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Cell-intrinsic and cell-extrinsic cues regulating lineage decisions in multipotent neural crest-derived progenitor cells

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ABSTRACT Multipotent stem cells must generate various differentiated cell types in correct number and sequence during neural development. In the peripheral nervous system (PNS), this involves the formation of postmigratory progenitor cell types which maintain multipotency and are able to give rise to neural and non-neural cells in response to instructive growth factors. We propose that fate restrictions in such progenitor cells are controlled by the combinatorial interaction of different extracellular signals, including community effects in response to both neurogenic and gliogenic factors. In addition, distinct progenitor cell types display intrinsic differences which modulate their response to the extracellular environment. Thus, a progenitor cell is apparently able to integrate multiple intrinsic and extrinsic cues and thereby to choose fates appropriate for its location. Fate analysis of genetically modified progenitor cells will help to identify the molecules involved. This approach appears promising given the identification of multipotent progenitor cells from the mouse PNS and the availability of genetics in the mouse system.

KEY WORDS: *PNS, neural crest, stem cell, multipotency, community effect*

Introduction

Multicellular tissues such as the central nervous system (CNS) and the peripheral nervous system (PNS) are generated from multipotent stem cells during vertebrate development (Gage, 2000). These cells have the competence to self-renew and to give rise to differentiated progeny in a manner appropriate to the embryonic location. How this is achieved is only partially understood. To identify the nature of the cellular and molecular mechanisms involved it is necessary to challenge stem cells by altering both their extracellular environment as well as their intrinsic genetic programs regulated by transcription factors. The neural crest has turned out to be a valuable system to address these issues.

The neural crest is a transient population of cells that detach from the neuroepithelium of the dorsal neural tube and migrate along defined pathways to various tissues, where they differentiate into their final phenotypes such as the neurons and glial cells of the PNS, as well as non-neural cell types such as melanocytes and smooth muscle-like cells (Anderson *et al.,* 1997; Le Douarin *et al.,* 1994). Transplantation experiments in avian embryos and *in vitro* analysis of avian and rodent neural crest cells revealed that many neural crest cells are multipotent and have some self-renewal capacity, while other precursors in migrating neural crest and in crest-derived tissues displayed lineage restrictions in these experiments (reviewed in Anderson, 2000; Sommer, 2001). Thereby, lineage commitment of individual crest cells had to be tested by exposing the cells to changing environmental conditions. Such experiments in the rat system led to the identification of multipotent neural crest stem cells (NCSCs) isolated from neural tube explants that generate glia, neurons and smooth muscle-like cells in response to instructive signaling by Notch, NRG1, BMP2, and TGFß (Morrison *et al.,* 2000; Shah *et al.,* 1996; Shah *et al.,* 1994; Stemple and Anderson, 1992). Such cells apparently persist during development, as NCSCs with self-renewal capacity have been identified in sciatic nerves (Morrison *et al.,* 1999). Similarly, multipotent progenitor cells characterized by the expression of P0 and PMP22 have been identified in rat sciatic nerve and DRG, and these cells respond, like NCSCs, to NRG1, BMP2, and TGFß (Hagedorn *et al.,* 1999; Morrison *et al.,* 1999). A similar cell type can be generated *de novo* from NCSCs cultures, demonstrating a lineage relationship between NCSCs and multipotent progenitor cells (Hagedorn *et al.,* 1999). Thus, postmigratory targets of the neural crest contain multipotent progenitor cells that can be instructed by various growth factors to

Abbreviations used in this paper: BMP, bone morphogenetic protein; CDK, cyclin-dependent kinase; DRG, dorsal root ganglia; FBS/F, fetal bovine serum and forskolin; NCSC, neural crest stem cell; NRG, neuregulin; PNS, peripheral nervous system; SMA, smooth muscle actin; TGF, tumor growth factor.

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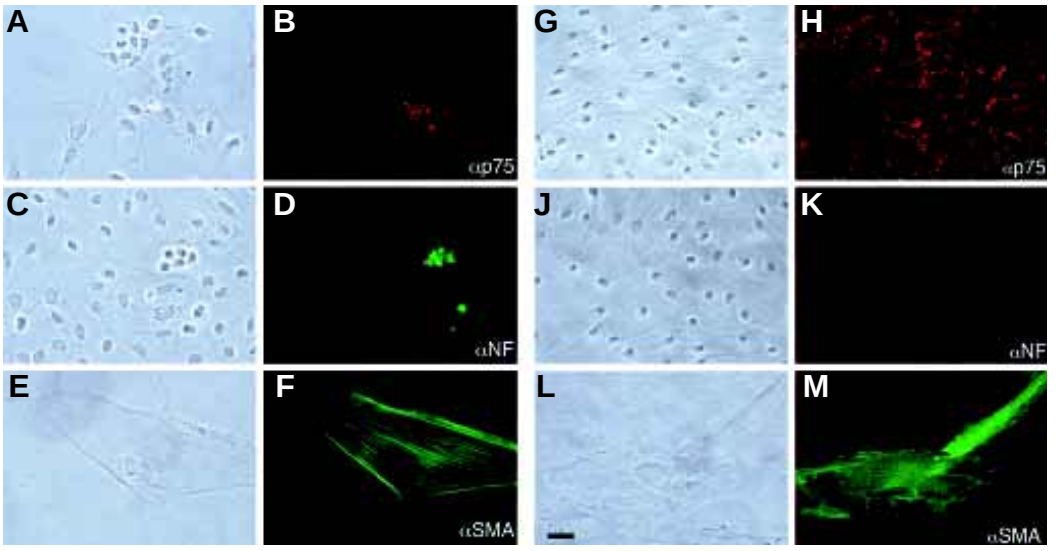

