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Prospective Identification, Isolation by Flow Cytometry, and In Vivo Self-Renewal of Multipotent Mammalian Neural Crest Stem Cells

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Summary

Multipotent and self-renewing neural stem cells have been isolated in culture, but equivalent cells have not yet been prospectively identified in neural tissue. Using cell surface markers and flow cytometry, we have isolated neural crest stem cells (NCSCs) from mammalian fetal peripheral nerve. These cells are phenotypically and functionally indistinguishable from NCSCs previously isolated by culturing embryonic neural tube explants. Moreover, in vivo BrdU labeling indicates that these stem cells self-renew in vivo. NCSCs freshly isolated from nerve tissue can be directly transplanted in vivo, where they generate both neurons and glia. These data indicate that neural stem cells persist in peripheral nerve into late gestation by undergoing self-renewal. Such persistence may explain the origins of some PNS tumors in humans.

Introduction

Stem cells are self-renewing multipotent progenitors with the broadest developmental potential in a given tissue at a given time (Morrison et al., 1997a). Neural stem cells have aroused a great deal of interest (reviewed by Cameron and McKay, 1998; Gage, 1998; Temple and Alvarez-Buylla, 1999) not only because of their developmental importance but also for their therapeutic potential (Gage et al., 1995). A major limitation in the study of neural stem cells has been the inability to identify them prospectively in vivo. This is because there have been no markers to isolate the stem cells or to distinguish them from restricted progenitors in vivo. Thus, multipotent, self-renewing neural stem cells have all been isolated after a period of growth in culture that could change their properties (Stemple and Anderson, 1992; Kilpatrick and Bartlett, 1993; Davis and Temple, 1994; Gritti et al., 1996; Johe et al., 1996; Kalyani et al., 1997; Palmer et al., 1997). It is therefore not yet clear whether such cells derive from cells with similar properties in vivo.

We have used the neural crest as a model system to study the biology of mammalian neural stem cells. Neural crest cells delaminate from the dorsal neural tube and migrate extensively before aggregating to form the ganglia and neuroendocrine tissues of the PNS, as well as mesectodermal tissues such as smooth muscle and bone (Le Douarin, 1980, 1982). In avian embryos, clonal marking experiments in vivo (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991), as well as clonogenic assays in vitro (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988), have shown that many individual neural crest cells are multipotent. Previously, we described the isolation of a multipotent progenitor from the mammalian neural crest (Stemple and Anderson, 1992). These cells have the capacity to self-renew and to generate neurons, glia, and smooth muscle (Shah et al., 1996) and have therefore been termed neural crest stem cells (NCSCs). As these cells were isolated from neural tube explant cultures, however, there is no evidence that multipotent NCSCs actually self-renew in vivo. To the contrary, the proliferation of neural crest cells has been associated with progressive restrictions in developmental potential (reviewed by Anderson, 1993).

We sought to prospectively identify and isolate post-migratory neural crest cells in order to determine whether they are multipotent and self-renewing in vivo as well as in vitro. To do this, we searched for such cells in fetal peripheral nerve, which is thought to contain only Schwann (glial) cell precursors (Jessen et al., 1994). Using antibodies against the cell surface antigens p75, the low-affinity neurotrophin receptor, and P_0, a peripheral myelin protein (Lee et al., 1997), we have fractionated the E14.5 sciatic nerve by flow cytometry into five distinct subpopulations. Surprisingly, the $p75^+P_0^-$ subfraction was highly enriched in cells that were functionally indistinguishable from NCSCs in vitro. Freshly isolated $p75^+P_0^-$ cells also generated neurons and glia after direct transplantation into chick embryos. Finally, in vivo cell cycle analysis and BrdU incorporation experiments indicated that the $p75^+P_0^-$ cells undergo self-renewing divisions in the sciatic nerve. Taken together, these data indicate that multipotent neural crest cells self-renew in vivo and persist into late gestation at least a week after the onset of neural crest migration. The persistence of NCSCs is of potential therapeutic importance and may explain the origin of some PNS tumors in humans.

Results

The Fetal Sciatic Nerve Contains Multipotent and Committed Neural Progenitors

To examine their cellular composition, E14.5–E17.5 rat sciatic nerves were dissociated and cultured at clonal density (see Experimental Procedures). After 14 days, the cultures were fixed and analyzed with immunocytochemical markers. We identified three cell types in such cultures: neurons, Schwann cells, and smooth muscle–like myofibroblasts. Neurons were typically identified by expression of peripherin, a marker of mature PNS neurons (Parysek and Goldman, 1988) (Figure 1B), but also expressed neuron-specific tubulin (TuJ1) and neurofilament 160 (data not shown). Schwann (glial) cells were typically identified by expression of glial fibrillary acidic protein (GFAP) (Figure 1D) but also expressed p75 and cytoplasmic S100β. Although S100β has been

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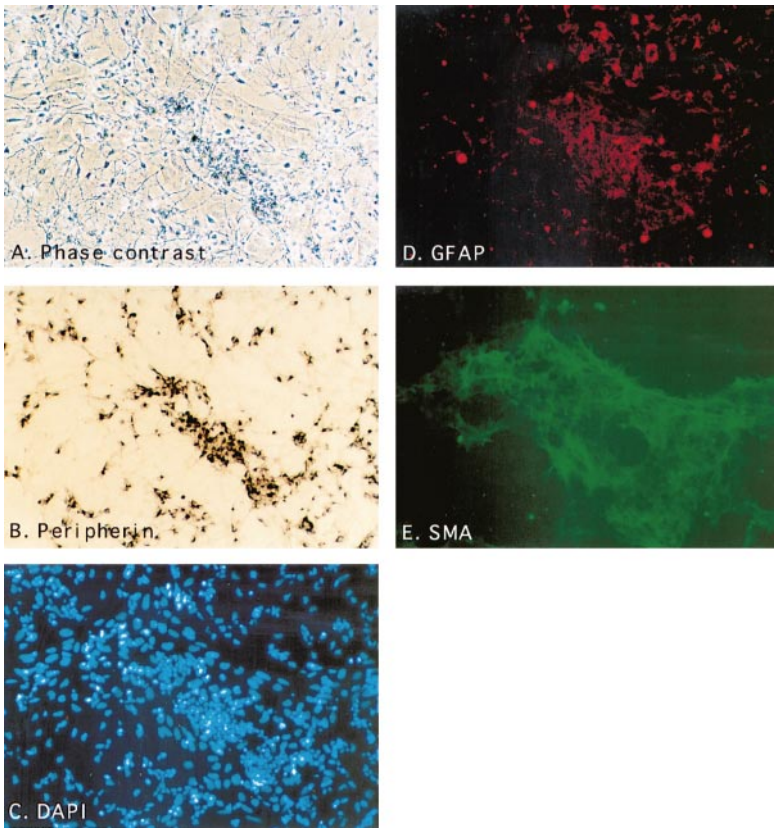

Figure 1. A Multipotent Colony after Culture in Standard Medium

Dissociated E14.5 rat sciatic nerve cells were plated at clonal density and cultured under standard conditions for 14 days and then fixed and immunohistochemically stained. The same field from a typical multipotent clone is shown in each panel, including phase contrast (A), bright field showing peripherin staining (B), DAPI fluorescence showing nuclei (C), glial fibrillary acidic protein (GFAP) (D), and smooth muscle actin (SMA) (E). Peripherin staining indicates neurons, GFAP staining indicates Schwann cells (glia), and SMA staining indicates myofibroblasts.

used as a Schwann cell marker in previous studies (Jessen and Mirsky, 1992), we found it was not glial specific in these cultures, consistent with earlier studies (Haimoto et al., 1987). Other cells coexpressed alpha–smooth muscle actin (SMA; Figure 1E), calponin, and S100β (not shown). Although similar NCSC-derived cells were previously referred to as smooth muscle cells (Shah et al., 1996), the sciatic nerve–derived cells did not express the smooth muscle markers desmin or myosin light chain kinase (data not shown), and we therefore refer to them as myofibroblasts (Sappino et al., 1990). Myofibroblasts did not express peripherin, GFAP, or p75.

By triple labeling with antibodies to peripherin, GFAP, and SMA, we identified five types of colonies in clonal cultures of sciatic nerves from different ages (Table 1). A substantial number of colonies contained neurons, Schwann cells, and myofibroblasts (N+S+M; Figure 1). These were the largest colonies observed, containing on average $1.07 \pm 0.33 \times 10^5$ cells (mean ± SD) after 14 days of culture. They represented almost 16% of colonies at E14.5, but their frequency declined significantly with each day of development, down to less than 2% of progenitors from the E17.5 sciatic nerve (Table 1). In a minority of experiments, we observed infrequent but very large colonies that contained only neurons and Schwann cells (N+S). More frequent, irrespective of developmental stage, were intermediate-sized (thousands of cells) colonies that contained Schwann cells and myofibroblasts (S+M) but no neurons (Table 1).

