

P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF- β family factors

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SUMMARY

Protein zero (P0) and peripheral myelin protein 22 (PMP22) are most prominently expressed by myelinating Schwann cells as components of compact myelin of the peripheral nervous system (PNS), and mutants affecting P0 and PMP22 show severe defects in myelination. Recent expression studies suggest a role of P0 and PMP22 not only in myelination but also during embryonic development. Here we show that, in dorsal root ganglia (DRG) and differentiated neural crest cultures, P0 is expressed in the glial lineage whereas PMP22 is also detectable in neurons. In addition, however, P0 and PMP22 are both expressed in a multipotent cell type isolated from early DRG. Like neural crest stem cells (NCSCs), this P0/PMP22-positive cell gives rise to glia, neurons and smooth-muscle-like cells in response to instructive extracellular cues. In cultures of differentiating neural crest, a similar multipotent cell type can be identified in which expression of P0 and PMP22

precedes the appearance of neural differentiation markers. Intriguingly, this P0/PMP22-positive progenitor exhibits fate restrictions dependent on the cellular context in which it is exposed to environmental signals. While single P0/PMP22-positive progenitor cells can generate smooth muscle in response to factors of the TGF- β family, communities of P0/PMP22-positive cells interpret TGF- β factors differently and produce neurons or undergo increased cell death instead of generating smooth-muscle-like cells. Our data are consistent with a model in which cellular association of postmigratory multipotent progenitors might be involved in the suppression of a non-neural fate in forming peripheral ganglia.

Key words: Protein zero, Peripheral myelin protein 22, Neural crest, Dorsal root ganglia, Multipotency, Community effect, TGF- β factors

INTRODUCTION

The multiple cell types of the vertebrate peripheral nervous system (PNS) are generated from a transient population of neural crest stem cells (NCSCs) derived from the dorsal portion of the developing neural tube (Le Douarin, 1982). A central issue in developmental biology is to understand how an initially multipotent stem cell becomes fate restricted giving rise to distinct cell types at precise embryonic locations (Edlund and Jessell, 1999). Cell type-specificity could be achieved by the differentiation of committed precursors, or could reflect the action of local cues imposing particular fates on pluripotent cells (reviewed in Anderson et al., 1997). Multipotency of neural crest cells has been demonstrated both in avian and rodent neural crest cells (Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988; Fraser and Bronner-Fraser, 1991; Ito et al., 1993; Sextier-Sainte-Claire Deville et al., 1992; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). Clonal analysis, transplantation and lineage-tracing experiments revealed that in vitro and in vivo pluripotent cells coexist with lineage-restricted precursors in migrating neural crest and in early crest-derived tissues (reviewed in Anderson et al., 1997; Le Douarin, 1986; Le Douarin and Ziller, 1993). The ratio of fate-restricted precursors to pluripotent cells

present in neural crest-derived structures increases with age. Thus, it is thought that fate restriction of neural crest cells is a progressive process occurring during migration and at sites of differentiation.

The molecular basis of fate restriction in neural crest cells is believed to involve exposure to environmental signals (reviewed in Anderson et al., 1997; Le Douarin and Ziller, 1993). However, it is often unclear whether the observed restriction of neural crest cells to particular fates also represents reduced developmental potential. Is a neural crest-derived cell that in lineage-tracing experiments or in clonal cultures gives rise only to a particular sublineage indeed committed to this lineage? Demonstrating lineage commitment requires a challenge to the precursor cells by changing the environment of the cell, a process that might induce alternative cell fates. Back-transplantation into avian embryos during early phases of neural crest migration suggested that late emigrating neural crest cells display restricted developmental capacities (Artinger and Bronner-Fraser, 1992). Clonal analysis of NCSC cultures identified instructive extracellular signals promoting particular sublineages at the expense of other fates (Shah et al., 1994, 1996). A neuregulin (NRG1) isoform, glial growth factor (Marchionni et al., 1993) induces gliogenesis, while bone morphogenetic protein 2 (BMP2)

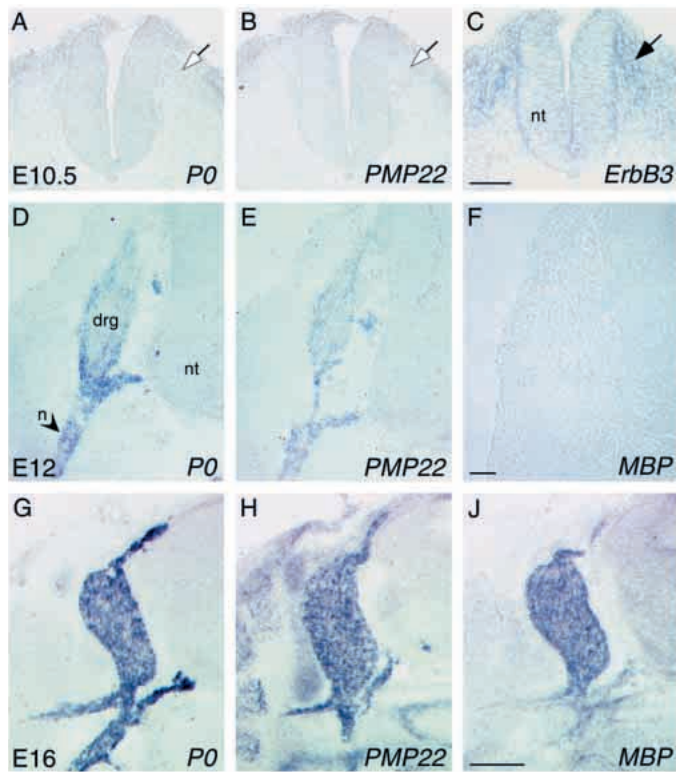


Fig. 1. mRNA expression of *P0* and *PMP22* at early stages of PNS development. Near-adjacent transverse sections of rat (A-C) E10.5, (D-F) E12 and (G-I) E16 embryos were processed for in situ hybridization. Migratory neural crest is marked by *ErbB3* mRNA expression (C; arrow) while *P0* and *PMP22* messages (A and B, respectively; open arrows) are not yet detectable. At E12, peripheral nerves (n) and the forming dorsal root ganglia (drg) express *P0* (D) and *PMP22* (E) but not *MBP* (F). At E16, nerves and drg are strongly labeled by all three riboprobes, *P0* (G), *PMP22* (H) and *MBP* (I). Note also the non-neural expression of *PMP22* (H). nt, neural tube. Scale bars: (A-F) 40 μ m; (G-I) 100 μ m.

promotes autonomic neurogenesis and, to a lesser extent, smooth muscle formation in NCSCs (Shah et al., 1994, 1996). A smooth muscle fate is also promoted by transforming growth factor- β (TGF- β ; Shah et al., 1996). Knowledge of such instructive cues enables testing of whether cellular stages succeeding migratory NCSCs are committed to particular sublineages or instead can be instructed to give rise to alternative fates (Lo and Anderson, 1995; Lo et al., 1997; Morrison et al., 1999).

The identification of instructive cues does not imply that selective mechanisms are not effective in neural crest

development. Fate selection might be achieved by promoting either the survival or the programmed cell death of particular neural crest-derived sublineages. For example, NRG1, endothelins and various neurotrophins have been shown to regulate survival of Schwann cell precursors, progenitor cells of the melanocyte lineage and peripheral neurons, respectively (Dong et al., 1995; Heuckeroth et al., 1998; Lahav et al., 1998; Ockel et al., 1996; Reid et al., 1996; Thoenen, 1991). The development of mature cell types from a multipotent stem cell is thus implemented by a combination of instructive and selective signals provided by extracellular growth factors, the extracellular matrix and cell-cell interactions. In addition, these extrinsic signals are involved in an interplay with cell-intrinsic mechanisms to regulate cellular differentiation (reviewed by Edlund and Jessell, 1999). Given the multiple environmental cues to which a neural crest cell is exposed during migration and at the site of its differentiation, the question arises whether and how different signals are integrated and whether convergence of different signaling pathways elicits differential cellular responses. When NCSC are challenged with combinations of TGF- β , BMP2 and NRG1, the former two factors are dominant (Shah and Anderson, 1997). Intriguingly, NCSCs display decreased sensitivity and delayed response to NRG1 despite the presence of functional receptor. Fibroblast growth factor (FGF) and α -forms of NRG1 depend on the activity of insulin-like growth factor (IGF) to promote survival but not DNA synthesis of Schwann cell precursors (Dong et

