

# Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling

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## SUMMARY

The transcription factor Sox10 is required for proper development of various neural crest-derived cell types. Several lineages including melanocytes, autonomic and enteric neurons, and all subtypes of peripheral glia are missing in mice homozygous for Sox10 mutations. Moreover, haploinsufficiency of Sox10 results in neural crest defects that cause Waardenburg/Hirschsprung disease in humans. We provide evidence that the cellular basis to these phenotypes is likely to be a requirement for Sox10 by neural crest stem cells before lineage segregation. Cell death is increased in undifferentiated, postmigratory neural crest cells that lack Sox10, suggesting a role of Sox10 in the survival of neural crest cells. This function is mediated by neuregulin, which acts as a survival signal for postmigratory neural crest cells in a Sox10-dependent manner. Furthermore, Sox10 is required for glial fate

acquisition, as the surviving mutant neural crest cells are unable to adopt a glial fate when challenged with different gliogenic conditions. In Sox10 heterozygous mutant neural crest cells, survival appears to be normal, while fate specifications are drastically affected. Thereby, the fate chosen by a mutant neural crest cell is context dependent. Our data indicate that combinatorial signaling by Sox10, extracellular factors such as neuregulin 1, and local cell-cell interactions is involved in fine-tuning lineage decisions by neural crest stem cells. Failures in fate decision processes might thus contribute to the etiology of Waardenburg/Hirschsprung disease.

Key words: Neural crest, Gliogenesis, Community effects, Sox10, Waardenburg/Hirschsprung disease, Mouse

## INTRODUCTION

Cellular diversity in the developing vertebrate nervous system is thought to be achieved by differentiation of multipotent neural stem cells in a spatially and temporally regulated fashion. In the developing and adult nervous system, multipotent stem cells co-exist with fate-restricted cell types, raising the question of how appropriate sizes of stem cell pools are maintained during development and how cell type specification from these pools is initiated (Gage, 2000; Morrison et al., 1997). The mechanisms involved probably encompass an interplay between extracellular cues and cell-autonomous intracellular signaling (Edlund and Jessell, 1999; Morrison et al., 1997). Moreover, it is conceivable that the decision of a stem cell to survive, self renew or differentiate is dependent on the combinatorial activity of multiple environmental signals (Sommer, 2001). To identify these signals, multipotent stem cells have to be challenged by altering both their extracellular environment and their intrinsic genetic programs regulated by transcription factors. In neural crest development, this is facilitated by the availability of clonogenic culture systems of multipotent cells (reviewed by

Anderson et al., 1997; Sommer, 2001). Clonal analysis of rat neural crest stem cells (NCSCs) allowed the characterization of cell-extrinsic factors that act instructively to promote specific fates at the expense of other lineages (Shah et al., 1996; Shah et al., 1994), although the cell-extrinsic cues that control maintenance of a stem cell state are not known. Similarly, transcription factors that control early steps in neuronal development have been described (Anderson, 1999; Brunet and Ghysen, 1999), but cell-intrinsic factors that regulate other features of neural crest development such as maintenance of a stem cell pool or early peripheral gliogenesis remain to be identified.

Several members of the basic helix-loop-helix (bHLH) transcription factor family have been shown to participate in cell type specification in the nervous system (reviewed by Anderson, 1999; Kageyama and Nakanishi, 1997; Lee, 1997). In the central nervous system (CNS), downregulation of bHLH genes required for neurogenesis promotes glial differentiation (Tomita et al., 2000). Likewise, misexpression of the Notch effectors *Hes1*, *Hes5* and *hesr2* induces gliogenesis (Furukawa et al., 2000; Hojo et al., 2000; Satow et al., 2001), and the bHLH proteins *Olig1* and *Olig2* might

be involved in the early development of oligodendrocytes (Lu et al., 2000; Zhou et al., 2000). However, it remains to be shown whether bHLH factors are required for gliogenesis. Moreover, in contrast to the CNS, bHLH genes that play a role in peripheral gliogenesis have not been described. In *Drosophila*, transcription factors other than bHLH proteins have been implicated in early glial development (reviewed by Granderath and Klämbt, 1999). The formation of lateral glia is dependent on *glial cell missing* (*gcm*) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), but there is no evidence so far that orthologs of *gcm* play a role in vertebrate gliogenesis (Anson-Cartwright et al., 2000; Kim et al., 1998; Schreiber et al., 2000). While *Drosophila* lateral glia are dependent on *gcm* function, midline glia are not regulated by this factor. By contrast, development of midline glia requires, among other genes, the activity of *Dichaete*, a Sox protein containing a high-mobility group (HMG) domain (Soriano and Russell, 1998). *Dichaete* interacts genetically with the POU domain transcription factor *ventral veinless* to specify midline glia and might also have a function at later stages of gliogenesis. Similarly, the vertebrate transcription factor Sox10 potentiates the activity of the POU domain factor Oct6 and other transcription factors involved in glial differentiation in transient transfection experiments (Kuhlbrodt et al., 1998). Furthermore, a role of Sox10 in peripheral gliogenesis is supported by its capacity to regulate PO gene expression (Peirano et al., 2000). In the developing vertebrate PNS, mRNA encoding Sox10 is expressed in neural crest cells, the enteric nervous system, peripheral ganglia and peripheral nerves (Bondurand et al., 1998; Kuhlbrodt et al., 1998; Schneider et al., 1999; Southard-Smith et al., 1998). Although the expression is transient in the enteric nervous system, it persists in other PNS structures and seems to become confined to peripheral glial cells at later stages (Kuhlbrodt et al., 1998). In accordance with this expression pattern, both the spontaneous mouse mutant *Dominant megacolon* (*Dom*) and mice carrying a targeted *Sox10* mutation display various neural crest defects (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). In homozygous animals, these include absence of enteric, sympathetic and parasympathetic ganglia, and loss of glia, melanocytes and adrenal chromaffin cells. While neural crest-derived cranial sensory ganglia are also missing, residual dorsal root ganglia (DRG) in the trunk are formed containing few differentiated neurons and seemingly undifferentiated cells but no glia. Haploinsufficiency of *Sox10* leads to enteric aganglionosis and pigmentation defects in heterozygous *Sox10* mutants (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; reviewed by Wegner, 2000). These symptoms are also found in humans heterozygous for *Sox10* mutations that suffer from Waardenburg/Hirschsprung syndrome (Pingault et al., 1998; Southard-Smith et al., 1999). Other mutations observed in humans cause additional neurocristopathies including myelin deficiencies beside the classical symptoms of Waardenburg/Hirschsprung disease (Bondurand et al., 1999; Inoue et al., 1999; Pingault et al., 2000; Touraine et al., 2000).

Although Sox10 plays a crucial role in neural crest development, its cellular function remains to be determined. Sox10 might be required in multipotent progenitor cells or in developmentally restricted neural crest-derived cell types to

regulate fate decision, early differentiation, survival or migration. We have addressed the cellular function of Sox10 by fate analysis of *Sox10* mutant cells in different contexts *in vitro* and *in vivo*. We demonstrate that *Sox10* is not only required for the survival of multipotent neural crest cells, but also to allow these cells to adopt a glial fate. Moreover, haploinsufficiency of *Sox10* affects fate decisions of neural crest cells in a context-dependent manner by altering their responsiveness to complex extracellular cues.

## MATERIALS AND METHODS

### Cell cultures

Timed matings were performed with mice heterozygous for a targeted deletion of *Sox10* that had been bred onto a C3HeB/FeJ background (Britsch et al., 2001). Neural crest cultures were derived from E9 embryos as previously reported (Stemple and Anderson, 1992) with modifications according to Hagedorn et al. (Hagedorn et al., 1999; Hagedorn et al., 2000b). To assay for endogenous Sox10 expression, glial cells were generated by incubation of neural crest explants in differentiation medium (containing 10% fetal bovine serum and 5  $\mu$ M forskolin) prepared as described in Sommer et al. (Sommer et al., 1995); neurogenesis was promoted by incubation of neural crest explants with differentiation medium supplemented with BMP2 (a gift from Genetics Institute, Cambridge MA) (Shah et al., 1996); non-neural smooth muscle-actin positive cells were generated by replating neural crest cells at low density as described (Shah et al., 1994), followed by incubation in differentiation medium. For comparison of wild-type and *Sox10*-mutant neural crest, neural crest explants were cultured in differentiation medium; the neural tubes were used for genotyping (Sommer et al., 1995). Cultures of postmigratory neural crest cells were obtained by dissection and dissociation of DRG from E13 embryos, as described by Hagedorn et al. (Hagedorn et al., 1999; Hagedorn et al., 2000a). Tissue from a forelimb was used for genotyping by PCR (Britsch et al., 2001; Sommer et al., 1995). In order to perform clonal analysis, single undifferentiated neural crest cells were inscribed with a circle on the bottom of the tissue culture plate. DRG cultures were maintained in differentiation medium (Sommer et al., 1995) between 5 and 13 days. In some experiments, 1 nM rhGGF2, a soluble NRG1 isoform (a gift from M. Marchionni, Cambridge Neuroscience; Marchionni et al., 1993), was added 4 hours after plating the cells.