In addition to such uncommitted progenitors, the sciatic nerve contained progenitors that gave rise to only

Table 1. The Frequencies of Different Progenitor Types from Dissociated E14.5–E17.5 Sciatic Nerve Preparations Based on the Types of Colonies that Form in Clonal Culture

Sciatic Nerve	Plating Efficiency	Frequency of Colony Types (% ± SD)				
		N+S+M	N+S	S+M	S Only	M Only
E14.5	63.7 ± 16.6	15.8 ± 7.0 a	0.4 ± 1.0	10.9 ± 11.9	19.2 ± 10.2 a	53.6 ± 20.2
E15.5	51.6 ± 13.8	6.8 ± 3.1 b	2.4 ± 3.3	6.8 ± 5.1	31.9 ± 14.0 a,b	52.1 ± 22.5
E16.5	52.0 ± 18.1	0.7 ± 1.4 c	0.0 ± 0.0	9.0 ± 4.1	36.6 ± 4.9 b	53.7 ± 5.6
E17.5	52.2 ± 21.5	1.7 ± 2.0 c	0.5 ± 1.0	9.8 ± 6.5	42.1 ± 14.3 b	45.8 ± 12.4

N, S, and M indicate the presence of neurons, Schwann cells, and myofibroblasts, respectively, in colonies. For example, N+S+M colonies contain neurons, Schwann cells, and myofibroblasts. Plating efficiency expresses the percentage of cells added to culture that went on to form colonies analyzed after 2 weeks of culture. Statistics within columns of colony-type data were compared by analyses of variance followed by post hoc t tests. Columns containing significantly different statistics ($p < 0.05$ by anova) include letters to designate the pairwise differences. Significantly different statistics are not followed by the same letter (e.g., a is different from b but not from a,b).

Table 2. Subcloning of Multipotent Colonies from E14.5 Sciatic Nerve after 7 or 11 Days in Culture

Day of Subcloning	Average Number of Subclones per Founder Colony				
	N+S+M	N+S	S+M	S Only	M Only
7	131 ± 57	0.6 ± 1	12 ± 13	10 ± 7	2 ± 2
11	133 ± 165	14 ± 14	100 ± 45	219 ± 138	56 ± 58

N, S, and M indicate the presence of neurons, Schwann cells, and myofibroblasts, respectively, in subcloned colonies. Ten colonies were subcloned at day 7. All colonies yielded N+S+M, S+M, and S-only subclones. Three of ten colonies also yielded N+S subclones, and five of ten colonies yielded M-only subclones. Ten colonies were subcloned at day 11. Seven of ten colonies gave rise to at least ten subclones of each type. One colony gave rise to subclones of all types except N+S. Finally, two colonies gave rise to subclones containing only S and/or M cells and may have been misidentified as multipotent progenitors.

a single cell type. As expected, a substantial number of colonies contained only Schwann cells (S only). The frequency of such S-only progenitors increased significantly with development, from 20% of colonies at E14.5 to 42% of all colonies at E17.5 (Table 1). In standard culture conditions, these colonies typically contained hundreds to thousands of cells. At all stages of development, around 50% of colonies contained only myofibroblasts (M only). These colonies ranged from less than 10 to more than 100 cells.

Sciatic Nerve Multipotent Progenitors Self-Renew in Culture

The colonies formed by the multipotent progenitors from the sciatic nerve were reminiscent of those formed by migrating NCSCs (Stemple and Anderson, 1992; Shah et al., 1994, 1996). We thus tested their self-renewal potential in subcloning experiments (Table 2). Single $p75^+$ cells dissociated from E14.5 sciatic nerves were cultured under standard conditions for 7 or 11 days. Multipotent colonies were distinguished by their appearance (see Experimental Procedures), subcloned in secondary cultures, and grown for an additional 14 days. In 18 out of 20 cases, each multipotent colony gave rise to many multipotent (N+S+M) subclones as well as to S+M subclones and S-only subclones (Table 2). In most cases, multipotent colonies also gave rise to M-only and N+S subclones as well. On average, each multipotent founder gave rise to more than 100 multipotent secondary clones irrespective of the day of cloning. Similar results were also obtained from E16.5 sciatic nerves (data not shown). Thus, the multipotent progenitors not only self-renewed in culture but also gave rise to all other classes of progenitors that were observed in freshly dissociated fetal sciatic nerve, including the M-only myofibroblast progenitors (Table 2).

Separation of Functionally Distinct Sciatic Nerve Progenitors by Flow Cytometry

In order to determine whether the self-renewing multipotent progenitors were functionally equivalent to NCSCs, we wanted to challenge them with factors that instruct NCSCs to differentiate into neurons or glia. To facilitate this analysis, we sought to separate the different classes

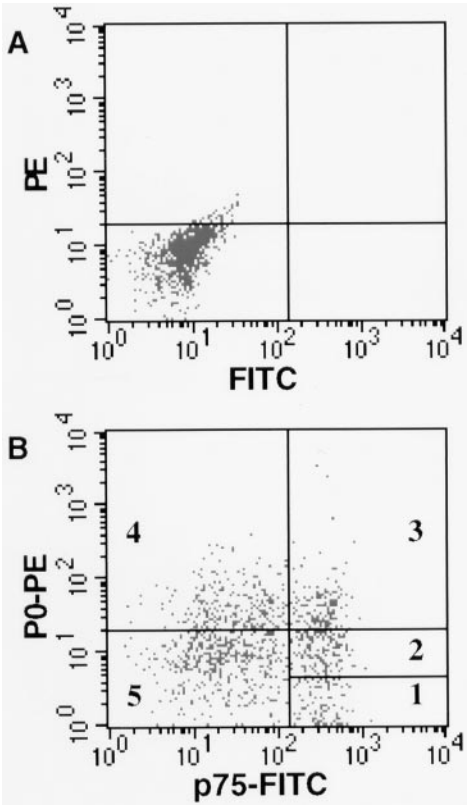

Figure 2. Fluorescence-Activated Cell Sorting (FACS) Profiles of E14.5 Rat Sciatic Nerve Cells

Sciatic nerves were dissociated by treating with trypsin and collagenase. Cells are either unstained (A) or stained with antibodies against p75 and P_0 (B). Sciatic nerve cells were divided into five populations based on differences in p75 and P_0 staining as shown in (B): 1, $p75^+P_0^-$; 2, $p75^+P_0^{-/low}$; 3, $p75^+P_0^+$; 4, $p75^{-/low}P_0^+$; 5, $p75^{-/low}P_0^{-/low}$. At the concentrations used, none of the antibodies exhibited nonspecific staining when tested by FACS on telencephalon or fetal liver cells (data not shown).

of sciatic nerve progenitors by flow cytometry, using p75 and P_0 as surface markers. Figure 2 shows fluorescence-activated cell sorting (FACS) plots of dissociated E14.5 sciatic nerve cells either unstained or stained with p75 and P_0. We divided such cells into five subsets (Figure 2B): (1) $p75^+P_0^-$ cells (12% ± 2%); (2) $p75^+P_0^{-/low}$ cells (18% ± 5%); (3) $p75^+P_0^+$ cells (11% ± 7%); (4) $p75^{-/low}P_0^+$ cells (20% ± 9%); and (5) $p75^{-/low}P_0^{-/low}$ cells (39% ± 10%).

The five phenotypically defined populations showed striking differences in developmental potential, as shown in Table 3. Most (60%) $p75^+P_0^-$ cells formed multipotent colonies, with smaller numbers of cells giving rise to the other classes of colonies. $p75^+P_0^{-/low}$ cells gave rise to a mixture of multipotent colonies (50%) and Schwann-only colonies (37%). Both of these fractions gave rise to a low percentage of M-only colonies (<10%). The $p75^+P_0^+$ population contained a mixture of multipotent progenitors (28%), Schwann-only progenitors (34%), and myofibroblast-only progenitors (22%). Thus, all $p75^+$ populations, including those expressing P_0, contained substantial numbers of multipotent progenitors, but the

Table 3. Developmental Potentials of Phenotypically Distinct Populations from the E14.5 Sciatic Nerve in Clonal Culture

Population	Plating Efficiency	Frequency of Colony Types (% ± SD)				
		N+S+M	N+S	S+M	S Only	M Only
$p75^{+}P_0^{-}$	24.9 ± 8.8	60.4 ± 3.5 a	4.8 ± 8.2	11.6 ± 16.1	18.4 ± 7.4 a	4.8 ± 8.3 a
$p75^{+}P_0^{-/low}$	36.8 ± 6.3	50.4 ± 11.9 a	0.0	5.7 ± 6.9	37.1 ± 12.8 b	6.8 ± 8.8 a
$p75^{+}P_0^{+}$	48.2 ± 16.4	27.8 ± 12.8 b	4.5 ± 6.5	11.7 ± 6.0	34.2 ± 15.8 a,b	21.8 ± 11.0 b
$p75^{-/low}\ P_0^{+}$	84.7 ± 17.5	0.0 c	0.0	0.4 ± 0.8	0.4 ± 0.8 c	99.2 ± 1.6 c
$p75^{-/low}\ P_0^{-}$	52.4 ± 9.9	0.0 c	0.0	0.0	0.0 c	100.0 ± 0.0 c

Statistics within columns of colony-type data were compared by analyses of variance followed by post hoc t tests. Columns containing significantly different statistics ($p < 0.001$ by anova) include letters to designate the pairwise differences. Significantly different statistics are not followed by the same letter (e.g., a is different from b but not from a,b).

apparent frequency of multipotent progenitors decreased as P_0 expression increased. Both of the $p75^{-/low}$ populations (P_0^{+} and $P_0^{-/low}$) were pure or nearly pure populations of progenitors that gave rise only to myofibroblasts.

