian trunk crest. These results highlight the danger in extrapolating all the findings from one species to another. Finally, studies using DiI have also shown an important pattern to neural crest differentiation in avian and mammalian embryos {(Serbedzija et al., 1989; Serbedzija et al., 1990); see also, (Weston, 1963)}. The entire neural tube was labeled with DiI and then embryos were fixed at progressively later times. This revealed that the more ventral derivatives such as sympathetic ganglia and adrenal medulla are populated by earlier migrating crest while more dorsal derivatives such as DRG and ventral roots are labeled by late emigrating crest cells.

Thus, DiI is a very useful tool in mapping neural crest fates. An advantage of using DiI instead of chimeras is that development can be studied in situ for a particular

species. This bypasses the necessity of combining tissues from different species, a perturbation that may affect the fate of either the host or the donor tissue. One limitation of using DiI is the progressive dilution of the dye with cell division. Highly mitotic cell populations may lose the label almost entirely, making it impossible to detect them. Moreover, the fixation protocol for DiI makes it difficult to use antibodies to identify the DiI+ cell types. Therefore, studies with DiI have generally involved using cell position and morphology to identify the different cell types. The availability of newer dyes (DiI-CM) with better fixation protocols should allow the simultaneous use of immunochemistry or in situ hybridization.

In summary, the fate mapping experiments have revealed that the neural crest differentiates into a strikingly diverse set of cell types. Furthermore, the fate of the neural crest is wedded to its origin along the AP axis. These studies also raise several critical issues concerning neural crest differentiation: Distinct derivatives differentiate from the neural crest at different levels of the neuraxis. Does this imply that neural crest cells at different levels are competent to give rise to derivatives only for that level of the AP axis? Another, and not necessarily mutually exclusive, possibility is that the environment imposes restrictions on what derivatives may develop. The ventral-to-dorsal filling pattern mentioned above raises another important question. Are cells migrating at different times capable of contributing to different derivatives or are they determined to certain fates? In other words, is the ventral-to-dorsal pattern of migration a reflection of the migratory pathways available to cells rather than developmental restrictions in crest cells that emigrate at different times? Several lineages differentiate from the crest at any level of the rostrocaudal axis. On the one hand, this phenomenon may reflect the existence of several cell type-committed precursors in the premigratory crest. Alternatively, the premigratory crest may consist of multipotential cells that then produce different cell type-committed precursors. To answer these questions, it is

important to know the developmental potential of neural crest cells. Studies addressing these and related issues are described below.

Developmental potential of the neural crest

The quail-chick chimera system has been extensively used to describe the developmental potential of premigratory neural crest cells. The approach is to take trunk neural tube (for example), before neural crest cells have started migration, from one species and transplant it to a different level, either as a replacement of the host tube or as a supernumerary structure, into the other species. The embryos are then allowed to develop and their crest derivatives examined to determine any donor neural crest contributions. With few exceptions (see later), such perturbations have revealed that neural crest cells possess greater developmental potential than is normally realized in situ {reviewed in (Le Douarin, 1982)}. For example, the trunk crest normally does not contribute to the formation of enteric ganglia or the ectomesenchyme of the cardiac outflow tract. When heterotopically transplanted to a more rostral level, however, trunk neural crest cells can differentiate into cells of the enteric plexus (Le Douarin et al., 1975) and the cardiac outflow tract (Kirby, 1989; Nakamura and Ayer-Le Lievre, 1982). Similarly, while the vagal crest does not normally form sympathetic ganglia or cells of the adrenal medulla, it can do so when transplanted more caudally.

Weston (Weston and Butler, 1966) used a clever method to understand the basis of the ventral-to-dorsal filling pattern in the avian trunk described above (Serbedzija et al., 1989; Serbedzija et al., 1990; Weston, 1963). Trunk neural tubes from "old" and "young" embryos were labeled with [^{3}H]-thymidine. Unlabeled trunk neural tubes from young embryos were replaced with "old" [^{3}H]-thymidine labeled neural tubes. Conversely, unlabeled trunk neural tubes from old embryos were replaced by "young" [^{3}H]-thymidine labeled tubes. If the orderly pattern of migration was imposed by the

environment, then donor tubes should behave according to the host environment. Alternatively, if the crest populations from the two ages were intrinsically different, then their behavior should not be affected by the novel environment. The striking result was that donor tubes behaved according to the host environment ruling out any intrinsic differences in the cell populations from these ages.

The foregoing studies demonstrate a powerful effect of the environment in modulating the fate of neural crest cells. These experiments and the fate mapping studies described earlier have involved the manipulation of a population of crest cells. Thus, the multilineage nature of differentiation of the neural crest may, in fact, result from the existence of unipotent precursors in the premigratory crest for all the different crest lineages. Alternatively, multilineage differentiation may be a property of the multipotential nature of individual neural crest cells. To distinguish between these possibilities, it is necessary to analyze the behavior of single neural crest cells. This problem has been tackled in a number of ways.

Since the pioneering experiments of Cohen and Konigsberg (Cohen and Konigsberg, 1975), a number of different laboratories have developed tissue culture protocols for growth of neural crest cells at clonal density. Such culture systems are now well established for quail, rat and mouse neural crest cells (Baroffio et al., 1988; Ito et al., 1993; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). These clonal cultures offer several advantages. It is possible to continuously monitor the growth and differentiation of clones developing in vitro. Furthermore, the culture medium can be easily modified to test the effects of different factors that may be encountered by neural crest cells in vivo. With the rapidly increasing availability of markers for different lineages, it is possible to assay for many cell types simultaneously. The results of numerous clonal analyses done in tissue culture, with crest cells from different species and from different levels of the AP axis, have demonstrated that many

single neural crest cells are multipotential. For example, studies done with quail cephalic neural crest cells have shown that a single clone can contain melanocytes, neurons (sensory and autonomic), glia, cartilage and smooth muscle (Baroffio et al., 1991; Baroffio et al., 1988; Ito and Sieber-Blum, 1991). An important advance in the cultures of neural crest has been the clonal cultures now available for mammalian crest cells. These systems offer the chance to genetically manipulate the embryo and assay for changes in differentiation of single cells in vitro. Another technical feat has been to successfully subclone crest cultures grown at clonal density (Stemple and Anderson, 1992). In these experiments, rat neural crest cells were plated at clonal density. The majority of primary clones exhibited multilineage differentiation. During the early culture period, however, most clones were undifferentiated. If daughter cells of a single undifferentiated primary clone were replated at clonal density, they generated subclones that were also multifatent. This demonstrated that rat neural crest cells were capable of self-renewal and differentiation, two properties considered to be the defining feature of stem cells. The implications of this finding will be discussed later. In most cases, investigators assay for differentiation after varying periods of growth in vitro. Rarely, the differentiation of single cells has been assayed by allowing them to form clones in vitro and then injecting all the progeny back into the embryo (Bronner-Fraser et al., 1980). Such experiments have also revealed the multipotential nature of neural crest cells. The latter sort of manipulation is important since tissue culture experiments may reflect the developmental potential of the cells and not necessarily their fate in the embryo. To illustrate, quail cephalic crest does not differentiate into adrenergic cells in the embryo. However, cephalic crest cells can differentiate into adrenergic cells in vitro (Ito and Sieber-Blum, 1991). Where possible, such latent developmental potentials should be distinguished from the normal fates of the cells in vivo.

The foregoing in vitro studies suggest the need to assay the developmental capacity of single crest cells in the embryo. Two approaches have been used to study the differentiation of individual neural crest cells in situ. In the first instance, single premigratory neural crest cells were injected in the developing chick and amphibian embryo using a fluorescent dextran (Bronner-Fraser and Fraser, 1988; Collazo et al., 1993). The membrane impermeability of dextran combined with the large size of the dextran used in these studies ensures that the dye is only passed to progeny of the injected cell. These experiments showed that many single neural crest cells are multipotential {reviewed in (Bronner-Fraser and Fraser, 1991)}. Progeny of single injected cells were often found in many derivatives including the dorsal root ganglia, ventral roots, sympathetic ganglia and under the epidermis (presumptive melanocytes). While the phenotypes were assigned mainly on morphology and cell location, in some experiments an antibody to neurofilament was used to demonstrate the neuronal nature of some of the progeny. In one study, single neural crest cells were labeled with the fluorescent dextran as they were migrating through the somite (Fraser and Bronner-Fraser, 1991). This study demonstrated that many migratory crest cells were still multipotential. Injection of fluorescent dextran has the limitation of dye dilution beyond detection after many cell divisions. This suggests that some progeny of a labeled cell may not have been observed. However, if anything, this leads to a conservative estimate of the multipotential nature of individual neural crest cells.

These results have been corroborated by a retroviral lineage analysis of chick crest cells (Frank and Sanes, 1991). In this study, the authors used replication-incompetent retroviruses carrying *lacZ* as a reporter to infect neural tubes in ovo. The retroviral construct is capable of integration into the genome of a cell. The integrated provirus is then passed on to progeny of the infected cell. Embryos were incubated for various times after infection and then analyzed using ß-galactosidase histochemistry.

Since the neural crest is a migratory population that disperses over a wide region of the embryo, clonality of infected neighboring cells is not assured. The authors used low viral titers, statistical calculations and two sets of virions with distinct intracellular localization of the *lacZ* gene product to demonstrate the clonal nature of infected populations. For these reasons, however, they were forced to limit their analysis to DRG development. Nevertheless, they observed many multipotential clones containing neuronal and non-neuronal cells in DRGs. Further, a single clone could contain morphologically distinct types of DRG neurons. Since the provirus is not lost upon multiple rounds of cell division, the authors could analyze development of the DRG after the period of neuronal death. This allowed them to confirm that the neurons that differentiated were appropriate for the location. It is important to realize that these single cell marking experiments are essentially clonal fate maps (Slack, 1991). Thus, the observation that all labeled progeny express the same phenotype does not imply that the founder cell was determined to that fate. For example, all progeny of a multipotential cell may end up in the same location in the embryo. In such a case, environmental signals could direct all the progeny to differentiate along one lineage {for a discussion, see (Bronner-Fraser and Fraser, 1991)}.

LINEAGE SEGREGATION IN THE NEURAL CREST

By "lineage segregation" I mean the pattern of differentiation of different lineages from crest cells. Two issues will be addressed in the following paragraphs. The first concerns the homogeneity of the premigratory crest population. In vivo and in vitro data suggest that many single crest cells are multipotent. However, are all crest cells comparable in their developmental potential? If not, can neural crest populations differing in developmental potential be identified prospectively by molecular markers?

Finally, what molecules are likely to impose restrictions in developmental capacity in the neural crest? The second issue is that of the differentiation of lineage committed progenitors from neural crest cells. Do multipotential neural crest cells undergo progressive restrictions in their developmental repertoire to generate lineage committed progenitors (Anderson, 1989) as has been suggested to occur in hematopoiesis? Furthermore, is such a progressive loss of developmental potential obligatory? Alternatively, do multipotential cells directly differentiate into unipotent cells? I will review the evidence that sheds light on these issues. It is instructive to start with a comparison of lineage segregation between cephalic and trunk neural crest as it highlights a number of important issues that can be addressed experimentally.

Rostral vs. caudal neural crest

The most obvious segregration of lineages in the neural crest is that of the mesectodermal cell types. As mentioned earlier, the neural crest differentiates into ectomesenchymal cell types only at rostral levels in avians. However, quail-chick chimera experiments have shown that the trunk crest can give rise to at least smooth muscle when transplanted rostrally. In such transplants, the trunk crest did not contribute to cartilage and bone formation (Le Douarin, 1982; Nakamura and Ayer-Le Lievre, 1982). The latter lineages are also normal cranial crest derivatives. This suggests that the developmental potential of the neural crest may differ at distinct levels of the AP axis. It has been suggested that the AP patterning in the neural tube set up by the *Hox* genes may, in part, be responsible for this difference {for reviews, see (Le Douarin et al., 1994; Wilkinson, 1993)}. Thus, there may be a molecular code that patterns the developmental potential of neural crest cells according to their level of origin along the AP axis. However, trunk and cranial crest cells use different migratory cues and pathways (Bronner-Fraser, 1993). Furthermore, the constituents of the

extracellular matrix present in the crest migratory pathways may differ between cranial and trunk levels. These environmental differences may also affect the ability of different populations of crest cells to undergo appropriate differentiation. Thus, the apparent rostrocaudal differences in neural crest developmental potential could be cell autonomously generated or due to differences in the environment. These models have been recently tested in the axolotl, *A. mexicanum*. By transplanting wild-type trunk crest cells into albino hosts, it was shown that trunk neural crest cells do not migrate into all the pathways utilized by the cranial crest (Graveson et al., 1995). However, the same study demonstrated that trunk neural crest cells do not form cartilage in vitro, even under conditions that promote chondrogenesis from cranial crest explants (Graveson and Armstrong, 1987). Thus, trunk neural crest cells may indeed lack the potential to form certain ectomesenchymal cell types.

When cranial neural tubes are transplanted caudally, donor neural crest-derived ectopic cartilage and bone differentiation is often seen (Kirby, 1989; Le Douarin, 1982). This was most striking in the case where rostral crest cells replaced crest cells that would have contributed to the second branchial arch (Noden, 1983). In this study, the donor crest cells formed a more or less complete host- and donor-appropriate set of ectomesenchymal structures. Such results have been taken to imply that there is a certain degree of predetermination in the neural crest found at cranial levels. However, it is not yet known if such predetermination is autonomously determined or a result of cell-cell interactions. In summary, it seems likely that there is a profound difference in the behavior of cranial vs. trunk neural crest. Furthermore, *Hox* and other genes involved in establishing the AP pattern may also influence neural crest developmental potential at different rostrocaudal levels.

Heterogeneity and developmental restrictions within the crest

Heterogeneity in the neural crest as revealed by different antibodies

Heterogeneity may be defined prospectively if the cells within the population can be shown to express different markers. A number of antibodies have been used to characterize subsets of migratory neural crest cells. In at least one case, it has been shown that HNK1-immunoreactivity on neural crest cells in vitro correlated positively with the ability of these cells to generate adrenergic cells (Maxwell et al., 1988). Melanocytes and other phenotypes, however, were generated by $HNK1^+$ as well as HNK^- cells. The authors were not able to distinguish whether the starting neural crest population itself was heterogeneous with respect to HNK1-immunoreactivity or whether the $HNK1^-$ cells differentiated from the $HNK1^+$ population in culture. It should be possible to do this by isolating a pure $HNK1^+$ population and then following the differentiation of any $HNK1^-$ cells that develop in these cultures. Nevertheless, these results argue for the existence of neural crest cells that have a reduced ability to produce adrenergic cells in vitro.

About 5% of chick mesencephalic neural crest cells have been shown to bind antibodies (CG1, 4 and 14) that recognize the high affinity choline transporter present in parasympathetic neurons (Barald, 1988). Since the mesencephalic crest does differentiate into parasympathetic neurons in the ciliary ganglion, it was of interest to determine if the CG^+ crest cells were committed to a parasympathetic neuronal fate. The author showed that the CG^+ crest cells were morphologically undifferentiated and under certain culture conditions gave rise to parasympathetic neurons (Barald, 1989). Moreover, crest depleted of CG^+ cells could not, under the same conditions, differentiate into parasympathetic neurons. The depleted population did differentiate into other types of neurons. While this study strongly suggests the existence of a parasympathetic precursor in migratory crest, it, like the previous study, does not rule out the possibility that such CG^+ cells arose from CG^- crest cells in vivo. That CG^+

cells did not differentiate de novo from CG^- cells under conditions which promoted differentiation of CG^+ cells into parasympathetic neurons may reflect the requirement of different factors for the generation of the CG^+ phenotype from CG^- cells. It should be possible to test this hypothesis by transplanting chick trunk crest to the mesencephalic region of quail hosts to see if the trunk crest cells (normally CG^-) can now generate such CG^+ cells in an appropriate environment. It should be noted that expression of an antigen on a subset of neural crest cells does not always correlate with a distinct developmental potential of such cells (Barbu et al., 1986; Maxwell et al., 1988).

Heterogeneity in the developmental potential of neural crest cells in vitro

Another approach to identify heterogeneity in early neural crest populations is to grow the cells at clonal density and assay for the various lineages that arise in different clones. In general, such clonal analyses have revealed a vast combination of possible phenotypes that can arise in different clones. This is true regardless of the rostrocaudal origin of the crest or of the species under examination. On the basis of such studies, many have concluded that the early migrating neural crest population indeed consists of cells with developmental capacities ranging from the unipotent to the pluripotent {reviewed in (Le Douarin et al., 1994)}. The assumption here is that the lineage diversity present in the clone at the end of the culture period is a faithful reflection of the developmental potential of the founder crest cell. This may not always be true: In most cases, the cultures were done in complex media containing serum or other undefined components, often on fibroblast feeder layers. Small changes in the local concentrations of growth/survival factors, or other random variations, could easily lead to variable development of different clones. Furthermore, the possibility that there could be cell-cell interactions within the developing clone was discounted. Finally, the possibility that there were undifferentiated cells in clones at the end of the culture period was also not excluded. This is especially important in light of the demonstration of the self-

renewal capacity of mammalian neural crest cells. As mentioned early on, explantation of cells into tissue culture reveals their specification state. To prove determination towards one or another lineage, it is essential to *challenge* the cells with different environments. This has only rarely been done (Bronner-Fraser et al., 1980; Stemple and Anderson, 1992). To illustrate, in clonal cultures of rat crest cells it was demonstrated that no neuronal differentiation was observed if the cells were grown on a fibronectin substrate (Stemple and Anderson, 1992). However, if the clone was split onto a poly-D-lysine/fibronectin substrate, a large number of subclones contained neurons. (The same result was obtained if, alternatively, cultures established on fibronectin were overlaid with poly-D-lysine.) Similar conclusions about the developmental potential of cultured multipotential cells have been reached by studies in hematopoiesis. Thus, in cultures of hematopoietic progenitors, none of the primary clones contained erythrocytes (Nakahata and Ogawa, 1982). However, simply subcloning primary clones revealed a latent potential in these founder cells to generate erythrocytes. These examples suggest the need to be cautious in extrapolating the developmental fate of founder cells in vitro to their developmental potential. It is necessary to challenge the cells with various differentiation conditions before conclusions are drawn regarding their developmental potential.

Using data obtained from the in vitro clonal analyses described above, an incredibly complex hierarchy of lineage segregation has been proposed (Le Douarin et al., 1994). Clones containing the most phenotypes are assumed to have differentiated from founder cells with the broadest developmental repertoire and such cells sit at the top of the hierarchy. After these cells are founder cells that gave rise to clones containing fewer lineages and so on. At the bottom of the hierarchy are the "unipotent" founder cells that differentiated into one lineage exclusively. For the reasons mentioned above, in vitro cultures may not reveal the full developmental repertoire of cells thus

rendering such hierarchies of sequential restrictions in developmental capacity potentially inaccurate. A corollary of the hierarchical model is the existence of cells with predictable restrictions in their developmental potential arising sequentially from pluripotential clones. While this may be addressed using serial subcloning experiments, it remains to be demonstrated.

Segregation of neuronal lineages in the neural crest

In one study, the developmental capacity of quail neural crest cells emigrating at different times from the neural tube was examined (Artinger and Bronner-Fraser, 1992). Early migratory cells were found to be capable of differentiating into neurons (as defined by morphology and neurofilament expression), melanocytes and adrenergic cells (as defined by catecholamine histofluorescence) in vitro as well as upon transplantation into host chick embryos. However, crest cells taken from significantly older tubes {as compared to (Weston and Butler, 1966)} generated neurons and melanocytes, but not adrenergic cells, both in vitro and upon transplantation into chick embryos. Thus, neural crest cells may lose the ability to generate certain neural phenotypes while retaining the potential to generate other types of neurons. The early and late neural crest cells were not examined immediately upon emigration from the neural tube for the expression of HNK1- immunoreactivity. It is, therefore, difficult to directly compare the inability of neural crest cells from "old" neural tubes to generate adrenergic cells with that previously reported for the HNK1$^-$ population. Importantly, the authors stressed the fact that the reason for this loss of adrenergic potential was not clear (Artinger and Bronner-Fraser, 1992). Thus, while the inability to generate adrenergic cells reflects a restriction in developmental capacity, either cell autonomous or environmental factors (such as prolonged exposure to neural tube and ectodermal signals) could bring about this cell state. Future studies should be able to distinguish between these possibilities.

Earlier studies have also reported a loss of the capacity to generate neurons (Girdlestone and Weston, 1985; Vogel and Weston, 1988). In this case, crest cells cultured as clusters in non-adhesive substrate conditions varied in their ability to generate neurons and melanocytes as follows. Many neurons and a few melanocytes differentiated when the clusters were dissociated and subcultured early. However, late subculture of the clusters generated mainly melanocytes. The same result was obtained regardless of whether the clusters were grown with or without the neural tube. These data suggest that neural crest cell-cell interactions mediate the change in developmental capacity observed in vitro. Such interactions could act instructively on pluripotent cells. Alternatively, these interactions may act to select for the survival of only a subset of cells present either at the time of cluster formation or generated within the cluster environment.

It has been suggested that the sensory neuronal lineage might segregate early from the autonomic neuronal lineage (Le Douarin, 1986; Sieber-Blum, 1989). In vitro observations consistent with this proposal include the early differentiation of sensory neurons from non-cycling precursors adjacent to the neural tube (Sieber-Blum, 1989; Ziller et al., 1983). These observations do not eliminate the possibility that neural tube-derived signals induced sensory differentiation from multipotent precursors. A stronger case has been made for an early segregation of the sensory and autonomic lineages using the quail-chick chimera system. In this series of experiments, various peripheral ganglia were dissected and back-transplanted into the crest migratory pathway of younger hosts. The observation was that while sensory ganglia contained precursors for other lineages (including the autonomic lineage), cells from autonomic ganglia never differentiated into sensory neurons (Dupin, 1984; Le Lievre et al., 1980). Furthermore, the age at which sensory ganglia stopped generating sensory neurons in vivo correlated with their inability to generate sensory neurons upon back-transplantation. However,

such sensory ganglia could still generate autonomic neurons both in vivo and in vitro (Xue et al., 1985). These results contrast sharply with studies in which quail autonomic ganglia were dissociated and the cells grown at clonal density. Many clones contained autonomic neurons, sensory neurons and melanocytes (Duff et al., 1991). A similar result was obtained when sensory ganglia were dissociated and grown at clonal density (Duff et al., 1991). Interestingly, clones containing exclusively sensory neurons or autonomic neurons were only obtained in cultures established from the respective ganglia. While the developmental potential of such unifatent clones was not tested, the results suggest that such unifatent cells may differentiate or survive only in the appropriate ganglia. What is clear is that the issue of an early segregating sensory lineage is far from resolved. Many genes that may be expressed exclusively in sensory neurons (or their precursors) in the mammalian PNS have been recently cloned (Gerrero et al., 1993; Lee et al., 1995; Ma et al., 1996; Saito et al., 1995; Sommer et al., 1996). It should therefore be possible to assay for sensory lineage segregation with multiple markers, using both cell culture and transgenic technology.

Recently, a precursor to enteric neurons has been identified. Enteric neurons are known to express the receptor tyrosine kinase, RET. Loss-of-function mutations in *c-ret* are associated with an almost complete absence of enteric neurons (Durbec et al., 1996; Schuchardt et al., 1994). Monoclonal antibodies to the extracellular domain of RET were used to isolate cells from embryonic rat gut with FACS (Lo and Anderson, 1995). A subset of the sorted cells were differentiated enteric neurons. However, many cells were morphologically undifferentiated and resembled early migratory neural crest cells. Upon clonal culture, many such undifferentiated cells rapidly become neurons. That the latter population of RET^{+} cells represents a precursor committed to the neuronal pathway was demonstrated by challenging these cells with a glial differentiation promoting environment (see Chapter 2). Even under such conditions,

many RET^+ cells differentiated into neurons. Thus, these experiments elegantly demonstrate a precursor committed to a neuronal fate in the presence of a gliogenic signal. It is not yet known how these RET^+ cells segregate from the vagal premigratory crest or from the SA lineage (see below).

The sympathoadrenal lineage

One of the best characterized restricted cell types in the PNS is the sympathoadrenal (SA) progenitor {reviewed in (Anderson, 1993)}. This cell type is probably committed to the catecholamine-synthesizing neural crest lineages. At the level of the trunk, these include the chromaffin cells and the sympathetic neurons. The SA progenitor, or a closely related cell, has also been postulated to exist in the gut where it may give rise to the serotonergic enteric neurons (Anderson, 1993; Carnahan et al., 1991). The SA progenitor in the rat is characterized by expression of tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis, and the SA antigens, SA1-5 (Carnahan and Patterson, 1991; Carnahan and Patterson, 1991). These and other markers have been used to isolate SA progenitors in vitro to assay their developmental potential. Single SA progenitors can differentiate into chromaffin cells or sympathetic neurons. Whether the same cell can differentiate into enteric neurons is not yet known.

How do single SA cells differentiate into different lineages? The multipotency of SA progenitors is correlated with a low level of transcription of genes expressed at high levels in different SA-derived lineages (Anderson et al., 1991; Vandenbergh et al., 1991). It remains to be determined whether this expression simply reflects the availability of multiple fates to SA cells or whether it actually participates in endowing SA cells with multilineage differentiation capacity. Using degenerate PCR on cDNA derived from an SA progenitor cell line (Birren and Anderson, 1990), rat homologs of *Drosophila achaete-scute* were isolated (Johnson et al., 1990). These genes encode basic helix-loop-helix (bHLH) transcription factors that regulate cell fate choice in many

Drosophila neural lineages. Of the two rat homologs isolated, MASH1 & 2, MASH1 is expressed early and transiently in all autonomic lineages and in the adrenal medulla (Lo et al., 1991). Mice homozygous null for *Mash1* fail to develop the majority of sympathetic and parasympathetic neurons (Guillemot et al., 1993). Furthermore, serotonergic neurons of the gut also do not develop in these mice (Blaugrund et al., 1996). Thus, some of the molecular mechanisms controlling SA differentiation are beginning to be understood. It is not clear, however, how SA cells differentiate from the neural crest. Some experiments relating to the differentiation of autonomic neurons from the neural crest and the control of MASH1 expression are described in Chapter 3 and Appendix I.

Segregation of non-melanocyte lineages in the neural crest

The posterior rhombencephalic (somites 1-3) neural crest normally participates in the formation of ectomesenchymal derivatives, melanocytes and enteric ganglia (Le Douarin, 1982). In fact, many single premigratory neural crest cells from this level can generate clones containing all the possible lineages that normally differentiate at this level in vitro (Baroffio et al., 1991; Ito and Sieber-Blum, 1991). These crest cells normally migrate to populate the posterior branchial arches. When crest cells from posterior branchial arches are cultured at clonal density under identical conditions (as the premigratory cells), they generate clones containing ectomesenchymal and neuronal lineages but no melanocytes (Ito and Sieber-Blum, 1993). This is true even when cells from the posterior branchial arches or the gut are backtransplanted into younger hosts (Ciment and Weston, 1983; Ciment and Weston, 1985; Rothman et al., 1990). This loss of melanogenic potential has been shown to be correlated with the gain of expression of an intermediate filament-associated protein, NAPA-73. These experiments suggest that at least some neural crest cells cannot generate melanocytes. However, these experiments do not prove that a pluripotential neural crest cell has lost

melanogenic potential after migrating into the posterior branchial arch. An alternative explanation is that the premigratory crest is a heterogeneous population and only cells with nonmelanogenic potential populate the posterior arches. Serial subcloning of premigratory rhombencephalic crest cells should reveal whether the pluripotential cells can generate progeny that exhibit multilineage differentiation but a loss of competence to differentiate into melanocytes.

Segregation of glia from the neural crest

A Schwann cell precursor has been identified in rat embryonic sciatic nerve (Jessen et al., 1994). The antigenic phenotype of this cell distinguishes it from both undifferentiated neural crest cells and mature Schwann cells. Further, it is present transiently in vivo and can differentiate rapidly into Schwann cells in vitro when cultured under the appropriate conditions. Consistent with previous demonstrations of the influence of neuron-derived signals on glial differentiation {for review, see (Mirsky and Jessen, 1996)}, there is evidence to suggest that the survival and differentiation of the Schwann cell precursor is mediated by axon-derived signals (Dong et al., 1995). However, a restriction to the glial lineage, either by a challenge with neuron-inducing conditions or by transplanting it into the crest migratory pathway, has not been demonstrated. Given that some neuronal precursors may exist in the sciatic nerve in the postnatal rat (Walter, 1994), this is an important experiment. Experiments relating to how Schwann cells may segregate from multipotential neural crest cells are described in Chapter 2.

Evidence for progressive restriction in developmental potential of neural crest cells

Numerous studies have isolated neural crest cells from various postmigratory sites and grown them in mass or clonal cultures {(Duff et al., 1991; Richardson and Sieber-Blum, 1993; Rohrer et al., 1986; Sextier-Sainte-Claire Deville et al., 1994; Xue et al., 1985); for a review, see (Sieber-Blum et al., 1993)}. Strikingly, such studies have

demonstrated the existence of cells with the broadest range of developmental potential at many sites, including the spinal, autonomic and enteric ganglia and the skin. Such cells have been observed well after neural crest cells have reached these locations. A related observation has been a progressive decrease in the proportion of such pluripotent cells isolated from post-migratory sites with increasing embryonic age. This loss is paralleled by an increase of cells with more restricted developmental capacity (as assayed in vitro). In general, the decrease in the number of multipotential cells along with an increase in the number of "committed" cells isolated from post-migratory sites has been taken as proof of the progressive restriction in developmental potential of pluripotential crest cells in vivo. While the observations are consistent with such an occurrence, an alternative explanation is the progressive decrease in the proportion of pluripotent cells in vivo. Such a decrease may come about due to the selective proliferation of more restricted precursors that may be present in early migratory crest cells.

The foregoing should illustrate the complexities involved in interpreting the results of experiments dealing with lineage differentiation from multipotential precursors. However, a few generalizations can be safely made. Some restrictions in developmental potential do seem to exist in the pre- and post-migratory neural crest population {(Anderson, 1993; Artinger and Bronner-Fraser, 1992; Lo and Anderson, 1995); for a review, see (Anderson, 1993)}. The mechanisms generating such restrictions in developmental potential are far from clear. One explanation seems to be pre-existing heterogeneity in the pre-migratory neural crest. Another explanation is the progressive restriction of developmental capacity in initially pluripotent crest cells; such a restriction might be generated in a stochastic (Baroffio and Blot, 1992) or a deterministic manner. However, a true progressive restriction in developmental capacity, as evidenced by the

sequential generation of progressively restricted progeny from an initially pluripotential precursor, has not been demonstrated. The availability of many different markers and the ability to subclone cells should allow one to test this possibility. The following paragraphs discuss the molecular mechanisms that may control lineage differentiation from the neural crest.

AUTONOMOUS VS. ENVIRONMENTAL CONTROL OF NEURAL CREST DIFFERENTIATION

Cell autonomous mechanisms

It has been suggested, based on the analysis of differentiation of many crest clones in vitro, that the neural crest may undergo restrictions in developmental potential in a stochastic manner (Baroffio and Blot, 1992). While this may be true, two issues need to discussed. The first is whether in vitro cultures reveal the full developmental repertoire of each cell. As discussed in the preceding paragraphs, this may not always be the case. The second issue is that a stochastic restriction in developmental potential does not imply the involvement of cell autonomous mechanisms. Cell-cell interactions may also bring about a stochastic pattern of differentiation (Félix and Sternberg, 1996; Morrison et al., 1997). Current studies have yet to rule out the latter possibility.

Studies of *Drosophila* neurogenesis have defined a role for asymmetric cell division in the differentiation of particular neuronal lineages. Further, the asymmetry may, in part, be mediated by an asymmetric partitioning of the product of *numb* (Knoblich et al., 1995; Rhyu et al., 1994). Studies of a mouse homolog of *numb* have demonstrated that mouse NUMB may also play a role in neuronal differentiation in the neural crest (Verdi et al., 1996). Thus, overexpression of NUMB increased the

proportion of clones containing neurons in an immortalized multipotential mouse neural crest cell line (MONC-1). Constitutive expression of a putative dominant negative form of NUMB promoted the differentiation of various non-neuronal lineages. However, no asymmetric partitioning of mouse NUMB was observed in the neural crest {but see (Zhong et al., 1996)}. Moreover, overexpression of NUMB did not enhance neuronal differentiation of MONC-1 cells on its own. This effect was only observed under culture conditions which promoted neurogenesis suggesting that environmental signals may also be required for neuronal differentiation in MONC-1 cells.

Molecular analyses have revealed the differential expression of transcription factors, belonging to various gene families such as the *Hox*, *Pax*, *Pou*, *bHLH*, etc., in many neural crest derivatives. Many of these genes are expressed very early and at times consistent with a role for them during neural crest lineage segregation. Furthermore, mutations in many of these genes leads to the elimination of the lineages in which they are expressed. A description of each gene, its pattern of expression and its loss-of-function phenotypes is beyond the scope of this Section. However, certain general issues are worth discussing. Early expression of a transcription factor does not imply a cell autonomous mechanism of cell fate specification. Once the transcription factor is expressed, it may lead to an autonomous lineage differentiation by setting in motion an auto-regulatory cascade of differentiation (Weintraub et al., 1991). However, what is critical to establish is whether the onset of expression of the transcription factor is cell autonomous. In many neural crest lineages, lineage differentiation may be controlled by surrounding tissues. For example, differentiation of sensory and sympathetic neurons may be directed by signals coming from the neural tube or the neighboring mesenchymal structures; similarly, the differentiation of some mesectodermal lineages may be initiated by the surrounding branchial endodermal tissues. The environment may also play a determinative role in melanocyte

differentiation. These data suggest that the onset of expression of transcription factors important in these lineages may be controlled by surrounding tissues. Thus, the early expression of a transcription factor in a lineage does not exclude a cell nonautonomous control of lineage differentiation. Recently, NRSF, a zinc finger protein, has been shown to repress the expression of several neuron-specific genes in non-neuronal lineages (Chong et al., 1995; Schoenherr and Anderson, 1995; Schoenherr et al., 1996). It is, however, expressed in neuroepithelial cells, suggesting that it may control neuronal differentiation in a negative fashion. It will be interesting to see if NRSF does play a role in this process, and whether it influences the segregation of non-neuronal lineages {for example, see (Ciment and Weston, 1985)} from multipotential neural crest cells.

Environmental regulation of neural crest lineage differentiation

There is a huge volume of literature on the effects of the environment in modulating proliferation, differentiation and survival of neural crest cells and their derivatives. I have restricted my discussion to the role of extracellular signals that may mediate the differentiation of the various lineages from crest cells. To understand the process of induction, it is necessary to define the inducing and the responding tissues, the nature of the inducing signal and the temporal and spatial dynamics of the inductive event (Jacobson, 1966). Furthermore, it is important to distinguish between instructive and selective actions of the inductive signal (Gurdon, 1992). An instructive signal should cause the responding cells to differentiate into a particular lineage at the expense of alternative fates. A selective signal leads to the amplification or survival of a particular lineage after it has already differentiated from multipotential cells. This distinction is even more critical if the starting population is heterogeneous in its developmental

potential. As will become clear, these issues have been satisfactorily resolved in a few cases only.

At least two different signals have been suggested to play a differentiative role in sensory neurons from neural crest cells. In early experiments, silastic membranes inserted between the DRG anlage and the neural tube in chick embryos led to the rapid disappearance of all cells in the DRGs (Kalcheim and Le Douarin, 1986). This could be reversed if the silastic membranes were coated with neural tube extract or BDNF, a polypeptide belonging to the NGF family of growth factors (Kalcheim et al., 1987). The authors attributed this solely to a trophic effect of BDNF, although proliferative and differentiative effects were not ruled out. In a related study, done in vitro using quail neural crest cells grown at clonal density, an increase in the number of sensory neurons (as identified by SSEA-1-immunoreactivity and lack of staining for dopamine ß-hydroxylase) per colony was noted in BDNF (Sieber-Blum, 1991). Since the absolute number of cells per colony remained unchanged, an argument was made for an instructive role for BDNF in influencing the commitment of neural crest cells to a sensory lineage. However, this is difficult to distinguish from the known differentiative effects of BDNF on sensory precursors (Wright et al., 1992). Furthermore, since clonal analysis was not performed (i.e., prospectively identified single cells were not exposed to BDNF), it is difficult to rule out an effect of BDNF on differential precursor plating and survival. In mass density experiments of mouse neural crest cells, the cytokine LIF was shown to increase the absolute number of neurons at the end of culture period (Murphy et al., 1991). (These neurons were assigned a sensory phenotype based on their morphology and expression of CGRP, a neuropeptide.) However, since this is a mass culture, it is difficult to rule out an indirect effect of LIF. Furthermore, the trophic effect of LIF on mature sensory neurons (Murphy et al., 1993) makes it difficult to rule out a selective mechanism in mass density experiments. Thus,

while sensory neurogenesis may be dependent on external signals, neither the identity of such a signal nor its mechanism of action are established. The source of such a signal may be the neural tube {see also (Perris et al., 1988)}.

The development of sympathetic neurons from the neural crest has been studied using many different approaches. In order to define the source of a signal that can influence sympathetic differentiation, ablation and tissue recombination experiments have been carried out. These experiments have revealed that the ventral neural tube or notochord and surrounding mesenchyme may play a role in directing the differentiation of sympathetic neurons (Cohen, 1972; Norr, 1973; Stern et al., 1991). Early in vitro experiments of quail cells also suggested that a specific type of extracellular matrix could enhance the numbers of adrenergic cells (Maxwell and Forbes, 1990; Sieber-Blum and Cohen, 1980). In all these experiments, sympathetic neuronal differentiation was assayed by using catecholamine histofluorescence (FIF). This is reasonable given that sympathetic neurons do synthesize norepinephrine, a catecholamine. However, a recent study in chick embryos has demonstrated that the floor plate/notochord is required for expression of TH but not for the development of sympathetic neurons (Groves et al., 1995). Thus, MASH1 and SCG10, a pan-neuronal marker, but not TH, were expressed in the sympathetic ganglia even in the complete absence of notochord and floorplate. It is not clear whether the notochord acts directly on the sympathetic anlage to induce TH. Since the notochord is thought to induce differentiation in a number of neighboring tissues, an alternative source of the TH-inducing signal could be such tissues that have been patterned by the notochord. Regardless of the exact mechanism involved, these results suggested that differentiation of a neuronal phenotype was separately controlled from the acquisition of neuron type-specific traits. In cultures of avian crest, a reverse dissociation is seen: adrenergic cells can develop in vitro, but in general they do not have a neuronal morphology and they do not express pan-neuronal

markers (Christie et al., 1987). The above studies have tried to identify the source and mechanism of action of the signals inducing sympathetic neurogenesis. Using a complementary approach, many investigators have tried to identify the signal that may mediate sympathetic neuronal differentiation. Most of them have continued to rely only on the FIF assay or TH immunocytochemistry to define sympathetic differentiation. However, the foregoing results also suggest that it is important to demonstrate whether cells expressing FIF/TH are sympathetic neurons. Retinoic acid has been identified as one such inducer of the sympathetic phenotype. Using quail clonal cultures, it was demonstrated that there was a significant increase in the number of clones containing TH^+ cells (Dupin and Le Douarin, 1995). However, since the survival of crest clones was very poor, it is hard to argue for an instructive effect of retinoic acid in commitment of neural crest cells to a sympathetic fate. Moreover, several groups have shown an effect of retinoic acid in selectively promoting the proliferation of TH^+ cells in both quail and mouse neural crest (Henion and Weston, 1994; Ito and Morita, 1995; Rockwood and Maxwell, 1996). The interpretation of the effects of retinoic acid on induction of TH is also complicated because retinoic acid can upregulate NGF receptors on immature chick sympathetic neurons and enhance their survival (Rodriguez-Tébar and Rohrer, 1991). Thus, while retinoic acid seems to play a role in the differentiation of the sympathetic phenotype, it is not clear whether it does so by affecting neural crest lineage commitment to a sympathetic fate. Several other molecules have been identified as being capable of modulating the number of TH expressing cells generated from the crest in vitro. I discuss these studies in greater detail in Chapter 5.

Numerous investigators have studied the development of melanocytes from the neural crest. The abundance of naturally occurring pigmentation mutants in many different species and the molecular cloning of many of the genetic loci has improved our understanding of the normal development and functioning of melanocytes (Barsh,

1996). The environment through which neural crest cells migrate to form melanocytes has been shown to have a powerful effect on various aspects of melanocyte differentiation (Fukuzawa and Ide, 1988; Hirobe, 1992; Hirobe, 1994; Mayer, 1973). Steel Factor, a polypeptide that may regulate melanocyte differentiation, is present in the dorsolateral pathway through which neural crest cells migrate. Furthermore, neural crest cells migrating in the dorsolateral pathway express c-Kit, the receptor for Steel Factor (Morrison-Graham and Takahashi, 1993). The genetics confirm the nonautonomous action of *Steel* and the autonomous nature of *Dominant Spotting* (encoding c-Kit) in melanocyte development. However, it is not clear if Steel Factor can direct the differentiation of neural crest cells to a melanocyte lineage. These studies are complicated by the fact that Steel factor may also be a survival factor for undifferentiated neural crest cells and for melanocyte precursors developing in neural crest cultures (Lahav et al., 1994; Langtimm-Sedlak et al., 1996; Morrison-Graham and Weston, 1993). α-melanocyte stimulating hormone (MSH) has been shown to increase the proportion of undifferentiated quail neural crest cells that forms melanocyte-only clones (Satoh and Ide, 1987). However, this study did not rule out an effect of MSH on differential survival and differentiation of melanocyte precursors. Indeed, gain- and loss- of-function mutations in the MSH receptor (*Extension* locus) suggest a role for MSH in late melanocyte differentiation (Jackson, 1993) rather than in commitment of crest cells to a melanocyte lineage. Other growth factors have also been implicated in melanocyte differentiation but, as their effects are poorly understood, they are not discussed here (Morrison-Graham et al., 1992; Takayama et al., 1996).

