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Neural Crest Stem Cells Persist in the Adult Gut but Undergo Changes in Self-Renewal, Neuronal Subtype Potential, and Factor Responsiveness

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Summary

We found neural crest stem cells (NCSCs) in the adult gut. Postnatal gut NCSCs were isolated by flow-cytometry and compared to fetal gut NCSCs. They self-renewed extensively in culture but less than fetal gut NCSCs. Postnatal gut NCSCs made neurons that expressed a variety of neurotransmitters but lost the ability to make certain subtypes of neurons that are generated during fetal development. Postnatal gut NCSCs also differed in their responsiveness to lineage determination factors, affecting cell fate determination in vivo and possibly explaining their reduced neuronal subtype potential. These perinatal changes in gut NCSCs parallel perinatal changes in hematopoietic stem cells, suggesting that stem cells in different tissues undergo similar developmental transitions. The persistence of NCSCs in the adult PNS opens up new possibilities for regeneration after injury or disease.

Introduction

Most of the neurons and glia of the peripheral nervous system (PNS) arise during fetal development from the neural crest, a heterogeneous collection of progenitors that migrates out of the neural tube in midgestation (LeDouarin, 1986). Migrating neural crest cells, which include neural crest stem cells (NCSCs), undergo progressive restrictions in developmental potential (Baroffio et al., 1991) and terminally differentiate soon after reaching postmigratory sites. The postnatal PNS was thought to lack stem cells.

Like the PNS, the adult CNS was once thought to lack stem cells. However, multipotent neural progenitors and neurogenesis do persist in certain regions of the adult CNS (Reynolds and Weiss, 1992; Palmer et al., 1997; Johansson et al., 1999; Doetsch et al., 1999; Altman, 1969; Eriksson et al., 1998; Gould et al., 1999). Stem cells from the adult CNS have recently been prospectively identified and purified by flow-cytometry, creating the possibility of studying their properties as they exist in vivo (Rietze et al., 2001). The discovery of stem cells in the adult CNS raises the question of whether stem cells also persist in the adult PNS.

It has not been clear whether neural stem cells undergo perinatal developmental changes. Stem cells in the adult CNS are sometimes thought of as left-over from fetal development or as deriving from the life-long self-renewal of fetal neural stem cells (reviewed by Morrison et al.,

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1997). These views assume that the important properties of neural stem cells, such as developmental potential and self-renewal capacity, do not change between fetal and postnatal life. Even assuming that postnatal neural stem cells are derived from fetal neural stem cells, it is possible that fetal stem cells undergo developmental changes that give rise to phenotypically and functionally distinct populations of postnatal neural stem cells. Adult hematopoietic stem cells are multipotent and self-renewing (Morrison et al., 1995b) but exhibit less self-renewal potential than fetal liver hematopoietic stem cells (Lansdorp et al., 1993; Morrison et al., 1995a) and lose the ability to make certain subtypes of lymphocytes that arise only during fetal development (Hayakawa et al., 1985; Ikuta et al., 1990; Kantor et al., 1992). This raises the question of whether postnatal neural stem cells undergo similar changes.

Postmigratory rat NCSCs persist into late gestation by self-renewing within peripheral nerves and the gut (Morrison et al., 1999; Bixby et al., 2002). At embryonic day (E)14.5, sciatic nerve NCSCs can be prospectively identified and isolated by flow-cytometry as $p75^{+}\mathbf{P}_{0}^{-}$ cells, and gut NCSCs can be isolated as $p75^{+}\alpha_{4}^{+}$ cells. Seventy to eighty percent of single cells in each population self-renew and form multilineage colonies in culture that contain neurons, glia, and myofibroblasts (Morrison et al., 1999, 2000a; Bixby et al., 2002). In this study, we have discovered that NCSCs do persist in the adult gut and that these cells undergo temporal changes in self-renewal potential and neuronal subtype potential. Post-natal gut NCSCs gave rise to neurons and glia in vitro and in vivo but, in contrast to E14.5 gut NCSCs, generated mainly glia upon transplantation into developing peripheral nerve. The reduced neuronal subtype potential and gliogenic bias of postnatal gut NCSCs may be caused by perinatal changes in their responsiveness to lineage determination factors.

Results

Multipotent Progenitors Persist Postnatally in the Gut

We have so far been unable to identify any multipotent progenitors from the postnatal sciatic nerve (data not shown), but we have found multipotent progenitors in the postnatal gut. Postnatal day (P) 5 to P15 rat gut was dissociated into single cell suspensions and plated in culture at clonal density. Clonal density allowed individual founder cells to form spatially distinct colonies so that the developmental potential of the founder cells could be assessed based on colony composition. After 14 days in culture, we consistently observed the formation of multilineage colonies containing neurons, glia, and myofibroblasts (Figure 1) that resembled colonies formed by embryonic NCSCs (Stemple and Anderson, 1992;Morrison et al., 1999;Bixby et al., 2002), though they tended to be somewhat smaller. Based on hemocytometer counts of trypsinized cells, multilineage colonies from E14.5 and P15 progenitors averaged 184,000 ± 69,000 and 82,000 ± 17,000 cells per colony, respectively.

To begin to assess the localization of these multipotent progenitors in vivo, we stripped the plexus/outer muscle layers from the gut epithelium and dissociated these tissues separately. We found that multipotent progenitors were consistently cultured from the plexus/outer muscle layers but never from the gut epithelium. This indicated that these multipotent progenitors were localized in vivo to the submucosal plexus, myenteric plexus, or outer muscle layers of the gut. All subsequent experiments on postnatal gut progenitors were performed using cells dissociated from the plexus/outer muscle layers.

Multipotent progenitors were infrequent among the unfractionated cells obtained from the preparations dissociated P15 plexus/outer muscle layers. Only 0.7% of dissociated (but unfractionated) P15 cells survived and formed multilineage colonies in culture. Since p75 has consistently been observed to mark NCSCs in the gut and other tissues (Stemple and Anderson, 1992; Morrison et al., 1999; Lo and Anderson, 1995; Bixby al., 2002), we tested whether these multipotent progenitors were enriched within the $p75^{+}$ fraction of cells. As in the E14.5 gut,

we found that multipotent progenitors were enriched among cells with high levels of p75 expression (see Figure 1D for a flow-cytometric profile p75 expression). Although $p75^{-}$ and $p75^{low}$ cells formed no multilineage colonies and $p75^{med}$ cells gave rise few multilineage colonies, $p75^{+}$ cells were highly enriched for multipotent progenitors. An average of fifty-two percent of $p75^{+}$ cells survived to form colonies culture, and 66% of these colonies were multilineage (Table 1). Interestingly, although E14.5 gut NCSCs are $p75^{+}\alpha_4^{+}$ (Bixby et al., 2002), Figure 1D shows that $p75^{+}$ cells were largely negative for α_4 integrin by P15. By staining sections of P15 gut with an antibody against p75, we found that $p75^{+}$ cells localized to the myenteric and submucosal plexi (see Supplemental Figure S1 online at http://www.neuron.org/cgi/content/full/35/4/657/DC1).

Prospective Identification and Isolation of Postnatal Gut NCSCs by Flow-Cytometry

By isolating the 1%–2% of cells that expressed the highest levels of p75 from the plexus/outer muscle layers of the gut, we found that we could isolate highly enriched populations of NCSCs from E14.5 through the oldest rat we examined at P110 (Table 1). Sixty to seventy percent of colonies formed by $p75^{+}$ cells from P5 to P22 gut contained neurons, glia, and myofibroblasts (N + G + M; Table 1). Additional colonies contained neurons and glia, but not myofibroblasts (N + G). It is uncertain whether these N + G colonies represent restricted progenitors or whether some multipotent progenitors sometimes failed to make myofibroblasts under these culture conditions. Only 0.2% of unfractionated cells from the P22 plexus/outer muscle layer preparations survived and formed multilineage colonies in culture (data not shown). Thus, taking into account plating efficiencies, multipotent progenitors were more than 150-fold enriched within the $p75^{+}$ fraction of P22 gut cells relative to unfractionated cells.

Multipotent progenitors continued to be present within the adult (250 g rats; P65–P110) rat gut (Figure 2). These multipotent progenitors were still highly enriched in the $p75^{+}$ fraction of cells and largely negative for α_4 integrin. However, the purity of multipotent progenitors among adult $p75^{+}$ cells was much lower than in the P22 gut, with only 9% of cells surviving to form colonies in culture and 17% of colonies being multilineage. Additional work will be required to improve our ability to purify adult gut multipotent progenitors by flow-cytometry.

Although we did not detect neuron only colonies from any of the postnatal gut $p75^{+}$ cells after 14 days of culture (Table 1), we wanted to be sure that this population was not substantially contaminated by committed neuronal progenitors or by immature neurons. To this end, we sorted P15 $p75^{+}$ cells into culture and examined the colonies 4 days later. 2.3% of colonies contained a single neuron, and 0.6% of colonies contained two neurons. No neuron only colonies contained more than two neurons. We also sorted P15 $p75^{+}$ cells into culture and stained these cells 17 hr later for the early neuronal marker neuron-specific (class III) β-tubulin (TuJ1 antibody). On average, 3.9% ± 5% of P15 gut $p75^{+}$ cells expressed β-tubulin. This suggests that around 4% of P15 $p75^{+}$ cells are immature neurons or committed neuronal progenitors with the ability to divide once in culture.