Neuronal Potentials Determined by BMP2 Challenge

BMP2 instructs NCSCs to differentiate into neurons (Shah et al., 1996). BMP2 (1.6 nM) was therefore added to standard cultures of unseparated sciatic nerve cells or cells from each subpopulation isolated by flow cytometry. After 24 hr with BMP2, some cultures were fixed and stained for MASH1, an early transcription factor marker of autonomic neurogenesis (Sommer et al., 1995; Lo et al., 1997). After 4 days with BMP2, sister cultures were fixed and stained for peripherin. On average, 18%–20% of unseparated sciatic nerve cells were capable of neuronal differentiation, as judged by either MASH1 (Figures 3A–3C) or peripherin expression (Figures 3D and 3E; Table 4). This is consistent with our observation that 16% of unseparated sciatic nerve cells formed colonies that contained neurons in standard cultures (Table 1).

In the $p75^{+}P_0^{-}$ population, over 80% of the cells formed neuronal colonies in the presence of BMP2 (Table 4), a higher proportion than in standard cultures (60%). In the $p75^{+}P_0^{-/low}$ and $p75^{+}P_0^{+}$ populations, substantial but lower numbers of cells (68% and 52%, respectively) formed neuronal colonies under the influence of BMP2, consistent with the presence of multipotent progenitors (Table 3). By contrast, in both of the $p75^{-/low}$ populations (P_0^{+} and $P_0^{-/low}$), few cells

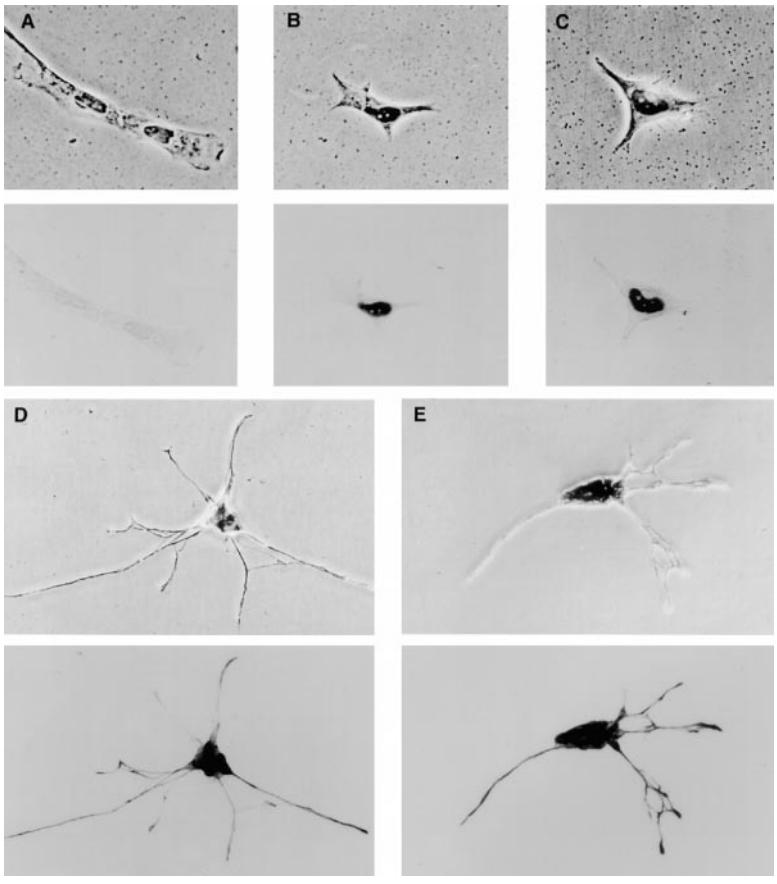

Figure 3. MASH1 and Peripherin Stained Cells in Culture after BMP2 Challenge

E14.5 rat sciatic nerve cells were dissociated and plated under standard culture conditions with BMP2 added. For each pair of panels, the phase contrast view is shown on top, and the bright field view revealing antibody staining developed by peroxidase precipitation of nickel-DAB product is shown on the bottom. (A–C) show cells after 24 hr in culture with BMP2. The cells in (A) did not stain for MASH1, whereas cells in (B) and (C) show typical nuclear staining for MASH1. (D) and (E) show cells after 4 days in culture with BMP2. All cells in both colonies stained for peripherin.

Table 4. Neuronal Potentials of Phenotypically Distinct Populations from the E14.5 Sciatic Nerve Challenged by BMP2 in Clonal Culture

	24 hr Challenge (% MASH1$^+$)		4 Day Challenge (% Peripherin$^+$)	
Population	No Add	+ BMP2	No Add	+ BMP2
Unseparated cells	0.0	19.6 ± 14.5 a	0.0	18.0 ± 13.6 a
$p75^+ P_0^-$	0.0	81.0 ± 11.5 b	0.0	82.2 ± 10.9 b
$p75^+ P_0^{-/low}$	0.0	68.0 ± 9.4 b	0.0	68.1 ± 18.8 b
$p75^+ P_0^+$	0.0	42.5 ± 20.7 c	0.0	52.9 ± 14.6 c
$p75^{-/low} P_0^+$	0.0	0.7 ± 1.2 d	0.0	0.9 ± 1.4 d
$p75^{-/low} P_0^-$	0.0	1.1 ± 2.1 d	0.0	3.8 ± 3.6 e

Cells from each population were sorted into culture with or without BMP2. After 24 hr, some cultures were fixed and stained for MASH1, a marker of neuronal differentiation, while other cultures were fixed at 4 days and stained for peripherin, a marker of mature neurons. BMP2 addition did not significantly affect the plating efficiency of any population at either time point. Statistics within columns were compared by analyses of variance followed by post hoc t tests. Columns containing significantly different statistics ($p << 0.001$ by anova) include letters to designate the pairwise differences. Significantly different statistics are not followed by the same letter (e.g., a is different from b or c).

were capable of neuronal differentiation even when challenged by BMP2 (Tables 3 and 4). Thus, most cells in these latter populations lack neuronal potential and may be restricted to myofibroblast fates.

BMP2 did not appear to either kill cells or promote the survival of subpopulations of cells, because in no case was there a difference in plating efficiency comparing side-by-side cultures with and without BMP2 (data not shown). Furthermore, BMP2 both accelerated (by 10 days) neuronal differentiation and dramatically increased the proportion of neurons in clones. These data suggested that it acted instructively on cells with neuronal potential. To confirm this, $p75^+P_0^-$ cells from the E14.5 sciatic nerve were sorted into clonal culture and marked by etching a circle on the underside of the culture plate, after which BMP2 was added to some cultures. Twenty-four hours after BMP2 addition, some cultures were fixed and stained for MASH1. In control cultures, an average of 88.9% of cells survived, and no cells expressed MASH1 (45 founder cells studied in two experiments). In BMP2-treated cultures, an average of 87.5% of cells survived, and 62.7% of those cells expressed MASH1 (40 founder cells studied in two experiments). Thus, BMP2 did not act selectively, but instructed multipotent progenitors to differentiate into the neuronal lineage, similar to its effect on NCSCs obtained from E10.5 neural tube explants (Shah et al., 1996).

Glial Potentials Determined by NRG1 Challenge

NRG1 instructs migrating NCSCs to differentiate into glia (Shah et al., 1994; Shah and Anderson, 1997). Cultures of each FACS-isolated population from E14.5 sciatic nerve were challenged by adding 1 nM NRG1 (in the soluble form also known as glial growth factor II [Marchionni et al., 1993]). After 14 days, the cultures were fixed and stained for peripherin, GFAP, and SMA. In contrast to the neuronal differentiation seen in cultures of $p75^+P_0^-$ cells under standard conditions (Table 3), in the presence of NRG1 no neuron-containing colonies were observed (Table 5; N+S+M), and 95% of colonies contained only Schwann cells. Furthermore, the frequency of colonies containing only Schwann cells was dramatically increased (compare S-only values in Tables 1, 3, and 5—note that the data in these tables were obtained in side-by-side cultures in the same experiments). Plating efficiencies were also significantly higher for $p75^+$ populations in the presence of NRG1 (Tables 3 and 5). Thus, NRG1 also acted as a survival factor for neural progenitors, as previously reported (Dong et al., 1995). Neither the plating efficiency nor the differentiation of $p75^{-/low}$ progenitors were affected by NRG1 challenge. All colonies derived from $p75^{-/low}$ progenitors contained only myofibroblasts, even in the presence of NRG1 (Table 5). Thus, these myofibroblast progenitors appear to have neither glial nor neuronal potential. NRG1 did, however, promote the proliferation of $p75^{-/low}$ progenitors (data not shown).