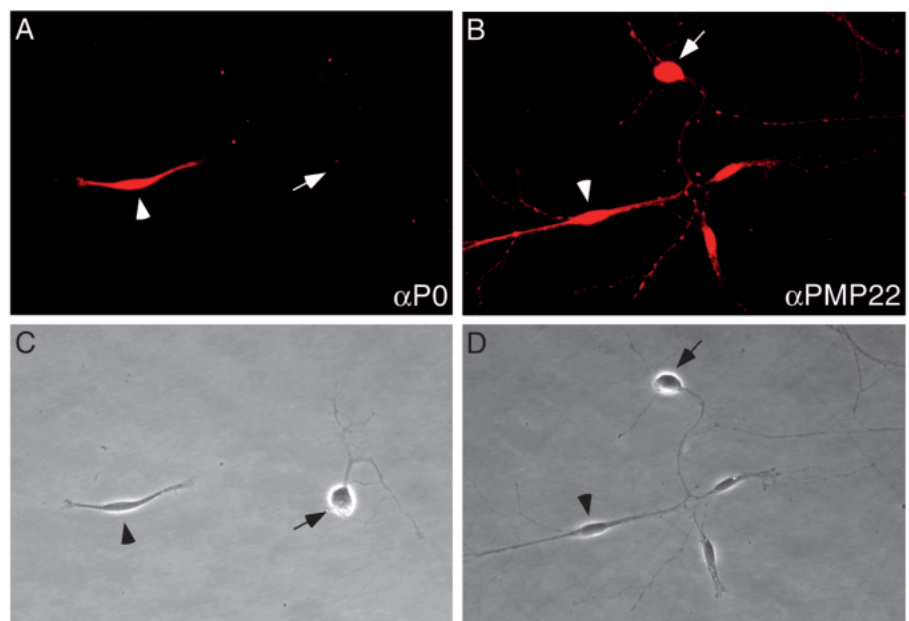


Fig. 2. PMP22 protein is expressed both in neurons and in glial cells of DRG at E16. Dorsal root ganglia isolated from rat E16 embryos were dissociated and the cells were fixed 3 hours after plating. Immunostaining for P0 (A) and PMP22 (B) using a fluorescent Cy3-coupled secondary antibody revealed expression of both proteins in glial cells (arrowheads). In contrast, neurons (arrows) are negative for P0 while they express PMP22 protein. (C,D) Phase-contrast views. Magnification, $\times 20$.

al., 1995; Gavrilovic et al., 1995), whereas glial growth factor alone (a β -form of NRG1; Burden and Yarden, 1997) regulates survival and proliferation (Dong et al., 1995). The molecular basis of these processes remains to be determined.

In this report, we describe the characterization of a multipotent progenitor cell type isolated from early developing dorsal root ganglia (DRG). A similar cell type can also be generated from NCSC cultures. This progenitor is characterized by the expression of P0 and PMP22, two major components of PNS myelin (reviewed by Suter and Snipes, 1995), while other differentiation markers are not yet expressed. The P0/PMP22-positive progenitor can be challenged to give rise to both neural and non-neural crest derivatives, but displays fate restrictions in cell communities.

MATERIALS AND METHODS

Riboprobes and non-radioactive in situ hybridization

Antisense riboprobes were labeled with digoxigenin according to the manufacturer's instruction (Roche Diagnostics). The following riboprobes were used: mouse ErbB3 (a gift from H. Werner, ZMBH, Heidelberg); rat P0 (Lemke and Axel, 1985); rat PMP22 (Welcher et al., 1991); mouse MBP (de Ferra et al., 1985; a gift from A. Gow, Cleveland Clinic Foundation, Ohio). Non-radioactive in situ hybridization with digoxigenin-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse embryos (Birren et al., 1993; Sommer et al., 1996) with the modifications described in Paratore et al. (1999).

Cell cultures

Time-mated OFA rats were obtained from Biological Research Laboratories (Fullinsdorf, Switzerland). DRG were dissected from E14 and E16 embryos, respectively, and dissociated by incubation in 0.25% trypsin (Gibco

BRL), 0.3 mg/ml collagenase type I (Worthington Biochemical, NJ) in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (Amimed) for 25 minutes. 1/10 vol of FBS was added and the cells were centrifuged for 3 minutes at 1800 revs/minute. The cells were washed once in standard culture medium (Stemple and Anderson, 1992), resuspended and plated at 150 cells per 35 mm culture dishes (Corning) coated with 0.5 mg/ml poly-D-lysine (pDL; Roche Diagnostics) and 0.25 mg/ml fibronectin (FN; Roche Diagnostics). DRG cultures were maintained in standard culture medium. In some experiments, the following growth factors were added 3 hours after plating the cells, as previously described (Shah et al., 1994, 1996): 10 nM rhGGF2, a soluble NRG1 isoform (a gift from M. Marchionni, Cambridge NeuroScience, Cambridge MA); 1.6 nM BMP2 (a gift from Genetics Institute, Cambridge MA); 4 pM TGF-

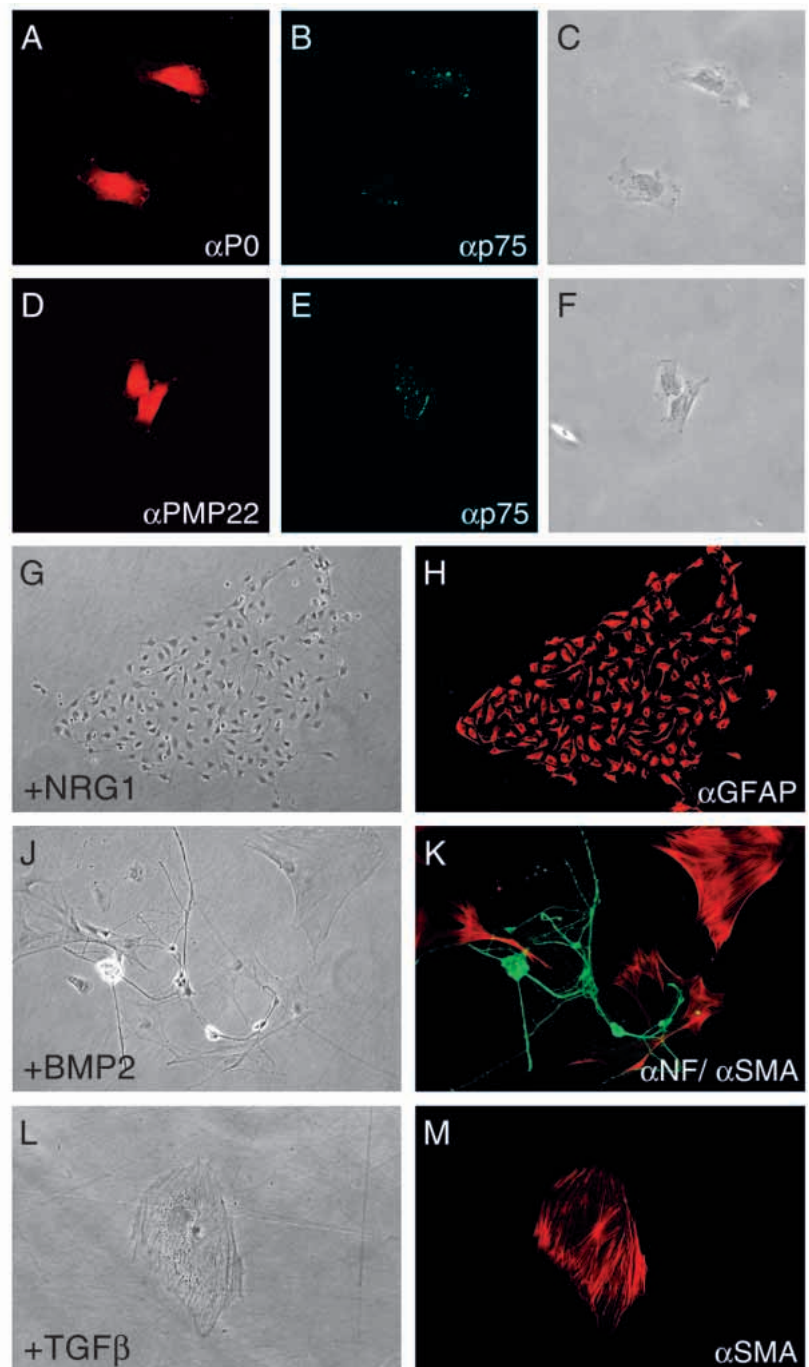


Fig. 3. Developmental potential of a P0/PMP22-expressing cell type isolated from DRG E14. Dissociated rat DRG E14 were plated and living cells were immunolabeled after 3 hours using anti-p75 antibody visualized by a FITC-conjugated secondary antibody (B,E). Subsequently, some culture dishes were fixed and stained for P0 (A) or PMP22 (D). Note that most of the p75⁺ cells with a non-neuronal morphology were double-positive for P0 and PMP22 (see Table 1). On sister culture dishes, with DRG cells plated at clonal density, the position of non-neuronal p75⁺ cells was mapped and the development of individual clones under various culture conditions was monitored. When grown in the presence of NRG1 for 6 days, clones were mostly composed of GFAP⁺ glial cells as detected with a Cy3-coupled secondary antibody (G,H). (J,K) A typical clone generated from a BMP2-treated progenitor cell. The clone is mixed, containing both NF⁺ neurons (visualized with FITC) and SMA⁺ non-neuronal cells (detected by Cy3 fluorescence; K). (L,M) SMA⁺ non-neuronal cells were also produced after TGF- β treatment, which usually suppressed cell division. (C,F,G,J,L) Phase-contrast images. Magnification, (A-F) $\times 32$; (G-K) $\times 10$; (L,M) $\times 20$.

$\beta 1$ (R&D Systems). Single $p75^+$ cells with a non-neuronal morphology were mapped by marking their position on a system of co-ordinates. To be able to trace single clones, the co-ordinate system was aligned relative to the culture dish by marks on the dish.