Postnatal Gut Multipotent Neural Crest Progenitors Self-Renew in Culture

The self-renewal capacity of the gut multipotent progenitors was assayed by depositing single $p75^{+}$ cells into individual wells of 96-well plates by flow-cytometry and then culturing for 8 days under standard conditions (Morrison et al., 1999). Multipotent colonies were then trypsinized and subcloned into secondary cultures at clonal density as previously described (Morrison et al., 1999). Almost all multipotent primary colonies gave rise to multipotent daughter colonies (20/20 at E14.5, 11/11 at P5, 16/18 at P15, and 6/6 at P22). Since the multipotent progenitors from postnatal gut were $p75^{+}$, formed colonies similar to embryonic NCSC colonies, appeared to localize to the myenteric or submucosal plexi, and had the capacity to self-renew, we conclude that they are postnatal NCSCs.

Although the postnatal gut NCSCs consistently self-renewed in culture, the extent of self-renewal quantitatively declined with increasing age. Each E14.5 gut NCSC produced an average of 730 multipotent daughter colonies (845 total daughter colonies) subclonable after 8 days in culture, while P22 gut NCSCs produced an average of only 70 multipotent daughter colonies (360 total daughter colonies) under the same conditions ($p = 0.002$; Table 2). NCSC self-renewal did not decline uniformly over time as some NCSCs at P15 and P22 self-renewed at a rate comparable to what was observed among E14.5 NCSCs. For example, one P15 NCSC gave rise to 370 multipotent daughter cells, while a P22 NCSC gave rise to 335 multipotent daughter cells.

Because of the impurity of multipotent progenitors in the adult $p75^+$ population, we cultured single adult gut cells for 15 days prior to subcloning in an attempt to distinguish multipotent colonies from restricted colonies based on size. Some of the large adult colonies gave rise to multipotent daughter cells while others did not. It is not yet clear whether this heterogeneity reflects the subcloning of restricted progenitor colonies or whether some multipotent adult progenitors may fail to detectably self-renew in this assay. Nonetheless, at least some of the adult multipotent progenitors self-renewed, producing an average of 35 multipotent subclones per founder cell (Table 2).

The 8–15 day culture assay represents one way of quantitating self-renewal potential, but it does not estimate the maximum self-renewal capacity of NCSCs. By culturing the postnatal gut NCSCs for longer periods of time, they give rise to larger numbers of multipotent daughter cells.

Postnatal Gut NCSCs Are Less Mitotically Active than E14 Gut NCSCs

In the hematopoietic system and the adult CNS, stem cells go from being highly mitotic during fetal development (Morrison et al., 1995a; Cai et al., 1997) to being relatively quiescent in adults (Morshead et al., 1994; Cheshier et al., 1999; Johansson et al., 1999). Since this is the first identification of NCSCs in the postnatal PNS, we used our ability to prospectively identify the gut NCSCs to ask whether their cell-cycle distribution changed perinatally. The cell-cycle distribution of uncultured NCSCs was assayed at isolation by flow-cytometry, using Hoechst 33342 staining to determine the DNA content of individual cells (Figure 3). E14.5 gut $p75^+\alpha_4^+$ NCSCs included 37% ± 7% of cells in S/G2/M phases of the cell cycle (>2N DNA content), as compared to only 26% ± 2% of P5 $p75^+$ cells (data not shown) and 10% ± 0.5% of P15 p75± cells (Figure 3; $p < 0.01$). When BrdU was administered for a 20 hr period in vivo prior to isolation of NCSCs, 87% ± 7% of E14.5 gut $p75^+\alpha_4^+$ cells incorporated BrdU while only 13.1% ± 2.5% of P15 gut $p75^+$ cells incorporated BrdU. Thus, while nearly all E14.5 gut NCSCs divided at least once in a 20 hr period in vivo, only 13% of P15 gut $p75^+$ cells divided in the same period. Either P15 gut NCSCs have a much longer cell-cycle time than E14.5 gut NCSCs, or a significant percentage of gut NCSCs are quiescent at P15.

NCSCs from the P15 Gut Differentiate into Neurons and Glia In Vivo

To test the ability of the P15 gut NCSCs to generate neurons and glia in vivo, freshly isolated, uncultured $p75^+$ cells were injected into two hindlimb bud somites of eight stage 17–18 chick embryos (Figure 4). After 72 hr, embryos were fixed, sectioned, and processed for in situ hybridization using probes against rat *SCG10* to identify neurons (Anderson and Axel, 1985) and rat P_0 to identify glia (Lemke et al., 1988). Chick neurons were identified by hybridizing with a chick *SCG10* probe in some sections. Of eight chicks analyzed, four showed engraftment with both neurons and glia, two showed engraftment of glia only, and two were not detectably engrafted. The failure of all eight chicks to detectably engraft with both neurons and glia may be due to differences between postnatal gut NCSCs and embryonic gut NCSCs or the relatively small numbers of cells injected (~310/somite; see Experimental Procedures). Neuronal

engraftment was found in sympathetic ganglia (2.2 cells/positive section; Figure 4A), and glia were present in peripheral nerves (17.5 cells/positive section; Figure 4B). Thus, uncultured postnatal gut NCSCs migrated to embryonic neural crest structures and formed neurons and glia.

Postnatal Gut NCSCs Differentiate into Neurons that Express a Variety of Neurotransmitters

We performed a clonal analysis of gut NCSCs to test whether they generate cells expressing a variety of neurotransmitters. We stained NCSC colonies that had been cultured for 14 days at clonal density using commercially available antibodies against Vasoactive Intestinal Peptide (VIP), Neuropeptide Y (NPY), and neuronal Nitric Oxide Synthase (nNOS) (Figure 5). Each of these antibodies specifically stained neurons based on analyses of adult rat gut sections and multilineage colonies in culture that were double labeled with these antibodies and antibodies against neuronal markers (data not shown). VIP, NPY, and Nitric Oxide are all expressed by subsets of enteric neurons in vivo (Pham et al., 1991;Jarvinen et al., 1999). Nearly all E14.5, P15, and adult gut NCSC colonies contained neurons expressing VIP, NPY, and nNOS (Figure 5D). Based on these experiments, adult gut NCSCs retain the ability to generate neurons that express a variety of neurotransmitters normally found in the ENS.

Gut NCSCs Undergo Developmental Restrictions in Neuronal Subtype Potential

Neurons with different neurotransmitter phenotypes are born at different intervals of gut development (Pham et al., 1991). Serotonergic progenitors last divide between E8 and E14.5 in mouse, while progenitors of NPY-expressing neurons last divide between E10 and P7. Since serotonergic differentiation is completed earliest during embryonic gut development, we examined whether gut NCSCs undergo restrictions between embryonic and postnatal stages in their potential to generate serotonergic neurons in culture.

Serotonergic neurons in the ENS can be identified by their expression of Tryptophan Hydroxylase, the initial and rate-limiting enzyme in the serotonin synthesis pathway (Gershon et al., 1977). We cultured E14.5 $p75^+\alpha_4{}^+$ cells or P5 or P15 $p75^+$ cells at clonal density for 14 days in standard medium and then stained with an antibody specific for Tryptophan Hydroxylase (Belin et al., 1991). Tryptophan Hydroxylase+ neurons were present at low density in most multilineage colonies formed by E14.5 gut NCSCs (66% ± 3%; Figures 6A and 6C) but only in a few multilineage colonies formed by P5 gut NCSCs (9% ± 5%; Figure 6C). No P15 progenitors formed Tryptophan Hydroxylase+ neurons in culture (0 ± 0; Figures 6B and 6C). Between E14.5 and P15, NCSCs lost the neuability to make serotonergic neurons based on this in vitro assay.

In addition to examining the ability of postnatal gut NCSCs to make subtypes of neurons that normally exist in the adult gut, we also wanted to examine their ability to make subtypes of neurons that differentiate only in other regions of the PNS. To this end, we examined the ability of gut NCSCs to make noradrenergic neurons. Although some gut neural crest progenitors transiently express a noradrenergic phenotype prior to E15 (Tyrosine Hydroxylase+ [TH+] and Dopamine-β-hydroxylase + [DβH+]), these cells continue to proliferate and TH can no longer be detected in the gut after E15 (Baetge et al., 1990).

NCSCs were isolated from rats ranging in age from E14.5 to P22 and cultured under conditions that promote noradrenergic differentiation (Morrison et al., 2000a). We double labeled these cultures with antibodies against Peripherin (to identify neurons) and either Tyrosine Hydroxylase (TH) or Dopamine-β-hydroxylase (DβH). All TH^+ or $D\beta H^+$ cells coexpressed Peripherin, but only a minority of Peripherin-positive neurons expressed TH or DβH (Figure 7). Double labeling experiments with antibodies against TH and DβH demonstrated that all of the TH^+ cells examined coexpressed DβH, but $D\beta H^+$ cells often did not express TH. The

percentage of DβH positive cells also expressing TH declined with age. At E14.5, an average of 51.7% ± 14.5% of DβH positive cells also expressed TH but at P15, only 23.4% ± 21.4% of DBH^+ cells did ($p < 0.05$). This is consistent with the fact that gut neural crest progenitors can give rise to both noradrenergic neurons ($TH^+D\beta H^+$) as well as $D\beta H^+TH^-$ neurons that are peptidergic, but not catecholaminergic (Baetge et al., 1990).

