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Postmigratory Neural Crest Cells Expressing c-RET Display Restricted Developmental and Proliferative Capacities

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Summary

c-RET is an orphan receptor tyrosine kinase essential for enteric neurogenesis in mice and is involved in several human genetic disorders. RET is also one of the earliest surface markers expressed by postmigratory neural crest cells in the gut. We generated anti-RET monoclonal antibodies to isolate such cells. We find that RET^+ cells are antigenically and functionally distinct from neural crest stem cells (NCSCs) characterized previously. Unlike NCSCs, which are RET^- and $MASH1^-$, most RET^+ cells express MASH1. Moreover, unlike NCSCs, which are multipotent and have high proliferative capacity, many RET^+ cells generate only neurons following a limited number of divisions. This behavior is observed even in the presence of glial growth factor, a polypeptide that suppresses neuronal and promotes glial differentiation by NCSCs. These data provide direct evidence for the existence of committed neuronal progenitor cells and support a model of neural crest lineage diversification by progressive restriction of developmental potential.

Introduction

The nervous system, like the immune system, develops from multipotent progenitor cells. The existence of neural progenitor cells that generate multiple types of neurons and glia has been well documented both in vivo and in vitro, in the CNS and in the PNS (for reviews, see McKay, 1989; Sanes, 1989; McConnell, 1991). In some cases, moreover, such multipotent cells have been shown to be capable of self-renewal at the single-cell level (Stemple and Anderson, 1992; Wren et al., 1992; Davis and Temple, 1994), suggesting that they may be analagous to self-renewing hematopoietic stem cells (Spangrude et al., 1988). In support of this idea, there is evidence in the CNS for the persistence of some kinds of neuronal and glial progenitors into adulthood (Altman, 1969; Kaplan and Hinds, 1977; Wolswijk and Noble, 1989; Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994).

The existence of multipotent neural progenitors raises the question of how these cells generate their differentiated derivatives. On the one hand, cell fate could be assigned by lineage or by other cell-autonomous mechanisms. On the other hand, cell fate could be influenced or determined by cell-extrinsic signals. A popular idea to explain hematopoiesis is that both types of mechanisms operate, so that multipotent stem cells generate progenitors committed to one or more sublineages, which then proliferate, survive, and differentiate in response to specific growth factors (Ogawa, 1993). Similar "neuropoietic" models have also been invoked to explain cell lineage diversification in the nervous system (Anderson, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991), although evidence in support of such models has been relatively scant and indirect (for review, see Anderson, 1993).

The neural crest represents a good model system in which to investigate the process of neural cell lineage diversification in vertebrates because it is relatively simple and experimentally accessible (Le Douarin, 1982). In vivo lineage-tracing studies (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991) and in vitro clonal analyses (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Stemple and Anderson, 1992; Ito et al., 1993) have demonstrated that many neural crest cells are multipotent at the time they emigrate from the neural tube in both avian and mammalian embryos. In the rat, moreover, serial cell cloning experiments have shown that such multipotent cells are capable of at least limited self-renewal in vitro (Stemple and Anderson, 1992). Furthermore, the fate of such multipotent cells can be influenced by environmental signals (for review, see Stemple and Anderson, 1993).

These experiments did not address the issue of whether neural crest cells undergo progressive restrictions in developmental potential. That such restrictions may occur has been suggested from studies of transplanted or cultured avian neural crest cell populations (Le Lievre et al., 1980; Le Douarin, 1986; Artinger and Bronner-Fraser, 1992) or from clonal analysis of postmigratory crest cells in peripheral ganglia (Duff et al., 1991; Hall and Landis, 1991; Deville et al., 1992, 1994). However, in the transplantation studies that manipulated the cells' environment, there was no analysis of single cells, and in the single cell culture experiments, there was no manipulation of the cells' environment. To date, there has been no study in which postmigratory neural crest cells in clonal culture have been challenged by exposure to environmental signals known to influence the fate of early migratory cells.

Here we have isolated postmigratory neural crest cells from fetal rat gut using newly generated monoclonal antibodies to c-RET (RET), an orphan receptor tyrosine kinase that is not expressed by early migratory cells (Pachnis et al., 1993; Lo et al., 1994). We have examined the developmental and proliferative capacities of these cells using a clonal culture system (Stemple and Anderson, 1992). Many of the RET^+ cells divide symmetrically to generate small clones containing only neurons. They do so even in the presence of glial growth factor (GGF)/neuregulin (Marchionni et al., 1993), a polypeptide that represses neuronal and promotes glial differentiation by early migrating neural crest stem cells (NCSCs; Shah et al., 1994). We suggest that such RET^+ cells are committed neuronal progenitors. Other RET^+ progenitors are multipotent but are different from NCSCs in that their progeny rapidly and asymmetrically segregate into neuronal and nonneuronal

lineages. These data support the idea that neural crest cells undergo sequential restrictions in their developmental capacity, and establish a system in which the mechanistic basis of such restrictions can be studied further.

Results

Generation of Monoclonal Antibodies to RET

We wished to generate a cell surface marker that could be used to isolate specifically postmigratory neural crest cells. To do this, we chose to produce monoclonal antibodies to the extracellular domain of RET because this orphan receptor tyrosine kinase is one of the earliest surface markers that distinguishes postmigratory from early migrating neural crest cells (Pachnis et al., 1993; Lo et al., 1994). Importantly, RET is not simply a marker for enteric progenitors but is also essential for their proper development, as shown by genetic studies in both mice (Schuchardt et al., 1994) and humans (Edery et al., 1994). To generate such antibodies, we immunized hamsters with Chinese hamster ovary (CHO) cells expressing the extracellular domain of murine RET in phosphatidyl inositol (PI)–linked form (Devaux et al., 1991) (see Experimental Procedures). An example of a positive antibody obtained from this immunization is illustrated in Figure 1. This antibody specifically labels the surfaces of live 293T cells transiently transfected with a full-length murine *Ret* cDNA (Figure 1A), indicating that it can recognize an epitope present on the intact receptor. The hybridomas were further screened on cell lines to select those antibodies recognizing both endogenous murine and rat RET. The reactivity of the antibody with the RET protein was confirmed by Western blotting (data not shown).

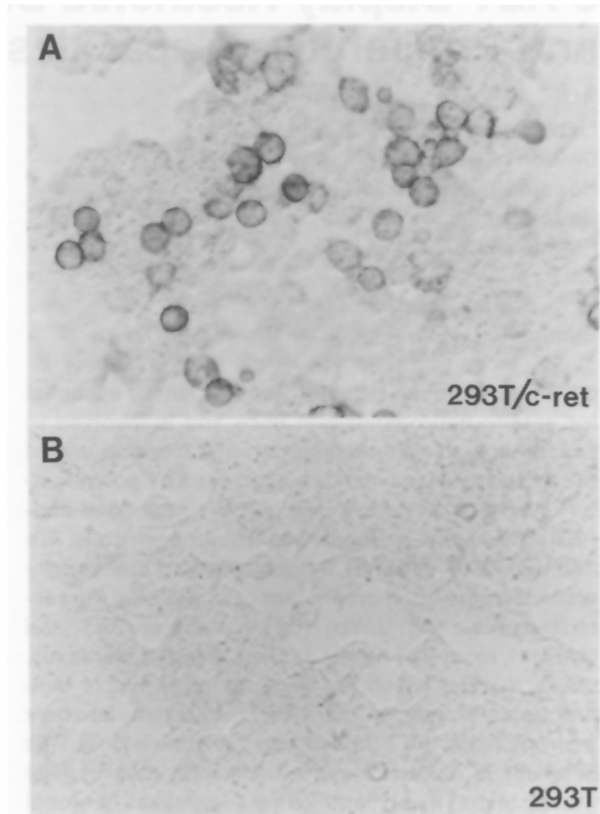

Figure 1. Monoclonal Antibody to RET-PI Also Detects Native RET
(A) Cells (293T) were transfected with an expression plasmid harboring the intact RET-coding sequence and then live-labeled with the anti-RET antibody. Positive cells are dark, indicating the horseradish peroxidase reaction product.
(B) Control 293T cells are not stained by the antibody.
Magnification, 22×.