Recently, a family of small polypeptides, the endothelins, has received attention because of its role(s) in differentiation of several neural crest lineages. Endothelins (EDN 1-3) bind to 7-helix receptors (EDNR A & B). Loss-of-function mutations in EDN 3 or in one of its receptors, EDNR-B, lead to hypopigmentation and aganglionic

megacolon (Baynash et al., 1994; Edery et al., 1994; Hofstra et al., 1996; Hosoda et al., 1994; Puffenberger et al., 1994). EDNR-B is known to be expressed by migratory neural crest cells at most levels of the AP axis and by neural crest cells in the gut (Nataf et al., 1996). In vitro, EDN-3 is mitogenic to naive neural crest cells and may promote their differentiation into melanocytes (Lahav et al., 1996; Reid et al., 1996). It is not known if enteric neurogenesis is induced from neural crest cells in vitro in the presence of EDN 3. The effect of a loss-of-function mutation of EDNR-B on the colonization of the gut by enteric neural crest cells is even more enigmatic since it has been shown to behave in a non-autonomous manner (Kapur et al., 1995). This suggests that the gut environment is also affected in such mutants. A targeted loss-of-function mutation in EDN-1 (which binds to both receptors) in mice leads to severe craniofacial and cardiac abnormalities (Kurihara et al., 1995; Kurihara et al., 1994). This suggests a role for EDN-1 in differentiation of neural crest cells into ectomesenchyme. Consistent with this idea, EDN-1 is expressed in the branchial arches populated by neural crest cells that form ectomesenchyme. The above experiments do not yet provide a clear picture of the exact mechanism of action of endothelins in neural crest differentiation. Forward (Pavan et al., 1995) and reverse genetic analyses combined with tissue culture experiments should lead to dramatic insights into this problem.

The differentiation of the SA progenitors and their derivatives is discussed next {for a detailed review, see (Anderson, 1993)}. Although this involves later steps in development, i.e., after the segregation of the SA lineage from the neural crest, differentiation of this lineage has served as a model system for vertebrate developmental biologists for a long time. It has been known for some time that chromaffin cells can differentiate into sympathetic neurons or retain their chromaffin phenotype depending on culture conditions {for example, see (Doupe et al., 1985; Doupe et al., 1985)}. This suggested that at earlier times in development the chromaffin and sympathetic neuronal

lineages might arise from a common progenitor. Indeed, serial observations of cells obtained from embryonic adrenal glands demonstrated the presence of bipotential SA progenitors. The majority of such progenitors differentiated into sympathetic neurons or into chromaffin cells depending upon the culture conditions, suggesting an instructive influence on cell fate determination in this lineage (Anderson and Axel, 1986). It has also been shown that FGF instructs the cells to become neurons, while glucocorticoids (GC) influence the cells to become chromaffin cells (Anderson and Axel, 1986; Claude et al., 1988; Stemple et al., 1988). Furthermore, it has been demonstrated in an elegant study that the influence of GC on chromaffin differentiation is two-fold (Michelsohn and Anderson, 1992). Initially, GCs inhibit neuronal differentiation of chromaffin precursors. Subsequent differentiation into mature chromaffin cells (as assayed for by the expression of phenyl N-methyl transferase, PNMT) requires higher doses of GCs. However, the onset of expression of PNMT by chromaffin cells in vitro is cell autonomous and coincides with the initiation of PNMT expression by chromaffin cells in vivo. These results suggest that while chromaffin precursors may need an environmental signal to differentiate, the timing of this differentiation is determined cell autonomously.

In a later step during development, a subset of sympathetic neurons acquire a cholinergic phenotype (Patterson, 1978). The change from a noradrenergic to a cholinergic phenotype in vivo is dependent on the target innervated, in this case the periosteal membranes and sweat glands {reviewed in (Rao and Landis, 1993)} and in vitro by the culture conditions (Patterson and Chun, 1977). This phenotypic switch of neurotransmitter expression in vivo requires the presence of the correct target and can be induced to occur in sympathetic neurons that normally do not innervate these targets (Schotzinger and Landis, 1988; Schotzinger and Landis, 1990). Furthermore, the switch can occur in single identified postmitotic sympathetic neurons in vitro and

probably also in vivo (Landis and Keefe, 1983; Reichardt and Patterson, 1977). Several approaches have been used to define this target-derived factor. PCR-based assays have been used to screen large numbers of different cytokines, belonging to different families, in order to identify candidate molecules (Fann and Patterson, 1993). While several such putative target-derived factors have been identified, these factors are not thought to be present at an appropriate time and place in vivo to mediate the phenotypic switch {for example, see (Francis et al., 1997)}. The search for this target-derived factor still continues. The studies of the SA lineage and their derivatives have beautifully demonstrated the intricate meshing of environmental and cell autonomous influences operative at the single cell level during development. Someday our understanding of the differentiation of other crest lineages will approach that attained for the SA lineage.

It is hoped that the above material has not left the reader in a state similar to Jacobson (Jacobson, 1966) who commented, "Embryonic induction is part of a continuum of developmental processes. But the concept of induction, once separated out and named, has suffered reification. A number of papers imply or refer to "the moment of induction," and attention has prematurely shifted from study of the *process* of embryonic induction to a search for "the inductor substance."" The neural crest provides an excellent model system to study inductive mechanisms. Progress in our understanding of the development of this transient tissue, although sometimes painfully slow, is inevitable.

CONCLUDING REMARKS

The foregoing data do not yet provide a clear idea of the logic of neural crest differentiation. However, certain conclusions, proposed at one time or another by

several investigators, can be made (Anderson, 1989; Baroffio and Blot, 1992; Le Douarin et al., 1994; Selleck et al., 1993). The first is that there may be significant differences in the developmental potential of neural crest cells along the AP axis. This may not be surprising given the widely different structures arising from the crest along the AP axis. It will be informative to understand how these differences are initiated and maintained. These processes are likely to be part of the mechanism(s) used to set up AP differences in the CNS. Furthermore, these studies will reveal how pattern generating mechanisms interact with those regulating cell fate specification. The availability of both cellular and molecular assays for crest development should provide unique insights into this problem. Knowledge of how the anterior neural crest produces such specialized structures may also improve our understanding of the evolution of vertebrates from their chordate ancestors.

At a given level of the neuraxis, it is probable that many of the premigratory neural crest cells are multipotential. While there is data suggesting that there may be developmental restrictions in some crest cells, it is not clear whether these represent autonomously distinct populations or a homogeneous population exposed to different environmental factors. The most contentious area of research is likely to be that demonstrating the progressive loss of developmental potential in multipotential crest cells. For reasons outlined above, it is difficult to prove that cells are oligopotent. The increase in the number of antigenic markers available may allow one to demonstrate the progressive generation of developmentally restricted precursors. Two other issues regarding oligopotent intermediates are worth raising. How is oligopotency determined at the molecular level? Are oligopotent intermediates obligatory in the differentiation program of multipotential neural crest cells? The recent use of zebrafish as an embryological and genetic organism will undoubtedly bring about an increased

understanding of mechanisms of neural crest differentiation (Henion et al., 1996; Raible and Eisen, 1994).

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Section B

Regulatory mechanisms in stem cell biology

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Regulatory Mechanisms in Stem Cell Biology

Review

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Introduction

Stem cells are a subject of intense and increasing interest because of their biological properties and potential medical importance. Unfortunately, the field has been difficult for the nonspecialist to penetrate, in part because of ambiguity about what exactly constitutes a stem cell. A working definition is useful in order to pose the important questions in stem cell biology. However, since different people define stem cells in different ways (for examples, see Hall and Watt, 1989; Potten and Loeffler, 1990), formulating a generally acceptable definition can lead to a conclusion similar to that of U. S. Supreme Court Justice Byron White's in regard to pornography: "It's hard to define, but I know it when I see it." A minimalist definition is that stem cells have the capacity both to self-renew and to generate differentiated progeny. Although this is in many respects inadequate, it immediately highlights some important problems: How at each cell division is a stem cell able to pass on its "stem" properties to at least one of its two daughters? And what determines whether stem cell divisions will be self-renewing, or differentiating?

In considering these and related questions, we will draw primarily on examples provided by stem cells in the mammalian hematopoietic and nervous systems, as well as by C. elegans. The focus on hematopoiesis and neurogenesis reflects the fact that these systems are the ones in which stem cells have been most rigorously and directly identified. Hematopoietic stem cells (HSCs) have been isolated using antibodies to cell surface antigens (Spangrude et al., 1988), and their functional properties have been established by transplantation into lethally irradiated host animals under conditions where the progeny of a single stem cell can be identified ("clonogenic" assays; for review, see Morrison et al., 1994). The self-renewal properties of these cells have been demonstrated by serial transfer into secondary recipients.

The brain has not traditionally been considered a stem cell system because of the dogma that this tissue is incapable of regeneration. Recently, however, there has been a rediscovery of Altman's original observations (Altman, 1969) that some regions of the adult brain exhibit ongoing neurogenesis, and this has been accompanied by a surge of activity in identifying the progenitor cells responsible for both embryonic and postnatal neural development (for reviews, see Alvarez-Buylla and Lois, 1995; Gage et al., 1995; Weiss et al., 1996). Stem cells in the neural crest (Stemple and Anderson, 1992) and embryonic central nervous system (CNS) (Davis and Temple, 1994; Johe et al., 1996; Reynolds and Weiss, 1996) have been identified using in vitro assays in which the differentiation and self-renewal capacity of single progenitor cells have been demonstrated by subcloning experiments. It is not yet clear, however, whether any of these neural stem cells can generate all the different classes of neurons found in the adult CNS or PNS, nor is it clear whether the stem cells isolated from adult brain tissue manifest their multilineage differentiation capacity under physiological conditions in vivo.

The existence of stem cells in the gut (Potten and Loeffler, 1990), gonads (Dym, 1994), skin (Lavker et al., 1993), and olfactory epithelium (Monti Graziadei and Graziadei, 1979) has been demonstrated indirectly by mosaic in vivo lineage–marking experiments, anatomical studies, or in vitro experiments. Although the standard of proof defined for HSCs or neural stem cells has not yet been achieved, one can proceed on the assumption that stem cells exist in these tissues. It has also been proposed that stem cells exist in the liver (Sigal et al., 1992), a tissue which can regenerate in response to injury, although this is controversial (Wilson, 1996) because under most conditions differentiated cell types reenter the cell cycle and contribute the preponderance of regeneration.

Properties of Stem Cells

A number of properties besides self-renewal and differentiation potential are frequently ascribed to stem cells, including the ability to undergo asymmetric cell divisions, exhibit extensive self-renewal capacity, exist in a mitotically quiescent form, and clonally regenerate all of the different cell types that constitute the tissue in which they exist (Hall and Watt, 1989; Potten and Loeffler, 1990). Below, we illustrate how many of these properties are exhibited by stem cells in some tissues or organisms, but not in others. This helps to distinguish the most fundamental questions in stem cell biology from questions that are highly relevant but specific to certain systems. It also illustrates the difficulty in arriving at a universally applicable definition of a stem cell. While some readers will undoubtedly take issue with this point of view, a certain tolerance of ambiguity in the definition of stem cells is necessary in order to remain focused on the mechanistic questions and avoid semantic arguments.

Symmetric Versus Asymmetric Divisions

Stem cells are often thought to undergo repeated, intrinsically determined asymmetric cell divisions that produce one differentiated (progenitor) daughter and another daughter that is still a stem cell (Figure 1A). While there are clear examples of such lineages in Hirudo medicinalis, Drosophila melanogaster, and Caenorhabditis elegans, in mammalian systems there is stronger evidence that stem cells divide symmetrically (Figures 1B and 1C). Symmetric divisions allow the size of the stem cell pool to be regulated by factors that control the probability of self-renewing versus differentiative divisions (for more detailed discussion, see Potten and Loeffler, 1990).

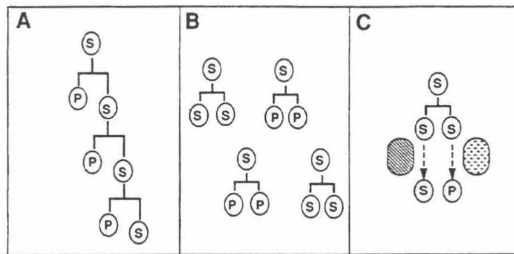

Figure 1. Possible Patterns of Cell Division in Stem Cell Lineages

"S" indicates stem cell; "P" indicates a committed or restricted progenitor cell.

(A) All divisions are obligatorily asymmetric and controlled by a cell-intrinsic mechanism. Note that no amplification of the size of the stem cell population is possible in this type of lineage.

(B) A population of four stem cells is shown in which all divisions are symmetric, but half the time are self-renewing. The steady-state behavior of this population is indistinguishable from that of a population of stem cells like that shown in (A). However, the probabilities of self-renewing versus differentiative divisions could in principle be different than 0.5 (see Potten and Loeffler, 1990, for further discussion).

(C) A lineage in which individual stem cell divisions are asymmetric with respect to daughter cell fate, but not intrinsically so, as in (A). The daughters behave differently owing to different local environments (shaded ovals). Examples of all of the patterns in (A)–(C) are found in nature, including combinations of (B) and (C).

Self-Renewal Capacity

Murine HSCs do not have unlimited self-renewal potential, although a subset is able to self-renew for the lifetime of a mouse (for review, see Morrison et al., 1994). However, in larger, longer-lived animals, such as humans, it is not at all clear that HSCs self-renew for an entire lifespan; rather, successive subsets of stem cell clones may become activated with increasing age (Abkowitz et al., 1990). Even in small, shorter-lived organisms, there is clear evidence that stem cells have lifetimes less than that of the entire animal. For example, one of the two somatic stem cells in the Drosphila ovary dies or differentiates after about 26 days (Margolis and Spradling, 1995). Thus, not all stem cells have unlimited self-renewal potential.

In tissues where serial transplantation of isolated cells is not technically possible, it is often difficult to assess the self-renewal capacity of putative stem cells in vivo. The mere existence of progenitor cells in an adult tissue is not de facto evidence that these cells have undergone extensive self-renewal, as is sometimes assumed, because they may simply have persisted in quiescent form. There are, moreover, clear cases of stem cells that exist only transiently during development, such as fetal and embryonic HSCs. Oocyte production ceases by birth, while that of sperm continues into adulthood, yet both cells derive from primordial germ cells (PGCs) whose stem cell properties are indistinguishable in males and females early in gestation (Donovan, 1994). Thus, not all stem cells self-renew into adulthood, and not all adult stem cells reflect self-renewal of fetal cells. Finally, in some cases, adult stem cells may derive neither by self-renewal nor by persistence of fetal cells, but rather may represent a distinct stem cell class that develops from a transient fetal stem cell population (Morrison et al., 1994). This makes the entire concept of self-renewal capacity "for the lifetime of the organism" precarious as a criterion for stem cells.

Mitotic Quiescence

Another property shared by some, but not all, stem cells is that they divide slowly or rarely. This is thought to be true for stem cells in the skin (Lavker et al., 1993) and bone marrow (Morrison and Weissman, 1994). Other kinds of stem cells, however, divide more rapidly. Somatic stem cells in the Drosophila ovary and mammalian intestinal crypt stem cells have been estimated to divide every 12 hr (Potten and Loeffler, 1990; Margolis and Spradling, 1995). It may be generally true that stem cells in adult tissues are more likely to cycle slowly, but this quiescence is not an obligatory property of stem cells.

"Mother of All Cells"

Another characteristic attributed to stem cells is the ability to regenerate clonally the entire adult tissue from which they derive, meaning all cell types that constitute that tissue (Potten and Loeffler, 1990). In practice, this is an extremely difficult criterion to satisfy. Even in the hematopoietic system, for example, certain classes of blood cells—such as some kinds of T cells—are only produced during fetal life and are maintained in the adult by proliferation of committed cells (Ikuta et al., 1990). Therefore, adult HSCs can replace most, but not all, blood cells found in the adult tissue (reviewed in Morrison et al., 1994). The mature olfactory epithelium consists of neurons and sustentacular (glial) cells, but retroviral lineage analysis has shown that only the neurons are regenerated from stem cells in the basal layer (Caggiano et al., 1994). These examples illustrate cases where stem cells regenerate only a subset of the differentiated cell types in a given tissue. We suggest that stem cells include all self-renewing progenitor cells that have the broadest developmental potential available within a particular tissue at a particular time.

Some authors do not consider all self-renewing pluripotent progenitors to be stem cells, reserving this category only for the subset with the "most primitive" characteristics. This results in a trend to restrict incrementally the stem cell definition to smaller and smaller subsets of cells. The concept of a most primitive progenitor is inherently ambiguous because it often is based on largely untested expectations about the properties that correlate with primitiveness. If we are to understand the biology of self-renewal and pleuripotency, then all self-renewing pluripotent progenitors in a given tissue should be studied.

Regenerative Capacity

It has been argued that only regenerative tissues can have stem cells. The most significant problem with this definition is that certain tissues or at least certain cell types exhibit regenerative capacity only during limited windows of ontogeny (e.g., the spinal cord [Sechrist et al., 1995], or female germ line [Donovan, 1994]). It seems arbitrary to exclude certain classes of progenitor cells from consideration simply because they display their regenerative capacity at one stage of development but not at others. The failure of regeneration in the adult may be due not to the absence of pluripotent, self-renewing cells, but to the inability of the injured tissue

to accomodate or promote their differentiation, as may well be the case in most areas of the brain (Alvarez-Buylla and Lois, 1995; Gage et al., 1995; Weiss et al., 1996).

These considerations reinforce the idea that there are basic common properties of stem cells that extend across diverse species, tissues, and developmental stages: the capacity to self-renew and to generate progeny that are fated to differentiate into mature cells. This raises the question of whether there are common molecular mechanisms, shared by all stem cells, that underly these properties. Other properties, such as the ability to divide asymmetrically, to undergo extensive self-renewing divisions, to exist in a quiescent rather than mitotically active state, and to generate a multiplicity of differentiated derivatives, are exhibited by some classes of stem cells, but not by others.

Control of Self-Renewal

Self-renewal potential is the most fundamental property of stem cells. However, to understand self-renewal it is not sufficient simply to understand how stem cell proliferation is controlled, because not all cell divisions involve self-renewal. Are there specific signals that couple mitogenesis to maintenance of the stem cell state? Or are proliferation and maintenance of the stem cell state regulated independently by distinct signals? These issues are important because although the size of the stem cell pool remains nearly constant in many tissues under steady-state conditions, it can expand rapidly in response to tissue damage (Harrison and Lerner, 1991; Paulus et al., 1992; Lavker et al., 1993; Grisham and Coleman, 1996).

Extrinsic Regulation of Self-Renewal

What limits the number of stem cells under steady-state conditions? One possibility is that stem cells can only exist in a restricted microenvironment in each tissue, which provides factors that maintain them and excludes factors that induce differentiation (Trentin, 1970). For example, intestinal epithelium stem cells appear to be localized to a narrow ring of tissue near the base of the crypts (Potten and Loeffler, 1990). If the amount of space in such microenvironments (or "niches") is limited, the number of stem cells would be limited by the number that can fit in that space. Stem cells generated in excess of the available space would differentiate (Williams et al., 1992). Evidence for such a mechanism is scant in mammals, but in C. elegans the self-renewal of germ line stem cells requires proximity to the distal tip cell (Kimble et al., 1992), which produces a ligand that promotes stem cell divisions (see below). Not all stem cell systems, however, utilize such local control mechanisms. For example, PGCs self-renew while migrating to the genital ridges (Tam and Snow, 1981).

The proliferation of stem cells also increases in response to tissue damage. For example, in the sensory epithelia of the nose (Monti Graziadei and Graziadei, 1979) and the inner ear (Forge et al., 1993), damage to the primary sensory neurons induces the proliferation of cells that regenerate the lost neurons. In principle, the induction of division in such systems could be promoted either by the release of mitogens from dying cells, or by relief from inhibitors normally produced by healthy neurons (or both); no evidence yet exists to distinguish among these possibilities. It is also assumed that such feedback control of stem cell proliferation is local, either by direct signaling to the stem cells or by indirect signaling via intermediate progenitor compartments (discussed in more detail in Potten and Loeffler, 1990).

Identity of Factors That Control Stem Cell Self-Renewal and Their Mechanisms of Action

In C. elegans, the germ line stem cells require activation of the Notch-related receptor GLP-1 to retain self-renewal potential. The ligand for GLP-1, LAG-2, is membrane bound and expressed only by the neighboring distal tip cell (Henderson et al., 1994). In *glp-1* mutants, germline stem cells not only cease self-renewing mitoses, but also undergo meiosis and differentiate into gametes (Crittenden et al., 1994). Thus, LAG-2 appears to be necessary both to maintain proliferation and prevent differentiation of stem cells. By contrast, genetic studies of *Notch* (a *glp-1*-related gene) in Drosophila have been interpreted to suggest that its primary role is to maintain cells in an undifferentiated state, whether or not those cells are actively dividing (Artavanis-Tsakonas et al., 1995). Consistent with this, activated forms of mNotch, a murine homolog of GLP-1, inhibit differentiation of myogenic and neurogenic cell lines without a detectable effect on cell proliferation (Kopan et al., 1994; Nye et al., 1994). However, lineage-specific expression of an activated form of human *Notch*, *tan-1*, is found in tumors of primitive lymphoid cells in humans (Ellisen et al., 1991). Taken together, these data suggest that Notch and its homologs can regulate proliferation or maintenance of the undifferentiated state, or both, depending on context.

Although a number of growth factors can drive quiescent HSCs into cycle, despite a vigorous search no factors have yet been identified that (singly or in combination) are capable of maintaining self-renewing divisions of these stem cells in vitro. In the nervous system, EGF promotes proliferation of stem cells from the adult CNS (Reynolds and Weiss, 1992), and basic fibroblast growth factor (bFGF) promotes the self-renewal of embryonic as well as adult CNS stem cells (Gritti et al., 1996; Johe et al., 1996). bFGF also promotes proliferation of primordial germ cells in culture (Resnick et al., 1992), although it also appears to broaden their developmental potential (Donovan, 1994). While these studies have been performed in vitro, they demonstrate that factors do exist that can cause stem cells to self-renew repeatedly when they would otherwise remain quiescent or differentiate.

Stem cell self-renewal can also be negatively regulated by locally acting or long-range factors. In tissues where stem cells have a restricted location, locally acting factors have been sought. For example, proliferation of primordial germ cells and intestinal crypt stem cells is thought to be inhibited by local sources of transforming growth factor β (TGFβ) (Godin and Wylie, 1991; Podolsky, 1993). Both short- and long-range feedback mechanisms are hypothesized to regulate negatively HSC self-renewal (Zipori, 1992). Macrophage inhibitory protein 1α, constitutively produced by macrophages, has been shown to inhibit the proliferation of multipotent progenitors (Graham et al., 1990); whether this inhibition occurs

locally or at long range is not yet clear. Since HSCs are segregated among different bones and organs throughout the body, at least some factors that regulate self-renewal must act at long range for the stem cell pool to be regulated in a coordinated fashion.

In summary, factors that regulate stem cell self-renewal can induce or inhibit proliferation, and can act locally or at long range. Few of the factors involved have been identified. In cases where factors have been identified, it is usually not known what cells produce them, or how their production is regulated. It will be interesting to determine whether there are systematic differences in stem cell regulation between tissues with relatively invariant architecture, like intestinal crypts, and those with more flexible architecture, like the hematopoietic system.

Do Stem Cells Have Intrinsic Limitations on Their Self-Renewal Capacity?

The self-renewal capacity of certain stem cells may exceed the extent of self-renewal that they actually undergo in vivo. Does that mean that self-renewal capacity is unlimited, or are there limitations on self-renewal capacity even when that capacity exceeds actual self-renewal fate? The hematopoietic system clearly exemplifies that not all pluripotent stem cells have equivalent self-renewal capacities. Individual HSCs can exhibit either transient (< 8 weeks) or long-term (> 16 weeks) self-renewal capacity (Harrison and Zhong, 1992). This difference was proposed to depend on the environment encountered by intrinsically similar cells (Uchida et al., 1993). However, fractionation of HSCs by surface marker expression has revealed distinct subpopulations that exhibit different self-renewal capacities even when the cells are exposed to equivalent environments in vivo (Morrison and Weissman, 1994), implying that these differences are cell intrinsic.

The molecular basis of self-renewal capacity remains to be elucidated. Even in cases where this has been shown to be an intrinsic property of stem cells, the molecules need not act in a purely cell-autonomous way. For example, differential expression of adhesion molecules could cause different HSC subpopulations to home to different bone marrow microenvironments that specify different self-renewal fates. Entirely cell-autonomous mechanisms may, however, be at work as well. Telomerase expression widely correlates with self-renewal potential in many cell types (Morrison et al., 1996a; Yasumoto et al., 1996). Recently, about 70% of fetal liver or bone marrow HSCs, but only rare non-self-renewing multipotent progenitors, were shown to exhibit telomerase activity (Morrison et al., 1996a). Unlike tumor cells, HSCs are not immortal (Ogden and Micklem, 1976), and human HSCs show decreasing telomere length with increasing age (Vaziri et al., 1994). Thus, telomerase may regulate self-renewal capacity by reducing the rate at which telomeres shorten. Stem cells with long telomeres could, nevertheless, be caused to differentiate and exit the stem cell pool by other factors.

Maintenance of the Uncommitted State by Intrinsic Factors

There is strong evidence for cell-intrinsic factors that can maintain the uncommitted nature of the stem cell state without influencing proliferation. Germline progenitors in the C. elegans embryo undergo asymmetric divisions that maintain the germline lineage and produce a series of progenitor cells that become committed to various somatic fates (for review, see Guo and Kemphues, 1996). This asymmetric segregation of daughter cell fates appears to be determined by the nuclear protein PIE-1, which is maternally inherited and asymmetrically distributed to the germline daughter cells (Mello et al., 1996). PIE-1 represses the transcription of embryonic genes that cause commitment to particular somatic fates (Seydoux et al., 1996). Thus, one mechanism for maintaining the stem cell state is to actively repress genes required for commitment. Transmission of this state to daughter stem cells would require a mechanism for maintaining expression of such active repressors.

Evidence for Asymmetric Cell Divisions

As mentioned earlier, it is often assumed (incorrectly) that all stem cell lineages necessarily involve intrinsically asymmetric divisions (Figure 1A). There are several well-documented examples of such lineages in invertebrates, including C. elegans germline blastomeres (Mello et al., 1996; Seydoux et al., 1996) and Drosophila neural precursors (Rhyu et al., 1994; Spana et al., 1995). However, in mammals, there are very few examples of asymmetric stem cell divisions. In the ferret cerebral cortex, time-lapse films have revealed that some progenitor cells divide to generate one daughter that remains in the ventricular zone, and another that migrates away, presumably to differentiate to a neuron (Chenn and McConnell, 1995). Such asymmetric divisions are correlated with an orientation of the mitotic spindle perpendicular to the surface of the ventricle. The further observation that a mammalian homolog of Notch1 is asymmetrically distributed on some ventricular zone cells prior to cytokinesis (Chenn and McConnell, 1995) suggests that at least some molecules are unequally distributed to the daughter cells (although it does not mean that the orientation of this distribution is independent of environment). Asymmetric divisions of multipotent hematopoietic progenitors have also been observed in clone-splitting experiments (Mayani et al., 1993).

Molecular Determinants of Asymmetry. In Drosophila neuroblasts, asymmetric cell divisions are dependent upon correct mitotic spindle orientation, as well as on the asymmetric distribution of several proteins, such as numb and prospero (reviewed in Doe and Spana, 1995). The asymmetric distribution of numb and prospero is in turn controlled by additional regulators, such as inscuteable (for review, see Doe, 1996). Mammalian homologs of numb have been isolated (Verdi et al., 1996; Zhong et al., 1996), and one is asymmetrically distributed in some cortical progenitor cells (as well as in cells in other, non-neural tissues) (Zhong et al., 1996), suggesting that some asymmetric divisions in mammals may also be intrinsically determined. Distinct molecular determinants of asymmetric cleavages have also been identified in C. elegans and yeast (reviewed in Horvitz and Herskowitz, 1992; Guo and Kemphues, 1996), but whether these have been conserved in mammals as well is not yet known. Apparently asymmetric divisions can also reflect intrinsically symmetric divisions that place the daughter cells in different environments that confer different fates (Figure 1C). While such a mechanism has

been shown to control the fate of somatic blastomeres in C. elegans embryos at the four-cell stage (Priess and Thomson, 1987; Mickey et al., 1996), direct evidence for such a process in vertebrates is lacking.

Are Asymmetric Cell Divisions the Rule or the Exception? Despite the recent attention to asymmetric stem cell divisions, the available evidence favors a predominance of symmetric divisions in mammalian stem cell systems (Figure 1B). In strictly asymmetric stem cell lineages (Figure 1A), no regulation of stem cell number is possible. But there is ample evidence for such changes in the size of stem cell populations in mammals, implying that symmetric divisions must occur. The absolute number of fetal liver HSCs doubles daily during midgestation (Morrison et al., 1995), and during adult life in mice there is a more than five-fold increase in the absolute number of long-term self-renewing HSCs (Morrison et al., 1996b). Primordial germ cells undergo at least five rounds of symmetric self-renewing divisions while they migrate into the genital ridges during fetal development (Tam and Snow, 1981).

Some mammalian stem cell populations may undergo both symmetric and asymmetric divisions, depending on their circumstances. Indeed, neural progenitors in the ferret cortex undergo both symmetric and asymmetric divisions (Chenn and McConnell, 1995). The relative proportion of symmetric divisions appears to change over time, with symmetric divisions predominating at early time points when the stem cell pool would be expected to be expanding (Chenn and McConnell, 1995; Takahashi et al., 1996). Whether this indicates that a single cell can switch from a symmetric to an asymmetric mode of cell division is not yet clear.

Control of Stem Cell Survival

As mentioned earlier, the persistence of stem cell populations throughout adulthood likely depends on the survival of quiescent cells, as well as on the ability of cycling cells to self-renew. Evidence for quiescent stem cells has been presented in the liver (reviewed in Grisham and Coleman, 1996), the brain (Morshead et al., 1994), and in bone marrow (Morrison and Weissman, 1994). However, it is still not clear whether such apparently quiescent cells are really in G_0 or whether they are just moving very slowly through G_1. Are there factors that promote stem cell survival, but not necessarily self-renewal? By itself, steel factor (also known as stem cell factor) promotes the survival, but not the proliferation, of HSCs (Keller et al., 1995) and primordial germ cells (Dolci et al., 1991; Godin et al., 1991); however, the regulation of these effects is likely to be complex, since steel factor is not required for the survival of HSCs and can synergize with other factors to promote stem cell proliferation (Ikuta et al., 1991; Resnick et al., 1992). Intestinal crypt (Leigh et al., 1995) and liver stem cells (Fujio et al., 1994) are also regulated by steel factor. These data raise further questions about the regulation of steel factor expression and its combinatorial action with other factors. As more factors are identified, the control of stem cell survival is likely to become an increasing focus of investigation.

Control of Stem Cell Differentiation

This section will address the main outstanding questions concerning the differentiation of stem cells. What sets the repertoire of potential fates available to a stem cell in a given tissue? How do stem cells choose to exit the stem cell state and begin to differentiate? In cases of multipotent stem cells, how is the choice of a particular differentiated fate made?

Determination of the Repertoire of Potential Stem Cell Fates

The overall developmental potential of a stem cell is defined by all the types of differentiated progeny it can ultimately give rise to. How is this property encoded in the stem cell in molecular terms? One possibility is that multipotent stem cells might express a set of transcription factors which individually specify different lineages or combinations of lineages. For example, mutations in the *ikaros* gene, which encodes a zinc finger protein present in HSCs, prevent the development of multiple lymphoid derivatives (Geogopoulos et al., 1994). However, it is not yet clear whether *ikaros* acts in HSCs themselves, or is independently required in multiple lymphoid sublineages at later stages of development. The entire developmental repertoire of a given multipotent stem cell could also, in theory, be specified by a single determining factor that sits at the top of a regulatory hierarchy. A targeted mutation in the bHLH transcription factor SCL prevents the development of all hematopoietic derivatives (Porcher et al., 1996), but it is not yet known whether SCL is expressed in HSCs, and, if so, required for their formation, self-renewal, or differentiation. From an evolutionary standpoint, mutations that increased the developmental repertoire of stem cells could lead to increased cellular diversity in a tissue by "duplication and modification" of cell types.

In tissues where different cell types are generated from a multipotent progenitor on a relatively precise schedule, such as the retina, multipotent cells may be competent to generate only one or two specific fates in a given period of development (for review, see Cepko et al., 1996). For example, all retinal cell types derive from multipotent progenitors (Turner and Cepko, 1987), but the competence of these progenitors to respond to environmental signals changes over time (Cepko et al., 1996). There are clear cases where competence is determined by the expression of receptors necessary to respond to fate-determining signals, but this need not always be so; in principle, competence may also be determined by expression of signal transduction molecules or transcription factors. However, there are few specific examples of this type.

How Do Stem Cells Initiate the Differentiation Process?

The differentiation of stem cells involves both exit from the uncommitted state and entry into a particular developmental pathway. Evidence from C. elegans indicates that these two aspects are independently controlled. Exit from the stem cell state requires loss of PIE-1, a zinc finger protein that represses the expression of genes involved in commitment to differentiation (Mello et al., 1996; Seydoux et al., 1996). This loss occurs by asymmetric distribution of PIE-1 to stem cell daughters at each blastomere division. However, the absence of PIE-1 in somatic blastomere daughters is insufficient to initiate a program of differentiation: positive-acting transcriptional regulators, such as SKN-1 (Bowerman

et al., 1993), are also required to promote entry into a particular somatic lineage.

It is not yet clear whether exit from the stem cell state and initiation of differentiation are also independently controlled in mammals. At one extreme, differentiation might be a "default" pathway executed by the stem cell when it is removed from a microenvironment that promotes maintenance of the uncommitted state. At the other extreme, specific signals might promote differentiation and consequently exit from the stem cell state. There is evidence that both mechanisms operate in the nervous system. In vitro, CNS stem cells undergo self-renewing divisions in bFGF, but upon withdrawal of this growth factor they rapidly differentiate to neurons (Johe et al., 1996). On the other hand, the differentiation of cultured neural crest stem cells to autonomic neurons is promoted by BMP2 (Shah et al., 1996; see below). These examples leave open the question of whether the effect of such environmental signals is to regulate transcription factors that maintain the stem cell state (analagous to PIE-1), or factors that promote entry into particular lineages, or both. In either case, such factors are likely to be subject to both negative and positive regulation by environmental signals, which may explain the different effects of such signals on cell fate decisions by CNS and PNS neural stem cells.

How Do Multipotent Stem Cells Select a Particular Differentiation Pathway?

The choice of fate by a multipotent stem cell could, in principle, be controlled from inside or outside the cell. There is ample evidence from invertebrate systems that such choices can be determined nonautonomously by local cell-cell interactions. For example, in C. elegans, an EGF-like signal produced by the gonadal anchor cell specifies the fate of vulval precursor cells (for review, see Kenyon, 1995). Similarly, in Drosophila, the choice between cone (glial) and photoreceptor cell fates is determined by a transmembrane ligand, BOSS, presented by the R8 photoreceptor (Zipursky and Rubin, 1994). While these examples concern cells that do not exhibit the self-renewal capability necessary to fit our definition of stem cells, they nevertheless provide important examples of how extrinsic signals can regulate fate determination in multipotent progenitors.

Selective Versus Instructive Actions of Growth Factors on Mammalian Stem Cells. In mammalian systems, there is considerable evidence that growth factors and cell-cell interactions can influence the outcome of fate decisions by multipotent progenitors at the population level. This raises a problem not encountered in invertebrate systems where the fates of individual cells are easily monitored. Specifically, growth factors could influence individual stem cells in a selective or instructive manner (Figure 2). In a selective mechanism, the stem cells commit to a particular lineage independently of the growth factors, and the factors act subsequently to control the survival or proliferation of such committed progenitors (Figures 2A and 2B). In an instructive mechanism, the growth factor causes the progenitor to choose one lineage at the expense of others (Figures 2C and 2D). In hematopoiesis, the relative contributions of these two mechanisms remain controversial (see Metcalf, 1991; Mayani et al., 1993). Forced expresson of *bcl-2* in an

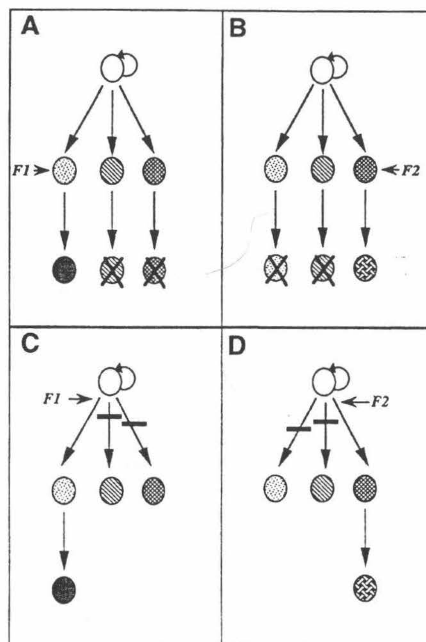

Figure 2. The Difference Between Selective and Instructive Mechanisms of Growth Factor Influences on Stem Cell Fate Decisions

(A and B) Selective mechanism in which two different factors (F1 and F2) allow the survival and maturation of lineage-committed progenitors generated by a cell-autonomous mechanism; "X" indicates death of the other progenitors. Erythryopoietin appears to work in this manner (Wu et al., 1995).

(C and D) Instructive mechanism in which the factors cause the stem cell to adopt one fate at the expense of others. Glial growth factor and BMP2 appear to work in this manner on neural crest cells (Shah et al., 1994, 1996).

immortalized hematopoietic progenitor cell line yielded multilineage differentiation in the absence of cytokines, implying that these growth factors act selectively (Fairbairn et al., 1993). In the neural crest, by contrast, serial observation of individual clones in vitro has indicated that differentiation to each of three cell types—autonomic neurons, Schwann (glial) cells, and smooth muscle—can be instructively promoted by three signals: BMP2, GGF (a neuregulin), and TGFβ, respectively (Shah et al., 1994, 1996). Similarly, the differentiation of CNS stem cells to astrocytes is instructively promoted by CNTF (Johe et al., 1996). It remains to be determined whether growth factors influence stem cells in the nervous system and hematopoietic system in fundamentally different ways, or whether instructive differentiation signals for HSCs have simply not yet been identified owing to lack of appropriate assays.

Instructive Factors Can Influence Differentiation Choices Whose Outcomes Are Stochastic. Instructive environmental signals may increase or decrease the probabilities of choosing a particular fate, rather than promote or repress them in an all-or-none manner. In nematodes, the binary decision between ventral uterine (VU) and anchor cell (AC) fates by neighboring precursor cells is controlled by lateral signaling, mediated by the

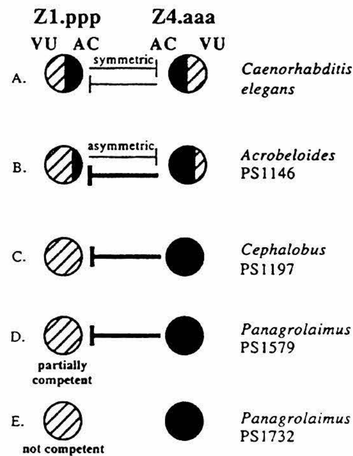

Figure 3. Phylogenetic Variation in the Control of a Binary Cell Fate Decision in Nematodes

In each case (A–E), a choice between ventral uterine (VU) and gonadal anchor cell (AC) fates is made by adjacent precursors (called "Z1.ppp" and "Z4.aaa"). In C. elegans (A), the decision is stochastic with a 50:50 probability and nonautonomously controlled by lateral signaling. In Acrobeloides (B), lateral signaling exerts a partial bias on a stochastic decision, so that the probability is about 80:20. In Cephalobus (C), the decision is deterministic yet nonautonomously controlled, while in Panagrolaimus PS1732 (E) it is both deterministic and autonomously controlled. (D) represents an intermediate case between (C) and (E) where the decision is deterministic, but displays autonomy only some of the time in laser-ablation experiments. Although the precursor cells involved do not meet our criteria for a stem cell, they illustrate how the same cell fate decision can be either stochastic or deterministic and controlled by autonomous or nonautonomous mechanisms. Reprinted with permission (from Felix and Sternberg, 1996).

NOTCH-like protein LIN-12 and its ligand LAG-2 (Figure 3). In some species, such as Cephalobus, this cell–cell interaction produces a deterministic (invariant) outcome (Figure 3C): the same precusor always adopts the VU fate in every animal of the species (Felix and Sternberg, 1996). In others (Acrobeloides), a similar cell–cell interaction produces a stochastic (probabilistic) outcome exhibiting bias (Figure 3B): one precursor becomes the anchor cell roughly 80% of the time (Felix and Sternberg, 1996). Finally, in C. elegans, the outcome is stochastic and unbiased: each precursor has a 50:50 probability of adopting either fate (Figure 3A). In all three cases, the cell–cell signaling is instructive, since in the absence of one precursor the other always adopts the AC fate (Felix and Sternberg, 1996). Thus, in different species, instructive signaling can exert a range of bias strengths on stochastic cell fate decisions. Similarly, it has been proposed that the engagement of MHC molecules with either the CD4 or CD8 coreceptors may exert a bias on a stochastic decision by T-cell progenitors between helper and killer cell fates (Davis and Littman, 1994).

It is sometimes assumed that if differentiation is stochastic and unbiased, a cell-autonomous mechanism must be at work. However, in C. elegans, the unpredictability of the outcome of the AC/VU decision derives from the equivalent strength of the reciprocal inhibitory interactions between AC/VU precursors (Felix and Sternberg, 1996) (Figure 3A). Similarly, where cell-autonomous mechanisms have been inferred from the apparently stochastic behavior of hematopoietic progenitors in vitro (see Suda et al., 1983; Mayani et al., 1993), the cells are usually cultured in complex media containing serum and other sources of undefined factors, and the collective influence of such environmental factors could cause the cells to behave in an apparently unpredictable (stochastic) manner.