Nearly all E14.5 gut NCSC colonies contained neurons that expressed TH (87% ± 3%) and/or DβH (85% ± 12%), but the percentage of gut NCSCs that formed such neurons declined with increasing stem cell age (Table 3). By P15, only 20% of NCSCs formed TH^+ neuability rons, and 38% formed $D\beta H^+$ neurons (both differences were statistically significant; $p < 0.01$). In addition to the reduction in the proportion of NCSCs that were able to form such cells, there was also a reduction in the number of such cells per colony (Figure 7; Supplemental Figure S1). While E14.5 gut NCSC colonies contained average of 444 ± 285 DβH+ cells and 307 ± 244 TH+ cells, P15 gut NCSC colonies averaged only 14 ± $D\beta H^+$ cells and 2 ± 1 TH^+ cells. This reduction in the number of noradrenegic neurons per colony cannot explained by a proportionate decline in the total number of neurons per colony as we estimated that E14.5 gut NCSC colonies averaged 41,000 ± 25,000 neurons/colony, while P15 gut NCSCs averaged 16,000 ± 13,000 neurons per colony. Thus the frequency of noradrenergic neurons declined much more precipitously than the total neurons per colony over this developmental interval.

Enteric neural crest progenitors have been observed transiently express TH and DβH in early embryonic development (before E15) prior to differentiating. To be certain that these NCSC cultures did not contain proliferating TH or DβH-positive progenitors, we added BrdU for the final 24 hr of culture prior to immunohistochemical staining. Consistent with their neuronal morphology, double labeling experiments with TH and BrdU indicated that the vast majority of TH^+ cells were not proliferative: while 22% of all cells in colonies containing TH^+ cells were $BrdU^+$, only 1.9% of the cells expressing TH had incorporated BrdU.

Reduced Neuronal Subtype Potential May Result from Decreased BMP Sensitivity

We investigated the mechanism by which postnatal NCSCs lose the potential to form serotonergic and noradrenergic neurons. BMPs are necessary and sufficient for the differentiation of noradrenergic neurons in vivo (Reissman et al., 1996; Schneider et al., 1999), and BMPs promote the differentiation of at least certain types of neurons from embryonic enteric progenitors in culture (Pisano et al., 2000). BMPs promote Mash-1 expression in NCSCs, and both serotonergic and noradrenergic differentiation are Mash-1-dependent (Guillemot et al., 1993; Blaugrund et al., 1996). Therefore, a possible mechanism by which NCSCs might lose the ability to generate serotonergic and noradrenergic neurons is by losing their sensitivity to the neurogenic effects of BMPs.

To test this, E14.5 and P15 gut NCSCs were cultured at clonal density with BMP4 for 24 hr or 5 days and stained for Mash-1 or Peripherin, respectively. Although BMP4 significantly increased neuronal differentiation in colonies formed by E14.5 gut $p75^+\alpha_4^+$ cells, as judged by either Mash-1 or Peripherin expression, it did not promote neuronal differentiation based on morphology or Mash-1 or Peripherin expression in colonies formed by P15 gut $p75^+$ cells (Table 4). To ensure that neurons were not dying from a lack of trophic factor support in the P15 cell cultures, we supplemented these cultures with GDNF, NT3, and NGF, but this did not increase the number of Peripherin+ neurons that arose in the presence of BMP4 (data not shown). This suggests that the mechanism by which P15 gut NCSCs lose the ability to make serotonergic and noradrenergic neurons in culture involves a loss of responsiveness to the neurogenic effects of BMPs.

Postnatal Gut NCSCs Are More Responsive to Gliogenic Factors Than Embryonic Gut NCSCs

Based on an analysis of rats that were administered BrdU from P14–P16, we detected many more newborn glial cells than neurons in the gut during this period (data not shown). This suggests that gliogenesis predominates in the P15 gut just as in the E14 sciatic nerve (Bixby et al., 2002). We thus tested whether P15 gut NCSCs exhibit increased sensitivity to gliogenic factors relative to E14 gut NCSCs. Figure 8 shows that postnatal gut NCSCs became increasingly responsive to the gliogenic effects of soluble Notch ligand Delta-Fc with increasing time after birth. Although few or no glial only gliocolonies were detected from E14.5 gut NCSCs treated with soluble Delta, P5 and P15 gut NCSCs did generate significantly increased numbers of glial only colonies after treatment with soluble Delta. Treatment with soluble Delta increased the formation of glia only colonies by P15 NCSCs by over 40% while increasing plating efficiency by only 10%–13%. These results are consistent with Notch promoting gliogenesis through an instructive mechanism as we previously documented (Morrison et al., 2000b). Postnatal gut NCSCs become responsive to the gliogenic effect of Notch activation in a way that is not observed among E14.5 gut NCSCs. P15 gut NCSCs are also more responsive to the gliogenic effects of Neuregulin than E14 gut NCSCs (data not shown).

Temporal Changes in the Responsiveness of Gut NCSCs to Lineage Determination Factors Affect Cell Fate Determination In Vivo

P15 gut NCSCs are less responsive to the neurogenic factor BMP4 (Table 4) but are more responsive to gliogenic factors (Figure 8) than E14.5 gut NCSCs. If temporal changes in the responsiveness of gut NCSCs to lineage determination factors affect cell-fate determination in vivo, then P15 gut NCSCs should give rise primarily to glia upon transplantation into developing chick nerves in contrast to E14.5 gut NCSCs, which gave rise primarily to neurons (Bixby et al., 2002). To test this, we examined the peripheral nerves of chicks injected with uncultured P15 gut NCSCs (Figure 4). Six of eight chicks exhibited glia in their peripheral nerves (17.5 cells/positive section) while one of eight chicks had a single neuron in a peripheral nerve. Thus P15 gut NCSCs are biased toward adopting a glial fate in developing peripheral nerves like E14.5 sciatic nerve NCSCs, but unlike E14.5 gut NCSCs. This demonstrates that NCSCs undergo perinatal changes in their responsiveness to lineage determination factors that affect cell fate determination in vivo.

Discussion

Individual cells from the postnatal (P5–P22) and adult gut (P65–P110) formed large colonies in culture containing neurons, glia, and myofibroblasts (Figure 1 and Figure 2), just as fetal NCSCs do (Stemple and Anderson, 1992;Shah et al., 1994;Morrison et al., 1999;Bixby et al., 2002). These multipotent progenitors were isolated by flow-cytometry as $p75^+$ cells (Table 1), and they self-renewed in culture (Table 2), confirming their identity as NCSCs. Uncultured P15 gut $p75^+$ cells gave rise to neurons and glia upon transplantation into the neural crest migration pathway of chick embryos (Figure 4). NCSCs, like CNS stem cells, persist in the adult nervous system.

Gut NCSCs Undergo Perinatal Developmental Changes that Parallel Changes Observed in Hematopoietic Stem Cells

Within the nervous system it has not been clear whether postnatal neural stem cells retain the ability to make subtypes of neurons that are normally produced during fetal development; however, studies of mixed populations of neural progenitors have suggested that they lose the ability to make some subtypes of neurons postnatally. Cells from the P5–P10 forebrain subventricular zone failed to form projection neurons upon transplantation into E15 lateral ventricle (Lim et al., 1997). Other studies of mixed populations of cortical progenitors also

suggested that progenitors undergo cell-intrinsic restrictions during fetal development in the types of neurons they can generate (Barbe and Levitt, 1991; Desai and McConnell, 2000). Even though these studies did not employ isolated stem cells, it is reasonable to expect that the observed restrictions were present within stem cells as well as restricted progenitors. Another possibility is that stem cells were rare within the cell populations that were studied and that the stem cells themselves may have retained a broader developmental potential.

In this study, we have prospectively identified and isolated both embryonic and postnatal NCSCs and compared their ability to generate different subtypes of neurons in culture. P15 gut NCSCs retained the ability to generate neurons that expressed a variety of neurotransmitters that are normally found within the ENS. Nearly all P15 gut NCSC colonies contained large numbers of neurons that expressed NPY, VIP, and/or nNOS (Figure 5). However, postnatal gut NCSCs gradually lost the ability to make serotonergic neurons in culture. Since serotonergic neurons become postmitotic by E15 and have not been observed to form postnatally (Pham et al., 1991), the data suggest that postnatal gut NCSCs lose the ability to form a subtype of neuron that forms only during fetal development. Gut NCSCs undergo perinatal restrictions in neuronal subtype potential that parallel the perinatal restrictions in lymphocyte subtype potential observed among hematopoietic stem cells (Hayakawa et al., 1985;Ikuta et al., 1990;Kantor et al., 1992). Gut NCSCs also underwent perinatal changes in self-renewal potential and mitotic activity analogous to those documented in hematopoietic stem cells (Table 2, Figure 3).

In addition to losing the ability to generate serotonergic neurons, postnatal gut NCSCs also partially lost the ability to generate neurons that expressed TH and/or DβH (Table 3, Figure 7; see Supplemental Figure S2 online at http://www.neuron.org/cgi/content/full/35/4/657/DC1). Embryonic quail gut neural crest progenitors were previously observed to give rise to noradrenergic neurons after backtransplantation into the embryonic chick neural crest migration pathway (Rothman et al., 1990). In another study, enteric neuroblasts retained the ability to undergo noradrenergic differentiation at E14.5, but by E19.5, most neuroblasts failed to undergo noradrenergic differentiation (Pisano and Birren, 1999). Since most P5 gut NCSCs retained the ability to generate TH+ and DβH+ neurons, our data suggest that NCSCs retain noradrenergic potential later in development than restricted neuronal progenitors, though even in stem cells this potential declines with increasing age.