RET Is Expressed by Neurons but Not by NCSCs In Vitro

In situ hybridization experiments have indicated that RET is not expressed by early migrating trunk neural crest cells in vivo but is expressed after these cells have aggregated to form the primordia of autonomic ganglia (Pachnis et al., 1993). To determine the pattern of RET protein expression by neural crest cells in vitro, we stained primary explants of rat neural crest cells with the monoclonal antibody to RET. No RET staining was detectable in the explants after 24 hr (Figures 2A and 2B), whereas staining was clearly detectable on some of the neurons that had developed in these cultures after 9 days (Figures 2C and 2D). These results indicate that in vitro, as in vivo, RET is not expressed by neural crest cells immediately after they emigrate from the neural tube. However, as expected, RET is expressed by at least some of the neurons that derive from the neural crest explants.

Antigenic Phenotype of RET$^+$ Cells Isolated from Fetal Rat Gut

We wished to isolate and characterize postmigratory neural crest cells using the anti-RET antibodies. To do this, we chose the embryonic day (E) 14.5 fetal gut as a source of tissue since it is extensively colonized by RET$^+$ neural crest–derived cells (Pachnis et al., 1993; Lo et al., 1994). We used anti-RET antibodies to isolate these cells because this marker represents the earliest detectable cell surface antigen that is expressed by neural crest–derived cells in the gut, but that is not expressed by NCSCs (see above). RET$^+$ cells could be readily separated from unlabeled gut cells by fluorescence-activated cell sorting (FACS), and these constituted about 1% of the population at this stage (Figures 3A and 3B).

We next characterized the morphology, antigenic phenotype, and functional properties of the RET$^+$ cells isolated from E14.5 gut. RET$^+$ cells examined 15 hr after plating fell into two morphologically distinct categories: neurons and undifferentiated (flat) cells. Approximately 30% of the cells were neurons, and these cells usually expressed higher levels of RET immunoreactivity than did the flat cells (data not shown). Since we were interested in the properties of progenitor cells isolated by anti-RET antibodies, the neuronal subpopulation was not examined further.

To establish their antigenic phenotype, RET$^+$ cells were fixed 15 hr after plating and stained with several antibody markers for neural crest cells and their derivatives; 95% of the flat cells could be relabeled with anti-RET antibody

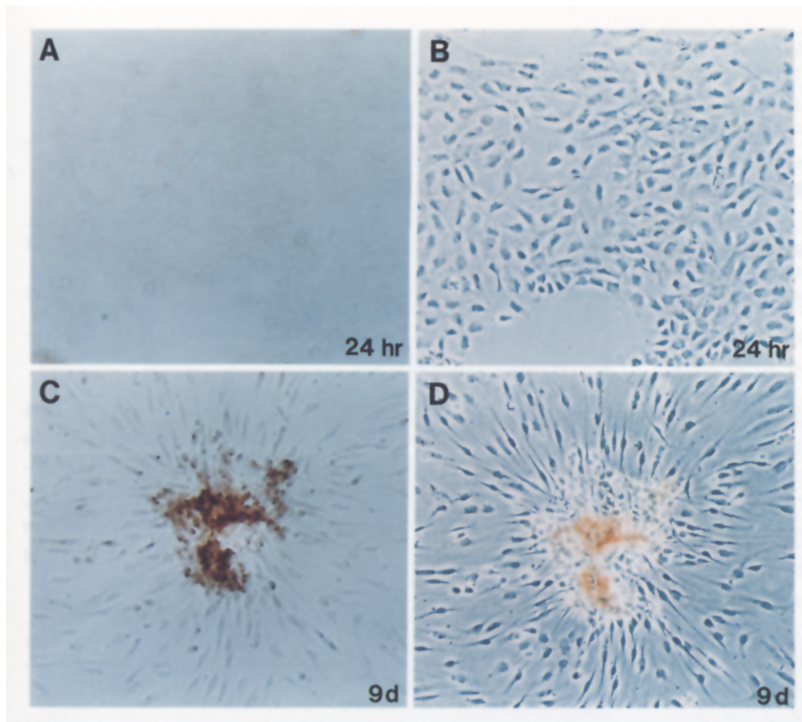

Figure 2. RET Is Expressed by Neural Crest–Derived Neurons but Not by NCSCs In Vitro

(A and B) A 24 hr rat neural crest explant, containing NCSCs, stained with anti-RET monoclonal antibody. No specific labeling is detected.

(C and D) The same antibody intensely stains neurons that developed in sister cultures incubated for 9 days.

Explants were viewed under bright-field (A and C) and phase-contrast (B and D) illumination. Magnification, 12 ×.

(Table 1) using the immunoperoxidase procedure. The unlabeled cells may represent a minor contaminant, or rather neural crest cells that down-regulated RET expression following their isolation. Almost 100% of the flat cells expressed nestin (Table 1), a neuroepithelial stem cell marker (Lendahl et al., 1990) also expressed by NCSCs (Stemple and Anderson, 1992). More than 70% of the cells expressed the low affinity nerve growth factor (NGF) receptor (p75), a surface marker of NCSCs (Stemple and Anderson, 1992). The majority of the cells were negative for the 160 kDa subunit of neurofilament (Table 1), a neuronal marker, and all of the cells were negative for the glial marker glial fibrillary acidic protein (GFAP; data not shown), as is the case for NCSCs (Stemple and Anderson, 1992). While the p75$^+$, nestin$^+$, lineage marker (e.g., GFAP, NF160) (lin)$^-$ phenotype is characteristic of NCSCs, as discussed above, NCSCs do not express RET (see Figure 2). Another difference between NCSCs and RET$^+$ cells was revealed by staining with an antibody to the basic–helix-loop-helix transcriptional regulator MASH1 (Lo et al., 1991). NCSCs do not express this marker; however, 87% of the RET$^+$ cells expressed detectable MASH1 immunoreactivity (Table 1). Thus, RET$^+$ cells isolated from fetal gut are antigenically distinct from both NCSCs and differentiated neural crest derivatives.

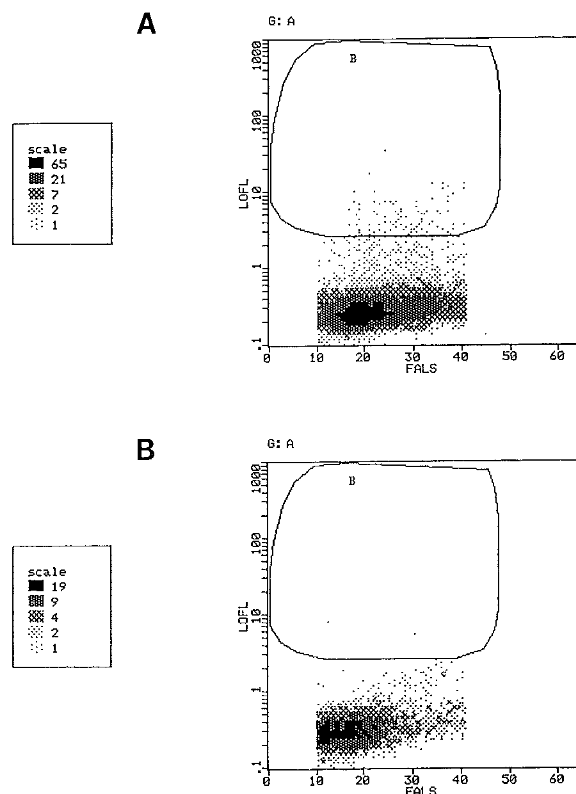

Figure 3. Typical FACS Profile of Dissociated E14.5 Rat Gut Following Live Cell Labeling with Anti-RET Monoclonal Antibodies

A cocktail of three different anti-RET monoclonals was used (see Experimental Procedures). RET$^+$ cells (A) were collected from the gated region "B". Approximately 1% of the input cells fell within this region. (B) shows a control sort in which the primary anti-RET antibody was omitted. LOFL, fluorescence intensity (log scale); FALS, forward angle light scatter (a measure of cell size). The gated cells were also selected for granularity (data not shown).

