The cellular function of the two transcription factors Sox10 and Erm in neural crest development

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Publication Date:
2002

Permanent Link:
https://doi.org/10.3929/ethz-a-004446394

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The Cellular Function of the Two Transcription Factors Sox10 and Erm in Neural Crest Development

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

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2002
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1. Zusammenfassung


Im nächsten Teil meiner Dissertation analysiere ich das Expressionsmuster und die zelluläre Funktion des Transkriptionsfaktors Sox10. Dieser Faktor spielt eine zentrale Rolle während der Neuralleistenentwicklung und es wurde bereits gezeigt, dass homozygot mutierte Sox10 Tiere keine Gliazellen entwickeln. Ich zeige zusätzlich, dass alle Neuralleistenzellen Sox10 Protein exprimieren und

2. Summary

A key question in developmental biology is how cellular diversity is established during development. This process involves restrictions in the developmental potential of initially multipotent cells at different developmental stages and at distinct embryonic sites. The entire vertebrate peripheral nervous system is derived from a migratory, multipotent population of cells termed neural crest cells (NCC). In vivo and in vitro experiments suggest that multipotent neural crest cells gradually undergo restriction in their developmental potential (Anderson, 2001). A process, which involves a complex interplay of environmental signals and intrinsic programs regulating fate restrictions and differentiation of NCC. The interactions between extrinsic signals and intrinsic programs are still poorly understood.

In a first part of my thesis, I illustrate intrinsic differences between ‘early’ NCC and developmentally ‘older’ dorsal root ganglia (DRG) progenitors in their developmental potential in response to diverse cellular contexts. Next, I analyzed the expression pattern and the function of the Ets domain transcription factor Erm. Ets genes were shown to be involved in many developmental processes. In the DRG, Erm is expressed in multipotent progenitor cells, in presumptive satellite glia and in sensory neurons. I show that Erm is the first mammalian marker distinguishing satellite glia from Schwann cells, which are devoid of Erm expression. In addition, the growth factor neuregulin1 (NRG1) maintains Erm expression in satellite glia and furthermore, is able to reinstate Erm expression in DRG-derived progenitors but not in Schwann cells of the sciatic nerve. These data demonstrate intrinsic differences of diverse glial subtypes in response to NRG1-signaling. Further, Erm-positive multipotent progenitors isolated from NC cultures give rise to satellite glia in response to NRG1 and to Schwann cells in the presence of serum and the adenylate cyclase activator forskolin. Therefore, given the expression pattern of Erm in vivo and the described NRG1/Erm signaling in NCC, we hypothesize that NCSC first give rise to Erm-positive progenitors that then differentiate into Erm-positive satellite glia in the forming DRG. Erm-positive progenitors might furthermore emigrate from the DRG and subsequently differentiate into Erm-negative Schwann cell precursors along the peripheral nerves.

Next, I investigate the cellular function of Erm in NC development by forced expression of a dominant-negative form of Erm in vitro. These experiments reveal a requirement of Erm for efficient neuronal fate acquisition, while progenitor survival and proliferation are not affected. Additionally, glial fate acquisition and differentiation are unaltered. However, the proliferation rate is drastically diminished in glial cells, suggesting a glia-specific role in controlling cell cycle progression. This indicates a dual, lineage specific, role of Erm during NC stem cell development.

In a subsequent part of my thesis, I analyze the expression pattern and the cellular function of the high mobility group (HMG) domain transcription factor Sox10 during NC development. This transcription factor is necessary for proper development of many NC derivatives. For example, Sox10-homozygous mutant mice lack all subsets of peripheral glia. I demonstrate that all NCC express Sox10 protein and require Sox10 function before lineage segregation. Postmigratory, undifferentiated progenitors in Sox10⁻/⁻ DRG display significantly
increased apoptosis suggesting a role of Sox10 in the survival of NCC. Furthermore, surviving mutant cells submitted to clonal analysis under gliogenic conditions show that Sox10 is essential for glial fate acquisition. In contrast, Sox10\(^{+/−}\) mutant NCC survive normally, while fate specification is drastically altered. Further, I illustrate that fate decision by mutant NCC is cell-context dependent. In sum, my data indicate that combinatorial signaling by Sox10, extracellular factors such as NRG1 and cell-cell interactions are involved in fine-tuning lineage specification.

Human patients with one mutated Sox10 allele suffer from Waardenburg/Hirschsprung disease, a congenital disorder of the enteric nervous system (ENS). This indicates that haploinsufficiency of Sox10 results in several NC defects characterized by aganglionosis along the gut. The cellular basis for this phenotype is not fully understood. I speculate that failures in fate decision processes might contribute to the etiology of Hirschsprung disease. For instance, I show that Sox10\(^{+/−}\) animals lack enteric neurons in the hindgut and therefore, are a suitable model for Hirschsprung disease. Early in development, the number of multipotent progenitors in the ENS of Sox10\(^{+/−}\) animals is highly reduced. Additionally, fate mapping experiments using persistent lacZ expression from the Sox10 locus demonstrate that the former Sox10-positive cells are still present in the gut but have lost Sox10 expression. Instead, many of these cells express the neuronal markers c-Ret and PGP9.5. Therefore, these data show that the maintenance of the progenitor state is impaired and that the Sox10\(^{+/−}\) enteric cells differentiate into enteric neurons. This process results in a depletion of the progenitor pool in the hindgut and in the onset of Hirschsprung disease.
3. Introduction

3.1 The development of neural crest stem cells (NCSC)

The entire peripheral nervous system (PNS) is derived from a migratory cell population entitled neural crest (NC). This migratory population of cells forms at the border between the neural plate and the future epidermis involving a complicated series of inductive interactions and morphogenetic movements (Le Douarin et al., 1982). During neurulation, the ectodermal cells in the prospective midline of the developing embryo thicken to form the neural plate and subsequently invaginate to form the neural tube. This cylindrical tube extends along the rostrocaudal axis of the embryo. Just prior or soon after neural tube closure, neural crest cells (NCC) delaminate from the neuroepithelium and start to migrate along distinct pathways within the developing embryo (Le Douarin et al., 1993; Bronner-Fraser et al., 1986). The very early steps of neural crest induction still remain to be elucidated. Classically, NCC were thought to be a separated population within the neural plate. However, single cell lineage labeling in avian embryos has shown that the progeny of single cells within the dorsal neural tube can contribute to both neural tube and neural crest derivatives suggesting that the cells are not yet committed at this stage (Krotoski et al., 1988). The differentiation of the cells within the dorsal neural tube requires signals from adjacent tissues. Several factors that are involved in this process have been described such as transforming growth factor β (TGF-β) family members (Dorsalin-1, BMP-4 and BMP-7). In vitro for example, BMPs are sufficient to induce neural crest from the neural plate (Baker and Bronner-Fraser, 1997a; Baker and Bronner-Fraser, 1997b). Additionally, inhibiting BMP activity expressed in the neural folds prevents neural crest formation. Further, Dorsalin-1 that is expressed in the dorsal neural tube is able to induce neural crest cells from neural plates in vitro (Basler et al., 1993). However, its late expression in the dorsal neural tube makes it unlikely to be involved in the initial induction of neural crest at the neural plate border. Besides TGF-β family members, the wnt molecules which have been associated with proliferation, maintenance and fate decisions of neural crest derivatives in mice and zebrafish (Ikeya et al., 1997; Dorsky et al., 1997) seem to be important players in early induction of neural crest. Wnts are able to induce the expression of neural crest markers in ectodermal explants in conjunction with BMP inhibition (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Taken together, wnt-, BMP- as well as FGF-generated signals seem to play a role at least in some early aspects of neural crest formation.

The delamination from the neural tube involves an epithelial to mesenchymal transition that comprises regulation of neural cell adhesion molecules such as N-CAM, N-cadherin and cadherin-6 (Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995). Changes in the expression pattern of cadherins have been suggested to be necessary for detachment from neighboring neuroepithelial cells and emigration from the neural tube. In addition to changes in cell-cell adhesion, a breakdown in the basement membrane surrounding the
neural tube occurs in areas from which neural crest emigrate (Erickson and Perris, 1993).

After detachment, the trunk neural crest cells migrate extensively along two main pathways within the developing embryo (Fig. 1): the dorsolateral pathway and the ventral pathway. Neural crest cells giving rise to the melanocyte lineage take the dorsolateral pathway. These cells migrate through the dermal mesoderm underlying the dermis and enter the ectoderm through minute holes in the basal lamina and colonize the skin and follicles (Erickson et al., 1992; Mayer, 1973). The second major pathway (ventral pathway) guides the cells ventrally through the anterior section of the sclerotome (Bronner-Fraser, 1986). Chick-quail grafting experiments revealed that the crest cells initially opposite the posterior regions of the somites migrate anteriorly or posteriorly along the neural tube and then enter the anterior region of their own or adjacent somites (Teillet and Le Douarin, 1983).

Figure 1: Migratory paths of neural crest cells

Neural crest cells migrate along two main pathways within the developing embryo. The cells on the dorsolateral pathway migrate underneath the dermis and give rise to melanocytes. The majority of neural crest cells migrates along a "ventral" route, between the neural tube and somite, the notochord and somite, and along the dorsal mesentery. (Figure adapted from Kandel, 1991)

At their final location, the crest cells start to differentiate into various cell types. These cell types exhibit diversity along the rostrocaudal extent of the neuraxis. For example, crest cells that emerge from the trunk level give rise to sensory and autonomic neurons, glia, melanocytes in the skin, chromaffin cells of the adrenal gland and smooth muscle cells, while enteric neurons are mainly generated from more anterior vagal neural crest (Fig. 2A, B). Nevertheless, heterotopic grafting showed that the developmental capacities of the crest cell population are qualitatively equivalent at all levels of the neural axis. Hence,
one of the key questions for developmental cell biologists is determining the molecular basis of cellular diversification. How is the generation of numerous spatially and temporally distinct fates controlled? How is the interplay between fundamental developmental events such as cellular proliferation, migration, growth, differentiation, and death regulated?

2A) Developmental differences along the rostrocaudal extent of the neuraxis

2B) Crest derivatives and structures within the developing embryo

Figure 2: Crest derivatives at different levels of the neuraxis (A). Schematic cross-section of a developing embryo (B).

*In vivo*, the positional origin of the neural crest cells within the neuraxis influences their future development (A). B) shows a schematic representation of different developmental stages. Neural crest gives rise to the entire peripheral nervous system such as the DRG, autonomic ganglia, peripheral nerves as well as the enteric nervous system.
3.2 Stem cells are multipotent and able to self-renew

It was shown that environmental signals as well as cell-intrinsic factors play an important role in the induction, determination, migration, proliferation and survival of neural stem cells (Anderson, 1997). Clonal analysis performed in vitro and transplantation experiments demonstrated that neuroepithelial cells and neural crest cells are multipotent and able to self-renew. In vitro, neuroepithelial cells from rat E10.5 spinal cord were dissociated and cultured. These cells give rise to the main cell types of the central nervous system (CNS) such as neurons, astrocytes, and oligodendrocytes (Kalyani et al., 1997). In the presence of BMPs, these neuroepithelial cells can generate neural crest derivatives such as Schwann cells, neurons and smooth muscle cells (Mujtaba et al., 1998). Furthermore, clonal analysis revealed that single neuroepithelial cells are multipotent and can give rise to both CNS and neural crest derivatives (Mujtaba et al., 1998).

Previous experiments described the isolation and growth of rat neural crest cells in clonal cultures (Stemple and Anderson, 1992). Therein, it was shown that crest cells are a population of multipotent cells that gives rise to different cell types under clonal cell culture conditions permissive for glia, autonomic neurons and smooth muscle cells. Moreover, in vivo labeling of individual premigratory or migratory neural crest cells and following the fates of their descendants supported these results (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989, Fraser and Bronner-Fraser, 1991). Furthermore, subcloning experiments demonstrated the self-renewal capacity of multipotent rat neural crest (Stemple and Anderson, 1992). Therefore, they were claimed to be true stem cells as defined by multipotency and self-renewal capacity. Such stem cells can be isolated from cultured peripheral nerve (Morrison et al., 1999), dorsal root ganglia (Hagedorn et al., 1999), migrating neural crest (Stemple and Anderson, 1992) and enteric nervous system (Lo and Anderson, 1995).