Fig. 1. Comparison between clonal cultures of rat NCSCs and DRG progenitor cells. *Rat NCSCs plated at clonal density in medium containing FBS/F gave rise to 45% glia-containing clones as marked by anti-p75 labeling* **(A,B)**. *The generation of neurons (labelled by an anti-NF160 antibody) was also observed in about 50% of all clones* **(C,D)**. *In addition, many NCSCs produced progeny consisting exclusively of smooth muscle actin (SMA)-positive cells* **(E,F)**. *In contrast, multipotent progenitors isolated from rat DRG differentiated predominantly into glia-containing clones (about 90% of all colonies)* **(G,H)**. *In these conditions, neurogenesis was not observed* **(J,K)** *while some SMA-positive cells formed* **(L,M)** *which were mostly associated with glia-containing clones (not shown). Representative fields of different clones are shown. (A,C,E,G,J,L) Phase images. Scale bar, 20μm.*

adopt a given cell fate at the expense of another fate.

The existence of multipotent progenitor cells in postmigratory targets of the neural crest poses the problem of how fates inappropriate to these locations are suppressed despite the presence of factors capable of inducing such fates. For instance, TGFß factors are expressed in peripheral ganglia (Flanders *et al.,* 1991; Hagedorn *et al.,* 2000a) but presumably do not induce smooth muscle-like cells in these structures, although they are capable of doing so in culture (Hagedorn *et al.,* 1999; Morrison *et al.,* 1999; Shah *et al.,* 1996). Recently, we were able to demonstrate that the activity of TGFß and BMP2 on multipotent progenitor cells is altered by community effects (Hagedorn *et al.,* 2000a; Hagedorn *et al.,* 1999). Individual progenitors give rise to smooth muscle-like cells in response to members of the TGFß family; in progenitor communities treated with TGFß-factors, however, such a non-neural fate is suppressed by short range cell-cell interactions and neurogenesis is promoted instead. In addition, TGFß but not BMP2 also induces apoptosis as an alternative fate to neurogenesis in progenitor communities (Hagedorn *et al.,* 2000a).

In this study, we have analyzed the relationship between different rat neural crest-derived progenitor cell types and provide evidence that these multipotent cells display intrinsic differences in their response to extracellular cues. Moreover, community effects acting on these progenitors are not only observed upon exposure to neurogenic but also to gliogenic signals. With the characterization of neural crest-derived progenitor cells in mouse embryos, it will be possible to further elucidate the nature of the molecules involved in these processes.

Results and Discussion

Intrinsic Differences Between Multipotent Progenitor Cell Types of the PNS

Although postmigratory neural crest-derived progenitors expressing P0 and PMP22 are similar to NCSCs isolated from neural tube explants in that they can give rise to neural and non-neural cell types, there seem to be intrinsic differences between these multipotent cell types. TGFß generates single cell clones in clonal cultures of P0/PMP22-positive progenitors, whereas NCSCs undergo cell divisions in the presence of TGFß (Hagedorn *et al.,* 1999). In the absence of instructive growth factors, NCSCs exhibit multiple fates when grown in standard conditions, producing mostly colonies containing neurons, glia and non-neural cells (Hagedorn *et al.,* 1999; Stemple and Anderson, 1992). In contrast, multipotent

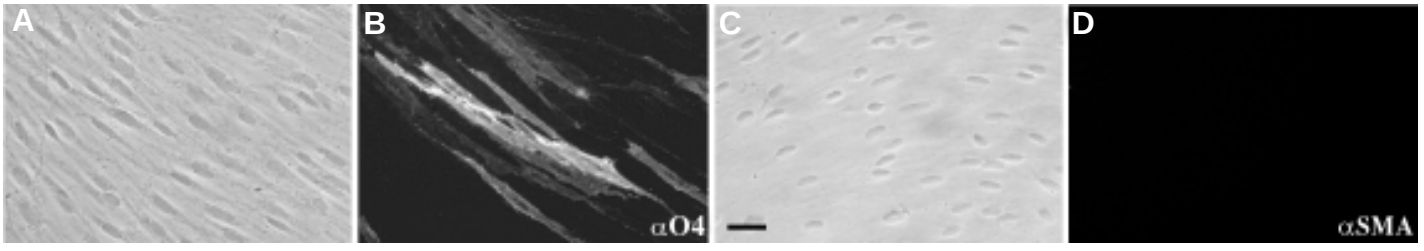

Fig. 2. Community effects restrict DRG-derived progenitor cells to a glial fate, suppressing a non-neural fate. *Neural crest-derived progenitor cells isolated from rat DRG at E14 were plated at high density and incubated in standard medium supplemented with FBS/F. Compared to clonal cultures (see Fig. 1), glial differentiation was accelerated in high density cultures, as analyzed by O4 expression* **(A,B)**. De novo *neurogenesis did not occur (data not shown). In addition, an almost complete suppression of a smooth muscle-like cell fate was observed* **(C,D)** *which might be due to community effects. A and C are phase images of B and D respectively. Scale bar, 20μm.*