Functional Properties of Undifferentiated RET$^+$ Cells

To determine the functional properties of the morphologically undifferentiated subset of RET$^+$ cells, individual flat cells were identified and circled 15 hr after plating, after which they were observed every day for the next 3–4 days. In addition, some cultures were allowed to develop for 12 days with or without the addition of 10% fetal bovine serum

Table 1. Antigenic Phenotype of E14.5 Undifferentiated RET⁺ Enteric Cells

	Percentage of Nonneuronal Cells Labeled				
	RET	p75	Nestin	MASH1	NF160
+	58.5% ± 0.5%	70% ± 1%	100%	54.5% ± 5.5%	16.5% ± 1.5%
+/−	36.5% ± 2.5%	7.5% ± 7.5%	0%	32.5% ± 7.5%	10% ± 10%
+ or +/−	95%	77.5%	100%	87%	26.5%

Isolated RET⁺ cells were plated at clonal density, fixed after 15 hr, and stained for the various antigenic markers indicated. The percentages of strongly labeled (+), weakly labeled (+/−), and unlabeled (−) cells were measured. Approximately 100 cells were scored for each determination. The results represent the mean ± range of two independent experiments. In a separate experiment, the cells were stained for GFAP and no expression was detected.

plus 5 μM forskolin (which have previously been shown to promote the expression of glial differentiation markers in clonal NCSC cultures; Stemple and Anderson, 1992) and were then fixed and stained with neuronal and glial antibody markers. This clonal analysis revealed that the population of undifferentiated RET⁺ cells contained three functionally distinct subsets. One subset produced clones that consisted of neurons and nonneuronal cells (Figure 4; Figure 5). In some cases, the first 1–3 divisions produced 2–8 flat cells similar to the founder cell (Figure 4B), followed by the generation of neurons from some of these cells. In other cases, process-bearing neurons and flat

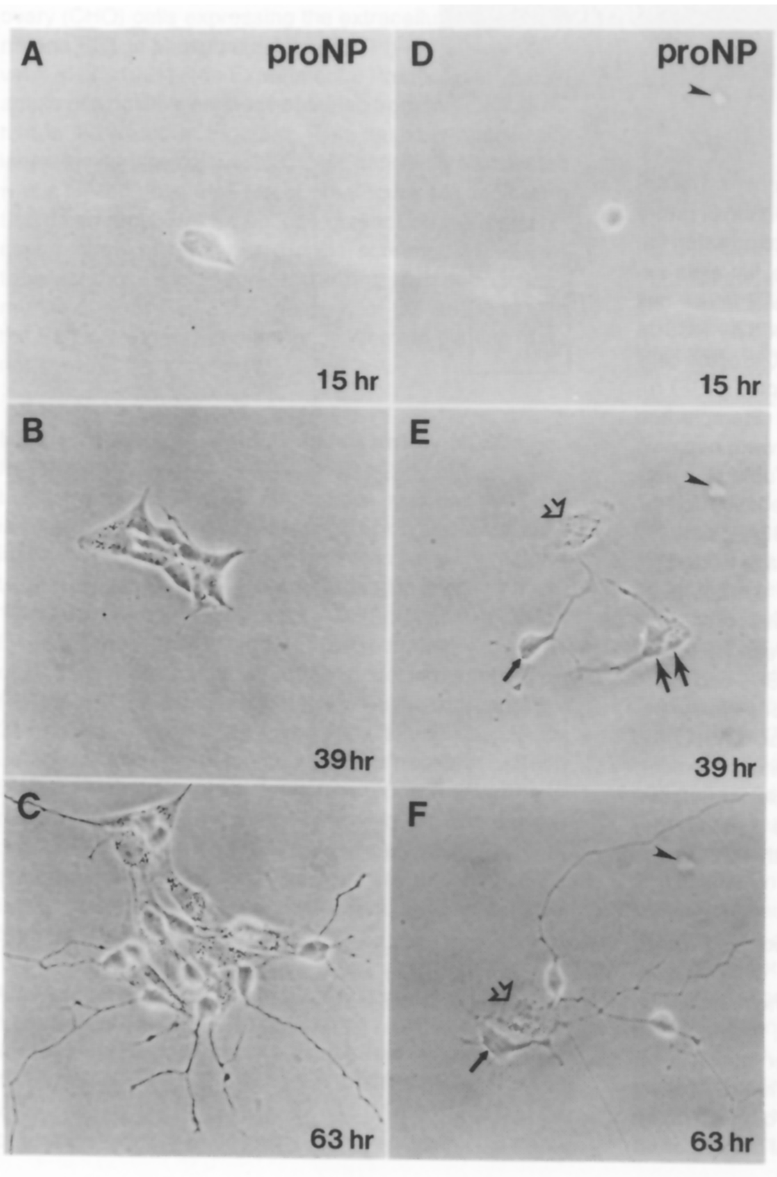

Figure 4. Two Examples of the Development of RET⁺ Multipotent ProNP Cells in Clonal Culture

Micrographs of the same microscopic fields in (A)–(C) and (D)–(F) were taken at the indicated times. In (D), a single cell apparently divided twice in 24 hr to produce an immature neuronal precursor (E, arrow), a neuronal progenitor cell that itself divided (double arrows), and an undifferentiated cell (open arrow), suggestive of 2 sequential asymmetric cell divisions. In (F), 24 hr later, 2 neurons have developed with long neurites and phase-bright cell bodies. The undifferentiated cell (open arrow) remains beside the immature neuronal precursor (solid arrow). Arrowheads in (D)–(F) indicate a mark on the substrate used to identify the microscopic fields. Magnification, 26× (A–C, E, and F), 20× (D).

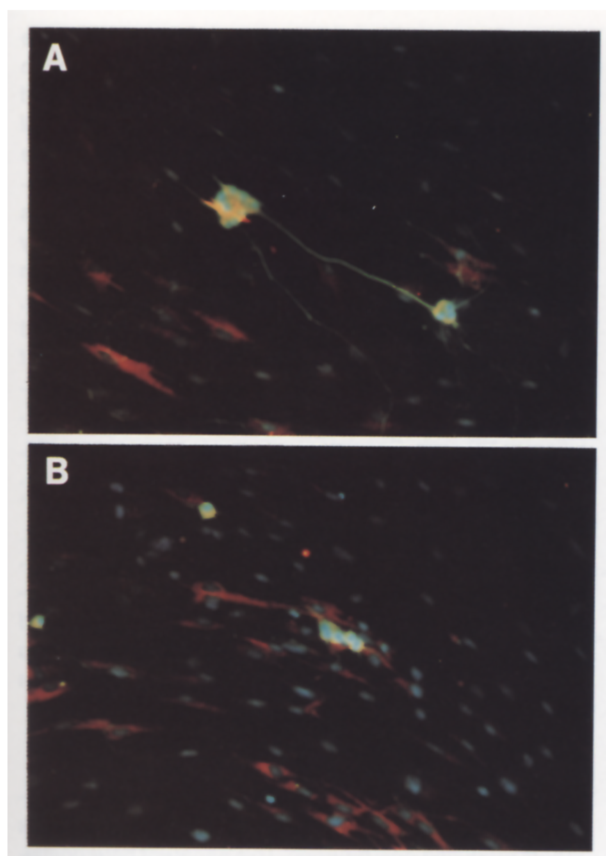

Figure 5. ProNP Clones Develop into Neurons and Glia

(A) and (B) illustrate two examples from a culture fixed after 12 days and stained for peripherin (green fluorescence), a neuronal marker, and GFAP (orange fluorescence), a glial marker. Note that both clones contain both neurons and glia. The specimens were counterstained with DAPI to reveal all cell nuclei (blue). Magnification, 11×.

cells were observed within 2 days after plating (Figure 4E), suggestive of an initial asymmetric cell division by the founder cell. In all cases, neuronal differentiation was detected morphologically within 3 days (Figures 4C and 4F). Double labeling of 12 day cultures exposed to serum and forskolin with antibodies to peripherin and GFAP revealed the presence of both neurons and glial cells (as well as other unidentified nonneuronal cells) in these clones (Figure 5). These cells, which we have termed proneuronal progenitors (proNPs), were present at a frequency of 5%–16% of the undifferentiated RET$^+$ cells in two different experiments (Table 2).