### Immunocytochemistry

After fixing the cells with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, the cells were permeabilized and blocked for 10 minutes at room temperature with blocking buffer (10% goat serum, 0.3% Triton X-100, 0.1% bovine serum albumin in PBS). Neural crest cells were labeled with rabbit anti-mouse p75 (Chemicon International) diluted 1:200 in blocking buffer (without Triton X-100) for 1 hour at room temperature. Live cell labeling was performed directly in cell culture medium. Staining was performed for 2 hours at room temperature diluted in blocking buffer: monoclonal Sox10 antibody (1:3 dilution; K. Kuhlbrodt and M. W., unpublished), rabbit anti-NF160 (1:500 dilution; Chemicon International), monoclonal anti-NF160 antibody NN18 (IgG) (1:500 dilution; Sigma), monoclonal anti-smooth muscle actin (IgG) (1:400 dilution; Sigma), rabbit anti-cow S100 (1:200 dilution, Dako) and mouse anti-peripherin (IgG) (1:200 dilution; Chemicon International). In order to carry out GFAP staining, the cells were fixed in 70% ethanol/50 mM glycine pH2.0 at  $-20^{\circ}\text{C}$  for 30 minutes. Then they were washed with PBS and incubated in methanol for another 30 minutes. After blocking, monoclonal anti-GFAP (1:200 dilution; Sigma) was applied for 2 hours at room temperature.

The immunostaining was visualized by incubation for 1 hour at

room temperature using the following reagents at a 1:200 dilution: Cy3-conjugated goat anti-mouse IgG; Cy3-coupled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories); and FITC-conjugated horse anti-mouse IgG (Vector Laboratories).

Staining with mouse anti-O4 antibody (1:30 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.

#### TUNEL assay and immunocytochemistry

Cryosections were fixed in 2% paraformaldehyde (PFA)/PBS for 10 minutes, washed three times for 5 minutes in PBS and blocked for 1 hour in 10% goat serum, 0.1% BSA and 1% Triton X-100 in PBS. The anti-p75 or anti-NF160 staining was carried out as previously described. The TUNEL staining was performed according to the manufacturer's instructions (Roche Diagnostics) and visualized by Cy3-conjugated streptavidin (1:200 dilution, Jackson ImmunoResearch Laboratories). In some experiments, immunostaining for an additional marker was detected using goat anti-mouse Cy5-coupled antibody (1:200 dilution; Jackson ImmunoResearch Laboratories). After washing in PBS, the sections were mounted in AF1 (Citifluor) and analyzed by confocal microscopy.

#### Nonradioactive in situ hybridization

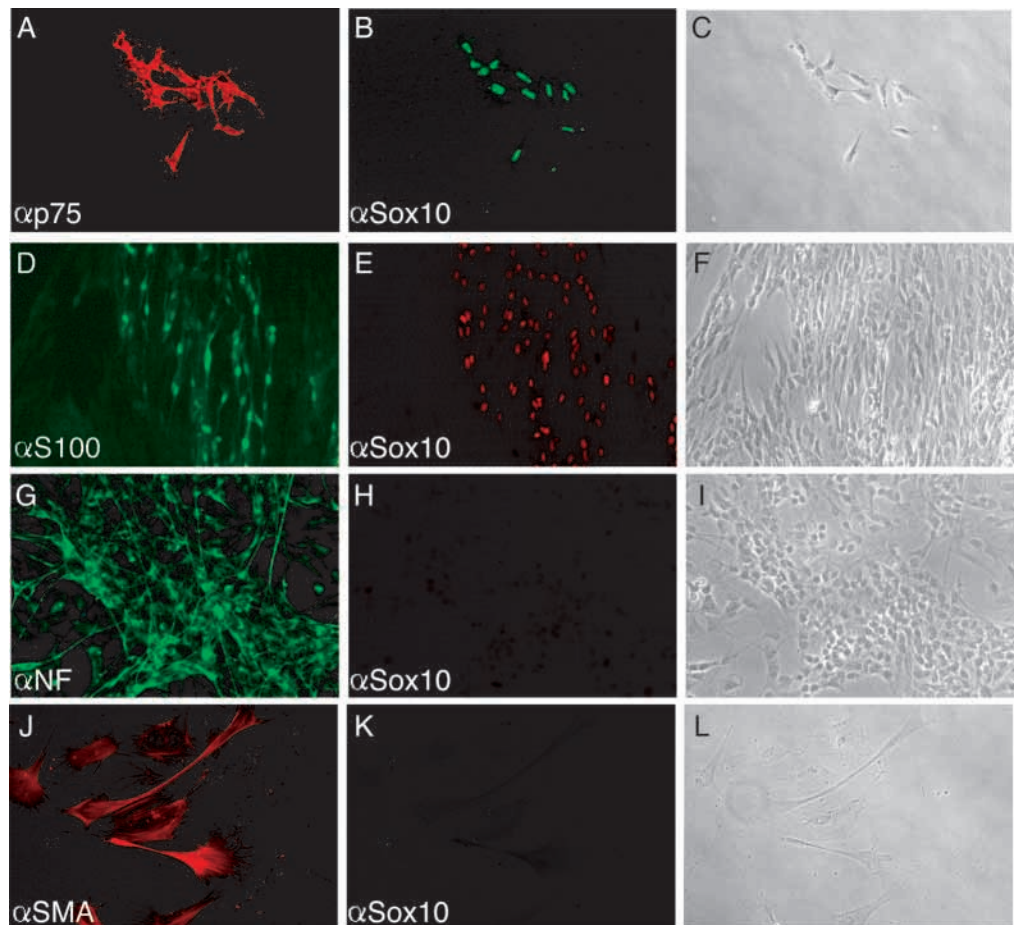
Nonradioactive in situ hybridization with digoxigenin-labeled *ErbB3* riboprobe (a gift from H. Werner, ZMBH, Heidelberg) was performed on frozen sections of paraformaldehyde-fixed mouse embryos as described in Paratore et al. (Paratore et al., 1999). NBT/BCIP were used as chromogens to visualize the hybridization signals.

## RESULTS

### Expression of Sox10 protein in neural crest stem cells

Previous in situ hybridization experiments have demonstrated that *Sox10* mRNA is expressed in the area of migrating neural crest and in postmigratory targets of the neural crest at early stages of embryonic development, and that expression is maintained in peripheral glia (Kuhlbrodt et al., 1998). It is not clear whether Sox10 is a marker for only subpopulations of neural crest cells or whether multipotent neural crest cells are homogeneous with respect to Sox10 expression. To address this issue, we performed immunocytochemistry on two populations of multipotent mouse neural crest cells: self-renewing NCSCs prepared from neural crest explants (Stemple and Anderson, 1992), and postmigratory neural crest cells isolated from early DRG (Hagedorn et al., 1999). Similar to NCSCs, undifferentiated cells isolated from rat or mouse DRG are marked by the low-affinity neurotrophin receptor (p75) and able to give rise to neurons, satellite glia, Schwann cells or smooth muscle-like cells in the presence of appropriate growth factors (Hagedorn et al., 2000b; Hagedorn et al., 1999; C. P. and L. S., unpublished). Labeling by an anti-Sox10 antibody revealed that all p75-positive NCSCs expressed Sox10 (Fig. 1A,B). Likewise, all undifferentiated p75-positive neural crest cells from DRG of mouse embryonic day (E) 13 embryos were also Sox10 positive (data not shown).

**Fig. 1.** Expression of Sox10 protein in neural crest stem cells and peripheral glia. Neural crest stem cells (NCSCs) were replated from neural crest explants, fixed after 4 hours, and immunolabeled using anti-p75 antibody (visualized by Cy3 fluorescence) (A) and anti-Sox10 antibody (visualized by FITC fluorescence) (B). Note that virtually all freshly isolated NCSCs are double-positive for p75 and Sox10. Sister cultures of replated NCSCs were allowed to develop in conditions (Materials and Methods) that predominantly generate S100-positive glia (D), neurofilament160 (NF)-positive neurons (G) or smooth muscle actin (SMA)-positive non-neural cells (J). Double-labeling of the cultures for Sox10 revealed that Sox10 expression is maintained in the glial lineage (E) while it is downregulated in neuronal (H) and non-neural (K) cells. (C,F,I,L) Corresponding phase-contrast pictures.