The loss of responsiveness of postnatal gut NCSCs to the neurogenic effects of BMPs may account for their reduced serotonergic and noradrenergic potential. The differentiation of serotonergic and noradrenergic neurons is Mash-1 dependent (Blaugrund et al., 1996), and BMPs induce Mash-1 expression in sympathoadrenal progenitors and enteric progenitors (Reissman et al., 1996; Schneider et al., 1999; Pisano et al., 2000). We found that while E14.5 gut NCSCs were highly responsive to the neurogenic effects of BMP4, P15 gut NCSCs did not detectably respond to BMP4 (Table 4). Although P15 gut NCSCs were unresponsive to BMP4 upon initial isolation, they were ultimately able to undergo at least limited noradrenergic differentiation after 18 days in culrons ture (Table 3, Figure 7, Supplemental Figure S2). This suggests that at least some progenitors within differentiating NCSC colonies may acquire BMP responsiveness. Further experiments will be required to determine the molecular mechanism that accounts for the reduced BMP responsiveness of postnatal gut NCSCs.

The insensitivity of postnatal gut NCSCs to the neurogenic effects of BMPs suggests that other factors likely promote neurogenesis in the postnatal ENS. This is consistent with the finding that the differentiation of some enteric neurons is Mash-1 independent (Blaugrund et al., 1996); however, the identity of these alternative neurogenic signals is unknown.

In Vivo Engraftment of Uncultured P15 Gut NCSCs

Postnatal gut NCSCs gave rise to only small numbers of neurons after transplantation into chick embryos, consistent with the observation that they are BMP insensitive at isolation. Nonetheless, they did form some neurons in the sympathetic ganglion, an environment in which neurogenesis is normally driven by BMP stimulation (Reissman et al., 1996). One possibility is that a subset of the transplanted P15 gut NCSCs acquired increased BMP sensitivity as a result of backtransplantation into the neural crest migration pathway and underwent neurogenesis in response to BMPs (Table 3, Figure 7, Supplemental Figure S2). An alternative possibility is that the P15 gut NCSCs formed a BMP-independent type of neuron in the sympathetic ganglion. Distinguishing between these possibilities will require further study.

In addition to exhibiting decreased sensitivity to the neurogenic effects of BMP4, P15 gut NCSCs also exhibited increased sensitivity to the gliogenic effects of Delta and Neuregulin. Thus gut NCSCs undergo perinatal changes in their responsiveness to lineage determination factors that are analogous to changes observed between E10.5 migrating NCSCs and E14.5 postmigratory NCSCs (White et al., 2001; Kubu et al., 2002). Consistent with our observation that sensitivity to gliogenic factors predisposes E14.5 sciatic nerve NCSCs toward gliogenesis in developing peripheral nerve (Bixby et al., 2002), the P15 gut NCSCs also preferentially underwent gliogenesis upon transplantation into developing chick nerves. Taken together, the data indicate that NCSCs exhibit spatial and temporal differences in responsiveness to lineage determination factors that affect the way the cells differentiate in vivo. Regulation of responsiveness to lineage determination factors may be a general mechanism by which stem cells intrinsically regulate the process of cell fate determination while remaining multipotent.

In this study, the P15 gut NCSCs engrafted only in structures proximal to the hindlimb bud somites (sympathetic chain, peripheral nerves) into which the NCSCs were transplanted. The P15 gut NCSCs did not detectably engraft in more distal neural crest structures such as gut or Remak's ganglion. This contrasted to E14.5 NCSCs, which consistently gave rise to neurons in Remak's ganglion and which sometimes engrafted in gut or pelvic plexus (Morrison et al., 1999; White et al., 2001; Bixby et al., 2002). Since α_4 integrin has been implicated in the migration of neural crest cells (Kil et al., 1998), postnatal gut NCSCs may have lost some of their ability to migrate as a result of losing α_4 integrin expression (Figure 1D). Unlike most embryonic gut NCSCs that must colonize the length of the gut (Burns and LeDouarin, 1998) postnatal gut NCSCs may not need to migrate long distances in order to differentiate within their normal environment. The loss of α_4 integrin expression by postnatal gut NCSCs may be an adaptive response to their altered need to migrate.

Why Are Neural Crest Stem Cells in the Adult ENS?

We do not yet know the functional significance of NCSCs in the adult gut. Additional studies will be required to examine whether there is neurogenesis or gliogenesis in the adult gut and whether there is any response to injury. The persistence of NCSCs in the adult ENS creates new possibilities for repair after gut injury or transplantation from the gut to other sites of injury in the PNS. Given the remarkable similarities between the CNS and the ENS (Gershon et al., 1994), it is particularly interesting that the adult ENS maintains NCSCs. It will be exciting to determine whether the persistence of adult neural stem cells in both the CNS and ENS is related to structural or functional similarities between these tissues.

Experimental Procedures

Isolation of Postnatal Gut Cells

Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). The small intestine was separated from attached mesentery and placed in cold Ca, Mg-free HBSS (Gibco, Grand Island,

NY). Outer muscle/plexus layers were peeled free of the underlying epithelium as previously described (Schafer et al., 1997), minced, and dissociated in 0.025% trypsin/EDTA (Gibco product 25300-054) plus 1 mg/ml type 4 collagenase (Worthington, Lakewood, NJ) in Ca, Mg-free HBSS for 8 min at 37°C. Adult gut was dissociated for 20 min in the same enzymes. The digestion was quenched with two volumes of staining medium (for recipe see Experimental Procedures in Bixby et al., 2002). After centrifuging, gut cells were triturated, filtered through a nylon screen (45 μm, Sefar America, Kansas City, MO) to remove aggregates of cells and undigested tissue, and resuspended in staining medium. E14.5 gut, including stomach, small intestine, and hindgut, were dissected and dissociated as described (Bixby et al., 2002).

Flow-Cytometric Isolation of NCSCs and Cell-Cycle Analysis

Flow cytometric isolation of gut NCSCs was performed as described (Bixby et al., 2002). For cell cycle analyses using Hoechst 33342, dissociated gut cells were suspended at a concentration of 1–2 × 10^6 cells/ml in staining medium containing 4 μg/ml Hoechst 33342 (Sigma) and 50 μg/ml verapamil (Sigma) to block MDR-mediated Hoechst efflux. Cells were incubated for 45 min at 37°C and agitated every 5–10 min to prevent settling. Immediately after incubation, the cells were put on ice and stained with antibodies as described above. Hoechst staining of NCSCs was assayed by flow-cytometry.

Cell Culture

Tissue culture plate preparation, culture medium, and culture conditions were as described (Bixby et al., 2002). To promote the differentiation of noradrenergic neurons, NCSCs were cultured in standard medium for 6 days, followed by 6 days in differentiation medium supplemented with 5 μM forskolin (Sigma) and 1 ng/ml BMP4 (R&D Systems), followed by a final 6 days in differentiation medium supplemented with 50 ng/ml nerve growth factor and neurotrophin-3 as previously described (Morrison et al., 2000a).

BrdU Labeling In Vivo

The mitotic activity of postnatal gut NCSCs in vivo was assayed by administering 5′-bromo-2′-deoxyuridine (BrdU, Sigma) to rat pups for 20 hr prior to dissecting guts at P15. 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 20, 16, and 2 hr before dissection. In one experiment, additional injections were performed 18 and 14 hr before dissection. NCSCs were isolated from the BrdU administered or control rats, allowed to adhere to tissue culture plates for 18 hr, and stained with an antibody against BrdU (Caltag product IU-4, Burlingame CA) (Raff et al., 1988).

Immunocytochemistry

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 (Chemicon AB1530; Temecula CA), GFAP (Sigma G-3893), and α SMA (Sigma A-2547) as described (Shah et al., 1994). Mash-1 staining was performed as described (Shah et al., 1994). Plates stained for neuronal subtype markers were fixed in fresh 4% paraformaldehyde for 10 min at room temperature, washed, and blocked as described (Shah et al., 1994). Antibodies were obtained as follows: Tyrosine Hydroxylase (TH) (Chemicon, product #MAB5280), Dopamine-β-hydroxylase (DβH; BD PharMingen, #556313), Neuropeptide Y (NPY; Peninsula Laboratories, #IHC 7161, San Carlos, CA), Vasoactive Intestinal Peptide (VIP; Peninsula Laboratories, #IHC 7180), neuronal Nitric Oxide Synthase (nNOS; Chemicon, #AB5380), and Tryptophan Hydroxylase (Chemicon, #AB1541). In some experiments, plates were colabeled with NeuN (Chemicon, #MAB377) to confirm that neuronal subtype antibodies were labeling neurons. For p75 (Promega prod. 1405-41-0) staining of gut sections, tissue was fixed in freshly prepared 4% paraformaldehyde and embedded in paraffin.