A second subset of RET$^+$ cells, called nonneuronal progenitors (NNPs), consisted of cells that produced progeny that failed to differentiate into neurons (Figure 6), even when the incubation was extended for nearly 2 weeks (data not shown). To determine whether these nonneuronal cells were glial precursors, we cultured them in 10% fetal bovine serum plus 5 μM forskolin. Under these conditions, some of the cells in the NNP clones expressed GFAP, indicating that these clones contain progenitors of glia and possibly other as yet unidentified nonneuronal cells. In two separate experiments, NNPs constituted 60%–67% of the undifferentiated RET$^+$ cells (Table 2).

A third subset of RET$^+$ cells, termed neuronal progenitors (NPs), consisted of cells that produced 2–8 progeny (1–3 divisions), all of which differentiated to neurons within the first 3–4 days of culture (Figure 7; Figure 8). Moreover, even within relatively large NP clones, neuronal differentiation appeared synchronous. For example, Figure 8 shows a NP cell that divided 3 times to produce a clone of 8 cells within 48 hr after identification. At this time, all cells in the clone have begun to extend processes, but their cell bodies are still flattened (Figure 8B). By 72 hr, however, all of the cells exhibit the round, phase-bright cell bodies and long, thin neurites characteristic of differentiated neurons (Figure 8C). NPs constituted 17%–50% of the undifferentiated cells examined, depending upon the experiment (Table 2; see below). Although the exact frequency of NP and proNP cells varied among experiments, the percentage of NPs was always greater than that of the proNPs (Table 2). We were unable to distinguish between these three different classes of progenitor cells by expression of any of the antigenic markers examined or by their morphology.

To determine the type(s) of neurons produced from NP and proNP cells, some of the cultures were fixed and stained with various antibody markers. Some, but not all, of the neurons were labeled by antibodies to tyrosine hydroxylase and B2 (data not shown), two markers that are transiently expressed by a subset of enteric neuronal progenitors as well as by sympathetic neurons (Carnahan et al., 1991). Unfortunately, there are no markers available that uniquely identify enteric neurons in vitro. All of the neurons that developed expressed higher levels of RET than did their progenitors (data not shown). In vivo, RET

Table 2. Developmental Potential of RET$^+$ Progenitor Cells in Clonal Culture

	% NP (n)	% ProNP (n)	% NP or ProNP	% NNP (n)
Exp. 1	17% (25)	16% (23)	33%	67% (96)
Exp. 2	35% (78)	5% (11)	40%	60% (135)
Mean ± range	26% ± 9%	10.5% ± 5.5%	36.5% ± 3.5%	63.5% ± 3.5%

Single RET$^+$ cells were identified 15 hr after plating and observed every 24 hr for the next 4 days. All of the cells initially circled survived this incubation. At the end of this incubation, they were classified as neuronal progenitors (NPs), proneuronal progenitors (proNPs), or nonneuronal progenitors (NNPs), depending upon whether they produced neurons only, neurons plus nonneuronal cells, or nonneuronal cells only, respectively. The numbers represent the percentage of each clone type scored, with the raw number of clones of each type given in parentheses. A total of 144 cells were examined in experiment 1, and 224 cells in experiment 2. Note that the variation in the percentage of NP plus proNP cells (±10%) is much smaller than the variation in the percentage of NP or proNP cells individually (±35% and ±52%, respectively).

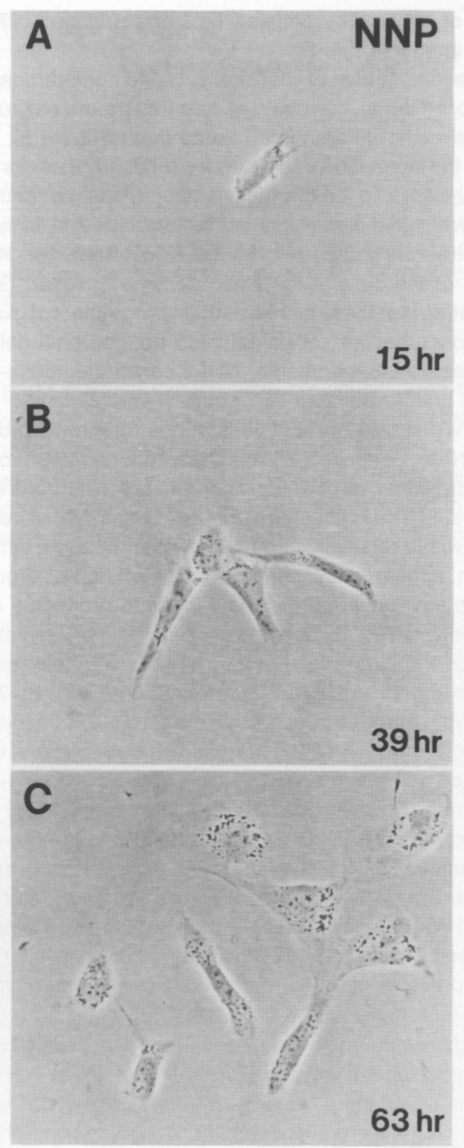

Figure 6. Development of a RET+ NNP Cell in Clonal Culture
Micrographs of the same microscopic field were taken at the indicated times. Note that the cell divided 3 times to produce 8 progeny in 48 hr (B and C) following its identification as a single cell at 15 hr (A). Magnification, 26×.

is expressed by most or all autonomic neurons and by only a small subset of sensory neurons in the dorsal root ganglia (unpublished data). These data are consistent with the idea that NPs and proNPs generate neurons in one or more autonomic lineages. However, in the absence of appropriate markers, we cannot exclude that these progenitors can give rise to sensory neurons as well.

Anti-RET and Anti-p75 Antibodies Select Different Populations of Enteric Precursors

Previously, we used monoclonal antibody 192Ig, directed against the low affinity NGF receptor ($p75^{LNGFR}$), as a surface marker for NCSCs isolated from E10.5 neural tube explants (Stemple and Anderson, 1992). We therefore wished to determine whether this antibody would bind to a similar or different population of neural crest-derived cells in the E14.5 gut than did the anti-RET monoclonal antibody. In parallel assays, approximately 11% of the dissociated E14.5 gut cells were $p75^+$, whereas only 1%–2% of the cells were RET^+. When FACS-isolated $p75^+$ cells were plated in clonal culture, identified, and followed every 24 hr, only 6.5% ± 0.5% of the cells were NPs (mean ± range of two independent experiments). By contrast, in parallel cultures seeded with RET^+ cells FACS-isolated from the same starting cell suspension, 50% ± 3% of the cells behaved as NPs. Thus, the RET^+ population appeared to be 8- to 9-fold enriched relative to the $p75^+$ population, in NP cells. Consistent with this functional analysis, only 5% of the $p75^+$ cells were RET^+ 15 hr after plating, and only 14% were $MASH1^+$. In contrast, 82% of the RET^+ cells isolated in parallel were $MASH1^+$ in this experiment. These data support the idea that anti-RET and anti-$p75^{LNGFR}$ antibodies enrich for distinct populations of neural crest-derived cells in the gut. RET^+ cells are enriched in both NPs and $MASH1^+$ cells. This correlation supports the idea that many (but not necessarily all) $MASH1^+$ cells are NPs.