3.3 Instructive factors influencing NC development

Several growth factors have been described to bias differentiation of neural crest cells along distinct lineages: glial growth factor (GGF, NRG1) which promotes gliogenesis (Shah et al., 1994); transforming growth factor β (TGF-β) (Shah et al., 1996), which promotes smooth muscle differentiation; bone morphogenetic proteins 2 and 4 (BMP2/4), which promote the differentiation of autonomic neurons and to a lesser extent smooth muscle (Shah et al., 1996), and most recently, Delta, a Notch ligand, which also promotes gliogenesis (Morrison et al., 2000). Observation of individual developing clones suggested that these growth factors act instructively rather than selectively in promoting the differentiation of one lineage at the expense of another (Shah et al., 1996; Shah et al., 1994; Morrison et al., 2000). All these factors were expressed at the appropriate places within the developing embryo at the correct time point: neuregulins and their receptors in the developing peripheral nerves (reviewed in Garratt et al., 2000); TGF-β in the major vessels of the heart (Millan et al., 1991); BMP2/4 in the dorsal aorta and gut (Shah et al., 1996); Notch family
genes in neural crest and their ligands including Jagged and Delta by neuroblasts in developing peripheral nervous system ganglia (Weinmaster et al., 1991; Williams et al., 1995; Bettenhausen et al., 1995; Lindsell et al., 1996; Lindsell et al., 1995). Targeted mutations of individual genes further corroborated the in vivo relevance of these factors. Mutations in the neuregulin gene reduce the number of Schwann cells along the peripheral nerves (Meyer and Birchmeier, 1995), while the TGF-β mutation displays a cardiac phenotype (Kingsley, 1994). Nevertheless, how these different factors act as lineage determinants, survival factors or mitogens has to be resolved in detail.

3.4 Signal integration by neural crest stem cells

Although virtually all neural crest cells can be driven into a specific lineage by instructive factors, newer results suggest that neural crest is a heterogeneous cell population. Specifically, clonal analysis demonstrated that while some neural crest cells give rise to multiple derivatives, other cells produce only one cell type under the same permissive culture conditions (Henion and Weston, 1997). This indicates that there is significant variation among neural crest cells and that at least some of them are restricted in fate (which is different from a restriction in potential). Though, the cells maintain their multipotency to produce different fates under diverse environmental conditions.

So far, the model is favored that neural crest development progressively generates restricted intermediates. Most of the crest cells emigrating from the neural tube contain the complete trunk crest potential and undergo partial restriction in their developmental potential during or after migration. Still, it is not yet clear whether these restrictions occur pre- or postmigratory. The time point of emigration seems to be a critical factor defining into which crest derivatives they differentiate. In the avian trunk, cells populating the sympathetic ganglia migrate before those that form the dorsal root ganglia (Serbedzija et al., 1989). Besides the positional heterogeneity, these temporal differences could reflect either pre- or postmigratory specification. Henion and Weston (1997) further addressed these issues in in vitro experiments. They showed that the initial neural crest population is already a heterogeneous population half of which generated single cell type clones. Distinct neurogenic and melanogenic sublineages were present in the outgrowth population almost immediately, but melanogenic precursors dispersed from the neural tube only after many neurogenic precursors had already done so. Taken together, this demonstrates that lineage restricted subpopulations constitute a major portion of the initial neural crest population. Neural crest diversification occurs well before overt differentiation by asynchronous restriction of distinct cell fates in a culture medium permissive for several fates.

In summary, most neural crest cells are multipotent and acquire different fates depending on the microenvironment or the growth factors they are exposed to. Additionally, there is growing evidence that some of the neural crest cells are already fate-restricted prior to migration. Nevertheless, several questions remain to be solved: how are partially restricted precursors generated in a “homogeneous” environment? Do slight variations exist in this “homogeneous”
environment or is restriction achieved by a stochastic mechanism? Another important question is when and where the restriction occurs.

3.5 Lateral inhibition mediated by Notch/Delta signaling pathway

Assuming an extrinsic mechanism for cell fate induction, the question how a group of cells within the same microenvironment produces heterogeneous cell fates still remains to be answered. In other systems an important mechanism was already described that might further elucidate this issue. This mechanism is called lateral inhibition which is mediated by the Notch/Delta signaling pathway (Artavanis-Tsakonas et al., 1999). During development of the sensory organ in Drosophila, one cell within the proneural cluster (equivalence group) is selected to develop into a neural precursor. The other cells surrounding this cell are prevented from doing so. This tightly regulated process is mediated by proneural genes. These genes activate the Notch signaling pathway in neural competent cells, which results in the selection of the future neural precursors. Thereby, a system can regulate the number of cells responsive to a given inductive signal by selecting particular cells to express a receptor molecule. This process is based on asymmetrically localized mRNAs and/or proteins during mitosis (Rhyu et al., 1994). Asymmetrically distributed cell fate determinants can interact with genes like Notch that influence cell fate specification by mediating cell-cell interactions (reviewed in Jan and Jan, 1995). Recent in vitro data demonstrated by adding soluble Delta, a Notch ligand and BMP2 to neural crest cultures that neural crest cells become progressively more gliogenic and less neurogenic during development (Kubu et al., 2002). The decrease in sensitivity to the instructive neurogenic signal BMP2 as well as an increase in sensitivity to the anti-neurogenic and gliogenic signal Delta come along with an increase in the ratio of expression of Notch1 to Numb mRNAs (Kubu et al., 2002). In Drosophila, a loss of function Numb mutation in the sensory organ precursor lineage has the same phenotype (fewer neurons) as a gain of function Notch mutation, and conversely, a gain of function Numb mutation within this lineage results in a phenotype (more neurons) indistinguishable from a loss of Notch mutation (Guo et al., 1996). These data, along with Numb overexpression studies in mammalian and avian systems have suggested that Numb function is to inhibit Notch signaling (Spana and Doe, 1996; Zhong et al., 1996; Wakamatsu et al., 1999; Sestan et al., 1999; Imai et al., 2001). Moreover, it has been shown that Numb physically interacts with the intracellular domain of Notch (Guo et al., 1996; Zhong et al., 1996), specifically the PEST domain of Notch1, thereby inhibiting Notch translocation into the nucleus (Wakamatsu et al., 1999; Berezovska et al., 2000). In summary, Delta-to-Notch signaling between neighboring cells, coupled with positive feedback control of Notch expression within “receiving” cells, gradually causes heterogeneity in the sensitivity of different progenitor cells to Notch signaling, even before they have committed to specific fates (Kubu et al., 2002). Previously, Notch signaling in Drosophila was thought to maintain cells in an undifferentiated state (Artavanis-Tsakonas et al., 1995; Kimble and Simpson, 1997). Whether or not Notch is also involved in maintenance of the stem cell
pool in mammals is still matter of investigation. However, mice deficient for Hes1, one of the downstream signaling effectors, display a decrease in the number of embryonic stem cells (Nakamura et al., 2000). Most recent data by Hitoshi et al. (2002) additionally support the idea that Notch signaling keeps cells in an undifferentiated state. Attenuated Notch signaling by means of a homozygous mutation in the Notch1 gene disrupts embryonic stem cell self-renewal. Less symmetrical and self-renewing divisions of the Notch signaling deficient neural stem cells take place and therefore, more neuronal and more astroglial differentiation of the neural progenitor cells happens. These progenitors are the asymmetric progeny of the neural stem cells. This finding of a premature neuronal division as a product of symmetric divisions of Notch-deficient neural stem cells in the forebrain can be seen as an alternative to the idea that Notch signaling is directly and instructively involved in cell fate decisions of neuronal and glial differentiation in the mammalian central nervous system (Gaiano et al., 2000). Therefore, Hitoshi et al. conclude that Notch signaling is primarily involved in symmetric stem cell self-renewal within the central nervous system. In the PNS, I think that these issues need further clarifying experiments in order to resolve whether and how Notch activation mechanistically regulates the size of the neural stem cell pool or fate decision processes during development.

3.6 Symmetric vs. asymmetric cell divisions

In general, a fundamental feature of stem cells is their ability to self-renew, to divide, and to give rise to at least one daughter cell that maintains the multipotent character of its parent (reviewed in Morrison et al., 1997). Often it is assumed that individual stem cells necessarily self-renew via asymmetric divisions. Nevertheless, asymmetry might also be produced by all symmetric divisions where 50% of the divisions are self-renewing and 50% are differentiative (Fig. 3, Morrison et al., 1997; Fuchs and Segre, 2000). So far, the available data favors a predominance of symmetric cell divisions in mammalian stem cell systems. Otherwise, in strictly asymmetric stem cell lineages no regulation of the stem cell number is possible. However, there is ample evidence for changes in the size of stem cell populations in mammals implying that symmetric divisions must occur (reviewed in Anderson, 2001; Morrison et al., 1997). Taken together, both, symmetric and asymmetric cell divisions might play a role in the development of the mammalian peripheral nervous system. It has to be further investigated which stem cells at which stage are generated by symmetric or asymmetric division.
3.7 Combinatorial signaling

Secreted or transmembrane signals present in a cell’s local environment and intrinsic signals that operate in a cell-autonomous manner influence a cell’s future fate. Besides lateral inhibition other mechanisms play a major role in extrinsic signaling and cell fate specification. 1) Inductive signals act on adjacent cells in exposing cells to a locally acting extrinsic signal. 2) Gradients of signals are able to induce different fates at different threshold concentrations. 3) Antagonist signaling factors/secreted inhibitory factors bind directly to signaling ligands and/or their receptors, blocking their signaling function (Schweitzer et al., 1995). Another example might be combinatorial signaling where cells acquire distinct identities through their exposure to several different signals.

Recently, Hagedorn et al. (1999) found that short-range cell-cell interactions termed community effect (Gurdon et al., 1993) can influence cell fate decision of multipotent neural crest cells. They showed that neural crest stem cells react differently as single cells in response to extrinsic factors than cell communities. Single neural crest cells produce mainly smooth muscle cells in response to TGF-β. In contrast, cell clusters of neural crest-derived progenitors display a reduced non-neural potential and preferentially choose cell death or a neuronal fate. Gurdon (1993) suggested that such community effects are required to eliminate or correct individual errant cells such as non-neural cells in developing peripheral ganglia. It is not clear how community effects are regulated, but they most probably take place due to the modulation of signaling pathways. Direct cell-cell contact, local accumulation of secreted signals or gap junction communication between adjacent cells might be involved (Gurdon et al., 1993).
However, what happens on the cellular level when a cell is exposed to multiple, competing instructive signals as this is the case in vivo? A variety of in vitro experiments using different combinations of growth factors tried to address this question. When neural crest stem cells are exposed to saturating concentrations of both factors, BMP2 and NRG1, neuronal differentiation predominates and glial differentiation is almost completely suppressed (Shah and Anderson, 1997). In contrast, when neural stem cells are exposed to BMP2 and Delta, glial differentiation instead predominates (Morrison et al., 2000). Furthermore, if the cells are exposed to saturating concentrations of both BMP2 and TGF-β1, the two factors are co-dominant (Shah and Anderson, 1997). These studies suggest that neural stem cells act like dynamic microprocessors that integrate different signals and produce an adequate output. This cellular internal integrator computes an output response to various inputs; in this case gliogenic and neurogenic signals, that the integrator interprets differently as a function of time in function to the relative influence of the inputs (Anderson, 2001; Fig. 4). The temporal changes were shown in postmigratory neural crest stem cells that are more sensitive to BMP2 and more responsive to Delta than developmentally 'younger' migratory neural stem cells (White et al., 2001; Kubu et al., 2002).

Figure 4: Neural stem cells as dynamic microprocessors.
Stem cells contain an internal integrator that computes the output as a result of different inputs. As a function of time this integrator can change so that the relative influence of the differentiation inputs changes. (Figure from Anderson DJ, 2001).
Therefore, cell identities are assigned through an interplay between extra- and intracellular signals. The context in which the signals are interpreted varies between cell types and developmental time. Such observations imply that neural progenitor cells gradually change their sensitivity to specific extrinsic signals. Additionally, neural progenitor cells can maintain their sensitivity to certain signals but over time produce distinct cell types (Michelsohn and Anderson, 1992; Liem et al., 1997). Generally, the basis of such temporal changes in progenitor cell competence is only poorly understood (Lillien, 1998a; Lillien, 1998b). Stochastic mechanisms or slight variations in microenvironments could also generate heterogeneity from otherwise equivalent cells in a homogenous culture environment. Otherwise, one might imagine a default pathway of differentiation that is changed by certain active signals (see before).