In Vivo Transplantation of Neural Crest Progenitors

Fertile White Leghorn eggs (Bilbie Aviaries, Ann Arbor, MI) were incubated to Hamburger and Hamilton stage 17–18 (Hamburger and Hamilton, 1951) and injected with P15 gut $p75^+$ cells. Approximately 70,000 $p75^+$ cells were isolated by flow-cytometry, backloaded into a drawn glass capillary tube (World Precision Instruments, Sarasota FL), centrifuged for 2 min at 52 × g, and injected into the anterior, promedial corner of two somites in the himblimb bud region of each embryo. Injections were performed using a Narishige H-7 pipette holder mounted on a MM-33 micromanipulator (Fine Science Tools, Forester City, CA) and very gentle air pressure. We estimated the number of cells injected per somite as 310 ± 20 cells by labeling sorted P15 $p75^+$ cells with DiI and then counting the number of DiI+cells/somite immediately after injection. Injected embryos were incubated for 3 days to stage 29, fixed, embedded in OCT, and cryostat sectioned. To control for nonspecific hybridization, sections from E16.5 rat and uninjected stage 29 chicks were processed in parallel as positive and negative controls, and sections adjacent to those showing engraftment were probed with the sense strands of *SCG10* and P_0. Since embryos were unilaterally injected, the contralateral side to engraftment also served as a negative control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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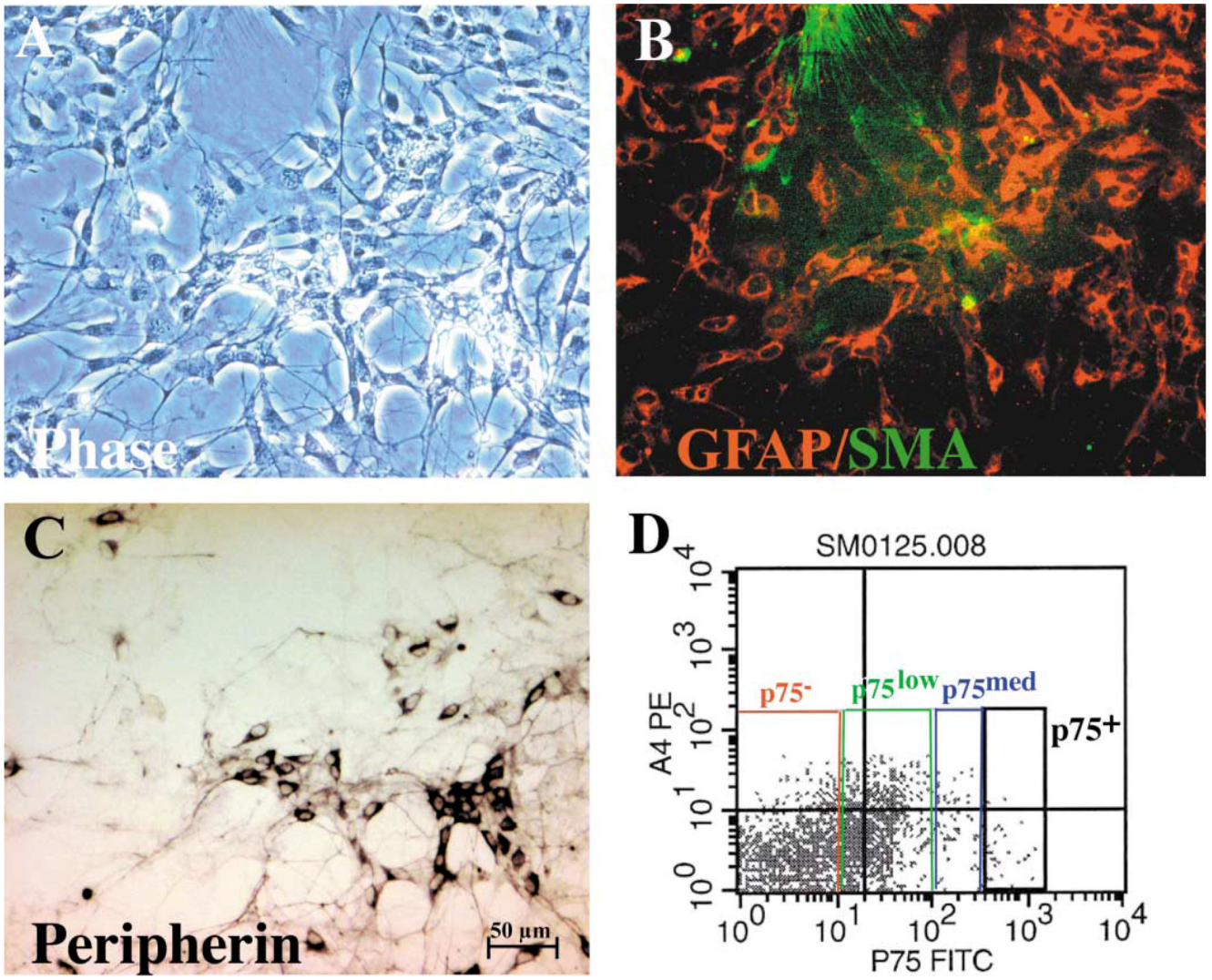

Figure 1. Multipotent Neural Progenitors Persist in the Postnatal Gut

Cells were dissociated from P15 gut and cultured at clonal density (40 cells/35 mm dish) for 14 days, then stained for neurons (Peripherin, [C]), glia (glial fibrillary acidic protein, red in [B]), and myofibroblasts (smooth muscle actin, green in [B]). One field of view from a typical multilineage colony is shown (A–C). Freshly dissociated P15 gut cells were also analyzed by flow-cytometry for expression of the neurotrophin receptor p75 and α_4 integrin (D). Cells in the regions marked on the flow-cytometry plot shown in (D) were sorted into clonal culture for analysis. Multipotent progenitors were enriched in the $p75^+$ fraction but depleted in other fractions (see Results).

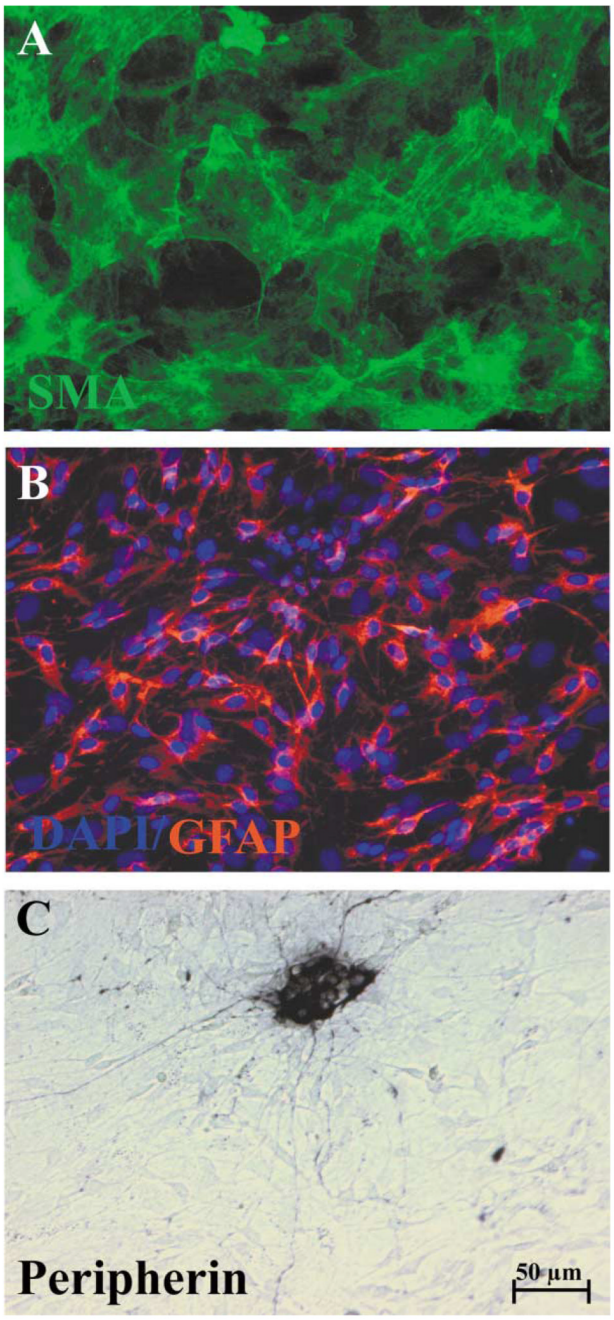

Figure 2. Multipotent Neural Crest Progenitors Are Present in Adult Gut

One field of view from a typical colony cultured from adult rat gut was stained for neurons (Peripherin; [C]), glia (GFAP; [B]), and myofibroblasts (SMA; [A]) as described in Figure 1. DAPI was used to label all cell nuclei (blue in [B]). Multipotent adult colonies were estimated to average 20,000 ± 7000 total cells/colony and 500±770 neurons/colony after 14 days of culture.