NCSC cultures were performed as reported (Stemple and Anderson, 1992) with the modification that dispaseII (1.4 u/ml; Roche Diagnostics) was used for 10 minutes at 4°C instead of collagenase to dissect neural tubes from rat embryonic E10.5 trunk sections. Clonal culture of NCSCs and addition of growth factors was as described above for DRG cultures. To characterize single neural-crest-derived progenitors, NCSCs were grown in standard medium for 3 days until clusters of about 10-15 progenitor cells were formed. Cells in clusters were removed from culture dishes by treatment with 0.25% trypsin (Gibco BRL) for 2 minutes and replated at clonal density onto pDL/FN-coated dishes. The plating efficiency was approx. 70%. The positions of individual $p75^+$ progenitors were mapped as described above. Cells were cultured in standard medium and factors were added to some dishes. To compare the developmental potential of single progenitor cells with that of cells in progenitor clusters, single NCSC at clonal density were mapped and allowed to develop into progenitor clusters in standard medium for 3 days. The original marking of single NCSCs allowed mapping the neural-crest-derived progenitor clusters. While sister plates were used for the analysis of single progenitors (see above), the medium on cell clusters was changed and, in some experiments, factors were added directly at the same time as they were to plates containing single progenitor cells at clonal density. On single progenitor cells and on progenitor clusters, dose-response experiments in 10-fold steps were performed with BMP2 concentrations ranging from 0.016 pM to 16 nM and with TGF- β concentrations ranging from 0.04 fM to 400 pM. If not otherwise indicated, concentrations above saturation were used (1.6 nM BMP2; 4 pM TGF- β ; see descriptions of DRG and NCSC cultures).

Immunocytochemistry

Labeling of the cell surface antigen LNGFR was performed on living cells in standard culture medium for 30 minutes using a monoclonal mouse anti-rat $p75$ antibody (Ig192; Roche Diagnostics) visualized by FITC-conjugated horse anti-mouse IgG (Vector Laboratories). To label intracellular antigens, cells were fixed in PBS containing 3.7% formaldehyde for 10 minutes at room temperature (RT). To reveal expression of P0, cells were permeabilized for 15 minutes at RT using 2% BSA, 0.1% gelatin type A, 2% goat serum, 0.1% saponin in TBS and stained for 2 hours at RT with rabbit polyclonal anti-P0 (Rab $\beta 2$, a gift from B. Trapp, Cleveland Clinic, Ohio) used at a 1:1000 dilution. Rabbit polyclonal anti-PMP22 peptide 2 (Snipes et al., 1992) was affinity-purified using Reacti-Gel HW-65 according to the manufacturer's instructions (Pierce). To visualize PMP22, cells were permeabilized with 10% goat serum, 1% Triton X-100 in PBS and incubated with the affinity-purified antibody (used at a 1:200 dilution) for 2 hours at RT. For all other immunoreactions, permeabilization was carried out for 15 minutes at RT using 10% goat serum, 0.3% Triton X-100, 0.1% BSA in PBS. Staining with the following antibodies was performed for 1 hour at RT: monoclonal anti-NF160 antibody NN18 (IgG; 1/100 dilution; Sigma); rabbit polyclonal anti-NF160 (1:200 dilution; Chemicon International); a mixture of monoclonal anti-GFAP antibodies Ab-1 and Ab-2 (IgG; NeoMarkers) used at a dilution of 1:100 each; monoclonal anti-SMA (IgG; 1:400 dilution; Sigma). Immunostaining was visualized by incubation for 1 hour at RT using the following reagents at a 1:200 dilution: Cy3-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories); Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories); FITC-coupled horse anti-mouse IgG (Vector Laboratories); FITC-coupled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories).

RESULTS

Expression of P0 and PMP22 at early stages of PNS development

The most abundant protein of peripheral myelin, P0, has previously been shown to be expressed in the developing PNS long before myelination (Bhattacharyya et al., 1991; Lee et al., 1997; Sommer et al., 1995; Zhang et al., 1995). A series of in situ hybridization experiments was performed on transverse sections of rat embryos to investigate whether another major constituent of compact myelin, PMP22, is also expressed at early stages of PNS development. While in chicken, P0 is expressed in migratory neural crest (Bhattacharyya et al., 1991), ErbB3 mRNA (Fig. 1C; Meyer and Birchmeier, 1995) but not yet *P0* or *PMP22* transcripts were detectable in migrating neural crest of rat embryos at E10.5 (Fig. 1A,B). *P0* and *PMP22* are expressed, however, both in peripheral nerves and in dorsal root ganglia (DRG) at E12 (Fig. 1D,E). Myelin Basic Protein (MBP), particularly its isoform Golli, is another marker of early neural crest derivatives (Landry et al., 1997). However, a riboprobe encoding sequences common to the known *MBP* isoforms did not generate hybridization signals at E12. Expression levels of *P0* and *PMP22* increase at later stages of development when *MBP* mRNA is also detectable (Fig. 1G-J). Overlapping expression of *P0* and *PMP22* is also seen in sympathetic ganglia as early as E12 (not shown). Thus, *PMP22* together with *P0* are among the earliest known markers present in cells of peripheral nerves and ganglia but absent in migrating neural crest cells.

The observation that *P0* and *PMP22* mRNA were found at early stages of rat development raised the question of whether the corresponding proteins are also expressed in early cell types of the PNS. Schwann cell precursors isolated from sciatic nerves of E14 embryos are P0-immunoreactive, but do not yet express glial differentiation markers (Lee et al., 1997; Mirsky and Jessen, 1996). Likewise, we detected PMP22 protein in most cells derived from E14 sciatic nerve (not shown). To investigate which cell types in peripheral ganglia express PMP22, we dissociated DRG from rat E16 embryos and analyzed protein expression 3 hours after plating the cells. Non-neuronal cells not only expressed P0 protein but were also positive for PMP22 (Fig. 2). Strikingly, however, PMP22 was also found in virtually all neurons of these DRG cultures while P0 was restricted to the presumptive glial cells (Fig. 2).

Multipotency of a P0 and PMP22 expressing cell type isolated from developing DRG

The expression of PMP22 in neurons and glial cells suggested that this protein not only marks peripheral neural lineages, but possibly also a progenitor cell having both neuronal and non-neuronal potentials. To test this hypothesis, we sought to investigate PMP22 expression and the developmental potential of non-neuronal cells isolated from DRG of E14 embryos. To monitor the development of single neural crest-derived cells, DRG were dissociated and plated, and live-cell staining was performed using an antibody to the low-affinity nerve growth factor receptor (LNGFR; $p75$), a marker for NCSCs (Stemple and Anderson, 1992). 3 hours after plating, some of the $p75$ -labeled cultures were fixed to establish the antigenic phenotype of cells with a non-neuronal morphology. 83% and 88% of the non-neuronal $p75^+$ cells isolated from E14 DRG were double-positive

Table 1. Clonal analysis of p75⁺ progenitor cells isolated from DRG E14

Condition	Phenotype of single p75 ⁺ cells 3 hours after plating (% ± s.d.)						
	PO ⁺ 83±6			PMP22 ⁺ 88±4			
	Phenotype of progeny (% ± s.d.)						
	G+N+S	G+N	N+S	G	N	S	†
No add	11±4	51±4	0	26±6	8±2	0	4±3
NRG1	0	0	0	84±5	11±2	0	5±2
BMP2	0	0	62±5	0	23±6	6±2	9±4
TGF-β	0	0	0	0	0	70±5	30±5

Cells dissociated from E14 rat DRG were prepared and cultured exactly as described in the legend of Fig. 3. 3 hours after plating, some dishes were fixed and the expression of P0 or PMP22 was analyzed in p75⁺ cells with a non-neuronal morphology. Sister dishes were cultured in standard medium ('no add') for 16 days. Alternatively, cultures were incubated in standard medium plus NRG1, BMP2 or TGF-β, and the phenotype of clones derived from the previously marked p75⁺ cells was assessed 6 days after factor addition. 'G' indicates clones containing at least one GFAP⁺ cell, 'N', colonies with at least one NF⁺ cell; 'S', colonies with at least one SMA⁺ cell; '†' represents clones that were lost during culture. The data are expressed as the mean ± s.d. of two independent experiments. 100 clones were scored per experiment.

for P0 and PMP22 protein expression, respectively (Table 1; Fig. 3A-F). None of the non-neuronal cells expressed differentiation markers such as glial fibrillary acidic protein (GFAP) or neurofilament 160 (NF160; data not shown). On unfixed sister dishes, the position of single non-neuronal p75⁺ cells plated at clonal density was mapped (Materials and Methods) and, in a first experiment, the cells were allowed to develop in standard culture conditions that have previously been used to reveal multipotency of NCSCs (Stemple and Anderson, 1992). In agreement with earlier studies performed in chicken (Duff et al., 1991; Sextier-Sainte-Claire Deville et al., 1992), DRG clonal cultures produced both heterogenous and homogenous colonies, revealing the co-existence of multipotent and fate-restricted cells in mammalian DRG (Table 1). 34% of the colonies were restricted to a single cell type, the vast majority being composed of glia only. Single NCSCs and P0-positive progenitors isolated from sciatic nerve have previously been shown to produce substantial numbers of non-neural cells labelled by smooth muscle actin (SMA; Morrison et al., 1999; Shah et al., 1996; Stemple and Anderson, 1992). In contrast, only very few clones of DRG progenitors contained SMA-positive cells and none of the colonies was composed exclusively of this cell type (Table 1) while, in control cultures of NCSCs at clonal density, the fraction of colonies containing SMA-positive cells was similar to that previously reported (Shah et al., 1996; Stemple and Anderson, 1992; not shown). In DRG cultures, the SMA-positive cells were part of the 11% of all colonies that also contained neuronal and glial cell types, revealing the existence of multipotent cells in DRG similar to NCSCs. However, in neural crest cultures, 64±1% of NCSCs generated clones containing all three cell types – neurons, glia and smooth-muscle-like cells. The vast majority of the uncommitted clones from DRG (51% of all colonies) were restricted to neural lineages comprising both NF160-positive neurons and GFAP-positive glia but not SMA-positive non-neural cells. Such neural colonies accounted for only 25±1% of the total in NCSCs cultures. These data indicate that DRG contain uncommitted cells, most of which exhibit, however, increased fate restrictions in standard conditions when compared to NCSCs.