To confirm that NRG1 also acted instructively to promote glial differentiation, we plated $p75^+P_0^-$ cells in the absence of NRG1, circled live individual cells, and then added NRG1. After 14 days in the absence of NRG1, 86% of the cells survived and formed colonies. Fifty-seven percent of these colonies were multipotent (N+S+M), and 37% were Schwann-only colonies (35 colonies observed). In the presence of NRG1, 96% of cells survived and formed colonies. No multipotent (N+S+M) colonies were observed, but 81% of colonies were Schwann only (76 colonies observed). Thus, NRG1 suppressed neurogenesis and promoted glial differentiation without killing multipotent progenitors, demonstrating that it acted instructively. The reduction in neuron-containing colonies in NRG1 cannot be explained by an increased survival of S-only progenitors, even though the increased plating efficiency suggests that it may also promote the survival of $p75^+$ cells.

The foregoing data suggested that the multipotent progenitors in the E14.5 sciatic nerve were phenotypically and functionally indistinguishable from migrating NCSCs isolated from E10.5 neural tube explants (Stemple and Anderson, 1992; Shah et al., 1996). The only other published functional characteristic of migrating NCSCs is that TGFβ instructs them to differentiate into SMA^+calponin$^+$ smooth muscle cells (Shah et al., 1996), which we refer to as myofibroblasts. NCSCs replated from neural tube explants and $p75^+P_0^-$ cells from the E14.5 sciatic nerve responded indistinguishably to TGFβ challenge under the standard culture conditions used in this study as well as in the conditions used in the original TGFβ studies (Shah et al., 1996) (data not shown). For reasons that are not clear, in the present experiments a higher proportion of cells failed to survive in TGFβ than was observed in the former study, but surviving cells were enriched for SMA^+ myofibroblasts,

Table 5. Glial Potentials of Phenotypically Distinct Populations from the E14.5 Sciatic Nerve as Determined by Challenge with NRG1 (Glial Growth Factor) in Clonal Culture

Population	Plating Efficiency	Frequency of Colony Types (% ± SD)			
		N+S+M	S+M	S Only	M Only
Unseparated	67.1 ± 0.8	3.3 ± 5.8	11.6 ± 5.7 a,d	38.5 ± 31.6 a	46.6 ± 31.6 a
$p75^+ P_0^-$	55.2 ± 3.8	0.0	5.0 ± 3.3 a	95.0 ± 3.3 b	0.0 b
$p75^+ P_0^{-/low}$	54.4 ± 10.7	0.7 ± 1.4	20.9 ± 21.0 a,b,c	79.1 ± 21.0 b,e	0.0 b
$p75^+ P_0^+$	64.8 ± 15.4	2.0 ± 3.1	12.0 ± 5.8 b,d	84.4 ± 7.3 c,e	1.6 ± 2.4 b
$p75^{-/low} P_0^+$	68.9 ± 17.5	0.0	0.0 c	0.0 d	100.0 ± 0.0 c
$p75^{-/low} P_0^-$	41.5 ± 20.4	0.0	0.0 c	0.0 d	100.0 ± 0.0 c

Cells from each population were sorted into cultures containing NRG1. After 2 weeks, the cultures were fixed and stained. No colonies containing only neurons and Schwann cells (N+S) were observed in these experiments. Statistics within columns of colony-type data were compared by analyses of variance followed by post hoc t tests. Columns containing significantly different statistics ($p < 0.05$ by anova) include letters to designate the pairwise differences. Significantly different statistics are not followed by the same letter (e.g., a is different from b but not from a,b).

consistent with an instructive role for TGFβ. We conclude that the multipotent progenitors observed in the fetal sciatic nerve are NCSCs.

$p75^+P_0^-$ NCSCs from the E14.5 Sciatic Nerve Give Rise to Neurons and Glia upon Transplantation In Vivo

We wanted to determine whether freshly isolated sciatic nerve $p75^+P_0^-$ cells could give rise to neurons in vivo to ensure that their neuronal potential was not acquired in vitro. To this end, we used a recently devised system for transplantation of rat neural crest cells into chick embryos (P. M. W. and D. J. A., submitted). $p75^+P_0^-$ cells from freshly dissociated sciatic nerves were isolated by FACS and injected into the ventral neural crest pathway at a developmental stage (St. 18) when host crest migration is well underway (Bronner-Fraser and Cohen, 1980). Three days after injection, the chick embryos were harvested, sectioned, and hybridized in situ with rat- and chick-specific probes against markers of neurons and glia.

E14.5 $p75^+P_0^-$ donor cells engrafted efficiently and gave rise to neurons and glia in diverse PNS locations. In two experiments, such cells were injected into a total of 22 chick embryos. We detected the engraftment of rat cells in 16 of 18 forelimb-injected chick embryos and 4 of 4 sacrally injected chick embryos, generating a total of 20 chimeras. Donor-derived neurons, identified by in situ hybridization with a rat-specific probe for the neuronal marker SCG10, were detected in the sympathetic ganglia of four chimeras (three forelimb and one sacral injection) (Figures 4A and 4E; arrows, purple stain) in close association with host neurons counterstained with a chick-specific SCG10 probe (orange stain). At least some rat cells that were found in sympathetic ganglia also expressed Phox2b (Figure 4B), a marker of autonomic differentiation appropriate to the sympathetic ganglion (Pattyn et al., 1997). Among chicks injected at sacral levels, rat neurons were always detected in Remak's ganglion (Figure 4D; arrow, purple stain), a component of the avian enteric nervous system.

Rat glia were found in all 20 chick embryos that were engrafted by rat cells. Cells expressing P_0 (Figure 4F) and the NRG1 receptor erbB3 (Riethmacher et al., 1997) (Figure 4C) were detected in the peripheral nerves of all chimeras, sometimes numbering into the hundreds. These cells did not express SCG10 in adjacent sections (data not shown). Thus, some rat cells differentiated appropriately in peripheral nerves by forming Schwann cells and not neurons. Taken together, the results demonstrate that sciatic nerve $p75^+P_0^-$ cells as a population can give rise to neurons and glia in vivo when transplanted directly after flow-cytometric isolation without any intervening period of growth in culture.

NCSCs Persist by Self-Renewing in the Sciatic Nerve

The persistence of NCSCs in the fetal sciatic nerve could reflect their survival in a mitotically quiescent state following immigration from the neural crest. Alternately, the cells could persist by undergoing self-renewing divisions. To distinguish these possibilities, we first examined the cell cycle status of $p75^+P_0^-$ cells from the E14.5 sciatic nerve. $p75^+P_0^-$ cells were isolated by FACS, stained with Hoechst 33342 and pyronin Y, and reanalyzed by FACS to determine their DNA and RNA contents. Using this approach, cells can be assigned to G_0, G_1, or $S/G_2/M$ phases of the cell cycle (Darzynkiewicz and Juan, 1997). Both unfractionated E14.5 sciatic nerve cells and $p75^+P_0^-$ cells appeared to be rapidly cycling populations with many cells in $S/G_2/M$ and few or no cells in G_0 (data not shown). About 10% of unfractionated sciatic nerve cells were in $S/G_2/M$, while about 15% of $p75^+P_0^-$ cells from the same nerves were in $S/G_2/M$.

To directly assay whether most NCSCs were self-renewing in vivo, pregnant rats were administered the thymidine analog bromodeoxyuridine (BrdU) for 18 hr prior to the harvest of fetal sciatic nerves at E14.5 (i.e., starting at E13.75; see Figure 5). Unfractionated sciatic nerve cells and $p75^+P_0^-$ cells isolated by FACS were plated, fixed, and stained for BrdU incorporation. Eighty percent of sciatic nerve cells and nearly 90% of $p75^+P_0^-$ cells incorporated BrdU over the 18 hr pulse in vivo (Table 6 and Figure 5). These data are consistent with the cell cycle analysis in demonstrating that the $p75^+P_0^-$ cells were dividing rapidly. We confirmed that the $p75^+P_0^-$ population from BrdU-administered rats remained enriched for NCSCs by observing that 86% of such cells expressed MASH1 after a 24 hr BMP2 challenge and that in standard culture conditions, an average

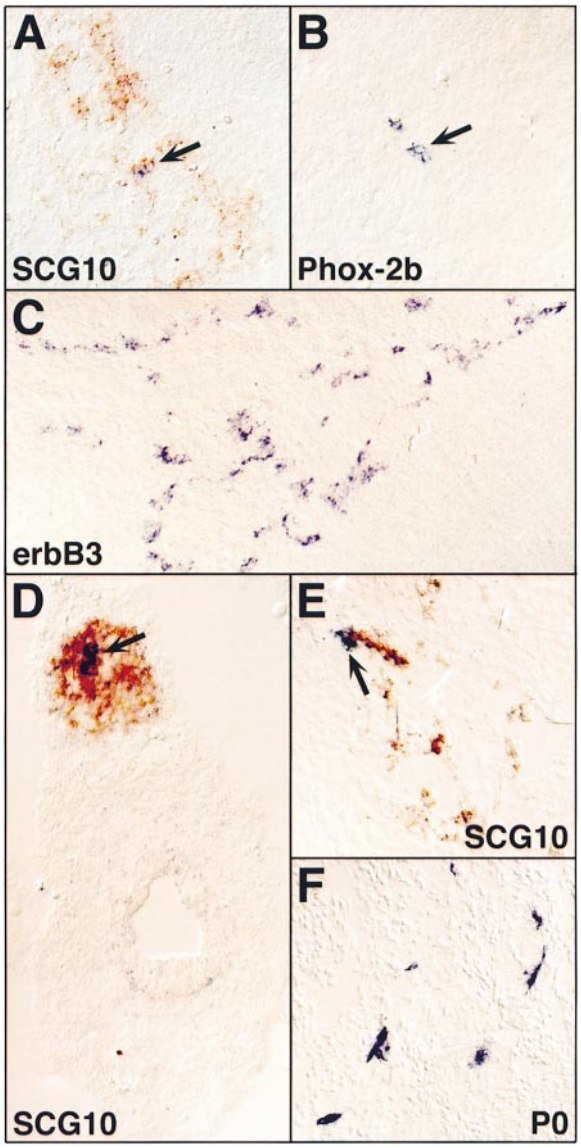

Figure 4. Sciatic Nerve $p75^{+}P_0^{-}$ Cells Give Rise to Neurons and Glia In Vivo