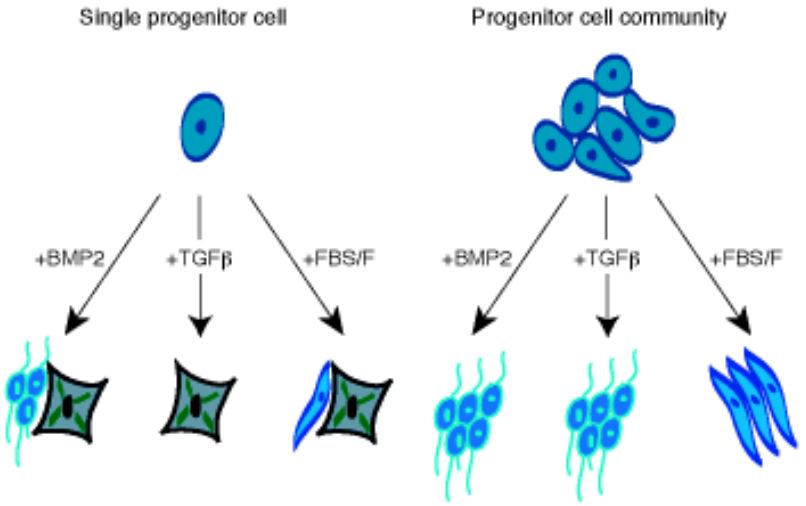

Fig. 3. Short range cell-cell interactions termed community effects influence fate decisions in neural crest-derived progenitor cells. *Our combined data indicate that community effects occur in response to several growth factors including BMP2, TGFß, and factors present in serum and forskolin (FBS/F) (this study and Hagedorn et al., 2000a; Hagedorn et al., 1999). Apart from neuronal or glial fates (promoted in the presence of BMP2 or FBS/F, respectively), these factors can also induce a non-neural, smooth muscle-like fate (indicated by grey, flat cells in the cartoon) in single progenitor cells derived from neural crest explants or from DRG. In contrast, the non-neural fate is suppressed in progenitor cell communities. In communities of neural crest cells derived from explant cultures, members of the TGFß-factor family induce neurogenesis. In addition, TGFß can promote cell death as an alternative fate (not shown; Hagedorn et al., 2000a). Factors contained in FBS/F promote gliogenesis in DRG-derived progenitor cells.*

P0/PMP22-positive progenitors residing in postmigratory neural crest derivatives of rat embryos are more restricted in such culture conditions (Hagedorn *et al.,* 1999; Morrison *et al.,* 1999). Although variations in the culture conditions can not be excluded, these data support the hypothesis that various neural crest-derived rat tissues contain similar but distinct multipotent progenitors early in development.

To further establish intrinsic differences between multipotent progenitors of the PNS, we compared the combined influence of serum and forskolin (which elevates intracellular cAMP levels) on rat NCSCs isolated from neural crest explants vs. DRG-derived progenitor cells. In mass culture, these conditions promote the generation of neurons and Schwann cells from neural crest explants (Stemple and Anderson, 1992; Hagedorn *et al.,* 2000b) while mainly Schwann cells are produced from DRG-derived progenitor cells (Hagedorn *et al.,* 2000b). Clonal analysis was performed to be able to follow and quantify the fates of individual neural crest-derived progenitor cells (Fig. 1). About 45% of all clones generated from single NCSCs in medium containing serum and forskolin (FBS/F) contained glial cells as defined by the expression of the low affinity neurotrophin receptor p75. In contrast, up to 90% of DRG-derived progenitors gave rise to clones with glial cells in these conditions. Moreover, although both NCSCs and DRG-derived cells were able to generate smooth muscle-like cells in response to FBS/F, such cells were mostly associated with glial cells in DRG cultures while many NCSCs gave rise to smooth muscle-only clones. Most strikingly, however, in contrast to NCSCs that produced neurons in about 50% of all colonies, neuron-containing clones were not detectable upon FBS/F treatment of DRG-derived progenitors, although these have the potential to generate neurons upon BMP2 treatment (Hagedorn *et al.,* 1999). Thus, neural crest-derived progenitor cells obtained from DRG maintain the competence to respond to instructive growth factors in a way similar to that exhibited by NCSCs, but are intrinsically biased towards a glial fate in response to other extracellular cues contained in FBS/F.

Recently, the *in vivo* transplantation of NCSCs isolated from neural crest explants and of neural crest cells derived from sciatic nerve also revealed cell-intrinsic differences in the developmental potential of these multipotent cells (White *et al.,* 2001). Thereby, the generation of different neuronal subtypes was apparently based on altered sensitivity to the neurogenic factor BMP2, suggesting an interplay between the extracellular environment and a progenitor type-specific cell-intrinsic program. Similarly, we recently demonstrated that multipotent progenitors isolated from DRG but not from sciatic nerve are competent to upregulate the Ets domain transcription factor Erm in response to NRG1 (Hagedorn *et al.,* 2000b). Thus, migratory and postmigratory neural crest cells, though multipotent, exhibit intrinsic changes during development which modulate their response to extracellular signals.

Community Effects Suppressing Non-Neural Fates in Progenitor Cells

Although intrinsically biased towards certain fates, various neural crest-derived progenitor cells still display a high degree of plasticity when challenged by instructive growth factors. As previously mentioned, we suggest that community effects represent a mechanism to control this plasticity and to restrict multipotent cells of the PNS to appropriate fates. So far, such effects have been

TABLE 1

BRDU INCORPORATION IN PROGENITOR CELLS UPON TGFβ TREATMENT

	Clones derived from single cells (%)	Clones derived from cell communities (%)
No add	98 ± 6	96 ± 3
+ TGFβ	22 ± 5	97 ± 4

TGFβ induces cell cycle arrest in single progenitor cells but not in progenitor cell communities. Single progenitor cells and progenitor cell communities were generated as described in the legend to Fig.4. TGFβ was added to some culture dishes, followed by BrdU addition. BrdU-positive clones contain at least 90% of BrdU-positive cells. The data are expressed as the mean ± SD of three independent experiments. 100 clones were scored per experiment. Single cell display a significantly reduced proliferative capacity in response to TGFβ compared to the control as assessed by BrdU incorporation. In contrast, proliferation of cells in clusters was not affected by TGFβ addition.