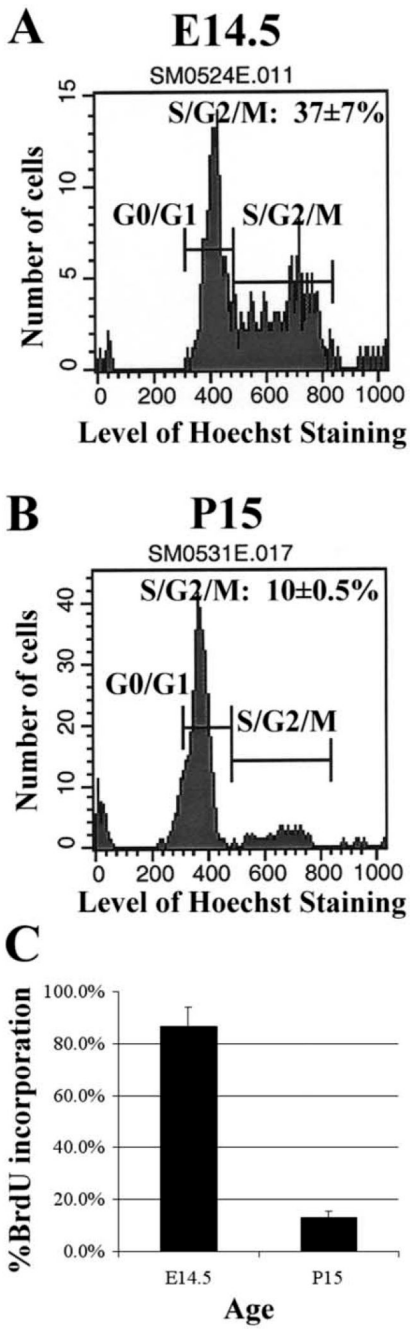

Figure 3. Postnatal Gut NCSCs Are Significantly Less Mitotically Active Than Embryonic Gut NCSCs

Cells were stained with antibodies against p75 and α_4 integrin to identify E14.5 and P15 gut NCSCs, and the amount of DNA per cell was quantitated by flow-cytometry using the DNA-intercalating dye Hoechst 33342 (Morrison and Weissman, 1994). The percentage of E14.5 (A) and P15 (B) gut NCSCs in G0/G1 (2N DNA content) and S/G2/M (>2N DNA content) phases of the cell cycle was determined. To compare the rate at which NCSCs divide in vivo, BrdU was administered to rats for a 20 hr period in vivo prior to isolating and assaying $p75^{+}\alpha_4{}^{+}$ E14.5 gut NCSCs or $p75^{+}$ P15 gut NCSCs for BrdU incorporation. An average of 87% of E14.5 NCSCs (but only 13% of P15 gut NCSCs) incorporated BrdU and therefore

divided over this period in vivo. These data indicate that E14.5 gut NCSCs divide more rapidly or are less frequently quiescent than P15 gut NCSCs.

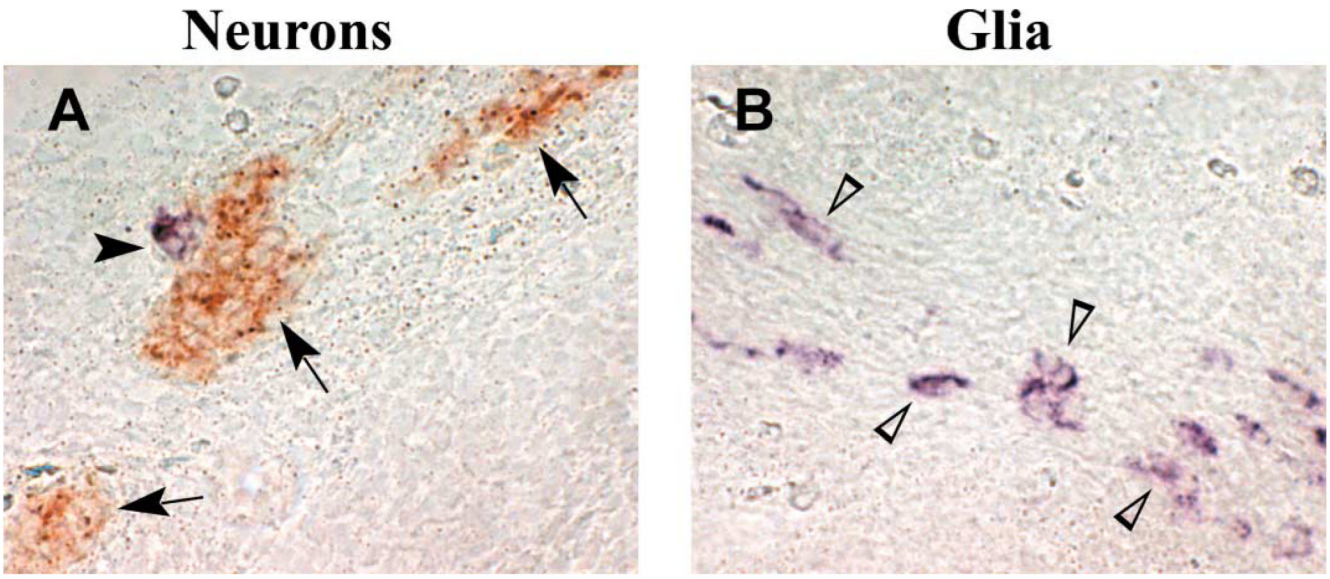

Figure 4. P15 Gut NCSCs Give Rise to Neurons and Glia In Vivo

Uncultured P15 rat gut p75^{+} cells were isolated by flow-cytometry and injected into two hindlimb bud somites of stage 18 chick embryos. After approximately 72 hr of development (stage 29), embryos were fixed and frozen and then sectioned at the level of the hindlimb. Sections were hybridized in situ with probes against the neuronal marker *SCG10* and the glial marker *P*$_0$. Hybridization with neuron-specific probes is shown in (A), where the purple signal indicates *SCG10*$^{+}$ rat neurons (arrowheads) and the orange signal indicates chick neurons (arrows) in a sympathetic ganglion. Hybridization with a rat-specific probe against the glial marker *P*$_0$ ([B], open arrowheads) showed rat glia in chick peripheral nerves. Both (A) and (B) are from the same embryo.

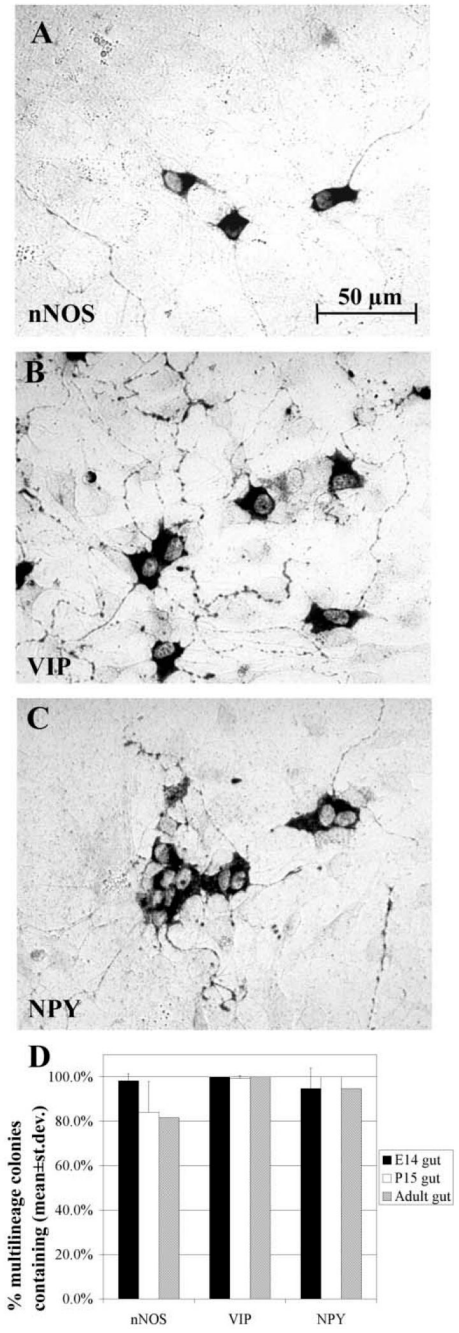

Figure 5. p75$^+$ NCSCs Give Rise to Neurons in Culture that Express a Variety of Neurotransmitters Normally Found in the Enteric Nervous System

Gut p75$^+$ cells were cultured under standard conditions for 14 days and then stained with antibodies against neuronal Nitric Oxide Synthase (nNOS) (A), Neuropeptide Y (NPY) (C), or Vasoactive Intestinal Peptide (VIP) (B). nNOS, NPY, and VIP were present within neuronal cell bodies and neurites as expected. (A)–(C) are photos taken of adult gut NCSC colonies. Nearly all multilineage colonies formed by E14.5 p75$^+\alpha_4{}^+$ NCSCs and P15 and adult gut p75$^+$ NCSCs contained nNOS+ cells, NPY+ cells, and VIP+ cells with neuronal morphologies (D). No differences were detected between E14.5 and P15 gut NCSCs in either the frequency of colonies containing cells that stained with these markers (D) or in the frequency of cells

within colonies that stained with these markers, though adult colonies appeared to contain a lower density of nNOS+ cells (data not shown).