$p75^{+}P_0^{-}$ cells were purified by FACS from E14.5 sciatic nerves and injected into stage 18 chicken embryos. After 3 days of incubation, the embryos were harvested, sections were made through peripheral ganglia and nerves, and sections were hybridized in situ with chick- and rat-specific markers. In (A), (D), and (E), both rat-specific (purple stain) and chick-specific (orange stain) SCG10 probes were included in the hybridization, allowing for the simultaneous identification of graft and host neurons. In (B), (C), and (F), sections were hybridized only with rat-specific probes (purple stain). (A) and (B) show sections through sympathetic ganglia of one engrafted chick. (A) A rat $SCG10^{+}$ cell (arrow) is detected in close association with chick $SCG10^{+}$ cells. (B) Rat Phox-$2b^{+}$ cells are also detected (arrow), indicating that the engrafted neurons were autonomic. (C) In the same embryo, hundreds of nonneuronal cells, expressing the NRG1 receptor subunit erbB3, were detected in peripheral nerve (adjacent sections were negative for SCG10). (D) A second engrafted chick exhibited rat neurons (arrow) in Remak's ganglion. (E and F) Engrafted chick embryos from a different experiment exhibited $SCG10^{+}$ rat neurons in a sympathetic ganglion (E, arrow) and $P0^{+}$ Schwann cells in the peripheral nerve (F). Rat cells were not observed in tissues that are not normally neural crest derived.

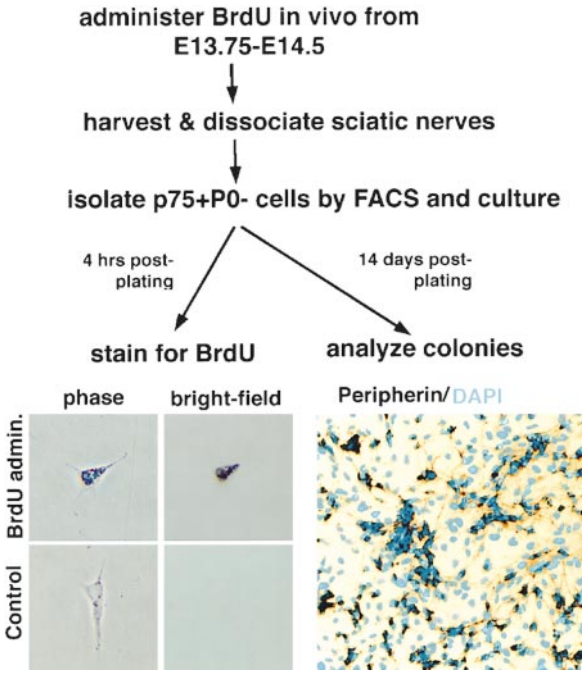

Figure 5. $p75^{+}P_0^{-}$ Cells Incorporate BrdU In Vivo while Remaining Multipotent

By plating $p75^{+}P_0^{-}$ cells from rats that had been administered BrdU in vivo from E13.75 to E14.5, it was determined that an average of 89% of $p75^{+}P_0^{-}$ cells incorporated BrdU (Table 6), and 50% of $p75^{+}P_0^{-}$ cells retained the ability to form multipotent N+S+M colonies. Peripherin and DAPI staining are shown for one field within a typical colony. This colony was multipotent, but GFAP and SMA staining are not shown.

of 50% of the colonies formed by such cells contained neurons, Schwann cells, and myofibroblasts (N+S+M; Figure 5). Since 90% of $p75^{+}P_0^{-}$ cells were $BrdU^{+}$, these data indicate that most or all cells that retained multilineage differentiation activity after isolation had previously incorporated BrdU in vivo. These data indicate that NCSCs undergo self-renewing divisions in vivo.

We confirmed in several ways that BrdU administration did not disrupt normal development within the sciatic nerve. Unseparated sciatic nerve cells from BrdU-administered and normal rats were indistinguishable in terms of their FACS profiles and their expression of p75 and P_0 (data not shown). Unseparated and $p75^{+}P_0^{-}$ cells from the sciatic nerves of normal and BrdU-administered rats also did not differ in terms of cell cycle status (data not shown). Unseparated cells from BrdU-administered rats also did not differ significantly from normal rats in terms of the number of cells that expressed MASH1 after a 24 hr BMP2 challenge or the number of N+S+M colonies that they formed after a 2 week culture (data not shown).

Discussion

Parallels are often drawn between our developing understanding of neural stem cells and the extensive characterization of hematopoietic stem cells (HSCs) (Morrison et al., 1995); however, there is a major distinction

Table 6. Cumulative BrdU Labeling of Neural Crest Cells in the Sciatic Nerve by Continuous Administration of BrdU for 18 hr prior to Harvest at E14.5

		$BrdU^+$ (%)
Unseparated SN	BrdU treated in vivo	79.8 ± 7.6
	normal	0.0
	normal, 6 hr BrdU in vitro[a]	18.9 ± 7.3
$p75^+ P_0^-$	BrdU treated in vivo	89.3 ± 6.0
	normal	0.0

[a] Normal, freshly dissociated sciatic nerve cells were cultured for 5 to 7 hr under standard conditions with NRG1 and 10 μM BrdU added.

in the approaches used to study the two stem cell types. HSCs can be identified prospectively based on surface marker expression, isolated by FACS, and transplanted in vivo without being cultured in vitro (Spangrude et al., 1988). In contrast, neural stem cells have been isolated from cultures of neural tissue. This is a critical difference, since it is precisely the ability to prospectively identify and isolate HSCs that has facilitated the rapid progress toward understanding their properties. This approach has allowed analyses of the genes expressed by HSCs (e.g., Matthews et al., 1991; Morrison et al., 1996), their lineage relationship with other multipotent progenitor populations (Morrison et al., 1997b), and aspects of their developmental potential (Ikuta et al., 1990; Geiger et al., 1998). The inability to isolate neural stem cells directly from tissue has made it impossible to study many of their properties in vivo.

Using cell surface markers and flow cytometry, we have isolated a population of $p75^+P_0^-$ cells from fetal rat sciatic nerve at E14.5, days after neural crest cell migration has ended. This population is highly enriched in cells that are functionally indistinguishable in vitro from NCSCs previously obtained from explant cultures of E10.5 rat neural tubes: they are multipotent, self-renewing, and respond to growth factors such as BMP2 and NRG1. Importantly, the neurogenic capacity of these cells can be manifested in a normal in vivo environment as well as in culture. $p75^+P_0^-$ cells incorporated BrdU in vivo while retaining multipotency, demonstrating that they undergo self-renewing divisions in vivo. Taken together, these data demonstrate the prospective identification and direct isolation from tissue of a neural stem cell using surface antigenic markers and indicate that such cells constitute a resident population of the fetal peripheral nervous system.

Self-Renewal of Neural Stem Cells In Vivo

Neural progenitor cells have often been demonstrated to divide in vivo, but the self-renewal of multipotent neural stem cells has not been directly addressed. For example, Nowakowski and colleagues extensively studied the cell cycle kinetics of dividing ventricular zone neural progenitors but did not address whether individual progeny of dividing cells were multipotent (Takahashi et al., 1996; Cai et al., 1997). Some cortical progenitor cells undergo asymmetric divisions with one daughter cell remaining in the ventricular zone, but it was not tested whether individual mother or daughter cells were multipotent (Chenn and McConnell, 1995). Retroviral lineage marking experiments in the fetal CNS and retina have provided evidence for proliferating cells that generate both neurons and glia (Turner and Cepko, 1987; Leber et al., 1990; Turner et al., 1990; Reid et al., 1995). However, the lack of markers to distinguish stem cells from committed progenitors made it impossible to distinguish whether such proliferation reflected self-renewing or restrictive divisions of the multipotent cells. The proliferation of cells within a ventricular zone is sometimes considered ipso facto evidence of self-renewal, based on the assumption that all ventricular zone cells are stem cells; however, this assumption is inconsistent with the data. When cortical ventricular zone cells are cultured, most progenitors give rise to small clones of neurons, and only a minority of clones form large multipotent colonies (Davis and Temple, 1994; Qian et al., 1997). These data suggest that the ventricular zone may contain a heterogeneous collection of progenitors, only a minority of which are multipotent (Temple and Alvarez-Buylla, 1999). Thus, the observation of proliferation within the ventricular zone does not demonstrate self-renewal: individual daughter cells must be shown to retain the multipotency characteristic of the mother cell.

