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

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Summary

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

Introduction

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

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

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

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

Results

BMP2 Is Expressed in the Dorsal Aorta Concomitant with Autonomic Neurogenesis

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

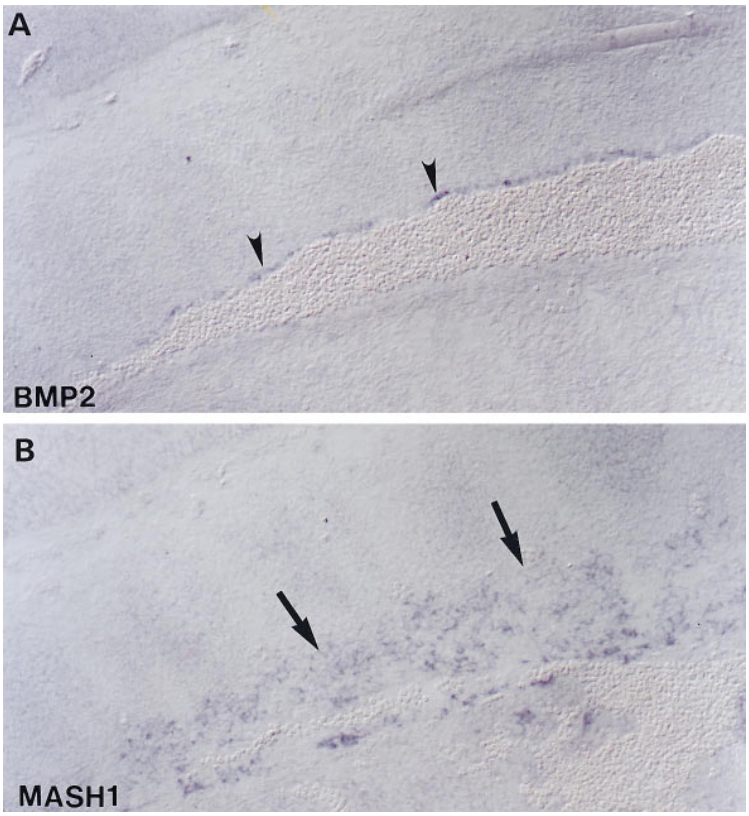

Figure 1. Expression of BMP2 Spatially and Temporally Overlaps MASH1 Expression in Autonomic Anlage In Vivo