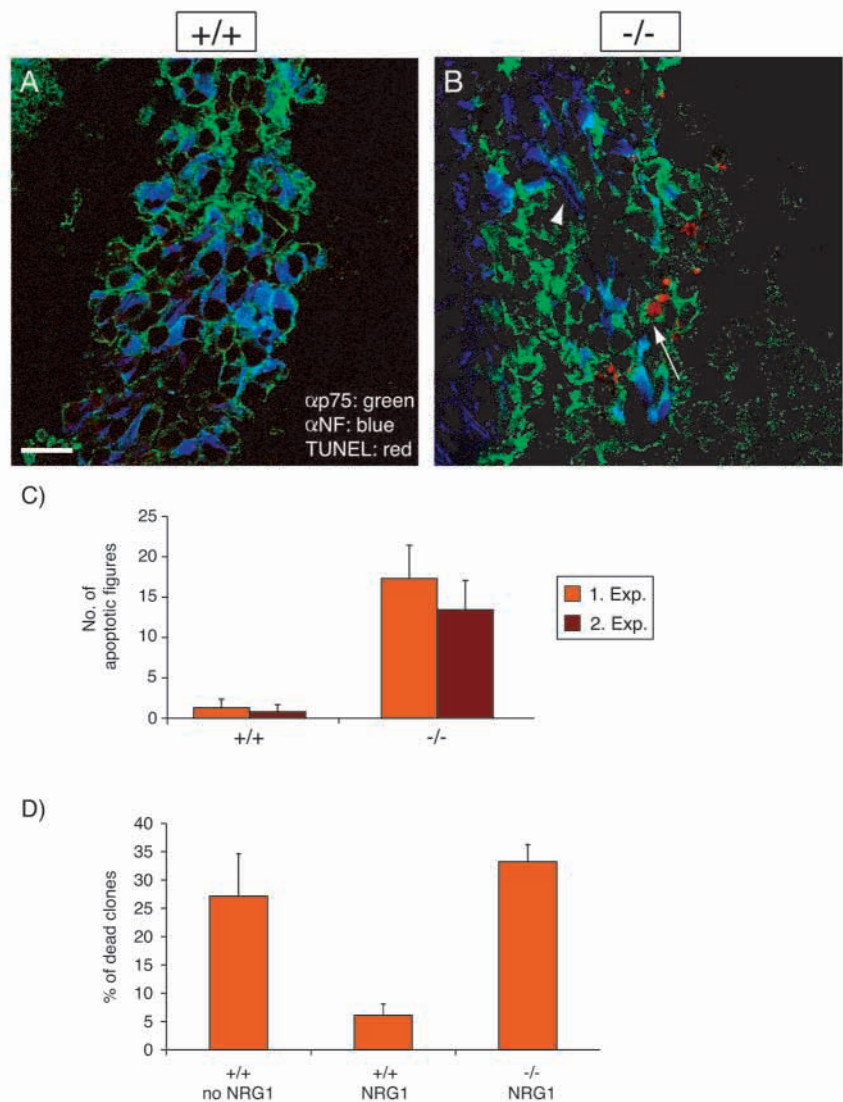
To investigate the cellular expression pattern of Sox10 protein during neural crest development, NCSCs were allowed to differentiate under conditions (Materials and Methods) that allow the generation of S100-positive glia (Fig. 1D), neurofilament (NF) 160-positive neurons (Fig. 1G), or smooth muscle actin (SMA)-immunoreactive smooth muscle-like cells (Fig. 1J), also called myofibroblasts (Morrison et al., 1999). While peripheral glia were Sox10 positive (Fig. 1E), Sox10 expression was not detectable in neural crest-derived neuronal and non-neural cells (Fig. 1H,K). Thus, Sox10 is homogeneously expressed in NCSCs and in multipotent, postmigratory neural crest cells. Sox10 protein expression is maintained in the glial lineage while it is downregulated upon neuronal and non-neural differentiation.

### Sox10 is required for NRG1-dependent survival of multipotent neural crest cells

The expression pattern of Sox10 is consistent with a role in NCSCs and during gliogenesis. In agreement with an early function of Sox10 in neural crest development, mice homozygous for *Sox10* mutations exhibit defects in multiple neural crest derivatives (Britsch et al., 2001; Herbarth et al.,

1998; Kapur, 1999; Southard-Smith et al., 1998). However, it remains unclear whether *Sox10* inactivation affects the early development of multipotent neural crest cells or of several distinct neural crest derivatives after lineage segregation. In particular, the phenotype of *Sox10* mutant embryos might reflect a requirement of *Sox10* in neural crest survival, migration, differentiation, or any combination thereof. Initial neural crest migration appears not to be affected in embryos homozygous for a targeted mutation in *Sox10* (Britsch et al., 2001). Moreover, we found no evidence for increased cell death in migratory trunk neural crest of *Sox10*<sup>-/-</sup> embryos using TUNEL labeling (data not shown). To analyze the survival capacity of multipotent postmigratory neural crest cells, we assessed apoptotic cell death in ganglionic cells of wild-type and *Sox10* mutant embryos. Transverse sections of wild-type embryos at E11 or of *Sox10*<sup>-/-</sup> littermates were processed for fluorescence double labeling with antibodies to p75 and to the neuronal marker NF160. Subsequently, TUNEL labeling was performed and the staining was analyzed by confocal microscopy. Very few apoptotic figures were detectable in wild-type DRG (on average 1.1 per embryonic section; Fig. 2A,C). By contrast, cell death was considerable

**Fig. 2.** *Sox10*-dependent survival of undifferentiated postmigratory neural crest cells. (A-C) Apoptotic cell death of undifferentiated postmigratory *Sox10*<sup>-/-</sup> neural crest cells. Transverse cryosections through DRG from E11 embryos were triple labeled for p75 (FITC fluorescence), NF (Cy5 fluorescence) and TUNEL (Cy3 fluorescence), and analyzed by confocal microscopy. A single confocal plane is shown. Apoptosis as assayed by TUNEL labeling was frequent in undifferentiated p75-positive DRG cells of *Sox10* homozygous (-/-) mouse mutants (arrow in B) but not of wild-type (+/+) littermates. The association of nuclear TUNEL staining with p75 labeling (on the cell surface) was particularly apparent when various confocal planes were analyzed (not shown). Note that NF-positive differentiated neurons were usually not associated with apoptotic figures at this stage (arrowhead in B). Scale bar: 10  $\mu$ m. (C) Quantification of apoptotic figures in DRG of wild-type (+/+) and *Sox10*<sup>-/-</sup> (-/-) embryos. Each bar represents the number (mean $\pm$ s.d.) of apoptotic nuclei in DRG per section. Two independent experiments using non-sibling embryos were performed, scoring 13 consecutive thoracic sections per experiment. (D) NRG1 is a *Sox10*-dependent survival factor of undifferentiated postmigratory neural crest cells. Postmigratory neural crest cells prepared from wild-type (+/+) or *Sox10*<sup>-/-</sup> (-/-) DRG were plated at clonal density, the position of single neural crest cells was mapped, and clones were allowed to develop in differentiation medium ('no NRG1') or in differentiation medium supplemented with NRG1 ('NRG1'). The numbers indicate the percentage of clones lost owing to cell death of the founder neural crest cell. The data were obtained from four independent experiments counting 60 clones each. Note that cell death of wild-type cells but not of *Sox10*<sup>-/-</sup> mutant cells was significantly reduced in the presence of NRG1.



in DRG of *Sox10*<sup>-/-</sup> embryos and was increased 13- to 17-fold in mutant when compared with wild type DRG (Fig. 2B,C). Apoptosis was prominent in undifferentiated cells expressing p75 (arrow) but not found in differentiated neurons (arrowhead). These data are compatible with a role of Sox10 activity in supporting survival of undifferentiated, postmigratory neural crest cells before lineage segregation.

The phenotype of mice mutant for the NRG1 receptor *ErbB3* is consistent with a role of *ErbB3* in survival and/or migration of neural crest cells (Riethmacher et al., 1997). Moreover, maintenance of *ErbB3* expression is regulated by Sox10 (Britsch et al., 2001). This suggests that NRG signaling might be involved in mediating the survival-promoting function of Sox10. To test this idea, an experimental system is required that allows to distinguish between the roles of Sox10 and NRG signaling in survival, migration and proliferation of multipotent, undifferentiated neural crest cells. In the rat, various clonogenic culture systems of NCSCs and neural crest-derived multipotent progenitor cells are available, in which the cellular behavior of individual cells can be monitored (Hagedorn et al., 1999; Lo and Anderson, 1995; Morrison et al., 1999; Stemple and Anderson, 1992). By contrast, clonal cultures of mouse multipotent neural crest cells have been difficult to perform due to massive cell death of dispersed cells at low density. This was not the case when we incubated multipotent neural crest cells isolated from mouse DRG in differentiation medium containing serum (see Materials and Methods). Using this experimental paradigm, we investigated whether: (1) NRG1 can indeed act as a survival factor for undifferentiated postmigratory neural crest cells; and (2) if so, whether this function is Sox10 dependent. DRG of wild-type E13 embryos were dissociated and the cells were plated at clonal density. The position of single non-neuronal p75-positive neural crest cells was mapped (Hagedorn et al., 1999), and the cultures were allowed to develop for 7 days in differentiation medium in the absence or presence of NRG1 (Marchionni et al., 1993). As shown in Fig. 2D, 27% of all neural crest cells underwent cell death in the absence of NRG1, while this number was reduced to 6% upon treatment with NRG1. Serial observation of developing clones suggested that cell death occurred at the level of single undifferentiated neural crest cells before proliferation and differentiation (data not shown). Thus, although survival of p75-positive neural crest cells is not solely dependent on NRG1 signaling in the chosen conditions, the number of surviving cells can be significantly increased by addition of NRG1.