A recent study argued that stem cells located in the subependymal layer of the adult CNS undergo asymmetric self-renewing divisions in vivo (Morshead et al., 1998). This conclusion assumed that large clones of retrovirally marked cells are necessarily derived from stem cells and that all marked cells that remained in the subependyma 31–33 days after retroviral infection or BrdU incorporation must be stem cells. However, no markers were available to identify stem cells in situ, and whether the persistent labeled subependymal cells retained multipotency was not assessed. The recent evidence that, to the contrary, at least some stem cells reside in the ependymal rather than the subependymal layer (Johansson et al., 1999) highlights the necessity of rigorously identifying stem cells with markers and functional assays. Even in that study, however, only about 5% of isolated ependymal cells behaved as stem cells in vitro, and that subpopulation could not be prospectively identified.

By combining in vivo BrdU labeling with functional assays of $p75^+P_0^-$ NCSCs isolated by FACS, we have demonstrated that the vast majority of these multipotent cells undergo at least one self-renewing division in vivo between E13.75 and E14.5. Almost 90% of $p75^+P_0^-$ cells that plated and survived in culture had previously incorporated BrdU in vivo, and at least 50% of these BrdU-labeled cells were multipotent in vitro (Table 6 and Figure 5). Although in principle it would be desirable to show that individual BrdU-labeled $p75^+P_0^-$ cells gave rise to both neurons and glia after transplantation in vivo, it has thus far not been technically possible to transplant single neural stem cells in vivo and reliably detect engraftment in either the CNS or PNS. Nevertheless, the fact that the developmental capacities of $p75^+P_0^-$ cells directly isolated from E14.5 sciatic nerve are indistinguishable from those of NCSCs isolated from 24 hr explants of E10.5 neural tube further argues that multipotent cells self-renew in vivo. The migration of trunk neural crest cells along the ventrolateral pathway in the rat is likely over by E11.5–E12.0 (Q. Ma and P. M. W., unpublished observations). Thus, NCSCs likely self-renewed

within the sciatic nerve itself, since most neural crest cells had arrived there before the BrdU was administered. Even if some $p75^+P_0^-$ cells incorporated BrdU while en route to the sciatic nerve, however, the data would still demonstrate their self-renewal in vivo.

The finding that NCSCs in the fetal sciatic nerve self-renew into late gestation raises the question of whether such cells persist into adulthood, which could have therapeutic importance. Small numbers of neurons have been reported to emerge from explants of postnatal sciatic nerves (Barakat-Walter, 1994). Whether these neurons arise from multipotent stem cells or from committed neuronal precursors was not determined, however. Even if they do not persist in adults, embryonic NCSCs could be transplanted to sites of PNS injuries or demyelination, especially if the environment of the adult nerve remains permissive for NCSC survival, self-renewal, and differentiation. It will be of interest to determine whether NCSCs persist in other crest-derived structures, such as the dorsal root ganglia and the gut.

Isolation and Enrichment of Peripheral Neural Stem Cells by FACS

Our ability to characterize the properties of freshly isolated NCSCs depends on their degree of enrichment in the $p75^+P_0^-$ population. Although this population is not pure, 60% of such cells formed self-renewing multipotent colonies in standard culture medium, and over 80% of such cells were capable of generating neurons in the presence of BMP2. These results suggest that some of the nonneurogenic colony types that formed under standard conditions may have been NCSCs that failed to "read out" their neuronal potential at the time point assayed. Thus, up to 80% of colony-forming $p75^+P_0^-$ cells may be NCSCs. Since only 15%–16% of unfractionated sciatic nerve cells behaved as NCSCs (Table 1), the $p75^+P_0^-$ fraction appears enriched for stem cells approximately 4- to 5-fold.

These calculations assume that the frequency of NCSCs in the in vitro assay is representative of their frequency in the freshly isolated $p75^+P_0^-$ fraction. The plating efficiency of the $p75^+P_0^-$ cells was around 25% under both standard conditions (Table 1) and in cultures supplemented with BMP2. While this figure compares favorably with previous clonal analyses of multipotent neural progenitors (Reynolds et al., 1992; Kilpatrick and Bartlett, 1993; Gritti et al., 1996; Johe et al., 1996), it raises the question of whether other progenitor types that do not form colonies under these culture conditions may be concealed within the $p75^+P_0^-$ population. This possibility seems very unlikely, given that all other progenitor types found within other subpopulations from the sciatic nerve efficiently formed colonies under our culture conditions. Indeed, these other progenitor populations had higher plating efficiencies than the $p75^+P_0^-$ cells (Table 3). Therefore, the frequency of NCSCs within the $p75^+P_0^-$ population may have been, if anything, underestimated.

The ability to directly isolate NCSCs from tissue by FACS has permitted us to test their developmental capacities by in vivo transplantation without a prior period of growth in culture. This approach has been used routinely in the analysis of HSCs but had not been possible with neural stem cells. Freshly isolated NCSCs were able to generate both neurons and glia following direct transplantation into the trunk neural crest migratory pathway of host chick embryos. This demonstrates that the neurogenic capacity of crest-derived cells isolated from a tissue (the sciatic nerve) where they do not normally manifest this potential is not a tissue culture artifact, but is exhibited when the cells are exposed to a normal in vivo environment permissive for neurogenesis. This further supports the idea that the fates of multipotent NCSCs are determined by the environments to which they migrate (Le Douarin, 1986). This in vivo assay will be useful to determine the full developmental potential of the $p75^+P_0^-$ population, and we are currently using neuronal subtype–specific markers to assay the classes of neurons they generate in vivo.

Lineage Restriction in the Peripheral Nerve Is More Complex and Dynamic Than Previously Anticipated

The PNS forms relatively quickly during early to midgestation, with neural crest progenitors differentiating rapidly after migrating. Although in chick, multipotent neural crest progenitors have been detected in postmigratory trunk crest derivatives, including the skin (Richardson and Sieber-Blum, 1993), dorsal root ganglion (Duff et al., 1991; Sextier-Sainte-Claire Deville et al., 1992), sympathetic ganglion (Duff et al., 1991), and gut (Sextier-Sainte-Claire Deville et al., 1994), these cells persist for only 2 days after crest migration has ceased. Thus, the approaches applied so far in avians suggest that within a short time after migration, all cell fates are determined within the PNS.

A similar view has been held for the mammalian PNS. For example, neural crest–derived cells in the E14 rat sciatic nerve were previously described as Schwann cell precursors (Jessen et al., 1994). The developmental potential of these Schwann precursors was not tested, but it was concluded that they were distinct from neural crest progenitors because $p75^+$ cells from the sciatic nerve were observed to stain with an antibody against GAP43, while neural crest outgrowth did not. We tested monoclonal and polyclonal anti-GAP43 antibodies under a variety of fixation and culture conditions but failed to observe staining of sciatic nerve progenitors other than in neurons that differentiated in NCSC colonies (data not shown). Whether or not they express GAP43, at E14.5 sciatic nerve cells comprise a heterogeneous collection of progenitors with respect to marker expression and developmental potential. A significant proportion of such cells are functionally indistinguishable from NCSCs, while only a minority of cells appeared committed to Schwann cell fates at this age.

We observed multipotent progenitors in the rat sciatic nerve at least until E17.5. Why should multipotent stem cells be retained in developing peripheral nerve? The types of progenitors cultured from the E14.5 sciatic nerve suggest that NCSCs may generate both myofibroblast derivatives and Schwann cells in this tissue. Fibroblastic components of peripheral nerve have previously been assumed to develop from mesodermally derived mesenchymal cells. That some myofibroblast progenitors are crest derived in vivo is suggested by

the fact that they can be recovered in fractions positive for P_0, a marker previously thought to reflect commitment to a Schwann cell fate (Lee et al., 1997). Myofibroblast derivatives in the nerve may include perineurium, epineurium, and vascular smooth muscle. Identification of the ultimate fates of crest-derived myofibroblasts will require the application of novel fate mapping technologies (Zinyk et al., 1998).

Role of NRG1 in the Developmental Restriction of NCSCs

NRG1 is expressed on motor axons in the peripheral nerve (Meyer and Birchmeier, 1994) and is genetically essential for proper Schwann cell development (Meyer and Birchmeier, 1995). Previously, we have shown that NRG1 promotes glial differentiation by NCSCs in an instructive manner (Shah et al., 1994), and more recently, that it can cause a rapid loss of neurogenic capacity by NCSCs in the absence of cell death (Shah and Anderson, 1997). Others have shown that neuregulin promotes the survival (Dong et al., 1995) and proliferation (Lemke and Brockes, 1984) of Schwann cells and their progenitors. These latter observations have led some to conclude that NRG1 acts primarily in a selective rather than an instructive manner in Schwann cell development.

In the present experiments, we confirmed that NRG1 acted instructively on NCSCs from sciatic nerve. We also found that it promoted the survival of all neural progenitors (see plating efficiencies, Tables 3 and 5) as well as the proliferation of Schwann (S-only) and myofibroblast (M-only) progenitors. These effects were independent and could be clearly distinguished from each other. Thus, there is no conflict between these different neuregulin functions. Taken together, these data indicate that NRG1 may play multiple roles in Schwann cell development in the peripheral nerve, including the instructive restriction of NCSCs to nonneurogenic fate(s). Such an action may account in part for the diminishing frequency of NCSCs in the peripheral nerve at developmental stages after E14.5 (Table 1).