observed in response to factors which promote neurogenesis in cell communities (Hagedorn *et al.,* 2000a; Hagedorn *et al.,* 1999; see Fig. 3). We therefore investigated whether short range cell-cell interactions would also be able to influence fate decisions of multipotent progenitor cells in response to gliogenic factors. As discussed above, single DRG-derived progenitor cells plated at clonal density generate mostly glia-containing clones in the presence of FBS/F. However, approximately 60% of these clones were associated with few smooth muscle-like cells. In contrast, when cell-cell interactions were allowed to take place in high density cultures of DRG-derived progenitor cells, we not only observed accelerated glial differentiation (as assessed by O4 expression) but also an almost complete suppression of a smooth muscle-like cell fate (Fig. 2). As in single cell cultures, FBS/F did not induce neurogenesis in high density cultures of DRG-derived progenitor cells (data not shown). Thus, cell-cell interactions provided in high density cultures of progenitor cells in conjunction with a hitherto unidentified activity present in FBS/F are glia-promoting whereas the induction of a non-neural fate by FBS/F is suppressed.

The nature of the glia-inducing activity contained in FBS/F remains to be elucidated. NRG1, which is a strong gliogenic signal for various neural crest-derived cell types (Hagedorn *et al.,* 1999; Morrison *et al.,* 1999; Shah *et al.,* 1994), is an unlikely candidate. In contrast to FBS/F, approximately 85% of all progenitor cells isolated from rat DRG give rise to glia-only clones in response to NRG1, even when plated at clonal density, while non-neural cells do not develop in such cultures (Hagedorn *et al.,* 1999).

Our combined data demonstrate that community effects occur in different types of culture conditions that allow short range interactions between progenitor cells. The fact that these effects act in conjunction with several growth factors including TGFβ, BMP2, and signals present in FBS/F (Fig. 3) suggests a general mechanism to suppress non-neural fates in neural tissues. Conceivably, signaling provided by cell-cell interactions not only acts in culture but also influences aggregating cells in the developing peripheral ganglia (Sommer, 2001). Accordingly, in these structures neurogenesis and gliogenesis can be promoted by specific growth factors while the aberrant generation of non-neural cells in peripheral ganglia is likely precluded by community effects.