Since 83% of the p75-positive progenitors isolated from DRG were P0 positive and 88% PMP22 positive, and since 62% of the clones derived from these cells gave rise to more than one

cell type in standard culture conditions (Table 1), a substantial fraction of P0- and of PMP22-immunoreactive cells must be uncommitted. However, it is not evident from the above data whether such cells are multipotent and capable of producing non-neural cells. Therefore, we sought to investigate the developmental potential of P0- and PMP22-positive cells from DRG by challenging single progenitors at clonal density with instructive extracellular cues (Lo and Anderson, 1995; Lo et al., 1997; Morrison et al., 1999; Shah et al., 1994, 1996). As above, single p75⁺ progenitors with a non-neuronal morphology were mapped and their fate in response to factor addition was monitored. Similar to NCSCs, NRG1 promoted a glial fate in 84% of DRG-derived progenitors (Table 1; Fig. 3G,H) while 11% of the cells were committed to a neuronal fate consistent with the analysis in standard conditions. BMP2 induces neurogenesis and smooth muscle in NCSCs (Shah et al., 1996). The same fates were also promoted in DRG-derived progenitors (Table 1; Fig. 3J,K), 23% of which gave rise to colonies consisting of NF160⁺ neurons only and 62% to colonies comprising both neurons and SMA-positive cells. A smooth-muscle-like fate was induced in 70% of progenitors treated with TGF-β (Fig. 3L,M) which, in addition caused cell death of 30% of the monitored p75⁺ cells (Table 1). However, neural lineages were not generated in the presence of TGF-β. In striking contrast to NCSCs, which gave rise to clones of 4.1±0.4 cells per colony upon treatment with TGF-β, DRG-derived progenitors exposed to TGF-β mostly did not undergo further cell divisions before differentiation (1.3±0.1 cells per clone). Given the high percentage of p75/P0 and p75/PMP22 double-positive progenitors, our data imply that many P0-positive and PMP22-positive cells in E14 DRG are multipotent, giving rise to glia, neurons and non-neural cells in response to NRG1, BMP2 and TGF-β, respectively. Thus, our data confirm a recent report (Morrison et al., 1999) that P0 is not a marker for committed glia. Moreover, although PMP22 is expressed in neural but not in non-neural crest derivatives (not shown), PMP22-positive progenitors have the potential to become non-neural cells.

NCSCs generate a multipotent P0/PMP22-positive progenitor that gives rise to non-neural cells in the presence of TGF-β factors

The existence of a multipotent cell type present in a

postmigratory neural crest derivative prompted us to investigate whether a similar cell type can also be generated de novo from NCSC cultures. We first sought to analyze the cellular stages that express P0 and PMP22 in developing neural crest cultures. Freshly isolated NCSCs were negative for both P0 and PMP22 immunoreactivity (Fig. 4A-F) consistent with our in situ experiments which did not reveal expression of these markers in migratory crest (Fig. 1). Glial and neuronal differentiation markers were not yet found in NCSCs (Fig. 5; Stemple and Anderson, 1992). However, GFAP⁺ glia produced from NCSCs were positive for P0 and PMP22 (Fig. 4G-K). PMP22 but not P0 was also detectable in peripheral neurons derived from neural crest (Fig. 4L-O), corroborating our observations made in DRG cultures (Fig. 2) that PMP22 marks both glial and neuronal peripheral lineages. Thus, in vitro P0/PMP22-negative NCSCs give rise to cell types expressing these proteins, providing a system to study the onset of P0 and PMP22 expression.

In the next experiment, NCSCs were seeded at clonal density and allowed to differentiate on a poly-D-lysine (pDL) / fibronectin (FN) substratum in standard culture conditions (Stemple and Anderson, 1992). Colonies were fixed at various time points and their antigenic phenotype was determined. As previously demonstrated (Shah et al., 1996; Stemple and Anderson, 1992), about 95% of all colonies contained NF160⁺ neurons and GFAP⁺ glia after 16 days in culture (Fig. 5). Similar numbers of colonies also contained P0⁺ and PMP22⁺ cells, respectively. In contrast, on sister plates that had already been fixed after 3 days in culture, the colonies very rarely contained cells exhibiting differentiation markers but labeling for PMP22 and P0 was positive in almost every colony (97%

for both stainings; Fig. 5). Moreover, in the vast majority of the colonies expressing P0 (91%) and PMP22 (93%), virtually all cells of the colony were labelled for both of these markers (Fig. 6) indicating a fairly homogenous distribution of P0 and PMP22 in 3 day cultures of neural crest. These results demonstrate that both P0 and PMP22 expression are induced in early neural crest cultures, preceding the expression of neuronal and glial differentiation markers.

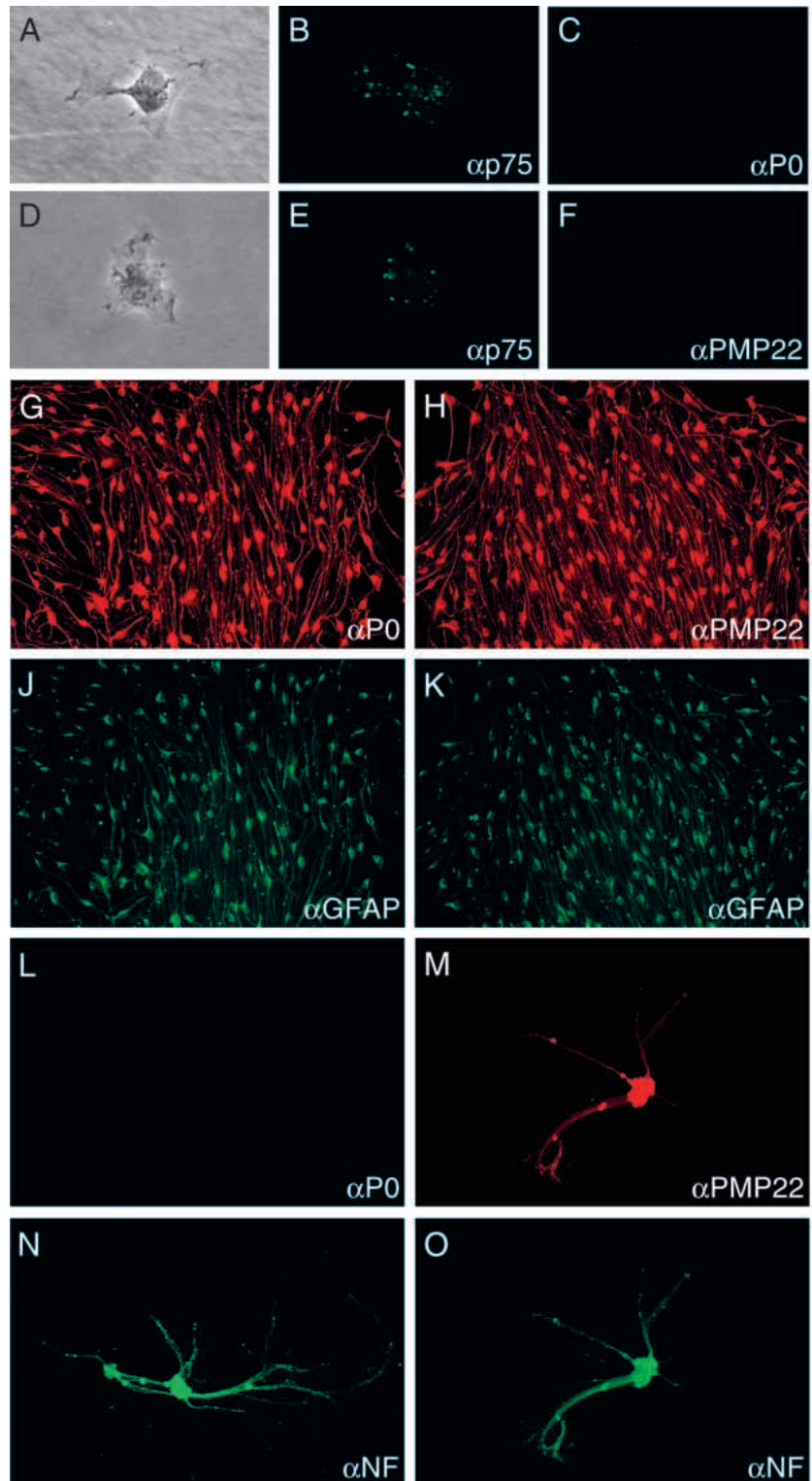


Fig. 4. Expression of P0 and PMP22 in neural crest cultures. Neural crest cells were allowed to emigrate from rat E10.5 neural tubes in culture, replated and living NCSCs were labeled immunocytochemically for p75 (B,E). Staining was visualized with an FITC-coupled secondary antibody. NCSCs do not express P0 (C) and PMP22 (F) protein as shown by immunoreactions on fixed p75-labeled NCSCs. (A,D) Phase-contrast views of B,C and E,F, respectively. (G-K) Neural crest cells were grown for 6 days in the presence of NRG1, which promotes gliogenesis. Cultures were fixed and double labeled for either GFAP (J) and P0 (G) or GFAP (K) and PMP22 (H). GFAP was detected by a secondary antibody conjugated to FITC while P0 and PMP22 stainings were demonstrated by fluorescent Cy3-coupled secondary antibody. Note that virtually all glial cells express P0 and PMP22. (N,O) Anti-NF antibody followed by a secondary antibody conjugated to FITC was used to reveal neuronal cells in neural crest cultures incubated with BMP2 for 6 days. Neurons generated from neural crest cultures were also marked by PMP22 (Cy3 fluorescence; M) but not by P0 (L). Magnification, (A-F) $\times 65$; (G-O) $\times 10$.