In sum, signal integration and cell fate decision of multipotent neural crest progenitors involving extrinsic signals and transcription factors are the main issues addressed in my thesis. The existence of multipotent neural crest progenitor cells in postmigratory targets raises the question of how fates inappropriate to these locations are suppressed despite the presence of factors capable of inducing aberrant fates. First, I will focus onto the community effect displayed by neural crest stem cells and their response to extrinsic signals by comparing migrating neural crest cells and multipotent progenitors isolated from the DRG. Multipotent progenitor cells have been identified in the rat sciatic nerve and DRG and were characterized by the expression of P0 and PMP22. These cells respond like NCSCs, to NRG1, BMP2, and TGFβ (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). In the following study, I 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. Further, the characterization of neural crest-derived progenitor cells in mouse embryos will make it possible to further elucidate the nature of the molecules involved in these processes.
4. Part I

Cell-Intrinsic and Cell-Extrinsic Cues

Regulating Lineage Decisions in
Multipotent Neural Crest-Derived Progenitor Cells

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Published 2002 in:
Int J Dev Biol 46, 193-200
4.1 Results and Discussion

4.1.1 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. TGFβ generates single cell clones in clonal cultures of P0/PMP22-positive progenitors, whereas NCSCs undergo cell divisions in the presence of TGFβ (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 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 cannot 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.
Part I, Cell-Intrinsic and Cell-Extrinsic Cues

Results

Figure 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 (labeled 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) that 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.

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., 2000). Thus, migratory and postmigratory neural crest cells, though multipotent, exhibit intrinsic changes during development that modulate their response to extracellular signals.
4.1.2 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 observed in response to factors that 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.

Figure 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 (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) that might be due to community effects. (A, C) Phase images of B, D. Scale bar: 20µm.
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 community effects likely preclude the aberrant generation of non-neural cells in peripheral ganglia.

Figure 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, TGFβ, 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 gray, 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 TGFβ-factor family induce neurogenesis. In addition, TGFβ 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.
4.1.3 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.

Table 1. BrdU incorporation in progenitor cells upon TGFβ treatment.

<table>
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<tr>
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<th>Clones derived from single cells (%)</th>
<th>Clones derived from cell communities (%)</th>
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<tr>
<td>No add</td>
<td>98 ± 6</td>
<td>96 ± 3</td>
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<tr>
<td>+ TGFβ</td>
<td>22 ± 5</td>
<td>97 ± 4</td>
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Table 1: 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.
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.

Figure 4. Cell-cycle-arrested communities of progenitor cells maintain their neuronal potential upon treatment with TGFβ.
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 TGFβ (4fM) (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 TGFβ alone proliferated normally (B, E). In low-density cultures, most of the cells stopped proliferating upon TGFβ treatment (A, 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 TGFβ treatment (G, K, N), TGFβ-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.
4.1.4 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; Paratore et al., 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 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.5 G; 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.5 H; 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.
Figure 5. Comparison of early markers of neural crest derivatives in developing rat and mouse embryos.

*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 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 rat and mouse PNS (C, F, J, M). Scale bars A-M: 100µm; inset in H: 25µm.

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.

Figure 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) that differentiated to NF160-positive neurons (E, F). TGFβ induced the differentiation of SMA-positive non-neural cells (G, H). (A, C, E, G) Phase images. Scale bar: 25µm.
4.2 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, 1997; Rothman et al., 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 (Rothman et al., 1993) and are revealed by transplantation experiments (Rothman et al., 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.
5. Part II

Erm expression and regulation

5.1 Introduction

5.1.1 Signal interpretation and procession

How do progenitor cells establish autonomous programs of differentiation and function? How do cells interpret and process extrinsic signals? One possible mechanism involves the generation of persistently active forms of intracellular transduction proteins. Typically, cytoplasmic proteins mediate the signal from the surface of the cell. These are often further processed by posttranslational mechanisms or by proteolytic cleavage of intracellular effector proteins (Schroeter et al., 1998). The above mentioned Notch signaling pathway for instance appears to require a ligand-dependent proteolytic cleavage of its intracellular domain (Schroeter et al., 1998; Struhl and Adachi, 1998). These ligand-dependent processed intracellular forms of proteins could have, if stable, a major impact in changing the state of cell differentiation. Additionally, the phosphorylation state of intracellular proteins might also be of some importance providing a potential link between long-term changes in protein kinases activity and transcriptional control of neural fate. For example, neuronal kinase activities implicated in memory storage are also subject to persistent activation by intracellular or intermolecular phosphorylation (Schwartz, 1993). Yet another mechanism might be regulated by transcriptional auto-activation. Transcription factors are activated by extrinsic signals and maintain the state of gene expression through a positive feedback loop that involves transcriptional auto-regulation. Finally, mechanisms might be involved that produce stable specification of cell identity by stabilizing gene expression. The disassembly of chromatin during cell division may permit extrinsic signals to efficiently alter programs of gene expression in dividing progenitor cells. It is possible that transcription factors regulating cell fate decisions do so in part through their ability to participate in the reorganization of chromatin structure (Gerber et al., 1997). Furthermore, the regulation of the cell cycle and its influence on cell fate decision should be considered. Often progenitor cells leave the cell cycle in order to differentiate. Nevertheless, the details of the interplay between extracellular signals/intrinsic programs and exit of the cell cycle directing the identity of specific neural cell types in the vertebrate nervous system remain to be elucidated.
5.1.2 Transcription factors in neural development

5.1.2.1 The basic helix-loop-helix family of transcription factors

Extrinsic signals are processed by intrinsic mechanisms involving signaling cascades mediated by transcription factors. Transcription factors have been described to play an central role in neural crest development and fate decision. Specifically, one important class of transcription factors in neural development is the basic helix-loop-helix transcription factor (bHLH) family. This family of transcription factors was shown to be involved in cell type specification in many developmental processes such as mammalian myogenesis (Weintraub, 1993), hematopoiesis (Porcher et al., 1996), and mammalian neurogenesis (Sommer et al., 1996). In Drosophila, proneural bHLH proteins are expressed in the proneural cluster and provide neural competence to ectodermal cells (Jan and Jan, 1993). The proneural genes achaete-scute and atonal are expressed in a tissue specific manner and work as positive regulators of the neurogenic program (reviewed in Jan and Jan, 1994). Further, based on conservation during evolution, a number of bHLH genes related to the Drosophila proneural genes have been isolated in vertebrates (reviewed in Lee, 1997). Their expression pattern, cellular function and regulatory pathways are conserved compared to their Drosophila counterparts. Mash1, the mammalian achaete-scute homologue was analyzed by targeted gene ablation in mice. These studies indicate that Mash1 function is required for the proper development of the autonomic nervous system and olfactory neurons (Guillemot et al., 1993). Mash1 plays a proneural role in determining neural fate and is also involved in the transition from a precursor to a differentiated cell. Additionally, other mammalian bHLH genes like neurogenin-1 and –2 (ngn1, 2) were identified and described to be involved in neural determination. Gene disruption experiments showed that these mice lack sensory neurons in the dorsal root ganglia and cranial ganglia (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). Additionally, downstream genes were not activated, including NeuroD and the Notch ligand Delta. Therefore, ngn genes and Mash1 have similar determination function in distinct neural lineages.

Additionally, bHLH transcription factors are known to react directly in response to extrinsic growth factors. Exposure of neural crest cells to BMP2 induces Mash1 expression and thereby neurogenesis (Shah et al., 1996). Forced expression of Mash1 in neural crest cells is not sufficient to promote neurogenesis, it rather maintains the competence of these cells to respond to BMPs (Lo et al., 1997). In summary, bHLH genes play an important role in lineage determination and differentiation. Furthermore, they control the ability of progenitors to respond to extrinsic factors and thus might integrate signals from the environment into transcriptional programs of differentiation. In the development of peripheral nervous system, bHLH transcription factors are mainly involved in neurogenesis. On the contrary, it remains obscure how glial fate in the vertebrate peripheral nervous system is specified.
5.1.2.2 The role of transcription factors in glial cell fate specification

One might argue that during embryonic neural development glial differentiation is a default pathway of differentiation and that neurogenesis has to be actively promoted instead. So far, there are no indications pointing towards this direction. Generally, between Drosophila and vertebrates, much less evolutionary conservation of function exists in gliogenesis than in neurogenesis. Therefore, it remains a very challenging question how the glial fate is specified and whether/which transcription factors play a key role in glial fate decision of the peripheral nervous system.

In the central nervous system (CNS), for instance, downregulation of bHLH genes that are 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 are involved in the early development of oligodendrocytes (Lu et al., 2000; Zhou et al., 2000). In contrast to the CNS, bHLH genes involved in the development of peripheral gliogenesis have not (yet) been described. It is likely that a different family of transcription factors than the bHLH factor family is involved in this process. For instance in Drosophila, transcription factors other than bHLH proteins are implicated in early glial development (reviewed in Granderath and Klambt, 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. In contrast, a mutation in the gene pointed (pnt) gives rise to a migration defect of the midline glia. Migrating midline glial cells are required for the separation of segmental commissures. During this process the glial cells recognize specific neuronal cells at the midline. The gene pointed is required for this glial-neuronal cell interaction (Klämbt, 1993). So far, the mammalian homologue of pnt has not yet been identified. Molecular analysis of the pointed gene reveals a high degree of homology to the Ets domain transcription factors suggesting that this family of transcription factors might play a significant role in mammalian neural development.

5.1.2.3 Ets domain transcription factors

The family of Ets domain transcription factors consists of transcriptional activators and repressors that are characterized by a conserved DNA-binding Ets domain (Sharrocks, 2001). The activity of Ets proteins is modulated by phosphorylation events and by their interaction with other co-regulatory proteins, which allows Ets domain transcription factors to act as nuclear effectors of multiple signal transduction pathways (Wasylyk et al., 1998). Ets proteins have been found in various organisms and cell types and are thought to be involved in various biological processes. In haematopoietic lineages,
proliferation, survival, cell type specification, and differentiation all involve control by Ets domain proteins (Remy and Baltzinger, 2000; Sharrocks, 2001). Thereby, a given Ets domain transcription factor can elicit distinct functions presumably dependent on the partner proteins it interacts with (DeKoter and Singh, 2000; DeKoter et al., 1998). Further, many Ets genes seem to be involved in the control of cellular proliferation. For instance, Ets-1, the founder of the Ets family is the precursor of a retroviral oncogene. Additionally, deregulation of various other Ets genes was reported to be associated with tumorigenic processes (Dittmer and Nordheim, 1998; Sharrocks et al., 1997). Commonly, Ets genes are expressed in migratory cells and regulate the epithelial to mesenchymal transition in several tissues such as the heart, somites, kidneys and the neural crest (Hay, 1995). E-cadherin, for instance, is closely associated with epithelial tissues and is downregulated during epithelial-mesenchymal transitions. Additionally, it is downregulated in many epithelial cancers and plays an important role in the development and progression of cancer (reviewed in Vallorosi et al., 2000; Nollet et al., 1999). Strikingly, the promoter of the E-cadherin gene contains a negative Ets regulatory element. Exogenous Ets-1 and Erm can circumvent the negative regulation by endogenous Ets protein(s), suggesting that E-cadherin level might be regulated by competition of different Ets proteins for its promoter (Rodrigo et al., 1999). Additionally, ICAM-1 an intercellular immunoglobulin-type adhesion molecule is also positively regulated by Ets-2 and Erm (de Launoit et al., 1998). In sum, many transcription-regulatory regions of genes that encode growth factors, oncoproteins, transcription factors and extracellular matrix metalloproteases (MMPs) implicated in the invasion and metastasis of tumor cells contain binding sites for Ets transcription factors (Crepieux et al., 1994).