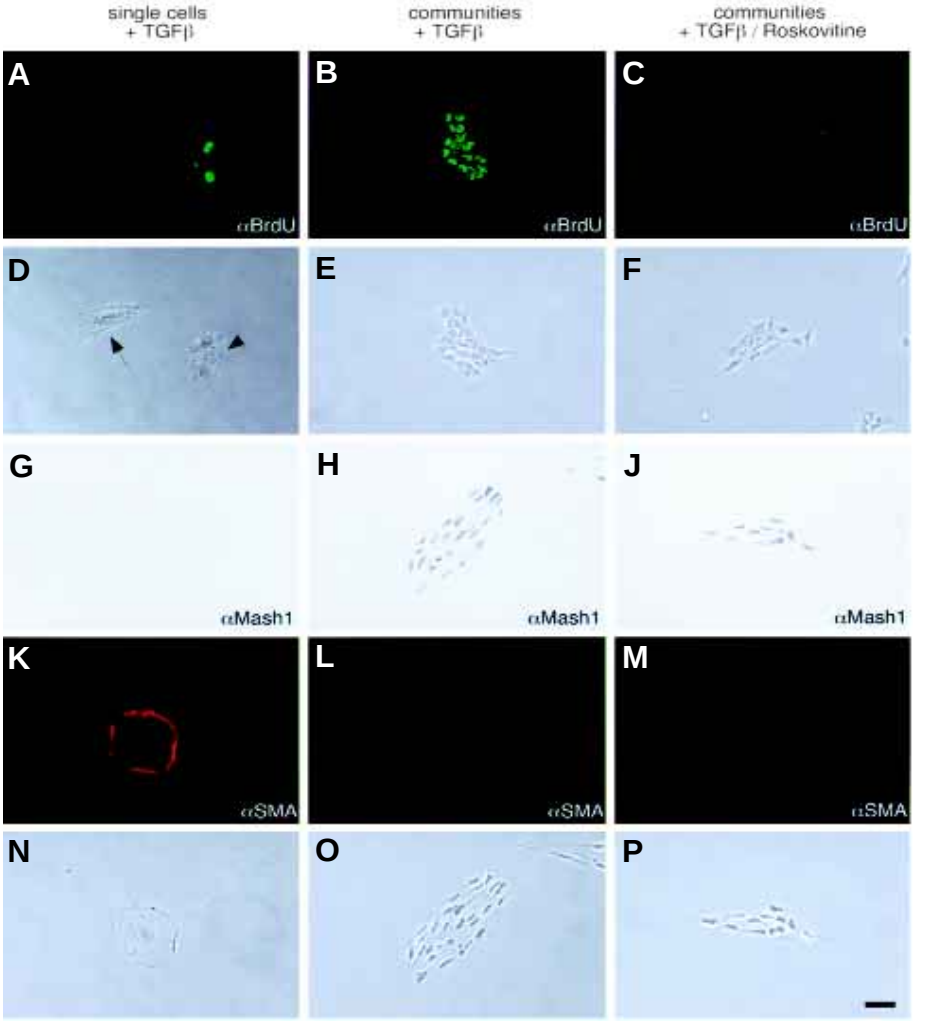

Fig. 4. Cell-cycle-arrested communities of progenitor cells maintain their neuronal potential upon treatment with TGFß. *Clonal cultures of NCSCs were allowed to differentiate in standard culture medium for three days to form neural crest-derived progenitor cells (Hagedorn et al., 1999). Subsequently, some cells were replated and some sister dishes containing clones of progenitors were maintained as cell clusters. To arrest the cell cycle in clusters, Roskovitine was added 30 minutes prior to the addition of TGFß (4 fM) (C,F,J,M,P). As shown by BrdU incorporation, the drug was able to abort the cell cycle in the clusters* **(C,F)**, *whereas clusters treated with TGFß alone proliferated normally* **(B,E)**. *In low density cultures, most of the cells stopped proliferating upon TGFß treatment* **(A)**; *see also arrow in* **(D)**, *and the cells that incorporated BrdU, arrowhead in* **(D)**, *underwent very few cell divisions. Whereas single cells differentiated into SMA-positive non-neural cells upon TGFß treatment* **(G,K,N)**, *TGFß-treated clusters started expressing the neuronal marker Mash1 both in proliferating communities* **(H,L,O)**, *or when the cell cycle was aborted* **(J,M,P)**. *Scale bar, 40μm.*

Differential Regulation of Cell Cycle Progression in Progenitor Cells Displaying Community Effects

In our experimental paradigms of neural crest development, fate restrictions in response to community effects were always found to affect neural vs. non-neural fate choices, as opposed to neuronal vs. glial fate decisions (Fig. 3). The mechanisms underlying community effects in the PNS might thus control processes specific to the development of non-neural, smooth muscle-like cells. The generation of smooth muscle-like cells from NCSCs appeared to correlate with reduced proliferation capacity (Shah *et al.,* 1996). Likewise, when single P0/PMP22-positive progenitors are challenged with TGFβ to adopt a smooth muscle-like fate, proliferation seems to be suppressed as indicated by an average clone size of 1.3 cells per progenitor-derived colony (Hagedorn *et al.,* 1999). In contrast, progenitor communities appear to proliferate upon TGFβ treatment

before they differentiate into neurons (Hagedorn *et al.,* 1999). The fates acquired by single multipotent progenitors versus progenitor communities in cultures of neural crest-derived cells thus suggest an association of cell proliferation with specific responses to TGFβ signaling. Moreover, it is known that TGFβ is able to interact with members of the CDK inhibitor family that are involved in cell cycle control (Amati *et al.,* 1998; Hannon and Beach, 1994; Polyak *et al.,* 1994). Therefore, we investigated whether lineage decisions in response to TGFβ signaling are coupled with the capacity to proliferate. To this end, we first confirmed differences in the proliferation rate of single neural crest-derived progenitors compared to clusters of progenitors in response to 4fM TGFβ by performing BrdU labeling. While in control cultures, 98% of the single cells incorporated BrdU, only 22% of the single cells on TGFβ-treated sister dishes did so (Table 1). In contrast, 96% of the clusters in control cultures and 97% of the clusters in TGFβ-treated cultures were BrdU-positive (Table 1). These data indicate that TGFβ signaling aborts the cell cycle in single progenitor cells but does not affect proliferation in communities of neural crest-derived progenitors.

Hence, progenitor communities normally proliferate before undergoing neuronal differentiation whereas single progenitor cells do not proliferate before adopting a non-neural, smooth muscle-like fate. If the cell cycle influences the differential fate decisions in progenitor communities compared to single cells, one might speculate that blocking cell cycle progression of TGFβ-treated progenitor cell communities prevents these cells from adopting a neuronal fate. The CDK inhibitor Roskovitine is able to block cell cycle progression efficiently. To test the influence of the cell cycle on cell fate decisions in progenitor communities, Roskovitine was directly added to the cultures 30 minutes prior to addition of BrdU and TGFβ. As shown by BrdU labeling, addition of the drug to the cultures was able to abort the cell cycle completely (Fig. 4 A-F). Moreover, while single non-proliferative progenitors differentiated into smooth muscle–like cells upon treatment with TGFβ (Fig. 4 G,K,N), virtually all communities of cell cycle-arrested progenitors upregulated expression of the early neuronal marker Mash1 but not of the non-neural marker smooth muscle actin (Fig. 4 J,M,P). Thus, despite its correlation with the developmental program adopted by progenitor cells, arrest of cell cycle progression does not appear to influence cell fate decisions in communities of neural crest-derived progenitors.

Characterization of Multipotent Progenitors Isolated from Mouse DRG: Establishing an Experimental System to Elucidate Intrinsic and Extrinsic Mechanisms Underlying Fate Restrictions in Neural Crest Development

Community effects that alter the response of multipotent progenitor cells to a given instructive growth factor conceivably reflect the capacity of the cells to integrate different signaling pathways. In particular, TGFβ signaling has been shown to be modulated by convergence with several other signal transduction pathways (reviewed in Massague and Chen, 2000; Piek *et al.,* 1999; Wrana, 2000). It will require a suitable experimental system to determine the molecular nature of the signaling mechanisms involved. Many aspects of fate decision processes in neural crest development, particularly concerning intrinsic developmental changes as well as community effects discussed in this study, have been investigated using rat or chicken neural crest-derived cells (Anderson *et al.,* 1997; Sommer, 2001). Mice in which gene ablation experiments can be performed might represent a valuable alternative model system to address the molecular mechanisms regulating fate restrictions in multipotent neural crest cells. Therefore, we sought to characterize neural crest-derived progenitor cells in developing mouse embryos and to compare features of these cells with their well-characterized rat counterparts.