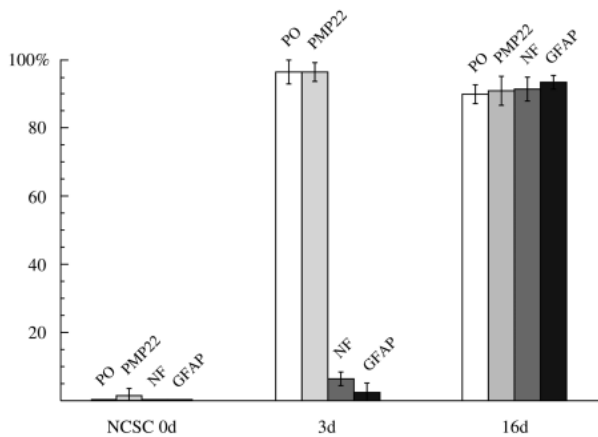


Fig. 5. P0 and PMP22 expression precede overt neuronal and glial differentiation in neural crest cultures. NCSCs were plated at clonal density, labeled live for p75 and incubated under standard culture conditions. Cells were fixed immediately after p75-staining (0d), after 3 days in culture (3d) or after 16 days in culture (16d). Fixed cultures were phenotyped with antibodies to P0, PMP22, NF and GFAP. Two independent experiments were carried out, scoring 100 clones for each staining per experiment. The proportion of colonies containing at least one cell labeled by the markers indicated is shown. Each bar represents the mean \pm s.d. Note that, in NCSCs at 0d, none of the markers are expressed while, at 3d, the majority of the colonies contain P0⁺ and PMP22⁺ cells but only very few colonies contain differentiation markers. Moreover, in 91% of the clones scored positive for P0 staining at 3d, virtually all cells expressed the marker. This number was 93% in the category of PMP22-positive clones. In contrast, at 3d, colonies labeled GFAP⁺ or NF⁺ never contained more than 2-3 cells positive for these differentiation markers.

To address the potential of P0/PMP22-positive progenitors generated from neural crest cultures, single NCSCs were allowed to form colonies for 3 days, as described above. Subsequently, the derivatives were replated at clonal density and stained for p75, which was used as a live cell marker to monitor the development of single progenitor cells. 3 hours after replating, some culture dishes were fixed, some dishes

were incubated in standard culture conditions and further sister dishes were treated with various growth factors. At the time of factor addition, approximately 90% of the p75⁺ cells were also positive for P0 and PMP22 (Table 2). In standard culture conditions, 76% of all clones produced by neural crest-derived progenitors were mixed colonies comprising neurons, glia and SMA⁺ cells (Table 2). This reveals multipotency of neural crest-derived progenitors very similar to NCSCs but distinct from most DRG-derived progenitors, which displayed more fate restrictions in standard culture conditions (Table 1).

Challenging single neural crest-derived progenitors with NRG1, BMP2 or TGF- β resulted in very similar phenotypes as previously obtained by exposing NCSCs or progenitors isolated from DRG to instructive cues (Table 2; Fig. 7). In particular, treatment with BMP2 led to the generation of colonies containing exclusively neurons (41%) and colonies containing SMA⁺ cells (56%; Fig. 7C,D). TGF- β produced no neural cells, and about 70% of the clones adopted a smooth-muscle-like fate (Fig. 7E,F). Interestingly, the clone size of TGF- β -treated neural-crest-derived progenitors was 1.2 ± 0.1 cells per clone indicating that, in contrast to NCSCs, progenitor cell division is almost fully suppressed in the presence of TGF- β . Together with the expression of P0 and PMP22 in the vast majority of the cells, this trait distinguishes neural-crest-derived progenitors from NCSCs and rather relates them to the predominant progenitor cell type isolated from E14 DRG.

Communities of P0/PMP22-positive progenitors produce neurons or undergo increased cell death in response to TGF- β factors but rarely give rise to non-neural cells

The expression of P0 and PMP22 indicate that the early multipotent progenitor derived from neural crest would be postmigratory, since in situ these markers start to be detectable in aggregating cells of the forming ganglia (Fig. 1). Hence, lineage decisions as analyzed above on single cells might be affected by community effects (Gurdon et al., 1993). We therefore investigated the fates of cell clusters challenged by extracellular signals that act instructively on single cells. The positions of single NCSC plated at clonal density were mapped

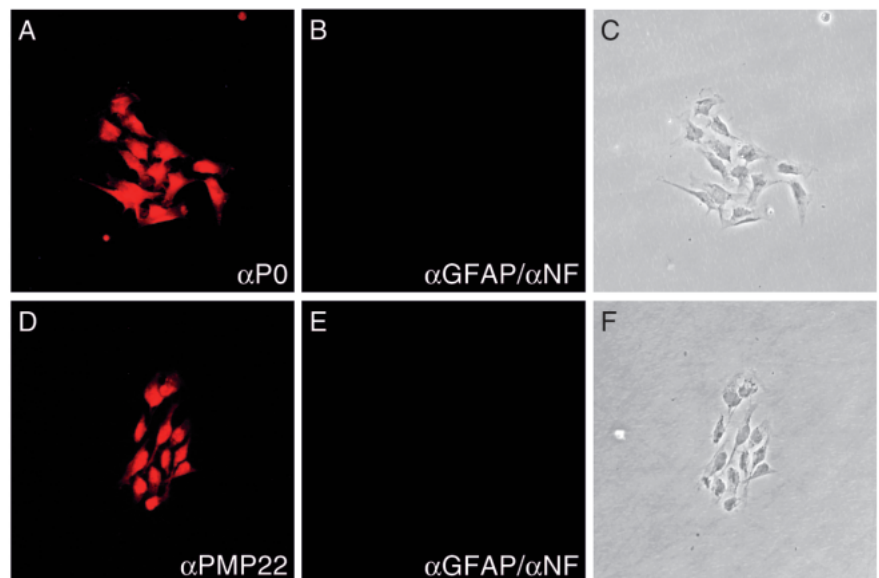


Fig. 6. Typical examples of clones (3d) from Fig. 5 consisting of either P0⁺ (A) or PMP22⁺ (D) cells (revealed by Cy3-coupled secondary antibody) that were usually negative for GFAP and NF (B,E) are shown. (C,F) Phase-contrast. Magnification, $\times 20$.

Table 2. Clonal analysis of p75⁺ progenitor cells derived from neural crest cultures

Condition	Phenotype of single p75 ⁺ cells 3 hours after replating (% ± s.d.)						
	PO ⁺ 89±4			PMP22 ⁺ 88±5			
	Phenotype of progeny (% ± s.d.)						
	G+N+S	G+N	N+S	G	N	S	†
No add	76±5	5±3	0	7±2	2±3	1±1	9±2
NRG1	0	5±4	4±2	85±5	0	0	6±2
BMP2	0	0	43±3	0	41±5	13±3	3±3
TGF-β	0	0	0	0	0	73±10	27±10

NCSCs were allowed to differentiate at clonal density in standard culture medium for 3 days. Subsequently, the cells were replated at clonal density and stained live with an anti-p75 antibody. Some dishes were fixed and analyzed for P0 and PMP22 protein expression in p75⁺ cells. On sister dishes, the position of the replated p75⁺ cells was mapped and clones were developed in standard culture medium ('no add') for 16 days, or in the presence of NRG1, BMP2 or TGF-β, respectively, for 6 days. The cultures were fixed and the cell types present in the colonies were characterized by immunocytochemistry for NF, GFAP or SMA. A colony was labeled 'N', 'G' or 'S' when at least one cell was expressing the corresponding differentiation marker NF, GFAP or SMA. '†' indicates lost clones. The figures represent the mean ± s.d. of four independent experiments. 100 clones were scored per experiment.

and the cells were allowed to differentiate in standard conditions for 3 days, as in the previously described experiments. At this time point, the clones formed clusters of 10-15 cells, the vast majority of which express P0 and PMP22, respectively (Fig. 6; Table 2). Instead of replating these cells (Fig. 7), the clones were now maintained in cell clusters and treated with different growth factors. The development of an individual cell cluster was monitored based on the positional mapping of the single NCSC that generated the cluster. Upon NRG1 treatment, progenitor cell clusters behaved comparably to single cells and predominantly gave rise to colonies consisting only of GFAP⁺ glia (86±4% of all colonies; Fig. 9 A,B). Monitoring individual clusters every 6 hours for the first day and subsequently every 24 hours did not reveal significant cell death occurring within the differentiating progenitor colonies, indicating that the cells responded to NRG1 as a virtually homogenous population (Table 3).

Apart from promoting neurogenesis, incubation of single progenitor cells with BMP2 leads to the formation of non-neural SMA⁺ cells (Fig. 3, 7). In contrast, when clusters of P0/PMP22-positive progenitors were treated with BMP2, the number of colonies containing SMA⁺ cells was drastically reduced (Figs 8, 9). While 43% of single neural-crest-derived progenitors gave rise to colonies with both neurons and smooth-muscle-like cells ('N+S'), only 6% of the progenitor clusters produced such colonies in response to BMP2. Likewise, communities of neural crest-derived progenitors generated 4% of colonies that consisted exclusively of smooth muscle, whereas such cell type-restricted colonies accounted for 13% of all clones derived from single progenitors. However, the proportion of colonies consisting solely of NF160⁺ neurons was substantially higher when cell clusters were developed in BMP2 than when single cells were BMP2 treated (83% of the progenitor clusters versus 41% of single progenitors gave rise to neuron-only clones, 'N'; Fig. 8). Since loss of colonies was comparable in both cases (2-7%), the different responses of single progenitors versus communities of progenitors cannot exclusively be due to selective mechanisms acting on factor-treated cell clusters that would favor neuronal colonies over smooth muscle-containing colonies. Rather, the generation of neuron-only colonies occurred at the expense of smooth muscle formation in cell clusters.