5.1.2.4 The Pea3 subgroup of Ets domain transcription factors

Initially, our laboratory planned to isolate mammalian counterparts of the Drosophila transcription factor pointed by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers that represent the conserved Ets domain of this gene (Hagedorn et al., 2000). A similar approach was already successful in isolating members of the neurogenin subfamily of bHLH transcription factors (Sommer et al., 1996). The RT-PCR was performed on cDNA derived from NRG1-treated gliogenic neural crest cultures and subsequently, 89% of the clones were identified as Ets domain transcription factors (Diploma thesis, N. Mercader) encoding mostly Pea3 (Xin et al., 1992) or Erm (Monté et al., 1994). These two genes form, together with Er81, the Pea3 subgroup of Ets domain factors (de Launoit et al., 1997). Sequence comparison revealed that the Ets domain of human Erm is 63% identical to that of Ets-1 and 95% identical to that of human (Higashino et al., 1993) and mouse Pea3 (Xin et al., 1992) and 96% to that of mouse Er81 (Brown and McKnight, 1992).

The high degree of conservation between members of the Pea3 subgroup raised the question whether they fulfill redundant functions during development.
Therefore, de Launoit and colleagues investigated the spatio-temporal expression patterns during murine embryonic development. Expression of all three genes starts at E9.5 and is maintained until birth and in some cases postnatally. The three genes are expressed in many developing tissues with numerous sites of co-expression mainly during early stages of development. As differentiation proceeds, expression patterns are modified so that some populations continue to express the genes while other subpopulations exhibit new specific expressions (Chotteau-Lelièvre et al., 1997).

More focused in situ hybridization studies on early stages of developing embryos revealed that Erm and Pea3 are not expressed in migrating neural crest cells, whereas expression can be detected in earliest neural crest-derived structures such as cranial ganglia, autonomic ganglia and dorsal root ganglia (DRG; Hagedorn et al., 2000). This expression pattern is opposite to the expression of another Ets gene, called Ets-1 (Fafeur et al., 1997; Maroulakou et al., 1994). Ets-1 expression is found in migrating neural crest cells and is later downregulated in its derivatives (Mercader, Diploma thesis, 1997). Therefore, Erm and Pea3 are probably involved in differentiation processes of neural crest cells, whereas Ets-1 might play a role in migratory events. In the DRG, quite strong expression of both, Erm and Pea3, can be detected although the characteristic punctuate expression pattern of Pea3 indicates that Erm and Pea3 are expressed in different subpopulations of sensory cells. In contrast, the prospective myelinating Schwann cells do not display detectable levels of Erm and Pea3. This demonstrates that at least the differentiation of some neural crest lineages is Erm and Pea3 independent.

In the nervous system, spinal circuits are marked by the coordinate expression of specific Ets proteins in motor and sensory neurons that contact the same peripheral muscle target (Lin et al., 1998). The Ets factors involved are Pea3 and Er81 that are expressed in neurons upon target-derived signaling. Targeted deletion of Er81 results in the loss of motor coordination due to reduced connections between a subgroup of sensory afferents and the corresponding motor neurons (Arber et al., 2000). Thus, Er81 controls a late step in sensory-motor circuit formation. In contrast, mice carrying a Pea3 mutation are phenotypically normal but display male sexual dysfunction, possibly due to an underlying neuronal abnormality (Laing et al., 2000). Functional compensation between Ets factors might prevent more severe phenotypes in these mutants. In addition to Pea3 and Er81, the transcription factor Erm is expressed in several areas of the nervous system (see above) but, unlike Pea3 and Er81, is absent in motor neurons of the spinal cord (Chotteau-Lelièvre et al., 1997; Hagedorn et al., 2000; Lin et al., 1998).
5.1.2.5 The Ets domain transcription factor Erm

Commonly, Ets family factors recognize specific sequences in the regulatory region of many genes. The core DNA sequence recognized by an 85 amino acid long DNA-binding Ets domain is GGAA/T (Wasylyk et al., 1993), although bases adjacent to this core motif might modulate the efficiency of binding. The total open reading frame of Erm encodes 510 amino acids, that would result in a calculated protein mass with a molecular weight of 57.8 kDa. Instead, *in vitro* translation revealed a molecular mass of 70 kDa indicating that Erm could be submitted to post-translational modifications (Monté et al., 1994). Analyzing the transactivation properties of Erm, Defossez et al. (1997) found two transactivating domains (TADs): AD1 at the N-terminus and AD2 at the C-terminus. These two transactivation domains are functionally dissimilar and exhibit a synergistic effect on transcription. AD1 displays functional features of the ‘acidic’ class of TADs. In addition, Erm binds via AD1 TAFII60 and via other regions TBP and TAFII40, which are proteins of the transcription machinery. Moreover, it was reported that the Ets domain of Erm binds stronger to its sequence specific target than full size Erm protein. This led to the conclusion that it might contain DNA-binding modulatory domains (Laget et al., 1996). Finally, the model was proposed that Erm has to undergo a conformational change induced by post-translational modification or interaction with a cofactor in order to bind to the DNA and activate transcription.

5.1.2.6 Downstream targets of NRG1 signaling

The fact that Erm and Pea3 were isolated from NRG1-treated gliogenic cultures raised the question whether Erm and Pea3 are downstream target genes of NRG1 signaling. Neuregulins are a family of epidermal growth factor-like (EGF-like) factors that activate receptor tyrosine kinases of the ErbB type. It is likely that the intracellular signal transduction cascade of NRG1 induces distinct transcription factors. *In situ* hybridization studies using a NRG1 probe that detects all NRG1 transcripts showed that Erm, Pea3 and NRG1 display an overlapping expression pattern within neural crest derivatives. At E10.5, NRG1 transcripts are only weakly detectable in the DRG Anlagen which do not express any Erm or Pea3 transcripts. Half a day later at E11, NRG1 is clearly detectable in the forming DRG that starts to display weak expression of Erm and Pea3. At E12, all three genes are highly expressed in the DRG (Hagedorn et al., unpublished). This spatio-temporal co-expression strengthens the hypothesis that Erm and Pea3 are potential downstream targets of NRG1 signaling. Additionally, semi-quantitative PCR revealed that Erm transcripts are 3- to 7-fold upregulated in NRG1-treated neural P19 cells as compared to untreated cells (C. Paratore, Diploma thesis, 1998). Interestingly, DRG and cranial ganglia all express Erm, Pea3 and NRG1 during development in a similar sequential expression pattern, although the cell types expressing these factors cannot be identified unambiguously using conventional *in situ* hybridization analysis.
Furthermore, the hypothesis that Erm and Pea3 might be downstream targets of NRG1 signaling has been corroborated for Pea3. As mentioned above, NRG1 function is mediated by the ErbB receptor tyrosine kinase family. The HER2/neu proto-oncogene encodes a 185 kDa receptor tyrosine kinase that is structurally related to the epidermal growth factor receptor (c-ErbB-1). 93% of HER2/Neu-overexpressing human breast tumors show increased transcription levels of Pea3 (Benz et al., 1997). Therefore, HER2/Neu receptor tyrosine kinase initiates an intracellular signaling cascade resulting in increased Pea3 transcriptional activity (Benz et al., 1997). O'Hagan and colleagues (1998) suggested that transcriptionally activated Pea3 stimulates its own transcription as well as the transcription of HER2/Neu. Furthermore, it activates the transcription of genes encoding matrix-degrading proteinases, enzymes required for tumor cell migration and invasion. Cotransfection of Pea3 and HER2/Neu in COS cells stimulated Pea3-dependend reporter gene expression to a much greater extent than did either protein alone suggesting that HER2/Neu upregulates the transcriptional activity of Pea3 (O'Hagan and Hassell, 1998). Additionally, the transactivation capacities of mouse Pea3, mouse Er81, and human Erm are dramatically increased by Ras, Raf-1, MEK, and MAPK ERK-1 and ERK-2. Therefore, these transcription factors seem to be targets of the Ras-dependent signaling cascades (O'Hagan et al., 1996). Apart from these described pathways, protein kinase A is able to increase Erm and Er81 transactivation (Janknecht, 1996; Janknecht et al., 1996). Taken together, this suggests that several independent signal transduction pathways regulate the transactivation ability of these factors although direct in vivo phosphorylation remains to be established.

In summary, there are indications that Erm and Pea3 are possible downstream targets of NRG1 signaling as shown by their spatio-temporal expression pattern and by the cotransfection studies of Pea3 and HER2/Neu. One goal of my thesis was to further address the cellular expression of Erm during neural crest development and to determine the function of Erm in the development of the peripheral nervous system by using in vitro and in vivo systems. The following study shows that Erm is not detectable in emigrating neural crest cells, while its expression is induced at early stages in aggregating peripheral ganglia. In peripheral ganglia, Erm expression is found in multipotent progenitors, neurons and satellite glia, whereas Schwann cells are devoid of Erm expression. Both, in cultured neural crest stem cells (NCSCs) and in multipotent progenitor cells isolated from dorsal root ganglia (DRG), Erm expression is regulated by NRG1 signaling. In such cultures, NRG1 instructs multipotent neural crest cells to adopt a glial fate (Hagedorn et al., 1999; Shah et al., 1994).
The Ets Domain Transcription Factor Erm
Distinguishes Rat Satellite Glia from Schwann Cells
and is Regulated in Satellite Cells by Neuregulin Signaling

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Published 2000 in:
Dev Biol 219, 44-58
5.2 Introduction

Glia cells of the vertebrate PNS consist of myelinating and non-myelinating Schwann cells of peripheral nerves, the satellite glia of sensory, sympathetic and parasympathetic ganglia, and the enteric glia. All of these cell types are derived from the neural crest, but it is still unclear how their phenotypic characteristics arise (Le Douarin et al., 1991; Le Douarin, 1982). In avian embryos, the Schwann cell myelin protein (SMP) is expressed only by Schwann cells but not by satellite cells or enteric glia (Dulac et al., 1988). Satellite and enteric glia have the potential to express SMP when withdrawn from their ganglionic or enteric environment, suggesting that inhibitory cues suppress a glial default pathway characterized by SMP expression (Cameron-Curry et al., 1993; Dulac and LeDouarin, 1991). Similarly, in mouse embryos, Schwann cells but not satellite glia express the transcription factor Krox20 (Topilko et al., 1994). The microenvironment of sensory ganglia appears to negatively regulate Krox20 expression since cultured satellite cells are able to upregulate Krox20 (Murphy et al., 1996). The conversion of satellite cells, as defined by the absence of Schwann cell markers, to SMP-positive or Krox20-positive Schwann cells indicates that peripheral glia are derived from a common precursor (Cameron-Curry et al., 1993; Le Douarin et al., 1991; Murphy et al., 1996). This hypothesis was supported by clonal analysis of neural crest cultures, in which bipotent precursors giving rise to both SMP-positive and SMP-negative glial cells were found (Dupin et al., 1990).

The nature of the signals that regulate subtype-specific glial markers is poorly understood, but both inhibitory and inducing factors may be involved. The neurotrophins BDNF and NGF exert a positive effect on SMP expression in glial cells derived from quail DRG, while NT3 prevents SMP expression (Pruginin-Bluger et al., 1997). A crucial role at several stages of gliogenesis has been attributed to NRG1. NRG1, a member of the Epidermal Growth Factor superfamily, influences growth and differentiation of various cell types including neurons and glia (Burden and Yarden, 1997; Gassmann and Lemke, 1997). In peripheral gliogenesis, the NRG1 isoform GGF2 has been shown to instructively bias multipotent neural crest stem cells and neural crest derivatives to choose a glial fate (Hagedorn et al., 1999; Morrison et al., 1999; Shah et al., 1994), although it was not determined which glial subtypes are produced from these cultures due to the lack of appropriate markers. In the Schwann cell lineage, NRG1 promotes the survival and maturation of Schwann cell precursors (Dong et al., 1995) and exerts a mitogenic and anti-apoptotic effect on postnatal Schwann cells (Goodearl et al., 1993; Grinspan et al., 1996; Levi et al., 1995; Marchionni et al., 1993; Syroid et al., 1996; Trachtenberg and Thompson, 1996). Mice with targeted mutations of NRG1, or of ErbB receptors transmitting NRG1 signaling, exhibit severely reduced numbers of both Schwann cell precursors in peripheral nerves, and neuronal and glial cells in cranial and sympathetic ganglia (Britsch et al., 1998; Erickson et al., 1997; Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995; Meyer and Birchmeier, 1995; Meyer et al., 1997; Morris et al., 1999; Riethmacher et al., 1997). However, the generation of presumptive satellite cells in DRG is apparently not affected in these mutants (Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). The analysis of ErbB2-null embryos, in which
glial cells appear to accumulate in the nerve roots of the DRG and Schwann cells are not found in peripheral nerves, led to the proposal that in wild type animals, satellite glia emigrate from the ganglia into peripheral nerves where they acquire a Schwann cell fate (Morris et al., 1999). According to this model, NRG1 signaling might be required to promote a glial differentiation state compatible with migration or might stimulate migration directly. An early marker expressed by developing satellite glia but not by Schwann cells would greatly facilitate investigations aimed at understanding the relationship between these neural crest-derived glial subtypes. Here we show that the Ets domain transcription factor Erm (Monté et al., 1994) is expressed by sensory neurons and satellite glia early in development but is not expressed at any time by Schwann cells. The NRG1-isoform GGF2 instructs Erm-positive progenitors in neural crest cultures to give rise to Erm-positive glia and regulates Erm expression in cultured satellite cells. Moreover, Erm-positive cells derived from either cultures of neural crest or from DRG are able to generate Schwann cells. Finally, while satellite glia are able to undergo a transition to Schwann cells, GGF2 does not convert Schwann cells to Erm-positive satellite glia at any stage investigated.