Autonomous Control of Cell Fate. A selective action of environmental factors implies that the initial choice of differentiated fate by a stem cell is controlled by a cell-autonomous mechanism. Such an intrinsic mechanism may yield a stochastic outcome, as has been suggested for HSCs, or a deterministic outcome. In yeast, the mating-type switch is a cell-autonomous fate decision that appears stochastic at the population level, but is deterministic for individual cells according to their previous history (Herskowitz, 1989). In early C. elegans embryos, the assignment of somatic blastomere fate is determined in an autonomous and deterministic manner by the asymmetric partitioning of transcription factors at successive cleavages (Bowerman et al., 1993; Hunter and Kenyon, 1996). Currently there are no clear examples of such cell-autonomous mechanisms operating in a mammalian stem cell.

There are, of course, many examples of transcription factors required for the development of particular mammalian lineages. Although once expressed these factors may impose a cell-heritable and autonomous state of determination on a progenitor cell, the initial decision to express such factors may be nonautonomously controlled. For example, the bHLH transcriptional regulator myoD is able to confer a cell-heritable state of myogenic determination, owing to its autoregulatory properties, when transfected into cultured fibroblasts (Weintraub et al., 1991). However, in vivo, the expression of this protein in somitic mesoderm is induced by a combination of signals from neighboring tissues, such as the notochord and neural plate (reviewed in Molkentin and Olson, 1996). Moreover, the execution of the muscle differentiation program in determined myoblasts is still regulated by growth factors (Molkentin and Olson, 1996). Thus, the involvement of lineage-specific transcription factors does not imply that either selection or execution of specific fates are autonomously controlled.

Order and Pattern in the Segregation of Different Lineages from Stem Cells

In principle, multipotent stem cells could generate different derivatives in a random manner (Figures 4A and 4C), or according to a predictable sequence or hierarchy (Figures 4B and 4D). There is evidence for both mechanisms in different systems. In grasshopper, the midline neuroblast sequentially produces neurons, glia, and neurons again (Condron and Zinn, 1994). In the vertebrate retina, different cell types emerge on a predictable schedule (Cepko et al., 1996), although whether individual progenitors generate their differentiated progeny in a fixed order is not yet clear. In contrast, clone-splitting experiments in vitro have suggested that there is no perceptible order or pattern to the emergence of different lineages from multipotent hematopoietic progenitors (Suda et al., 1983), although since no lymphoid

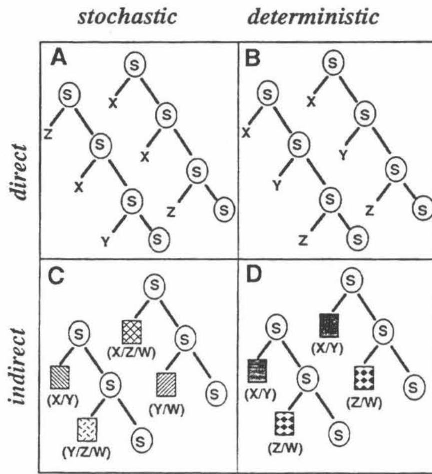

Figure 4. Alternative Modes of Differentiation by Multipotent Stem Cells

In each panel, two equivalent stem cells in a population are shown. In a "direct" mode (A and B), the immediate progeny of stem cell divisions are committed to a single fate. This mode frequently operates in invertebrates. In an "indirect" mode (C and D), stem cell progeny are partially restricted to a subset of potential fates. This mode operates in hematopoiesis. In either case, the segregation of different lineages can exhibit no perceptible order or pattern ("stochastic;" A and C), or can occur according to a defined sequence or hierarchy ("deterministic;" B and D). For convenience, all examples are shown with asymmetric stem cell divisions; however, symmetrically dividing stem cells could operate with each mode as well. Furthermore, hierarchical restrictions, as shown in (B) and (D), could occur by progressive loss of developmental potentials from partially restricted intermediates, rather than by sequential production from a self-renewing stem cell. Finally, all four modes could be controlled either cell-autonomously or nonautonomously. (For an example of a stochastic decision that is nonautonomously controlled, see Figures 3A and 3B.)

differentiation was detected it is not clear whether these conclusions apply to HSCs.

A related question is whether the immediate progeny of stem cells are committed to a single fate ("direct" differentiation; Figures 4A and 4B), or restricted to a subset of fates ("indirect" differentiation; Figures 4C and 4D). CNS stem cells generate some progeny fated to produce only neurons (Davis and Temple, 1994), but whether these unifatent cells are truly committed was not determined. Committed neuronal progenitors have been identified in the PNS (Lo and Anderson, 1995), but whether these are directly generated from stem cells is not yet clear. In the hematopoietic system, progenitors committed to single lineages (e.g., B cell or T cell) have been shown to be derived from partially restricted lymphoid progenitors (Galy et al., 1995; Wu et al., 1996). Analagous partially restricted progenitors have been suggested to exist in the neural crest based on in vitro clonal analyses (Le Douarin et al., 1991), but whether these cells are truly committed to a subset of lineages has not been rigorously tested by exposure to appropriate instructive signals. The existence of partially restricted intermediates raises the additional question of whether their developmental potentials are assorted randomly (Figure 4C), or in an ordered, hierarchical manner (Figure 4D). The hematopoietic system may employ both strategies, depending upon the stage of lineage diversification (Suda et al., 1983; Wu et al., 1996). An ordered or hierarchical segregation of lineages at the cellular level may reflect the action of transcription factors that coordinately specify multiple sublineages; for example, there are lymphoid progenitors restricted to B and T sublineages (Wu et al., 1996) and several transcription factors, such as ikaros and E2A, required for both sublineages (for review, see Kehrl, 1995).

Formation of Stem Cells

Stem cells in the hematopoietic system, nervous system, gonads, liver, and intestine form de novo during fetal life. The progenitors of stem cells are sometimes referred to as pre-stem cells. Pre-stem cells can be defined as cells whose progeny contribute to tissues other than that derived from the particular stem cell they generate, and that produce stem cells only during a defined interval of development. While the sites of stem cell formation during mammalian fetal development are generally known, the identities of the pre-stem cells are usually not known; furthermore, little is known about the events that regulate the acquisition of stem cell competence.

Are there any genes identified that are required for the formation of stem cells? In Drosophila, asymmetrically dividing CNS progenitors, which are in many ways like stem cells, delaminate from a group of neuroectodermal precursor cells. Within this group, the bHLH transcription factors ACHAETE-SCUTE confer competence to generate the progenitor (Campuzano and Modolell, 1992). A single progenitor is selected from the group of competent cells by lateral inhibition, mediated by Notch proteins and their ligands (Ghysen et al., 1993). Recent data indicate that a similar process underlies the selection of neuronal precursors during primary neurogenesis in Xenopus (Chitnis et al., 1995; Ma et al., 1996). Although such amphibian neuronal precursors have not been defined as stem cells, a similar mechanism may be employed in the mammalian CNS, where stem cells have been clearly identified. Genes encoding both transcription factors and extracellular signals that are involved in the formation of the hematopoietic system have been identified (Maeno et al., 1996; Porcher et al., 1996), but whether these act at the level of stem cell formation is not yet known. Genetic screens in zebrafish may identify more such molecules (for review, see Zon, 1995).

There is evidence that different classes of stem cells can exist simultaneously in the same tissue. Stem cells from different positions along the cephalocaudal axis of the gut exhibit position-specific differences in terms of the differentiated cells they give rise to. When explants from different portions of the intestine were transplanted subcutaneously, the regional differences appeared to persist, providing some evidence that the differences may be intrinsic to the stem cells (Rubin et al., 1992). There is also evidence for regional differences among central nervous system progenitor cells. Mouse basal ganglion progenitors, but not ventral mesencephalic progenitors, were able to differentiate into striatal

cells upon transplantation into rat striatum, suggesting that the progenitors differed in their ability to adopt the fates of their new tissues (Campbell et al., 1995). Such differences are correlated with the region-specific expression of transcriptional regulators in the neuroepithelium from the earliest stages of brain development (for review, see Puelles and Rubenstein, 1993), suggesting an intrinsic component to such progenitor cell diversity. On the other hand, there are several cases where neural precursors adopt a correct identity when transplanted from one region into another (reviewed in Temple and Qian, 1996), suggesting that intrinsic differences may not always irreversibly commit such cells to a given fate.

The developmental potential of stem cells for a given tissue can differ in time as well as in space. Fetal liver HSCs are thought to give rise to adult bone marrow stem cells (Fleischman et al., 1982). Yet fetal liver stem cells are able to give rise to several classes of blood cells that adult bone marrow stem cells do not themselves produce (Ikuta et al., 1990; reviewed in Morrison et al., 1994). These differences are intrinsic to the stem cells since they persist even when fetal liver stem cells are transplanted into adult bone marrow, or when both stem cell types are transplanted into culture. The mechanisms underlying such stage-specific differences in developmental potential are not known.

Perspective

In this review, we have tried to raise and address some of the key mechanistic questions in stem cell biology. A few salient points emerge. First, molecules that maintain the stem cell state are beginning to be identified: ligands of Notch family receptors do this from outside the cell, and factors like PIE-1 do it from within. At least some of these mechanisms appear conserved in mammals. Second, we are beginning to gain insight into the mechanisms that may regulate stem cell self-renewal capacity, such as expression of telomerase. Third, it is now clear that at least some stem cells can be instructed to choose one pathway of differentiation, at the expense of others, by growth factors. In other systems, however, stem cells may make this choice stochastically, and growth factors may act mainly as survival factors or mitogens for committed cells. Understanding the interplay between extracellular and intracellular regulatory factors in controlling lineage determination remains an important challenge for the future.

A great deal of effort in the near term is likely to be invested in identifying self-renewal and survival factors for stem cells in various tissues. This in turn will allow investigation of the way in which these factors interact with cell-intrinsic molecules to maintain the uncommitted state and transfer it to daughter cells at each stem cell division. Some of the most interesting future questions will involve understanding the control of stem cell behavior at the population level, e.g., in tissues undergoing regeneration in response to injury. What feedback mechanisms operate to maintain the steady state in such tissues, to initiate the regenerative response and to restore the system back to steady state once regeneration is achieved? Stem cells in the adult brain present a particularly intriguing subject for study. What is the normal function of these cells? Can the system be manipulated to exploit the regenerative potential implied by the existence of these cells, as a recent study (Craig et al., 1996) suggests? The answers to such questions will advance our understanding of basic developmental mechanisms, and may open new avenues for therapeutic intervention in humans.

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Chapter 2

Glial growth factor restricts mammalian neural crest stem cells to a glial fate

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Glial Growth Factor Restricts Mammalian Neural Crest Stem Cells to a Glial Fate

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Summary

Growth factors and cytokines are thought to influence the development of uncommitted progenitor cell populations, but the issue of how these factors act on individual cells remains controversial. Such factors may act simply as selective mitogens or survival factors for cells that undergo lineage restrictions stochastically. Alternatively, they may instruct or bias multipotent cells to choose one lineage at the expense of others. Here we show that glial growth factor (GGF), previously defined as a Schwann cell mitogen, strongly suppresses neuronal differentiation of rat neural crest stem cells while promoting or allowing glial differentiation. Quantitative clonal analysis suggests that the action of GGF is likely to be instructive rather than selective. Taken together with the expression pattern of GGF, these data suggest a lateral signaling model for the diversification of cell types within developing peripheral ganglia.

Introduction

A central issue in developmental biology is to understand how the fate of uncommitted progenitor cells is influenced by factors in their local environment (Jessell and Melton, 1992). In higher vertebrates, this problem has been investigated intensively in both the immune system and the nervous system. The neural crest provides a neurobiological system to analyze the effects of environmental signals on cell fate determination. Neural crest cells migrate from the dorsal aspect of the neural tube and differentiate to a variety of cell types in different embryonic locations. These cell types include peripheral neurons and glia, melanocytes, endocrine cells, smooth muscle, and bone (LeDouarin, 1982). In vivo lineage tracing and in vitro clonal analyses in avian embryos have indicated that many neural crest cells are multipotent (for recent reviews see Anderson, 1993a; LeDouarin et al., 1993; Selleck et al., 1993). Transplantation and culture studies suggest that the fate of multipotent neural crest cells can be dictated by the environment (LeDouarin, 1986; Patterson, 1990). However, with few exceptions (Patterson and Nawa, 1993) the identity of these factors and their mechanisms of action are unknown. In the sympathoadrenal sublineage of the neural crest, it has been clearly demonstrated that cytokines can act instructively to alter the neurotransmitter phenotype of postmitotic neurons (Patterson and Chun, 1977; Yamamori et al., 1989). However, it has been difficult to distinguish instructive from selective effects of environmental factors on proliferating multipotent neural crest cells at the earliest stages of lineage diversification (for review see Stemple and Anderson, 1993).

The general problem of how growth factors influence multipotent cells has also been studied intensively in hemopoiesis, in which both cloned cytokines and isolated progenitor cell populations have been available for many years (Metcalf, 1989; Dexter et al., 1990). Analyses of cytokine and growth factor influences on hemopoietic progenitor cells have led to at least two models whereby these factors may influence lineage commitment (for discussion see Just et al., 1991). In one model, multipotent cells are instructed to choose a particular lineage, at the expense of others, in response to a given cytokine. In a second model, multipotent cells choose their fate stochastically, and cytokines simply support the survival or proliferation (or both) of appropriate lineage-restricted cells, after the fact of their decision.

It has proven difficult to distinguish between these models experimentally. Clone-splitting experiments have provided evidence for a partial influence, albeit incomplete, of colony-stimulating factors on lineage commitment of myeloid progenitor cells (Metcalf, 1980, 1991). By contrast, forced expression of cytokines in multipotent hemopoietic progenitor cell lines has suggested that different cytokines may alter the balance between proliferation and differentiation but not the type of differentiation (Just et al., 1991). More recently, expression of the antiapoptotic gene *Bcl-2* in hemopoietic progenitors has indicated that such progenitors can commit to several lineages in the absence of cytokines or growth factors if they are prevented from undergoing programmed cell death (Fairbairn et al., 1993). Such results appear to support the idea that hemopoietic lineage decisions can be made autonomously, but do not rule out the possibility that growth factors or cytokines may exert a biasing influence on such stochastic decisions. In the development of T lymphocytes, experiments in transgenic mice have supported the idea that the T cell receptor–mediated cell–cell interactions that occur in the thymic microenvironment instructively control the choice between CD4 and CD8 lineages (Robey et al., 1991).

The analysis of growth factor effects on individual progenitor cells requires in vitro clonogenic assay systems. In an earlier paper, we described the isolation, character-

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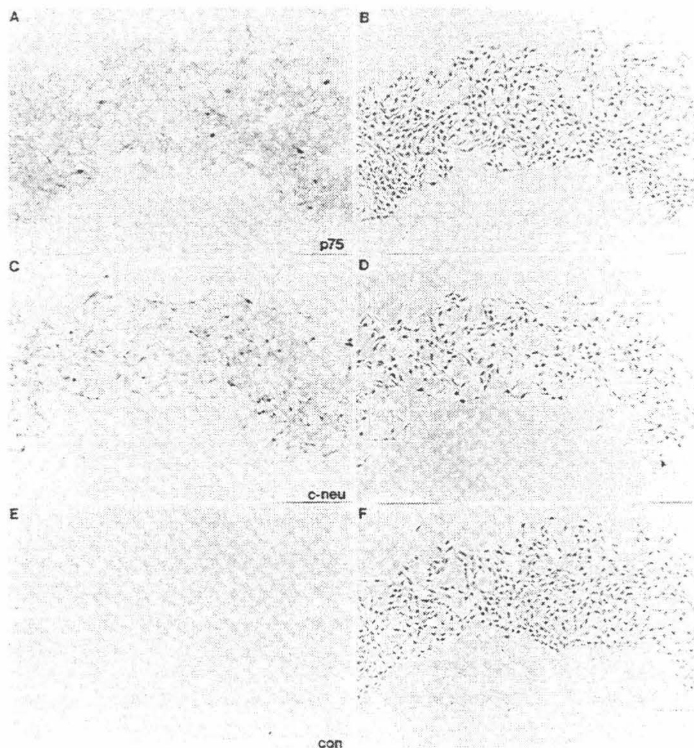

Figure 1. Migrating Neural Crest Cells Express c-Neu Immunoreactivity In Vitro

Explants (24 hr) of rat E10.5 neural tubes were fixed and labeled with monoclonal antibodies to p75 (A), c-Neu (C), or a control (con) incubation (E) and were developed using an avidin–biotin–HRP detection system (see Experimental Procedures). The neural tube was scraped from the plate with tungsten needles prior to fixation so that only the emigrated neural crest cells are shown. (A and B), (C and D), and (E and F) are paired bright-field ([A], [C], and [E]) and phase-contrast ([B], [D], and [F]) views. A similar extent of labeling is observed with the c-Neu and p75 antibodies. The arrowhead in (D) indicates a cell that does not express c-Neu, demonstrating the specificity of the staining. Direct double-labeled immunofluorescent staining of neural crest cells with anti-p75 and anti-c-Neu confirmed that many p75$^+$ cells coexpress c-Neu (data not shown).

ization, and clonal culture of multipotent neural crest cells from rat embryos (Stemple and Anderson, 1992). Subcloning experiments indicated that such cells not only differentiate to neurons and glia, but also undergo at least limited self-renewal, a characteristic of stem cells. This system provides an assay for environmental factors that control lineage commitment. In an initial examination of such factors, we have focused on glial growth factor (GGF), a Schwann cell mitogen of neuronal origin (Lemke and Brockes, 1984). GGF has recently been cloned (Marchionni et al., 1993) and shown to be a member of a family of ligands in the epidermal growth factor/transforming growth factor α (TGFα) superfamily for the receptor tyrosine kinases p185^{erbB2}/HER2/c-Neu and p180^{erbB4}/HER4 (Peles et al., 1992; Plowman et al., 1993b). These ligands, also named heregulins, Neu differentiation factor, ARIA, and neuregulins, are encoded by alternatively spliced transcripts from the same gene and include both membrane-bound and secreted forms (Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993). Since GGF acts on neural crest–derived Schwann cells, we sought to determine when such cells first acquired responsiveness to GGF. On the one hand, GGF could act simply as a mitogen for committed Schwann cells; on the other, it could act earlier in the lineage to affect the commitment of multipotent cells to a glial fate. Strikingly, we find that GGF suppresses neuronal differentiation while promoting or allowing glial differentiation by neural crest stem cells (NCSCs). These results argue that environmental factors can exert an instructive influence on lineage determination by stem cells in this system.

Results

Early Migrating Neural Crest Cells Express c-Neu Immunoreactivity In Vitro

The tyrosine kinase p185^{erbB2}/HER2/c-Neu (hereafter referred to as c-Neu) has been shown to undergo tyrosine phosphorylation in response to GGF/heregulins/Neu differentiation factor/neuregulins in certain cell types (Peles et al., 1992, 1993). As a first step toward investigating whether NCSCs might respond to GGF, therefore, we stained them with a monoclonal antibody to rat c-Neu, previously shown to stain differentiated Schwann cells in peripheral nerve (Jin et al., 1993). This antibody stained a majority of neural crest cells 24 hr after their emigration from the neural tube in vitro (Figure 1C). A similar proportion of cells were labeled by a monoclonal antibody to p75 (low affinity nerve growth factor receptor) (Figure 1A), previously shown to be a cell surface marker for multipotent NCSCs (Stemple and Anderson, 1992). These data suggested that many, if not all, p75$^+$ neural crest cells also express p185^{erbB2}/c-Neu, an idea confirmed by double-labeled immunofluorescent staining experiments (data not shown). Recently, HER4 has been shown to be necessary for GGF-induced tyrosine phosphorylation of c-Neu (Plowman et al., 1993b). Unfortunately, immunocytochemical staining reagents are not yet available to examine HER4

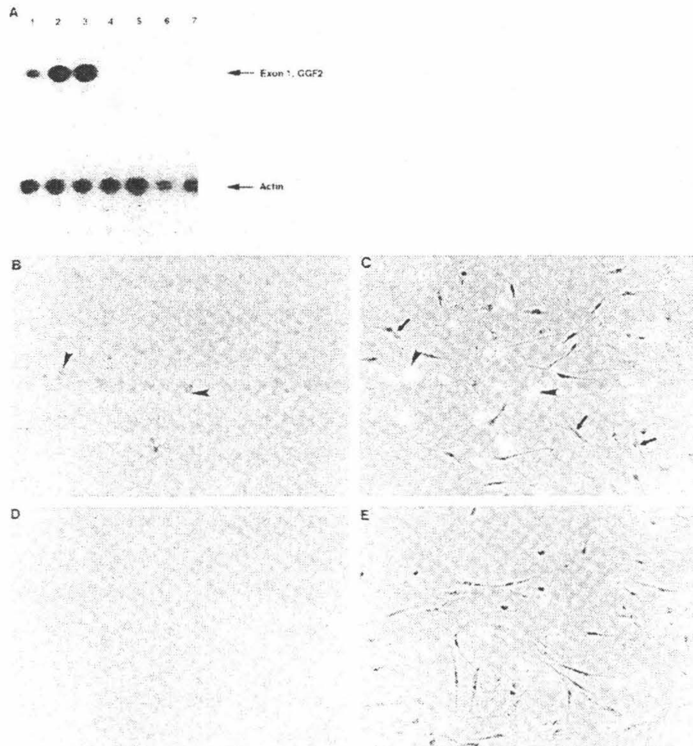

Figure 2. Expression of *GGF* mRNA and Protein in Developing Sensory Ganglia

(A) Detection of exon I–containing *GGF2* mRNA(s) in embryonic spinal ganglia by RT–PCR. PCR amplification was carried out using 0.25 μl, 0.5 μl, and 1 μl of cDNA reaction from E13.5 rat dorsal root ganglia (lanes 1–3, respectively); using 1 μl and 5 μl of cDNA from NCM-1 cells (a glial progenitor cell line) (lanes 4 and 5, respectively); and using 1 μl and 5 μl of cDNA from Rat-1 fibroblasts (lanes 6 and 7, respectively). The cDNA was amplified using primers specific for either exon I of *GGF2* (upper panel) or β-actin (lower panel) in separate reactions (see Experimental Procedures). The GGF PCR products were detected by Southern blotting using a [γ-^{32}P]ATP end-labeled exon I–specific oligonucleotide probe internal to the primers used. The β-actin PCR products were detected using a random-primed PCR product as a probe. No specific amplified products were detected in controls in which reverse transcriptase was omitted from the cDNA synthesis step (data not shown); therefore, the bands are not derived from contaminating genomic DNA.

(B–E) GGF2 protein is expressed specifically by neurons in dissociated embryonic peripheral ganglia. E15.5 rat dorsal root ganglia were dissociated, plated, fixed 24 hr later, and stained with monoclonal antibody D3-2 to GGF2 (see Experimental Procedures). Arrowheads in (C) indicate neurons that express GGF2 (B); arrows in (C) indicate flat nonneuronal cells that do not (see [B]). (D and E) show a control in which the anti-GGF2 antibody was omitted to demonstrate the specificity of staining. Similar results were obtained using an independent affinity-purified rabbit polyclonal anti-GGF2 antibody (data not shown). Neuron-specific expression of GGF2 was also detected using these two antibodies in cultures of neonatal superior cervical sympathetic ganglia (data not shown).

expression in neural crest cells. Nevertheless, the expression of c-Neu by these cells raised the possibility that they might be responsive to one or more forms of GGF.

GGF2 Is Expressed during Early Peripheral Gangliogenesis In Vivo

Previous studies have identified a secreted form of GGF, GGF2, as particularly effective in stimulating DNA synthesis by cultured Schwann cells (Marchionni et al., 1993). We wished to determine whether GGF2 is expressed at an appropriate place and time in vivo to influence the development of neural crest derivatives. Earlier in situ hybyridization experiments using epidermal growth factor domain–containing probes (detecting all forms of GGF) revealed expression in neural crest–derived rodent peripheral ganglia at the initial stages of ganglion condensation (Marchionni et al., 1993; Meyer and Birchmeier, 1994). To determine whether *GGF2* mRNA is expressed in these embryonic ganglia, we performed reverse transcription polymerase chain reaction (RT–PCR) experiments using primers specific to exon I, which is unique to *GGF2* (Marchionni et al., 1993). The results indicated that *GGF2* mRNA could be easily detected in rat embryonic day 13.5 (E13.5) spinal ganglia (Figure 2A, lanes 1–3). By contrast, expression was not detected in Rat-1 fibroblasts (Figure 2A, lanes 6 and 7) or from NCM-1 cells (Figure 2A, lanes 4 and 5), a rat neural crest–derived cell line with glial progenitor properties (Lo et al., 1990). To determine which cell types express GGF, dissociated cultures of embryonic spinal ganglia were stained with a monoclonal antibody to GGF2. Staining was clearly detected in neurons (Figure 2B, arrowheads) identifiable by their round, phase-bright cell bodies and neurites (Figure 2C, arrowheads). In contrast, little or no staining was detected in flat nonneuronal cells (Figure 2C, arrows; compare Figure 2B). Similar results were obtained in cultures of neonatal sympathetic ganglia (data not shown). Taken together, these data are consistent with the suggestion (Marchionni et al., 1993; Meyer and Birchmeier, 1994) that within the embryonic nervous system GGF is expressed primarily by neuronal cells. The early expression of GGF2 and of a possible component of its receptor by neural crest–derived cells encouraged us to examine the actions of this factor on the behavior of NCSCs in vitro.

GGF Suppresses Neuronal Differentiation and Promotes Glial Differentiation of NCSCs

When grown at clonal density in standard medium (SM), the majority of NCSCs ultimately form colonies containing both neurons and glia (Stemple and Anderson, 1992). Neuronal differentiation can be detected by staining with antibody to peripherin, a neuron-specific intermediate filament

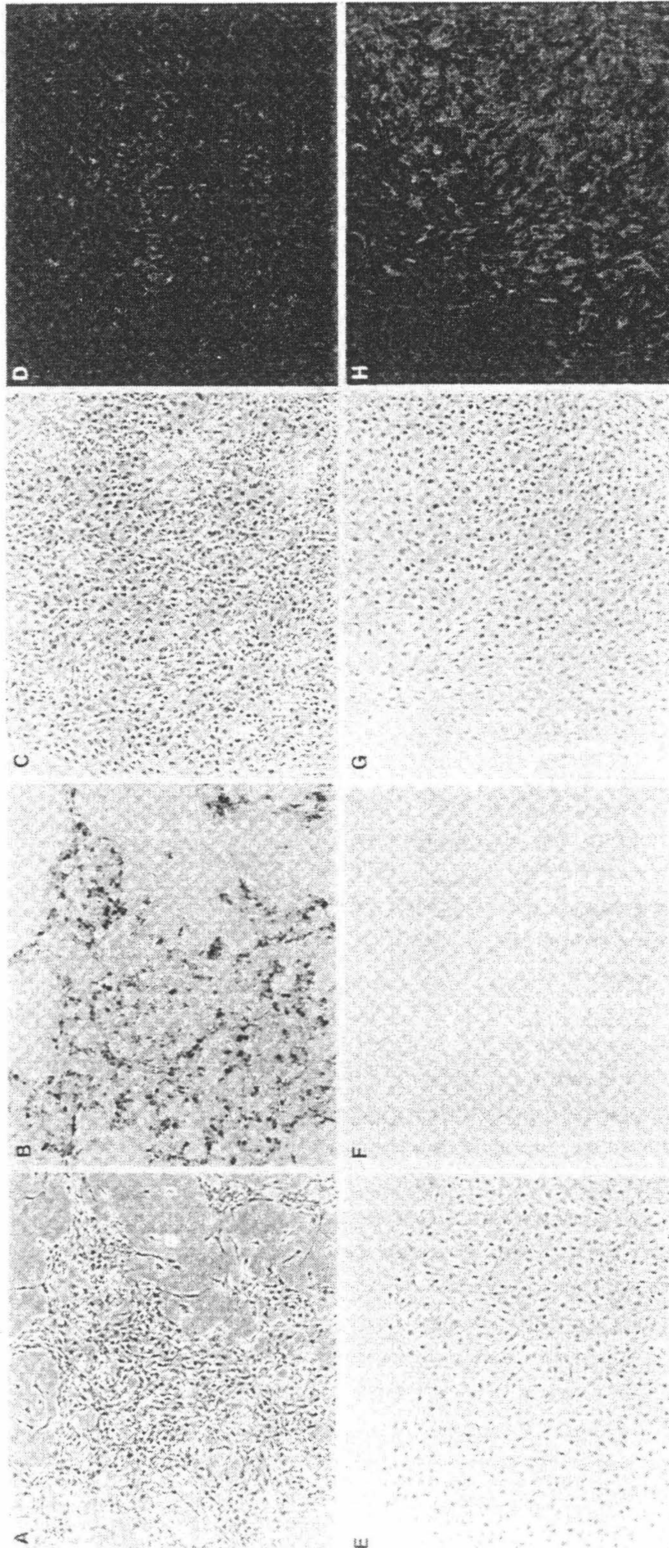

Figure 3. NCSCs Differentiate to Glia but Not Neurons in rGGF2

Shown are examples of colonies grown for 16 days with (E–H) or without (A–D) rGGF2. (A), (C), (E), and (G) represent phase-contrast views of the immunostained fields shown in (B), (D), (F), and (H), respectively. (B) and (F) represent staining with anti-peripherin visualized using an HRP-conjugated secondary antibody and bright-field optics; (D) and (F) represent staining with anti-GFAP visualized using a phycoerythrin-conjugated secondary antibody and epifluorescence optics. Matched exposures are shown in (D) and (F) to permit direct comparison of staining intensities. For optimal photography, the peripherin and GFAP staining were performed on separate cultures, but double-labeling experiments confirmed the results shown here. rGGF2 was used at a concentration that gave an approximately half-maximal mitogenic response in primary Schwann cells (see Figure 4).

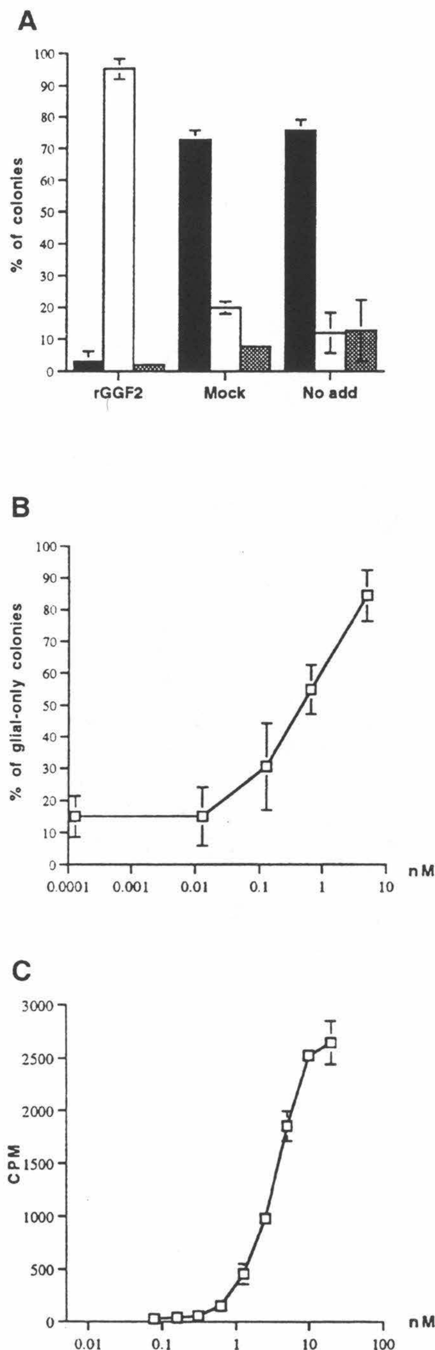

Figure 4. Quantification of the Effects of rGGF2 on Neural Crest Cell Differentiation

(A) Colony analysis of NCSCS grown at clonal density in SM containing rGGF2 purified from CHO cell supernatants (rGGF2), equivalently purified material from nontransfected CHO cells (Mock), or no additions (No add). rGGF2 was used at a saturating concentration as determined by dose-response experiments on Schwann cells (compare [C]). Colonies were phenotyped after 16 days using anti-peripherin and anti-GFAP antibodies. Closed bars, neuron plus glia-containing colonies; open bars, colonies containing glia but not neurons; hatched bars, colonies that died during the incubation. Data are expressed as the percentage of all colonies initially identified at day 4 that exhibited a given phenotype at day 16. Bar heights represent mean ± SEM of two independent experiments.

(B) Dose-response curve for rGGF2 on NCSC differentiation. The open squares denote the percentage of colonies containing glia but not neurons after 16 days of incubation in each of the indicated concentrations of rGGF2. The rGGF2 concentration is plotted on a logarithmic scale. Each point represents the mean ± SEM of two independent experiments. Because rGGF2 was added only at the start of the incubation and not replenished during the experiment and because the stability of rGGF2 under these conditions is not known, the effective concentration of rGGF2 may have been less than the initially added value.

(C) Dose-response curve for stimulation of DNA synthesis in Schwann cells by rGGF2 (Marchionni et al., 1993), carried out using the same preparation of purified rGGF2 as in (B).

protein (Figure 3B), while differentiation of immature Schwann cells can be detected by staining with antibody to glial fibrillary acidic protein (GFAP), a glial-specific intermediate filament protein (Figure 3D). The formation of differentiated neurons and glia is typically detected following 2 weeks of growth in culture.

In striking contrast with this behavior, there was a complete abolition of neurogenesis in the majority of colonies obtained from NCSCs grown in recombinant human GGF2 (rGGF2) (Figure 3F; compare with Figure 3B). However, glial differentiation was unsuppressed as evidenced by strong staining for GFAP (Figure 3H). If anything, GFAP expression appeared more intense (or brightly GFAP$^+$ cells more numerous) in rGGF2-treated cultures compared with control cultures (compare Figure 3D with Figure 3H). Double labeling with GFAP and DAPI (to reveal all cell nuclei) indicated that most or all cells in rGGF2-treated colonies expressed GFAP immunoreactivity (data not shown). Although GGF2 was originally identified as a Schwann cell mitogen, it does not appear to act as a mitogen for neural crest cells: after 4 days in vitro, the average number of cells per clone in control medium was 23.7 ± 1.8 while in rGGF2 it was 24.4 ± 1.1 (mean ± SD; n = 20 clones counted per condition from two independent experiments); after 5 days in vitro, there were 65.8 ± 2.3 cells per clone in control medium and 65.9 ± 1.8 cells per clone in rGGF2 (mean ± SD; n = 14 clones per condition from two independent experiments). Preliminary experiments with other Schwann cell mitogens such as TGFβ1 and β2, platelet-derived growth factor BB, and fibroblast growth factor (Ridley et al., 1989; Davis and Stroobant, 1990), as well as other members of the TGFα family (including TGFα), indicated that these factors did not mimic the effects of GGF2. Thus, GGF2 appears to be unique among growth factors so far tested in its ability to drive NCSCs to a glial fate.

The effect of rGGF2 was quantified by growing NCSCs at clonal density and by identifying viable colonies 4 days after plating. These colonies were then cultured for 12 more days and their phenotype analyzed. This analysis revealed that the majority (90%) of colonies grown in rGGF2 survived to form glial clusters without any neurons (Figure 4A, open bars marked rGGF2). As expected, the

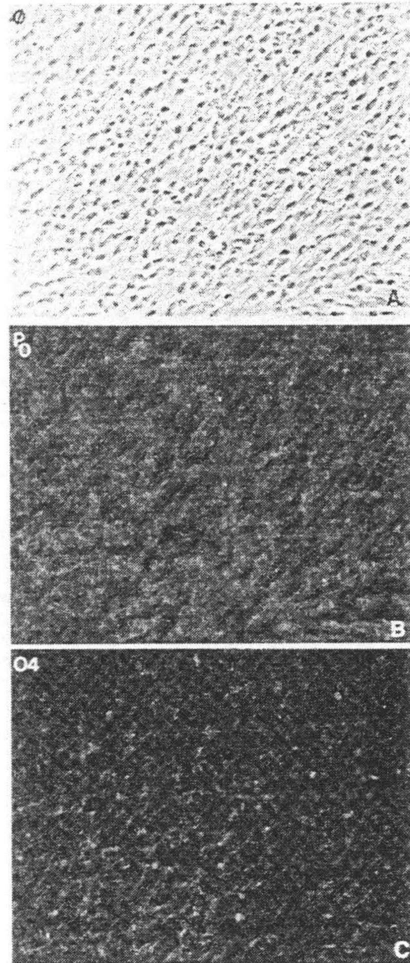

Figure 5. Differentiation of rGGF2-Treated Cells to Mature Schwann Cells

NCSCs were grown in SM containing rGGF2 for the first 8 days in culture, and then fetal bovine serum and forskolin were added to a final concentration of 10% and 5 µm, respectively. Similar amounts of forskolin were then added every other day for a period of 8 days and the cells then fixed and stained for O4 and P_0 as described previously (Stemple and Anderson, 1992). Visualization of the labeling was done using secondary antibodies conjugated to fluorescein for P_0 (B) and to rhodamine for O4 (C). The cells obtained after this procedure resembled mature Schwann cells in their morphology (A). Shown are phase-contrast (A), fluorescein (B), and rhodamine (C) views of the same microscopic field. While P_0 and O4 are not expressed by $GFAP^+$ cells grown in rGGF2 (data not shown), the subsequent induction of these markers by serum and forskolin uniquely identifies these $GFAP^+$ cells as immature Schwann cells (Jessen and Mirsky, 1991) and distinguishes them from central nervous system glial cells such as oligodendrocytes or astrocytes.

majority (80%) of colonies grown in SM survived to form clusters containing both neurons and glia (Figure 4A, closed bars marked No add). Dose-response experiments were also done at clonal density to measure the sensitivity of NCSCs to rGGF2; a half-maximal response was obtained at about 0.2 nM rGGF2 (Figure 4B). Although the large number and high density of cells per clone after 16 days (500–1000) precluded accurate quantitation of the number of neurons and glia per clone, our impression was that the number of clones containing any neurons at all, rather than the number of neurons per clone, increased with decreasing concentrations of rGGF2. The neuron-containing clones that developed in subsaturating doses of rGGF2 appeared indistinguishable from control clones in terms of the approximate number of neurons they contained. This suggests that rGGF2 acts to influence an all-or-none decision to generate neurons early in clone evolution. The dose-response experiments also indicated that rGGF2 suppressed neuronal differentiation at half-maximal doses that were slightly lower than those required for mitogenic activity on Schwann cells (Figure 4C).

NCSC colonies cultured in rGGF2 express GFAP, but do not express later Schwann cell differentiation markers such as myelin protein zero (P_0) and the sulfated ganglioside antigen recognized by monoclonal antibody O4 (Jessen and Mirsky, 1991) (as shown previously for NCSCs cultured in SM [Stemple and Anderson, 1992]). To confirm that these $GFAP^+$ cells are immature Schwann cells and not astrocytes (a central nervous system glial cell type that also expresses GFAP), we further incubated NCSC colonies initially grown in rGGF2 in medium containing 10% fetal bovine serum plus 5 µM forskolin. Such culture conditions have previously been shown to elicit expression of P_0 and O4 by NCSCs following growth in SM (Stemple and Anderson, 1992). As shown in Figure 5, rGGF2-treated NCSC cultures also expressed both P_0 and O4 following culture in serum plus forskolin. This combination of markers uniquely identifies these cells as Schwann cells. Since the majority of cells in the colonies were $GFAP^+$ prior to the shift to serum plus forskolin (see above), it is likely that the Schwann cells derive by phenotypic conversion of the $GFAP^+O4^-P_0^-$ cells obtained following growth of NCSCs in rGGF2. In further support of this conclusion, continued GFAP expression can be detected in many cells following subculture in serum plus forskolin (Stemple and Anderson, 1992; data not shown).

GGF Exerts an Instructive Influence on Neural Crest Cell Lineage Determination

The preceeding experiments left open the possibility that rGGF2 selected for the survival of committed glial progenitor cells during the first 4 days of culture. To address this issue, we identified individual NCSCs (50–70 per condition) and circled them 6 hr after plating. This procedure ensured that each colony examined constituted a clonal population of cells derived from a single NCSC. Some cultures then received rGGF2 while the others were maintained as control cultures. All clones were examined every day for the next 15 days. This day-to-day analysis failed to reveal even a transient appearance of morphologically identifiable neurons in clones grown in rGGF2 (Table 1). Moreover, most of the NCSCs (80%–90%) survived to form clones in control as well as in rGGF2-containing medium (Table 1). Furthermore, the majority of NCSCs grown in rGGF2 formed glial clones devoid of neurons, while the

Table 1. Clonal Analysis of NCSCs Grown in rGGF2

Condition	Clones Identified	G Clones	N plus G Clones	Nonviable Clones[a]
rGGF2	68	79.4% (54)	8.8% (6)	11.8% (8)
No addition	56	5.4% (3)	82.1% (46)	11.6% (7)
Mock GGF[b]	56	7.1% (4)	71.4% (40)	21.4% (12)

Individual p75⁺ NCSCs were identified 6 hr after plating, and their positions were inscribed on the bottom of the tissue culture plate. rGGF2 was added after 12 hr, to avoid effects on cell attachment. Clones were observed every 24 hr for 16 days and scored for survival and appearance of morphologically identifiable neurons. After 16 days, the clones were fixed and labeled with anti-peripherin plus anti-GFAP and their phenotype determined. The criterion for a neuron (N) plus glia (G) clone was very stringent: clones containing even a single neuron were placed into this category. This criterion would, if anything, underestimate the inhibition of neurogenesis produced by rGGF2. A small number of clones failed to develop either neurons or glia; these other (O) clones (Stemple and Anderson, 1992) constituted a small proportion of the total clones and for simplicity were omitted from the analysis. In no case did we observe neuron-containing clones that died or clones that survived in which morphologically identifiable neurons appeared but then died over the course of the 16-day incubation.

[a]Nonviable identifies those clones that died during the 16-day incubation.

[b]Mock indicates that a sample subjected to a mock rGGF2 purification protocol and containing the same final salt and buffers was added to the culture medium at the same dilution as GGF. Numbers in parentheses indicate the total number of clones scored for each phenotype.

majority of NCSCs grown in control (SM) formed clones that contained both neurons and glia (Table 1). Thus, the striking reduction in neuron-containing clones observed in this and in the preceeding colony analysis (see Figure 4) cannot be explained by a failure of neurons or neuron-containing clones to survive in rGGF2; rather, overt neuronal differentiation is inhibited.