NPs Appear Insensitive to GGF and Fibronectin

As described above, some RET^+ cells produced only nonneuronal cells or neurons plus nonneuronal cells, whereas others produced only neurons. This apparent heterogeneity could reflect the existence of distinct progenitor cell compartments at different and sequential stages in the lineage segregation process, as suggested for avian neural crest cells in clonal culture (Baroffio et al., 1988; Le Douarin et al., 1991). Alternatively, it may suggest a uniform progenitor population that exhibits clonal variation in developmental fate due to stochastic properties or to subtle variations in the local culture microenvironment. To distinguish between these possibilities, we examined the effect of recombinant human GGFII (rhGGFII; also called neuregulin; Marchionni et al., 1993) on the behavior of these cells. rhGGFII/neuregulin has previously been shown to exert an instructive influence on trunk-derived NCSCs, repressing neuronal differentiation and promoting glial differentiation by most or all of the cells (Shah et al., 1994). If the RET^+ enteric progenitor population were developmentally homogeneous but sensitive to local microenvironmental factors, then in the presence of a uniform environmental influence such as GGF, all clones might behave similarly (i.e., neuronal differentiation would be suppressed). On the other hand, if the RET^+ population contained some cells committed to a neuronal fate, these cells might be insensitive to the influence of GGF.

Two separate experiments were performed, in which clones derived from morphologically undifferentiated founder cells were followed with (n = 97) and without (n = 75) GGF. Each clone was examined every day for 3 days to determine whether neurons developed and survived or developed and died. The proportion of NNPs in the cohort of clones examined was virtually identical with or without

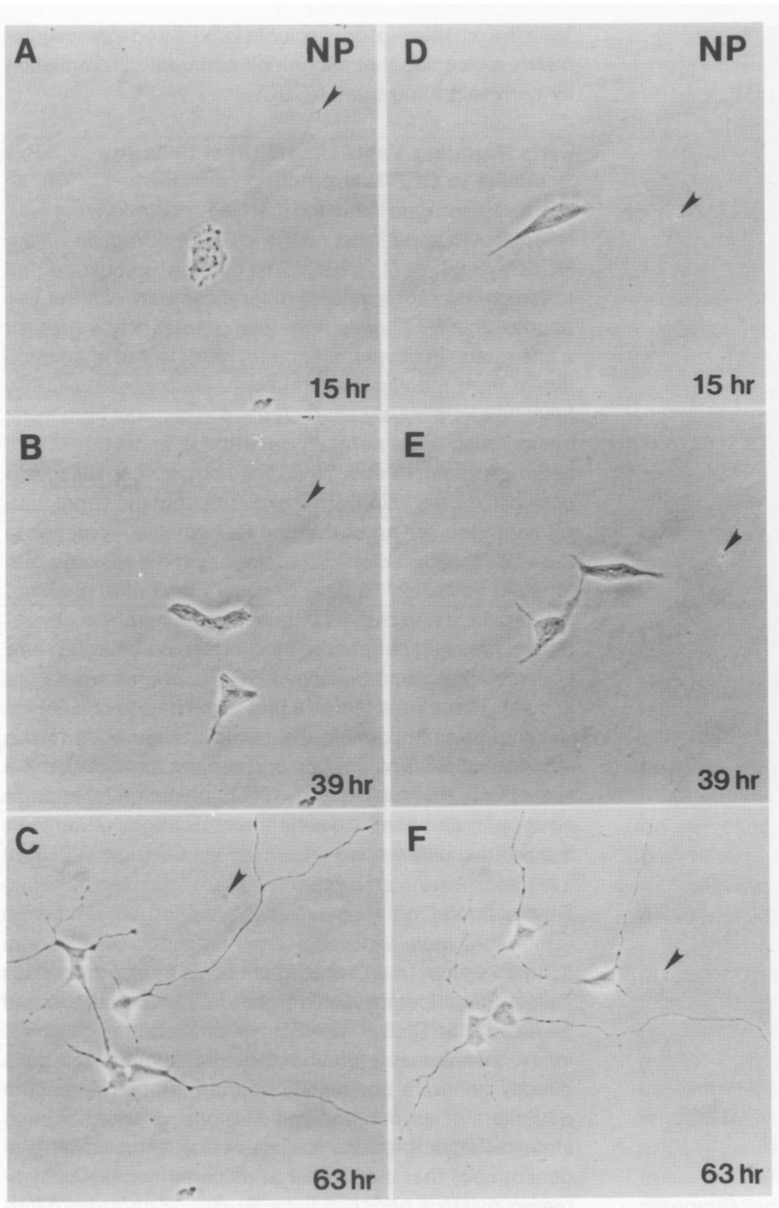

Figure 7. Two Examples of the Development of RET+ NP Cells in Clonal Culture

Micrographs of the same microscopic fields in (A)–(C) and (D)–(F) were taken at the indicated times. In both cases, the founder cells (A and D) divided twice to produce clones containing 4 neurons (C and F) within 48 hr. Arrowheads indicate marks on the plate used to identify the microscopic fields. Magnification, 26×.

GGF (62% with GGF versus 57% without GGF; Table 3), suggesting that GGF was unable to convert proNPs to NNPs by completely inhibiting neuronal differentiation. Similarly, the ratio of NPs to proNPs was comparable in the two experiments (6% versus 9%; Table 3), suggesting that GGF was unable to convert NPs to proNPs by partially inhibiting neuronal differentiation. Positive controls indicated that the preparations of rhGGFII used in these experiments were active in suppressing neuronal differentiation in both primary neural crest explants and in clonal NCSC cultures (data not shown). The concentrations of rhGGFII used in these experiments were always greater than that required to achieve half-maximal inhibition of neuronal differentiation in clonal cultures of NCSCs (Shah et al., 1994). Together, these data indicate that NPs, proNPs, and NNPs maintain their distinct developmental capacities in the presence of rhGGFII, suggesting that they are intrinsically different from one another as well as from neural crest stem cells.

As a further test of the extent of commitment of NPs, we compared their behavior on the standard poly-D-lysine/fibronectin substrate and on a fibronectin substrate. It has previously been demonstrated that neuronal differentiation from NCSCs is strongly inhibited or delayed on a substrate that contains fibronectin but not polylysine (Stemple and Anderson, 1992). In contrast, when RET+ progenitors were plated on fibronectin, a significant number of clones generated neurons after only 48 hr in culture (Table 3, NP clones), indicating that this substrate is unable to inhibit or delay neuronal differentiation of this progenitor cell type. An apparent reduction in the frequency of proNPs and an increase in the frequency of NNPs were observed (Table 3), however, suggesting that neuronal differentiation in multipotent proNP clones might be susceptible to inhibi-