5.3 Results

5.3.1 Spatiotemporal expression of Erm in neural crest derivatives

In order to address whether Erm might be involved in the development of the PNS, we investigated the expression pattern of Erm in a series of in situ hybridization experiments focusing on the neural crest and its derivatives at various developmental stages. At early stages (E8.5), Erm is not expressed in migrating neural crest cells (Fig.1, open arrow in B) that are positive for the Ets domain transcription factor Ets-1 (arrow in Fig.1 A) (Fafeur et al., 1997; Maroulakou et al., 1994). As soon as neural crest cells aggregate in the presumptive dorsal root ganglia (DRG), Ets-1 expression is downregulated (data not shown). In E11 embryos, a small population of cells at the ventrolateral margin of the forming DRG starts to display Erm expression (arrow in Fig.1 C); by E12, Erm mRNA is abundant (Fig.1 D). Erm expression persists in the DRG up to postnatal day (P) 4, the latest time point examined (Fig.1 E). However, Erm mRNA was not detectable in presumptive Schwann cells found along peripheral nerves at any developmental stage analyzed (E9 to P4) (open arrow in Fig.1 D,E). Apart from its expression in DRG, Erm mRNA was also present in other peripheral ganglia, such as in developing sympathetic and cranial sensory ganglia (data not shown).
Figure 1. Erm expression in developing dorsal root ganglia.

(A, B) Erm is not detectable in migrating neural crest cells. Adjacent transverse sections through an E8.5 mouse embryo were hybridized with Ets-1 and Erm riboprobes. Migrating neural crest cells arising from the dorsal part of the neural tube (nt) are positive for Ets-1 (arrow in A) but not for Erm (open arrow in B). Transverse sections of E11 (C), E12 (D) and P4 (E) mouse trunk regions hybridized with Erm riboprobe. A few cells within the dorsal root ganglia (drg) start to express Erm at E11 (arrow in C). At E12, transcripts encoding Erm are strongly expressed in the dorsal root ganglia (arrow in D) and persist to postnatal stages (arrow in E). Note that Erm is not expressed in presumptive Schwann cells along peripheral nerves (open arrows in D, E). Scale bars: (A, B) 50µm; (C, D) 100µm; (E) 1mm.

5.3.2 Erm is expressed in neurons and satellite glia of developing DRG but not in presumptive Schwann cells

The in situ hybridization analysis did not settle the question whether Erm is expressed in neurons and/or presumptive glia within the developing DRG. Unfortunately, the available antibodies to Erm (see below) were not sensitive enough to be suitable for immunohistochemistry. Therefore, we applied a fluorescence hybridization method that we have recently established, to address the cellular expression of Erm in the developing PNS in situ (Paratore et al., 1999). The procedure is based on tyramide signal amplification (TSA) using fluorophore-labeled tyramide. When combined with confocal microscopy, this allows the simultaneous detection of distinct mRNA species or of mRNAs together with proteins on the cellular level. In the PNS, the NRG1-receptor ErbB3 is expressed in migrating neural crest and in developing glial cells (Meyer and Birchmeier, 1995). In agreement with previous studies (Meyer et al., 1997; Morris et al., 1999), prominent ErbB3-positive cells were found at the ventrolateral margins of the DRG at E12 (Fig.2 A). Hybridization of E12 mouse embryo sections with an antisense Erm riboprobe followed by ErbB3
immunofluorescence demonstrated that the ErbB3-positive presumptive glial cells of DRG coexpressed Erm mRNA (Fig. 2 A-C). A sense Erm probe did not produce fluorescent signals (data not shown). In contrast, ErbB3-positive glial precursor cells along peripheral nerves did not express Erm mRNA (Fig. 2 D-F), corroborating the conventional *in situ* hybridization experiments (Fig. 1). Thus, Erm is expressed in presumptive satellite glia but not in developing Schwann cells. In the DRG, however, Erm transcripts are not restricted to the glial lineage but are also found in neuronal cells positive for neurofilament (NF160) protein (Fig. 2 G-I). Fluorescence *in situ* hybridization on motor neurons did not reveal Erm expression (data not shown).

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**Figure 2.** Cellular localization of Erm mRNA in ErbB3-positive presumptive satellite glia and in NF160-positive sensory neurons.

Sections through mouse E12 DRG (A-C, G-I) and peripheral nerve (D-F) were hybridized with Erm riboprobe (A, D, G). Fluorescence *in situ* hybridization using Cy3-coupled tyramide was followed by immunohistochemistry using anti-ErbB3 antibody (B, E) or anti-neurofilament (NF)160 antibody (H). Immunoreactions were visualized by FITC-conjugated secondary antibodies and the stainings were analyzed by confocal microscopy. Single confocal planes are shown. (C), (F) and (I) represent the confocal overlays of (A, B), (D, E) and (G, H), respectively. Yellow color indicates double positive cells (arrows in C) revealing individual presumptive satellite glia coexpressing Erm and ErbB3. Likewise, NF-positive neurons in the DRG are double positive for Erm mRNA expression (arrow in I). However, Erm signals were not detectable in ErbB3-positive presumptive Schwann cells in peripheral nerves (D-F). Note that at early stages in development, neuronal and glial cell types can hardly be distinguished on ganglionic sections solely based on their morphology (Hall and Landis, 1992). Scale bars: (A-C), (G-I) 10µm; (D-F) 20µm.
To investigate the cellular expression of Erm protein in DRG, an antibody directed to a peptide corresponding to the N-terminus of Erm (Janknecht et al., 1996) was affinity purified. In transfection experiments, the antibody did not crossreact with Er81 and Pea3, the most closely related Ets domain transcription factors (data not shown) (Brown and McKnight, 1992; de Launoit et al., 1997; Xin et al., 1992). DRG dissected from rat E16 embryos were dissociated and fixed 4 hours after plating. In such cultures, Erm was detectable by a biotin/avidin-based immunoreaction both in virtually all NF160-positive neurons and in satellite glia identified by the expression of glial fibrillary acidic protein (GFAP) (Fig.3 A,C), consistent with the fluorescence in situ hybridization results. Strikingly, virtually all GFAP-positive cells expressed Erm suggesting that this Ets domain transcription factor is a marker for the vast majority of developing glia in E16 DRG. In contrast, the POU transcription factor Oct-6 (also called SCIP or Tst-1; He et al., 1989; Meijer et al., 1990; Monuki et al., 1989), which is expressed in promyelinating Schwann cells found along peripheral nerves (Arroyo et al., 1998; Blanchard et al., 1996; Scherer et al., 1994; Zorick et al., 1996), was barely detectable in satellite glia (Fig.3 B,D).

Figure 3. Erm protein is expressed in satellite glia and in neuronal cells of E16 DRG.

DRG isolated from rat E16 embryos were dissociated and the cells were fixed four hours after plating and double-labeled for Erm and GFAP (A, C, E), Erm and NF160 (insets in A, C, E), or for Oct-6 and GFAP (B, D, F). Immunostaining for Erm (A) was visualized using a biotin/avidin amplified horseradish peroxidase (HRP) reaction and bright-field optics. Note that Erm is expressed both in NF160-positive neurons (detected by Cy3-fluorescence; inset in C) and in GFAP-positive glia that were detected by FITC-immunofluorescence. In contrast, Oct-6 (B) is not detectable in freshly isolated cells of the DRG. (E, F) are phase-contrast views. Scale bars: 20µm.
5.3.3 Differential regulation of Erm and Oct-6 in peripheral glia: role of the NRG1 isoform GGF2 in maintenance of Erm expression

In cultures of DRG, satellite cells can acquire characteristics of Schwann cells. Krox20 and SMP are expressed by developing Schwann cells but not by satellite cells (Dulac et al., 1988; Topilko et al., 1994). Both Krox20 and SMP are upregulated in glia of serum-treated DRG cultures, revealing that at least in vitro, a transition from satellite cells to Schwann cells is possible (Cameron-Curry et al., 1993; Murphy et al., 1996). To investigate the regulation of Erm protein expression in glia acquiring Schwann cell features, cultures of dissociated DRG (E16) were incubated for 6 days in the presence of serum and forskolin which is known to result in upregulation of myelin genes (reviewed by Mirsky and Jessen, 1996; Zorick and Lemke, 1996). In such cultures, the GFAP-positive glial cells (Fig.4 D,K) underwent massive proliferation, became positive for the Schwann cell marker Oct-6 (Fig.4 G) and lost Erm expression (Fig.4 A). These results strongly suggest that serum/forskolin promoted the transition of Erm⁺/Oct-6⁻ satellite glia (Fig.3) into Erm⁻/Oct-6⁺ Schwann cells (Fig.4).

Figure 4. Differential regulation of Erm and Oct-6 in glial subtypes derived from DRG.
Dissociated DRG were cultured for 6 days in standard medium (no add) (B, E, H, L), supplemented with fetal bovine serum and forskolin (A, D, G, K), or in the presence of the NRG1 isoform GGF2 (C, F, J, M). Cultures were double immunolabeled for GFAP (D-F, K-M) and for Erm (A-C) or Oct-6 (G-J). Cells cultured in standard medium were also double labeled for NF160 (inset in E) and Erm (inset in B). Erm staining was revealed by a biotin/avidin amplified HRP reaction, Oct-6 and NF160 by Cy3-fluorescence, and GFAP by FITC-fluorescence. In serum/forskolin, the Schwann cell marker Oct-6 was strongly expressed (G),
while Erm expression (A) was not detectable in GFAP-positive glia (D). In ‘no add’ conditions, glial Erm expression is abolished while occasional neurons still express Erm (arrows and inset in B). Oct-6 is not expressed in these conditions (H). Upon addition of GGF2, glial cells express Erm protein at high levels (C), comparable to the Erm expression in satellite cells of freshly isolated DRG (see Fig.3). In contrast, Oct-6 expression is barely detectable in the presence of exogenous GGF2 (J). Note that glial cells obtained in standard culture medium, in GGF2-treated cultures or upon serum/forskolin treatment display distinct morphologies. Scale bars: (F,M) 50µm; (inset) 20µm.