To examine whether the survival activity of NRG1 on neural crest cells is dependent on Sox10, we tested the survival capacity of *Sox10*<sup>-/-</sup> mutant neural crest cells in clonal cultures treated with NRG1. In contrast to cultures of wild-type cells and consistent with our in vivo data, cell death was markedly increased in cultures of p75-positive *Sox10*<sup>-/-</sup> mutant cells, despite the presence of NRG1 (33% of all clones died when compared with 6% in wild-type cultures; Fig. 2D). Cell death appeared to affect single undifferentiated neural crest cells before they were able to form clones of differentiated cells. Moreover, the number of *Sox10*<sup>-/-</sup> mutant clones surviving in the presence of NRG1 was very similar to that of wild-type clones in the absence of NRG1. Similar to mutant cells treated with NRG1, 33% of *Sox10*<sup>-/-</sup> mutant neural crest cells were lost in the absence of NRG1 (data not shown; two independent

experiments, counting 60 clones each). Thus, Sox10 supports survival at least of a subpopulation of multipotent neural crest cells, and this function appears to be exerted by regulating the responsiveness of the cells to the survival-promoting activity of NRG1.

### Sox10 is required for glial fate acquisition

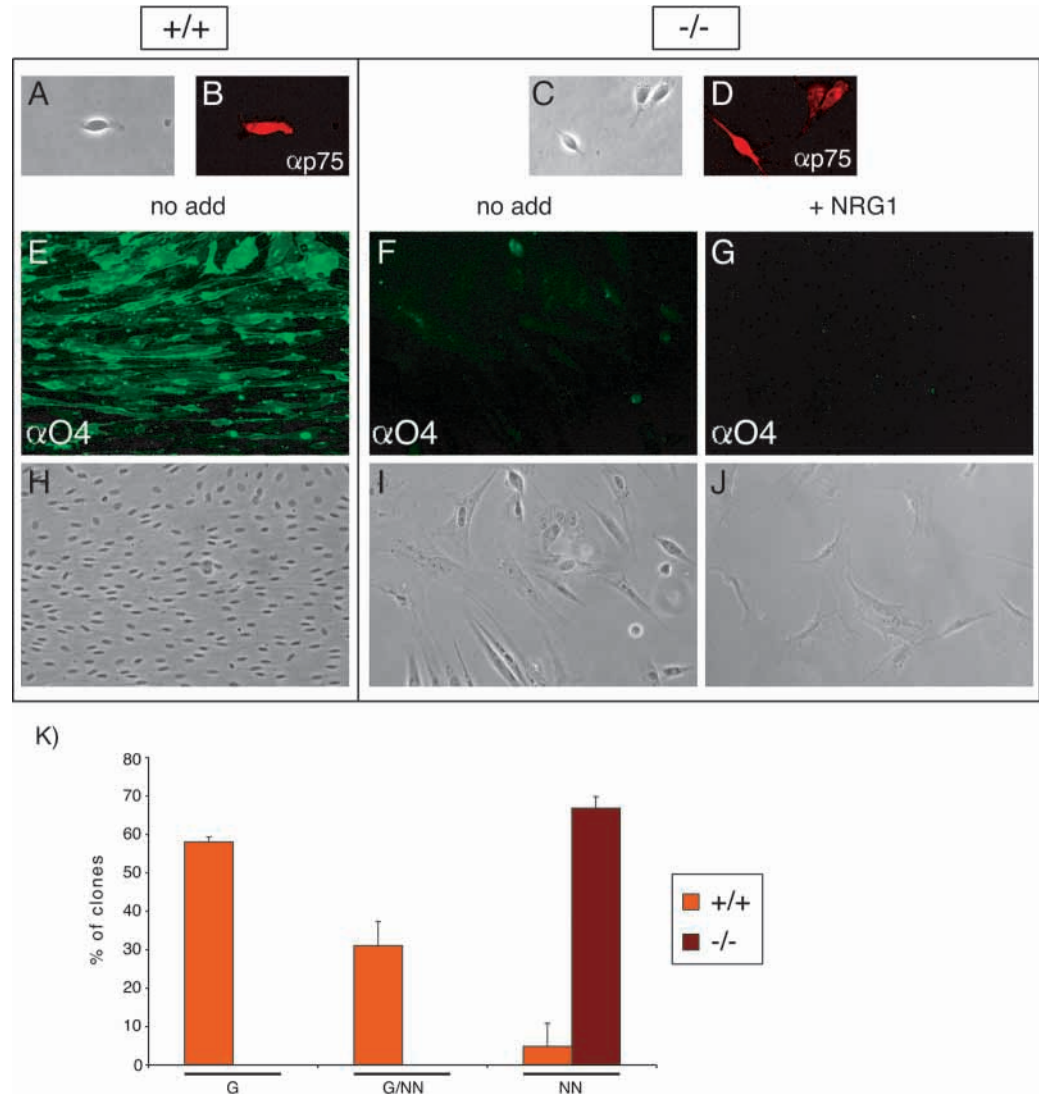
Our data suggest that cell death of multipotent neural crest cells might explain why differentiation of glia and other lineages was not observed by in situ analysis of *Sox10* homozygous mutant embryos (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999). This does not exclude additional roles of *Sox10* in neural crest development. To elucidate the cellular function of *Sox10* in gliogenesis, we monitored the fate of undifferentiated *Sox10*<sup>-/-</sup> mutant neural crest cells in various culture conditions that promote gliogenesis in wild-type neural crest-derived progenitor cells. Undifferentiated p75-positive cells isolated from DRG of wild-type and *Sox10*<sup>-/-</sup> mutant E13 embryos (Fig. 3A-D) were plated in mass cultures and either incubated in differentiation medium, which promotes the generation of O4-positive Schwann cells in wild-type cultures of postmigratory neural crest cells (Fig. 3E; Hagedorn et al., 2000b), or in differentiation medium supplemented with NRG1, which instructs multipotent neural crest cells to adopt a glial fate (Hagedorn et al., 1999; Shah et al., 1994). In both conditions, the surviving mutant cells did not give rise to any glia (Fig. 3F,G), but instead adopted the morphology of non-neural cells (Fig. 3I,J) that were negative for p75 and for NF160, and often expressed smooth muscle actin (SMA) (data not shown). Moreover, while wild-type cells proliferated to form high density arrays of glia (Fig. 3H), the generation of non-neural cells from mutant progenitors was accompanied by reduced proliferation, as has been described before for neural crest-derived smooth muscle-like cells (Shah et al., 1996; C. P. and L. S., unpublished).

To quantify this phenomenon and to assess whether the phenotype was due to a fate switch of *Sox10*-mutant postmigratory neural crest cells, the fate of individual p75-positive cells was monitored in clonogenic cultures that were incubated in differentiation medium supplemented with NRG1. While the vast majority of the wild-type progenitors gave rise to either clones containing exclusively glia ('G'-clones; 58% of all clones) or clones containing glia plus few smooth muscle-like cells ('G/NN'; 31%), none of the surviving *Sox10*<sup>-/-</sup> mutant neural crest cells was able to produce glia. Instead, all surviving clones (67% of all clones) were composed solely of smooth muscle-like cells ('NN') (Fig. 3K). Thus, *Sox10* is not only required for survival of undifferentiated, postmigratory neural crest cells but also for surviving cells to adopt a glial fate.

### Haploinsufficiency of Sox10 affects fate specification but not survival of neural crest cells

Haploinsufficiency has previously been demonstrated in embryos heterozygous for *Sox10* mutations that display several deficiencies in neural crest derivatives including melanocytes and the enteric nervous system (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). Therefore, we investigated the developmental potential of *Sox10* heterozygous postmigratory neural crest cells isolated from E13 DRG and cultured at clonal density. Three hours after

**Fig. 3.** Loss of glial potential in *Sox10*<sup>-/-</sup> undifferentiated neural crest cells. (A–J) Cells isolated from DRG of wild-type or *Sox10*<sup>-/-</sup> mutant E13 embryos were either incubated in differentiation medium ('no add') or in differentiation medium supplemented with NRG1 ('+NRG1'). In both conditions, surviving undifferentiated *Sox10*<sup>-/-</sup> mutant p75-positive cells (C,D) were not able to produce any O4-positive glia (F,G) but adopted the morphology of smooth muscle-like non-neural cells (I,J). Wild-type cells generated mainly O4-positive glia cells (E). (K) Quantitative clonal analysis of undifferentiated cells incubated in the presence of NRG1. G indicates clones containing exclusively glial cells; G/NN, clones containing glia and non-neural smooth muscle-like cells; NN, clones containing exclusively non-neural smooth muscle-like cells. For the quantitative analysis, expression of p75, NF160 and absence of these markers were used to distinguish between different fates. Numbers are given as percentage of all founder cells originally plated. Note that the indicated numbers of clone phenotypes do not sum to 100% because of cell death of a proportion of the founder cells (see Fig. 2). The data are expressed as mean±s.d. of four independent experiments. 60 clones were scored per experiment.