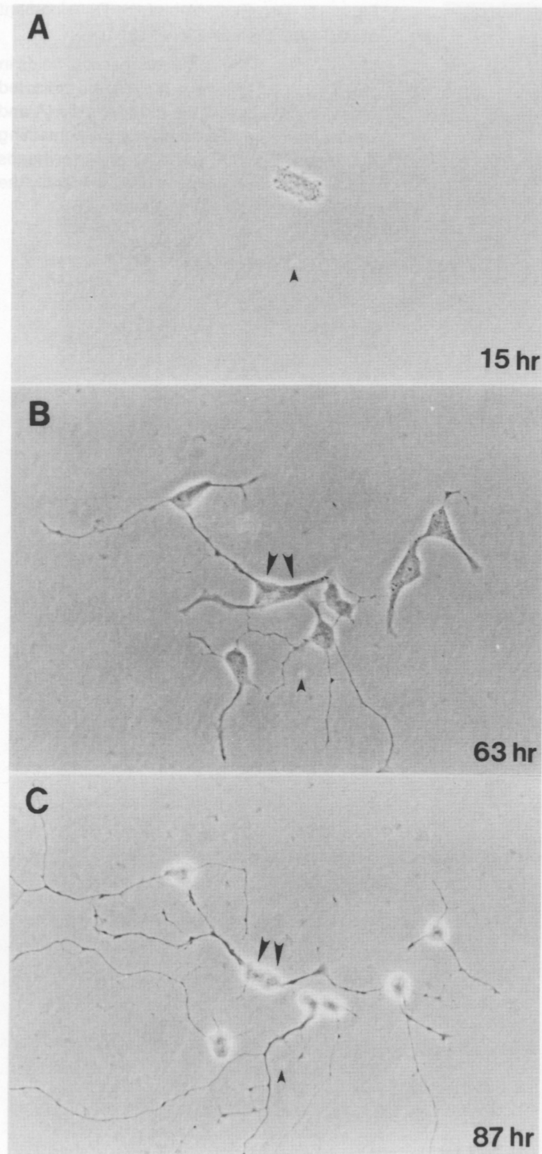

Figure 8. Apparently Synchronous Neuronal Differentiation of the Progeny of a NP Cell

The single cell identified 15 hr after plating (A) divided 3 times in 48 hr to produce 8 progeny (B). By 72 hrs (C), all 8 cells have developed the phase-bright cell bodies and long, branched neurites characteristic of mature neurons. Large arrowheads in (B) indicate 2 different cells that are poorly resolved, but which are clearly distinct in (C). Small arrowheads indicate marks on the substrate used to identify the microscopic field at successive time points. Magnification, 26×.

tion by fibronectin. On the other hand, these differences could simply reflect differences in the initial attachment of proNPs versus NNPs, rather than a conversion of proNPs to NNPs. The important point, however, is that there were any neurons that differentiated on fibronectin at all; in positive control experiments, neuronal differentiation by NCSCs was completely inhibited by these batches of fibronectin (data not shown). Together, therefore, these data indicate that RET$^+$ NPs differentiate to neurons despite the presence of both soluble factors and extracellular matrix molecules that can inhibit neuronal differentiation by early migrating trunk NCSCs.

Early Migrating Vagal Neural Crest Cells Are Sensitive to GGF/Neuregulin

The foregoing data indicated that RET$^+$ postmigratory neural crest cells appear insensitive to GGF/neuregulin, unlike NCSCs (Shah et al., 1994). This difference could be due to temporal or to positional differences between the two populations: RET$^+$ cells from fetal gut not only represent a later stage in development than NCSCs, but in addition derive from the vagal neural crest. By contrast, NCSCs have been previously characterized in cultures from trunk neural crest, which normally does not generate the enteric nervous system in vivo. To distinguish between these two possibilities, we established explants from the vagal neural crest (the region of the first 6–7 somites posterior to the otic placode in E10 rat embryos) and exposed them to GGF. Following 12 days of culture in control medium, the vagal crest explants contained large numbers of neurons expressing peripherin; by contrast, no neurons were observed in explants grown in GGF/neuregulin (data not shown). These data indicate that vagal neural crest cells, like their counterparts in the trunk, are sensitive to the influence of GGF/neuregulin and support the idea that the lack of GGF responsiveness in RET$^+$ postmigratory neural crest cells represents a developmental change rather than a positional difference.

Discussion

It is well-accepted that many vertebrate neural progenitor cells are multipotent, able to generate both neurons and glia in both the CNS and PNS. It remains a matter of controversy, however, as to whether these multipotent stem cells directly generate postmitotic neurons as the immediate daughters of asymmetric cell divisions, or whether such stem cells first produce lineage-restricted neuronal progenitor cells that then undergo a limited number of symmetric divisions prior to mitotic arrest and neuronal differentiation. In vivo lineage-tracing experiments (Fraser et al., 1990; Grove et al., 1992; Luskin et al., 1993; Birgbauer and Fraser, 1994) as well as some in vitro clonal analyses (Vescovi et al., 1993; Davis and Temple, 1994) have provided evidence for CNS progenitors that divide to generate clones containing phenotypically similar cells, e.g., neurons only. While such data are suggestive of the existence of committed neuronal progenitor cells, they do not exclude the possibility that the apparent restriction in cell fate is a property of the progenitor cell's local environment rather than of its intrinsic developmental capacities.

Here we have used newly generated monoclonal antibodies to the orphan receptor tyrosine kinase RET to isolate a population of postmigratory neural crest cells from the fetal rat gut. Two lines of evidence suggest that this population is enriched for a cell (which we call a NP) that is committed to a neuronal fate. First, NPs divide and differentiate relatively synchronously into neurons, while other

Table 3. Effect of GGF and Fibronectin on RET+ Progenitors in Clonal Culture

	rhGGFII			Control[a]			Fibronectin Only[b]		
	NP	ProNP	NNP	NP	ProNP	NNP	NP	ProNP	NNP
Ex.1/p1	9	3	14	9	3	8	4	2	16
Ex.1/p2	11	0	12	10	3	12	3	2	16
Ex.1/p3	7	3	14	4	1	11	4	0	21
Ex.2/p1	12	2	19	5	0	20	2	0	18
Ex.2/p2	6	1	22	6	3	12	2	0	14
Ex.2/p3	4	1	16	11	2	12	6	0	23
Total	49	10	97	45	12	75	21	4	108
Percentage of all clones	31%	6%	62%	34%	9%	57%	16%	3%	81%

RET+ enteric cells were plated at clonal density and then cultured for 12 days in the presence or absence of rhGGFII (lot #92893) at a concentration of 89 ng/ml (~1.5 nM). This dose is 5 times that required to achieve half-maximal inhibition of neuronal differentiation in NCSC clonal cultures (Shah et al., 1994); similar results were obtained in other experiments (data not shown) using twice this concentration. At the end of the incubation, the proportions of NP, proNP, and NNP clones were determined retrospectively as in Table 2. The results are derived from two independent experiments in which cultures were analyzed in triplicate (e.g., "Ex.1/p1" indicates experiment 1, plate 1, etc.). Note that the average percentage of each progenitor cell type is virtually identical with or without rhGGFII. Similar results were obtained with a second independent lot of rhGGFII (data not shown). Note that the results with fibronectin derive from two independent experiments (Ex.1 and Ex.2). Positive control experiments indicated that these batches of fibronectin produced effective inhibition of neuronal differentiation in clonal cultures of NCSCs.

[a] Cultures were grown on a standard fibronectin/poly-D-lysine substrate in the absence of rhGGFII.

[b] Plates were coated with fibronectin only, rather than with fibronectin plus poly-D-lysine.

cells in the same culture dish generate clones of different composition (i.e., neurons and nonneuronal cells, or nonneuronal cells only). Second, and more importantly, when the cells are challenged by exposure to environmental signals (GGF and fibronectin) shown to suppress neuronal and promote glial differentiation by early migrating cells from both trunk and vagal neural crest, NPs nevertheless generate neurons. Together, these data provide direct evidence that neural crest cells exhibit temporal restrictions in their developmental capacities, consistent with neuropoietic models of neural crest cell lineage diversification (Anderson, 1989; Sieber-Blum, 1990; Le Douarin et al., 1991), in which self-renewing stem cells give rise to their differentiated derivatives via the generation of lineage-restricted progenitors.

Identification of a Multipotent Neural Progenitor in the Developing Gut

RET+ neural crest cells isolated from E14.5 gut contained four distinct but apparently related cell types: postmitotic, process-bearing neurons; multipotent progenitors of neurons and nonneuronal (glial) cells (proNPs); nonneuronal progenitor cells (NNPs); and committed neuronal progenitors (NPs). The simplest interpretation of these data is that the four cell types represent distinct stages in a common lineage that are present contemporaneously in the developing gut (Figure 9). Although this is not formally proven, it is well established that the differentiation of the enteric nervous system is asynchronous (Pham et al., 1991), so that at E14.5 both differentiated neurons and undifferentiated progenitors should coexist in the gut.