Cell death is a common fate of TGF-β-treated neural crest derivatives (Table 2; Morrison et al., 1999; Shah et al., 1996). This effect was even stronger when communities of P0/PMP22-positive progenitors were exposed to TGF-β (62% of all clones were lost, '†'; Fig. 8). Moreover, cell death was present within many clusters. In 9% of all colonies, most of the cells died, leaving two to three SMA⁺ cells. A smooth-muscle-like lineage is the fate predominantly promoted when single cells are treated with TGF-β (Table 2). We never observed, however, that whole progenitor clusters would survive with all cells giving rise to smooth-muscle-like cells. In striking contrast, a considerable proportion (29%) of communities of P0/PMP22-positive cells interpreted TGF-β signaling differently, giving rise to clones containing neurons ('N+S' and 'N'). Moreover, most of these clones were composed entirely of neurons ('N'; Figs 8, 9).

A trivial explanation for the observed effect might be that clusters of cells are exposed to different local concentrations of TGF-β factors compared to single cells, and that this would be sufficient to cause the differential response to BMP2 and TGF-β, respectively. Therefore, we performed dose-response experiments (Materials and Methods), exposing both single progenitors and progenitor communities to a range of either BMP2 or TGF-β concentrations including doses that exceed saturation. At no concentration tested did single cells respond like cell clusters undergoing predominantly neurogenesis at the expense of smooth muscle differentiation (data not shown). Thus, neural crest-derived progenitors display community effects in response to TGF-β factors: clusters of cells, generated from single NCSCs, suppress a smooth-muscle-like fate and rather undergo neurogenesis; as shown above (Fig. 7), dissociation of such clusters allows the progenitor cells to also adopt a non-neural fate.

Gurdon (1993) has coined the term community effect to designate developmental processes whereby a community of cells eliminates or corrects the differentiation of individual aberrant cells. We assume that it is aberrant for the developing PNS to generate smooth-muscle-like cells in early ganglionic anlagen where P0/PMP22-positive progenitors aggregate. Thus, communities of neural crest-derived progenitors might prevent smooth muscle formation by either eliminating or correcting cells that could otherwise adopt a smooth-muscle-

like fate. With regard to TGF- β signaling on neural crest-derived progenitors, it is difficult to distinguish between these mechanisms, since TGF- β induces significant cell death both with single cells and within cell clusters (Fig. 8). However, only few colonies derived from either single cells or cell clusters are lost after BMP2 treatment (Table 2; Fig. 8). We therefore addressed the question of whether neurogenic colonies derived from P0/PMP22-positive cell clusters in response to BMP2 can prevent smooth muscle formation within the cell community by either eliminating or by correcting cells with a smooth-muscle-like potential. Elimination of cells would result in considerable cell death within a colony, since 56% of the progenitors exhibit a smooth-muscle-like potential when exposed to BMP2 as single cells (Table 2). As in the experiments described above, NCSCs at clonal density were differentiated for 3 days to generate clusters of P0/PMP22-positive progenitors. BMP2 was added to the cultures and individual clusters were observed every 6 hours for the first day and subsequently every 24 hours. Virtually no dying cells or cell debris were observed within the developing clones (Fig. 10). To confirm this result, BMP2-treated sister dishes were fixed every 6 hours for a period of 36 hours and were analyzed for the presence of fragmented nuclei. Such structures are revealed by diamidinophenylindole (DAPI) staining and are typical for apoptotic cells (Raff, 1992). Again, cell death within the factor-treated cell clusters was minimal and not increased in BMP2-treated cultures (Table 3), while apoptotic figures were unambiguously detectable in TGF- β -treated cell clusters (data not shown). These data suggest that communities of P0/PMP22-positive progenitors react as a homogenous population to BMP2. Progenitors that have a smooth muscle potential (Fig. 7; Table 2) are instructed by community effects to suppress this non-neural fate.

DISCUSSION

In the present study, we show that P0 and PMP22, both prominent components of peripheral myelin, are not only expressed in glial cells long before myelination but also mark a multipotent cell type in the early dorsal root ganglion (DRG). Comparable to neural crest stem cells (NCSC), this P0/PMP22-positive progenitor can be induced with extracellular growth factors to give rise to neurons, glia and smooth-muscle-like cells. A similar progenitor cell type can be generated from NCSC cultures in which the expression of P0 and PMP22 precedes the appearance of overt glial and neuronal differentiation markers. Single cell analysis identifies this neural-crest-derived cell as a multipotent progenitor of the PNS. Strikingly, the P0/PMP22-positive progenitor displays community effects when exposed to extracellular signals. In cell clusters, it adopts a neuronal fate or undergoes cell death in response to TGF- β -factors, but will not generate smooth-muscle-like cells. In contrast, the same factors can promote the formation of non-neural cells from single P0/PMP22-positive progenitors.

Possible roles of P0 and PMP22 in early PNS development

Our findings may reflect a function of P0 and PMP22 in cell clustering, thereby influencing signaling pathways in neural crest derivatives. The classical roles described for P0 and PMP22 have been initiation and maintenance of myelin

(Mirsky and Jessen, 1996; Suter and Snipes, 1995), based on the predominant expression of these proteins in myelinating Schwann cells and their localization to compact myelin (Lemke and Axel, 1985; Spreyer et al., 1991; Welcher et al., 1991). In agreement with such roles, the phenotypes of various mutants of P0 and PMP22 are associated with hereditary motor sensory neuropathies such as Charcot-Marie-Tooth (CMT1), Dejerine-Sottas syndrome (DSS) and hereditary neuropathy with liability to pressure palsies (HNPP; reviewed in Sommer and Suter, 1998; Suter, 1997; Suter and Snipes, 1995). However, the embryonic expression of both P0 and PMP22 (this study and Baechner et al., 1995; Bhattacharyya et al., 1991; Bosse et al., 1994; Lee et al., 1997; Parmantier et al., 1995, 1997) suggests alternative functions of these membrane proteins. PMP22 has been implicated in regulating cell cycle and apoptosis (Fabbretti et al., 1995; Manfioletti et al., 1990; Zoidl et al., 1995, 1997). Moreover, both P0 and PMP22 might mediate cell-cell interactions during development (Naef and Suter, 1998; Sommer and Suter, 1998). It remains to be determined whether adhesive properties of P0 and/or PMP22 are required for fate restrictions in clusters of progenitor cells identified in this study. Furthermore, the expression of P0 and PMP22 in this progenitor and of PMP22 in developing neurons might point to possible developmental contributions to the disease mechanisms in peripheral neuropathies.

Neural crest stem cells generate multipotent PNS progenitor cells

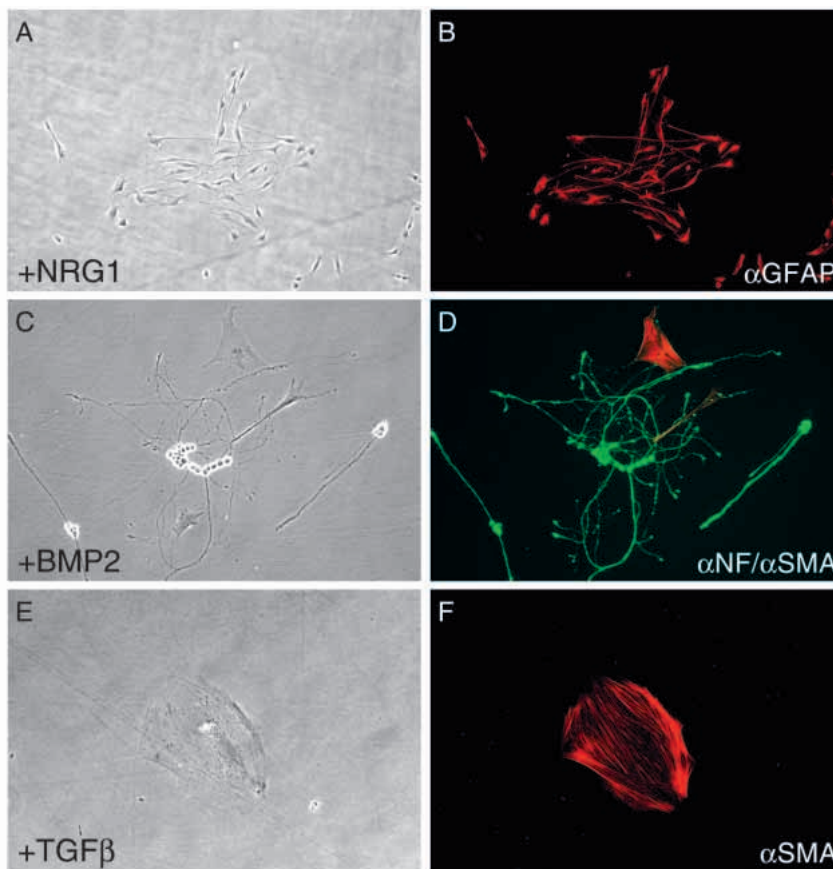
The expression of P0 and PMP22 in multipotent postmigratory progenitors isolated from early DRGs and from NCSC cultures reveal that these molecules are not markers for committed

Table 3. Cell death within clusters of progenitor cells

Condition	Time after addition of growth factors (hours)	Clusters with no dying cell (%)	Clusters with 1-2 dying cells (%)	Clusters with >3 dying cells (%)
No add	6	98.5	1.5	0
	12	94.5	5.0	0.5
	18	98.0	2.0	0
	24	96.5	3.5	0
	30	94.0	6.0	0
	36	95.5	4.0	0.5
NRG1	6	95.0	5.0	0
	12	99.0	1.0	0
	18	97.5	2.5	0
	24	96.0	3.5	0.5
	30	95.0	4.5	0.5
	36	94.0	6.0	0
BMP2	6	97.0	2.5	0.5
	12	97.0	3.0	0
	18	98.0	2.0	0
	24	95.5	4.0	0.5
	30	95.0	5.0	0
	36	97.0	2.0	1.0

Progenitor cell clusters were obtained from NCSCs grown for 3 days at clonal density. Thereafter, clusters were incubated in standard medium ('no add'), or in standard medium containing NRG1 or BMP2. Cultures were fixed every 6 hours and nuclear morphology of cells within the clusters was investigated by DAPI staining. The numbers indicate percentage of all clusters analyzed. For each condition and time point, 200 colonies were scored. Note that cell death within colonies is minimal. In particular, treatment of progenitor communities with BMP2 does not result in increased elimination of cells within the clusters.