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

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

BMP2 Induces Rapid Neurogenesis in NCSC Clonal Cultures

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

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

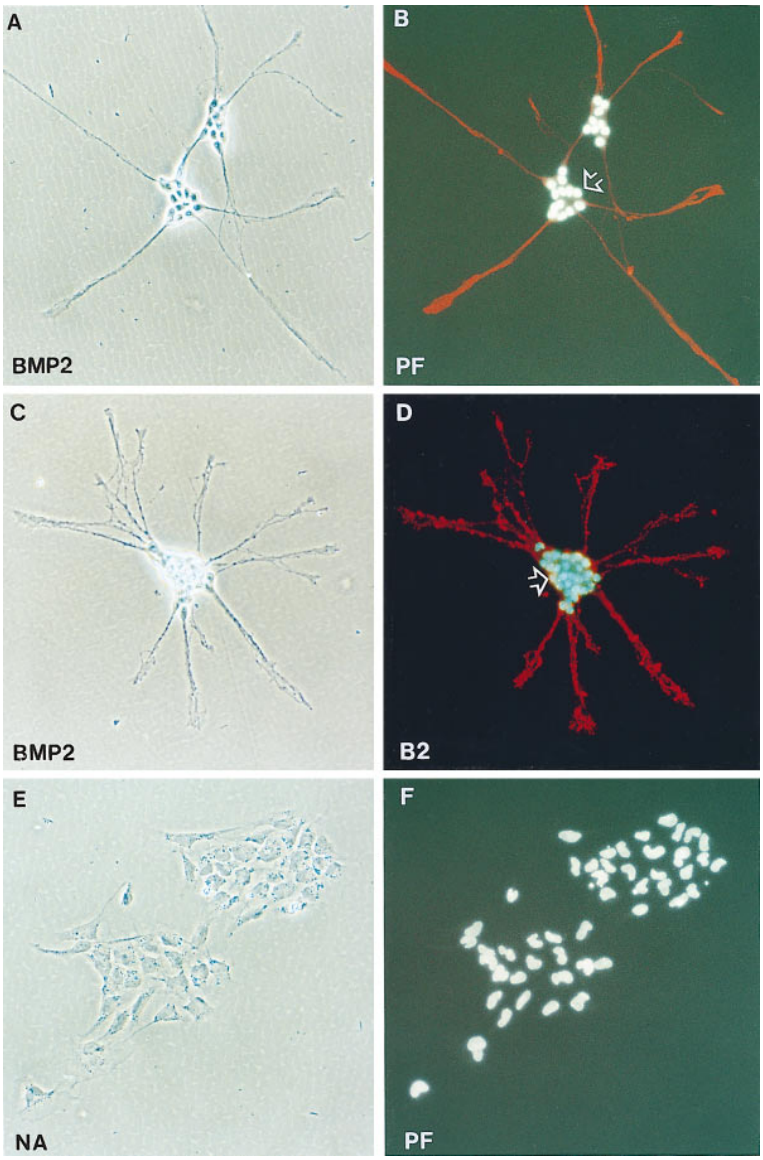

Figure 2. rBMP2 Induces Autonomic Neuronal Differentiation

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

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

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

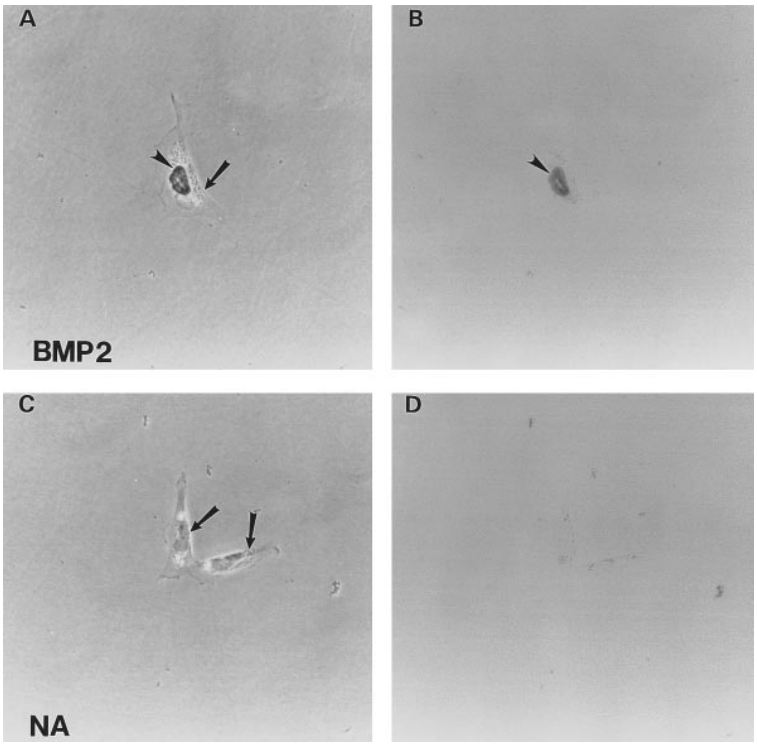

Figure 3. BMP2 Induces MASH1 Expression in NCSCs

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

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

The Flat Cells That Develop in rBMP2 Are Smooth Muscle Cells

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

Table 1. Kinetics of MASH1 Induction in NCSCs by rBMP2

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

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

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

TGFβ1 Promotes Smooth Muscle Differentiation of NCSCs

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

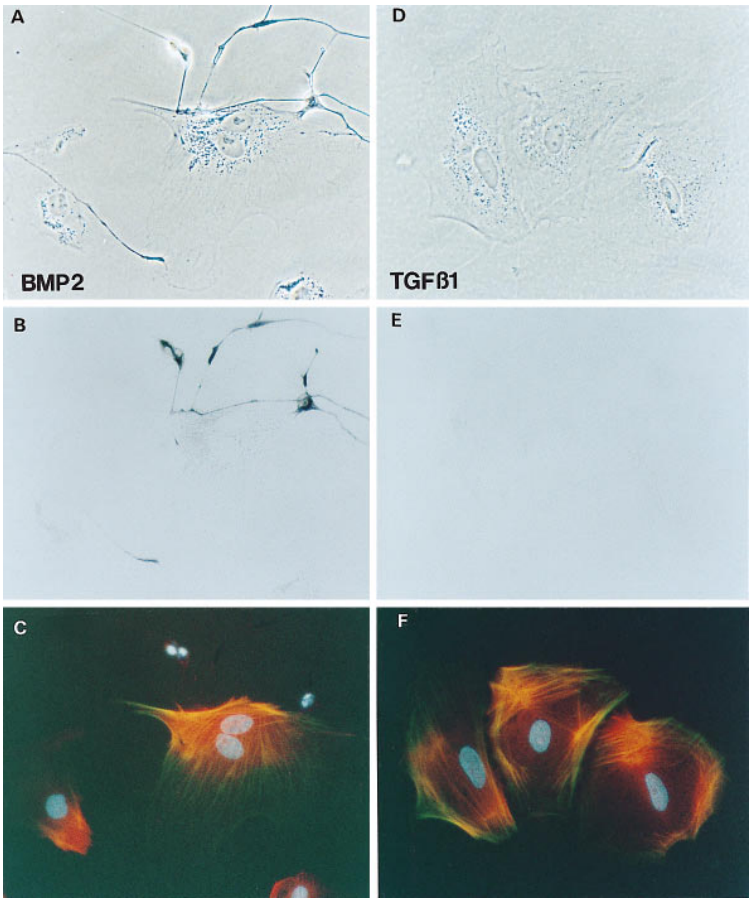

Figure 4. Differentiation of Smooth Muscle Cells in rBMP2 and rTGFβ1