The identification of proNPs in the E14.5 gut provides direct evidence that multipotent progenitors of neurons and glia persist in the mammalian gut long after neural crest migration has ended, consistent with recent results in the avian system (Deville et al., 1994). Previous studies have reported the development of neurons and glia from populations of crest-derived cells immunoselected from fetal rat gut using other antibody markers, but no clonal analysis was performed to determine whether neurons and glia arose from separate or common progenitors (Pomeranz et al., 1993; Chalazonitis et al., 1994). It will be interesting to determine whether multipotent neural progenitors in the gut persist into adulthood, as has been demonstrated for their counterparts in the CNS (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993).

While the developmental potential of proNPs from E14.5 gut is similar to that of NCSCs isolated from E10.5 neural tube explants, several lines of evidence suggest that these two multipotent progenitor cell types are functionally and antigenically distinct. First, proNPs were isolated on the basis of RET expression, and NCSCs do not express RET immunoreactivity (see Figure 4). Second, at least some proNPs may express MASH1 (see below). NCSCs, by contrast, are MASH1− (Shah et al., 1994). Third, neuronal differentiation in NCSC clones is repressed by GGF and fibronectin, whereas in proNPs it is apparently insensitive to these environmental influences. Finally, NCSCs appear to undergo at least 6–10 rounds of symmetric, self-renewing division before the emergence of distinct neuronal and glial lineages (Stemple and Anderson, 1992). By contrast, proNPs generate progeny that differentiate to neurons after only a few divisions (see Figure 4). Together, these data suggest that the properties of proNPs are distinct from those of NCSCs. The fact that vagal neural crest cells respond to GGF/neuregulin as do their trunk counterparts, moreover, argues that these distinct properties reflect differences in developmental stage rather than in position of origin along the neuraxis.

Isolation of a Committed Neuronal Progenitor Cell Derived from the Neural Crest

The use of anti-RET antibodies allowed enrichment for and identification of an apparently committed neuronal

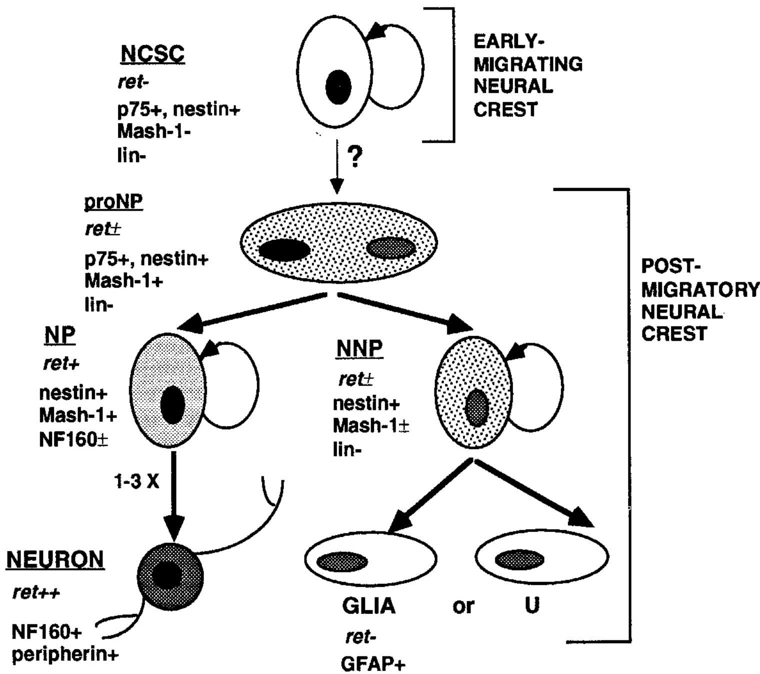

Figure 9. Model Representing the Putative Lineage Relationships between the Progenitor Cell Types Identified in This and in Earlier Studies

The antigenic phenotype of each progenitor cell type is indicated. The proposed lineage relationships between these cells have not been demonstrated directly; in particular, it has not yet been shown that NCSCs can generate RET$^+$ proNPs or NPs in vitro. The two differently shaded ovals in the proNP indicate the daughter nuclei of an asymmetrically dividing cell and are speculative. The progressively darker stippling in the cell bodies of proNPs, NPs, and neurons indicates that the expression of RET is progressively higher in these three cell types. U, unidentified nonneuronal cell(s).

progenitor cell (called NP) in the enteric precursor population. For comparison, such NPs were 8–9 times more enriched in the RET$^+$ population than in a population isolated using another surface marker of neural crest cells, p75^{LNGFR}. The fact that NPs can be recovered at all using anti-p75 antibodies makes it highly unlikely that the anti-RET antibodies induced neuronal committment, e.g., by mimicking ligand activation of the receptor. Furthermore, if this were the case, one might have expected 100% of the RET$^+$ cells to behave as NPs; in fact, many (50%–60%) did not.

The existence of committed neuronal progenitors in the CNS has been suggested previously, based on studies of cortical (Davis and Temple, 1994) or striatal (Vescovi et al., 1993) neuroepithelial cells grown in clonal cultures. However, in those cases the cells were not "challenged" by exposure to environmental factors known to suppress neuronal differentiation by multipotent stem cells. Here we have shown that NPs appear insensitive to GGF and fibronectin, environmental factors that suppress neuronal and promote glial differentiation by trunk NCSCs (Stemple and Anderson, 1992; Shah et al., 1994). This strongly suggests that NPs are committed to a neuronal fate, although whether these neurons are committed to an enteric or autonomic lineage is presently unclear.

A number of earlier studies have been interpreted to suggest that neural crest cells undergo progressive restriction in their developmental capacities (reviewed in Anderson, 1993). However, in cases where neural crest cell populations were challenged by exposure to a different environment (e.g., by in vivo transplantation [Le Lievre et al., 1980] or explantation in vitro [Artinger and Bronner-Fraser, 1992]), analysis was not performed at the single-cell level. Conversely, in cases where postmigratory neural crest cells were analyzed in clonal cultures (Duff et al., 1991; Deville et al., 1992, 1994) or by retroviral marking (Hall and Landis, 1991), the cells were not challenged by exposure to different environmental signals. In the present study, we have challenged postmigratory neural crest cells in clonal cultures with environmental signals previously shown to control the fate of multipotent cells. The identification of committed neuronal progenitors using RET as a marker now opens the way to reconstituting the committment process in vitro, beginning with uncommitted NCSCs.

Symmetrically and Asymmetrically Dividing Progenitors

Like NCSCs, proNPs are multipotent; unlike NCSCs, however, their progeny rapidly segregate into neurogenic and nonneurogenic lineages. This implies that either division or differentiation of these cells must be asymmetric. Such asymmetry may not be intrinsic to the cell division itself, but rather may be conferred by differences in the local microenvironment encountered by 2 otherwise equivalent daughter cells. Alternatively, the division could be intrinsically asymmetric and generate 2 nonequivalent daughter cells, such as has been demonstrated for the first division of the sensory mother cell in the Drosophila PNS (Posakony, 1994). It will be interesting to see whether proNPs express vertebrate homologs of *numb* (W. M. Zhong and Y. N. Jan, personal communication), a Drosophila gene required for the asymmetric division of the sensory mother cell (Uemura et al., 1989) whose protein product is asymmetrically distributed prior to cytokinesis (Rhyu et al., 1994).