Fig. 7. P0- and PMP22-expressing progenitor cells derived from neural crest cultures are multipotent. Clonal cultures of NCSCs were allowed to differentiate in standard culture medium for 3 days. At this time, clones immunoreactive for P0 and PMP22 have formed on sister dishes. The cells were replated at clonal density and stained live with an anti-p75 antibody. Some dishes were fixed and analyzed for P0 and PMP22 protein expression, showing that the vast majority of the replated p75⁺ cells were P0/PMP22⁺ (not shown; see Table 2). On other dishes, the position of p75⁺ cells was mapped and the development of the clonal cultures under various conditions was observed daily. After 6 days, the cultures were fixed and immunoreactions were performed to identify the cell types present in the colonies. After NRG1 treatment, colonies consisted exclusively of GFAP⁺ cells (A,B; fluorescent signals in B by Cy3-coupled secondary antibody). (C,D) Anti-NF and anti-SMA antibodies followed by secondary antibodies conjugated to FITC (for NF) or Cy3 (for SMA) were used to label neuronal and smooth-muscle-like cells in mixed clones generated in the presence of BMP2. (E,F) TGF- β treatment of clonal cultures resulted in the formation of single SMA⁺ cells (revealed by Cy3 fluorescence). Magnifications, (A-D) $\times 10$; (E,F) $\times 20$.



neural-crest-derived sublineages. These data are in agreement with previous reports on pluripotent cells found in postmigratory neural crest-derived structures both in avian and rodent embryos (Artinger and Bronner-Fraser, 1992; Duff et al., 1991; Frank and Sanes, 1991; Hall and Landis, 1991; Henion and Weston, 1997; Richardson and Sieber-Blum, 1993; Sextier-Sainte-Claire Deville et al., 1992). In particular, grafts of quail DRGs produced sensory and autonomic derivatives as well as Schwann cells when transplanted into the chick neural crest migration pathway (Le Douarin, 1986; Le Douarin and Ziller, 1993). However, changing environmental signals in order to investigate developmental potentials of single cells has not been performed in these studies. Such experiments have been carried out on progenitors isolated from the developing enteric nervous system (Lo and Anderson, 1995; Lo et al.,

1997) and from sciatic nerve (Morrison et al., 1999). In this way, postmigratory progenitors were identified that generate clones of non-neuronal cells but that can be challenged to

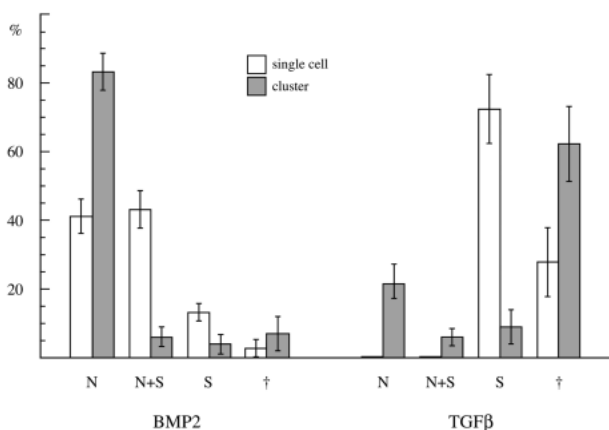


Fig. 8. Community effects of P0/PMP22-positive progenitor cells in response to TGF- β factors. NCSCs were cultured for 3 days to form clones of P0/PMP22⁺ progenitors. The cells on some dishes were subsequently replated at clonal density and labeled live with anti-p75 antibody, as described in the legend of Fig. 7. Sister dishes containing clones of progenitors derived from NCSCs were maintained as cell clusters, the medium was exchanged and various factors were added at the same time that they were presented to the replated single cells. It was possible to follow the fate of individual communities of progenitors since the position of the single NCSCs that generated them had been mapped previously. A comparison between the fates of single cells versus clusters of progenitors is shown. 'N' represents colonies consisting exclusively of NF⁺ neurons; 'N+S' are mixed clones of NF⁺ neurons and SMA⁺ non-neuronal cells; 'S' are colonies of smooth-muscle-like cells only; '†' represents colonies that were lost during culture. Each bar represents the mean \pm s.d. of four independent experiments, in each of which 100 clones were scored. Note that upon BMP2 treatment single progenitor cells gave rise to 56% of colonies containing smooth-muscle-like cells ('N+S' plus 'S') while only 10% of the progenitor clusters generated such clones. The category 'N' was, however, doubled in communities as compared to single progenitor cells. Communities of progenitors underwent increased cell death ('†') or formed neuron-only colonies in response to TGF- β , which on single cells mainly induced colonies exclusively composed of SMA⁺ cells ('S'). Moreover, it was never observed that all cells within a community would adopt a smooth-muscle-like fate; rather, for progenitor clusters the group 'S' represents clones of 1-3 cells with the remaining cells having died.

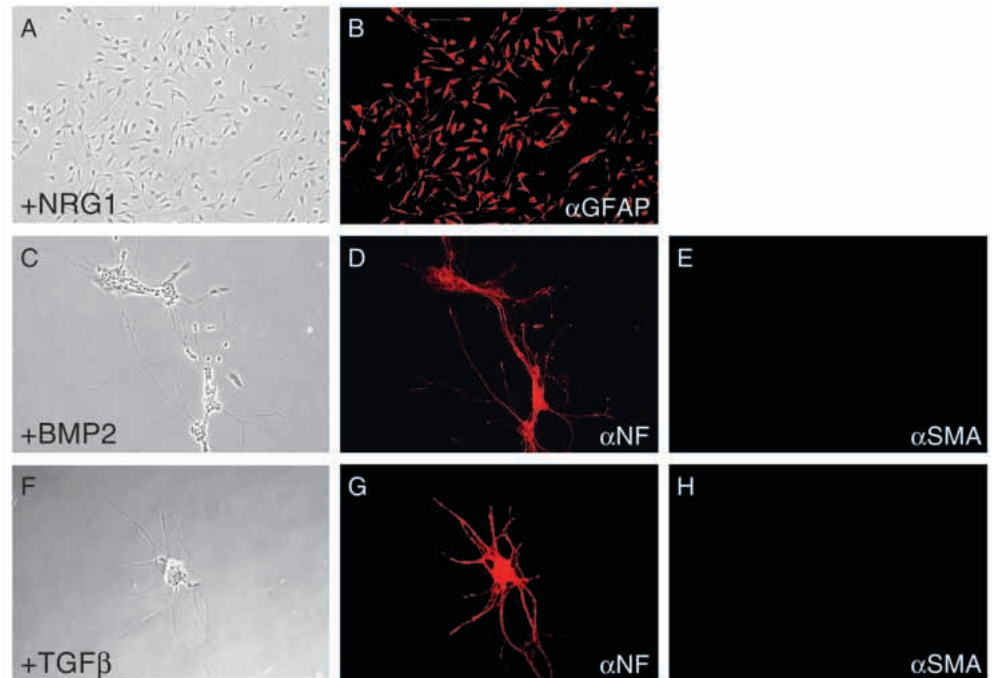


Fig. 9. Typical examples of colonies produced from progenitor clusters upon NRG1 (A,B), BMP2 (C-E) and TGF- β (F-H) treatment as described in Fig. 8 are shown. While NRG1 induced gliogenesis in virtually all cells of a cluster (as assayed by GFAP staining), BMP2 and TGF- β gave rise to neuronal colonies containing NF⁺ cells (D,G) but not SMA⁺ cells (E,H). Magnification, $\times 10$.

produce neurons in response to BMP2. Likewise, our clonal analysis revealed that the vast majority of P0/PMP22-immunoreactive cells, whether isolated from DRG or generated from neural crest cultures, are multipotent and responsive to instructive extracellular cues. Final proof of the multipotency of the P0/PMP22-positive progenitor will require *in vivo* experiments, for example back-transplantation of labeled cells into embryos at early stages of neural crest migration (Le Douarin and Ziller, 1993).