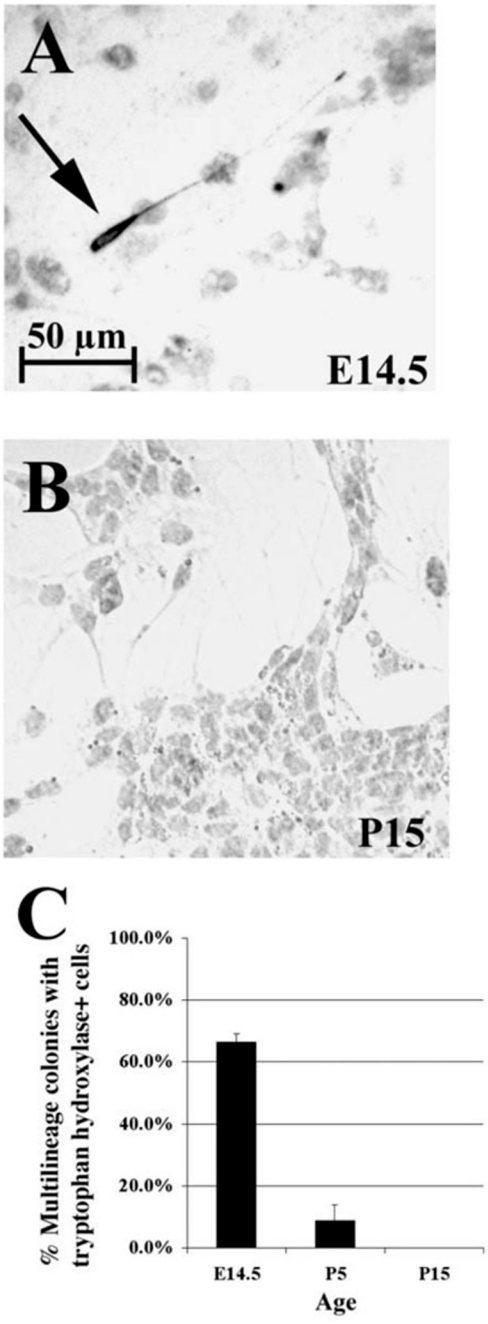

Figure 6. E14.5, But Not P15, Gut NCSCs Generate Serotonergic Neurons in Culture

E14.5, P5, and P15 gut NCSCs were cultured under standard conditions for 14 days and stained for the presence of serotonergic neurons using an antibody against Tryptophan Hydroxylase. Serotonergic neurons were present at low density in the majority of multilineage E14.5 gut colonies (arrow in [A]) but were not detected in P15 gut multilineage colonies (B). The decline with age in the percentage of multilineage colonies containing any serotonergic neurons is shown in (C). A significantly higher fraction of E14.5 gut multilineage colonies contained serotonergic neurons than did either P5 or P15 gut colonies ($p < 0.001$).

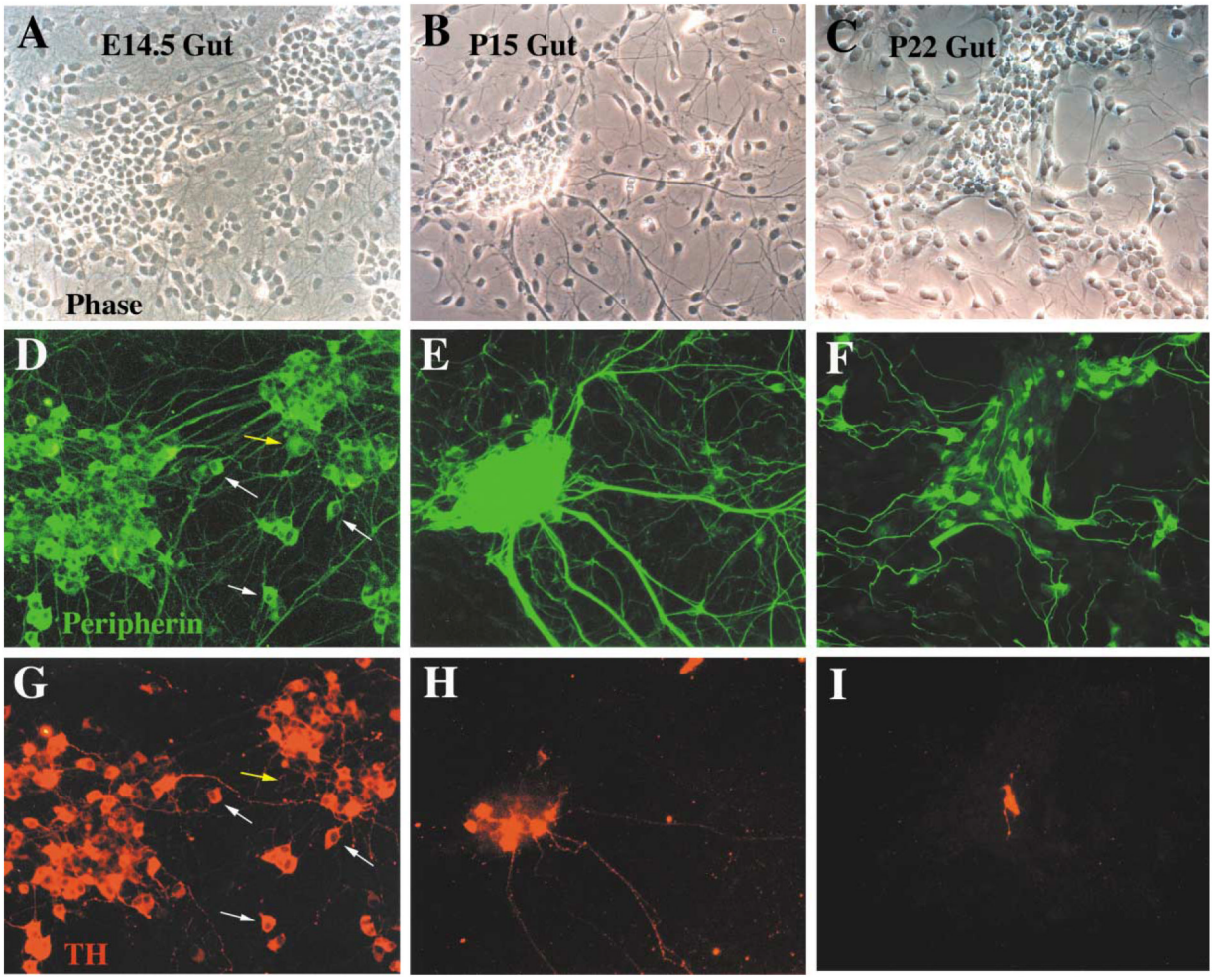

Figure 7. Postnatal Gut NCSCs Decline in Their Ability to Form Tyrosine Hydroxylase-Expressing Neurons with Increasing Age

NCSCs from E14.5, P15, and P22 gut were cultured at clonal density under conditions that support the differentiation of noradrenergic neurons (Morrison et al., 2000a). One field of view from a typical E14.5 gut NCSC colony (A, D, and G), P15 NCSC colony (B, E, and H), or P22 NCSC colony (C, F, and I) is shown in phase contrast (A–C), with Peripherin epifluorescence (D–F), or with tyrosine hydroxylase (TH) epifluorescence (G–I). Double labeling of similar cultures with Peripherin and Dopamine-β-hydroxylase (DβH) is shown in Supplemental Figure S2. In both cases, the number of neurons per colony that expressed TH or DβH declined significantly with increasing NCSC age. Among colonies that contained TH+ cells, E14.5 gut NCSC colonies averaged 307 ± 244 TH+ neurons/colony, P5 gut NCSC colonies averaged 11 ± 10 TH+ neurons/colony, P10 NCSC colonies averaged 7 ± 10 TH+ neurons/colony, and P15 gut colonies had only 2 ± 1. Typical neurons that costained for TH and Peripherin are shown with white arrows (D and G), and a neuron labeled with Peripherin, but not TH, is shown with a yellow arrow (D and G). Note that TH+ cells tended to be clustered within part of each colony, so although most Peripherin+ neurons appear TH+ in (D) and (G), only a minority of Peripherin + neurons within the overall colony were TH+.