Sciatic Nerve Stem Cells and the Origins of PNS Tumors

The persistence of NCSCs may provide an important insight into the etiology of certain cancers associated with the PNS. Peripheral neuroectodermal tumors and Ewing's sarcomas often contain primitive cells with the potential to differentiate into several different neuronal and mesectodermal lineages (Cavazzana et al., 1987). While these tumors have been hypothesized to be associated with the transformation of neural crest progenitors (Thiele, 1991), this association was mysterious given the expectation that neural crest progenitors terminally differentiate early in fetal development. The persistence of NCSCs raises the possibility that Ewing's sarcomas, which occur predominantly in the bones of children, may derive from the immortalization of NCSCs present in the peripheral nerve fibers that innervate the periosteum. Similarly, neurofibromas containing cells with Schwann and myofibroblast properties occur in the peripheral nerves of children (Riccardi and Eichner, 1986). These tumors might also derive from the transformation of NCSCs (or of S+M progenitors) during late fetal or postnatal development. The finding that NCSCs persist in rodent peripheral nerve may therefore have implications for diagnosis and treatment of diseases of the PNS.

Experimental Procedures

Rats

Pregnant Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). For timed pregnancies, animals were put together in the afternoon, and the morning on which the plug was observed was designated E0.5.

Dissociation of Sciatic Nerve

E14–E17 sciatic nerves were dissected into ice-cold Ca, Mg-free HBSS with 10 mM HEPES (pH 7.4), pelleted, and dissociated by incubating for 4 min at 37°C in 0.025% trypsin (GIBCO [Grand Island, NY] product 25300-054 diluted 1:1 in Ca, Mg-free HBSS) plus 1 mg/mL type 3 collagenase (Worthington, Lakewood, NJ). The digestion was quenched with 2 vol of staining medium: L15 medium containing 1 mg/mL BSA (GIBCO product 11019-023), 10 mM HEPES (pH 7.4), penicillin/streptomycin (Biowhittaker, Walkersville, Maryland), and 25 μg/mL deoxyribonuclease type 1 (Sigma, St. Louis, MO, D-4527). After centrifuging, nerve cells were triturated and resuspended in staining medium.

Flow Cytometry

All sorts and analyses were performed on a FACSVantage dual-laser flow cytometer (Becton-Dickinson, San Jose, CA). In order to isolate NCSCs, dissociated E14.5 sciatic nerve cells were stained with antibodies against p75 and P_0. Neither of these antibodies exhibited nonspecific staining at the concentrations used to stain sciatic nerve cells when tested by FACS on telencephalon or fetal liver control cells (not shown).

Dissociated sciatic nerve cells were suspended in a 1/2000 dilution of P07 monoclonal antibody against P_0 (J. J. Archelos, Munich) for 20–25 min on ice, washed, and incubated in phycoerythrin-conjugated anti-mouse IgG1 secondary antibody (Southern Biotechnology Associates, Birmingham, AL). After washing, the cells were resuspended in 192 IgG antibody (against p75) directly conjugated to fluorescein in the presence of 0.1 mg/mL mouse IgG1 (Sigma). The cells were washed and resuspended in staining medium containing 2 μg/mL 7-aminoactinomycin D (7-AAD; Molecular Probes, Eugene, OR), a viability dye. Dead cells were excluded by gating on forward and side scatter as well as by eliminating 7-AAD-positive events. Prior to and after sorts, tissue culture plates were kept in sealed plastic bags gassed with 5% CO_2 to prevent the culture medium from becoming basic by equilibrating with the air.

Cell cycle analyses of NCSCs were performed by staining with Hoechst 33342 (Sigma) to measure DNA content and pyronin Y (Sigma) to measure RNA content (Darzynkiewicz and Juan, 1997). $p75^+P_0^-$ cells from E14.5 sciatic nerve were sorted into staining medium and then pipetted into ice-cold 70% ethanol. The cells were left in ethanol at 4°C overnight and then resuspended in 1 μg/mL Hoechst 33342 plus 2 μg/mL pyronin Y 20 min before flow-cytometric reanalysis.

Culture of Sciatic Nerve Cells

Sciatic nerve progenitors were typically cultured in 6-well plates (Corning, Corning, NY) at clonal density (fewer than 30 clones per well for 14 day cultures, or 60 clones per well for 1 to 4 day cultures). Plates were sequentially coated with poly-d-lysine (PDL) (Biomedical Technologies, Stoughton, MA) and 0.15 mg/mL human fibronectin (Biomedical Technologies) as described (Stemple and Anderson, 1992). The culture medium contained DMEM-low (GIBCO, product 11885-084) with 15% chick embryo extract (prepared as described [Stemple and Anderson, 1992]), 20 ng/mL recombinant human bFGF (R & D Systems, Minneapolis, MN), 1% N2 supplement (GIBCO), 2% B27 supplement (GIBCO), 50 μM 2-mercaptoethanol, 35 mg/

mL (110 nM) retinoic acid (Sigma), and penicillin/streptomycin (Biowhittaker). Throughout the results, this medium composition is described as our standard medium. Under standard conditions, cells were cultured for 6 days in this medium and then switched to a similar medium (with 1% CEE and 10 ng/mL bFGF) that favors differentiation for another 8 days before immunohistochemical analysis of colony composition. Cultures were maintained in humidified incubators with 6% to 8% CO_2.

Self-Renewal Experiments

Single $p75^+$ cells from E14.5 sciatic nerve were sorted by FACS into individual wells of 96-well plates and cultured in standard medium without refeeding. After 7 and 11 days, the wells were examined and multipotent colonies were identified by their appearance: multipotent progenitors gave rise to larger colonies than S-only or S+M progenitors even by 7 days of culture. Colonies were trypsinized and quenched in staining medium with chick embryo extract added. The cells were spun down, resuspended in staining medium, replated in multiple 6-well plate wells at clonal density, and cultured under standard conditions.

Self-renewal was assayed in vivo by administering BrdU (Sigma) to pregnant rats for 18 hr prior to harvest of sciatic nerves from pups at E14.5. Doses of BrdU equivalent to 50 μg/g body weight were dissolved in 1 mL D-PBS with 0.007 M NaOH and injected intraperitoneally at harvest −18 hr, −16 hr, −14 hr, −4 hr, and −2 hr (Cai et al., 1997). Additionally, at harvest −14 hr, the rat's normal water was replaced by water containing 2 mg/mL BrdU. After dissecting sciatic nerves, the cells were stained as described above and $p75^+P_0^-$ cells were sorted into culture. The cells were plated for 3–4 hr and stained with an antibody against BrdU (IU-4, Caltag, Burlingame, CA) as described previously (Raff et al., 1988).

Immunohistochemistry

Cultures were fixed in acid ethanol (5% glacial acetic acid in 100% ethanol) for 20 min at −20°C, washed, blocked, and triply labeled for peripherin, GFAP, and alpha-SMA essentially as described (Shah et al., 1996). After washing, nuclei were stained by incubating in 10 μg/mL DAPI for 10 min. When cultures were stained with combinations of antibodies that did not include anti-GFAP or anti-BrdU, they were fixed in 4% paraformaldehyde for 10 min at room temperature. MASH1 staining was performed as described (Shah et al., 1994).

In Vivo Transplantation of Sciatic Nerve Progenitors

Fertile white Leghorn eggs were incubated to Hamburger and Hamilton stage 18. Twenty to ninety thousand $p75^+P_0^-$ cells from E14.5 sciatic nerve were isolated by FACS, added to a drawn glass capillary tube, and injected into the anterior, medial corner of one or two somites of each embryo using an MM33 micromanipulator (Fine Science Tools, Foster City, CA) and very gentle air pressure. Details of the procedure will be reported elsewhere (P. M. W. and D. J. A., submitted) and are available on request (squirrel@cco.caltech.edu). Injected embryos were incubated for an additional 3 days to stage 29, fixed, embedded in OCT, and cryostat sectioned at 15 μm.

In Situ Hybridization

Antisense probes for rat- and chick-specific genes were synthesized with digoxygenin- and fluorescein-conjugated nucleotides, respectively. In situ hybridization was performed as described (Birren et al., 1993); detailed protocols are available upon request. Normal rat and chick embryos were processed in parallel as positive and negative controls for in situ hybridization. Since embryos were unilaterally injected with rat cells, the contralateral side of the same embryos served as an additional internal control for staining specificity.

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References

Anderson, D.J. (1993). Cell and molecular biology of neural crest cell lineage diversification. Curr. Opin. Neurobiol. *3*, 8–13.

Barakat-Walter, I. (1994). Neuronal potential of cells in early postnatal rat sciatic nerve. Dev. Biol. *161*, 263–273.

Baroffio, A., Dupin, E., and Le Douarin, N.M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. Proc. Natl. Acad. Sci. USA *85*, 5325–5329.

Birren, S.J., Lo, L., and Anderson, D.J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development *119*, 597–610.