GGF Inhibits Expression of Mammalian Achaete-Scute Homolog 1

To determine the earliest identifiable stage of neurogenic development at which rGGF2 acts, we used mammalian achaete-scute homolog 1 (MASH1) (Johnson et al., 1990) as a marker. MASH1, a basic-helix-loop-helix transcriptional regulator (Johnson et al., 1992), is expressed in neuroepithelial precursors in vivo several days prior to overt neuronal differentiation (Lo et al., 1991). This early expression of MASH1 reflects an essential role in neurogenesis in the peripheral nervous system, as shown recently by targeted mutagenesis experiments in mice (Guillemot et al., 1993). In cultures of rat neural crest cells, expression of MASH1 protein is not detectable by antibody staining for the first 4–5 days in vitro (data not shown). On days 5–6, MASH1 immunoreactivity becomes detectable in the nuclei of morphologically undifferentiated cells (Figures 6A and 6B). Thus, expression of MASH1 precedes overt

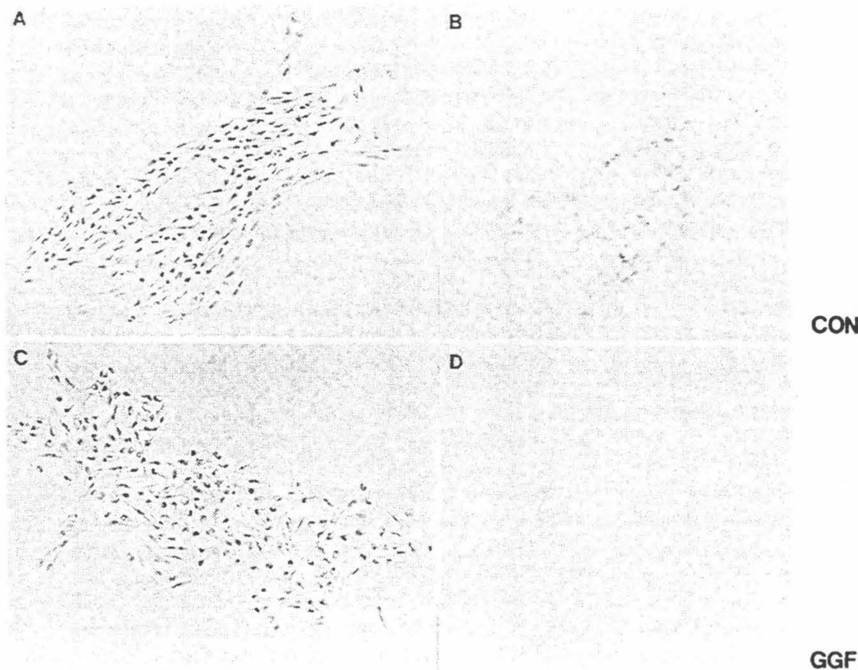

Figure 6. Inhibition of MASH1 Expression in Clonal Density Cultures of NCSCs Grown in a Saturating Dose of rGGF2

See Figure 4B. NCSCs were grown with (C and D) or without (A and B) rGGF2 for 6 days before being fixed in 4% formaldehyde for immunostaining. (A) and (C) represent phase-contrast views of the immunostained fields shown in (B) and (D), respectively. Immunostaining was performed using a monoclonal antibody to MASH1 (Lo et al., 1991) and visualized using a secondary antibody conjugated to HRP. While many cells in (C) show nuclear MASH1 immunoreactivity, none of the cells in (D) do. Note that the MASH1⁺ cells (B) show an undifferentiated (i.e., nonneuronal) morphology (A). No staining with antibodies to S100 or GFAP was detected at this stage of culture.

neuronal differentiation in vitro as it does in vivo, thereby providing an early marker of neurogenesis.

In contrast with control colonies, NCSC colonies grown in rGGF2 did not express detectable MASH1 immunoreactivity (Figures 6C and 6D) at 6 days in vitro, a time when most colonies in control sister cultures contained numerous cells with MASH1$^+$ nuclei (Figure 6B). Quantification indicated that 83.6% $\pm$ 3.7% of the colonies in SM contained MASH1$^+$ cells, while only 1.8% $\pm$ 2.6% of the colonies in SM containing rGGF2 contained MASH1$^+$ cells (mean $\pm$ SD of two independent experiments). The majority of colonies in rGGF2 were still MASH1$^-$ after 8 and 10 days in vitro (data not shown). This inhibition of MASH1 expression is not accompanied by a precocious expression of glial markers such as S100 and GFAP in colonies grown in rGGF2 (data not shown). Thus, the earliest detectable effect of rGGF2 on NCSC differentiation is an inhibition of expression of *MASH1*, a neurogenic lineage determination gene.

To confirm that the inhibition of MASH1 expression does not reflect an effect of rGGF2 to kill MASH1-expressing cells, we performed the following experiment. Neural crest colonies were grown for 8 days in control medium, by which time a majority of the colonies expressed nuclear MASH1 immunoreactivity. Subsequently, rGGF2 was added at a saturating dose to half the dishes; the others were maintained as controls. After 24 hr, the cultures were fixed and double labeled with anti-MASH1 antibody and DAPI to reveal fragmented nuclei indicative of apoptotic cells. We observed no evidence of selective apoptotic fragmentation of MASH1$^+$ nuclei in rGGF2-treated cultures under these conditions; in fact, there was little evidence of apoptotic nuclei in any of the cells under either condition (data not shown). Moreover the percentage of colonies containing MASH1$^+$ cells was similar in control (86.8% $\pm$ 9.5%; mean $\pm$ SD) and in rGGF2-treated cultures (82.8% $\pm$ 8.6%; mean $\pm$ SD). The overall intensity of anti-MASH1 antibody staining also appeared similar under the two conditions. These data support the idea that rGGF2 does not act to promote apoptosis of MASH1-expressing cells. Rather, it appears to influence an all-or-none decision to enter the neuronal pathway of differentiation, an early consequence of which is the induction of MASH1 expression.

Discussion

We have utilized a clonal culture system for multipotent self-renewing rat neural crest cells to examine the actions of GGF2, one of a family of polypeptide growth factors previously defined as Schwann cell mitogens (Lemke and Brockes, 1984). Our results indicate that GGF2 can exert a strong influence on the early development of these cells, inhibiting neurogenic lineage commitment and promoting glial development. Clonal analysis indicates that the action of GGF2 most likely involves an instructive or biasing effect on the choice of fate by these cells rather than an effect to select for survival or proliferation of precommitted glial progenitors. We cannot distinguish yet whether the primary effect of GGF2 is to repress neurogenesis, promote gliogenesis, or a combination of both. Nevertheless, the data present a clear case in which a molecularly defined environmental factor controls a choice of fate by a multipotent progenitor cell. Interestingly, GGF shows sequence similarity to the product of the *LIN-3* gene in Caenorhabditis elegans, which has been demonstrated by genetic analysis to control a choice of fate by multipotent vulval precursor cells (Hill and Sternberg, 1992). Our results indicate that a member of the epidermal growth factor/TGFα superfamily can play an analagous role in controlling a choice between alternative pathways of differentiation in a mammalian system. Since c-Neu and HER4 are expressed in other nonneural tissues (such as mammary epithelium) (Plowman et al., 1993a), it is possible that the various ligands of this receptor influence the fate of multipotent progenitors in other lineages as well.

Instructive versus Selective Effects of Growth Factors on Progenitor Cells

As mentioned earlier, two models have been proposed to explain cytokine influences on lineage determination by hemopoietic progenitor cells. In one model, cytokines (or growth factors) determine the direction of differentiation. In the second model, multipotent cells choose their fate stochastically, and cytokines selectively support the proliferation or survival (or both) of appropriate lineage-restricted cells. The latter model implies that in the absence of appropriate lineage-specific factors, differentiated cells (or their progenitors) of inappropriate lineages will form and then die. As mentioned earlier, it has been difficult to distinguish experimentally between these models in the hemopoietic system, in part because the effect of cytokines such as granulocyte/macrophage colony-stimulating factor to alter the differentiative behavior of progenitor cells is incomplete (Metcalf, 1980, 1991). By contrast, we observed a profound and essentially complete repression of neuronal differentiation by rGGF2. Moreover, serial observation of identified clones grown in rGGF2 failed to reveal any evidence that neurons differentiate (as assessed by morphologic criteria) and then die. More importantly, we also observed that expression of *MASH1*, a neurogenic lineage determination gene, was prevented by rGGF2 and that rGGF2 does not cause the death of MASH1-expressing precursor cells when added late in the culture period. Taken together, these data argue against the interpretation that MASH1-expressing neurogenic progenitors are generated stochastically, but fail to survive or proliferate in rGGF2. While we cannot exclude the possibility that in rGGF2, lineally restricted neuronal progenitors are generated but then die prior to the stage marked by expression of MASH1, we feel that this is unlikely. This is because previous serial subcloning experiments have indicated that the majority of cells in NCSC clones remain multipotent during the first 5 days of culture (Stemple and Anderson, 1992; D. Stemple and N. M. S., unpublished data); thus, there is little evidence to support the existence of a restricted neurogenic progenitor cell compartment that precedes the onset of MASH1 expression. The simplest interpretation of our data, therefore, is that GGF2

acts instructively to control the choice of fate by multipotent NCSCs.

The presence of glial cells in our control colonies may reflect the production of endogenous GGF by the neurons that develop in these conditions. Consistent with this notion, in such control colonies, the most intensely stained GFAP+ cells are usually located adjacent to neurons (data not shown). Indeed, previous studies have indicated that neurons can promote expression of a glial phenotype (Holton and Weston, 1982), but have not distinguished effects on overt differentiation of committed cells from an influence on lineage commitment of multipotent progenitors. It is also possible that GGF is not essential for glial differentiation but simply increases the probability that NCSCs will differentiate into glia. The idea that neural crest cells may undergo lineage restriction by probabilistic (i.e., stochastic) mechanisms has been suggested based on quantitative analysis of avian clonal cultures (Baroffio and Blot, 1992). Our results suggest that if such mechanisms indeed operate, environmental factors such as GGF must be able to alter the probabilities assigned to commitment towards a particular fate. Similar models have been invoked in the hemopoietic system (Ogawa, 1993).

There is good experimental evidence that environmental factors can influence the phenotype of neural crest-derived cells at later stages in the lineage diversification process. The cytokines leukemia inhibitory factor and chick neurotropic factor have been shown to control a switch from a noradrenergic to a cholinergic neurotransmitter phenotype by postmitotic sympathetic neurons (Nawa et al., 1990). Glucocorticoids have been demonstrated to promote the differentiation of sympathoadrenal progenitors to an endocrine (chromaffin) rather than a neuronal phenotype (for review see Anderson, 1993b). However the chromaffin phenotype appears to be part of a continuum of phenotypes between endocrine cells and neurons, since postnatal and even adult adrenal medullary endocrine cells can be caused to transdifferentiate to sympathetic neurons by nerve growth factor and fibroblast growth factor (Doupe et al., 1985a, 1985b; Stemple et al., 1988). By contrast, the neuron–glial decision controlled by GGF appears to involve discontinuous differences in cell phenotype, since Schwann cells, once differentiated, appear unable to convert to a neuronal phenotype under any conditions tested thus far both in vivo and in vitro (Ciment, 1990). Previously, the neurotrophin brain-derived neurotrophic factor was proposed to control the commitment of avian neural crest cells to the sensory neuron lineage in vitro (Sieber-Blum, 1991); however, an effect of the neurotrophin selectively to maintain the survival of a subset of committed sensory progenitors was not excluded, nor was sensory development demonstrated to occur at the expense of other lineages. GGF thus appears to be unique in its ability to influence an early and fundamental lineage determination event by multipotent NCSCs.

Role of GGF in the Diversification of Peripheral Ganglionic Cells In Vivo

During development in vivo, multipotent neural crest cells migrate from the neural tube and aggregate to form peripheral sensory and autonomic ganglia that eventually develop both neurons and glial (satellite) cells. The notion that the fate of multipotent neural crest cells is determined by signals in their local environment raises the apparent paradox of how two distinct cell types can be generated in a common microenvironment (Frank and Sanes, 1991). One possible resolution to this paradox is the suggestion that neuronal and glial progenitors are precommitted prior to the condensation of migrating cells into ganglion primordia (Weston, 1991). However, in vivo lineage tracing experiments have indicated that even migrating neural crest cells are multipotent (Fraser and Bronner-Fraser, 1991). If peripheral ganglia are initially composed of multipotent cells (Duff et al., 1991), then mechanisms must exist to ensure the generation of appropriate numbers of neurons and glial cells within these ganglia. Our data suggest a role for GGF in mediating local cell–cell interactions that may regulate this diversification process.

In vivo, neurons in peripheral ganglia differentiate prior to glial cells. Moreover, each developing neuronal cell body is eventually surrounded by glia called satellite cells. Taken together with the present data, these facts suggest a model of gangliogenesis in which developing neurons, acting via secreted or membrane-bound forms of GGF, inhibit neighboring uncommitted cells from adopting a neuronal fate and may, in addition, commit them to a glial lineage. Such a lateral signaling mechanism is similar to the lateral inhibition process that controls the segregation of neuronal and epidermal lineages in the insect neuroectoderm (Ghysen et al., 1993). However, the latter case involves the products of the *Notch* and *Delta* genes, which participate in as-yet-undefined signaling pathways, whereas GGF acts through a receptor tyrosine kinase. It is possible that a *Notch*-like system is also involved in the neuron–glial decision, as suggested by the expression of mammalian *Notch* homologs in developing peripheral ganglia (Weinmaster et al., 1991). Indeed, in the aforementioned C. elegans vulval induction system, the GGF-homologous *LIN-3* gene product acts on a cell fate decision that is also regulated by the *Notch* homolog *LIN-12* (Greenwald et al., 1983). Whatever the precise molecular circuitry involved, our data suggest an explanation for how multipotent neural crest cells, aggregating to form ganglia in a common microenvironment, can nevertheless acquire different fates. The availability of a clonal culture system for NCSCs should permit tests of this hypothesis using reagents that perturb the expression or function of endogenous forms of GGF, of its receptor, and of *Notch*-related genes.

Experimental Procedures

Culture of NCSCs

Rat neural crest cells were isolated and cultured in complete ("standard") medium containing (among other additives) insulin, epidermal growth factor, basic fibroblast growth factor, nerve growth factor, and 10% chick embryo extract as described previously (Stemple and Anderson, 1992). All cultures were established at clonal density (75 cells per each 35 mm diameter Corning tissue culture dish or 140 cells per each 60 mm diameter Corning tissue culture dish). For clonal analysis, NCSCs were identified by surface labeling live cells with anti-p75

monoclonal antibody (Chandler et al., 1984) and drawing a circle around the labeled single cells on the bottom of the culture dish (Stemple and Anderson, 1992).

Immunocytochemistry

Colonies were fixed using acid–ethanol and stained with rabbit anti-peripherin (Chemicon) at 1:1000 and with mouse anti-GFAP (clone GA5; Sigma) at 1:100, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (TAGO) at 1:200 and phycoerythrin-conjugated goat anti-mouse IgG (Jackson Laboratories) at 1:200, respectively. Both primary and secondary antibody incubations were done at room temperature for 30 min. The histochemical reaction for HRP was performed using diaminobenzidine (DAB) and nickelous sulfate as substrates.

MASH1 antibody staining was carried out on colonies fixed in 4% formaldehyde and permeablized using 0.1% NP-40. Cultures were incubated for 4–8 hr at 4°C in a 1:1 dilution of anti-MASH1 hybridoma supernatant (Lo et al., 1991) followed by a HRP-conjugated goat anti-mouse IgG (TAGO) (1:200 dilution) for 30 min at room temperature. The histochemical reaction was performed as described for peripherin staining.

Immunocytochemical staining for c-Neu was performed by labeling neural crest explant cultures fixed in 4% formaldehyde with anti-c-Neu monoclonal antibody AB-4 (Oncogene Sciences) at a 1:10 dilution using an overnight incubation at 4°C (Jin et al., 1993). p75 expression was detected by an overnight incubation in undiluted culture supernatant of monoclonal antibody 192Ig (Chandler et al., 1984). Staining was visualized using a Vectastain ABC kit (Vector Labs) with HRP development and the nickel sulfate–DAB reaction as described above. Staining for rGGF2 in dissociated cultures of embryonic dorsal root ganglia was carried out using a mouse monoclonal antibody to GGF2 (D3-2) produced against rGGF2 isolated from CHO cells. The specificity of the antibody was demonstrated by Western blotting and staining of rGGF2-transfected CHO cells but not CHO cells transfected with an irrelevant expression plasmid. Incubation with hybridoma supernatant diluted approximately 1:1 was carried out for 1 hr at room temperature, and development methods were carried out as for MASH1 staining. In some experiments, an affinity-purified rabbit polyclonal anti-human GGF2 antibody was used. Absorption controls using an excess of the immunogen indicated that the staining observed was specific. Similar results were obtained using both the polyclonal and monoclonal anti-GGF antibodies.

Purification of rGGF2

rGGF2 was produced in a stably transfected CHO cell line grown in roller bottles. Conditioned media was harvested, and rGGF2 was purified by two chromatographic steps: cation exchange followed by hydrophobic interaction chromatography. The recovery of biologically active rGGF2 was monitored by a Schwann cell proliferation assay (Marchionni et al., 1993). The final preparation was judged to be >95% pure based on Coomassie blue staining of SDS gels.

RT-PCR

Reverse transcription of total RNA (1 μg) (Chomczynski and Sacchi, 1987) was performed at 37°C for 1 hr followed by 20 min at 42°C. The primers used for the PCR for *GGF2* exon I were GGT GGT GAT CGA GGG AAA (5′ primer) and ATG TAC CTG CTG TCC TCC TTG A (3′ primer). Primers used to detect β-actin in the PCR were TCA TGA AGT GTG ACG TTG ACA TCC (5′ primer) and GAC TGT TAC TGA GCT GCG TTT TAC (3′ primer). The source of these rat *GGF* exon I sequences is a rat genomic clone (J. Lucas and M. A. M., unpublished data). Denaturation temperature was 94°C and extension temperature was 72°C for both primer sets; the annealing temperatures for *GGF2* exon I and β-actin were 55°C and 60°C, respectively. PCR was performed for 35 cycles for *GGF2* and for 25 cycles for β-actin. Transfer to GeneScreen Plus membranes and hybridization with appropriate probes were performed according to instructions of the manufacturer. The oligoprobe used to detect *GGF2* exon I was GGT TTG AAG AAG GAC TCG CTA. End-labeling of the probe with [γ-^{32}P]ATP was done using the T4 polynucleotide kinase under standard conditions. Random primer labeling of the β-actin PCR product with [α-^{32}P]dATP was done using a kit from Boehringer Mannheim.

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Chapter 3

Alternative neural crest cell fates are instructively promoted by TGFß superfamily members

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Alternative Neural Crest Cell Fates Are Instructively Promoted by TGFβ Superfamily Members

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Summary

How growth factors influence the fate of multipotent progenitor cells is not well understood. Most hematopoietic growth factors act selectively as survival factors, rather than instructively as lineage determination signals. In the neural crest, neuregulin instructively promotes gliogenesis, but how alternative fates are determined is unclear. We demonstrate that bone morphogenic protein 2 (BMP2) induces the basic–helix-loop-helix protein MASH1 and neurogenesis in neural crest stem cells. In vivo, MASH1$^+$ cells are located near sites of BMP2 mRNA expression. Some smooth muscle differentiation is also observed in BMP2. A related factor, transforming growth factor β1 (TGFβ1), exclusively promotes smooth muscle differentiation. Like neuregulin, BMP2 and TGFβ1 act instructively rather than selectively. The neural crest and hematopoietic systems may therefore utilize growth factors in different ways to generate cellular diversity.

Introduction

The mechanisms controlling binary fate decisions by developmentally equivalent cells are being rapidly elucidated by genetic analysis (Greenwald and Rubin, 1992; Ghysen et al., 1993). In contrast, the process whereby a multiplicity of differentiated cell types is generated from pluripotent stem cells is less well understood. This problem has been studied in the context of hematopoiesis, in vertebrates (for reviews, see Dexter et al., 1990; Ikuta et al., 1992). In this system, growth factors such as erythropoietin (EPO) have been isolated that influence the development of cells in a particular lineage (Clark and Kamen, 1987; Metcalf, 1989; Krantz, 1991). A major issue has been whether these growth factors act by instructing multipotent cells to commit to one lineage at the expense of others (Metcalf and Burgess, 1982), or whether they prevent the death of lineage-committed progenitors that have been generated by a stochastic mechanism (see Ogawa, 1993, and references therein). The available evidence favors the latter alternative. Thus, when apoptosis is autonomously blocked in a myeloid progenitor cell line by forced expression of *bcl-2*, all possible lineages differentiate in the absence of any exogenous growth factors (Fairbairn et al., 1993). Selective rather than instructive mechanisms of lineage commitment have also been inferred from clonal analyses of paired hematopoietic progenitor cells (Suda et al., 1984), as well as of T lymphocyte development in transgenic mice (Davis and Littman, 1994).

The neural crest is another vertebrate system in which multiple lineages arise from pluripotent progenitor cells (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988). It generates most of the peripheral nervous system (PNS), skin melanocytes, and mesectodermal derivatives such as smooth muscle (SM) cells, bone, and cartilage (Horstadius, 1950; Le Douarin, 1982). Transplantation and cell culture experiments have indicated that extracellular signals can influence the fate of neural crest cells (for reviews, see Bronner-Fraser, 1993; Stemple and Anderson, 1993; Le Douarin et al., 1994). However, with few exceptions (e.g., see Sieber-Blum, 1991; Dupin and Le Douarin, 1995) most of the relevant signals have not yet been identified, nor have their actions been examined in clonal cultures.

Previously, we demonstrated that neuregulin/GGF can instructively influence multipotent, self-renewing rodent neural crest stem cells (NCSCs) (Stemple and Anderson, 1992) to differentiate to glia in vitro (Shah et al., 1994); while this study demonstrated that one fate could be promoted by an environmental signal, it left open the question of how alternative fates might be chosen. For example, the neuronal fate of NCSCs, like the glial, might be promoted by other extrinsic cues. Alternatively, neural crest cells might be predisposed to select a neuronal fate in the absence of extrinsic influences. Indeed, in many systems developmental decisions have been suggested to involve one fate that is promoted by an extracellular signal and an alternative fate that is assumed in the absence of that signal, as if by default (Raff, 1989; Kelly and Melton, 1995).

Members of the TGFβ superfamily of growth factors are expressed at sites where autonomic neurons differentiate. For example, bone morphogenic protein 2 (BMP2) is expressed in the dorsal aorta (Lyons et al., 1995), near which sympathetic ganglia form. We show that BMP2 promotes rapid induction of the autonomic lineage-specific basic–helix-loop-helix protein MASH1 and autonomic neurogenesis in vitro. Some SM cell differentiation is also observed in BMP2. In contrast, TGFβ1, the prototypic member of the TGFβ superfamily, drives virtually all NCSCs to an SM fate. Both TGFβ1 and BMP2 act instructively to influence cell fate decisions, rather than selectively to support survival of lineage-committed progenitors. These data indicate that the choice of each of several alternative fates available to NCSCs can be instructively promoted by different environmental signals. Thus, the neural crest and hematopoietic systems appear to use growth factors in different ways to generate cellular diversity.

Results

BMP2 Is Expressed in the Dorsal Aorta Concomitant with Autonomic Neurogenesis

Previous studies have suggested that the dorsal aorta may be a source of signals that influence the differentiation of neural crest–derived autonomic neurons in the sympathetic sublineage (Stern et al., 1991; Groves et

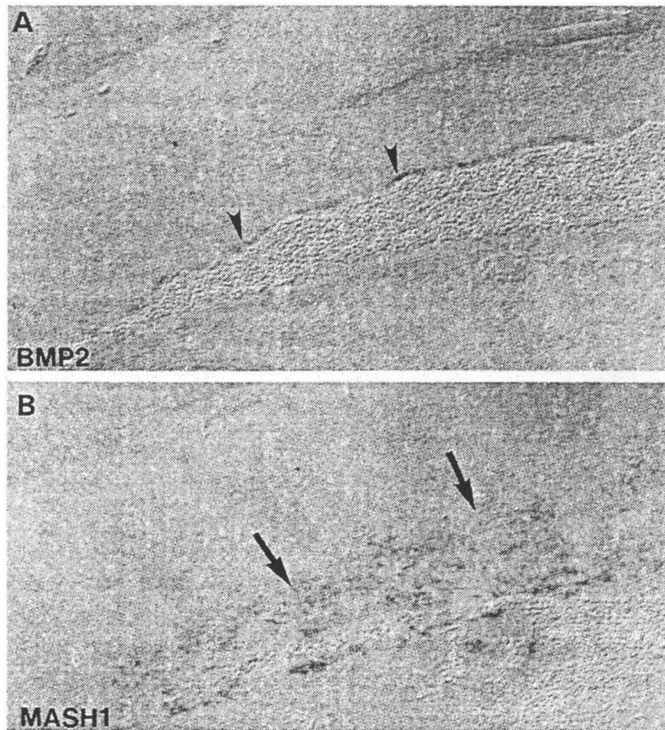

Figure 1. Expression of BMP2 Spatially and Temporally Overlaps MASH1 Expression in Autonomic Anlage In Vivo

Shown are sagittal sections of embryonic day 10.5 mouse embryos processed for in situ hybridization using probes specific for BMP2 (A) or MASH1 (B). At this stage the aorta is essentially a tube lined by a single layer of cells, and these cells can be seen to express BMP2 transcripts (arrowheads). MASH1 mRNA (arrows) can be clearly seen extending for several cell diameters away from the aorta in a near-adjacent section.

al., 1995). In mammals, BMP2 (a member of the TGFβ superfamily of growth factors) is expressed in tissues where autonomic neurons develop, such as the heart, lung, and dorsal aorta (Bitgood and McMahon, 1995; Lyons et al., 1995). A similar but nonidentical pattern is observed for the closely related factor BMP4. To confirm that BMP2 mRNA is expressed in the dorsal aorta at a time when autonomic progenitors are developing nearby, we hybridized sections of embryonic day 10.5 mouse embryos with cRNA probes for *Bmp2* and *Mash1*. MASH1, a basic-helix-loop-helix transcription factor (Johnson et al., 1990, 1992) required for autonomic neurogenesis (Guillemot et al., 1993), is transiently expressed by neural crest precursors in all autonomic sublineages (Lo et al., 1991). BMP2 mRNA was detected in a single layer of cells lining the lumen of the dorsal aorta, which are likely to be endothelial cells (Figure 1A, arrowheads). On near-adjacent sections, MASH1-expressing cells were clearly seen aggregating adjacent to the aorta (Figure 1B, arrows). These data raised the possibility that BMP2 could act as a diffusible signal to influence the developing sympathetic ganglia. We therefore examined the effect of this factor on the development of isolated NCSCs in vitro.

BMP2 Induces Rapid Neurogenesis in NCSC Clonal Cultures

NCSCs grown at clonal density in standard culture medium undergo symmetrical, self-renewing divisions for at least 5–6 days in vitro (Stemple and Anderson, 1992). Neurons do not begin to differentiate in such cultures until 10–15 days of incubation. Moreover, clones containing only neurons are never observed; rather the neurons differentiate together with nonneuronal cells such as glia (Stemple and Anderson, 1992).

In striking contrast, when NCSCs were grown in 1.6 nM recombinant BMP2 (rBMP2), many neuron-only colonies (identified by their neurite-bearing morphology and expression of peripherin) developed within 3–4 days (Figures 2A and 2B). At this dose, ~50% of the colonies contained only neurons; 20%–25% contained neurons (about as many per colony as in the neuron-only colonies) as well as large flat cells; the remainder consisted only of such flat cells. Thus, 75% of colonies grown in rBMP2 contained neurons after 4 days. By contrast, none of the colonies grown in the absence of rBMP2 contained any neurons at this time point (Figures 2E and 2F). Glial fibrillary acidic protein–positive (GFAP^{+}) cells were not detected at any time in BMP2-containing cultures. The phenotype of the large, flat cells is described below. Comparable results were obtained using rBMP4 (data not shown), which is known to have virtually indistinguishable biological activities as BMP2 in most assays examined (Kingsley, 1994). No clear effect was seen with rBMP7 used at 100 ng/ml, although in some systems this factor has overlapping effects with BMP2 or -4 (Liem et al., 1995; Varley et al., 1995). Similarly, BMP6, activin, and glial cell line–derived neurotrophic factor had no detectable influence on NCSC differentiation (not shown).

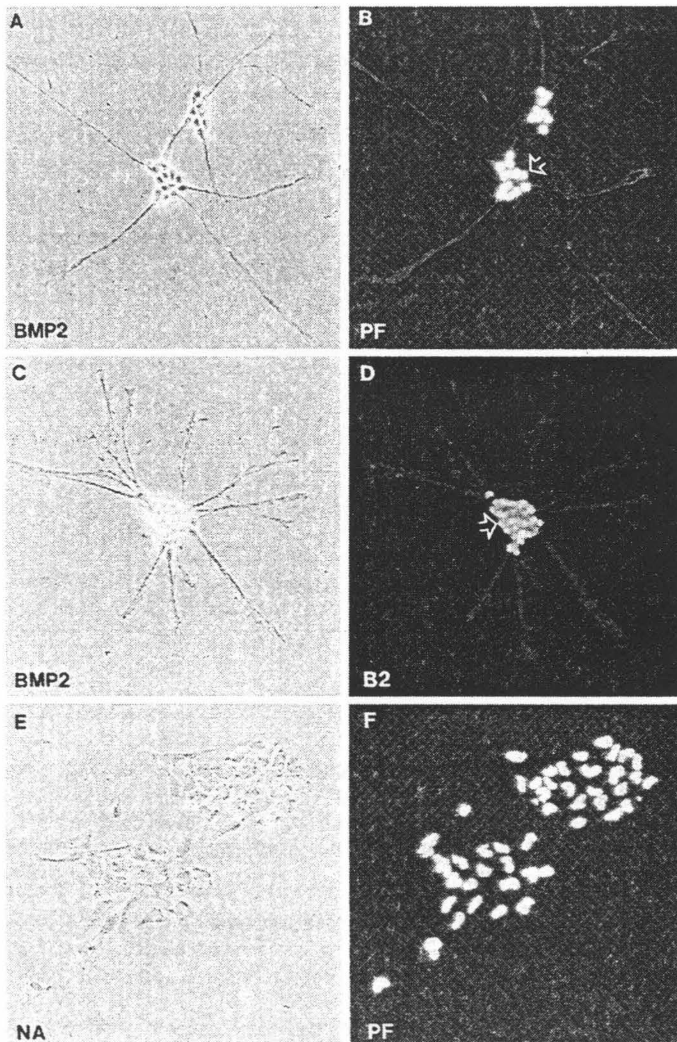

Figure 2. rBMP2 Induces Autonomic Neuronal Differentiation

NCSCs were grown in 1.6 nM rBMP2 (A–D) (a concentration routinely used with other cell types that respond to rBMP2) or in control medium (NA) (E and F) for 4 days. The cultures were fixed and immunostained with antibody to peripherin (B and F) or monoclonal antibody B2 (D), an autonomic lineage-specific marker, developed with a phycoerythrin-conjugated secondary antibody and counterstained with DAPI. (A), (C), and (E) represent phase-contrast views of the immunostained fields (B), (D), and (F), respectively. Matched exposures were taken in (B) and (F) to permit comparision of peripherin (PF) staining intensities. Note the neuronal phase-bright cell bodies with processes and absence of non-neuronal cells in (A); all these cells were peripherin$^+$ confirming that they were differentiated neurons (B). In contrast, the colony shown in (E) and (F) resembles an undifferentiated stem cell colony and none of the cells stained for peripherin (F). The nuclear morphology of the neurons (arrows in [B] and [D]), as revealed by DAPI, was characteristic and differed from that of the stem cells in (F).

Most of the neurons that developed in rBMP2 stained positively with monoclonal antibody B2 (Figure 2D), which is only expressed by autonomic neurons (Anderson et al., 1991). However, these neurons did not express catecholamine biosynthetic enzymes such as dopamine β-hydroxylase or tyrosine hydroxylase, at any concentration of the factor tested. These data suggest that rBMP2 promotes the differentiation of autonomic neurons, which are either nonsympathetic or which require additional signals (Groves et al., 1995) to express markers characteristic of the sympathetic sublineage (for review, see Patterson and Nawa, 1993).

As overt neuronal differentiation was not apparent until 3–4 days after addition of rBMP2, we sought evidence for an earlier influence of this factor on neurogenesis. To do this, we examined expression of MASH1, whose expression precedes that of neuronal markers by several days both in vivo (Lo et al., 1991) and in vitro (Shah et al., 1994). At 12 hr after addition of rBMP2 to NCSCs, over 70% of the colonies (many of which were still single cells) expressed MASH1 (Figures 3A and 3B; Table 1); by 24 hr, ~85% of the colonies were MASH1$^+$. The effects of rBMP2 were apparent even by 6 hr, the earliest time tested, when ~30% of the colonies expressed MASH1 (Table 1). By contrast, at these time points very few of the NCSCs in control medium were MASH1$^+$ (Figures 3C and 3D; Table 1). Rather, MASH1 is expressed by NCSCs in control cultures only after 7–8 days (Shah et al., 1994) (Table 1, legend, this study). Moreover, within such control colonies, MASH1 is expressed by subsets of cells; by contrast, within rBMP2-treated colonies most or all cells expressed MASH1. These data indicate that in the presence of rBMP2 the majority of NCSCs express MASH1, and do so on a far

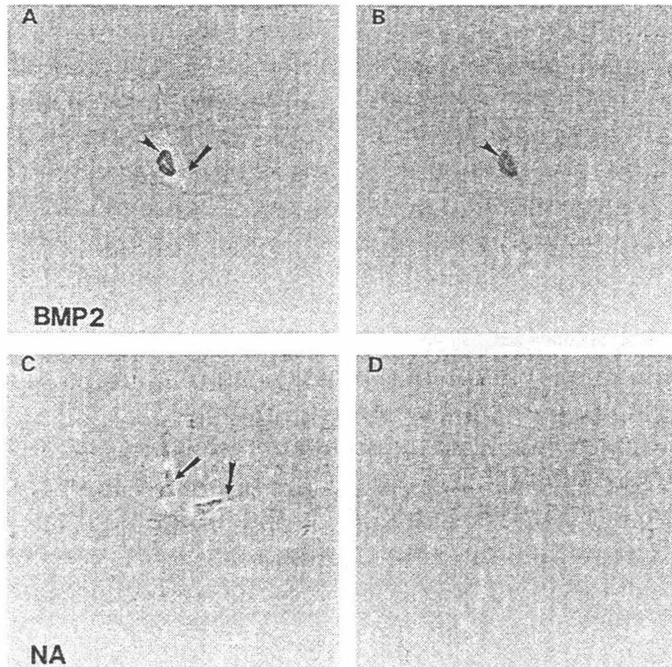

Figure 3. BMP2 Induces MASH1 Expression in NCSCs

NCSCs grown at clonal density in 1.6 nM rBMP2 (A and B) or in control medium (NA) (C and D) for 12 hr were fixed and labeled with anti-MASH1 monoclonal antibody. Staining was visualized using a secondary antibody conjugated to horseradish peroxidase. (A) and (C) are phase-contrast views of the bright-field images shown in (B) and (D), respectively. Since the cells were plated at clonal density and fixed within 18 hr, it is likely that the single cell in (A) and (B) did not undergo mitosis before expressing MASH1 (arrowheads). The faint signal in (D) represents cytoplasmic vesicles (C, arrows) and not MASH1 staining. Similar vesicles were observed in cells that were stained with the secondary antibody alone (not shown).

earlier schedule than under control conditions. Moreover, they support the idea that the expression of MASH1 in autonomic neuronal precursors in vivo may reflect its induction by BMP2 derived from neighboring tissues (see Figure 1).

The Flat Cells That Develop in rBMP2 Are Smooth Muscle Cells

As mentioned above, a subset of the colonies in rBMP2 also contained large, flat cells that were not glia (Figure 4A and data not shown); this suggested that they could be a mesectodermal derivative of the neural crest, such as smooth muscle (Chamley-Campbell et al., 1979; Ito and Sieber-Blum, 1993). Many of the flat cells expressed

Table 1. Kinetics of MASH1 Induction in NCSCs by rBMP2

Time of Analysis (hr)	Culture Condition	MASH1^{+} Colonies (%)
6	BMP2^{-}	30 ± 1
6	No add	5 ± 1
12	BMP2^{+}	74 ± 9
12	No add	2 ± 2
24	BMP2^{+}	84 ± 1
24	No add	1 ± 1

NCSCs were grown at clonal density and 1.6 nM rBMP2 was added to some dishes 6–8 h after the replating. Cells were fixed 6, 12, and 24 h after the addition of growth factor and stained with anti-MASH1. The staining was visualized using a secondary antibody conjugated to horseradish peroxidase. The data are expressed as the mean ± SEM of 2 independent experiments. Although essentially none of the colonies in No add expressed MASH1 at these time points, by 9 days in vitro 70 ± 4 colonies were MASH1^{+}.

α smooth muscle actin (αSMA), a well-characterized SM marker (Owens, 1995) (Figure 4C, green fluorescence, and data not shown). Further, most of these flat cells expressed calponin, another SM-specific protein that may regulate contractility (Owens, 1995) (Figure 4C, red fluorescence). Of all the nonneuronal cells observed in rBMP2, 93% expressed αSMA, calponin, or both. The remaining cells displayed a similar SM-like morphology (Figure 4A) despite their lack of expression of these two SM-specific markers. These data therefore suggest that most or all of the flat cells observed in rBMP2 are SM cells at various stages of differentiation.

TGFβ1 Promotes Smooth Muscle Differentiation of NCSCs

The preceding results raised the question of whether all members of the TGFβ superfamily would similarly promote both neuronal and SM differentiation. We therefore screened other available family members for their effects on NCSCs. In recombinant TGFβ1 (rTGFβ1) virtually all NCSC colonies differentiated to SM cells (Figure 4D). Of the colonies, 82.4% ± 0.6% (mean ± SEM, n = 2) consisted exclusively of cells that were αSMA^{+}, calponin^{+}, or both (Figure 4F); 12% had at least one αSMA^{+} or calponin^{+} cell together with SM-like, marker-negative cells, while 5.6% ± 1.8% of the colonies contained only marker-negative but SM-like cells. Less than 1% of the colonies contained any low affinity nerve growth factor receptor–positive (LNGFR^{-}) NCSCs. No neurons or glial (GFAP^{+}) cells were observed to develop under these conditions. Furthermore, staining of clones grown for 12 or 24 hr in TGFβ1 failed to detect even transient expression of MASH1 (data not shown). In cultures grown in the absence of TGFβ1 for a similar period,

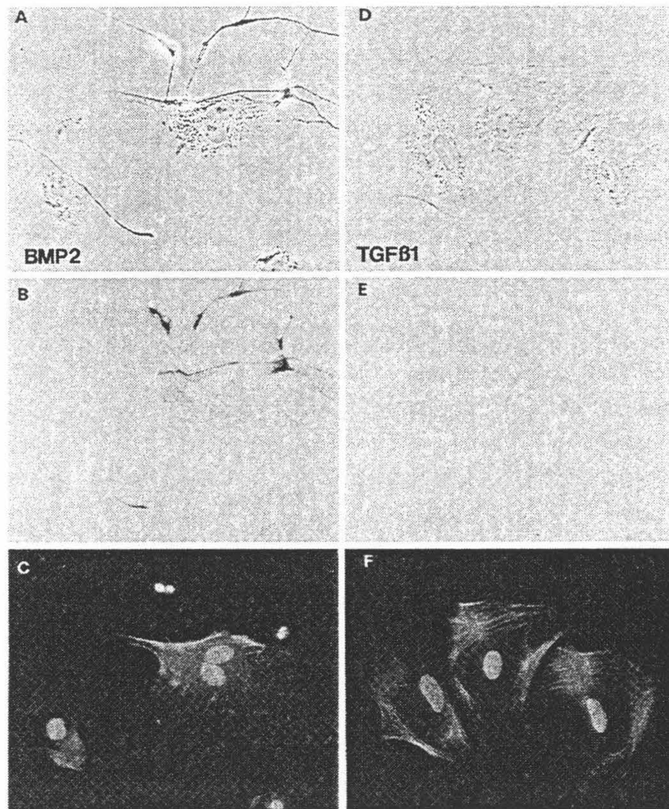

Figure 4. Differentiation of Smooth Muscle Cells in rBMP2 and rTGFβ1

NCSCs were grown at clonal density as described in the experimental procedures for 4 days in either 1.6 nM rBMP2 (A–C) or 20 pM rTGFβ1 (D–F). The cells were then fixed and triply labeled for peripherin, αSMA, and calponin followed by counterstaining for DAPI to reveal cell nuclei. Staining for peripherin was visualized with a secondary antibody conjugated to horseradish peroxidase and bright-field optics (B and E). Staining for αSMA and calponin was visualized with subtype-specific secondary antibodies conjugated to fluorescein and rhodamine, respectively, using epifluorescence optics (C and F). The orange staining reflects the expression of both SMA (green) and calponin (red). (A) and (D) represent phase-contrast images of fields shown in (B) and (C), (E) and (F), respectively. Note that ~25% of all colonies in rBMP2 contained SM together with neurons, as illustrated in (A)–(C), whereas 99% of colonies in rTGFβ1 (D)–(F) contained SM-like cells.

95% of the colonies consisted primarily of undifferentiated NCSCs, although some SM cells were present. TGFβ2 and TGFβ3 yielded similar results as TGFβ1 (data not shown).

The fact that BMP2 produced neurons and some SM cells, while TGFβ1 produced only SM cells, could simply reflect the different concentrations at which these related factors were initially used. However, dose–response experiments (Figure 5) did not support this idea: there were no doses at which the factors elicited identical responses, or at which BMP2 elicted a homogeneous response. Thus, at no concentration of BMP2 did we obtain exclusively SM or neuronal differentiation; rather, the proportion of both neuronal and SM colonies increased as a function of BMP2 dose (Figures 5A and 5B). Similarly, varying the concentration of TGFβ1 over three orders of magnitude did not cause the appearance of mixed (i.e., neuronal + SM) colonies at any dose (Figure 5C and data not shown). These data suggest that the mixed response observed with BMP2 cannot be explained by a suboptimal or excess concentration of the factor used.

rBMP2 and rTGFβ1 Act Instructively Rather Than Selectively

The foregoing colony assays did not distinguish whether BMP2 and TGFβ1 act to influence differentiation by multipotent NCSCs, or rather to support survival of subpopulations of pre-committed neuronal or SM precursors, respectively. To address this issue, we performed a clonal analysis: in this experiment, individual NCSCs were identified shortly after plating, growth factors were added to some, and their subsequent survival and differentiation assessed after 4 days. If BMP2 and TGFβ1 allowed survival and proliferation of different subpopulations of neural crest cells, then only a small proportion of the founder cells should form clones under each of the two conditions.