As previously mentioned, P0 and PMP22, abundant proteins of peripheral myelin, are expressed on multipotent progenitors of the rat PNS (Hagedorn *et al.,* 1999; Morrison *et al.,* 1999). We first

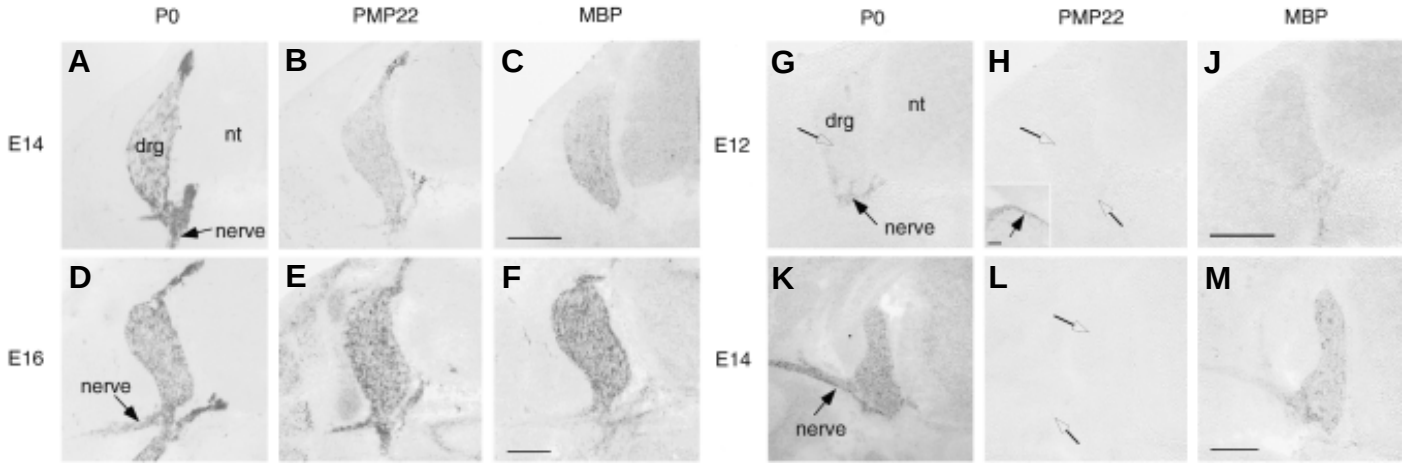

Fig. 5. Comparison of early markers of neural crest derivatives in developing rat (A-F, left panel) and mouse (G-M, right panel) embryos. *(A-C), E14; (D-F), E16; (G-J), E12; (K-M), E14.* In situ *hybridization analysis on transverse sections of rat and mouse embryos revealed that P0 and PMP22 are expressed in rat embryonic development both in peripheral nerves and in DRG from E12 onwards* **(A,B,D,E)***; shown are data from rat E14 and E16. P0 mRNA expression is not detectable in mouse DRG at E12; open arrow in* **(G)***, but is clearly present in the peripheral nerves early in mouse development* **(G)***. At E14 in the mouse, P0 expression is found both in DRG and in peripheral nerves* **(K)***. More strikingly, PMP22 expression is not found in the early mouse PNS at E12 and E14; open arrows in* **(H,L)***, while it is detectable in non-neural tissues such as the liver capsule (inset in H, arrow). The expression pattern of MBP is comparable in the rat and mouse PNS* **(C,F,J,M)***. Scalebars A-M, 100μm; inset in H, 25μm.*

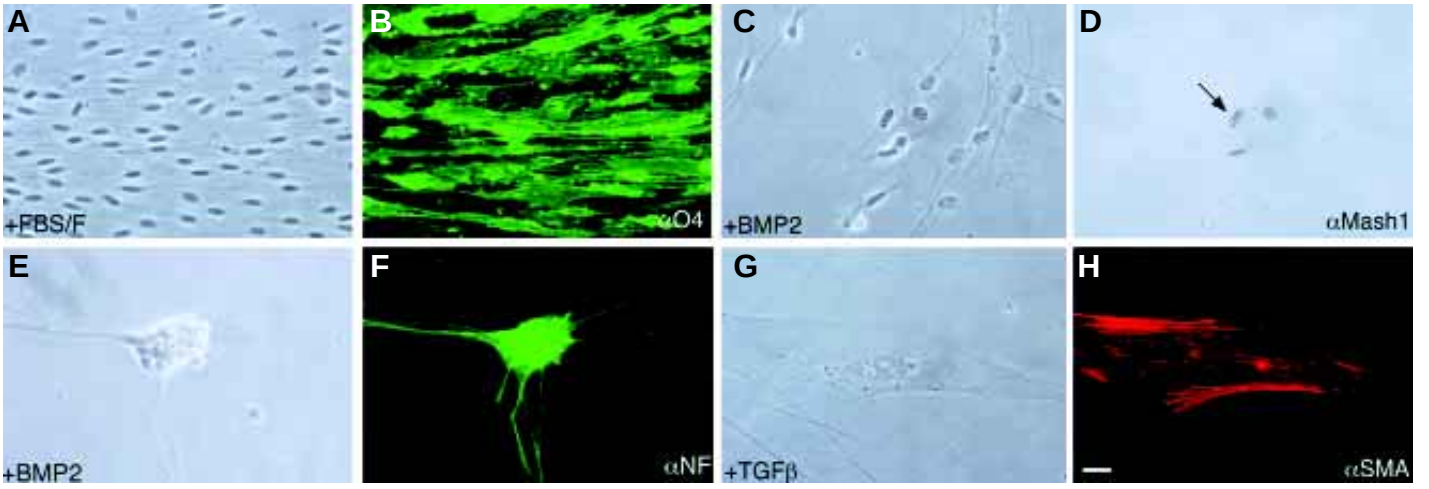

Fig. 6. Mouse DRG-derived progenitor cells display glial, neuronal and non-neural potential. *Undifferentiated progenitor cells isolated from mouse DRG at E12 were challenged with different growth factors and the fates of their progeny were assessed by immunocytochemistry. Gliogenesis was promoted in response to FBS/F as shown by O4 staining* **(A,B)**. *BMP2 treatment led to the formation of neuronal cells expressing Mash1 (arrow)* **(C,D)** *which differentiated to NF160-positive neurons* **(E,F)**. *TGFß induced the differentiation of SMA-positive non-neural cells* **(G,H)**. *(A,C,E,G) Phase images. Scale bar, 25μm.*