Bronner-Fraser, M., and Cohen, A.M. (1980). Analysis of the neural crest ventral pathway using injected tracer cells. Dev. Biol. *77*, 130–141.

Bronner-Fraser, M., and Fraser, S. (1988). Cell lineage analysis shows multipotentiality of some avian neural crest cells. Nature *335*, 161–164.

Cai, L., Hayes, N.L., and Nowakowski, R.S. (1997). Local homogeneity of cell cycle length in developing mouse cortex. J. Neurosci. *17*, 2079–2087.

Cameron, H.A., and McKay, R. (1998). Stem cells and neurogenesis in the adult brain. Curr. Opin. Neurobiol. *8*, 677–680.

Cavazzana, A.O., Miser, J.S., Jefferson, J., and Triche, T.J. (1987). Experimental evidence for a neural origin of Ewing's sarcoma. Am. J. Pathol. *127*, 507–518.

Chenn, A., and McConnell, S.K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell *82*, 631–641.

Darzynkiewicz, Z., and Juan, G. (1997). Differential staining of DNA and RNA. In Current Protocols in Cytometry, J.P. Robinson, ed. (New York: John Wiley and Sons), pp. 7.3.1–7.3.13.

Davis, A., and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature *372*, 263–266.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., and Jessen, K.R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation and maturation of rat Schwann cell precursors. Neuron *15*, 585–596.

Duff, R.S., Langtimm, C.J., Richardson, M.K., and Sieber-Blum, M. (1991). *In vitro* clonal analysis of progenitor cell patterns in dorsal root and sympathetic ganglia of the quail embryo. Dev. Biol. *147*, 451–459.

Frank, E., and Sanes, J.R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis *in vivo* with a recombinant retrovirus. Development *111*, 895–908.

Gage, F.H. (1998). Stem cells of the central nervous system. Curr. Opin. Neurobiol. *8*, 671–676.

Gage, F.H., Ray, J., and Fisher, L.J. (1995). Isolation, characterization and use of stem cells from the CNS. Annu. Rev. Neurosci. *18*, 159–192.

Geiger, H., Sick, S., Bonifer, C., and Muller, A.M. (1998). Globin gene expression is reprogrammed in chimeras generated by injecting adult hematopoietic stem cells into mouse blastocysts. Cell *93*, 1055–1065.

Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D.J., Roisen, F., Nickel, D.D., and Vescovi, A.L. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci. *16*, 1091–1100.

Haimoto, H., Hosoda, S., and Kato, K. (1987). Differential distribution

of immunoreactive S100-alpha and S100-beta proteins in normal nonnervous human tissues. Lab. Invest. *57*, 489–498.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y.H., and Weissman, I.L. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. Cell *62*, 863–874.

Jessen, K.R., and Mirsky, R. (1992). Schwann cells: early lineage, regulation of proliferation and control of myelin formation. Curr. Opin. Neurobiol. *2*, 575–581.

Jessen, K.R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The Schwann cell precursor and its fate—a study of cell death and differentiation during gliogenesis in rat embryonic nerves. Neuron *12*, 509–527.

Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U., and Frisen, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. Cell *96*, 25–34.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D.G. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. *10*, 3129–3140.

Kalyani, A., Hobson, K., and Rao, M.S. (1997). Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. Dev. Biol. *186*, 202–223.

Kilpatrick, T.J., and Bartlett, P.F. (1993). Cloning and growth of multipotential neural precursors—requirements for proliferation and differentiation. Neuron *10*, 255–265.

Le Douarin, N.M. (1980). The ontogeny of the neural crest in avian embryo chimeras. Nature *286*, 663–669.

Le Douarin, N.M. (1982). The Neural Crest (Cambridge, UK: Cambridge University Press).

Le Douarin, N.M. (1986). Cell line segregation during peripheral nervous system ontogeny. Science *231*, 1515–1522.

Leber, S.M., Breedlove, S.M., and Sanes, J.R. (1990). Lineage, arrangement, and death of clonally related motorneurons in chick spinal cord. J. Neurosci. *10*, 2451–2462.

Lee, M.-J., Brennan, A., Blanchard, A., Zoidl, G., Dong, Z., Tabemero, A., Zoidl, C., Dent, M.A.R., Jessen, K.R., and Mirsky, R. (1997). P0 is constitutively expressed in the rat neural crest and embryonic nerves and is negatively and positively regulated by axons to generate non-myelin-forming and myelin-forming Schwann cells, respectively. Mol. Cell. Neurosci. *8*, 336–350.

Lemke, G.E., and Brockes, J.P. (1984). Identification and purification of glial growth factor. J. Neurosci. *4*, 75–83.

Lo, L., Sommer, L., and Anderson, D.J. (1997). MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. Curr. Biol. *7*, 440–450.

Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature *362*, 312–318.

Matthews, W., Jordan, C.T., Wiegand, G.W., Pardoll, D., and Lemischka, I.R. (1991). A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell *65*, 1143–1152.

Meyer, D., and Birchmeier, C. (1994). Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. Proc. Natl. Acad. Sci. USA *91*, 1064–1068.

Meyer, R., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. Nature *378*, 386–390.

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. *11*, 35–71.

Morrison, S.J., Prowse, K.R., Ho, P., and Weissman, I.L. (1996). Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity *5*, 207–216.

Morrison, S.J., Shah, N.M., and Anderson, D.J. (1997a). Regulatory mechanisms in stem cell biology. Cell *88*, 287–298.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997b). Identification of a lineage of multipotent hematopoietic progenitors. Development *124*, 1929–1939.

Morshead, C.M., Craig, C.G., and van der Kooy, D. (1998). In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. Development *125*, 2251–2261.

Palmer, T.D., Takahashi, J., and Gage, F.H. (1997). The adult rat hippocampus contains primordial neural stem cells. Mol. Cell. Neurosci. *8*, 389–404.

Parysek, L.M., and Goldman, R.D. (1988). Distribution of a novel 57 kDa intermediate filament (IF) protein in the nervous system. J. Neurosci. *8*, 555–563.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J.-F. (1997). Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. Development *124*, 4065–4075.

Qian, X., Davis, A.A., Goderie, S.K., and Temple, S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron *18*, 81–93.

Raff, M.C., Lillien, L.E., Richardson, W.D., Burne, J.F., and Noble, M. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature *333*, 562–565.

Reid, C.B., Liang, I., and Walsh, C. (1995). Systematic widespread clonal organization in cerebral cortex. Neuron *15*, 299–310.

Reynolds, B.A., Tetzlaff, W., and Weiss, S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. *12*, 4565–4574.

Riccardi, V.M., and Eichner, J.E. (1986). Neurofibromatosis: Phenotype, Natural History, and Pathogenesis (Baltimore: Johns Hopkins University Press).

Richardson, M.K., and Sieber-Blum, M. (1993). Pluripotent neural crest cells in the developing skin of the quail embryo. Dev. Biol. *157*, 348–358.

Riethmacher, D., Sonnerberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G.R., and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the erbB3 receptor. Nature *389*, 725–730.

Sappino, A.P., Schurch, W., and Gabbiani, G. (1990). Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. Lab. Invest. *63*, 144–161.

Sextier-Sainte-Claire Deville, F., Ziller, C., and Le Douarin, N.M. (1992). Developmental potentialities of cells derived from the truncal neural crest in clonal cultures. Brain Res. Dev. Brain Res. *66*, 1–10.

Sextier-Sainte-Claire Deville, F., Ziller, C., and Le Douarin, N.M. (1994). Developmental potentials of enteric neural crest-derived cells in clonal and mass cultures. Dev. Biol. *163*, 141–151.

Shah, N.M., and Anderson, D.J. (1997). Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness. Proc. Natl. Acad. Sci. USA *94*, 11369–11374.

Shah, N.M., Marchionni, M.A., Isaacs, I., Stroobant, P.W., and Anderson, D.J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell *77*, 349–360.

Shah, N.M., Groves, A., and Anderson, D.J. (1996). Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. Cell *85*, 331–343.

Sieber-Blum, M., and Cohen, A. (1980). Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in vitro in the absence of non-neural crest cells. Dev. Biol. *80*, 96–106.

Sommer, L., Shah, N., Rao, M., and Anderson, D.J. (1995). The cellular function of MASH1 in autonomic neurogenesis. Neuron *15*, 1245–1258.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58–62.

Stemple, D.L., and Anderson, D.J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell *71*, 973–985.

Takahashi, T., Nowakowski, R.S., and Caviness, V.S. (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium:

a general model of neocortical neuronogenesis. J. Neurosci. *16*, 6183–6196.

Temple, S., and Alvarez-Buylla, A. (1999). Stem cells in the adult mammalian central nervous system. Curr. Opin. Neurobiol. *9*, 135–141.

Thiele, C.J. (1991). Biology of pediatric peripheral neuroectodermal tumors. Cancer Metastasis Rev. *10*, 311–319.

Turner, D.L., and Cepko, C.L. (1987). Cell lineage in the rat retina: a common progenitor for neurons and glia persists late in development. Nature *328*, 131–136.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Zinyk, D.L., Mercer, E.H., Harris, E., Anderson, D.J., and Joyner, A.L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr. Biol. *8*, 665–668.