Our study reveals a direct lineal relationship between multipotent NCSCs and distinct but still multipotent progenitors generated from neural crest. The latter appear to be different from NCSCs not only because of P0 and PMP22 expression, but also based on their characteristic response to extracellular growth factors. Like NCSCs, single neural crest-derived progenitor cells give rise to glia, neurons and smooth muscle in response to NRG1, BMP2 and TGF- β , respectively. However, TGF- β suppressed proliferation of neural crest-derived progenitors in clonal cultures, whereas NCSCs undergo cell divisions in the presence of TGF- β . With respect to the response to instructive signals, the progenitor obtained from neural crest cultures is very similar to the P0/PMP22-positive cell type isolated from E14 DRG and, in addition, displays similarities to a recently characterized P0-positive cell type isolated from E14 sciatic nerve (Morrison et al., 1999). There are, however, also considerable differences among the various multipotent P0/PMP22-positive progenitors described so far (this study; Morrison et al., 1999). The majority of single progenitor cells generated in culture from NCSCs exhibit multiple fates when grown in standard conditions, producing colonies containing neurons, glia and non-neural cells. This trait relates such progenitors to NCSCs. By contrast, most of the progenitors residing in postmigratory neural crest derivatives of E14 rat embryos are more restricted in standard culture conditions. While progenitors from sciatic nerves generate predominantly glia and smooth-muscle-like cells

termed myofibroblasts (Morrison et al., 1999), we mainly observed the formation of neurons and glia from DRG-derived progenitors. Although we can not exclude that these differences are due to variations in the culture conditions, they support the idea that similar but distinct multipotent progenitors populate various neural crest-derived tissues early in development. We do not yet know the factors that induce the development of the progenitor cells described in this report. P0- and PMP22-expressing cells are generated from NCSCs in standard culture conditions without the addition of any defined instructive cues. The extracellular matrix might play an important role since, at least in short-term cultures, P0/PMP22-positive cells are only obtained on a poly-D-lysine/fibronectin substratum but not on fibronectin alone (data not shown).

Fate restrictions in response to TGF- β factors due to community effects

Although neural crest-derived progenitors exhibit neural and non-neural developmental potentials when challenged with extracellular factors, their fate map might be more restricted. As discussed above, many clones of postmigratory progenitors isolated from DRG produce mostly neurons and glia, whereas non-neural cell types are generated to a lesser extent in the absence of specific instructive cues. More strikingly, cell clusters of neural crest-derived progenitors, in contrast to single cells, display a reduced non-neural potential when exposed to TGF- β factors. The predominant response of single neural crest-derived progenitors to TGF- β is to choose a smooth-muscle-like fate. In contrast, TGF- β promotes mainly cell death or, surprisingly, neurogenesis in communities of progenitors generated from NCSCs. Likewise, BMP2 induces both neurogenesis and smooth muscle formation in single progenitors but only neurogenesis in progenitor cell clusters. The notion that embryonic cells can have a broader developmental potential than their fate map would suggest is not novel. The neural crest generates distinct cell types at

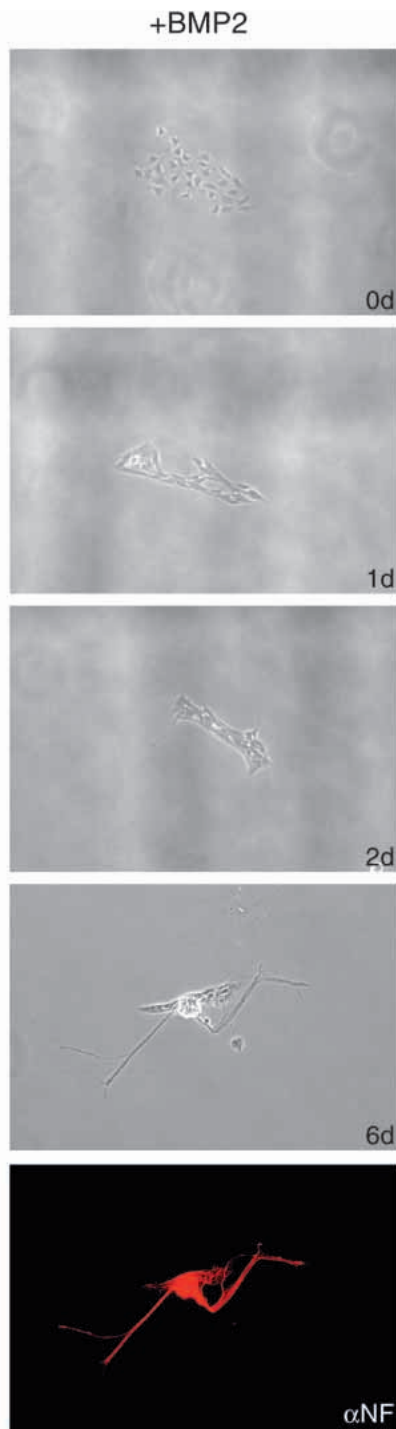


Fig. 10. Cells within a cluster of progenitors are instructed to choose a neuronal fate at the expense of a non-neuronal fate in response to BMP2. Neural crest-derived cultures were treated with BMP2 and individual clusters of progenitors were monitored every 6 hours for the first 36 hours and then every 24 hours. Shown is one clone out of 20 that was rephotographed every 24 hours. '0d' illustrates the neural-crest-derived progenitor cluster just before addition of BMP2. 6 days after addition of the factor (6d), the cells were fixed and stained for SMA (not shown) and NF expression. No dying cells or debris of dead cells were observed in the developing clone, indicating that cells with a smooth-muscle-like potential were promoted to generate neurons only. Magnification, $\times 10$.

different axial levels but its potential is fairly homogeneous along the neuraxis (Anderson, 1997; Le Douarin, 1986; Le Douarin and Ziller, 1993). This has been attributed to distinct environmental signals encountered during migration of crest cells from different positions along the anteroposterior axis. Our data are consistent with an additional mechanism of fate restriction in neural crest development. A single signal elicits differential fate responses in a multipotential cell depending on whether the signal is presented to individual cells or to a community of cells. This may explain differing reports on lineage restrictions in neural crest: lineage-tracing experiments in mass cultures identified more restricted neural crest derivatives (Henion and Weston, 1997) than were obtained by limiting dilution clonal analysis (Baroffio et al., 1988; Ito et al., 1993; Le Douarin and Ziller, 1993; Sextier-Sainte-Claire Deville et al., 1992; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1992). Our finding that *in vitro* neurogenesis is promoted at the expense of smooth muscle formation in cell clusters of progenitors appears plausible, since *in vivo* a non-neuronal fate has presumably to be suppressed in P0/PMP22-positive progenitors that aggregate in forming peripheral ganglia. Consistent with a role in peripheral neurogenesis, both BMP2 and TGF- β isoforms are expressed in, or adjacent to, developing ganglia of the PNS (Bitgood and McMahon, 1995; Flanders et al., 1991; Millan et al., 1991; Shah et al., 1996).

Coordinated fate specification of cell populations has been referred to as a community effect and proposed to be dependent on cell-cell communications (Gurdon et al., 1993). In aggregates of animal cap cells from *Xenopus*, increasing amounts of a mesoderm-inducing TGF- β factor produce as many as five different fates (Green et al., 1992; Green and Smith, 1990). However, not all of these fates are promoted in dissociated animal cap cells, indicating that cellular association is required to specify distinct cell types (Green et al., 1994; Wilson and Melton, 1994; reviewed in Harland and Gerhart, 1997; Hogan, 1996). Our data suggest that the response to TGF- β factors reflects a community effect in clusters of neural crest-derived progenitors. As proposed by Gurdon (1993), community effects are required to eliminate or correct individual errant cells such as non-neuronal cells in developing peripheral ganglia. Frequent observation of developing cell clusters in the presence of BMP2 did not reveal selective survival of a subset of cells, but rather indicated the coordinated neuronal differentiation of virtually all of the cells within a progenitor community. It is not clear how such community effects are regulated, but they are likely due to modulation of signaling pathways. Members of the TGF- β superfamily signal through heteromeric complexes of type I and type II serine/threonine kinase receptors, which phosphorylate signal transducing proteins of the SMAD family (reviewed in Attisano and Wrana, 1998; Heldin et al., 1997; Massague, 1996). Signaling diversity and modulation is achieved by changing concentrations of ligand antagonists (reviewed by Weinstein and Hemmati-Brivanlou, 1997), by the activation of distinct type I receptors (Krishna et al., 1999; Zou et al., 1997) or by regulated expression of inhibitory SMAD proteins (Topper et al., 1997). In addition, cross regulation has been reported between TGF- β factor signaling and other signal transduction pathways (reviewed by Whitman, 1998). The receptor tyrosine kinase (RTK) and mitogen-activated protein kinase (MAPK) pathway converges with TGF- β signaling,

having synergistic or antagonistic effects dependent on the signal transduction molecules involved (de Caestecker et al., 1998; Kretzschmar et al., 1997; Yamaguchi et al., 1995, 1999). Pathway convergence might also occur at the level of target promoters when signaling by a TGF- β factor and by additional signals is integrated at distinct promoter elements (Whitman, 1998). The molecular mechanisms underlying the differential interpretation of TGF- β factors by single neural crest-derived progenitors versus communities of progenitors remain to be elucidated. Direct cell-cell contact, local accumulation of secreted signals or gap junction communication between adjacent cells might be involved (Gurdon et al., 1993). Dependent on the cell type or cellular stage, SMAD proteins that are otherwise pathway-restricted seem to be shared by both TGF- β and BMP2 signaling (Lechleider et al., 1996; Yingling et al., 1996). Such a pathway cross talk might be a reason why in cell clusters of progenitors both BMP2 and TGF- β elicit a neuronal response. The experimental accessibility of neural crest cultures should allow us to dissect the molecular events leading to community effects of multipotent P0/PMP22-positive progenitors in response to TGF- β factor signaling.

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