While serum/forskolin was required to induce Oct-6 expression in DRG-derived glia, down-regulation of Erm expression was serum/forskolin-independent. In dissociated DRG, most glial cells lost Erm expression even when cultured without serum/forskolin (Fig.4 B). However, the neurons identified by their morphology and by NF160 expression (inset in Fig.4 E), continued to express Erm (inset in Fig.4 B). Thus, different regulatory mechanisms govern Erm expression in neurons and glia. Since Erm expression is detectable in non-neuronal components of DRG both in situ and in freshly isolated cultures, we considered that glial expression is lost upon cellular dissociation due to dilution of a positively acting signal. NRG1 is a good candidate for such an activity given its coordinate expression with Erm in DRG and in other developing tissues (data not shown) and since Ets domain transcription factors have been suggested to be potential targets of NRG signaling (Galang et al., 1996; Janknecht, 1996; Janknecht et al., 1996; Khurana et al., 1999; Marais et al., 1993; O'Hagan and Hassell, 1998; O'Hagan et al., 1996; Sapru et al., 1998; Schaeffer et al., 1998; Yang et al., 1996). To test this hypothesis, we examined the effect of the NRG1 isoform GGF2 on cultures of dissociated DRG. In contrast to serum/forskolin, GGF2 maintained Erm protein expression in virtually all GFAP-positive glial cells (Fig.4 C,F). Based on in vivo expression data, the presence of Erm indicates that these DRG-derived, GGF2-treated cells are satellite glia. In agreement with this, GGF2 did not upregulate expression of the Schwann cell marker Oct-6 (Fig.4 J). Hence, the expression of transcription factors that characterize distinct glial subtypes is differentially regulated by extracellular signals: endogenous Erm protein expression is maintained by the NRG1 isoform GGF2 in satellite glia without induction of Oct-6 expression. In contrast, treatment with serum/forskolin abolishes Erm expression and upregulates the Schwann cell marker Oct-6.

5.3.4 Generation of two distinct glial subtypes from Erm-positive progenitor cells in neural crest cultures

The transition from GFAP-positive satellite glia to GFAP-positive Schwann cells might reflect a general plasticity of glial subtypes (Le Douarin et al., 1991) or might point to a lineage relationship of Erm-positive cells and Schwann cells. In particular, neural crest cells might produce Erm-positive progenitor cells that generate satellite glia in the presence of GGF2, and Schwann cells in the presence of unidentified factors. Previously, cells marked by the low affinity
neurotrophin growth factor receptor (LNGFR; p75) have been isolated from neural crest explants, shown to be multipotent and to be instructed to a glial fate by GGF2 (Hagedorn et al., 1999; Shah et al., 1994; Stemple and Anderson, 1992). Our analysis of similar neural crest cultures revealed that approximately 85% of the p75-positive cells were also positive for Erm expression (Fig. 5 A-C), indicating that the early expression of Erm in neural crest cultures is independent of exogenously added GGF2. Thus, this culture system allowed us to investigate whether undifferentiated Erm-positive progenitors are able to give rise to both satellite glia and Schwann cells, independent of previous association with a DRG environment. We confirmed that addition of GGF2 to cultured neural crest cells promoted gliogenesis in the absence of neurons (Hagedorn et al., 1999; Shah et al., 1994), and found that the glia displayed features of satellite cells, as defined by the presence of Erm and GFAP immunoreactivity and the absence of Oct-6 staining (Fig. 5 H-L). In contrast, when Erm-positive neural crest cells were treated with serum and forskolin, neurons and glia were generated (Sommer et al., 1995; Stemple and Anderson, 1992), and virtually all of the glia were Oct-6-positive but devoid of Erm expression, i.e. displaying features of Schwann cells (Fig. 5 D-G). Thus, cultured neural crest cells can give rise to two distinct glial sublineages, satellite and Schwann cells, and these Schwann cells are generated from Erm-positive progenitors.
Figure 5. Generation of distinct glial subtypes from Erm-positive progenitor cells in neural crest cultures.

Neural crest cells were allowed to emigrate from rat E10.5 neural tubes in culture and were replated. In some culture dishes, cells were labeled after 3 hours with anti-p75 antibody visualized by Cy3-fluorescence (B) and subsequently fixed and stained for Erm expression (A). (C) Phase-contrast image. In other culture dishes, neural crest cells were grown for 11 days in the presence of serum/forskolin (D-G) or of GGF2 (H-L) that promotes gliogenesis. These cultures were fixed and double labeled for either Erm (D, H) and GFAP (F, K) or Oct-6 (E, J) and GFAP (G, L). Erm labeling was revealed by a horseradish peroxidase reaction while GFAP was detected by a secondary antibody conjugated to FITC and Oct-6 by a fluorescent Cy3-coupled secondary antibody. Note that glial cells present in serum/forskolin-treated cultures were Erm−/Oct-6+ while glial cells generated from GGF2-treated cultures expressed Erm but not Oct-6. Scale bars: (C) 20µm; (G,L) 50µm.

5.3.5 Erm-negative glia derived from DRG, but not developing Schwann cells, display competence to induce Erm expression in response to GGF2

The aforementioned experiments show that Erm-positive progenitor cells and Erm-positive satellite glia have the potential to adopt a Schwann cell fate. This could reflect sequential steps in a common lineage in which presumptive satellite cells – i.e. non-neuronal Erm-positive cells residing in the developing DRG – serve as a source for Schwann cells. Alternatively, the transition from satellite cells to Schwann cells could simply represent plasticity between peripheral glial subtypes. In the latter case, early Schwann cells would presumably also have the capacity to generate Erm-positive satellite glia. To test this hypothesis, we exposed Schwann cell precursors and early Schwann cells (Jessen et al., 1994) isolated from rat E14, E16 and E18 sciatic nerves to the NRG1 isoform GGF2 and analyzed Erm expression in such cultures. In accordance with the in situ hybridization analysis (Figs 1 and 2) freshly isolated developing Schwann cells did not exhibit Erm expression (data not shown). Importantly, Erm protein expression was neither induced in Schwann cell precursors nor in early Schwann cells even after prolonged incubation with GGF2 (Fig.6 A,D; shown are the data from E16 sciatic nerve cells).
Part II, Expression of Erm and NRG signaling

Results

Figure 6. The NRG1 isoform GGF2 induces Erm expression in DRG-derived glia but not in early Schwann cells.

(A, D) Schwann cells were isolated from E16 sciatic nerve and cultured for 6 days (d) in the presence of GGF2. Cultures were fixed and double labeled for both Erm (A) and GFAP (D). Note that the HRP immunoreaction did not reveal Erm expression in GFAP-positive Schwann cells (visualized by Cy3-immunofluorescence). (B, C, E, F) Dissociated E16 DRG were incubated in standard medium (n.a., no add) for three days. While some culture dishes were fixed thereafter, sister dishes were cultured for an additional three days in the presence of GGF2. Double labeling for Erm (B, C) and GFAP (E, F) revealed that in standard medium Erm expression is downregulated in DRG-derived glia, but is reinduced upon treatment with GGF2. Scale bar: 20µm.

A possible explanation for this result might be that GGF2 is only able to maintain Erm expression in glial cells but not to induce it. To identify whether GGF2 is able to induce Erm expression in glial cells, we made use of the DRG culture system. As described above (Fig.4), satellite cells lose Erm expression in standard medium, and were negative for both Erm (Fig.6 B) and Oct-6 (data not shown) after three days in culture. Upon treatment with GGF2 for an additional three days, however, Erm protein expression was reinduced to high levels in virtually all cells (Fig.6 C). In principle, these data could be accounted for by differential selection of residual Erm-positive cells upon GGF2 treatment of three-day cultures rather than by induction of Erm expression in Erm-negative cells. Given that in three-day cultures most glial cells were Erm-negative (Fig. 6B), such an unexpected selective activity of GGF2 (Hagedorn et al., 1999; Shah et al., 1994) would have to be accompanied by substantial cell death, which we did not observe (data not shown). Hence, the NRG1 isoform GGF2 not only maintains Erm expression in satellite cells but can also reinduce it. The differential response to NRG1 signaling of glia isolated from DRG compared to developing Schwann cells prepared from peripheral nerves indicates an intrinsic difference of these glial subtypes and is consistent with an irreversible developmental step from presumptive satellite cells to Schwann cells.
5.4 Discussion

Identifying traits that distinguish between neural crest-derived glial subtypes is a prerequisite to study their developmental relationship. We have shown here that the Ets domain transcription factor Erm is differentially expressed and regulated in distinct subsets of peripheral glia (Fig. 7). To our knowledge, Erm is the first marker shown to be expressed in presumptive satellite glia of sensory ganglia at early developmental stages but not in Schwann cells of the peripheral nerves. Maintaining Erm expression in cultured satellite cells requires the continuous presence of a positive signal that can be provided by the NRG1 isoform GGF2. GGF2 also promotes the generation of satellite cells from Erm-positive precursor cells in neural crest cultures, but does not induce the Schwann cell marker Oct-6. Serum and forskolin cause cultured Erm-positive neural crest cells and presumptive satellite glia to generate Oct-6-positive Schwann cells, while a similar transition from developing Schwann cells to satellite glia upon treatment with GGF2 was not observed. Because in vivo Erm-positive cells are detected in developing peripheral ganglia but not in peripheral nerves, our combined data suggest that Schwann cells are likely derived from ganglionic Erm-positive progenitors.

![Diagram](image)

Figure 7. Model of lineage relationship between Erm-positive cells and Schwann cells.

Erm-negative migrating neural crest cells locate to the Anlage of peripheral ganglia and give rise to Erm-positive progenitor cells that are devoid of differentiation markers such as GFAP and NF. These progenitors generate Erm-positive neurons and satellite cells. Erm expression in neurons is constitutive while it is regulated in satellite glia by NRG1/GGF2. The model predicts that Erm-positive progenitors can emigrate from peripheral ganglia and have the potential to generate Erm-negative Schwann cells in peripheral nerves. This competence is maintained by GFAP-positive presumptive satellite glia. The factor(s) inducing Schwann cell traits are not yet defined but are likely to cause an irreversible progression in glia development.
The response of presumptive satellite cells to the NRG1 isoform GGF2 is reflected by regulated expression of endogenous Erm. Signaling of NRG1 is transmitted by the receptors ErbB3 and ErbB4 and their coreceptors EGFR/ErbB1 and ErbB2 (Burden and Yarden, 1997; Gassmann and Lemke, 1997; Riese and Stern, 1998). All ErbB receptor combinations activate the Ras/MAP-kinase signaling cascade (Ben-Levy et al., 1994; Hynes and Stern, 1994; Kim et al., 1995; Marte et al., 1995; Pinkas-Kramarski et al., 1996; Si et al., 1996; Tansey et al., 1996). Members of the Ets domain transcription factor family are candidates for playing a role in NRG1 signaling since several vertebrate Ets domain transcription factors are transactivated by Ras/MAP-kinase activity (de Launoit et al., 1997; Janknecht, 1996; Janknecht et al., 1996; Karin, 1994; Marais et al., 1993; O’Hagan et al., 1996; Wasylyk et al., 1993; Yang et al., 1996). The Ets domain transcription factors Pea3 and Ets-2 can also be transactivated by overexpression of ErbB2 in a heterologous cell system (Galang et al., 1996; O’Hagan and Hassell, 1998) and Pea3 was found to be upregulated concomitantly with ErbB2 in breast tumors (Benz et al., 1997; Trimble et al., 1993). Moreover, Ets factor-binding sites were shown to be crucial for NRG1- as well as MAP-kinase-dependent regulation of utrophin and acetylcholine receptor (AChR) δ and subunit gene expression (Khurana et al., 1999; Sapru et al., 1998; Schaeffer et al., 1998). The effect of NRG1 signaling on AChR δ and ε transcription appears to be mediated via the Ets domain transcription factor GABPα (Schaeffer et al., 1998). The identity of cell-endogenous Ets factors that mediate NRG1 signaling in neural development, however, has not been elucidated. Our data demonstrating regulation of Erm expression by NRG1 are consistent with the hypothesis that the Ets domain transcription factor Erm might mediate cellular responses to NRG1 signaling during development of satellite glia. This is also supported by our findings that Erm is concomitantly expressed with NRG1 at several different embryonic sites including the developing DRG (data not shown). Moreover, NRG1 signaling and Erm may share common targets. In cultured mammary tumor cells, NRG1 induces the expression of the cellular adhesion protein ICAM-1 (Bacus et al., 1993) whose promoter can be activated by Erm (de Launoit et al., 1998). We would expect that a direct target of NRG1 signaling should be induced in DRG-derived glia within a few hours after addition of GGF2. Reinduction of Erm in DRG cultures, however, was observed only after at least one day of treatment with GGF2 (data not shown), suggesting that Erm might not be an immediate early target of NRG1 signaling. Alternatively, the inhibitory mechanisms that led to Erm downregulation in DRG cultures might counteract the Erm inducing activity of GGF2. Thus, the detailed molecular processes relating NRG1 signaling to Erm expression remain to be determined.