NP clones contain only neurons. In principle, such clones could be produced either by asymmetric divisions

of a stem cell that generated a postmitotic neuroblast and another stem cell at each division until the stem cell was consumed or died, or rather by symmetric divisions of a committed neuroblast. A log plot of NP clone size as a function of time yields a straight line with a slope of 2 (data not shown). This indicates that NP clones expand by symmetric rather than asymmetric (stem cell–like) divisions, analagous to erythroblasts (for example) in the hematopoietic lineage (Briegel et al., 1993). Symmetrically dividing progenitors have also been identified in the CNS oligodendrocyte lineage (Temple and Raff, 1986). Whether here, as in that system, an intrinsic limitation on the number of cell divisions represents a mechanism for controlling the timing of differentiation remains to be determined.

Functions of RET and MASH1 during Neural Crest Development

Both *Ret* and *Mash1* are regulatory genes essential for the development of subsets of autonomic neurons, as shown by targeted gene disruption experiments in mice (Guillemot et al., 1993; Schuchardt et al., 1994). In addition, both genes are initially expressed in otherwise morphologically and antigenically undifferentiated neural crest cells (Lo et al., 1991; Guillemot and Joyner, 1993; Pachnis et al., 1993). While *Ret* is genetically essential for the development of all enteric neurons, the precise developmental operation it controls is not yet established. Our data indicate that some RET$^+$ cells (proNPs and NNPs) are not yet committed to a neuronal fate. This leaves open the possibility that RET signaling could trigger the commitment of multipotent neural crest cells to a neuronal fate, analagous to the role of the sevenless protein in Drosophila photoreceptor cell fate determination (for review, see Rubin, 1991). By contrast, if RET were expressed only by neurons or by NPs, a function in neuronal lineage committment could be excluded.

Similarly, the fact that the majority of RET$^+$ cells express MASH1 suggests that at least some of these MASH1$^+$ cells are multipotent as well. In this case, however, the argument is indirect (statistical) because MASH1 is a nuclear protein and its expression cannot be assessed without fixing and killing the cells. Nevertheless, since almost 90% of RET$^+$ cells are MASH1$^+$, and since close to 70% of RET$^+$ cells are either NNPs or proNPs (Table 2), it is apparent that MASH1 is expressed by some cells that are not yet committed to a neuronal fate. As in the case of RET, this would allow a potential function for MASH1 in the commitment of cells to a neurogenic lineage. However, recent data using cell lines derived from *Mash1* mutant mice suggest that MASH1 function is required only after cells are committed to a neuronal fate (L. Sommer, N. Shah, M. Rao, and D. J. A., unpublished data), although the present data suggest that the protein is expressed before such commitment occurs.

The fact that *Ret* and *Mash1* are expressed sequentially (Guillemot et al., 1993; Lo et al., 1994) in the same cells and that both are required for the differentiation of at least a subpopulation of peripheral autonomic neurons raises the possibility that there is an interaction between these two genes. For example, signaling through RET could lead to the expression of MASH1; conversely, MASH1 could be required for the maintenance or up-regulation of RET expression. However, though *Ret* is required for the differentiation of all enteric neurons (Schuchardt et al., 1994), it is not essential for the initial differentiation of sympathetic neurons (V. Pachnis, personal communication). Conversely, *Mash1* is required for sympathetic neuron differentiation (Guillemot et al., 1993) but not for the differentiation of some enteric neurons. These data suggest that *Mash1* expression does not require *Ret* function in sympathetic neurons, and that *Ret* function does not require *Mash1* expression in late-generated enteric neurons. Nevertheless, recent evidence indicates that early-generated enteric neurons, including the serotonergic subset, require *Mash1* function (Blaugrund et al., submitted) as well as *Ret* function (Schuchardt et al., 1994). This leaves open the possibility that there is a genetic interaction between *Ret* and *Mash1* within this enteric sublineage. The ability to isolate RET$^+$ neural crest cells from embryos of various genotypes should permit a more detailed analysis of the functions and interactions of *Ret; Mash1*, and other regulatory genes involved in neural crest development, as well as of the mechanistic basis of developmental restriction within this population.

Experimental Procedures

Construction of Lipid-Linked form of c-RET

Molecular cloning manipulations were performed using standard methods. PI-anchored RET was constructed by methods similar to those used for the expression of a lipid-linked form of the T cell antigen receptor (Devaux et al., 1991). In brief, a DNA segment encoding the murine RET extracellular domain was ligated to a DNA fragment encoding the HPAP-PI anchoring signal (Affimax) and cloned into the expression vector pBJ5 GS. PCR primer sequences used in these manipulations are available on request. pBJ5 GS contains the glutamine synthetase (GS) gene as a selectable marker and provides a means of gene amplification in the presence of the drug methionine sulfoximine (MSX), a system developed by Celltech, Inc. Amplification in CHO cells was accomplished using 25 μM MSX followed by 100 μM MSX after cloning.

Immunization Procedures and Antibody Screening

Armenian hamsters (Cytogen Research & Development) were immunized with 5×10^6 CHO cells per injection and a total of four injections. Three days after the boost, the hamster was sacrificed, and its spleen cells were fused with P3X63Ag8u.I mouse myeloma cells. Hybridoma supernatants were screened on a subline of murine NIH 3T3 cells stably expressing a high level of the c-RET–PI protein. Positive clones were further tested on transiently transfected 293T cells expressing a cDNA encoding intact RET. Out of five subclones, three clones were able to stain both mouse neuroblastoma Neuro-2a and rat MAH cells (Birren and Anderson, 1990).

Isolation and Culture of Primary Rat Enteric Precursor Cells

The fetal gut (including stomach, midgut, and hindgut) was dissected from embryonic albino rats (Simonsen Laboratories) at E14.5 and dissociated using 1.5 mg/ml collagenase (Worthington), 1.0 mg/ml elastase (Sigma), and 50 μg/ml DNase I (Sigma). The cells were incubated with a cocktail of three different hamster anti-RET hybridoma supernatants (3A61D7, 3A61C6, and 2C42H1) plus 50 μg/ml DNase I for 30 min at room temperature, followed by a 1:200 dilution of phycoerythrin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). RET$^+$ cells were isolated on an Epics Elite Fluorescence Acti-

vated-Cell Sorter (Coulter) using a multiparametric gate based on fluorescence intensity, size, density, and granularity.

The cells were collected into a single round-bottomed well of a 96-well plate. Viable cells were plated at 300 cells per 35 mm dish that had been treated with poly-D-lysine (Biomedical Technologies) and fibronectin (New York Blood Center). Cells were grown in complete NCSC medium containing (among other additives) insulin, epidermal growth factors, basic fibroblast growth factor, NGF, and 10% chick embryo extract as described previously (Stemple and Anderson, 1992). After 15 hr in culture, each individual flat (non-process-bearing) cell was identified by morphology and inscribed with a circle on the bottom of the tissue culture plate. Cells that underwent division during the first 15 hr were rejected from the analysis. Clones were observed and photographed every 24 hr for the first 4 days and scored for the presence of process-bearing neurons. For some experiments, the cultures were carried for 12–14 days, and the medium was further supplemented with rhGGFII (Marchionni et al., 1993) or 10% fetal bovine serum plus 5 μm forskolin (Sigma) to promote Schwann cell differentiation. GGF was added at the time of plating, and fetal bovine serum plus forskolin was added 4 days after plating. Similar results were obtained using either rhGGFII or a partially purified preparation of native bovine GGF from pituitary extracts.

Immunocytochemistry

For internal staining of RET protein, cells were fixed with freshly prepared 4% paraformaldehyde and permeablized using 0.1% Nonidet P-40. Cells were incubated with anti-RET hybridoma supernatants for 18 hr at 4°C, followed by a 2 hr incubation at room temperature with RG 7/7, a mouse monoclonal anti-rat K chain 1B that is cross-reactive with Syrian and Armenian but not Chinese hamster K chain, followed by a goat anti-mouse tertiary antibody. Staining was visualized using a Vectastain ABC Kit (Vector Labs) with horseradish peroxidase development using diaminobenzidine as substrate. Immunocytochemical staining for MASH1, p75, nestin, and neurofilament was carried out as described previously (Stemple and Anderson, 1992; Shah et al., 1994).

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