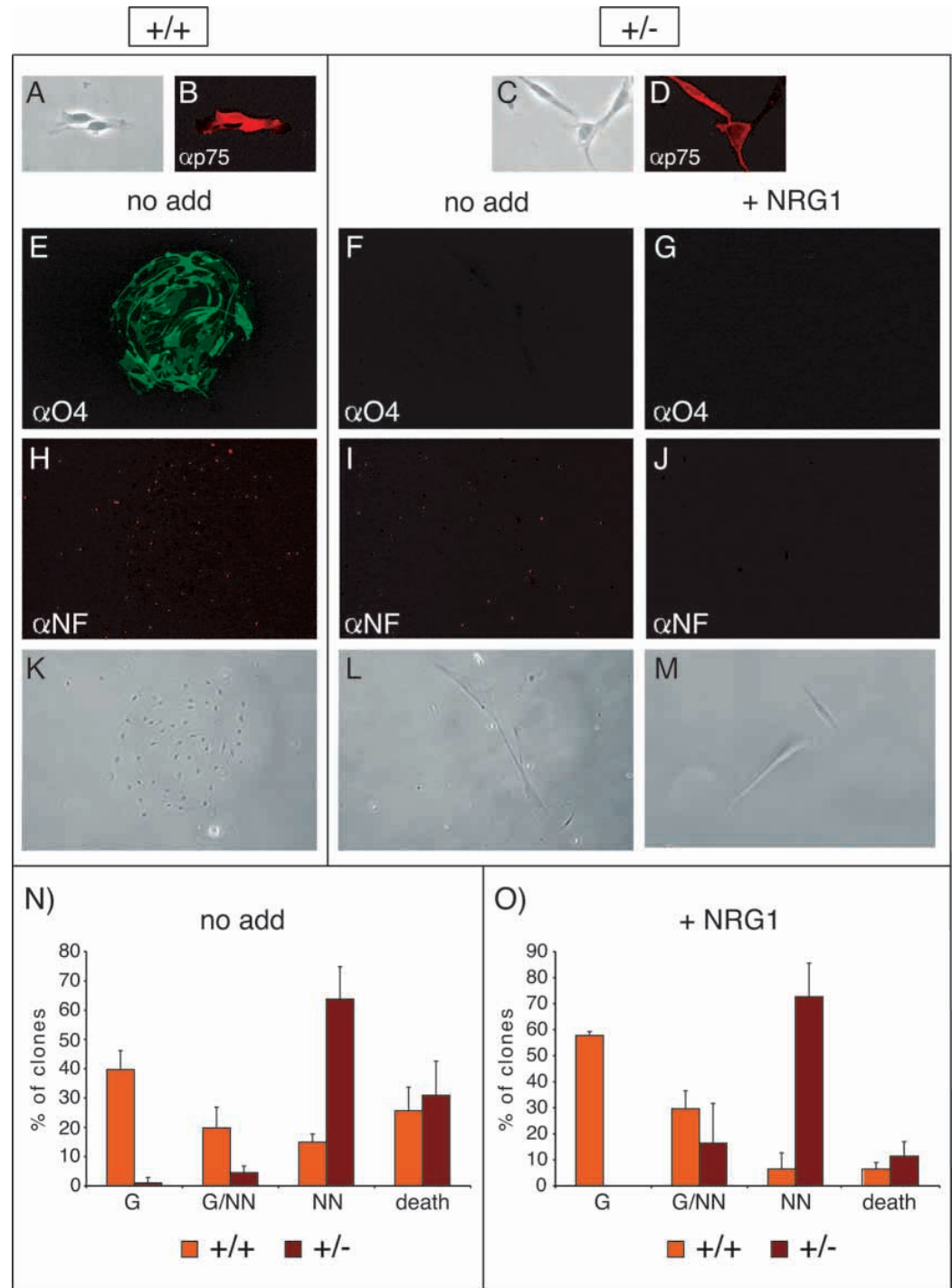
plating, some of the culture dishes were fixed and labeled for p75 (Fig. 4A–D) and Sox10 (data not shown). Similar to their wild-type counterparts, all p75-positive *Sox10*<sup>+/-</sup> mutant neural crest cells also expressed Sox10, indicating that allelic exclusion does not account for haploinsufficiency of *Sox10* in neural crest development (Nutt et al., 1999).

We then examined the survival capacity of postmigratory neural crest cells heterozygous for the *Sox10* mutation. The position of individual cells was mapped (Hagedorn et al., 1999) and loss of cells was monitored upon incubation in differentiation medium or in medium that has been supplemented with NRG1. In contrast to *Sox10* homozygous mutant neural crest (Fig. 2), *Sox10*<sup>+/-</sup> cells were responsive to the survival-promoting activity of NRG1 and cell death was suppressed by NRG1 to similar levels as in wild-type cells (Fig. 4N,O).

Surprisingly, however, while their survival capacity was comparable with wild-type neural crest cells, the fate of heterozygous cells was drastically altered (Fig. 4). Single multipotent neural crest cells derived from wild-type DRG at E13 proliferated and predominantly generated glia-containing clones in differentiation medium (Fig. 4E,H,K).

40% of the colonies were restricted to a glial fate whereas 20% of the colonies were heterogeneous containing glia associated with few smooth muscle-like cells (Fig. 4N). Relatively few clones (15% of all colonies) that derived from wild-type neural crest cells consisted exclusively of non-neural cells. In contrast to wild-type cultures, p75-positive cells prepared from *Sox10*<sup>+/-</sup> DRG proliferated only poorly in differentiation medium when plated at clonal density, and only 1% of the mutant colonies were composed solely of glia (Fig. 4F,I,L,N). Similarly, progeny consisting of both glia and smooth muscle-like cells were rare in mutant cultures (5% of all colonies). Instead, the majority of single *Sox10*<sup>+/-</sup> cells (64% of all colonies) gave rise to clones that were composed only of smooth muscle-like cells (Fig. 4N). These non-neural cells were characterized by downregulation of Sox10 and p75 expression, as well as by acquisition of SMA immunoreactivity in some cells (data not shown). Neurogenesis was not observed in clonal cultures of wild-type or mutant neural crest cells derived from DRG under the culture conditions used. As cell death was not significantly increased in *Sox10*<sup>+/-</sup> cell cultures when compared to wild-type cultures, our data demonstrate a fate switch in





*Sox10* mutant neural crest cells. At clonal density, haploinsufficiency of *Sox10* causes postmigratory neural crest cells to predominantly adopt a non-neural fate at the expense of a glial fate, even in conditions that are gliogenic for wild-type multipotent cells.

To investigate whether different glia-inducing growth factors elicit similar responses in *Sox10*<sup>+/-</sup> mutant cells, we also treated clonogenic cultures of postmigratory neural crest cells with NRG1 that promoted survival of both wild-type and *Sox10* heterozygous cells (Fig. 4O). Again, gliogenesis was significantly reduced in mutant cultures (Fig. 4G,J,M,O). No

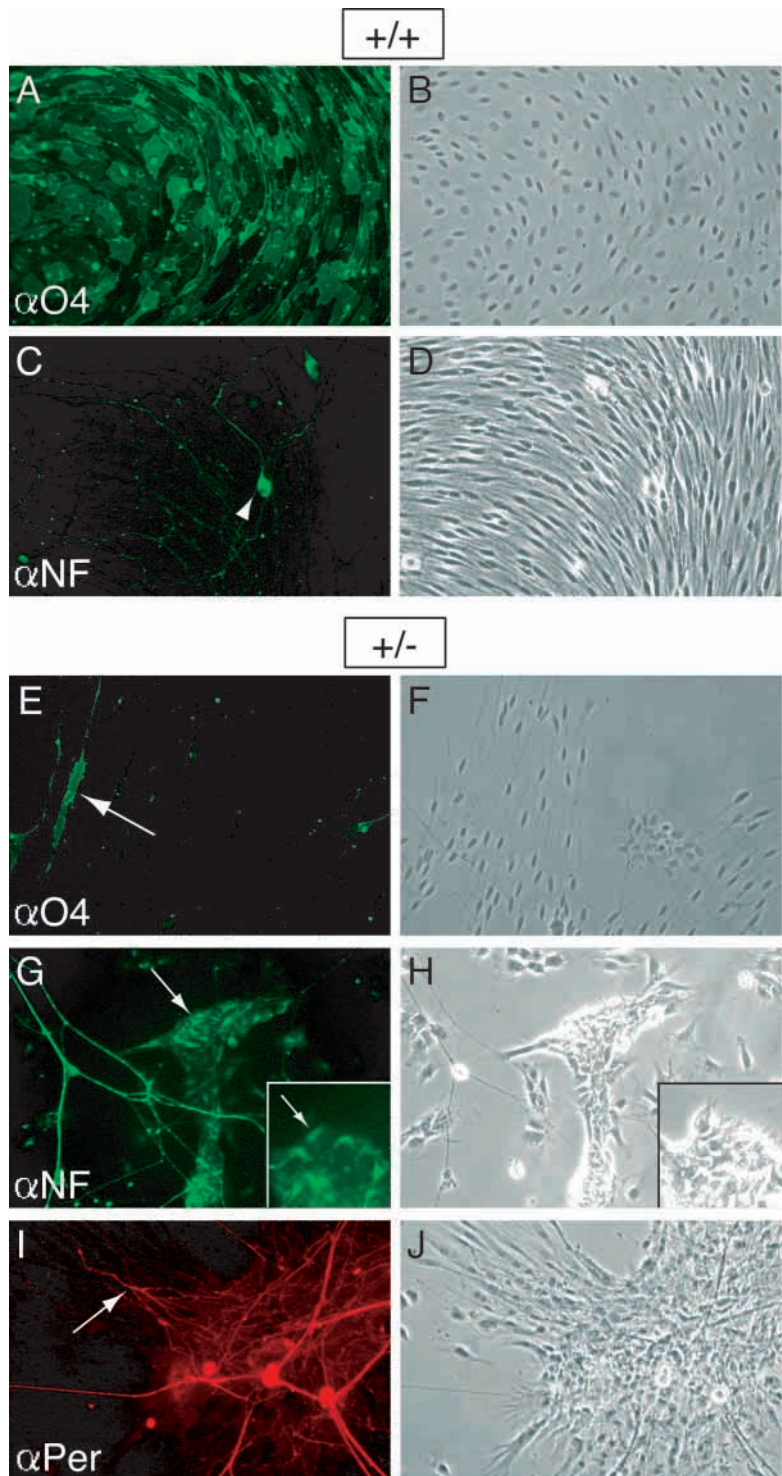
glia-only clones were generated (compared with 58% in wild type; see also Fig. 3K) and only about 16% mixed clones consisting of glia and smooth muscle-like cells were derived from individual *Sox10*<sup>+/-</sup> postmigratory neural crest cells (30% in wild type). In contrast, 73% of all mutant clones were exclusively composed of smooth muscle-like cells (Fig. 4O), suggesting that most mutant neural crest cells had undergone a fate switch towards a non-neural fate. Thus, even when treated with instructive gliogenic conditions, acquisition of a glial fate was severely hampered in *Sox10*<sup>+/-</sup> neural crest cultures.