It is not clear whether NRG1 signaling is associated with Erm expression at all stages of neural crest development. In neural crest cultures, initial Erm protein expression appears to be independent of exogenously added NRG1. Cultured peripheral neurons constitutively express Erm irrespective of whether or not the medium contains NRG1. Moreover, in situ analysis of homozygous embryos (E12) containing a targeted mutation in the ErbB3 locus (Riethmacher et al., 1997) suggests that the disruption of NRG1 signaling in vivo does not prevent...
the early expression of Erm in DRG (L.H., C.P., D.Riethmacher, C.Birchmeier, U.S., L.S.; unpublished). However, redundancies of signaling pathways could also account for this result.

5.4.2 Intrinsic differences between peripheral glial subtypes

The differential response to GGF2 of DRG-derived glia compared to developing Schwann cells isolated from sciatic nerves at various developmental stages reveals intrinsic differences between presumptive satellite glia and Schwann cells. Apparently, these differences are established early in development, since neither Schwann cell precursors (E14) nor early Schwann cells (E16-E18) (Jessen et al., 1994) were competent to induce Erm expression upon NRG1 treatment. Likewise, back-transplantation experiments in avian embryos indicated differences between glial cells isolated from distinct peripheral ganglia (Le Douarin et al., 1991). The molecular basis for this is unclear. Although ErbB receptors are able to undergo ligand-induced combinatorial interactions with potential functional implications (Vartanian et al., 1997), all neural crest-derived glia are thought to transduce NRG1 signaling via the receptor combination ErbB2 / ErbB3 (Grinspan et al., 1996; Levi et al., 1995; Shah et al., 1994; Vartanian et al., 1997). Nevertheless, NRG1 signaling in developing Schwann cells and satellite glia might recruit different signal transduction molecules. As mentioned above, activated ErbB receptors stimulate the Ras/MAP-kinase signaling cascade, but additional pathways appear also to be involved (Hynes and Stern, 1994; Tansey et al., 1996).

5.4.3 Schwann cells generated from Erm-positive progenitor cells

Our study on Erm expression and regulation is consistent with the hypothesis (Fig.7) that peripheral neurons, satellite glia and Schwann cells might derive from a common Erm-positive progenitor in which Erm expression is downregulated upon acquisition of a Schwann cell fate. Since in vivo Erm expression first appears in developing peripheral ganglia, this model predicts that Schwann cells are generated from ganglionic progenitor cells. The differential regulation of Erm in glial subtypes raises the question whether Erm might play a role in maintaining satellite cell properties in glia of peripheral ganglia. Moreover, downregulation of Erm might be linked with competence of glial precursors to activate Schwann cell traits. In view of the proposed lineage relationship, the activation of Schwann cell features in Erm-positive cells might be regarded as a progressive step in peripheral glial development. Accordingly, a transition from Schwann cell precursors or early Schwann cells to satellite cells would correspond to a de-differentiation step, which is unlikely to occur and was not observed in our cultures. Consistent with our model (Fig.7), we have recently isolated a multipotent P0/PMP22-positive progenitor cell type from rat E14 DRG that expresses Erm but not yet the differentiation markers GFAP and NF160 (Hagedorn et al., 1999;
and data not shown). In culture, these cells can give rise to neurons, non-neural cells and GFAP⁺ glia (Hagedorn et al., 1999). Depending on the extracellular factors added to the cultures, the glia generated from these progenitors were either satellite cells expressing Erm but not Oct-6, or Schwann cells expressing Oct-6 but not Erm (data not shown). Our data that Erm-positive progenitor cells derived from neural crest cultures or from E14 DRG, and GFAP⁺ satellite glia from E16 DRG display the competence to become Schwann cells corroborate the previous findings that demonstrated transitions of satellite cells to SMP-positive or Krox20-positive Schwann cells (Cameron-Curry et al., 1993; Murphy et al., 1996; reviewed by Le Douarin et al., 1993). Moreover, the idea that Schwann cells can develop from DRG-derived non-neuronal cells has also been supported by the phenotype of mutant animals in which ErbB2⁻/⁻ presumptive glia appear to stall in the nerve roots of DRG and, as an assumed consequence, Schwann cells in peripheral nerves are not generated (Morris et al., 1999). Based on the combined data we suggest that Erm-positive presumptive satellite glia might be able to emigrate from the forming ganglia and to contribute to the Schwann cell lineage along peripheral nerves. Emigration might result in the loss of a local signal that is provided by the ganglionic environment and required to maintain Erm expression and the satellite glia state. Likely, this signal is the NRG1 isoform GGF2 that is produced by neurons (Marchionni et al., 1993; Shah et al., 1994) and might act in a paracrine manner to regulate Erm expression in neighboring satellite cells. Our hypothesis predicts that in vivo the cells emigrating from the forming ganglia encounter axons that do not yet produce local NRG1 or express NRG1 isoforms not able to maintain Erm expression. Alternatively, downregulation of Erm in vivo might require a repressive signal present along peripheral axons that is capable of overriding the NRG1 effect on Erm expression. Our model does not exclude alternative cellular pathways in Schwann cell development. Clonal analysis of avian neural crest identified some founder cells which give rise exclusively to SMP-positive Schwann cells, independent of the generation of SMP-negative satellite cells or neurons (Dupin et al., 1990). However, precursor cells restricted to the Schwann cell lineage were rare in lineage-tracing studies of single premigratory and migratory neural crest cells in vivo (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Whether Erm-immunoreactive cells indeed give rise to Schwann cells in vivo has to be addressed in future fate-mapping experiments (Zinyk et al., 1998).
6. Part III

Functional analysis of the transcription factor Erm in neural crest development

6.1 Gain of function experiments

The relatively brief temporal expression of Erm in glial satellite cells and its consequent downregulation in Schwann cells raised the question whether this downregulation is a prerequisite for further glial differentiation. Additionally, we hypothesized based on the early expression of Erm during neural crest development that it might also play a role in neuronal or glial fate acquisition. Therefore, gain of function experiments in multipotent neural crest stem cells were performed in vivo and in vitro.

Clonal cultures, in which the fate of individual progenitor cells can be followed upon genetic manipulation, allow distinguishing between roles of regulatory molecules in survival, proliferation, fate decision, or differentiation processes (Anderson et al., 1997; Sommer, 2001). Such clonal experiments were performed in vitro using retroviral overexpression of full length Erm and revealed increased neurogenesis in BMP2-treated cultures compared to control cultures (data not shown, Lo et al., 1997). In contrast, in a medium permissive for glia, neurons and smooth muscle cells no striking phenotype was observed. However, a potential role of Erm in gliogenesis was difficult to address in this experimental setup due to downregulation of the retroviral transgene before overt glial differentiation. Therefore, we decided to apply an in vivo approach instead. A transgenic mouse was constructed expressing full length Erm under the control of the proteolipid protein (PLP) promoter (Bongarzone et al., 1999). The activity of this promoter in migrating neural crest, in presumptive satellite glia, Schwann cells and sensory neurons was optimal for our purpose. In order to follow Erm transgene overexpression, a lacZ reporter gene was cloned downstream of Erm, that was separated by an internal ribosomal entry site (PLP-Erm-IRES-LacZ). 36 founder animals were obtained out of which six PCR-positive animals were identified. Subsequent breeding of these founder animals yielded six transgenic mouse lines (#664-669) that expressed the transgene at different expression levels and at the correct sites. Analysis of cross-sections from developing embryos (E9 to E16) by in situ hybridization using numerous neural crest markers and X-Gal stainings revealed no obvious difference between wild type and transgenic animals. Further, it is likely that the increase in neurogenesis, which was observed in vitro might be regulated in vivo and compensated by enhanced apoptosis. However, TUNEL staining at different developmental stages indicated no change in the survival capacity of neuronal precursors in the developing DRG. Furthermore, the proliferation rate as assessed by a proliferation marker was not affected (data not shown). We hypothesized that any phenotype might be compensated in vivo and so, we preformed in vitro experiments, which might reveal changes on the cellular level. Nevertheless, analysis of high density as well as clonal cultures using diverse cell culture conditions revealed no significant difference between the
developmental potential of cells isolated from wild type and transgenic mice. Additionally, adult transgenic mice bred and behaved completely normally. Finally, a possible phenotype in Schwann cell maturation could also not be confirmed by histological analysis of semithin sciatic nerve sections. In summary, we were not able to see any change in migration, differentiation, proliferation and apoptosis by our in vivo Erm gain of function analysis.

6.2 Loss of Erm function analysis in neural crest cells

Therefore, we decided to investigate the effect of a loss of function Erm mutation in neural crest development. Mice carrying a targeted deletion of Erm are lethal at early embryonic stages (S. Arber and J. Hassell, personal communication), precluding an analysis of Erm function in neural development in these mutants. Therefore, mice in which Erm can conditionally be ablated will be generated in order to overcome this early lethality. In parallel, we have chosen a different approach by fusing the Ets domain of Erm to the engrailed repressor (EnR) that is a general strong transcriptional repressor (Han and Manley, 1993; Lo et al., 1999). Driven by the LTR of a retroviral expression vector, this form, called EnR-Erm, was used for retroviral infection of neural crest stem cells and should therefore interfere with endogenous Erm function. Concomitant expression of EGFP from an internal CMV promoter allowed monitoring living cells upon infection with the retroviral vector. This approach represents a fast and reliable system to address a potential role of Erm or any related Ets gene in neural crest development. The Ets domain is a highly conserved structure and therefore, it is possible that the EnR-Erm construct also represses the function of other Ets genes. So far, no additional Ets genes besides Erm have been identified that are expressed at this early stage in neural crest development. Furthermore, we screened for Ets domain transcription factors in neural crest stem cells using degenerate primers directed against the well conserved Ets domain (see above) and were not able to identify additional Ets factors besides Erm and Pea3. In particular, Pea3 and ER81, the Ets factors most closely related to Erm are, in contrast to Erm, not expressed in early neural crest development. Therefore, we claim that EnR-Erm mainly interferes with the function of Erm and not a putative other Ets domain transcription factor.

The regulation of Erm by NRG1 raises the question of whether Erm might regulate glial fate acquisition and survival of multipotent neural crest cells, similar to the function of the transcription factor Sox10 in neural crest development (Paratore et al., 2001). Alternatively, Erm might control other processes such as survival, proliferation, or differentiation of peripheral glia, given its continuous expression in satellite glia (Hagedorn et al., 2000). In contrast to its expression in satellite glia, Erm expression in peripheral neurons is independent of NRG1 signaling. The goal of the following study was to elucidate the developmental processes regulated by Erm in the peripheral nervous system (PNS). The data presented in this study suggest multiple roles of Erm in neural crest development.
The Role of the Ets Domain Transcription Factor Erm in Modulating Differentiation of Neural Crest Stem Cells

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in press
Developmental biology (2002)
6.3 RESULTS

6.3.1 Neural expression of Erm in cultured neural crest cells

In situ hybridization experiments revealed expression of the Ets domain transcription factor Erm in neural cells of dorsal root ganglia (DRG), cranial sensory ganglia and sympathetic ganglia (Chotteau-Lelièvre et al., 1997; Hagedorn et al., 2000). Moreover, multipotent neural crest cells from neural crest explants and from DRG as well as presumptive satellite glia derived from these cells have been shown to be Erm-immunoreactive (Hagedorn et al., 2000). In order to further elucidate the expression pattern of Erm protein, neural crest stem cells (NCSCs) derived from rat neural crest explants were allowed to differentiate into satellite glia in the presence of NRG1, neurons in the presence of BMP2, and smooth muscle-like cells in the presence of TGFβ. In agreement with our previous report (Hagedorn et al., 2000), undifferentiated p75-positive NCSCs and satellite glia expressing glial fibrillary acidic protein (GFAP) derived from NCSCs were positive for Erm (Fig.1 A-F). Likewise, Erm was expressed in neurofilament 160 (NF)-positive neurons generated de novo in cultures of neural crest cells (Fig.1 G-I). In contrast, smooth muscle actin (SMA)-positive non-neural cells derived from NCSCs were negative for Erm expression (Fig.1 J-L). Thus, the progression of NCSCs to neurons and glia is marked by the continued expression of the Ets domain transcription factor Erm.