Such selective survival of subsets of clones was not observed. In the presence or absence of rBMP2, ~90% of the identified founder cells survived to form clones (Table 2). Of these, the majority (75%) grown in rBMP2 contained neurons, whereas none of the clones in control medium contained neurons at this time (Table 2). Moreover, two-thirds of the neuronal clones contained only neurons and no other cell type; the remainder (as discussed above) contained neurons and SM cells. Similarly, in rTGFβ1 the majority (~65%) of NCSCs survived, and of these 99% contained cells that were either αSMA^+ or that had an SM-like morphology (Table 3). Only 1% of the clones contained any undifferentiated $LNGFR^+$ NCSCs. Although 35% of the clones did not survive the 4-day incubation in rTGFβ1, daily observation indicated that none of them contained neurons prior to death; in

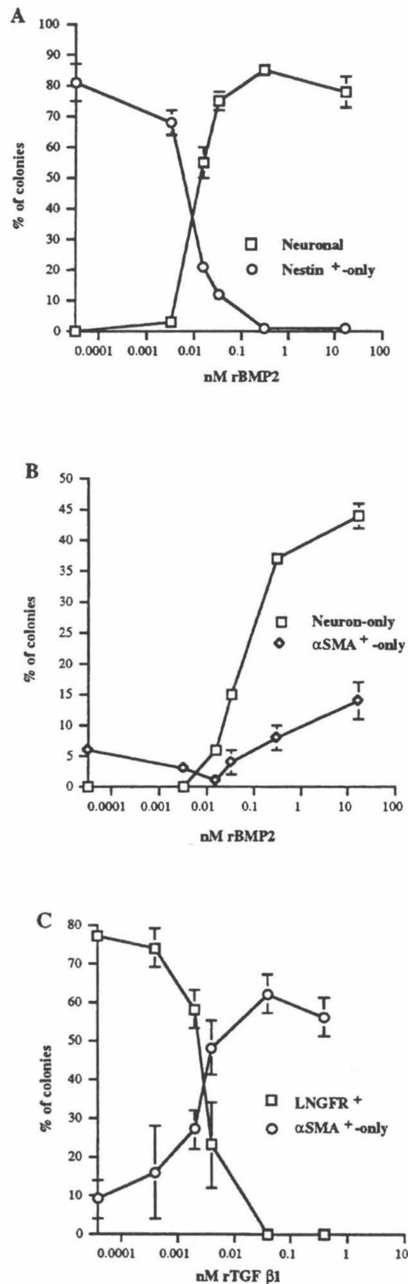

Figure 5. BMP2 and TGFβ1 Produce Distinct Responses over a Wide Range of Doses

NCSCs were plated at clonal density and various concentrations of rBMP2 or rTGFβ1 were added 6–8 hr after the replating. Cells were fixed 4 days after the addition of factors, stained for the markers indicated, and the proportion of different colony types determined. Each point represents the mean ± SEM of 2 independent experiments. Shown in (A) is the percentage of colonies containing any neurons at all (squares) or any nestin$^+$ NCSCs (circles) in rBMP2. Note that the percentage of neuronal and NCSC colonies shows a roughly inverse relationship. Shown in (B) is the percentage of neuron-only (squares) versus αSMA$^+$-only (diamonds) colonies in rBMP2. Note that both neuron-only and SM-only colonies are obtained at essentially all doses. Shown in (C) is the percentage of NCSC (LNGFR$^+$) (squares) and αSMA$^-$-only (circles) colonies in TGFβ1. At no concentration of TGFβ1 were neurons observed (data not shown). The maximal percentage of SM-only colonies (62%) is an underestimate, because for technical reasons calponin$^+$ cells could not be scored in this experiment and therefore SM cells that are calponin$^+$ but αSMA$^-$ (see text) are missed.

Table 2. Clonal Analysis of NCSC Survival and Differentiation in rBMP2

Condition	Survival	Phenotype of Surviving Clones: Neuronal	Phenotype of Surviving Clones: Nonneuronal
BMP2	88 ± 4	75 ± 3	25 ± 3
No add	91 ± 2	0	100

Individual NCSCs were identified 6–8 hr after replating, at which time rBMP2 (1.6 nM) was added to some cultures. This avoided effects of the growth factor on cell attachment. The phenotype of the clones was analyzed after 4 days. The category Neuronal includes both clones that consisted exclusively of peripherin$^+$ neurons, as well as clones containing both neurons and smooth muscle (SM)-like cells. Neuron-only clones constituted 67% of all these neuronal clones. Nonneuronal clones consisted mainly of SM; in rBMP and undifferentiated cells in No add. The data represent the mean ± SEM of 3 independent experiments.

fact many contained cells with an SM-like morphology. These data argue that in the presence of rBMP2 or TGFβ1, multipotent neural crest cells that would eventually have generated neurons, glia, and SM cells (in control medium) instead generated only neurons and SM cells, or SM cells alone, respectively.

While the foregoing data indicated that BMP2 and TGFβ1 act instructively on the founder cell population, they did not exclude the possibility that these factors act selectively on the clonal progeny of these founder cells. For example, within individual clones, cells in all available lineages might initially be generated by a stochastic mechanism, with different lineages surviving depending upon the growth factor added. We performed two kinds of experiments to address this issue. In one experiment, we made sequential observations of live, identified clones, every 24 hr for the 4-day incubation period (Figure 6). This experiment should have revealed if there was significant death occurring within clones in either the presence or absence of the growth factors. To the contrary, in rBMP2, many instances were observed in which a founder cell divided several times and all of its progeny then differentiated into neurons (Figure 6B). Similarly, in rTGFβ1 many cases were documented in which a founder cell divided to produce a clone of SM-like cells without any noticeable cell death (Figure 6C). The behavior of the clones in rBMP2 and rTGFβ1 was in clear contrast to that observed in control medium over the same culture period, in which NCSCs divided to produce clones containing NCSC-like cells (Figure 6A) (Stemple and Anderson, 1992). No death of SM-like or other nonneuronal cells within neuron-only clones that developed in rBMP2 was detected, by criteria of either pycnotic nuclei or cell carcasses (usually visible

Table 3. Clonal Analysis of NCSC Survival and Differentiation in rTGFβ1

		Phenotype of Surviving Clones			
			LNGFR⁻/αSMA⁺		
Condition	Survival	LNGFR⁺	αSMA⁺-Only	αSMA⁺-Containing	LNGFR⁻/SM-like
TGFβ1	64 ± 4	1 ± 1	52 ± 10	22 ± 5	25 ± 8
No add	96 ± 2	87 ± 2	5 ± 3	8 ± 4	0

Cells were grown in the presence or absence of rTGFβ1 (20 pM, added as described in Table 2). Clones were phenotyped using anti-LNGFR and anti-αSMA⁺ to identify NCSCs and SM-like cells, respectively. LNGFR⁻/αSMA⁺-only clones contained exclusively αSMA⁺ cells. LNGFR⁻/αSMA⁺-containing clones had at least one αSMA⁺ cell; the remaining cells were LNGFR⁻/αSMA⁻. As explained in the text, these marker-negative cells had an SM-like morphology and were separately determined to be calponin⁺ in many cases (for technical reasons, triple labeling with antibodies to LNGFR, calponin, and αSMA was not feasible). The column LNGFR/SM-like denotes clones negative for both LNGFR and αSMA; again, these cells had an SM-like morphology and many were calponin⁺ in separate experiments. A clone containing even one LNGFR⁺ cell was included in the LNGFR⁺ column. The data are expressed as the mean ± SEM of 3 independent experiments.

on the substrate following death). Moreover, neurons were never observed to differentiate and then die, in either control medium or in TGFβ1.

To address the possibility that some cells might have died and escaped detection between the 24 hr time points assayed in the continuous observation experiments, we performed a second experiment in which diamidinophenylindole (DAPI) staining was used to visualize fragmented nuclei, characteristic of apoptotic cells (Raff, 1992), in colonies fixed every 6 hr after the addition of growth factors. If the effects of the growth factors are due to intraclonal selection, then a large proportion of clones should contain a subset of dying cells. As shown in Table 4 there was minimal cell death detectable in any condition over the 36 hr period examined. Moreover, when cell death was detected it usually affected entire colonies (Table 4, last column). The percentage of such dying colonies, as detected by this DAPI analysis (10%–12%) was similar to that observed in the clonal analysis (Tables 2 and 3). Importantly, very few cases in any condition were observed in which a subset of cells died within a colony (Table 4, "Some in Colony," n ≈ 70 colonies analyzed per time point per condition).

To ensure that this analysis was performed over a sufficient period to allow at least some differentiation to occur, cultures were stained with an antibody to neurofilament 160 kDa subunit (NF160), an early marker of neuronal differentiation (Sommer et al., 1995). By 36 hr in rBMP2, 29% of colonies consisted of purely NF160⁺ cells, and none of these colonies contained dying cells as assessed by counterstaining for DAPI (data not shown). An additional 43% of colonies contained a mixture of NF160⁺ and NF160⁻ cells in rBMP2, but again fragmented nuclei were rarely if ever detected within such colonies. None of the colonies in control medium contained NF160⁺ cells at these time points. These data indicate that the development of colonies homogeneously expressing neuronal markers at several successive stages of differentiation, including MASH1, NF160, and peripherin, cannot be explained by selective intraclonal cell death in either rBMP2 or control medium. Qualitatively similar results were obtained in TGFβ1 for expression of SM-specific markers (data not shown). Together, these experiments support the idea that BMP2 and TGFβ1 act instructively to influence the direction of differentiation taken by the founder cell and its immediate progeny, rather than to support differential survival of lineage-restricted cells that are first generated stochastically within the clones.

The average clone size after 4 days was significantly smaller in TGFβ1 (2 cells per clone, mean of 2 experiments), compared with controls (45 cells per clone, mean of 2 experiments). The average size of neuron-only clones in rBMP2 (19 cells per clone, mean of 2 experiments) was also smaller than in controls, although much higher than in TGFβ1. The results from serial observation of identified clones (see above) suggest that this difference in cell number cannot be accounted for by cell death; rather, the reduced clone size in TGFβ1 and rBMP2 is most likely due to inhibited or slowed proliferation. Whether such effects on proliferation are a cause or a consequence of differentiation remains to be determined; however, TGFβ1 is known to inhibit proliferation of SM cells in low density cultures (Majack, 1987).

Discussion

We have developed and exploited a clonal culture system (Stemple and Anderson, 1992) that has permitted detailed investigation of the action of growth factors on rodent neural crest cells. Initially, we demonstrated that neuregulin/GGF promotes the glial fate at the expense of neuronal differentiation (Shah et al., 1994). We have now added SM differentiation to the NCSC repertoire and have shown that this fate is promoted by TGFβ1. In contrast, a related factor, BMP2/4, promotes primarily autonomic neuronal differentiation although some SM differentiation is observed. Clonal analysis and serial observations of living clones strongly indicate that both TGFβ1 and BMP2 act instructively rather than selectively. Thus, the differentiation of a multipotent, self-renewing cell along each of three different lineages can be instructively promoted by different growth factors in vitro (Figure 7). Moreover, the expression patterns of BMP2/4 (Bitgood and McMahon, 1995; Lyons et al., 1995), TGFβ1 (Akhurst et al., 1990; Millan et al., 1991; Dickson et al., 1993) and neuregulin (Marchionni et al., 1993; Meyer and Birchmeier, 1994; Shah et al., 1994) in vivo are consistent with the roles suggested for them by these in vitro experiments.

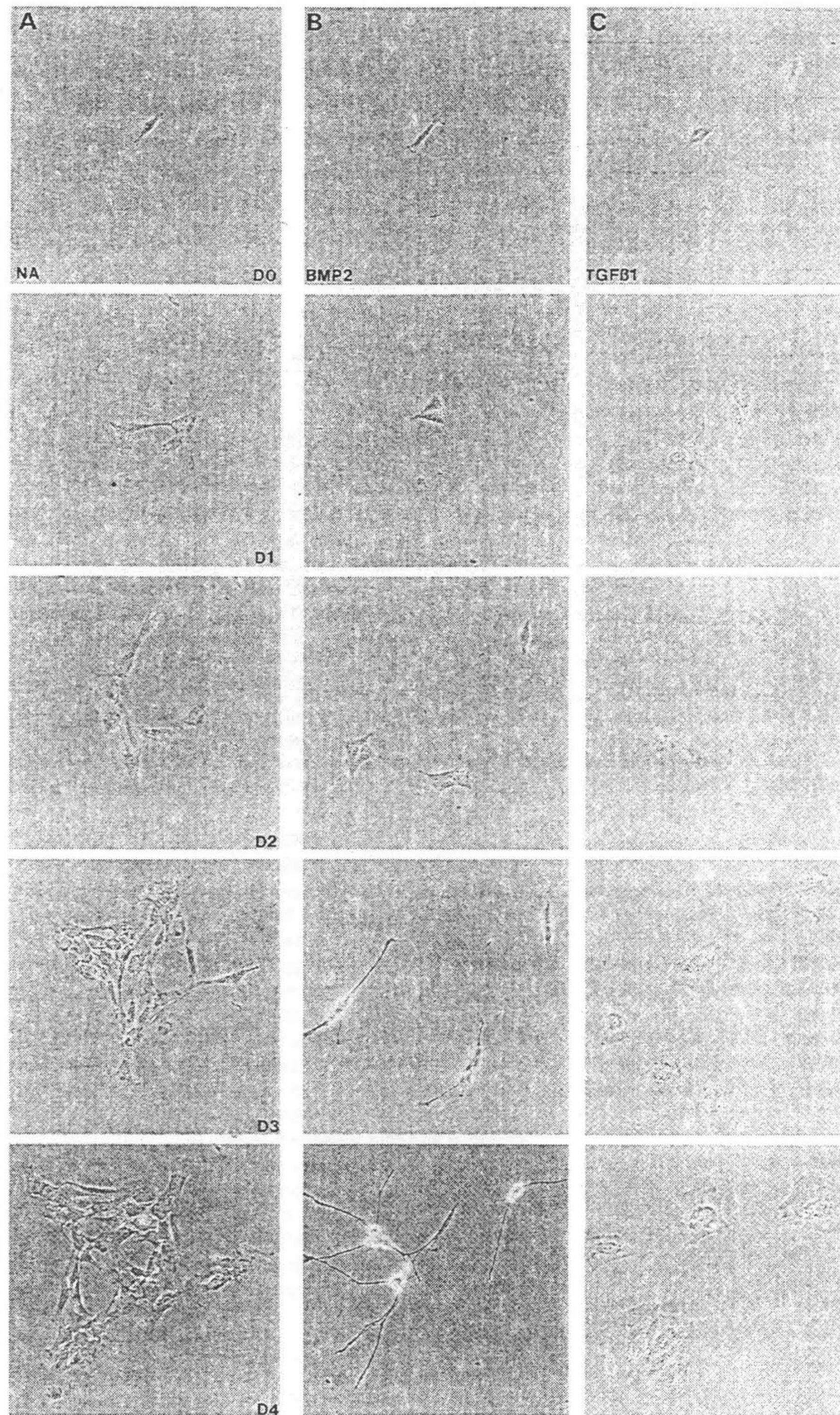

Figure 6. Serial Observation of Identified Clones Demonstrates Instructive Effects of rBMP2 and rTGFβ1

Individual founder cells were identified and photographed at day 0 (D0) in control medium, and then rBMP2 (B) or rTGFβ1 (C) was added to some plates while others were maintained as controls (A). The same clones were rephotographed every 24 hr for the next 4 days (D1, D2, etc.). Note that in rBMP2 ([B] column), the founder cell divides and all of its progeny become neurons, whereas in rTGFβ1 ([C] column), all the founder cell progeny become SM-like cells. Note that no dying cells or cell carcasses are observed in any of the developing clones. The

Table 4. Analysis of Cell Death within NCSC Colonies at Early Time Points

Condition	Time after Addition of Growth Factors (hr)	Colonies Containing Apoptotic Cells (% of total)		
		None in Colony (%)	Some in Colony (%)	Entire Colony (%)
rBMP2	6	91	0	9
	12	93	0	7
	18	90	3	7
	24	88	3	9
	30	87	1	12
	36	85	1	14
NA	6	92	0	8
	12	92	3	5
	18	89	1	10
	24	89	1	10
	30	90	0	10
	36	86	5	9
rTGFβ1	6	90	6	4
	12	86	4	10
	18	83	7	10
	24	91	3	6
	30	92	3	5
	36	84	4	12
NA	6	94	1	4
	12	96	3	1
	18	95	0	5
	24	96	1	3
	30	97	0	3
	36	93	3	4

NCSCs were grown at clonal density in the presence or absence of the indicated growth factors. Cultures were fixed every 6 h for 36 hr, stained for DAPI and analyzed for intact nuclear morphology under epifluorescence optics. None in Colony indicates the percentage of colonies with no dying cells; Some in Colony indicates the percentage of colonies that contained both dying (usually a single cell) and viable cells; Entire Colony is the percentage of colonies consisting of only dead cells (usually 1–2 cells). See text for data on the expression of NF160 and SM markers in this experiment. In each culture condition, 72 ± 2 (mean ± SD) colonies were analyzed per time point.

Role of BMP2 in Neuronal Differentiation of NCSCs

rBMP2 increases both the rate and extent of neuronal differentiation. In addition, this differentiation is prefigured by a rapid induction of MASH1, in some cases before the initial division of the founder cell has occurred (Figures 3A and 3B). This suggests that BMP2 is likely to act directly to promote neuronal differentiation. Although neurons eventually differentiate in the absence of rBMP2, this factor does not only accelerate neurogenesis: half of the clones grown in rBMP2 contain only neurons; by contrast no such clones are ever observed in control conditions. Sequential observation of individual clones (Figure 6) makes it unlikely that this is due to the intraclonal death of nonneuronal cells that are initially generated despite the presence of rBMP2.

Previously, we proposed a model in which the first neurons to differentiate within ganglia signal neighboring uncommitted cells to adopt a glial fate (Shah et al., 1994). Such a model might seem to predict that no neuron-only clones should ever develop under any conditions. However, in these experiments the NCSCs are exposed to rBMP2 while they are still single cells; therefore by the time there are multiple cells within a clone, they may have all been committed to a neuronal fate, precluding lateral signaling interactions that could promote gliogenesis. Although SM cells do develop together with neurons in some clones grown in rBMP2, this fate may be less sensitive to lateral signaling influences. Finally, the absence of glial cells in neuron-containing clones could reflect a hierarchy of influences, in which

results represent examples from each of 24 cells followed in rBMP2 and TGFβ1 and 19 cells followed in control medium. In some cases, founder cells or some of their progeny in TGFβ1 died (see also Tables 3 and 4), but the dying cells often had an SM-like morphology; no cases of dying neurons were observed in TGFβ1, nor was MASH1 expression observed when clones grown in TGFβ1 were fixed and stained at early times (data not shown). Magnifications: all images in (A) column, images D0–D3 in (B) column, and images D0 and D1 in (C) column: 44×; other images 33×.

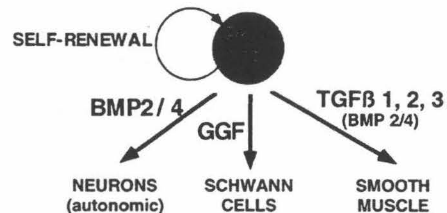

Figure 7. Summary of Instructive Effects of Growth Factors on Rodent Neural Crest Stem Cell Lineage Commitment In Vitro

The diagram illustrates results obtained in the present study combined with those of Shah et al., 1994. Individual factors that promote self-renewal of the neural crest cells have not yet been identified. The illustration should not be taken to imply that the three differentiated fates shown are the only ones available to NCSCs, nor that all three fates are necessarily available to the cells at every division.

BMP2 is dominant over neuregulin. Preliminary experiments in which NCSCs are exposed to saturating concentrations of both factors appear to support this idea; however, more detailed experiments will be required to understand how these cells integrate the influences of opposing signals as a function of relative concentration or time of exposure.

The neurons that develop in rBMP2 appear primarily autonomic. However, they do not express sympathetic markers such as the catecholaminergic biosynthetic enzymes tyrosine hydroxylase and dopamine-β-hydroxylase. This is consistent with other evidence that separate signals control the expression of panneuronal and neurotransmitter-synthetic aspects of the sympathetic phenotype (Groves et al., 1995). Alternatively, the neurons could derive from one or more noncatecholaminergic autonomic sublineages; unfortunately, there are as yet no definitive markers to identify these neuron types in vitro.

Our results appear to contrast with recent data obtained in avian systems, in which BMP2, -4, and -7 have been shown to promote expression of catecholaminergic properties by neural crest cells (Varley et al., 1995; Reissmann et al., 1995, Soc. Neurosci., abstract; Varley and Maxwell, 1995, Soc. Neurosci., abstract). This may simply represent a species difference in the actions of these growth factors or a difference in the culture medium used (the avian cultures contain horse serum, which is absent from our medium). Furthermore, the avian experiments are performed in high density mass cultures, in some cases as explants containing the neural tube. This could permit indirect effects of the factors or interactions with other factors produced by the cultures themselves. For example, retinoic acid has been shown to promote expression of catecholamines in clonal cultures of avian neural crest cells (Dupin and Le Douarin, 1995). As mentioned above, a direct effect of rBMP2 in our system seems likely given the rapid induction of MASH1.

Heterogeneity of the Response to BMP2

Although rBMP2 increases the rate and extent of autonomic neurogenesis, not all clones respond identically. The reason for this is not clear. It could reflect the presence of TGFβ1-like signals within our culture medium, especially since this factor is active on NCSCs at concentrations 10-fold lower than rBMP2 (Figure 5). Alternatively, it could reflect intrinsic heterogeneity within the NCSC population or stochastic differences between clones.

As BMP2 and TGFβ1 bind to structurally related serine-threonine kinase receptors (Massagué, 1992; Lin and Lodish, 1993), the fact that both factors promote SM differentiation seems more than coincidental. It is possible that BMP2 weakly activates the TGFβ1 receptors on NCSCs, although this has not been observed in other systems (J. Massagué, personal communication). More likely, BMP2 may activate some component of the TGFβ1 signal transduction pathway leading to SM differentiation, in addition to a separate pathway that leads to neuronal differentiation. Such cross-talk would have to be one-way, however, since TGFβ1 never generates neurons, even at low concentrations. There is precedence for such cross-activation of biological responses by distinct TGFβ superfamily members in other systems (Carcamo et al., 1994).

BMPs Control Multiple Stages of Vertebrate Neurogenesis

Efforts have recently been made to unify the genetic circuits involved in vertebrate neurogenesis by combining data from several systems. This has led to models in which BMPs are suggested to repress MASH1 (Simpson, 1995). Our results demonstrate that, to the contrary, BMP2/4 actually induces MASH1 in the lineage in which it functions (Guillemot et al., 1993). The inhibitory action of BMPs on MASH1 was extrapolated from the ability of these factors to inhibit primary neural induction in Xenopus (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). However, this involves a much earlier stage of neural development than that examined here. Our data indicate that BMP2 and -4 can act as positive regulators of neurogenesis for neural crest cells. Thus, these growth factors do not play a unitary role in vertebrate neural development: rather, they may control several sequential stages of this process (see also Liem et al., 1995), in either a positive or a negative manner.

Smooth Muscle Differentiation of the Neural Crest

Our identification of the large, flat cells that developed in TGFβ1 (and, to a lesser extent, in rBMP2) as SM cells is based on their morphology (Chamley-Campbell et al., 1979) and expression of two SM-specific markers, calponin and αSMA (Skalli et al., 1986; Gimona et al., 1990). Although each of these markers can occasionally be expressed by some non-SM cells, the coexpression of both markers by many individual cells in our cultures makes it likely that these cells are in fact SM. Nevertheless, in the absence of additional markers this identification should be considered tentative. It is important to point out, however, that SM cells are one of the normal derivatives of the neural crest, although in avians they derive from an anterior region of the neural crest (the cardiac neural crest; Kirby, 1987), rather than from the trunk region (which corresponds to the region from which our NCSCs are obtained). However, the trunk crest has the capacity to give rise to SM if transplanted to anterior regions (Nakamura and Ayer-Le Lievre, 1982). Therefore, the ability to elicit SM differentiation from rodent trunk NCSCs may reflect a developmental potential that is available to these cells in vivo. The available fate mapping data (Serbedzija et al., 1990) do not exclude a contribution of trunk neural crest to SM in mammals. Since sympathetic neurons develop next to the dorsal aorta, it is interesting to consider that in mammals some crest cells derived from the trunk region might contribute to the SM layer of that blood vessel as well.

Although the development of SM cells is of considerable relevance for human disease (Kirby and Waldo, 1990), their development from precursor cells in mammals is poorly understood (see Schwartz et al., 1990; Owens, 1995 and references therein). While SM cell differentiation has been obtained from cell lines such as

ES-like cells (Edwards et al., 1983), the present study represents the first case in which de novo differentiation of these cells from a naturally occuring precursor has been elicited in vitro. Such a system should open the way to further studies aimed at understanding the factors that control the differentiation and maturation of this important cell type (Chamley-Campbell et al., 1979).

Neuropoiesis versus Hematopoiesis: a Different Strategy?

There are a number of seeming parallels between the development of the neural crest and that of the blood, including the existence of migratory multipotent progenitors that are capable of self-renewal (stem cells), of proliferating blast cells, and of growth factors that influence the development of different sublineages (Anderson, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991). As in studies of hematopoiesis (Metcalf, 1980), we have asked the question of how growth factors influence lineage commitment of multipotent cells. However, we have obtained a different answer: rather than acting selectively, as survival factors for committed progenitors, GGF, TGFβ1, and BMP2 all appear to act instructively, to bias lineage decisions by multipotent NCSCs (Figure 7). While instructive factors for hematopoiesis may simply not yet have been identified, neuropoiesis and hematopoiesis may utilize different mechanistic strategies for generating cellular diversity.

Why should the two systems work differently? Hematolymphoid cells float in the bloodstream, whereas neurons must be precisely positioned in order to make correct connections. Such positional constraints may favor the use of spatially restricted instructive signals (Yamada et al., 1993) to direct location-appropriate patterns of differentiation. Another difference is that blood cells are renewed throughout life, allowing the hematolymphoid system to rapidly and repeatedly respond to the environment by adjusting the proportion of different blood cell types, through selective expansion of committed progenitors. By contrast, the nervous system has only one opportunity to develop and responds to the environment by modulating the activity or connectivity of existing cell types. Although cases of neuronal turnover have been described in the adult mammalian nervous system (Altman, 1969), these appear to be exceptions rather than the rule. Instructive mechanisms of lineage commitment would increase the precision with which appropriate neural cell types are generated in correct numbers during development. While selective survival mechanisms certainly operate during neuropoiesis (Cowan et al., 1984; Raff et al., 1993), they occur on a much smaller scale (1 in 2) than in hematopoiesis (e.g., 1 in 100 in the thymus). Thus, the brain and the blood have likely evolved different mechanisms for generating cellular diversity, reflecting the different biological constraints under which they develop and function.

Experimental Procedures

NCSC Cultures

Rat neural crest cells were isolated and cultured as described (Stemple and Anderson, 1992), with minor modifications (available on request).

All comparisons between rBMP2-treated cultures and controls were carried out on a pdL/FN substrate. In the case of rTGFβ1, we found that better cloning efficiency was obtained on an FN-only substrate. Therefore comparisons between TGFβ1 and controls were carried out on FN alone. Importantly, changing the substrates did not alter the overall pattern of phenotypic differentiation of NCSCs in either rTGFβ1 or rBMP2.

Recombinant human BMP2, −4, −6, and −7 were a gift from Genetics Institute. Recombinant human TGFβ1 and -3 were purchased from R&D Systems while recombinant human TGFβ2 was obtained from Genzyme. Commercially available recombinant human activin (Austral Biologicals) and glial cell line–derived neurotrophic factor (Alomone Labs) were used.

Immunostaining and In Situ Hybyridization Studies

Monoclonal antibodies to LNGFR (192 IgG), GFAP (Sigma), NF160 (Sigma), and MASH1 (Lo et al., 1991) were used as described (Stemple and Anderson, 1992; Shah et al., 1994; Sommer et al., 1995). Monoclonal antibody B2 (mouse IgM) was used at a 1:2 dilution and monoclonal antibodies to αSMA and calponin (Sigma) at 1:200 and 1:100–150 dilutions, respectively. Detailed immunostaining protocols are available upon request. DAPI was used at 10 μg/ml for 10 min at room temperature.

In situ hybridization was performed as described previously (Harland, 1991; Birren et al., 1993). Detailed protocols are available upon request.

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Chapter 4

Integration of multiple instructive signals reveals differences in growth factor responsiveness of multipotential rat neural crest cells

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ABSTRACT

The neural crest differentiates into almost the entire peripheral nervous system and many non-neural derivatives such as melanocytes and smooth muscle (Le Douarin, 1982). Single crest cells are multipotential in vivo and in vitro (Bronner-Fraser and Fraser, 1991; Sieber-Blum et al., 1993). We have tested whether rat neural crest stem cells (NCSCs) undergo multilineage differentiation in response to multiple inductive signals. Individual NCSCs form clones containing glia, neurons and smooth muscle (SM) in vitro (Shah et al., 1996; Stemple and Anderson, 1992). The growth factors Neuregulin-2 (GGF2), Bone Morphogenetic Protein-2 (BMP2) and Transforming Growth Factor-ß1 (TGFß1) are likely to instructively induce most NCSCs to differentiate into glia, neurons and smooth muscle (SM), respectively (Shah et al., 1996; Shah et al., 1994). We have exposed NCSCs simultaneously to either BMP2 plus TGFß1, or to BMP2 plus GGF2. Saturating concentrations of TGFß1, but not GGF2, can compete with saturating concentrations of BMP2 in eliciting NCSC differentiation. GGF2 can compete with BMP2 provided the latter is applied at subsaturating concentrations. Although NCSCs possess some functional signaling pathways for GGF2 from the earliest time in culture, GGF2 does not commit NCSCs to a glial fate for at least 48 hours. By contrast, commitment to a neuronal and SM fate occurs within 24 hours, in most cases, in the presence of the appropriate factor. These differences in dosage sensitivity and kinetics of differentiation may influence NCSC lineage determination in response to environmental signals.

RESULTS AND DISCUSSION

We presented BMP2 (2 nM) and TGFß1 (20 pM) either singly or in combination (BMP2+TGFß1) to NCSCs. As expected at these saturating concentrations (Shah et al., 1996), NCSCs grown in BMP2 or TGFß1 yielded predominantly neuronal and SM differentiation, respectively (Fig. 1A). However, in BMP2+TGFß1, an intermediate response was obtained: ~45% of colonies contained neurons and the remainder contained only SM (Fig. 1A). Undifferentiated NCSCs, glia or glial precursors, as assayed for by immunoreactivity for the low affinity neurotrophin receptor (LNTR), were not observed (Fig. 1A). A five-fold increase in the concentration of TGFß1 (100 pM; "BMP2+5xTGFß1" in Fig. 1A) led to an increase in the percentage of SM colonies and a corresponding drop in the proportion of neuronal colonies. There was a smaller increase in the percentage of neuronal colonies when the dose of BMP2 was raised five-fold (10 nM; "5xBMP2+TGFß1" in Fig. 1A). Thus, when NCSCs are grown in BMP2+TGFß1, a response to both growth factors is obtained. Further, the response of NCSCs can be titrated by changing the concentrations of TGFß1 and, to a smaller extent, BMP2.

Since both BMP2 and TGFß1 affect NCSC differentiation upon simultaneous presentation, NCSCs are likely to be competent to respond to these factors on a similar time-scale. To test this prediction and to define the time-scale of NCSC response to these factors, we added either BMP2 or TGFß1 first followed, at various times later, by the second factor. These experiments revealed that rat neural crest cells respond by committing to the appropriate lineage rapidly (usually within the first 24 hours) in either BMP2 or TGFß1. The results of these experiments are described below.

First, NCSCs were cultured in TGFß1 and then BMP2 was added at various times later. We used MASH1 as a rapid molecular indicator of NCSC responsiveness

to BMP2. *Mash1* encodes a bHLH protein that is expressed transiently in autonomic neuronal precursors (Lo et al., 1994). Targeted deletion of *Mash1* reveals that MASH1 is essential for neurogenesis in most autonomic ganglia (Lo et al., 1994; Sommer et al., 1995). BMP2, but not TGFß1, induces MASH1 expression in NCSCs (Shah et al., 1996). SM derived from NCSCs grown in TGFß1 also do not express MASH1 after exposure to BMP2 (not shown). Thus, if TGFß1 promotes a rapid differentiation of NCSCs to SM, then subsequent delayed addition of BMP2 should not induce MASH1 expression in these cells. As expected from experiments described in Fig. 1A, when BMP2 (2 nM) and TGFß1 (20 pM) were added simultaneously to clonal density cultures of NCSCs, about 45% of colonies expressed MASH1 (Fig. 1B, 0 hours). However, if BMP2 followed TGFß1 by 24 hours, ~ 85% of colonies were MASH1$^-$ (Fig. 1B, 24 hours). BMP2 added 48 hours after TGFß1 presentation induced MASH1 in virtually no colonies (Fig. 1B, 48 hours). In contrast, at any of these time-points, the addition of BMP2 induced MASH1 in most NCSC-derived colonies that had not been pretreated with TGFß1 (Fig. 1B).

We wished to determine whether a similar kinetics of NCSC differentiation would be obtained when BMP2 addition preceded that of TGFß1. Since there is no early marker for an effect of TGFß1 on NCSCs, in these experiments we assayed for differentiation markers after allowing NCSCs to grow for 4 days at clonal density in TGFß1. The results are summarized in Fig. 1C. When BMP2 and TGFß1 were added simultaneously, ~ 35% of colonies contained neurons while the rest contained only SM (Fig. 1C, Group B, 0 hours). By contrast, 65% of colonies contained neurons when BMP2 preceded TGFß1 by 24 hours (Fig. 1C, Group B, 24 hours). This was only slightly less than the 75% neuronal colonies obtained in BMP2-only (Fig. 1C, BMP2 at t = 0). If BMP2 addition preceded that of TGFß1 by 48 hours, neuronal differentiation

was indistinguishable from that obtained in BMP2-only conditions (compare Group B at 48 hours with BMP2 at t = 0 in Fig. 1C).

The foregoing results demonstrate that: i) NCSCs respond to both TGFß1 and BMP2 upon simultaneous presentation of these two signals and, ii) The differentiation response of NCSCs to either growth factor occurs, in most cases, within 24 hours. The cell cycle time of NCSCs is 12-18 hours and early NCSC cell divisions are likely to be self-renewing (Stemple and Anderson, 1992). Taken together, these data suggest that NCSCs (or their immediate progeny) can initiate a response to either BMP2 or TGFß1 within 24 hours in culture. Furthermore, these experiments suggest that if NCSCs are exposed to BMP2+TGFß1 in vivo, small differences in the order of presentation and concentration of these factors could play a decisive role in NCSC lineage decisions.

We next compared the response of NCSCs to BMP2 and GGF2 presented singly or in combination. In saturating concentrations of GGF2 (1 nM), ~ 95% of NCSCs formed colonies containing glia but not neurons (see Fig. 2B) while in BMP2 (2 nM), NCSC-derived colonies contained neurons but not glia (Fig. 2B). When NCSCs were cultured at clonal density in BMP2 (2 nM) plus GGF2 (1 nM) (BMP2+GGF2), glial differentiation was not observed and the majority of colonies were neuronal (Fig. 2 A & B). (An increase in the percentage of SM colonies was seen in BMP2+GGF2 compared to BMP2 alone (Fig. 2A); we cannot explain this observation yet.) To assess glial differentiation more definitively, NCSC colonies were allowed to develop for 15 days in vitro and stained for glial fibrillary acidic protein (GFAP), an antigen present in most peripheral glia. GFAP$^+$ glia were observed in control and GGF2-containing cultures (Fig. 2B). However, GFAP$^+$ cells were not seen in BMP2+GGF2 or BMP2-only conditions (Fig. 2B). To rule out the possibility that the apparent dominance of BMP2 over GGF2 reflected the use of sub-optimal concentrations of GGF2, we raised the concentration of this factor to 5 nM, a five-fold

increase. This concentration of GGF2 is well above saturation for the glial differentiation response of NCSCs, as well as for Schwann cell mitogenesis. However, even at this concentration of GGF2, LNTR+ non-neuronal cells, representing glia or their precursors, were not observed and the majority of NCSCs differentiated into neuron-containing colonies ("BMP2+5xGGF2" in Fig. 2A). GGF2 (1 nM) could compete with subsaturating doses of BMP2 because, under such conditions, the proportion of colonies containing neurons decreased if GGF2 was present. For example, at 20 pM of BMP2, the proportion of colonies containing neurons dropped from ~ 60% in BMP2-alone to ~ 15% if GGF2 (1 nM) was added.

The foregoing experiments suggest that NCSCs can exhibit a differentiation response to saturating concentrations of TGFß1, but not GGF2, in the presence of saturating concentrations of BMP2 (2 nM). A differentiation response of NCSCs to GGF2 is seen only when the concentrations of BMP2 are decreased below saturation. At least two models can explain the latter observation. NCSCs may not be competent to commit to a glial fate in response to GGF2 at early times in vitro. Since NCSCs can rapidly commit to a neuronal fate in 2 nM BMP2 (see Fig. 1), inability of NCSCs to respond to GGF2 would allow them to commit to the neuronal lineage to the exclusion of a glial fate. However, if subsaturating BMP2 doses cannot rapidly and synchronously commit NCSCs to neurons, then GGF2 should be able to exert a differentiative effect on uncommitted NCSCs when they gain competence to commit to a glial fate in the presence of the latter factor. In the other model, NCSCs are competent to commit to glia rapidly in the presence of GGF2. However, they may be prevented from doing so if BMP2 can interfere with GGF2-initiated signaling. Subsaturating doses of BMP2 may not be as effective in interfering with GGF2 signaling pathways. This would lead to differentiation of some NCSCs to glia in GGF2 with a concomitant reduction in neurogenesis. If the second model is true, then addition of GGF2 prior to

the addition of 2 nM BMP2 should suffice to commit NCSCs to a glial fate. However, response to a delayed addition of BMP2 would suggest that NCSCs may be unable to commit rapidly to a glial fate at early times in vitro.

To distinguish between these models, NCSCs were grown at clonal density in saturating GGF2 and BMP2 (2 nM) was added later. Cells were fixed 24 hours later and stained for MASH1. Glia obtained from rat sciatic nerve do not express MASH1 when exposed to BMP2 (N.M.S., personal observations). Thus, if NCSCs have differentiated into glia they should remain MASH1$^-$ upon subsequent exposure to BMP2. As expected (see Fig. 2), in BMP2+GGF2, ~85% of NCSC-derived colonies expressed MASH1, a proportion indistinguishable from that of NCSC-derived colonies in BMP2 alone (Fig. 3A, 0 hours). Moreover, when BMP2 was added 12 or 24 hours after GGF2, 85% of colonies expressed MASH1 (Fig. 3A, 12 & 24 hours), a response similar to that obtained in BMP2 alone at these time-points (Fig. 3A, 12 & 24 hours). To see if fewer cells within colonies exposed to GGF2 expressed MASH1 upon subsequent addition of BMP2, we calculated the proportion of MASH1$^+$ cells within each MASH1$^+$ colony. However, the percentage of MASH1$^+$ cells was high and did not decline even if cells had been exposed to GGF2 for 12 hours prior to exposure to BMP2 (Fig. 3B, 0 & 12 hours). The proportion of MASH1$^+$ cells decreased if GGF2 was added 24 hours prior to BMP2. Even at this time-point, however, the majority of cells (~70%) within colonies expressed MASH1. These data suggest that most NCSCs cannot commit to a glial fate (as assayed by the induction of MASH1 by BMP2) within 24 hours of exposure to GGF2.

The percentage of MASH1$^+$ colonies declined if GGF2 addition preceded that of BMP2 by 48-96 hours (Fig. 3A, 48 & 96 hours). At these time-points, the proportion of MASH1$^+$ cells within each colony decreased even more dramatically (Fig. 3B, 48 & 96 hours). Thus, NCSCs (or their progeny) need to be exposed to GGF2 for at least 48

hours before they lose competence to respond to neuron-inducing signals. Taken together with our previous demonstration of an instructive effect of GGF2 on glial differentiation from NCSCs, these data suggest that specification of NCSCs to a glial fate may occur after 2-3 days in GGF2.

The simplest explanation for the requirement of a prolonged exposure to GGF2 before glial determination can occur is the absence, at early times in vitro, of a GGF2-initiated signal transduction pathway in NCSCs. While NCSCs express erbB2 and erbB3 (Meyer and Birchmeier, 1994; Shah et al., 1994), receptor tyrosine kinases that bind GGF2, such receptors may be non-functional. Signal transduction components downstream of the receptors could also be absent. We therefore assayed for activation of MAP kinase (MAPK), a known effector of ligand-bound receptor tyrosine kinases, in NCSCs in response to GGF2 by using an antibody that recognizes only the doubly-phosphorylated, active MAPK (MAPK/PO_4) (Marshall, 1994). MAPK/PO_4 was detected by 10 minutes, the earliest time-point tested, in ~80% of NCSCs treated with GGF2 (Fig. 4 A, 10 minutes; Fig. 4B, Panel B-2). A similar proportion of NCSCs were also positive at 20 minutes (Fig. 4A, 20 minutes). In many cells, MAPK/PO_4 was observed in the nucleus. However, by 60 minutes of GGF2 treatment, expression of MAPK/PO_4 in NCSCs was no longer detectable (Fig. 4 A, 60 minutes; Fig. 4B, Panel B-4). NCSCs treated with either BMP2 or vehicle-only did not stain for MAPK/PO_4 at these time-points (Fig. 4A; Row C in Fig. 4B). One explanation for the dominance of 2 nM BMP2 over GGF2 is that this dose of BMP2 inhibits GGF2-mediated MAPK activation. However, no difference in MAPK/PO_4 was detected between NCSCs exposed to GGF2 or to BMP2+GGF2 (Fig. 4 A; Fig. 4B, compare Rows B & D). Thus, GGF2 can activate signaling pathways in NCSCs, presumably by binding erbB3 and erbB2. Further, this signaling can occur in the presence of saturating doses of BMP2.