**Fate decisions of Sox10 mutant cells are context dependent: community effects promote neurogenesis and suppress a non-neural fate in Sox10<sup>+/-</sup> mutant neural crest cells**

The generation of a non-neural cell type by single Sox10<sup>+/-</sup> mutant neural crest cells (Fig. 4) and by the surviving Sox10<sup>-/-</sup> mutant neural crest cells in culture (Fig. 3) only partially correlates with the phenotype observed in mutant embryos *in vivo* (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999).

In particular, neural development in peripheral ganglia and nerves appears to occur in Sox10 heterozygous mutant embryos (Britsch et al., 2001). This suggests that the fate acquired by single isolated Sox10 mutant cells might be different from the lineage chosen by mutant cells during embryogenesis, and that fate decisions by Sox10 mutant cells might be dependent on their cellular context. Recently, we have found that short-range cell-cell interactions termed community effects (Gurdon et al., 1993) can influence lineage decisions by multipotent postmigratory neural crest cells, suppressing a non-neural fate (Hagedorn et al., 2000a; Hagedorn et al., 1999). To investigate whether community effects affect the fate chosen by Sox10 mutant cells, wild-type and Sox10<sup>+/-</sup> mutant postmigratory neural crest cells were incubated in differentiation medium, exactly as described previously for the clonal analysis of postmigratory neural crest cells (Fig. 4). However, to allow short range cell-cell interactions to take place during the culture period, cells were plated at high density. Upon incubation for 7 days in these conditions, wild-type postmigratory neural crest cells proliferated and differentiated to form arrays of O4-positive glial cells (Fig. 5A,B). The generation of smooth muscle-like cells was almost completely suppressed (Fig. 5B,D, and data not shown). As in clonal cultures of wild-type cells, *de novo* neurogenesis was not detectable by daily observations of the cultures, and the only NF160-positive neurons found in wild-type high density cultures were postmitotic sensory neurons (arrowhead in Fig. 5C) present in the DRG preparation at the time of isolation (data not shown). By contrast, few cells with glial morphology developed from Sox10<sup>+/-</sup> mutant neural crest cells plated at high density (Fig. 5F). Some of these cells acquired features of differentiated glia and expressed

O4 (arrow in Fig. 5E). Non-neural cells, the predominant cell type derived from clonal cultures of Sox10 mutant cells (Figs 3, 4), were rarely generated in these conditions (data not shown). Rather, many Sox10<sup>+/-</sup> mutant progenitors started to aggregate in high density cultures (Fig. 5H,J), forming clusters of NF160-positive cells (arrow in Fig. 5G; insets in Fig. 5G,H). Prolonged incubation of such neuronal cells for 13 days allowed their differentiation into neurite-extending neurons that expressed the late differentiation marker peripherin (arrow



**Fig. 5.** Neurogenesis of Sox10<sup>+/-</sup> mutant cells in high density cultures. Undifferentiated p75-positive cells isolated from DRG of wild-type and Sox10<sup>+/-</sup> mutant E13 embryos were incubated at high density in differentiation medium. Wild-type cells produced O4-positive glia (A) while *de novo* neurogenesis did not occur. Arrowhead in C indicates a NF-positive sensory neuron present at time of isolation. Only few O4-positive glia were generated in Sox10<sup>+/-</sup> mutant cultures (arrow in E). Many mutant cells formed aggregates of NF-positive neuronal cells (arrow in G). Insets in G,H show a neuronal cluster at higher magnification. After prolonged incubation, neuronal cells acquired features of fully differentiated neurons expressing peripherin (Per) (arrow in I).



in Fig. 5I). Similarly, when NCSCs were allowed to emigrate from cultured neural tubes to form high-density explants (Sommer et al., 1995; Stemple and Anderson, 1992), *Sox10*<sup>+/-</sup> mutant neural crest cells predominantly generated neurons and gave rise to very few O4-positive glia in differentiation medium, while many glial cells were produced in wild-type NCSC explants (data not shown). The lack of glia in the mutant cultures was not simply due to a failure of *Sox10*<sup>+/-</sup> glial progenitor cells to differentiate, as p75 staining that marked undifferentiated neural crest cells and glial progenitors was also drastically reduced (data not shown). Thus, a frequent, though not exclusive, fate adopted by *Sox10*<sup>+/-</sup> mutant neural crest cells in cultures allowing short range cell-cell interactions is neuronal while their wild-type counterparts mostly generate glia.

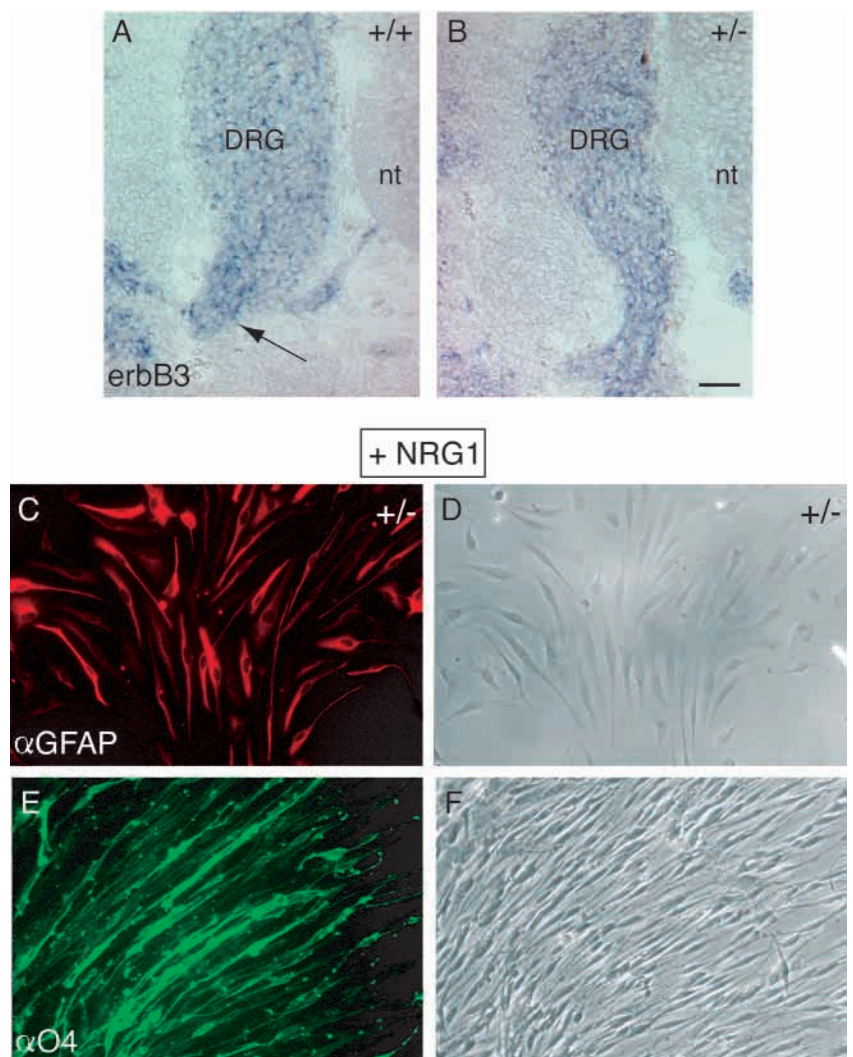
### Glial fate decision by *Sox10*<sup>+/-</sup> mutant cells is dependent on community effects and the instructive growth factor NRG1