performed a series of *in situ* hybridization experiments on transverse sections of rat and mouse embryos to investigate whether *P0* and *PMP22* are expressed at early stages of mouse PNS development, as they are in rat embryos. Surprisingly, while *P0* and *PMP22* are expressed in rat embryonic development both in peripheral nerves and in dorsal root ganglia (DRG) from E12 onwards (Fig. 5; Hagedorn *et al.,* 1999), expression in the early developing mouse DRG is weak or even absent. *P0* mRNA is clearly expressed in peripheral nerves of young mouse embryos but not yet detectable in the DRG at E12 when multipotent progenitors would be expected to populate this neural crest derivative (Fig. 5G; Sommer *et al.,* 1995). More strikingly, at these early stages *PMP22* expression is found in non-neural tissues such as the liver capsule (inset in Fig. 5H; Baechner *et al.,* 1995) but was not observed in the mouse PNS (open arrows in Fig. 5 H,L). In contrast, the expression pattern of Myelin Basic Protein (MBP), another marker of early neural crest derivatives (Landry *et al.,* 1997), is comparable in rat and mouse embryos (Fig. 5 C,F,J,M). Thus, P0 and PMP22 are valuable markers for the well characterized PNS progenitors isolated from rat embryos but not necessarily for early mouse neural crest derivatives. It has been suggested that these proteins not only have a role in the initiation and maintenance of myelin (Mirsky and Jessen, 1996; Suter and Snipes, 1995) but might also mediate cell-cell interactions during development (reviewed in Naef and Suter, 1998; Sommer and Suter, 1998). However, the expression pattern presented here makes them unlikely candidates for playing a role in mediating community effects in PNS progenitor cells.

The differential marker expression in early rat and mouse PNS raises the question of whether, in addition, there might be species differences in the processes that determine cell lineages in neural crest development. Do multipotent progenitors exist in postmigratory targets of mouse neural crest? If so, can they be challenged by instructive growth factors, as in the rat (Hagedorn *et al.,* 1999; Lo and Anderson, 1995; Morrison *et al.,* 1999; Shah *et al.,* 1996; Shah *et al.,* 1994)? To address these issues, we analyzed the potential of undifferentiated cells obtained from mouse E12 DRG. This stage is comparable to the developmental stage of rat E14 DRG from which rat multipotent progenitors have previously been isolated (Hagedorn *et al.,* 1999). Mouse E12 DRG were dissociated, plated at low density and challenged with a variety of growth factors. As in the rat, non-neuronal cells freshly isolated from early DRG were p75-positive but negative for glial and neuronal differentiation markers (data not shown). However, clonal cultures of mouse multipotent neural crest cells turned out to be difficult to maintain due to considerable cell death, with the exception of cell cultures treated with serum-containing medium. Nevertheless, the overall response of mouse cultures to instructive growth factors was similar to that seen with their rat counterparts. Gliogenesis was promoted in response to NRG1 (data not shown) and to FBS/F (Fig. 6 A,B). As in the rat (Fig. 1), the latter condition also induced smooth muscle formation to a certain extent from single progenitor cells. BMP2 treatment of mouse DRG cells led to the formation of non-neural cells and cells positive for Mash1, a marker for the autonomic neuronal lineage (Lo *et al.,* 1991) (Fig. 6 C,D). Moreover, the generation of differentiated neurons was observed in these conditions (Fig. 6 E,F). Finally, TGFβ induced the differentiation of smooth muscle-like cells from single mouse DRG cells (Fig. 6 G,H). These data demonstrate that undifferentiated cells derived from mouse DRG display the developmental potential to generate neuronal, glial, and non-neural cells, suggesting that, as in the rat, multipotent progenitor cells exist in derivatives of mouse neural crest.

Conclusion

The availability of appropriate *in vitro* culture systems allowed the identification of postmigratory multipotent progenitors from various structures of the PNS and to challenge these cells by changing their extracellular context. Such experiments, in combination with *in vivo* approaches (Anderson *et al.,* 1997; Le Douarin and Ziller, 1993), revealed that individual progenitor cells are competent to generate multiple lineages and that fate restrictions are regulated by a combinatorial interplay between cell-intrinsic and cell-extrinsic cues. The ability to generate progenitor cells with

similar features *de novo* from NCSCs (Hagedorn *et al.,* 1999) suggests a direct lineal relationship between multipotent NCSCs and distinct but still multipotent progenitors of the PNS. According to this model, neural crest development might be seen as a sequential production of distinct multipotent cell types. Neuroepithelial stem cells can self renew, differentiate into neurons and glia of the central nervous system (CNS), and generate limited but multipotent NCSCs (Kalyani *et al.,* 1997; Mujtaba *et al.,* 1998). NCSCs then produce neural cell types of the PNS and non-neural derivatives by intermediate postmigratory progenitors that are still multipotent. These progenitors, located in different crest derivatives such as DRG or peripheral nerves, display fate restrictions imposed by cellular association termed community effects. In addition, however, multipotent cell types of the PNS are intrinsically different and exhibit fate restrictions independent of cellular associations. These specific intrinsic programs have probably been implemented by distinct environments (Le Douarin and Ziller, 1993) and are revealed by transplantation experiments (Le Douarin and Ziller, 1993; White *et al.,* 2001) or by clonal analysis in conditions permissive for the generation of several neural crest-derived lineages. It will be interesting to investigate whether the various multipotent progenitor cells isolated from the CNS (reviewed in Fuchs and Segre, 2000; Rao, 1999) can also be distinguished by similar experiments.