The results from the preceding experiments demonstrate that while NCSCs can initiate signaling in response to GGF2 rapidly (as assayed by MAPK/P04), they do not lose responsiveness to neuron-inducing signals for 48-96 hours in GGF2. This could be due to the absence of components other than MAPK/P04 in NCSCs at an early time in vitro. The present unavailability of early molecular indicators of glia precludes a direct test of the existence of a functional gliogenic pathway triggered by GGF2 in NCSCs. Since GGF2 can block some neurogenesis from NCSCs cultured in subsaturating BMP2, we tested whether this inhibition of neurogenesis is preceded by an attenuation of MASH1 induction. If so, this would indicate the presence of a GGF2 signaling pathway that is functional at early times. In this experiment, NCSCs were simultaneously exposed to GGF2 (1 nM) and subsaturating doses of BMP2. Cells were then fixed 24 hours later and assayed for MASH1-immunoreactivity. We found that the proportion of MASH1$^+$ colonies decreased significantly in subsaturating BMP2+GGF2 compared to subsaturating BMP2 alone. For example, the proportion of MASH1$^+$ colonies in 20 pM BMP2 decreased from ~ 75% of total colonies to ~ 30% if 1 nM GGF2 was added. This result strongly suggests that NCSCs possess some functionally intact GGF2-initiated signaling from the earliest times in vitro. Such signaling can interfere with MASH1 induction by low, but not saturating, concentrations of BMP2. However, this early signaling by GGF2, as measured by an inhibition of MASH1 induction in low doses of BMP2, is not sufficient to specify NCSCs to a glial fate because NCSCs exposed to GGF2 alone for 24-48 hours can still respond to saturating doses of BMP2 by expressing MASH1 (Fig. 3). Thus, an early response of NCSCs to GGF2 is to inhibit neurogenesis by low doses of BMP2. However, NCSCs do not lose responsiveness to higher doses of neuron-inducing signal until later, suggesting that glial specification occurs over a protracted period in culture.

In summary, in BMP2+TGFß1 lineages appropriate for both factors differentiate, albeit in a lower proportion compared to the particular factor alone. This outcome may be expected if the cells can commit to neuronal and SM fates on similar time scales. Using tissue recombination experiments in amphibians, it has been demonstrated previously that simultaneous exposure to multiple inductive signals can lead to the differentiation of lineages appropriate for all of the inductive cues (Jacobson, 1963).

In saturating concentrations of BMP2, neuronal, but not glial, differentiation is observed even if GGF2 is present. Two lines of evidence suggest that NCSCs can respond to GGF2 at early times in vitro. GGF2 addition leads to phosphorylation of MAPK by 10 minutes in most NCSCs. Further, GGF2 can inhibit MASH1 induction by subsaturating doses of BMP2 in many NCSCs. However, our sequential addition experiments suggest that glial determination (as assayed by a loss of responsiveness to a neurogenic signal) occurs 48-96 hours after exposure to GGF2. This is in contrast to neuronal and SM determination which occurs within 24 hours of exposure to the appropriate growth factor. In summary, in the presence of multiple instructive cues, NCSCs reveal a differential dosage sensitivity to different growth factors as well as a temporal difference in the differentiation response elicited by these signals. We cannot as yet rule out that, in vivo, these instructive cues may be presented in such a manner that, for example, GGF may be able to compete even with saturating concentrations of BMP2. To illustrate, when NCSCs are exposed to other splice-variants of GGF, they might respond by committing to a glial fate more rapidly. In such a case, BMP2+GGF exposure may lead to the differentiation of both neurons and glia. However, since the various splice forms of GGF are thought to bind and activate erbB2 and erbB3 equivalently, such a possibility seems unlikely at this time (Pinkas-Kramarski et al., 1996; Weiss et al., 1997). Cell autonomous biases in the response to inductive signals

have been described in both invertebrate and vertebrate systems (Clandinin et al., 1997; Dickson et al., 1992; Sokol and Melton, 1991). Furthermore, such biases may change during development (Cepko et al., 1996; Freeman, 1996; Grainger and Gurdon, 1989; Klämbt, 1997; Michelsohn and Anderson, 1992; Watanabe and Raff, 1990).

Our experiments raise the question of how glial cells differentiate in the embryo. Since glia differentiate after neurons in vivo in the PNS (Carr and Simpson, 1978), presumably the crest cells have been exposed to neuron-inducing signals such as BMP2 prior to differentiating into glia. Some mechanisms must exist to ensure that the precursor pool is not depleted by signals promoting neurogenesis. These could include transient or limited secretion of BMP2 by the endogenous source(s) of this factor. The extracellular matrix can also sequester growth factors making it available only in limited amounts (Taipale and Keski-Oja, 1997). Recently, noggin, a polypeptide that binds BMP2 (and related molecules) with high affinity and prevents BMP2 from activating its receptors, has been described (Zimmerman et al., 1996). Although it is not known whether noggin is present in peripheral ganglia, presumably it, or related molecules, could limit the amount of BMP2 available to crest cells. Crest migration occurs over 24-36 hours and the transient or limited availability of BMP2 may ensure that later-arriving cells can respond to glial inducing signals such as GGF2. GGF2 or other splice forms of GGF that bind to the extracellular matrix may be presented more efficiently to crest cells leading to gliogenesis even in the presence of neuron-inducing signals. It is not known which of these mechanisms is operative in vivo. Our data suggest that if BMP2 is present at subsaturating concentrations in vivo, then GGF2 may be able to prevent some cells from adopting a neuronal fate. Our sequential addition experiments suggest that glial determination does not occur for 48-96 hours of exposure to GGF2. Such a protracted response time to GGF2 may be one reason why glial differentiation occurs much later than neuronal differentiation in the embryo.

MATERIALS AND METHODS

NCSC cultures

Neural crest stem cells were obtained from embryonic day 10.5 rat neural tubes and cultured at clonal density essentially as described previously (Shah et al., 1996; Stemple and Anderson, 1992). All experiments were on a poly-D-lysine+fibronectin substrate (pdL/FN) (Shah et al., 1996; Stemple and Anderson, 1992). In experiments involving only TGFß1 or TGFß1 followed by BMP2, NCSC colonies were established on a fibronectin-only substrate since this allowed better survival of cells. Qualitatively similar results (not shown) were obtained if the cultures were grown in TGFß1 on pdL/FN.

NCSCs were always allowed to attach for 6-8 hours before adding BMP2, GGF2 or TGFß1. In BMP2+TGFß1 or BMP2+GGF2 experiments, the growth factors were mixed prior to addition to culture medium. Recombinant human BMP2 and GGF2 were gifts from Genetics Institute and Cambridge Neuroscience, Inc., respectively. Recombinant human TGFß1 was purchased from R&D Systems. Differentiation of NCSCs in BMP2, GGF2 and TGFß1 was optimized by evaluating dose-responses to each of these factors. Saturating responses were obtained at 50 ng/mL for either GGF2 (~ 1 nM) or BMP2 (~2 nM) and at 0.5 ng/mL for TGFß1 (~ 20 pM) (not shown). These concentrations are consistent with our previous results using other lots of these proteins.

Immunocytochemistry

Mouse monoclonals to LNTR, MASH1, GFAP and α-smooth muscle actin (αSMA) and rabbit polyclonal to peripherin were used as previously described (Shah et al.,

1996; Shah et al., 1994; Stemple and Anderson, 1992) followed by appropriately conjugated secondary antibodies. Not all SM are αSMA+ (Shah et al., 1996); in such cases, SM were identified on the basis of their morphology and lack of immunoreactivity for neuronal, glial and NCSC markers.

For the MAPK assay, 250-1000 NCSCs were plated in the center of a pdL/FN coated well of a 12-well Corning dish. After 8-10 hours incubation in standard conditions (see above), the appropriate factors were added to each well without removing the dish from the incubator. Equal volume of carrier solution without factor (Vehicle) was added to some wells as a control. Dishes were agitated to ensure even distribution of the growth factor. (This entire procedure took 1-2 minutes.) The timer was started and dishes were fixed 10, 20 and 60 minutes later. Activated MAPK was detected with a rabbit polyclonal antibody (1 μg/mL) as per manufacturer's (Promega) recommendations. Staining was visualized with an HRP-conjugated antibody and nickelous sulfate/DAB histochemistry.

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Figure 1:

The response of NCSCs to treatment with BMP2 and TGFß1.

NCSCs were grown at clonal density. In Panels "A" and "C," cultures were analyzed for NCSCs (anti-LNTR), neurons (anti-peripherin) and SM (anti-αSMA): "$LNTR^+$" colonies (empty bars) contained $LNTR^+$ cells and no neurons; "Neuronal" colonies (black bars) contained neurons but no $LNTR^+$ non-neuronal cells; "SM" colonies (hatched bars) contained only SM. "NA" is culture in medium not supplemented with BMP2 or TGFß1.

A. The response of NCSCs to treatment with BMP2 and TGFß1.

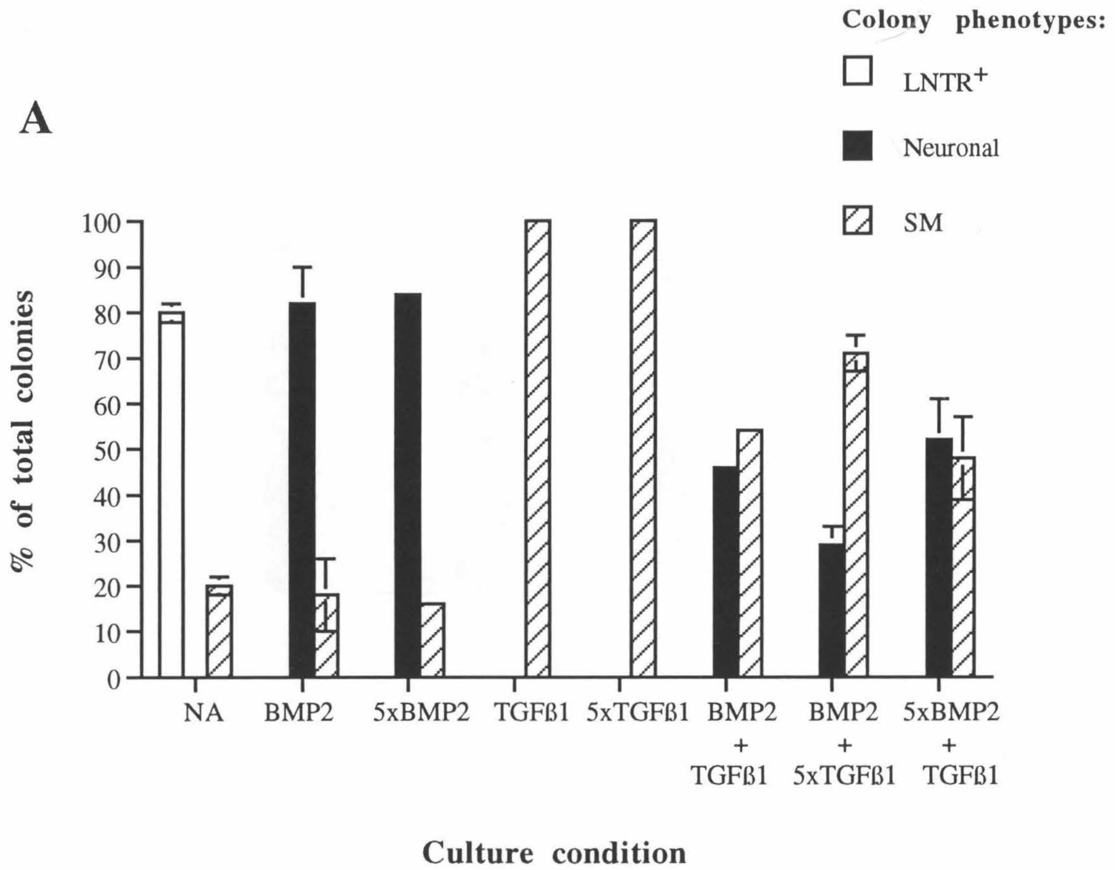
Abscissa shows culture conditions. Concentrations of BMP2 and TGFß1 are in the text.

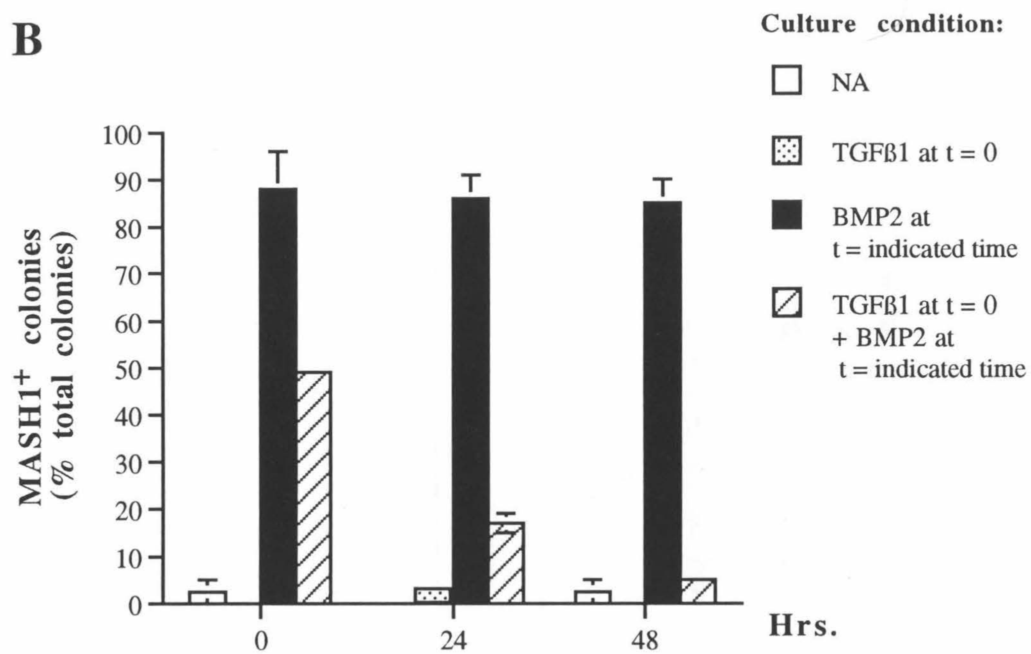
B. The response of NCSCs, as assayed by MASH1, when TGFß1 addition precedes BMP2. Cells received TGFß1 at the start (stippled bars) or BMP2 at the time indicated on the abscissa (black bars). In the experimental condition (hatched bars), TGFß1 was added at the start of the experiment and BMP2 was added at different times as indicated. NA (empty bars) received no treatment. In all cases, cultures were analyzed for MASH1 24 hours after addition of BMP2.

C. The differentiation of NCSCs when BMP2 addition precedes TGFß1.

As controls, cells received no treatment ("NA"), only BMP2 from the start ("BMP2 at t=0") or TGFß1 at different times (Group A) as indicated on the X-axis. In the experimental condition (Group B), cells were first treated with BMP2 at the start and then TGFß1 was added to the cultures at different times as indicated on the abscissa. Cultures were fixed 4 days after the addition of TGFß1 and analyzed for the phenotypes depicted.

(For each panel, mean ± sem of 2 independent experiments shown; ~ 15-20 colonies were analyzed for each time-point and culture condition per experiment.)


A
Colony phenotypes:
LNTR+
Neuronal
SM
% of total colonies
0
10
20
30
40
50
60
70
80
90
100
NA
BMP2
5xBMP2
TGFß1
5xTGFß1
BMP2
+
TGFß1
BMP2
+
5xTGFß1
5xBMP2
+
TGFß1
Culture condition


B
Culture condition:
NA
TGFß1 at t = 0
BMP2 at
t = indicated time
TGFß1 at t = 0
+ BMP2 at
t = indicated time
MASH1+ colonies
(% total colonies)
100
90
80
70
60
50
40
30
20
10
0
0
24
48
Hrs.

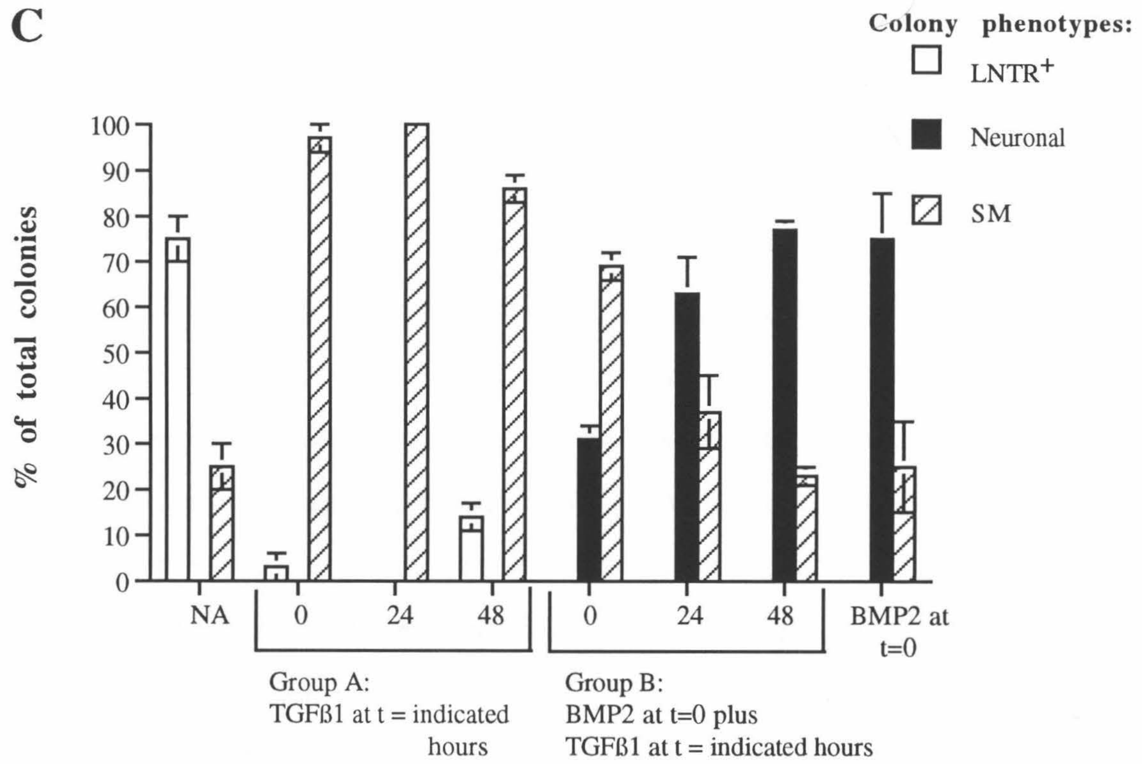
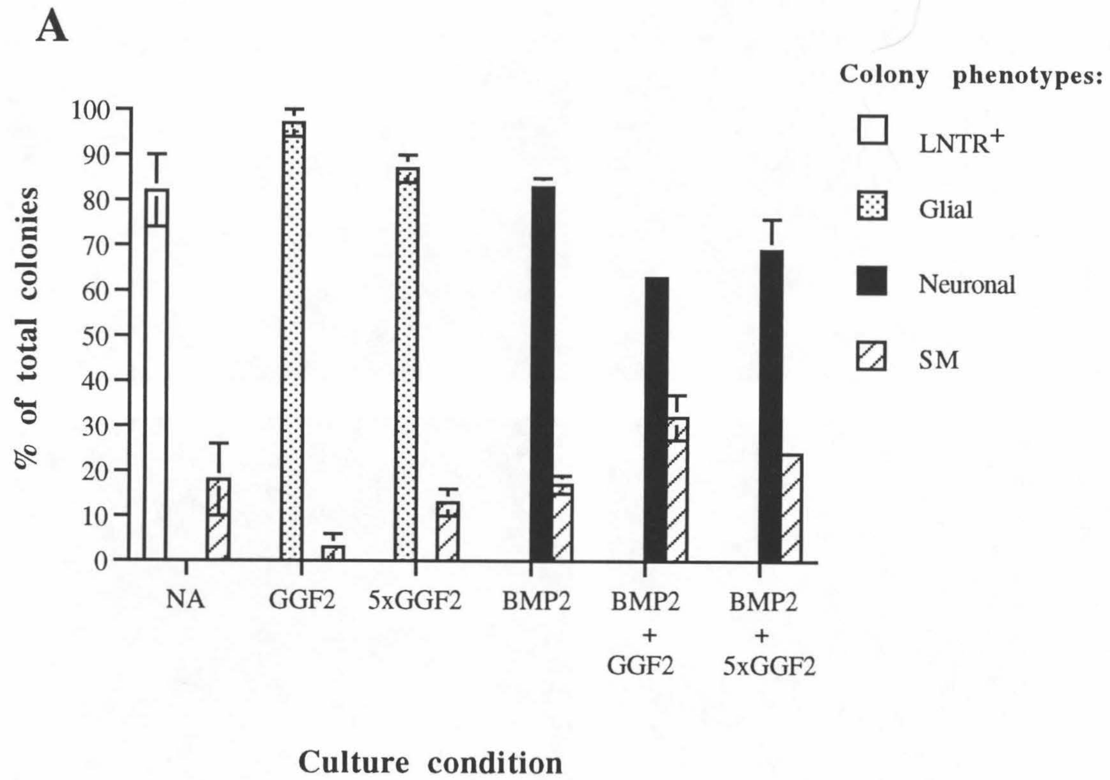

C
Colony phenotypes:
LNTR+
Neuronal
SM
% of total colonies
100
90
80
70
60
50
40
30
20
10
0
NA
0
24
48
0
24
48
BMP2 at t=0
Group A:
TGFß1 at t = indicated hours
Group B:
BMP2 at t=0 plus
TGFß1 at t = indicated hours

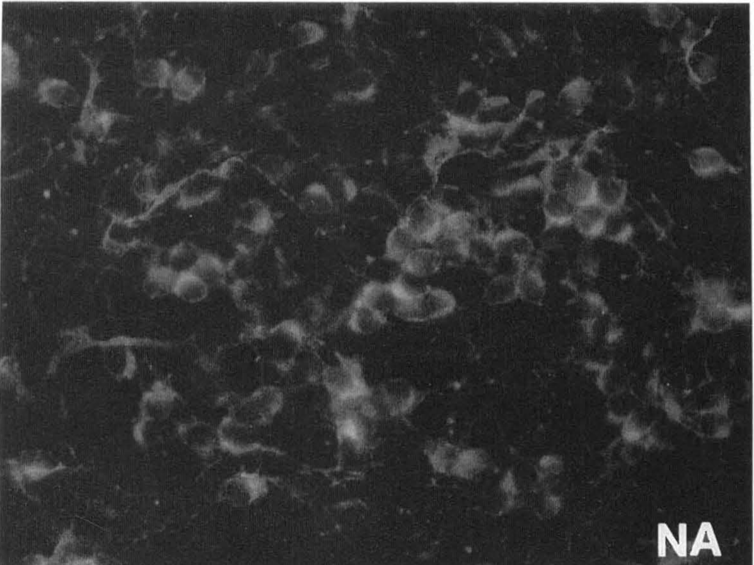
Figure 2:

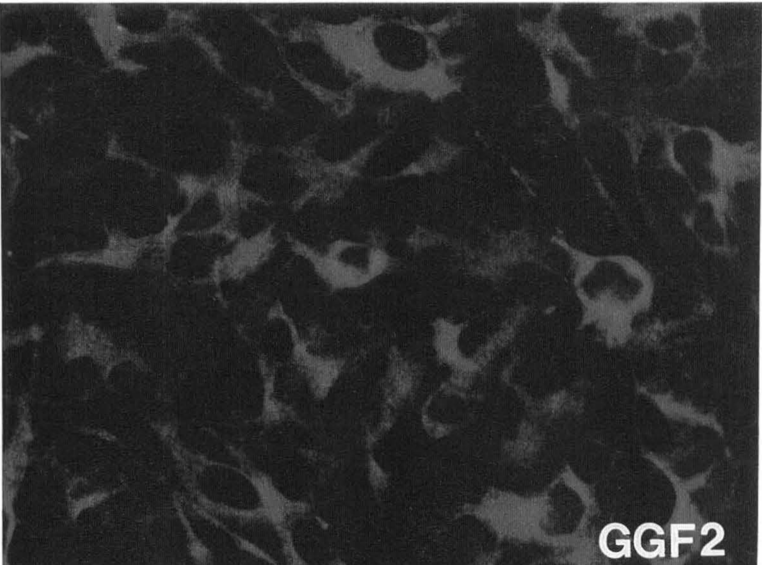
The response of NCSCs to treatment with BMP2 and GGF2

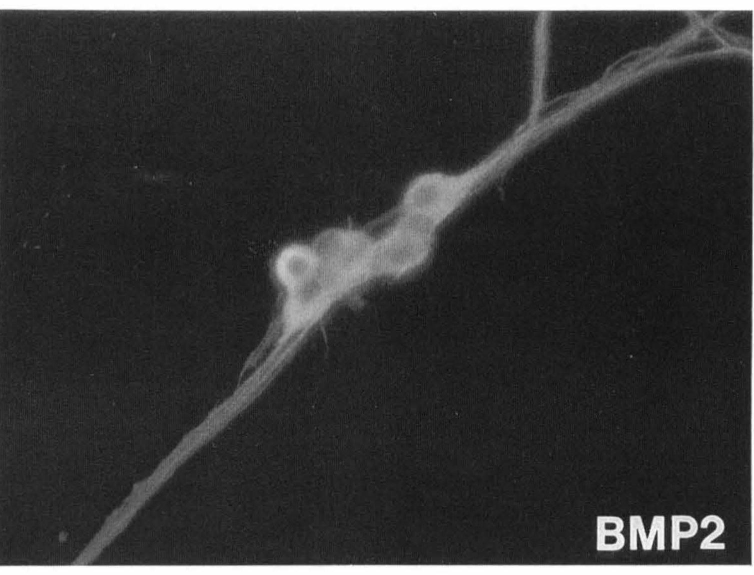
A. Crest colonies were analyzed with antibodies for NCSCs and glia ($LNTR^+$), neurons ($Peripherin^+$) and SM (αSMA^+). The antigen LNTR does not distinguish between NCSCs and glia. Our classification of colonies in GGF2 as "Glial" is prospective. It is based on many separate experiments in which colonies in GGF2 predominantly differentiate into colonies containing glia but no neurons. To elicit this behavior, however, colonies must be cultured for 10-15 days. In the present experiments colonies were cultured for 5 days, a time-point by which BMP2 has induced differentiation. "$LNTR^+$" (empty bars) or "Glial" (stippled bars) colonies contained no neurons. "Neuronal" colonies (black bars) contained neurons but no $LNTR^+$ non-neuronal cells; colonies containing only SM are labeled "SM" (hatched bars). Rarely, $LNTR^+$ $peripherin^+$ clumps were seen in BMP2+GGF2; these resembled autonomic neuronal precursors and are not shown here. A small reduction in the proportion of neuronal colonies with a corresponding increase in SM colonies is seen in BMP2+GGF2. We have no explanation for this phenomenon. Importantly, however, no glial colonies differentiate in this condition and the majority of colonies are still neuronal. (Mean $\pm$ sem of 2 independent experiments; ~ 20 colonies per condition per experiment analyzed.)

B. Clonal density crest cultures in different conditions were grown for 15 days and analyzed for GFAP (red/Phycoerythrin) and peripherin (green/FITC) with immunocytochemistry. Due to the large size of the colonies, only parts of colonies are depicted. Both neurons and glia have differentiated in No Add controls (NA). In GGF2 treated cultures, only glia are seen while neurons, but no glia, are observed in either BMP2 or BMP2+GGF2.


A
Colony phenotypes:
LNTR+
Glial
Neuronal
SM
% of total colonies
100
90
80
70
60
50
40
30
20
10
0
NA
GGF2
5xGGF2
BMP2
BMP2
+
GGF2
BMP2
+
5xGGF2
Culture condition


NA


GGF2


BMP2

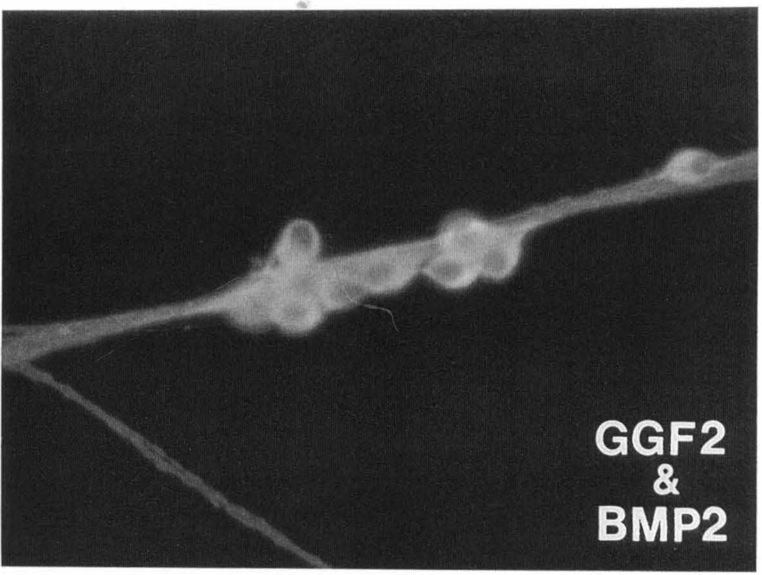

GGF2
&
BMP2

Figure 3:

Response of GGF2 treated NCSCs to late addition of BMP2.

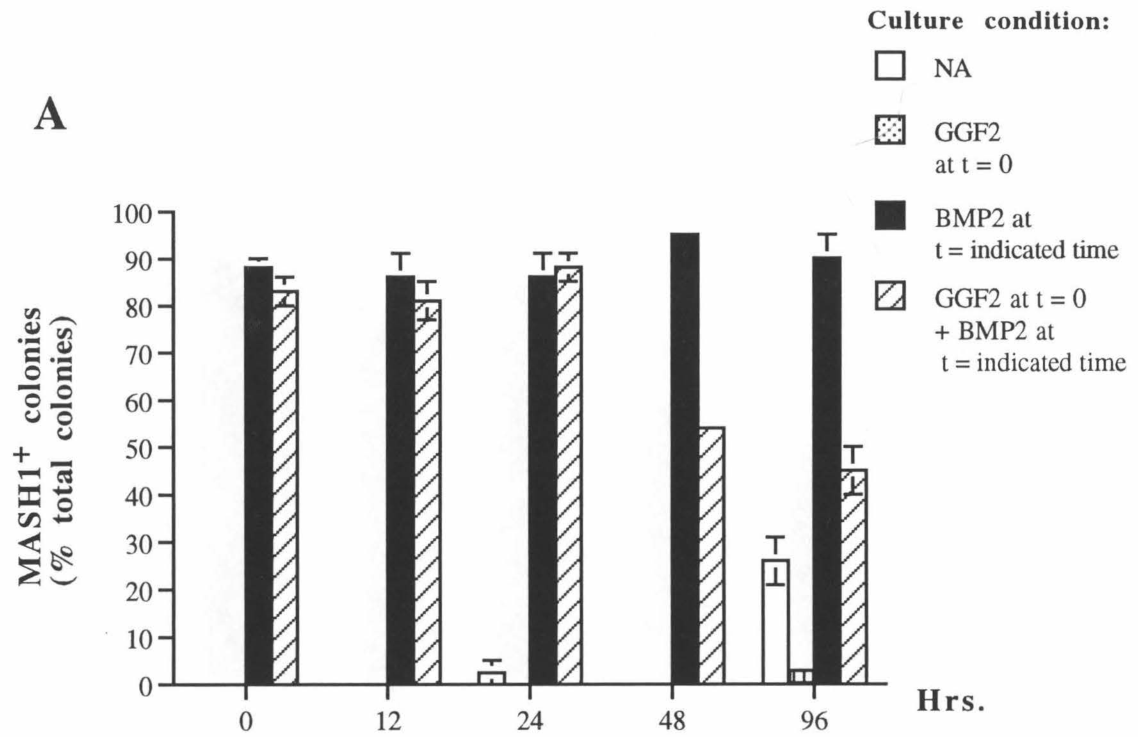
NA controls (empty bars) did not receive BMP2 or GGF2. Other controls included cells treated with GGF2 from the start (stippled bars) or cells treated with BMP2 (black bars) at the times indicated on the abscissa. In the experimental condition (hatched bars), dishes received GGF2 at the beginning and then BMP2 at different times (shown on abscissa). MASH1-immunoreactivity was analyzed 24 hours after addition of BMP2.

A. The percentage of colonies containing MASH1$^+$ cells is shown.

Note that by 96 hours, some colonies in NA have started expressing MASH1. (Mean ± sem of 2 independent experiments shown; ~ 20 colonies for each time-point and condition analyzed per experiment.)

B. The proportion of MASH1$^+$ cells within colonies expressing MASH1 is shown.

The total number of cells per colony at any time-point was comparable between the two conditions (not shown). Therefore, the same trend would be observed if the absolute number of MASH1$^+$ cells per colony was plotted for the two conditions. (Mean ± sem of 2 independent experiments shown; cell counts were done for ~9 colonies for each time-point and condition per experiment.)


A
Culture condition:
NA
GGF2
at t = 0
BMP2 at
t = indicated time
GGF2 at t = 0
+ BMP2 at
t = indicated time
MASH1+ colonies
(% total colonies)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
48
96
Hrs.

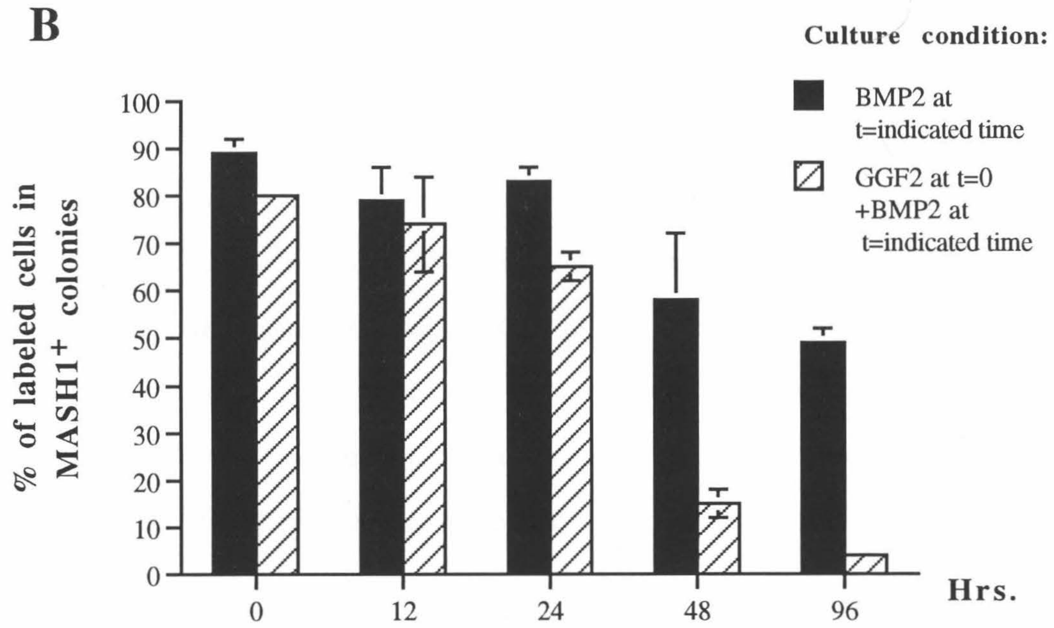

B
Culture condition:
BMP2 at
t=indicated time
GGF2 at t=0
+BMP2 at
t=indicated time
% of labeled cells in
MASH1+ colonies
100
90
80
70
60
50
40
30
20
10
0
0
12
24
48
96
Hrs.

Figure 4:

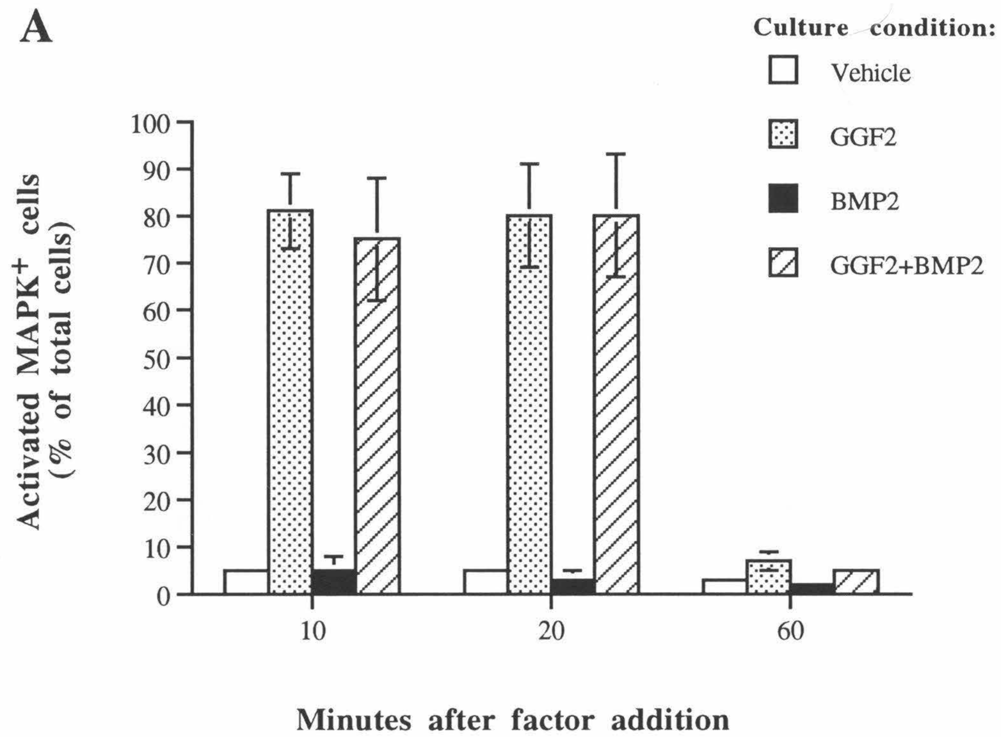
GGF2, but not BMP2, activates MAPK transiently in NCSCs.

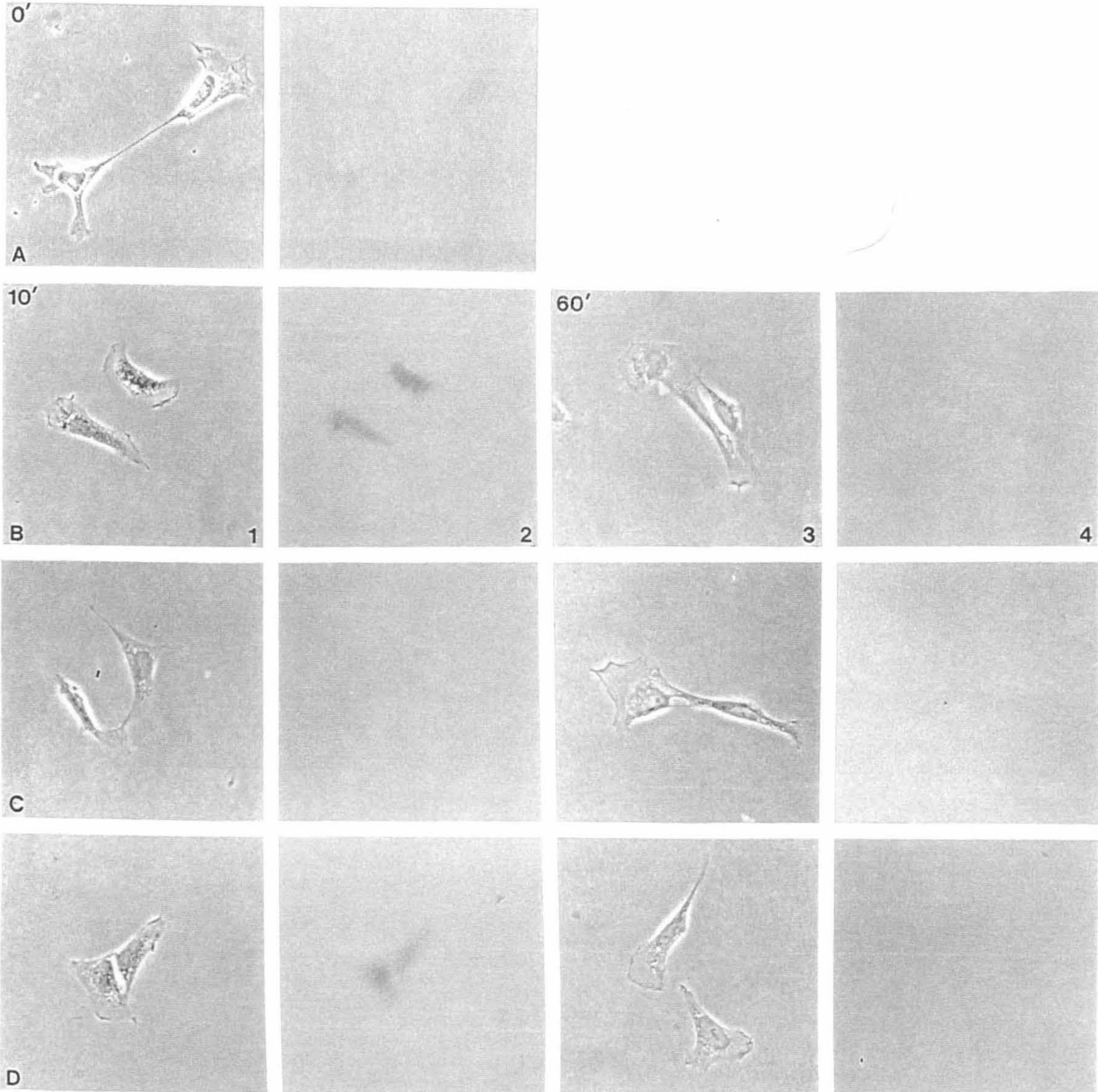
A. The percentage of cells expressing activated MAPK after different treatments is shown.