Our analysis of the developmental potential of *Sox10*<sup>+/-</sup> mutant cells in culture might predict impaired gliogenesis during embryonic development of animals heterozygous for the *Sox10* mutation. However, several markers including *erbB3*, P0, MBP, PMP22 and S100 were expressed in a normal spatiotemporal expression pattern during glial development of *Sox10*<sup>+/-</sup> mutant embryos, and TUNEL staining did not reveal excessive loss of glial cells (Fig. 6A,B; and data not shown). The appearance of glial cells in the PNS of *Sox10*<sup>+/-</sup> embryos indicate that *Sox10*<sup>+/-</sup> mutant progenitor cells have glial potential, despite their impaired glial fate acquisition in various culture systems. This suggests that, similar to the suppression of a non-neural fate shown above, realization of the glial potential is context dependent. NRG1 promotes gliogenesis and is thought to be presented to glial progenitors along peripheral nerves (reviewed by Adlkofer and Lai, 2000). As NRG1 did not induce gliogenesis in single cell cultures of *Sox10*<sup>+/-</sup> neural crest cells (Figs 3, 4), we investigated whether community effects modulate the response of mutant cells to NRG1. As described in the experiments

presented in Fig. 5, postmigratory neural crest cells isolated from DRG at E13 were plated at high density, allowing short range cell-cell interactions to occur during incubation. Cells were then treated with NRG1 and their fates was analyzed after 5 and 10 days, respectively. In contrast to clonal cultures in the presence of NRG1 (Fig. 4) or high density cultures in the absence of NRG1 (Fig. 5), high density cultures in the presence of NRG1 allowed *Sox10*<sup>+/-</sup> mutant neural crest cells to generate many cells positive for glial fibrillary acidic protein (GFAP) (Fig. 6C,D), that formed arrays of O4-positive glia after 10 days in culture (Fig. 6E,F). Thus, glial fate specification is influenced by multiple signals that include the transcription factor Sox10, the extracellular factor NRG1, and local cell-cell interactions provided by community effects (Fig. 7).

### DISCUSSION

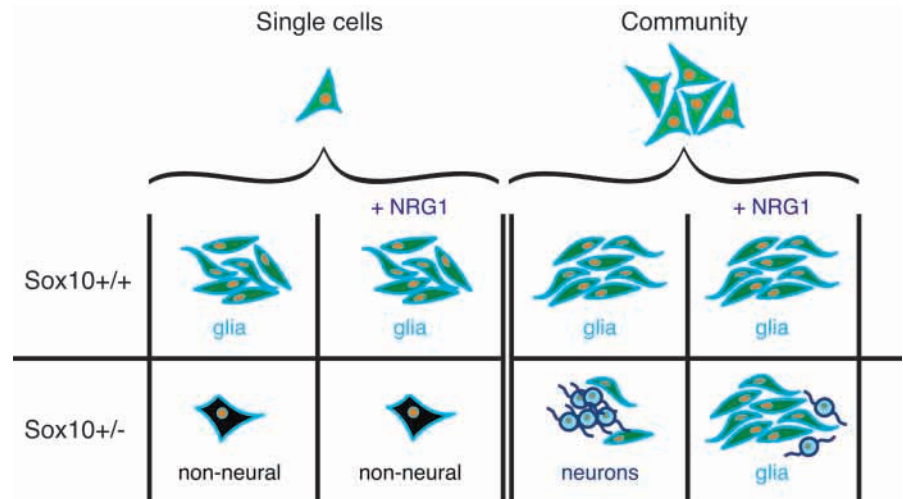
In situ analysis of mutant mice revealed a requirement of the transcription factor Sox10 for the proper development of various neural crest-derived lineages including melanocytes, the enteric nervous system and peripheral glia (Britsch et al.,



**Fig. 6.** Glial fate decision by *Sox10*<sup>+/-</sup> mutant cells is dependent on community effects and the presence of the instructive growth factor NRG1. (A,B) Normal expression of *ErbB3* mRNA in wild type and *Sox10*<sup>+/-</sup> mutant embryos at E13. In situ hybridization analysis on transverse sections showed normal expression in DRG and peripheral nerves (arrow) of wild type and *Sox10*<sup>+/-</sup> animals. nt, neural tube. Scale bar: 20  $\mu$ m. (C-F) Undifferentiated neural crest-derived cells isolated from DRG of wild type and *Sox10*<sup>+/-</sup> mutant E13 embryos were incubated at high density in differentiation medium supplemented with NRG1. Under these conditions, *Sox10*<sup>+/-</sup> mutant cells were able to differentiate into GFAP-positive glia (C,D) that after prolonged incubation expressed the glial differentiation marker O4 (E,F).

**Fig. 7.** Summary of context-dependent fates: fate decisions are dependent on levels of Sox10, presence of soluble extracellular factors such as NRG1 and short-range cell-cell interactions. Summary of the observed phenotypes. Postmigratory neural crest cells isolated from wild-type DRG produce predominantly glia-containing clones in differentiation medium and in differentiation medium supplemented with NRG1, irrespective of their cellular context. By contrast, single *Sox10* heterozygous cells undergo a fate switch and produce only non-neural cells at the expense of the glial fate. Moreover, in high density cultures that allow short range cell-cell interactions to occur, *Sox10* heterozygous cells undergo limited gliogenesis while neurogenesis is a prominent fate. The promotion of gliogenesis in *Sox10*

heterozygous cells is dependent on the synergistic activities of short range cell-cell interactions and the presence of NRG1. In summary, extracellular signals influencing cell fate decisions are differentially interpreted by postmigratory neural crest cells, depending on the cellular context and the level of Sox10.



2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). In the present study, we used a combination of in vivo analysis and primary cultures of mutant and wild-type neural crest derivatives to elucidate the cellular function of Sox10. This approach allowed us to distinguish between potential roles of Sox10 in survival, migration, fate specifications before lineage segregation, and early differentiation processes in separate lineages. Such distinctions would not have been possible on the basis of in situ analyses alone. Analysis of the developmental potential of undifferentiated neural crest cells homozygous for a targeted deletion of *Sox10* reveal that a primary function of Sox10 is to sustain survival of multipotent postmigratory neural crest cells before lineage segregation. Moreover, Sox10 is required for glial fate acquisition by multipotent neural crest cells as, even when challenged with various gliogenic conditions, surviving *Sox10*<sup>-/-</sup> mutant cells acquire an alternative fate at the expense of becoming glial. Haploinsufficiency of *Sox10* also affects the ability of multipotent neural crest cells to adopt a glial fate, but seems not to impair their survival capacity. Challenging *Sox10*<sup>+/-</sup> cells by exposing them to changing environmental conditions reveals that cell fate specification in multipotent neural crest cells involves the combinatorial activity of multiple extracellular signals (Fig. 7), and that Sox10 modulates the responsiveness of multipotent cells to this combinatorial signaling.

### Sox10 regulates survival of multipotent neural crest cells during PNS development

During neural crest development, a population of multipotent stem cells gives rise to various differentiated cell types in a controlled spatiotemporal manner. This requires a fine-tuned balance between persistence of an appropriate size of a stem cell pool and the differentiation of derivatives from this pool (reviewed by Morrison et al., 1997). Maintenance of multipotent progenitors in an uncommitted state can be achieved by the control of stem cell renewal, survival and differentiation. Our data suggest that Sox10 plays a crucial role in this process in the PNS. The significant cell death of

undifferentiated p75-positive neural crest cells that lack *Sox10* demonstrates a requirement of Sox10 in neural crest cells before they have initiated cellular differentiation. The observed cell death could be secondary to migratory defects in *Sox10* mutant cells. However, our analysis of postmigratory neural crest cells in early DRG in situ and in culture reveals an important role of Sox10 in controlling the survival of multipotent neural crest cells independently of migratory processes. Thus, we propose that in the absence of Sox10, the pool of multipotent neural crest cells is depleted by cell death contributing to the lack of multiple neural crest-derived lineages in *Sox10*-homozygous mutant embryos (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). As an exception, some sensory neurons, but no glial cells, develop in the DRG of *Sox10*-homozygous mutant embryos. This suggests that in *Sox10*<sup>-/-</sup> mutants a subset of sensory neurons can form from progenitors that are independent on Sox10 for survival, either because they possess compensatory mechanisms or because they represent a separate, Sox10-negative neural crest lineage that we are not able to reveal in cultures of Sox10-positive neural crest cells. Alternatively, neurogenesis might represent a preferred fate of the surviving *Sox10*-mutant neural crest cells in vivo.

NRG1 represents a candidate signaling molecule to mediate the survival function of Sox10 in vivo. Sox10 controls maintenance of the expression of the NRG1 receptor *ErbB3* in neural crest cells (Britsch et al., 2001). Similar to *Sox10*<sup>-/-</sup> mice, animals mutant for *ErbB3* lack several neural crest derivatives including Schwann cells and the autonomic nervous system (although glial fate acquisition is normal), indicative of a role of *ErbB3* in either survival and/or migration of neural crest cells (Britsch et al., 1998; Riethmacher et al., 1997). We demonstrate that in vitro NRG1 acts as a survival factor for a considerable subpopulation of undifferentiated postmigratory neural crest cells, and this activity is dependent on the presence of Sox10. Moreover, survival of heterozygous and wild-type cells is promoted by NRG1 irrespective of the fate chosen by the cells, as clonogenic cultures of *Sox10*<sup>+/-</sup> mutant cells produce mainly non-neural cells in conditions which are



gliogenic for wild-type cells. Thus, our analysis shows a role of *Sox10*-dependent NRG1 signaling in promoting survival of multipotent neural crest cells independently from migration and fate decision processes.