The molecular basis for both the intrinsic features that distinguish multipotent progenitor cell types and the extrinsic mechanisms regulating fate decisions appropriate to a given embryonic location remains to be elucidated. Membrane-associated signaling molecules, locally accumulated secreted signals, or signaling via gap junctions are likely to mediate the community effects observed in neural crest-derived cells (Sommer, 2001; Paratore *et al.,* 2001). With the ongoing characterization of multipotent progenitor cells in the mouse PNS it will be feasible to address the cellular function of candidate molecules in lineage decision and early neural differentiation using genetic approaches. The combination of *in vitro* analysis of mutant cells (Sommer *et al.,* 1995; Paratore *et al.,* 2001) with *in vivo* assays promises to become a very valuable tool to elucidate how fates are regulated in multipotent cells during neural development.

Materials and Methods

Riboprobes and Non-Radioactive In Situ *Hybridization*

Antisense riboprobes were digoxigenin-labeled according to the manufacturer's instruction (Roche Diagnostics). The following riboprobes were used: rat P0 (Lemke and Axel, 1985); rat PMP22 (Welcher *et al.,* 1991); mouse PMP22 (Suter *et al.,* 1992); mouse MBP (de Ferra *et al.,* 1985; a gift from A.Gow, Wayne State University, Detroit). Non-radioactive in situ hybridization with Dig-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse and rat embryos as described (Paratore *et al.,* 1999; Sommer *et al.,* 1996).

Cell Cultures

Time-mated OFA rats and mice were obtained from Biological Research Laboratories (Fullinsdorf, Switzerland). NC and DRG cultures were prepared as described (Hagedorn *et al.,* 2000b; Hagedorn *et al.,* 1999; Stemple and Anderson, 1992). NCSCs were replated at clonal density (300 cells per 35mm dish) onto 0.5 mg/ml poly-D-lysine (pDL) (Roche Diagnostics) and 0.25 mg/ml fibronectin (FN) (Roche Diagnostics) coated dishes (Corning) after neural crest outgrowth. In some experiments, the cells were treated with standard culture medium supplemented with 10% fetal bovine serum (FBS) and 5μM forskolin (Sommer *et al.,* 1995). In other experiments, NCSCs were allowed to differentiate into multipotent progenitor cells that were then further incubated as single cells or cell communities exactly as described before (Hagedorn *et al.,* 1999). DRG were dissected from rat E14 and mouse E12 embryos, respectively, dissociated as previously reported (Hagedorn *et al.,* 1999), and plated either at 300 cells (clonal density) or at 20'000 cells per 35mm pDL/FN-coated culture dishes. The cells were maintained in standard culture medium supplemented with 10% FBS and 5μM forskolin. In some experiments, 1.6 nM BMP2 (a gift from Genetics Institute, Cambridge MA) or 4 fM TGFß1 (R&D Systems) were added three hours after plating the cells.

BrdU Labeling and Cell Cycle Arrest

The BrdU labelings were carried out according to the manufacturer's instructions (Roche Diagnostics). In brief, prior to addition of BrdU, some of the cells were incubated in 4 fM TGFß for 8 hours while control cells on sister dishes were maintained in standard medium. BrdU was added directly to the cultures (final concentration 10 μM) and after 15 - 24 hours of incubation, the cells were fixed in 70% ethanol (in 50 mM glycine buffer, pH 2.0) for 20 minutes. BrdU incorporation was visualized using the anti-BrdU working solution for 30 minutes at 37°C, followed by incubation with FITC-coupled horse anti-mouse IgG (Vector Laboratories) for 1 hour at RT. To arrest the cell cycle, Roskovitine (25 μM) (Calbiochem) was directly added to the medium 30 minutes prior to the addition of TGFß. Cells were fixed after 24 hours.

Immunocytochemistry

Labeling of the cell surface antigen LNGFR was performed on living cells in standard culture medium for 30 minutes using a rabbit anti-mouse p75 nerve growth factor (NGF) receptor polyclonal antibody (Chemicon International) visualized by Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research Laboratories). To label intracellular antigens, cells were fixed in PBS containing 3.7% formaldehyde for 10 minutes at RT. For Mash-1, cells were permeabilized for 15 minutes at RT with 2% goat serum, 0.1% NP40 in PBS and stained o.n. at 4°C with mouse anti-Mash1 monoclonal antibody (1:1 dilution of hybridoma supernatant; a gift from D.J.Anderson, Caltech, Pasadena). The staining was visualized by incubation for 1 hour with horse raddish peroxidase (HRP)-coupled goat anti-mouse IgG (1:200 dilution; Pierce) and HRP development using diaminobenzidine (DAB) as substrate. 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) (1:200 dilution; Sigma); rabbit polyclonal anti-NF160 (1:200 dilution); 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); FITC-coupled horse anti-mouse IgG (Vector Laboratories); FITC-coupled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories). Staining with mouse anti-O4 antibody (1:50 dilution; a gift from M.Schwab, Brain Research Institute, Zurich) was performed without detergent. The staining was detected by FITC-coupled goat anti-mouse IgM (Sigma) used at a 1:200 dilution.

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