"Vehicle" is carrier medium without growth factor. BMP2 and GGF2 were used at 2 nM and 1 nM, respectively. (Mean ± sem of 2 independent experiments; ~ 100 cells analyzed for each time-point and culture condition per experiment.)

B. Expression of activated MAPK is shown in NCSCs.

Columns 2 and 4 represent bright-field views of the phase-contrast photographs in columns 1 and 3, respectively. Cells in Row A were fixed before any factor was added; activated MAPK is not expressed in cells at this time. Cells in Row B received GGF2, those in Row C received BMP2 and cells in Row D were treated with GGF2+BMP2. Except for Row A (see above), cells in Columns 1, 2 and 3, 4 were treated with growth factor(s) for 10 and 60 minutes, respectively.


A
Culture condition:
Vehicle
GGF2
BMP2
GGF2+BMP2
Activated MAPK+ cells
(% of total cells)
100
90
80
70
60
50
40
30
20
10
0
10
20
60
Minutes after factor addition


0′
A
10′
B
1
2
60′
3
4
C
D

Chapter 5

Summary and future directions

The neural crest is an excellent model system in which to study multilineage differentiation. Many individual neural crest cells are multipotential, both in vivo and in vitro. Most of the lineages that arise from the crest can be readily identified using currently available molecular markers. It is not surprising then that there are a vast number of studies that have analyzed the differentiation of particular lineages from the neural crest. A prevalent notion in the neural crest field is that the environment plays a significant role in differentiation of neural crest cells. However, it has been difficult to understand exactly what the environment does during neural crest lineage diversification. On one hand, cell nonautonomous signals could act instructively to induce lineage commitment of multipotential neural crest cells. Alternatively, an environmental signal may select for the survival of a lineage that has already differentiated from crest cells. In the latter case, the environment acts after lineage determination has occurred. Thus, such lineages must be generated in a cell autonomous manner. Instructive and selective environmental signals therefore act on fundamentally different stages of lineage differentiation.

My experiments provide further evidence for an influence of environmental signals in cell fate specification of multipotential neural crest cells (Shah et al., 1996; Shah et al., 1994). In control conditions, such crest cells form clones that contain neurons, glia and smooth muscle (SM) (Shah et al., 1996; Stemple and Anderson, 1992). However, under appropriate conditions, the vast majority of crest cells form clones containing only one phenotype. In the presence of GGF2, most clones contain glia; in BMP2, most clones contain neurons (SM differentiation is also observed in some clones); and in TGFß1, most clones contain smooth muscle (SM). My own studies, and results from other laboratories, suggest that each of these three growth factors is present at the appropriate time and place in vivo to be able to influence cell fate decisions by multipotential neural crest cells.

I have done various experiments which suggest that these polypeptides may influence crest differentiation in an instructive manner. In all cases in my experiments, neural crest cells were plated in identical conditions: The appropriate growth factors were added only after the cells had attached to the substrate. This ruled out differential plating of precursors and ensured that the starting population in each experimental condition was similar. I also identified cells prospectively on the dish before the addition of growth factors. This allowed me to follow the survival of clones arising from each founder cell. The survival of clones was significantly greater than 50% in all conditions. However, the phenotype of cells in the presence or absence of growth factor differed dramatically. This suggested that each growth factor acted instructively rather than selectively at the level of the clone-founder cell. In other words, the growth factors did not lead to preferential survival of a subset of founder cells. Finally, I performed serial observations on prospectively identified neural crest cells that had then been cultured in conditions with or without the particular growth factor. Such daily monitoring of clones suggested that the different growth factors did not lead to the death of "inappropriate" lineages. To illustrate, neurons were not observed to differentiate and then die when neural crest cells were cultured in TGFß1. In some cases, cell death was further ruled out by DAPI staining of clones at different time-points after treatment with the growth factor. This set of experiments revealed that even within clones generated by founder crest cells, the growth factors were likely to exert an instructive rather than a selective influence on lineage specification. Consistent with these data is the demonstration that BMP2 can induce MASH1 expression very rapidly in the majority of neural crest cells, sometimes even before they have undergone cell division. This rapid induction makes it unlikely that BMP2 exerts a selective influence on neural crest differentiation. Similar experiments could not be performed with GGF2 or TGFß1 since early markers of glial and SM differentiation are presently unavailable.

Recent studies of the vertebrate CNS have identified several growth factors that may instructively influence the lineage specification of neuroepithelial cells (Gross et al., 1996; Johe et al., 1996; Qian et al., 1997). PDGF was shown to promote neuronal differentiation from these CNS neuroepithelial cells. CNTF and various BMPs can instructively promote the differentiation of astrocytes, while thyroid hormone and FGF may promote oligodendrocyte differentiation. Thus, an instructive mechanism of differentiation may be a general phenomenon in the differentiation of many cell types in the vertebrate nervous system.

While growth factors may exert an instructive influence on neural crest differentiation, results presented in Chapter 4 suggest that neural crest cells may not be completely naive in their response to extracellular signals. In other words, the environment may not dictate absolutely the behavior of neural crest cells. Determination of NCSCs to a glial fate in the presence of GGF2 occurs over 48-96 hours (as measured by a loss of responsiveness of NCSCs to saturating concentrations of BMP2). While this is significantly faster than determination to a glial lineage in the absence of exogenous GGF2, most NCSCs are determined to a neuronal and SM fate within ~24 hours of exposure to BMP2 and TGFß1, respectively. The delayed response in GGF2 is unlikely to be due to a complete lack of functional signaling pathways at early times in vitro. GGF2 can activate MAPK in the majority of NCSCs from the earliest time in culture. Further, GGF2 can also attenuate MASH1 induction (as well as neuronal differentiation over a longer time period) by a subsaturating dose of BMP2. Saturating doses of BMP2, however, can override this inhibitory effect of GGF2 on neurogenesis. In fact, saturating concentrations of BMP2 strongly suppress glial differentiation even in the presence of GGF2. By contrast, saturating concentrations of BMP2 and TGFß1 elicit neuronal as well as SM differentiation from NCSCs; preferential differentiation of one lineage at the expense of a lineage appropriate for the other factor is not seen.

BMP2 and TGFß1, therefore, appear to exert equivalent influences on NCSCs. These experiments suggest a previously unknown temporal difference in NCSCs in their response to differentiation signals as well as a differential dosage sensitivity to different instructive signals. It will be interesting to understand the molecular basis of these observations.

GGF2, BMP2 AND TGFß IN NEURAL CREST DIFFERENTIATION IN VIVO

What is the evidence that these growth factors influence neural crest cell fate decisions in vivo? As mentioned earlier, the expression pattern of each of these polypeptides is consistent with such a function in the embryo. However, this is hardly definitive proof of a role for these particular growth factors in modulating neural crest lineage differentiation in situ. The most direct way to test for such function is to generate mice carrying targeted loss-of-function mutations in genes encoding these growth factors or their receptors. As detailed below, such mice have already been generated for all the growth factors I have identified. Before I describe their phenotypes, it is interesting to speculate on the possible developmental outcomes of deleting genes encoding these growth factors or their receptors.

A complete failure of the predicted lineage(s) to develop would suggest an important role for these growth factors in neural crest cell fate specification. This would complement my tissue culture studies very nicely and provide a relatively simple explanation for the differentiation of multiple lineages in the neural crest. It is, of course, hard to distinguish between failure of neural crest cells to commit to a particular lineage and failure of maturation of lineage committed progenitors that have differentiated from the multipotential crest cells. However, careful examination of

mutant mice with a broad panel of lineage-specific markers combined with detailed in vitro analyses can distinguish between these possibilities {for example, see (Sommer et al., 1995) in Appendix 1}.

An alternative hypothesis, also compatible with my data, is that the growth factors I have identified act to bias the probability of differentiation of neural crest cells. As mentioned earlier, a stochastic model has been proposed to explain the patterns of lineage segregation seen in clonal cultures of quail crest {(Baroffio and Blot, 1992); also see Section A of Introduction}. Assuming that this model is correct and implies cell autonomous lineage specification of the crest, then differentiation into various lineages may occur with certain probabilities in the absence of extracellular signals. In such a scenario, the phenotypes seen in "knock-out" mice may be more subtle. One possible phenotype is a delay in differentiation of the particular lineage. To illustrate why this may happen, in vitro, autonomic neurons differentiate after 10-15 days of culture of neural crest cells in control medium. In the presence of exogenous BMP2, however, autonomic neurons differentiate within 3 days. Thus, in the BMP2 -/- mice, while committed autonomic precursors may develop, this may occur only gradually. Another possible phenotype is a reduction in the number of precursors generated for the lineage. This may occur since neural crest cells now have more opportunities to differentiate into other lineages. However, such a reduction could be compensated for by increased proliferation of the precursors making this phenotype hard to detect at later times in embryogenesis.

The following "knock-out" mice have been generated: GGF and erbB2 (Kramer et al., 1996; Lee et al., 1995; Meyer and Birchmeier, 1995); TGFß1 (Kulkarni et al., 1993; Letterio et al., 1994; Shull et al., 1992) and; BMP2 and BMP type IA receptor (Mishina et al., 1995; Zhang and Bradley, 1996). Both the GGF and erbB2 heterozygous mice appear phenotypically normal while the -/- mice are embryonic lethal

at ~ day 10 of gestation (E10). The lethality is most likely due to cardiac maldevelopment resulting in poor circulation of blood. Unfortunately, at E10, neural crest cells have just reached their final locations. Glia do not differentiate until much later. Thus, it has not been possible to analyze glial development thoroughly in these embryos. In the GGF -/- embryos, a significant reduction in the number of Schwann cell precursors lining the peripheral nerves was seen (Meyer and Birchmeier, 1995). This suggests that peripheral gliogenesis is affected in GGF -/- embryos. However, erbB3, the marker used to identify Schwann cell precursors, is present on both undifferentiated neural crest cells and mature Schwann cells. Further, GGF is a known mitogen for mature Schwann cells (Lemke and Brockes, 1984; Raff et al., 1978). It is, therefore, unclear whether the reduction in "precursors" lining the peripheral nerves is due to a lack of commitment of neural crest cells to the glial lineage. The alternative possibility, which has yet to be ruled out, is that neural crest cells have committed to the glial fate in the absence of GGF. The reduction in the number of cells in the peripheral nerves in such a scenario is due to reduced proliferation and/or survival (Dong et al., 1995) of committed glial precursors.

SM normally derives from the neural crest and mesoderm. The neural crest differentiates into SM in the cardiac outflow tract while all other SM (at least in avians) is of mesodermal origin (Le Douarin, 1982). TGFß has been implicated in SM differentiation from both the neural crest and the mesoderm {my data; (Darland and Leblanc, 1996; Folkman and D'Amore, 1996; Leblanc et al., 1995)}. It is, therefore, interesting to examine the phenotype of TGFß1 -/- embryos. However, TGFß1 -/- embryos are born, are viable for the first few weeks of life, and appear histologically normal. It has recently been shown that these embryos receive TGFß1 transplacentally from the heterozygous mother (Letterio et al., 1994). Therefore, to examine the requirement for fetal sources of TGFß1, null embryos developing in TGFß1 null

mothers were analyzed. These embryos are uniformly lethal at about E15 due to severe cardiac malformations (although abnormal development of other tissues was not excluded). However, these embryos have not yet been examined to see if SM differentiate normally. The developing heart expresses TGFß 1-3 and my data suggest that TGFß 2 & 3 can also influence neural crest cells to differentiate into SM. Thus, SM differentiation may only be subtly affected in the TGFß1 -/- embryos.

Deletion of the gene encoding either BMP2 or BMP type IA receptor leads to very early embryonic lethality due to widespread defects in extraembryonic (BMP2) and embryonic mesodermal (BMP2 and BMP type IA receptor) tissues. Since autonomic precursors are just starting to develop at E10 in wild-type embryos and almost none of the mutant embryos survive beyond E10, it is hard to analyze defects in autonomic neurogenesis in -/- embryos. Furthermore, the extreme growth retardation present in older mutant embryos makes interpretation of any phenotype in the PNS tricky at best. For example, a loss/reduction of autonomic precursors could either be due to a specific effect of BMP2 on PNS neurogenesis or due to a general maldevelopment of all tissues. Obviously, it is critical to distinguish between these two possibilities. These caveats should be considered when analyzing BMP2 and BMP type IA receptor -/- embryos for defects in autonomic neurogenesis.

The foregoing results suggest that other approaches will be needed to analyze the necessity of GGF, TGFß and BMP2 in the differentiation of glia, SM and autonomic neurons from the neural crest. Such approaches may include neural crest-specific gene targeting strategies and expression of the appropriate dominant negative receptors either in rat neural crest cells in vitro or using replication competent avian retroviral constructs (RCAS) in ovo. At least some of these experiments are already in progress.

DIFFERENTIATION OF SYMPATHETIC NEURONS EXPRESSING TH

Recent studies have suggested that a BMP-like molecule may induce the expression of TH in cultures of avian neural crest (Varley and Maxwell, 1996; Varley et al., 1995). Overexpression of BMP4 in ovo can, in some experiments, also lead to an increase in the number of TH^+ cells (Reissman et al., 1996). These results have led the authors to conclude that the primary effect of BMPs on the neural crest is to induce TH expression in autonomic cells. Other reports have suggested that TGFß family members may influence the expression of neuropeptides and choline acetyltransferase in various populations of differentiated autonomic neurons (Darland et al., 1995; Fann and Patterson, 1994; Fann and Patterson, 1994). These latter studies, however, did not report any induction of TH by the TGFß ligands. My data show that autonomic neurons differentiate in the presence of BMP2. However, these neurons are TH^-. What is the explanation for this apparent discrepancy? Given that the neurotransmitter phenotype of autonomic neurons can depend on culture conditions (Patterson and Nawa, 1993), it is possible that other growth factors present in the culture medium used for quail neural crest induced TH expression in cells that differentiated in the presence of BMP2. Consistent with such a possibility, when grown in culture conditions (serum-containing medium and high density cultures) similar to those employed in the quail studies, rat neural crest cells also express TH in the presence of BMP2 (Groves, A.K., personal communication). Experiments are underway to identify the possible signals present in serum that may induce TH expression in rat neural crest cells.

My experiments have suggested that the dorsal aorta may be an endogenous source of BMP2. Since exogenous BMP2 induces MASH1 expression in neural crest cells, a co-culture with the aorta should also induce expression of MASH1 in crest cells. Furthermore, sequestering the BMP2 secreted by the aorta should block the induction of MASH1 in such co-culture experiments. This is precisely what happens (Groves,

A.K., personal communication). Trans-filter experiments, in which the aorta is separated from crest cells by a porous membrane allowing the diffusion of molecules (but not cells), demonstrate a rapid induction of MASH1 in rat neural crest cells. Moreover, addition of noggin to these cultures prevents the induction of MASH1 by the dorsal aorta. Noggin, a novel polypeptide, is known to bind to BMP2 (and BMP4) with picomolar affinity and prevent it from activating its receptors (Zimmerman et al., 1996). Noggin has no known receptor and it is thought to function primarily by sequestering BMP2 (and BMP4) in vivo. These results demonstrate that the aorta can induce MASH1 in rat neural crest cells, most likely via BMP2 or a BMP2-related molecule.

In summary, the earliest effect of BMP2 on neural crest cells is likely to be an induction of the autonomic neuronal phenotype. During a later step in development BMP ligands have been implicated in the control of dendritic outgrowth from sympathetic neurons (Lein et al., 1995). Additional signals are probably required for expression of TH. These signals were most likely present in the culture medium used for quail neural crest. These conclusions are also consistent with a perturbation study of sympathetic neuronal development in the chick embryo (Groves et al., 1995). In these experiments, autonomic neurons could be induced to develop adjacent to the aorta. However, in the absence of the notochord/floorplate complex, no TH expression was seen. These studies demonstrate that it is possible to dissociate the autonomic neuronal phenotype from expression of TH. Further, the results suggest that the most likely in vivo source of the TH-inducing signal is the notochord/floorplate or surrounding mesenchyme that is patterned by the notochord.

DEFINED VS. COMPLEX CULTURE MEDIA

All of my experiments were performed in medium containing an undefined component, chicken embryo extract. Thus, it is possible that either some ingredient in the embryo extract (or in the basal defined medium) may act as a co-factor to GGF, TGFß or BMP2. The existence of such a putative co-factor, however, should not detract from the main conclusions of my experiments: GGF, TGFß and BMP2 each may exert an instructive influence on neural crest lineage specification in vitro. My experiments do not address whether other co-factors are required for these effects. Since survival of neural crest cells in medium lacking embryo extract is poor, it may be hard to study the effects of GGF, TGFß and BMP2 in such a basal medium. It is, therefore, worthwhile developing a basal defined medium that will support neural crest survival and then assaying for the effects of the various growth factors. Experiments to develop such defined media are underway (Kim, J., personal communication). It should be noted that few clonal culture systems exist in which it is possible to get a high cloning efficiency of multipotential progenitors in defined medium and in the absence of feeder layers. Development of such a medium would represent a significant advance in the cell biological manipulation of multipotential cells.

REGULATION OF GLIAL DIFFERENTIATION IN THE NEURAL CREST

Why do neural crest cells not respond to GGF2 by immediately committing to a glial fate? One possibility is that the signaling initiated by GGF2, although capable of activating MAPK in NCSCs and inhibiting MASH1 induction by low doses of BMP2, is quantitatively limiting at early times in vitro. Thus, in neural crest cells, MAPK activation lasts less than 1 hour in the presence of GGF2. Previous studies have suggested that MAPK can also be activated for prolonged periods (many hours)

{reviewed in (Marshall, 1995)}. Furthermore, such prolonged activation of MAPK can lead to dramatically different cellular read-outs (mitogenesis vs. differentiation). Prolonged activation of MAPK can be achieved by over-expressing the upstream receptor tyrosine kinase (RTK) that initiates MAPK activation. Thus, it is possible that at early times in vitro, neural crest cells express low levels of GGF2 receptor(s). (Such low levels might be cell autonomously up-regulated over time or the up-regulation may be mediated by a feed-forward loop initiated by GGF2 or via some other mechanism.) A simple test of this hypothesis would be to overexpress GGF2 receptors (or alternatively, a constitutively active GGF2 receptor) using retroviral constructs in primary neural crest cells. Addition of GGF2 to the medium (if constitutively active receptors are not used) should then lead to a rapid differentiation of neural crest cells to a glial fate. Such a hypothesis also predicts that over-expressing the GGF2 receptor may allow glial differentiation even in saturating BMP2+GGF2.

Certainly, many other models can be postulated to explain why neural crest cells do not rapidly commit to a glial fate in the presence of GGF2. However, it is intriguing that a model similar to the one proposed above may operate in the generation of retinal Müller glial cells (Lillien, 1995). In this case, however, it is the EGF receptor that is present in limiting amounts on retinal multipotential progenitors. Overexpression of the EGFR promotes rapid differentiation of Müller cells. Furthermore, the expression of endogenous EGFR may increase in retinal precursors at a time when Müller cells are normally generated in vivo.

It is clear that many exciting experiments remain to be done in the neural crest. These experiments should lead to novel insights into the mechanisms of cell fate specification. In my mind, the great advantage of working on the neural crest is that the various embryologic, cell, molecular and transgenic technologies now offer the opportunity to

pose sophisticated questions of a vertebrate developmental system. I will be following future progress in this field with an undiminished interest for many years to come.

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Appendix 1

The cellular function of MASH1 in autonomic neurogenesis

Lukas Sommer, Nirao M. Shah, Mahendra Rao and David J. Anderson

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The Cellular Function of MASH1 in Autonomic Neurogenesis

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Summary

Using primary cultures and immortalized multipotential stem cell lines derived from wild-type and *Mash1* mutant neural crest cells, we have analyzed the cellular function of MASH1 in autonomic neurogenesis. We present evidence for the existence of a precursor expressing MASH1 and neuronal markers such as neurofilament, neuron-specific tubulin, and tetanus toxin receptor. This cell has a nonneuronal morphology. Differentiation of this precursor to neurons that express markers such as SCG10, peripherin, and neuron-specific enolase is dependent upon MASH1 function. These data imply that the differentiation of autonomic neurons from uncommitted neural crest cells occurs in several sequential steps. Moreover, they suggest that MASH1 does not commit multipotent cells to a neural fate, like its Drosophila *achaete-scute* counterparts, but rather promotes the differentiation of a committed neuronal precursor.

Introduction

Relatively little is known about the progression of cellular events that underlies the generation of neurons and glia from their progenitor cells in the developing nervous system. Lineage analysis has revealed that many neural progenitors are multipotent, able to give rise to both neurons and glia, in the CNS and PNS of both vertebrates and invertebrates (Anderson, 1989; McConnell, 1991; Udolph et al., 1993; Condron and Zinn, 1994; Jan and Jan, 1994). However, there is also evidence for the existence of progenitors with more restricted developmental capacities (Duff et al., 1991; Luskin et al., 1993; Lo and Anderson, 1995). These observations have suggested that the generation of cellular diversity during neural development involves a progressive or stepwise restriction in the developmental capacities of progenitor cells, analagous to what is thought to occur during the segregation of hematopoietic lineages (Anderson, 1989; McKay, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991). However, the control of this restriction process remains poorly understood.

In invertebrate systems such as Drosophila melanogaster or Caenorhabditis elegans, it has been possible to identify genes that act at different stages in a neurogenic developmental pathway (Ghysen and Dambly-Chaudiere, 1989; Sternberg et al., 1992). Thus, for example, in Drosophila the initial segregation of a neural precursor from uncommitted neuroectodermal cells is controlled by proneural genes, such as *achaete–scute* (*ac–sc*), while other genes such as *asense* may act at later stages to control the further division and differentiation of the neural precursor (Jan and Jan, 1994). However, the analysis of mutant phenotypes needs to be complemented with mechanistic studies to understand fully the developmental function of a given gene. In Drosophila and C. elegans, such studies have been limited by the lack of cell isolation techniques and in vitro culture systems.

The development of the PNS provides an experimentally accessible model system to dissect the genetics and cell biology of vertebrate neurogenesis. The PNS develops from the neural crest, a transient population of migratory precursor cells that derives from the dorsal margins of the neural tube (Le Douarin, 1982). A number of mutations, both naturally occurring and engineered, affect the development of neural crest derivatives in the PNS (Marusich and Weston, 1991). Moreover, many molecular markers are available to identify neural crest cells and their derivatives at different developmental stages. In addition, culture systems have been established for mammalian (Boisseau and Simonneau, 1989; Smith-Thomas and Fawcett, 1989; Morrison-Graham et al., 1990; Stemple and Anderson, 1992; Ito et al., 1993) as well as avian (Cohen and Konigsberg, 1975) neural crest cells. Together, these advances present the opportunity to investigate the cellular function of genes required for neural crest development.

One gene that is essential for the development of a subset of neural crest derivatives is *Mash1*, a basic–helix-loop-helix (bHLH) transcription factor (Johnson et al., 1992) that is a mammalian homolog of the *ac–sc* genes (Johnson et al., 1990). MASH1 is specifically and transiently expressed in subsets of neuronal precursors, in both the CNS and PNS (Lo et al., 1991; Guillemot and Joyner, 1993). Analysis of homozygous embryos containing a targeted mutation in *Mash1* has revealed that this gene is required for the generation of both peripheral autonomic (sympathetic, parasympathetic, and some enteric) and olfactory sensory neurons (Guillemot et al., 1993). However, this analysis did not define a cellular function for MASH1; thus, MASH1 could function in the commitment of multipotent neural crest cells to a neuronal fate, or it could be required at some stage in the differentiation, proliferation, or survival of developmentally restricted precursors.

Using primary cultures and immortalized cell lines from both wild-type and *Mash1* mutant neural crest, we present evidence for an autonomic neuronal precursor that expresses a number of neuron-specific genes, such as neurofilament and neuron-specific β-tubulin, and that is MASH1 independent. The subsequent differentiation of this precursor to neurons expressing other markers, such as peripherin and SCG10, requires MASH1. Isolation and reculture of these precursors suggest that they are most

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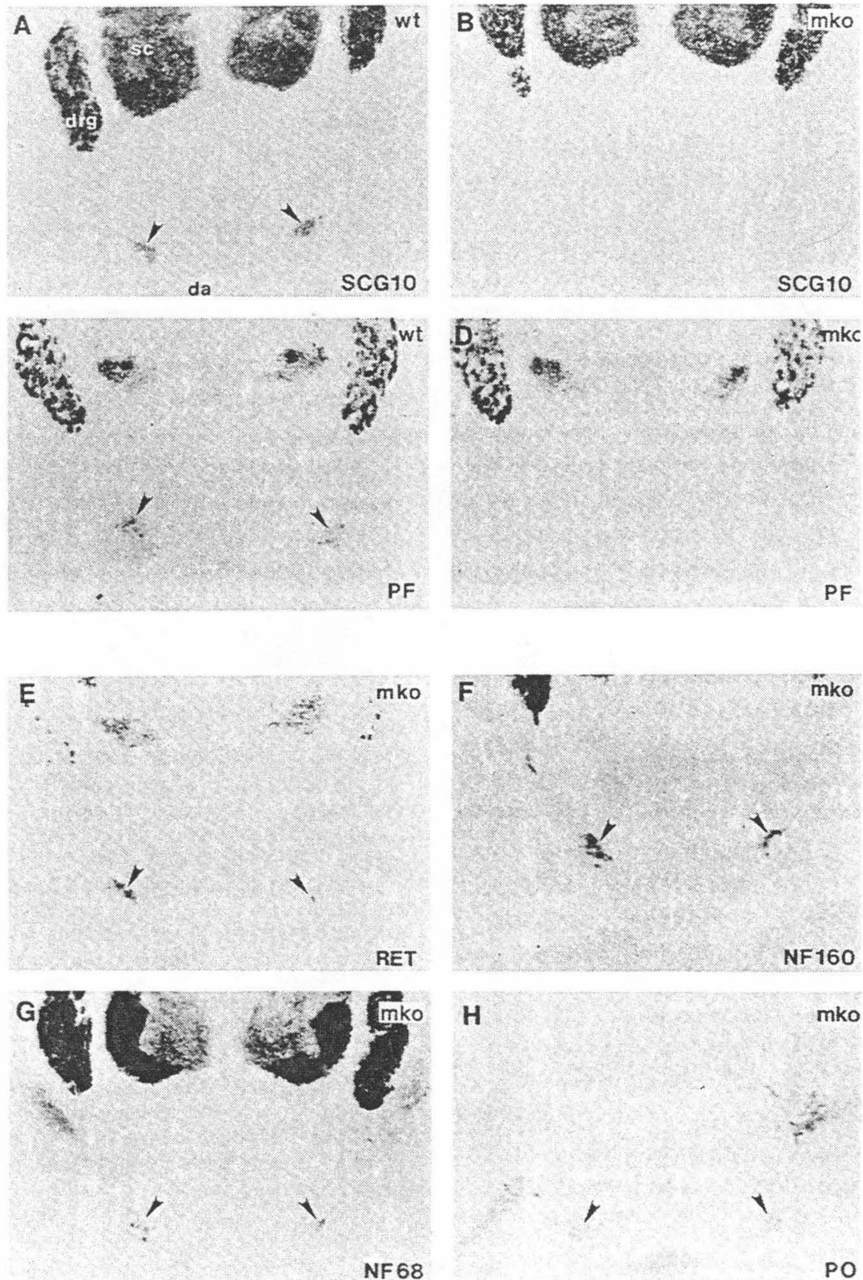

Figure 1. Cells Expressing Some Neuronal Markers and Glia Are Found in the Sympathetic Anlagen of *Mash1*$^{-/-}$ Embryos In Vivo

Near-adjacent serial transverse sections through an anterior region of a wild-type E12 embryo (A and C) and two E12 *Mash1*$^{-/-}$ (mko) embryos (B, D, and E–H) were processed for in situ hybridization, revealing the expression of mRNAs encoding SCG10 (A and B), peripherin (PF; C and D), c-RET (E), NF160 (F), NF68 (G), and P_0 (H). The sympathetic anlagen are marked by arrowheads. sc, spinal cord; drg, dorsal root ganglia; da, dorsal aorta.

likely committed to a neuronal fate. The phenotype of *Mash1* mutant embryos can therefore be explained by the arrest of neuronal development at this precursor stage, in various autonomic ganglia. These results identify both a novel intermediate in the autonomic neuronal differentiation pathway and the developmental step at which MASH1

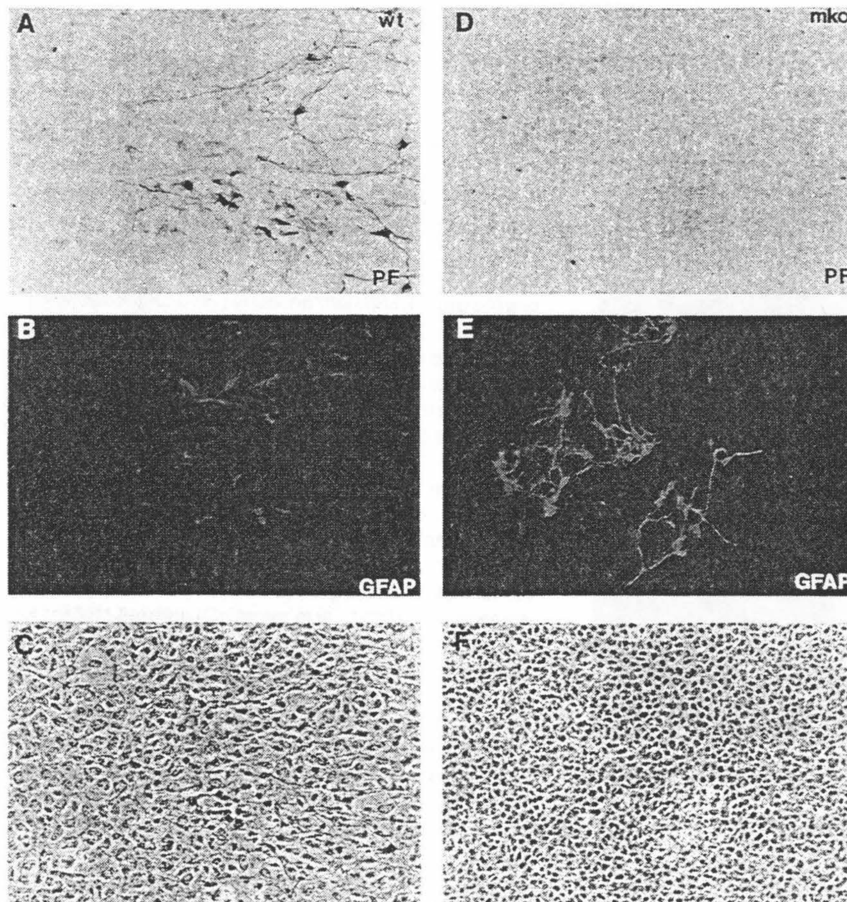

Figure 2. $Mash1^{-/-}$ Neural Crest Explants Do Not Produce Neurons but Give Rise to Glial Cells

Neural crest explants grown for 7 days were fixed and labeled with antibodies to peripherin (PF), and staining was visualized using a horseradish peroxidase–conjugated secondary antibody and bright-field optics (A and D). The same explants were then stained with anti-GFAP and visualized using a fluorescent phycoerythrin-conjugated secondary antibody (B and E). (A)–(C) show identical fields of neural crest explants derived from wild-type (wt) mice. (D)–(F) represent identical fields of $Mash1^{-/-}$ neural crest explants (mko). (C) and (F) are phase-contrast views. Note the absence of peripherin^{+} neurons in mko explants (D).

function is required. Furthermore, they indicate that the cell biological function of MASH1 is distinct from that of its Drosophila *ac–sc* homologs.

Results

Sympathetic Ganglia of *Mash1* Mutant Mice Express a Subset of Neuronal Markers

Previously, it was shown that neurons fail to develop in the sympathetic ganglia of *Mash1* mutant mice, as demonstrated by markers such as tyrosine hydroxylase, SCG10 (Guillemot et al., 1993; also see Figures 1A and 1B), and the PNS-specific intermediate filament protein peripherin (Figures 1C and 1D). Surprisingly, however, we found that two other neuronal markers, the 160 and 68 kDa subunits of neurofilament (NF160 and NF68, respectively), were expressed in the mutant ganglia (Figures 1F and 1G) at levels similar to those detected in wild-type embryos (data not shown). As shown previously, the mutant ganglia also expressed c-RET, an orphan receptor tyrosine kinase that is expressed by both autonomic neurons and their precursors (Pachnis et al., 1993; Lo and Anderson, 1995). The expression of P_0, a marker of peripheral glial cells, confirmed that glia develop in the mutant sympathetic ganglion anlagen (Figure 1H), as suggested earlier using less specific markers (Guillemot et al., 1993).

The finding that a subset of neuronal markers is expressed in the sympathetic ganglia of *Mash1* mutant mice raised the question of the nature of the cells expressing these markers. The presence of multiple markers could imply the existence of a single cell population coexpressing these markers, or a mixed population of cells each expressing a subset of the markers. The cells could represent neurons of a different lineage; they could represent arrested neuronal precursors; or they could represent an aberrant cell type that forms as a consequence of the loss

of MASH1 function. These possibilities could be distinguished by examining the phenotype of *Mash1* mutant neural crest cells in dissociated cultures.

Mash1 Mutant Neural Crest Cells in Primary Culture Express NF160 but Not Peripherin and Have a Nonneuronal Morphology

We first examined the phenotype of *Mash1* mutant neural crest cells in primary explants that had been allowed to differentiate for several days. Explants from wild-type embryos developed both peripherin⁺ neurons (Figure 2A) and glia (detected by expression of glial fibrillary acidic protein [GFAP]; Figure 2B). (In addition, the explants contained other nonneuronal cells, including undifferentiated neural crest stem cells [Figures 2C and 2F].) In contrast, explants from *Mash1* mutant embryos failed to develop neurons (Figure 2D) but did develop glia (Figure 2E). This result suggested that the phenotype of *Mash1* null mutants observed in vivo could be reconstituted in vitro.

The absence of any neurons at all in mutant explants was unexpected, because sensory neurons, which also derive from the neural crest, develop in vivo independently of MASH1 function (Guillemot et al., 1993). This observation suggests that the neurons which develop in our cultures under these conditions are exclusively autonomic. The differentiation of sensory neurons in vitro may require the neural tube, which is routinely removed from these explant cultures and has been shown to be a necessary source of differentiation or survival factors for sensory neurons in avian embryos (Kalcheim and Le Douarin, 1986).

Although *Mash1* mutant explants did not contain peripherin⁺ neurons, they did contain numerous NF160⁺ cells (data not shown), like autonomic ganglia in mutant embryos. To visualize the morphology of these NF160⁺ cells more clearly, explants were dissociated and replated at a lower density, then fixed and stained for NF160 together with GFAP. This revealed that the NF160⁺ cells in mutant cultures did not have a neuronal morphology (Figure 3B, NF160), but rather had a flattened, fibroblast-like morphology and contained bundles of elongated NF160⁺ filaments (Figure 3B, arrows). Moreover, these NF160⁺ cells were clearly distinct from the GFAP⁺ cells observed in the same cultures (Figure 3B, GFAP), indicating that they were not merely glial cells that had begun to express some neuronal properties. Furthermore, closer inspection of wild-type cultures revealed that they, too, contained some NF160⁺ cells with a nonneuronal morphology (Figure 3A, arrows; and data not shown). This suggested that the NF160⁺ cells seen in *Mash1* mutant cultures were not simply an aberrant cell type caused by the mutation but rather a normal cell type that forms in wild-type cultures as well.

Together, these data indicated that the explant cultures reconstituted the *Mash1* mutant phenotype of autonomic ganglia in vivo: they contained NF160⁺ cells and glia but not peripherin⁺ or SCG10⁺ (data not shown) neurons. Furthermore, the ability to visualize the morphology of the NF160⁺ cells clearly in mutant cultures indicates that they are not neurons of another lineage. Such nonneuronal NF160⁺ cells can be identified in wild-type cultures as well,

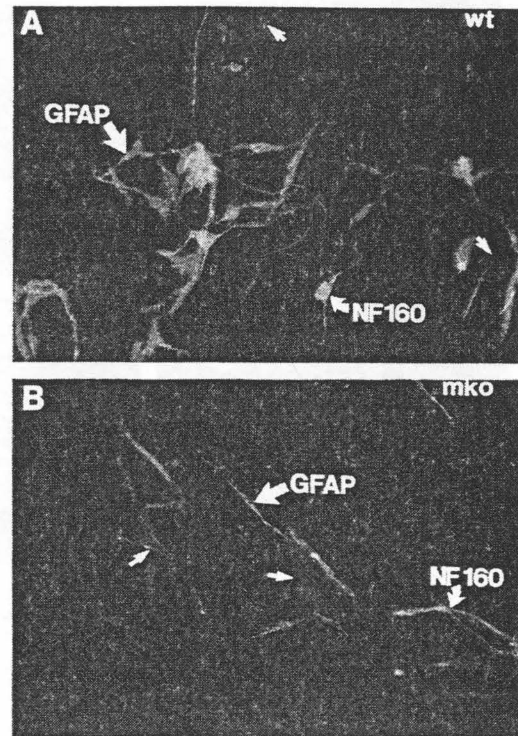

Figure 3. NF160⁺ Nonneuronal Cells Are Present in *Mash1*$^{-/-}$ Neural Crest Cultures

Neural crest cells from wild-type (A) and *Mash1*$^{-/-}$ (mko; B) explants were dissociated, replated, and allowed to differentiate for another 6 days on poly-D-lysine/fibronectin in differentiation medium. Anti-NF160 and anti-GFAP antibodies followed by secondary antibodies conjugated to fluorescein (for NF160) or phycoerythrin (for GFAP) were used to label neuronal cells and glia, respectively. Note that the NF160⁺ cells in mko (B) have a flat, nonneuronal morphology. Note also that the NF160⁺ cells in the mko culture are distinct from the GFAP⁺ cells. Small arrows in (B) indicate additional NF160⁺ nonneuronal cells with elongated neurofilament bundles; note that similar cells are present in wild-type cultures (A, small arrows). These cells have a flattened, fibroblast-like morphology in phase-contrast views (not shown).

suggesting that they may be neuronal precursors whose further differentiation is arrested in the absence of MASH1. To test this hypothesis, it was necessary to characterize these cells further and to demonstrate directly their conversion into neurons.

Immortalized Cell Lines from Wild-Type and *Mash1* Mutant Neural Crest Cells Reproduce the Phenotype Seen in Primary Cultures

To manipulate the NF160⁺ nonneuronal cells, we required greater numbers of cells than are available from primary explants. We therefore took advantage of recently developed methods for immortalizing mouse neural crest stem cells (M. R. et al., unpublished data) and applied them to explants from *Mash1*$^{-/-}$ embryos as well. To identify these cells, we relied on their expression of two antigens, the low affinity nerve growth factor receptor ($p75^{LNGFR}$) and nestin, previously shown to be expressed by neural crest

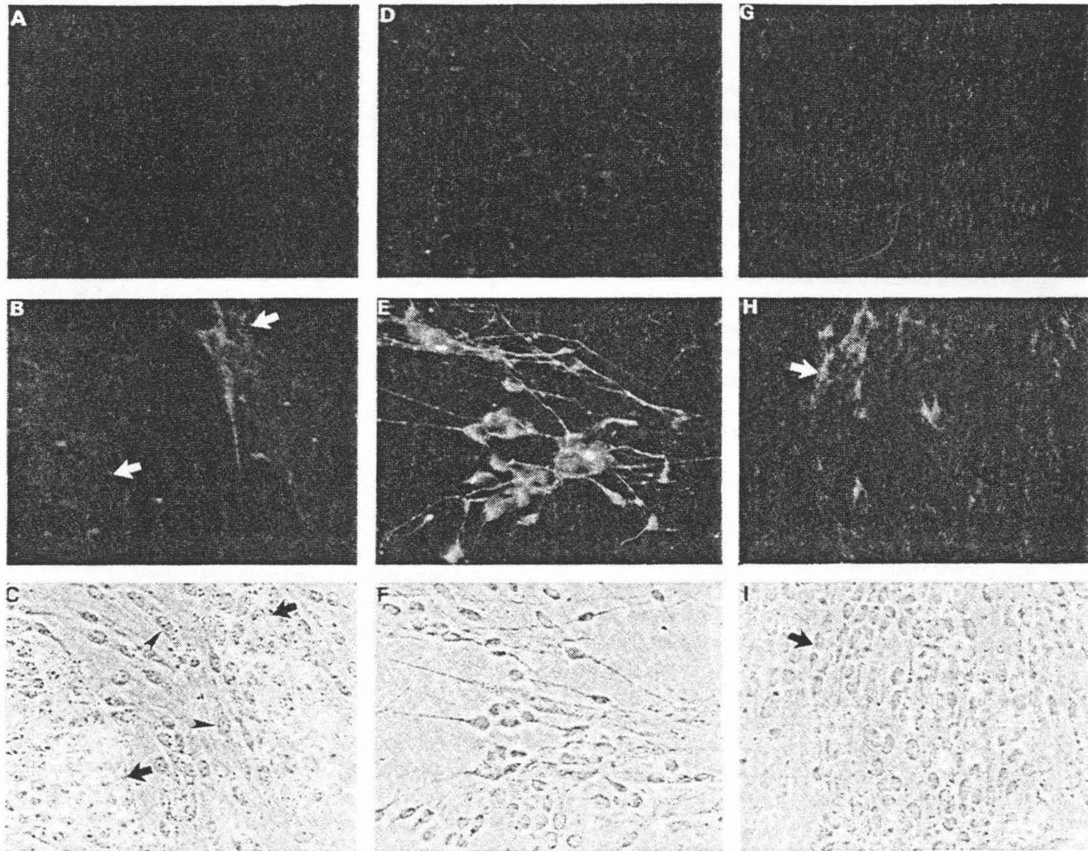

Figure 4. NF160$^+$ Cells in Immortalized Cell Lines from Wild-Type and *Mash1* Mutant Neural Crest Are Similar to Those Observed in Primary Cultures

(A–C) Mko-4 (*Mash1* mutant) cultures differentiated for 5 days were fixed and double labeled for peripherin (A) and NF160 (B). Note that the NF160$^+$ cell clusters (B and C, arrows) are peripherin$^-$ and are interspersed with NF160$^-$ nonneuronal cells (C, arrowheads), many of which are GFAP$^+$ glia (data not shown).