### Multipotent neural crest cells require Sox10 to adopt a glial fate

The lack of multiple differentiated neural crest-derived lineages in *Sox10*<sup>-/-</sup> mutant animals could be secondary to death of neural crest cells before overt differentiation. In addition, the absence of early differentiation markers such as the glial marker B-FABP (Britsch et al., 1998) might also be the consequence of direct gene regulation by Sox10, independently of actual lineage decisions. Indeed, several target genes of Sox10 that are expressed early in PNS development have recently been identified (Bondurand et al., 2000; Britsch et al., 2001; Peirano et al., 2000; Potterf et al., 2000). Yet another function of Sox10, however, might consist of regulating fate specification processes, which would also lead to disturbed appearance of neural crest-derived lineages in *Sox10*<sup>-/-</sup> mutant animals. The availability of clonogenic cultures systems, in which the fate of individual neural crest cells can be followed, allowed us to discriminate between these scenarios. In culture, surviving *Sox10*<sup>-/-</sup> mutant neural crest cells were not able to generate any cells with glial features, even in conditions in which their wild-type counterparts predominantly adopted a glial fate. Similarly, single *Sox10*<sup>+/-</sup> mutant cells challenged with glia-promoting cues were strongly biased towards a non-glial fate. These experiments indicate that neural crest cells depend on Sox10 activity to choose a glial fate, and identify Sox10 as the first transcription factor known to be required for PNS glial fate acquisition in vertebrate development.

The comparative analysis of *Sox10*<sup>-/-</sup> and *Sox10*<sup>+/-</sup> mutant cells indicates that survival and glial fate acquisition of multipotent neural crest cells are independently controlled by two separate functions of Sox10. While the complete absence of Sox10 in homozygous mutant neural crest cells leads to cell death, as well as deficiency in gliogenesis, Sox10 protein levels present in cultured heterozygous mutant cells are sufficient to support neural crest cell survival but not normal glial development. This indicates that different downstream mechanisms mediate the separate functions of Sox10. While NRG1 signaling is presumably involved in *Sox10*-dependent survival of crest cells, the role of Sox10 in gliogenesis cannot be mediated solely by NRG1 signaling, as lack of the NRG1 receptor *ErbB3* does not interfere with glial fate acquisition (Riethmacher et al., 1997). It remains to be determined whether and how Notch signaling might take part in *Sox10*-dependent glial fate acquisition. *Notch1* expression is controlled by Sox10 (Britsch et al., 2001), and Notch activation in single neural crest cells instructs these cells to adopt a glial fate (Morrison et al., 2000). However, it is not known whether Notch signaling is required for glial fate acquisition. In the CNS, the Notch effectors Hes1 and Hes5 suppress neurogenesis and promote gliogenesis, but neural precursor cells are still able to differentiate into glia in animals mutant for *Hes1* and *Hes5* (Hojo et al., 2000; Satow et al., 2001). It is, therefore, possible that Notch signaling adjusts the ratio of neuronal versus glial cell numbers without being required for glial fate acquisition per se.

The involvement of Sox10 in glial fate acquisition raises the question of whether Sox10 can be perceived as a glial determination factor. This would not only require that glial fate decision is impaired in a *Sox10* loss-of-function mutation but also that gain-of-function experiments induce gliogenesis from multipotent neural crest cells. This appears not to be the case, however, because neural crest development is apparently not affected by overexpression of Sox10, either in cultured rat neural crest stem cells (C. P. and L. S., unpublished) or in chicken embryos (H. Rohrer, personal communication). These findings are consistent with the idea that Sox10 acts as a modulator of other transcription factors (Kuhlbrodt et al., 1998) and, therefore, is necessary but not sufficient to regulate glial fate. In *Drosophila*, the generation of CNS lateral glia and peripheral glia is promoted by the activity of *gcm*. Intriguingly, the specification of midline glia is independent of *gcm* and rather requires the activity of the Sox protein Dichaete, which is, similar to Sox10, first expressed in multipotent progenitor cells and then maintained in glia (Soriano and Russell, 1998). *Dichaete* genetically interacts with *ventral veinless*, which encodes a POU domain transcription factor. Based on these data, we speculate that vertebrate peripheral glial cells might be specified by the combined activity of Sox10 and a yet to be identified POU domain transcription factor.

### Sox10 regulates the responsiveness of multipotent neural crest cells to combinatorial environmental signaling

Investigating the developmental potential of *Sox10* heterozygous mutant neural crest cells revealed that lineage decisions of *Sox10* mutant cells are dependent on the cellular environment. As discussed above, *Sox10*<sup>+/-</sup> mutant cells display a similar survival capacity as their wild-type counterparts, but they are biased to give rise to non-glial cells. Fate decisions are influenced by community effects (Hagedorn et al., 2000a; Hagedorn et al., 1999), which suppress the non-neural fate of single mutant cells (Fig. 7). Thereby, factors present in differentiation medium containing serum, in conjunction with short-range cell-cell interactions, induce neurogenesis in *Sox10*<sup>+/-</sup> cells while the same signal combination promotes glial formation in wild-type postmigratory cells that contain higher amounts of Sox10 (Fig. 7). Likewise, NRG1 is not sufficient to induce a glial fate in single mutant cells, although it is gliogenic for wild-type cells. Rather, for a *Sox10*<sup>+/-</sup> mutant cell to adopt a glial fate, the combined activities of the instructive signal NRG1 and short range cell-cell interactions are required (Fig. 7). These data reveal that NRG1 signaling interacts with signaling provided by community effects, which in cells with lower Sox10 levels leads to realization of the gliogenic activity of NRG1.

Thus, the fate adopted by a multipotent neural crest cell reflects the combinatorial activity of a specific set of multiple signals that include Sox10. Changes in the signal composition or in the levels of signals that participate in such signaling networks can affect survival and fate specification of multipotent neural crest cells. Similarly, unique cell fates in multipotent progenitor cells of the *Drosophila* eye are specified by context-dependent integration of several signaling pathways (Flores et al., 2000). However, our data also reveal a certain functional redundancy of signaling networks in that more than

one signal combination can elicit a similar biological response in multipotent neural crest cells (Fig. 7). Such flexibility in signal integration presumably allows a multipotent neural crest cell to adapt to changing environmental conditions in a finely tuned manner. Moreover, it is likely the reason why in vivo *Sox10*<sup>+/-</sup> neural crest cells seem to be able to compensate for reduced Sox10 levels at sites of peripheral gliogenesis and to give rise to glia. Such gliogenic sites, in which aggregating cells are exposed to NRG1 expression (Ho et al., 1995; Marchionni et al., 1993; Meyer and Birchmeier, 1994), are apparently mimicked by culture conditions that allow short-range cell-cell interactions in the presence of NRG1.

Unlike in peripheral nerves, neurogenic and gliogenic signals coexist in peripheral ganglia. Therefore, according to our in vitro experiments, we might possibly expect increased neurogenesis to occur at the expense of gliogenesis in peripheral ganglia of *Sox10*<sup>+/-</sup> embryos. There is ample evidence that the regulation of the correct neuronal cell number involves programmed cell death both in a target-dependent (reviewed by Deshmukh and Johnson, 1997) and possibly target-independent manner (Hagedorn et al., 2000a; Raoul et al., 2000). Thus, the putative production of supernumerary neurons in *Sox10* heterozygous embryos would presumably be compensated by increased programmed cell death. Accordingly, in two out of three *Sox10*<sup>+/-</sup> embryos, we observed a significant increase in neuronal cell death in DRG (data not shown). However, techniques allowing the monitoring of fate decisions of single multipotent cells in vivo would be required to examine whether fate switches by *Sox10* mutant neural crest cells indeed occur in the context of the developing embryo. The difficulty of performing such experiments demonstrates the value of investigating the developmental potential of mutant cells in vitro in varying conditions, in order to elucidate the cellular function of a given regulatory molecule.

In summary, two main conclusions can be drawn from our experiments. First, cell fate specification in neural crest cells is regulated by the integration of multiple signals present in the environment. Such processes conceivably involve crosstalk between distinct signal transduction pathways that act in a signaling network to inhibit, potentiate or modulate each other. Second, in the developing vertebrate PNS, changes in Sox10 dose modulate the specific biological response of multipotent neural crest cells to this complex extracellular signaling. Based on this finding, we predict that in wild-type embryos, controlled regulation of Sox10 expression allows a neural crest cell to alter its responsiveness to environmental cues. Consequently, we suggest that in *Sox10* heterozygous mutant cells the interpretation of the extracellular environment might be affected. According to this model, inappropriate fate decisions might cause the deficiencies in neural crest development that are characteristic for Waardenburg/Hirschsprung disease in individuals heterozygous for a *Sox10* mutation.

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