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

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

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

rBMP2 and rTGFβ1 Act Instructively Rather Than Selectively

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

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

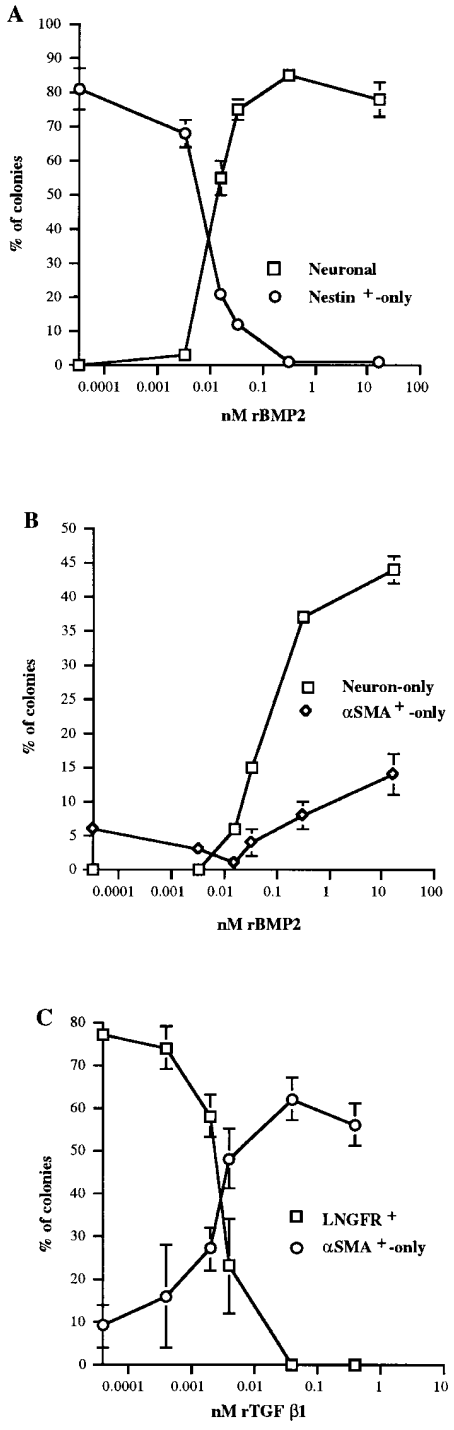

Figure 5. BMP2 and TGFβ1 Produce Distinct Responses over a Wide Range of Doses

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

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

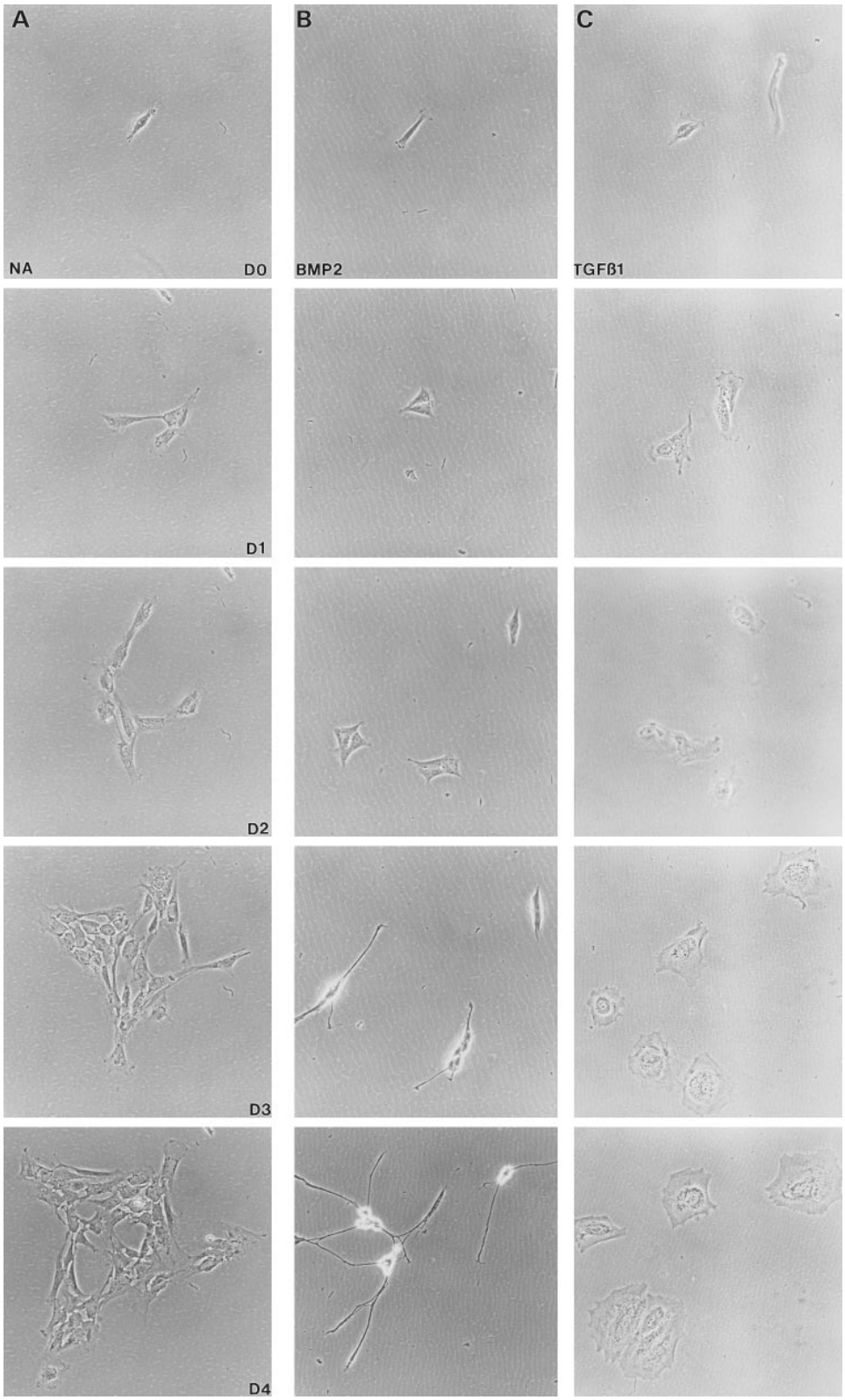

Figure 6. Serial Observation of Identified Clones Demonstrates Instructive Effects of rBMP2 and rTGFβ1

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

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

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

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

Role of BMP2 in Neuronal Differentiation of NCSCs

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

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

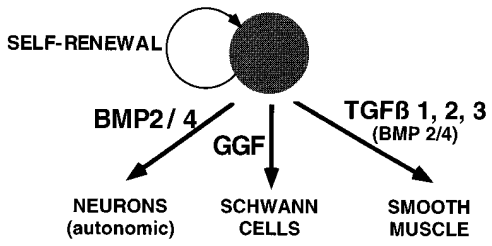

Figure 7. Summary of Instructive Effects of Growth Factors on Rodent Neural Crest Stem Cell Lineage Commitment In Vitro

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

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

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

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

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

Heterogeneity of the Response to BMP2

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

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

BMPs Control Multiple Stages of Vertebrate Neurogenesis

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

Smooth Muscle Differentiation of the Neural Crest

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

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

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

Neuropoiesis versus Hematopoiesis: a Different Strategy?

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

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

Experimental Procedures

NCSC Cultures

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

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

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

Immunostaining and In Situ Hybyridization Studies

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

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

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