(D–F) Monc-1 (wild-type) cultures differentiated for 5 days. Note the presence of NF160$^+$ cells (E) with a neuronal morphology (F) that coexpress peripherin (D).

(G–I) After 2 days of differentiation, NF160$^+$ (H), peripherin$^-$ (G) cells with a nonneuronal morphology (I) can also be seen in wild-type (Monc-1) cultures.

stem cells in the rat (Stemple and Anderson, 1992), and on their fibroblast-like morphology and lack of expression of lineage markers such as neurofilament and GFAP (M. R., unpublished data). Cells of a similar morphology and antigenic phenotype were identified in *Mash1* knockout neural crest cultures as well (data not shown).

Clonal lines were established from both wild-type and *Mash1* mutant immortalized neural crest cells by infection with a retrovirus harboring v-*myc* (see Experimental Procedures) and are called Monc-1 (Mouse neural crest-1) and Mko-4 (*Mash1* knockout-4), respectively. Monc-1 cells can be propagated under conditions where they remain undifferentiated, or they can be replated under different conditions (see Experimental Procedures) where they differentiate after 5 days to neurons expressing both peripherin (Figure 4D) and NF160 (Figure 4E) as well as glia (data not shown). At 2 days, however, NF160$^+$, peripherin$^-$ nonneuronal cells can be seen in these wild-type cultures (Figures 4G–4I). The fact that these NF160$^+$ nonneuronal cells appear earlier than the neurons is consistent with the idea that they are neuronal precursors.

In contrast to Monc-1 cells, Mko-4 cells plated under differentiation-promoting conditions for 5 days did not generate peripherin$^+$ neurons (Figure 4A) but did produce NF160$^+$ cells (Figure 4B, arrows) that exhibited a nonneuronal morphology (Figure 4C). These cells appeared similar to those that developed after 2 days in wild-type Monc-1 cultures (Figures 4G–4I), providing further evidence that they are not an aberrant phenotype produced by the mutation. The NF160$^+$ cells typically formed dense clusters that were interspersed among nonneuronal cells (Figure 4C, arrowheads). When such cultures were double labeled for NF160 and GFAP, the two cell populations appeared mutually exclusive (data not shown), again indicating that the

Table 1. Expression of Markers in Wild-Type and *Mash1*$^{-/-}$ Immortalized Neural Crest Stem Cell Lines

Marker	Monc-1 U	Monc-1 D	Mko-4 U	Mko-4 D
Panneuronal markers				
MASH1-dependent				
SCG10	−	+	−	−
Peripherin	−	+	−	−
NSE	−	+	−	−
MASH1-independent				
NF160	−	+	−	+
NF68	−	+	−	+
N-CAM (5A5)	−	+	−	+
Ttx receptor	−	+	−	+
c-RET	−	+	−	+
Cl. III β-tubulin	−	+	−	+
Stem cell markers				
LNGFR	+	−(−/+)	+	−
Nestin	+	−	+	−

Comparison of marker expression in Monc-1 and Mko-4 as assayed by immunocytochemistry. Positive expression (+) in differentiated Monc-1 indicates marker expression either in neurons (MASH1-dependent markers) or in neurons and neuronal precursors (MASH1-independent markers); positive expression in differentiated Mko-4 refers to expression in precursors only. U, undifferentiated; D, differentiated.

NF160$^+$ cells are not glia that express some neuronal markers. Together, these data indicate that the Monc-1 and Mko-4 cell lines reproduce the phenotypes seen in primary explants of wild-type and *Mash1* mutant neural crest cells, respectively.

We next examined the expression of a panel of additional neuron-specific markers in differentiated Monc-1 and Mko-4 cultures. These markers fell into two classes: one class was expressed in both mutant and wild-type cells; the other was expressed in wild-type cells but not in the mutant. The first class included NF68, neuron-specific β-tubulin, neural cell adhesion molecule (N-CAM), and tetanus toxin receptor (Table 1). In all cases, these markers were expressed in the same morphologically identifiable cell clusters as expressed NF160, as illustrated by tetanus toxin receptor (Figure 5). The second class of markers included peripherin, SCG10, and neuron-specific enolase (Table 1); these markers were not expressed in the clusters in Mko-4 cultures but were expressed by neurons in Monc-1 cultures (data not shown). These data indicate that neuron-specific genes can be divided into two categories according to their dependence on MASH1 function. Moreover, expression of the MASH1-independent subset of genes is apparently insufficient to confer a neuronal morphology.

Coexpression of c-RET and MASH1 in NF160$^+$ Clusters

The foregoing data were consistent with the idea that the NF160$^+$ cells represent precursors that express some panneuronal markers, but whose progression to a fully differentiated neuronal phenotype is dependent upon MASH1. This implies that in wild-type cultures these putative precursors should express MASH1, or at least derive from a MASH1-expressing lineage. To address this issue, double labeling was performed with antibodies to NF160 and MASH1. We found that 100% of NF160$^+$ cell clusters contained MASH1$^+$ cells; conversely, 85% ± 5.7% of MASH1$^+$ cells were associated with NF160$^+$ clusters ($n = 2$ independent experiments). Moreover, many individual cells coexpressing both MASH1 and NF160 could easily be detected (Figures 6A and 6B), although the high cell density within clusters precluded precise quantitation.

The coexpression of MASH1 and NF160 suggested that the NF160$^+$ cells in mutant cultures should represent precursors that would normally express MASH1. However, it was not possible to demonstrate this directly since the targeted mutation in *Mash1* completely eliminates the coding sequence. We therefore needed an independent marker to link the NF160$^+$ cells in mutant cultures to the MASH1$^+$, NF160$^+$ cells in wild-type cultures. This marker was provided by c-RET. Unlike NF160, N-CAM, and tetanus toxin receptor, which are panneuronal markers, c-RET expression is tightly associated with that of MASH1 (Lo et al., 1994). For example, in a population of c-RET$^+$ autonomic precursors isolated by fluorescence-activated cell sorting (FACS) from embryonic gut, 87% of the cells expressed MASH1 (Lo and Anderson, 1995). The only other cells in the PNS that express c-RET are a small subset of postmitotic sensory neurons (Pachnis et al., 1993); however, since sensory neurons are not generated under the culture conditions used here (as mentioned earlier), c-RET provides a marker of MASH1-expressing autonomic precursors. Importantly, in *Mash1*$^{-/-}$ mutant embryos, c-RET$^+$ cells are still found in the sympathetic ganglia (see Figure 1E), indicating that c-RET expression is not dependent upon MASH1.

In wild-type cultures, c-RET-immunoreactive cells coexpressed both MASH1 (Figure 6D) and NF160 (Figure 6F). Of the c-RET$^+$ clusters, 99.2% ± 0.8% were associated with MASH1$^+$ cells ($n = 2$ independent experiments). All NF160$^+$ clusters (100%) contained c-RET$^+$ cells, while 74% of c-RET$^+$ cells were associated with NF160$^+$ clusters. (That some c-RET$^+$ cells do not express NF160 most likely reflects the fact that the former marker is first expressed before the latter [Lo et al., 1994].) Similarly, in mutant Mko-4 cultures, 98% of NF160$^+$ clusters contained c-RET$^+$ cells, while 76% of c-RET$^+$ cells were associated with NF160$^+$ clusters (Figures 6G and 6H). Individual cells coexpressing c-RET and NF160 could be seen in both wild-type and mutant clusters (Figures 6F and 6H, arrowheads), although the fact that both antigens are cytoplasmic obscures this coexpression in many cases. The expression of c-RET by NF160$^+$ cells in mutant cultures suggests that these cells correspond to wild-type precursors that would normally express MASH1.

Neurotag$^+$ Cell Clusters Contain Neuronal Precursors

If, as suggested by the foregoing data, the NF160$^+$ non-neuronal cells in mutant cultures represented arrested neuronal precursors, their counterparts in wild-type cultures should be capable of neuronal differentiation. To test this idea, we used the tetanus toxin receptor as a surface

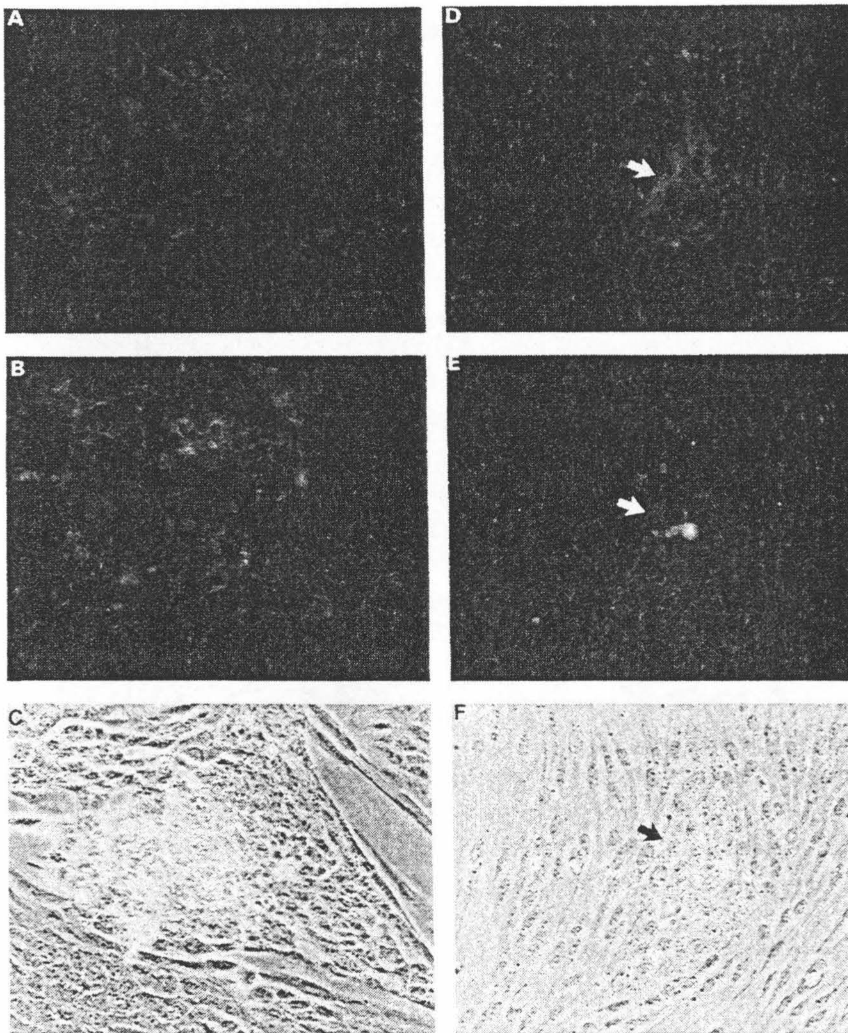

Figure 5. NF160$^+$ Clusters in Differentiated Monc-1 and Mko-4 Cultures Express Tetanus Toxin Receptor

Monc-1 (A–C) and Mko-4 (D–F) cells were differentiated for 2 days, and living cells were labeled with Neurotag and FITC-conjugated tetanus toxoid derivative (B and E). Cells were then fixed and double labeled for NF160 (A and D). Note that most of the Neurotag$^+$ cells in clusters of Monc-1 as well as Mko-4 (arrow) coexpress NF160.

marker to live-label these cells and follow their development into neurons. Receptor-bearing cells can be labeled using a fluorescein isothiocyanate (FITC)–conjugated derivative of tetanus toxoid (Raju and Dahl, 1982) called Neurotag. As mentioned above, Neurotag$^+$ cells colocalized with NF160$^+$ cells in both mutant and wild-type cultures (see Figures 5A–5D). When Neurotag$^+$ clusters in wild-type (Monc-1) cultures were followed, they generated neurons as well as dead cells (detected by propidium iodide staining; data not shown). In contrast, in mutant (Mko-4) cultures, Neurotag$^+$ clusters never generated neurons; rather, many of the cells died. These observations indicated that Neurotag$^+$ cells are unable to generate neurons in mutant cultures. However, owing to the cell death observed, it was not possible to conclude unequivocally by this in situ analysis that Neurotag$^+$ cells are neuronal precursors in wild-type cultures. To do this, it was necessary to isolate the Neurotag$^+$ population from wild-type cultures and follow the fate of individual cells.

Isolated Neurotag$^+$ Cells Are NF160$^+$ Neuronal Precursors

When Monc-1 cultures were dissociated to single cells 2 days after being placed under differentiation-promoting conditions (see Figures 4G and 4H), 95% (n = 302 cells counted) of the NF160$^+$ cells were Neurotag$^+$; furthermore, 78% of the NF160$^+$ cells coexpressed MASH1. This indicated that Neurotag labels virtually all NF160$^+$ cells, including the MASH1$^+$ cells. When Neurotag$^+$ cells were isolated by FACS, 73.2% of the cells were NF160$^+$ immedi-

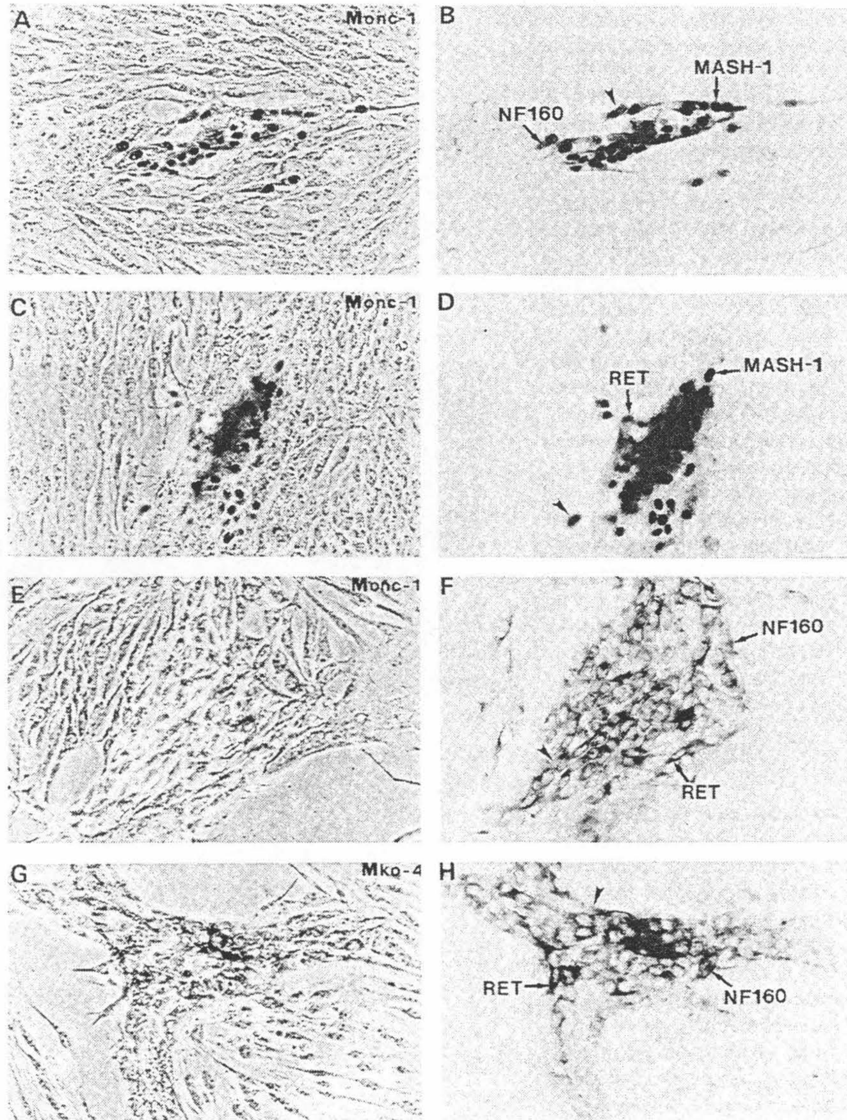

Figure 6. Colocalization of MASH1 and c-RET in NF160⁺ Cell Clusters

Monc-1 (A–F) and Mko-4 (G and H) cells were differentiated for 4 days and then double labeled for NF160 and MASH1 (A and B), c-RET and MASH1 (C and D), or c-RET and NF160 (E–H). In (A), (B), and (E)–(H), the NF160 staining is visualized by a red precipitate, while the counterstain (c-RET or MASH1) is purple. In (C) and (D), the c-RET staining is red, and MASH1 is purple. Arrowheads in (B), (D), (F), and (H) indicate examples of double-labeled cells. (A), (C), (E), and (G) represent phase-contrast micrographs of the bright-field views shown in (B), (D), (F), and (H), respectively. Note that $NF160^+$ cell clusters coexpress c-RET in both Monc-1 (F) and Mko-4 (H) cells. Double labeling of individual cells is obscured by the fact that both markers are cytoplasmic, although in some cases a perinuclear accumulation of c-RET staining can be seen on a more diffuse background of NF160 staining (F and H, arrows).

ately after sorting, while only 25.5% of these cells were $peripherin^+$ (average of 3 independent experiments). This indicates that the Neurotag-isolated cells are enriched for the $NF160^+$, $peripherin^-$ cells seen in clusters in the mass cultures. The small percentage of the cells that are $NF160^-$ may represent the occasional $Neurotag^+$ cells that lie outside of the $NF160^+$ clusters (see Figures 5A and 5B; and data not shown).

To maintain the viability of FACS-purified $Neurotag^+$ cells, it was necessary to reculture them on a monolayer of unlabeled, differentiated Monc-1 cells. To distinguish the isolated $Neurotag^+$ cells from the bulk Monc-1 population, the cells were labeled with the lipid-soluble dye PKH26 prior to Neurotag labeling and cell sorting. When isolated double-labeled cells (Figure 7A) were cultured on Monc-1 feeder layers and then fixed and stained for periph-

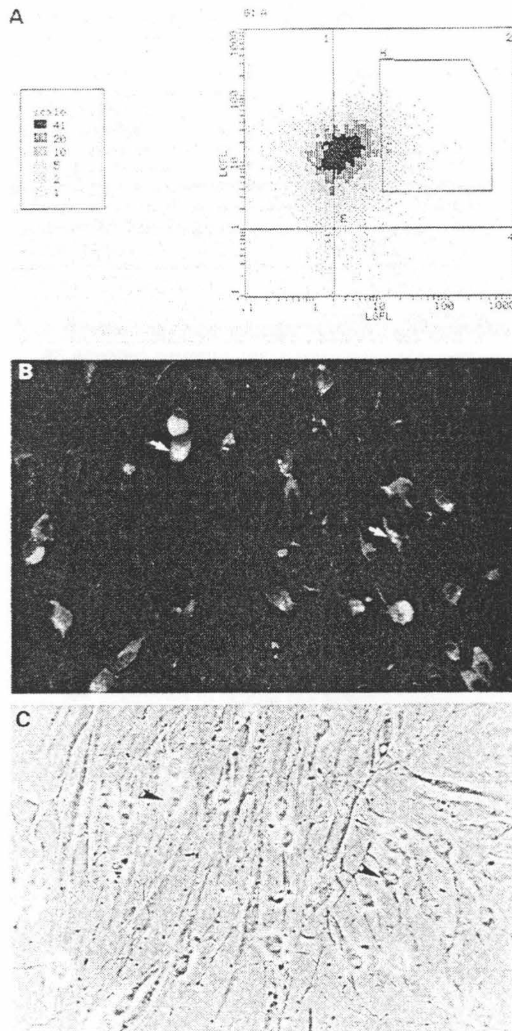

Figure 7. The NF160$^+$, Peripherin$^-$ Cell Type in Monc-1 Is a Neuronal Progenitor

Monc-1 cells were differentiated for 2 days as described before and labeled with Neurotag and the fluorescent dye PKH26 (see Experimental Procedures). Neurotag$^+$, PKH26$^+$ cells were isolated by FACS and plated onto unlabeled Monc-1 cells in differentiation-promoting conditions. The cultures were then allowed to differentiate for 5 days and labeled with anti-peripherin antibody. A typical FACS profile is shown in (A). Neurotag$^+$, PKH26$^+$ cells (about 5% of input cells) were collected from gate H. (B) shows a double exposure of peripherin$^+$ (green), PKH26$^+$ (orange) neurons (arrows) that differentiated from the sorted cells. (C) represents the phase-contrast view of the micrograph shown in (B).

erin after several days, many of the PKH26$^+$ cells had differentiated into peripherin$^+$ neurons (Figures 7B and 7C, arrows). These data suggested that the isolated Neurotag$^+$ cell population indeed contains neuronal precursors.

To assess quantitatively the differentiation capacity of the isolated Neurotag$^+$ population, a clonal analysis was performed (see Experimental Procedures). This analysis indicated that many of the Neurotag$^+$ cells divided to generate clones of peripherin$^+$ neurons similar to those found in unsorted Monc-1 cultures (36.8% ± 3%; Table 2). Since 73% of the cells were NF160$^+$ and only 25% were peripherin$^+$ immediately after sorting (see above), at least some of the Neurotag$^+$ cells that generated peripherin$^+$ neurons must have been initially NF160$^+$ and peripherin$^-$. Moreover, the value of 36.8% is likely to be an underestimate, because a portion of the Neurotag$^+$ cells generated clusters that contained peripherin$^-$ cells, but in which individual PKH26$^+$, peripherin$^+$ cells could not be distinguished due to high cell density. These cluster-forming cells were therefore assigned to a different category (27.3% ± 10%; Table 2, clusters). However, if these two categories are combined, 64% of the isolated cells adopted a neuronal fate.

Some of the Neurotag$^+$ cells (21.8% ± 13.6%; n = 3 experiments; Table 2) gave rise to cells with a nonneuronal morphology; these may have been glia or glial precursors. Such nonneuronal cells may derive from the subset of Neurotag$^+$ cells that were NF160$^-$ at the time of plating (see above). More importantly, however, such nonneuronal cells were rarely if ever found in clones that also contained neurons (Table 2, mixed). Since the culture conditions are permissive for both neuronal and glial differentiation, and yet most Neurotag$^+$ cells appear to give rise exclusively to neuronal progeny, the data suggest that these NF160$^+$ cells are likely committed to a neuronal fate.

Discussion

The process whereby multipotent neural stem cells generate their various differentiated derivatives remains poorly understood. We have taken a combined genetic and cell biological approach to analyze the cellular function of MASH1, a gene essential for the differentiation of autonomic neurons from uncommitted neural crest cells. We have shown that autonomic neurogenesis proceeds via a precursor that expresses a subset of neuronal genes and whose further differentiation requires MASH1 (Figure 8). This finding supports the idea that the genesis of autonomic neurons from undifferentiated neural crest cells occurs in several sequential steps. The behavior of isolated neuronal precursors indicates that they are most likely committed to a neuronal fate. This in turn suggests that MASH1 does not restrict multipotent cells to a neuronal fate but rather promotes the differentiation of committed neuronal precursors, perhaps analagous to the role of the myogenic bHLH protein myogenin in muscle development (Weintraub et al., 1991).

In Vitro Reconstitution of Wild-Type and Mutant Neural Crest Development

The in vitro reconstitution of neural crest cell differentiation from wild-type and *Mash1* mutant embryos has allowed us to infer a cellular function for MASH1 in a way that would not have been possible simply from an analysis of the *Mash1* mutant phenotype in vivo. Specifically, the identification of NF160$^+$ and c-RET$^+$ cells in sections of mutant embryos could not determine whether these markers were expressed in the same cells or in different cells,

Table 2. Clonal Analysis of Neurotag⁻ Cells

	# Clones Examined	% PF⁺ Neurons	% in PF⁺ Clusters	% Nonneuronal	% Mixed	% Dead
Experiment 1	61	36% (22)	36% (22)	10% (6)	1.6% (1)	16.4% (10)
Experiment 2	43	39.5% (17)	18.6% (8)	28% (12)	4.6% (2)	9.3% (4)
Experiment 3	40	35% (14)	27.5% (11)	27.5% (11)	0% (0)	10% (4)

Clonal analysis of FACS-isolated PHK26⁻, Neurotag⁺ cells. Clones derived from identified cells were classified as either neuronal (peripherin [PF]⁺, associated with neuronal clusters, nonneuronal, mixed (neuronal plus nonneuronal cells), or dead. The total number of clones examined in each experiment is given in the first column. In the other columns, the percentage of clones in each category is given, with the total number listed in parentheses.

or whether they represented neurons of a different lineage, an arrested neuronal precursor, or an aberrant cell type that forms as a consequence of the mutation. The in vitro analysis has allowed us to determine that NF160 and c-RET define a single cell population rather than two distinct cell types; that these NF160⁺, c-RET⁺ cells are not neurons of a different lineage, but rather cells that have an undifferentiated, nonneuronal morphology; that cells with this morphology and antigenic phenotype are also found in wild-type cultures, indicating that the cells in mutant cultures are not an aberrant phenotype caused by the loss of MASH1 function; and that the NF160⁺ nonneuronal cells in wild-type cultures are in fact proliferating, autonomic neuronal precursors.

We attempted to rescue the arrested neuronal precursors in mutant cultures by transfecting *Mash1* cDNA expression constructs into Mko-4 cells. Although transfected cells expressing exogenous MASH1 in their nuclei could clearly be identified by antibody staining, none of these cells differentiated to neurons (unpublished data). There are a number of possible explanations for this failure to rescue the *Mash1* mutant phenotype in vitro, including the level, timing, and duration of MASH1 expression, the presence of inhibitors, and the need for cofactors; these are currently being investigated.

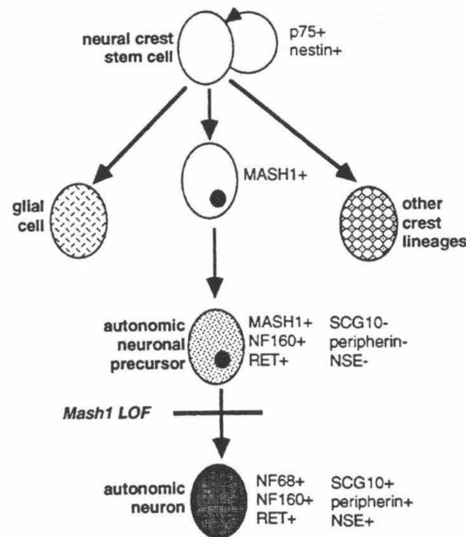

Figure 8. Model Representing the Function of MASH1 in the Context of Trunk Neural Crest Differentiation

Neural crest stem cells give rise to glial, sensory, and autonomic neuronal sublineages. Initially, these cells give rise to cells that express MASH1, but appear otherwise undifferentiated (N. S., L. S., and D. J. A., unpublished data). Such cells may retain some multipotency (Lo and Anderson, 1995). Subsequently, MASH1⁺ cells express a subset of neuronal markers, including NF160, in a MASH1-independent manner. Cells expressing these markers appear committed to a neuronal fate. The progression of this precursor to a fully differentiated neuron expressing additional neuronal markers (e.g., SCG10, peripherin, and neuron-specific enolase) requires MASH1.

Our data indicate that the NF160⁺ cells seen in the mutant are arrested neuronal precursors that eventually die. This conclusion is based on a combination of in vivo and in vitro data using both normal and immortalized neural crest cells. The ability to immortalize neural crest cells permitted us to demonstrate directly a conversion of wild-type NF160⁺ precursors to neurons, something that would not have been possible with primary cultures alone, owing to the limited number of cells available. Furthermore, the identification of NF160⁺, peripherin⁻ cells in nonimmortalized primary crest cultures indicates that these precursors are not an artifact of immortalization. Finally, the detection of cells expressing NF160 but not peripherin in sections of $Mash1^{-/-}$ embryos indicates that the NF160⁺, peripherin⁻ cells seen in vitro are not a culture artifact. Thus, the in vivo and in vitro approaches we have employed complement each other and are internally consistent.

Two Classes of Neuron-Specific Genes Can Be Distinguished by Their Dependence on MASH1 Function

A surprising result from this study was that the products of neuron-specific genes could be divided into two categories according to their dependence on MASH1 function. Those in the first category, including NF68, NF160, N-CAM, neuron-specific β-tubulin, c-RET, and tetanus toxin receptor, were expressed in *Mash1* mutant cells and in wild-type neuronal precursors; those in the second category, including peripherin, SCG10, and neuron-specific enolase, were expressed only in differentiated neurons. This result is unexpected because NF68, NF160, and neuron-specific β-tubulin have generally been considered to be markers of terminally differentiated neurons. The results presented here indicate that, at least in the autonomic lineage, these proteins are already expressed in proliferating neuronal precursors. Recent studies have demonstrated β-tubulin and N-CAM expression in proliferating precursors of olfac-

tory bulb granule cells located in the rostral migratory stream of the forebrain (Tomasiewicz et al., 1993; Menezes and Luskin, 1994). Furthermore, proliferative cells expressing some neuronal markers have been identified in cultures of spinal cord precursors grown in basic fibroblast growth factor (Ray and Gage, 1994). Whether or not these cells are committed to a neuronal fate is not yet known. These data suggest that the stepwise expression of neuron-specific genes is not a unique feature of the peripheral autonomic lineage but is exhibited by at least some CNS lineages as well.

The observation that some but not all neuron-specific genes are expressed in arrested neuronal precursors in *Mash1* mutants implies that the expression of the genes that define a neuronal phenotype is not controlled by a single genetic program, but rather by a series of "subprograms." These subprograms could run either in series or in parallel. We favor the former simply because of the fact that expression of the MASH1-independent genes precedes that of the MASH1-dependent genes in wild-type cultures. However, this does not imply that the two subprograms are obligatorily coupled. In other experiments, we have provided evidence that subprograms controlling the expression of neurotransmitter-synthesizing enzymes and of some neuronal genes (such as SCG10) can be experimentally uncoupled by manipulation of the neural crest cells' environment (Groves et al., 1995). If the expression of the final neuronal phenotype in a given lineage indeed reflects the operation of different subprograms that are controlled by different environmental signals, it could provide a way to generate cellular diversity in both development and evolution, by using different combinations of subprograms to generate related but distinct cellular phenotypes, in different lineages or in different organisms.

MASH1 and Neural Crest Cell Lineage Segregation

In the present study, we have shown that MASH1 function is required in a committed NF160⁺ neuronal precursor derived from migratory trunk neural crest cells in vitro. Previously, we have identified committed neuronal or neuroendocrine progenitor cells in two different populations of postmigratory neural crest-derived cells in vivo. One progenitor, called NP, gives rise only to neurons and has been identified in a population of c-RET⁺ cells isolated from the E14.5 gut (Lo and Anderson, 1995). Another progenitor, called the SA progenitor, gives rise to both sympathetic neurons and chromaffin cells and has been isolated using several different antibodies from E14.5 adrenal glands (Michelsohn and Anderson, 1992) or sympathetic ganglia (Carnahan and Patterson, 1991). The relationship between these various progenitor cell types is not yet clear because they have been isolated from different tissues with different antibodies and, in some cases, cultured under different conditions. Moreover, progenitors in the gut derive from the vagal neural crest, whereas the SA lineage derives from the trunk neural crest. Nevertheless, all three progenitors represent lineages that require MASH1 function in vivo. A simple interpretation is that MASH1 function is required at a similar stage of neurogenesis in several distinct autonomic sublineages. Further studies will be required to determine whether the progenitors representing these sublineages are interconvertible or committed to producing different types of neurons.

In addition to its expression in committed neuronal precursors, MASH1 appears to be expressed in more primitive progenitors as well. In trunk neural crest primary cultures, for example, expression of MASH1 is first detected prior to the onset of NF160 expression, in morphologically undifferentiated cells expressing nestin and p75 (N. S., L. S., and D. J. A., unpublished data). Similarly, MASH1 is expressed in most of the the c-RET⁺ cells isolated from fetal gut, and this population contains some multipotent cells (proNPs) as well as the committed NP cells mentioned above (Lo and Anderson, 1995). Together, these data indicate that MASH1 can be expressed by cells at several different stages of lineage commitment. However, our data demonstrate an essential function for this gene only in committed neuronal precursors. The apparent lack of a requirement for MASH1 in more primitive cells may reflect the presence of other, functionally redundant bHLH genes or simply the fact that the protein begins to accumulate to detectable levels before it actually carries out its requisite function.

Evolution of *ac–sc* Gene Function

The sequence of the MASH1 bHLH domain is highly related to those of the *ac–sc* complex genes in Drosophila (Johnson et al., 1990). Moreover, the expression of MASH1, like that of AC–SC, is restricted to the developing nervous system, where it appears transiently in subsets of precursor cells (Lo et al., 1991; Guillemot and Joyner, 1993). This parallel evolutionary conservation of amino acid sequence and cell type specificity of expression suggested an evolutionary conservation of function as well, a conclusion supported by the fact that the *Mash1* knockout prevents the development of specific subsets of neurons (Guillemot et al., 1993). However, the cellular analysis of the *Mash1* mutant phenotype presented here suggests that this apparent conservation obscures a difference in the cell biological functions controlled by *Mash1* and the *ac–sc* genes.

Mutations in *ac* and *sc* prevent the initial generation of the sensory mother cell, a multipotent progenitor of neurons and several different nonneuronal cell types (Ghysen and O'Kane, 1989). By contrast, the present studies indicate that MASH1 acts after the segregation of neuronal and glial lineages, to control the differentiation of a precursor that is likely committed to a neuronal fate. The *ac–sc* complex contains another bHLH gene related to *ac* and *sc*, called *asense*, that appears to be expressed immediately after the sensory mother cell has formed and therefore may act downstream of *ac–sc* (Brand et al., 1993; Jarman et al., 1993). However, the cells in which *asense* is expressed are still multipotent, so this Drosophila gene may still act at a comparatively earlier stage in its lineage than does MASH1. These data suggest that MASH1 exerts a different cell biological function than do its Drosophila *as–sc* homologs (Ghysen and Dambly-Chaudiere, 1989), de-

spite its similarities in sequence and expression. This difference is not due to intrinsic structural features of *Mash1*, however, as this mammalian gene efficiently complements the *ac–sc* mutations in Drosophila (A. Singson, J. Posakony, and D. J. A., unpublished data).

Together, these results indicate that, although *Mash1* and *ac-sc* perform different cellular functions, these genes are functionally interchangeable. This implies that cellular context may determine the biological roles played by different bHLH proteins, as much as primary structure. This conclusion is underscored by the fact that MASH2, which is 95% identical to MASH1 (Johnson et al., 1990), controls the development of extraembryonic membranes (Guillemot et al., 1994). Moreover, MASH2 complements *ac–sc* mutations in Drosophila as efficiently as MASH1 (A. Singson, unpublished data). The evolutionary conservation of AC–SC and MASH1 amino acid sequence must bear some relationship to the fact that these genes both function during neurogenesis. However, the cellular functions of these bHLH genes may have diverged as a consequence of evolutionary changes in the regulatory sequences controlling the time and place of their expression.

Upstream and Downstream of MASH1

In both Drosophila neurogenesis and mammalian myogenesis, bHLH genes act in cascades (Jan and Jan, 1993). It is therefore likely that there are additional bHLH genes acting in neural crest development, both earlier and later than MASH1. A novel bHLH gene, *eHAND/Th1* (Cserjesi et al., 1995; Hollenberg et al., 1995), is expressed in the same autonomic lineage as *Mash1*. Preliminary data indicate that *eHAND/Th1* mRNA is not expressed in *Mash1*$^{-/-}$ embryos, suggesting that it functions downstream of MASH1 (L. S., P. Cserjesi, E. N. Olson, and D. J. A., unpublished data). It is also likely that there are other bHLH genes that act earlier than MASH1 and function more analagously to *ac–sc* in Drosophila. However, such genes have yet to be identified in vertebrates. Extensive searches in mammals have failed to identify additional AC–SC homologs besides MASH1 and MASH2 (K. Zimmerman, J. E. Johnson, and D. J. A., unpublished data). XASH-3, another AC–SC homolog identified in Xenopus (Zimmerman et al., 1993; Turner and Weintraub, 1994), is expressed earlier than XASH-1, the Xenopus MASH1 homolog (Ferreiro et al., 1992), but searches for mammalian XASH-3 homologs have thus far been unsuccessful (K. Zimmerman and D. J. A., unpublished data). The availability of clonal cell lines blocked at the step before MASH1 function is required may facilitate the identification of novel bHLH genes that act at earlier stages in neural crest development.

Experimental Procedures

Culture of Primary Mouse Neural Crest Cells

Timed pregnant mice with a mixed C57Bl/6J × 129/Sv//Ev background carrying a null allele in the *Mash1* locus were obtained from the breeding facility at the California Institute of Technology. Mouse neural crest cells were isolated from gestational day 9 (E9) embryos and cultured essentially as previously described for rat neural crest cell cultures (Stemple and Anderson, 1992); details of minor modifications are available upon request. Secondary cultures were replated at a density of about 4000 cells per 35 mm dish (Stemple and Anderson, 1992) and differentiated in standard medium supplemented with 10% fetal bovine serum (FBS) and 5 μM forskolin (differentiation medium).

Genotyping Mice with a Targeted Null Mutation in the *Mash1* Locus

To distinguish *Mash1*$^{-/-}$ embryos from *Mash1*$^{+/-}$ and *Mash1*$^{+/+}$ embryos, the neural tube (from neural crest cultures; see above) or a forelimb (in the in situ hybridization experiments described below) was genotyped by polymerase chain reaction as described (Blaugrund et al., 1996), with slight modifications (details available upon request).

Generation of Immortalized Neural Crest Stem Cell Lines

A detailed characterization of immortalized neural crest stem cells will be published elsewhere (M. R. and D. J. A., unpublished data). In brief, neural crest cells were infected with a retroviral vector harboring the avian v-*myc* and neomycin resistance genes (Birren and Anderson, 1990). At 24 hr after infection, cells were placed under neomycin selection (500 μg/ml) for a period of 4 days. Neomycin-resistant clones expressing LNGFR immunoreactivity were isolated and expanded. Clonal cell lines Monc-1 (wild-type neural crest stem cell line) and Mko-4 (*Mash1*$^{-/-}$ neural crest stem cell line) were used to perform the experiments described in this study. To differentiate Monc-1 and Mko-4, cells were trypsinized and replated onto dishes sequentially coated with poly-D-Lysine (0.5 mg/ml) and fibronectin (0.25 mg/ml) in standard medium (Stemple and Anderson, 1992) containing 10% FBS and 5 μM forskolin. Neuronal and glial differentiation occurred within 2–5 days.

Immunocytochemistry

Labeling of cell surface antigens on living cells was performed as described in Stemple and Anderson (1992), using a monoclonal rat anti-mouse LNGFR antibody (IgG) (M. R. and D. J. A., unpublished data); a monoclonal anti-N-CAM antibody 5A5 (IgM) (from hybridoma cells obtained from Developmental Studies Hybridoma Bank; Dodd et al., 1988); and Neurotag, a FITC-labeled tetanus toxoid derivative (Boehringer Mannheim; Raju and Dahl, 1982). Phycoerythrin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used.

Labeling of intracellular antigens was performed as described (Stemple and Anderson, 1992; Shah et al., 1994) using the following reagents: rabbit polyclonal anti-SCG10 antibody (used at a 1:800 dilution; Stein et al., 1988); mouse monoclonal anti-peripherin antibody (IgG) (1:200 dilution; Chemicon; Parysek et al., 1988); monoclonal anti-neuron-specific enolase antibody (IgG) (1:10 dilution; Chemicon; Marangos and Schmechel, 1987); monoclonal anti-NF160 antibody NN18 (IgG) (1:40 dilution; Sigma Immuno Chemicals); monoclonal anti-NF68 antibody NR4 (IgG) (1:100 dilution; Sigma Immuno Chemicals; Cochard and Paulin, 1984); monoclonal anti-Class III β-tubulin antibody TuJ1 (IgG) (1:500 dilution; Geisert and Frankfurter, 1989); monoclonal anti-nestin antibody Rat 401 (IgG) (1:2 dilution; Developmental Studies Hybridoma Bank; Friedman et al., 1990); and rabbit polyclonal anti-GFAP antibody (1:500 dilution; Accurate Chemical and Scientific Corporation; Jessen et al., 1990). Detection of c-RET and MASH1 in fixed cells was performed as described (Shah et al., 1994; Lo and Anderson, 1995). Staining was developed with either fluorescent secondary antibodies or horseradish peroxidase–conjugated goat anti-mouse IgG (Chemicon) and goat anti-rabbit IgG (Vector Laboratories) using nickelous sulfate and diaminobenzidine (NiDAB) or 3-amino-9-ethyl carbazole (AEC) as substrates.

Isolation and Culture of Neuronal Precursors

Monc-1 cells were allowed to differentiate for 2 days and then labeled with FITC-conjugated Neurotag (see above). Subsequently, cells were removed from the culture dishes by trypsinization, washed once in medium containing 10% FBS, and labeled with the fluorescent dye PKH26 using the PKH26 Red Fluorescent General Cell Linker Kit (Sigma Immuno Chemicals; Horan and Slezak, 1989), according to the manufacturer's instructions. Neurotag$^+$, PKH26$^+$ cells were isolated on an Epic Elite Fluorescent Activated-Cell Sorter (Coulter) using a multiparametric gate based on fluorescent intensity, size, density, and granularity. To increase their survival, Neurotag$^+$, PKH26$^+$ cells were plated onto a monolayer of unlabeled Monc-1 cells that had previously

been cultured under differentiating conditions for 6–12 hr. Cultures were fixed 5 days after plating of the sorted cells and permeabilized by a freeze–thawing method (Temple and Davis, 1994) prior to immunocytochemical analysis of intracellular markers.

In Situ Hybridization

Nonradioactive in situ hybridization with digoxigenin-labeled cRNA probes was carried out on frozen sections of paraformaldehyde-fixed mouse embryos according to Birren et al. (1993). Detailed protocols are available upon request. Antisense cRNA probes used in this study were the following: SCG10 (Stein et al., 1988), peripherin (Parysek et al., 1988), NF160, NF68 (Julien et al., 1986), c-RET (Pachnis et al., 1993), and P_0 (Lemke et al., 1988).

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