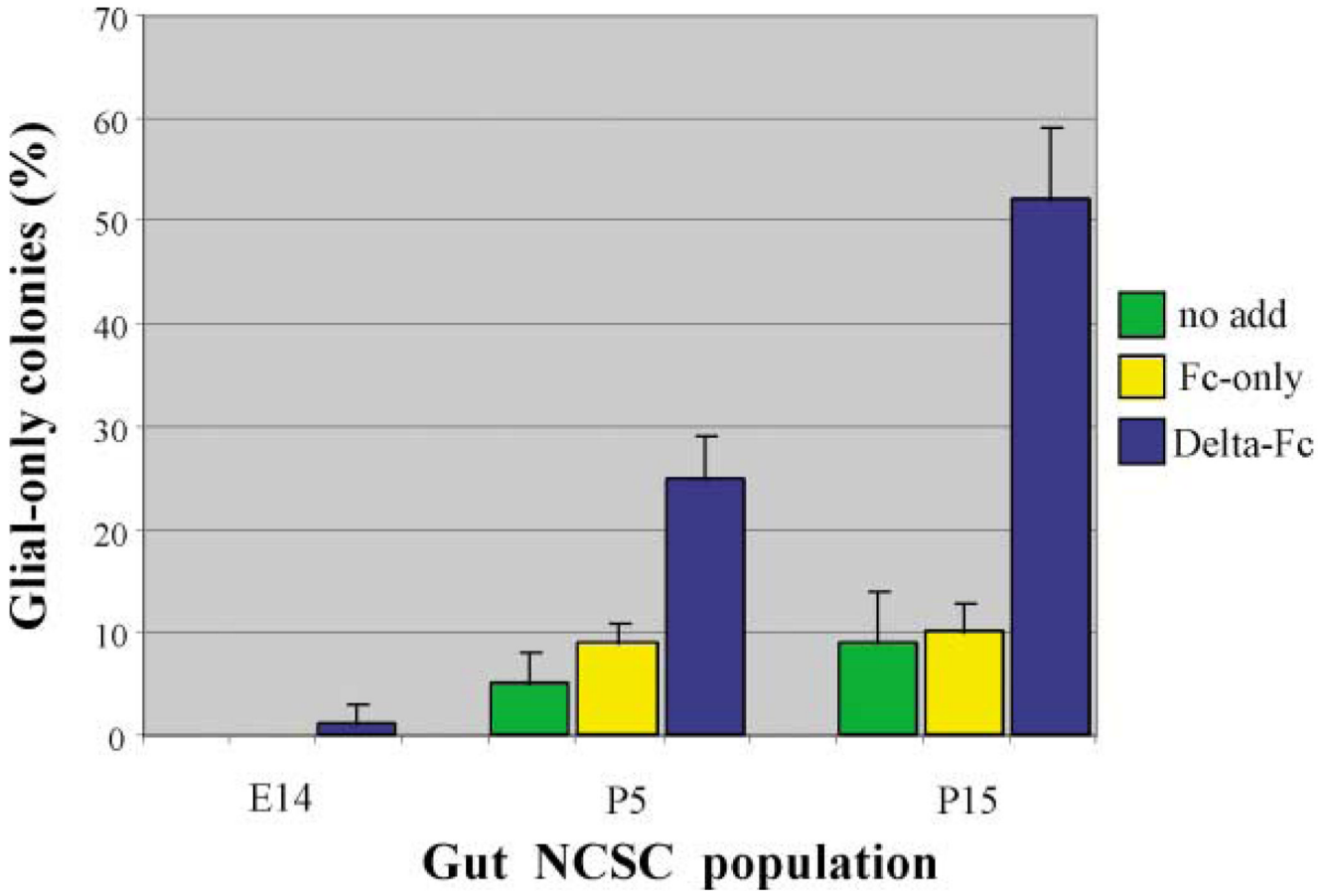

Figure 8. Gut NCSCs Become Increasingly Responsive to the Gliogenic Effect of Soluble Delta as Development Proceeds

Freshly isolated NCSCs were cultured for 24 hr at clonal density (40 cells/well) in standard medium (no add) or standard medium supplemented with either Fc or Delta-Fc (Morrison et al., 2000b). These factors were then washed out of the cultures, and the cells were cultured under standard conditions for another 13 days. There were no significant differences between NCSCs cultured in no add and Fc only conditions. Delta treatment did not significantly increase the frequency of glial only colonies from E14 gut NCSCs, but it did significantly increase the frequency of glial only colonies from P5 and P15 gut ($p < 0.05$). 29%–43% of E14 cells, 45%–67% of P5 cells, and 54%–67% of P15 gut cells survived to form colonies in culture. The data are based on three independent experiments.

Table 1

Multipotent Neural Crest Progenitors Can Be Isolated as $p75^+$ Cells from the Postnatal and Adult Gut

		Colonies that Contain the Indicated Cell Types (%)					
	Plating Efficiency (%)	**N + G + M**	**N + M**	**N + G**	**G + M**	**G Only**	**M Only**
E14.5 $p75^+$ α_4^+	45 ± 9	61 + 21	3 ± 4	5 ± 5	11 ± 13	4 ± 4	15 ± 15
P5 $p75^+$	51 ± 9	65 ± 18	1 ± 1	14 ± 11	7 ± 7	6 ± 7	6 ± 4
P10 $p75^+$	44 ± 6	58 ± 25	0 ± 0	29 ± 35	6 ± 8	3 ± 3	4 ± 5
P15 $p75^+$	52 ± 12	66 ± 11	0 ± 0	12 ± 8	7 ± 5	11 ± 8	4 ± 5
P22 $p75^+$	50 ± 14	67 ± 18	0 ± 0	1 ± 2	13 ± 7	17 ± 7	1 ± 1
P65-P110 $p75^+$	9 ± 7	17 ± 23	0 ± 0	1 ± 1	22 ± 25	53 ± 28	7 ± 14

E14.5 gut $p75^+$ $\alpha4^+$ cells or postnatal gut $p75^+$ cells were isolated by flow-cytometry and cultured at clonal density (40 cells/35 mm dish) for 14 days under standard conditions, then stained with antibodies against neurons (N; Peripherin+), glia (G; GFAP+), and myofibroblasts (M; SMA+). Although postnatal gut cells were routinely stained for α4 integrin, the great majority of $p75^+$ cells were $\alpha4^-$, so use of the α4 marker did not increase NCSC enrichment. Therefore, postnatal gut NCSCs were routinely isolated based only on their expression of high levels of p75 (see Figure 1). N + G + M colonies contained neurons, glia, and myofibroblasts, while M only colonies contained only myofibroblasts. Each data point is shown as mean ± standard deviation. Percentages do not necessarily add up to 100 because up to 2% of colonies did not stain with any marker. At P15, 6% ± 2% of unfractionated cells formed colonies and 13% ± 4% of colonies were N + G + M. At P22, 4% ± 1% of unfractionated cells formed colonies, and 5% ± 4% of colonies were N + G + M.

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Table 2

Postnatal Gut NCSCs Self-Renew in Culture But the Extent of Self-Renewal Declines Significantly with Increasing Age

	Subclones Per Multipotent Founder Cell					
Population	**Total**	**N + G + M**	**N + G**	**G + M**	**G Only**	**M Only**
E14.5 gut $p75^+\alpha_4^+$	845 a	730 ± 459 a	81 ± 151	27 ± 67	2 ± 5 a	5 ± 10
P5 gut $p75^+$	311 b	146 ± 90 b	15 ± 5	52 ± 23	96 ± 107 b	2 ± 3
P15 gut $p75^+$	386 b	143 ± 143 b	29 ± 50	69 ± 50	142 ± 100 b	3 ± 7
P22 gut $p75^+$	360 b	70 ± 132 b	22 ± 35	24 ± 31	242 ± 100 c	2 ± 4
Adult gut $p75^+$	366	35 ± 49	3 ± 3	110 ± 155	193 ± 230	24 ± 34

Single gut progenitors were deposited into individual wells of 96-well plates. After 8 (E14-P22) or 15 (adult) days in culture, individual colonies were subcloned into secondary cultures at clonal density. Secondary clones were cultured for 14 days and stained with antibodies to identify neurons (N), glia (G), and myofibroblasts (M) as described in Table 1. P22 gut NCSCs gave rise to significantly fewer total subclones and multipotent subclones but significantly more glial only subclones than E14.5 NCSCs ($p < 0.05$). Significantly different statistics are followed by different letters, except for adult subclones, which were not compared because they were subcloned after 15 days in culture.

Table 3

The Ability of Gut NCSCs to Generate Sympathoadrenal Neurons Declines Significantly during Development

	Multilineage Colonies Containing (% ± SD)	
Population	**TH+ Neurons**	**DβH+ Neurons**
E14.5 Gut	87 ± 3	85 ± 12
P5 Gut	58 ± 5*	82 ± 4
P10 Gut	43 ± 6*	63 ± 21
P15 Gut	20 ± 19*	38 ± 22*

Noradrenergic neurons were identified by colocalization of either Tyrosine Hydroxylase (TH) or Dopmaine-β-hydroxylase (DβH) with the neuronal marker Peripherin. The fraction of NCSCs capable of producing TH+ neurons was significantly decreased in all postnatal NCSCs compared to those at E14.5 (*, $p < 0.005$). The ability of NCSCs to form $D\beta H^+$ neurons did not decline as rapidly with development, but significantly fewer P15 gut NCSCs were capable of forming $D\beta H^+$ neurons than either E14.5 or P5 NCSCs (*, $p < 0.005$).

Table 4

Postnatal Gut NCSCs Are Not Responsive to the Neurogenic Effects of BMP4

		Treatment	% Mash1 + Colonies	Plating Efficiency (%)
24 hr assay	E14.5 $p75^+\alpha_4^+$	No add	36 ± 17	74 ± 13
		+ BMP4	66 ± 3*	68 ± 12
	P15 $p75^+$	No add	0 ± 0	41 ± 5
		+ BMP4	0 ± 0	43 ± 7
		Treatment	% Peripherin + Colonies	Plating Efficiency (%)
5 day assay	E14.5 $p75^+\alpha_4^+$	No add	23 ± 20	45 ± 17
		+ BMP4	90 ± 2*	57 ± 13
	P15 $p75^+$	No add	10 ± 5	48 ± 7
		+ BMP4	6 ± 1	44 ± 12

Gut NCSCs were cultured at clonal density in standard medium with or without 50 ng/ml BMP4. Some cultures were fixed after 24 hr and stained for Mash-1, an early marker of neuronal lineage determination. Other cultures were fixed after 5 days and stained for Peripherin, a marker of mature PNS neurons. Although BMP4 significantly increased Mash-1 and Peripherin expression by E14.5 gut $p75^+\alpha4$+ cells (*, $p < 0.01$), BMP4 did not promote either Mash-1 or Peripherin expression by P15 $p75^+$ cells. Plating efficiency indicated the percentage of cells added to culture that survived to form colonies. BMP4 addition did not significantly affect plating efficiency in any case.