Figure 1. Expression of Erm in neural crest cells and derivatives.
Neural crest stem cells (NCSCs) replated from neural crest explants were fixed after 4 hours and immunolabeled using anti-Erm antibody (visualized by a biotin/avidin-amplified HRP reaction) (A) and anti-p75 antibody (visualized by Cy3 fluorescence) (B). Sister cultures of replated NCSCs were allowed to develop in the presence of NRG1. In these cultures, Erm expression (D) was maintained in differentiated glia that are positive for GFAP (visualized by Cy3 fluorescence) (E). In BMP2-treated cultures, crest cells differentiated into neurofilament (NF)-positive neurons (visualized by FITC fluorescence) (H), while TGFβ -treated cultures gave rise to smooth muscle actin (SMA)-expressing non-neural cells (K). The latter did not express Erm (J) whereas neurons maintained Erm expression (G). (C, F, I, L) show the corresponding phase pictures.
6.3.2 Forced expression of a dominant-negative form of Erm in neural crest stem cells

Erm has been shown to be a transcriptional activator (Laget et al., 1996). To address whether Erm plays a role in regulating processes of neuronal and glial development, we designed a dominant-negative form of Erm that would act as a transcriptional repressor and should therefore interfere with Erm function in NCSCs. This form, called EnR-Erm, consisted of the DNA-binding domain of Erm that was tagged by a nuclear localization signal and a c-Myc epitope (NLS-MT) and fused to the engrailed repressor (EnR) (Han and Manley, 1993) (Fig. 2 A). EnR-Erm expression was driven from the LTR of a retroviral expression vector. Concomitant expression of EGFP from an internal CMV promoter allowed monitoring living cells upon infection of the retroviral vector. A control vector encoded EGFP but no Erm fusion protein (Fig. 2 A). Immunostaining of infected NCSCs with an anti-Myc antibody confirmed the nuclear expression of EnR-Erm, concomitant with expression of EGFP (Fig. 2 B). A very similar approach involving a dominant-negative form of Pea3 fused to the engrailed repressor was recently used successfully by Hassell and colleagues to inhibit Pea3 function in mammary tumorigenesis (Shepherd et al., 2001). Such inhibitory fusion constructs are thought to act by competing for binding sites in promoters of target genes. The inhibitory activity of EnR-Erm on the transactivation potential of Erm was confirmed in a transient transfection assay performed in HEK cells. Previously, full size Erm has been shown to transactivate a reporter gene containing an artificial promoter with three Ets binding sites (Defossez et al., 1997). Co-transfection of full size Erm and this reporter construct together with a 10-fold molar excess of the EnR-Erm construct reduced the transactivation capacity of Erm to approximately 20% of that displayed by Erm alone, as measured by a luciferase assay (Fig. 2 C).
Figure 2. Construction and analysis of a retroviral vector expressing a dominant-negative form of Erm.

(A) Schematic structure of full length Erm, retroviral EnR-Erm vector and control vector. Erm encompasses two activation domains (AD1, AD2) and a DNA-binding Ets domain (Ets). To construct dominant-negative Erm, its Ets domain was fused to the engrailed repressor (EnR). EnR possesses a nuclear localization sequence (NLS) and a Myc epitope tag (MT) at its N-terminus. This repressor construct was subcloned into a retroviral expression vector encoding an EGFP reporter gene. EGFP expression is driven from a CMV promoter. The EGFP-expressing vector without EnR-Erm served as control vector. (B) Test infections performed on NCSC demonstrated transport of the fusion protein into the nucleus (visualized by DAPI-labeling), as assessed by anti-Myc staining. Infected cells were also marked by EGFP reporter gene expression. (C) The repressor activity of EnR-Erm was analyzed in a luciferase transactivation assay. Two independent experiments were performed (Exp.1 and Exp.2). The reporter construct 3xTORU Luc that contains several Ets binding sites was transfected either alone, together with pSV-Erm encoding full length Erm (Erm), or together with pSV-Erm and EnR-Erm. The luciferase activity measured upon transactivation of the reporter construct by pSV-Erm alone was set to 100%, while the baseline represents the activity of the reporter construct alone.

In a next experiment, forced expression of EnR-Erm in NCSCs was performed to investigate whether repression of Erm activity would have an effect on neural crest development. To this end, NCSCs from rat neural crest explants were replated and infected either with virus encoding EnR-Erm or with control virus (Materials and Methods). Subsequently, the cells were replated at clonal density and the position of individual infected cells was mapped. Infected NCSCs were
identified by virtue of EGFP expression from the retroviral vectors. The cultures were then incubated in standard medium that allows differentiation of multipotent neural crest cells into neurons, glia, and non-neural smooth muscle-like cells (Hagedorn et al., 1999; Stemple and Anderson, 1992). After 8 days of incubation, the clonal cultures were fixed. The cellular composition of the clones was analyzed by immunostaining for Sox10 to label presumptive glia (Paratore et al., 2001) and for NF to mark neuronal cells. Non-neural cells were identified by the absence of both of these markers. Upon infection of EnR-Erm virus, neural crest cells generated slightly decreased numbers of mixed clones, i.e. clones that contained more than one cell type, as compared to control-infected neural crest cells (29% and 38% of all colonies, respectively) (Fig.3). Should Erm have multiple influences on number and type of cells produced in mixed clones, the cellular role of Erm in NCSCs and their derivatives might be difficult to address by the analysis of such colonies. However, in addition to mixed colonies, many infected clones were restricted to a single cell type. Thereby, the fates chosen by restricted neural crest cells were significantly altered upon forced expression of EnR-Erm. In control cultures, 16% of all colonies contained exclusively glia, 10% only neurons, and 15% were composed solely of smooth muscle-like cells (Fig.3). In contrast, the vast majority of EnR-Erm-expressing cells gave rise to glia-only clones (35% of all colonies) and 15% of all colonies contained only smooth muscle-like cells (Fig.3). Strikingly, only 1% of all colonies were neuron-exclusive clones in cultures infected with EnR-Erm virus, indicating that the dominant-negative form of Erm impaired the generation of clones restricted to a neuronal lineage. Since cell death of founder neural crest cells was not increased in EnR-Erm-infected cultures as compared to control cultures, the low frequency of neuron-restricted clones in cultures expressing EnR-Erm was not simply due to selective elimination. Rather, the loss of purely neuronal clones and the concomitant increase of glia-only colonies suggests an instructive role of dominant-negative Erm in biasing fate decisions of NCSCs.
Figure 3. Clonal analysis of neural crest stem cells expressing dominant-negative Erm.
NCSCs were infected with either control or EnR-Erm virus, and single EGFP-positive cells were mapped and allowed to differentiate in standard conditions permissive for neural and non-neural fates. After differentiation for 8 days, the cellular composition of the clones was analyzed. For the quantitative analysis, expression of Sox10, NF160, and absence of these markers were used to distinguish between different fates. When compared to control infected clones, EnR-Erm infected clones showed a strongly reduced amount of clones consisting solely of neurons (N-clones). In contrast, the number of clones that consisted of glia only (G-clones) was increased. 'S' indicates the number of clones containing exclusively non-neural smooth muscle-like cells, 'Mixed' the number of clones containing more than one cell type, and 'Death' indicates loss of clones. The numbers are given as the mean ± s.d. of three independent experiments. In each experiment, 50 clones were analyzed. Significant differences between Control and Erm-EnR infected cultures are marked by an asterisk (P<0.0005, Student’s t-test).

In addition to altered cell type composition in EnR-Erm-expressing colonies, we found that the clone size of glia-restricted colonies was affected. In particular, the majority of neural crest cells that gave rise to glia-restricted colonies in control cultures proliferated considerably, so that very few control-infected clones comprised less than 4 cells (11% in a first experiment, 0% in a second experiment; Table 1). In contrast, upon forced expression of dominant-negative Erm, 80 to 100% of all glia-only clones contained less than 4 cells (Table 1). Loss of founder cells and of cells within clones was not increased upon EnR-Erm infection indicating that. These data indicate a markedly decreased proliferation rate in EnR-Erm-infected in cells of the glial lineage. Neuronal and non-neural clone types, however, were of comparable size in EnR-Erm-infected and in control cultures (data not shown).

### Table 1. Erm is required for glial proliferation

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Table 1. Erm is required for glial proliferation.

Analysis of the number of cells within glia-only clones showed a massive reduction of the cell number in EnR-Erm-infected clones cultured in standard medium. In two independent experiments (Exp.1 and Exp.2), most of the EnR-Erm-infected glial clones were smaller than 4 cells, while the vast majority of control-infected glial clones consisted of more than 4 cells. Approx. 50 clones were analyzed per experiment. A $\chi^2$ square analysis revealed P values of less than 0.001 in both experiments.
6.3.3 Dominant-negative Erm impairs neuronal fate decision in neural crest stem cells

The abovementioned experiments were performed with NCSCs incubated in standard medium that is permissive for the formation of neurons, glia, and non-neural smooth muscle-like cells. Our results obtained by forced expression of dominant-negative Erm in such cultures were consistent with differential roles of Erm in fate decision and proliferation. Thus, in mixed clones containing several cell types, opposing and overlapping effects might influence the number of a given cell type, which makes these clones difficult to analyze. Therefore, to better distinguish the effects of functional inhibition of Erm in different neural crest lineages, EnR-Erm-expression assays were carried out in NCSCs promoted to generate specific lineages. To this end, NCSCs were infected with control virus or virus encoding EnR-Erm and replated at clonal density as described above. In a first set of experiments, the cultures were thereafter treated with BMP2 that stimulates neurogenesis and to a lesser extent the generation of smooth muscle-like cells in NCSCs (Shah et al., 1996). Mapping of EGFP-expressing cells allowed us to determine the progeny of individual infected founders. Typically, control-infected clones treated with BMP2 contained NF-positive neurons, and many of these clones consisted exclusively of neurons (Fig. 4 A, C, E). In contrast, the majority of clones expressing dominant-negative Erm did not adopt a neuronal fate and were composed of SMA-positive non-neural cells only (Fig. 4 B, D, F). Quantification of these experiments revealed that in control cultures, 34% of all colonies consisted of neurons only, 29% were mixed containing neurons and smooth muscle-like cells, and 25% were composed of smooth muscle-like cells only (Fig. 4 G). In these conditions, 12% of all colonies were lost due to cell death. Inhibition of Erm activity in BMP2-treated NCSCs reduced the number of neuron-containing clones, with only 17% of all colonies consisting exclusively of neurons and 15% containing neurons and smooth muscle-like cells (Fig. 4 G). Instead, 56% of all neural crest founder cells overexpressing EnR-Erm gave rise to clones that were composed solely of smooth muscle-like cells. Similar to control cultures, only 12% of all mapped cells expressing EnR-Erm underwent cell death. Thus, as in cultures incubated in standard medium (Fig. 3), blocking Erm function affected neurogenesis in BMP2-treated NCSCs. In the presence of BMP2, this led to the formation of non-neural cells at the expense of neurons, demonstrating a fate switch of NCSCs. Cell cycle progression in neuronal clones appeared not to be affected, however, since the neuronal clones generated despite the expression of EnR-Erm consisted of similar cell numbers as control-infected neuronal clones (Table 2).
Figure 4. Repression of Erm activity impairs neuronal fate decision of NCSCs in neurogenic conditions.

Infected neural crest stem cells plated at clonal density were grown in standard medium supplemented with BMP2. The phenotype of clones derived from previously marked NCSCs was assessed by immunocytochemistry 4 days after factor addition. Control-infected EGFP-positive clones (A) often consisted of NF-positive neurons only (C). EnR-Erm-infected clones (B) displayed a reduced neuronal capacity and mostly adopted a non-neural fate marked by smooth muscle actin (SMA) expression (D). (E, F) phase contrast pictures. (G) Quantitative clonal analysis of infected cells incubated in the presence of BMP2 confirmed a bias of EnR-Erm infected cells to give rise to non-neural cells. The number of neuron-only clones (N-clones) and of clones containing both neurons and smooth muscle-like cells (N/S-clones) was reduced, while that of smooth muscle-only clones (S-clones) was increased. Loss of clones was not affected upon EnR-Erm infection. Numbers are shown as the mean ± s.d. of three independent experiments. 50 clones were scored per experiment. Student’s t-test revealed a significance difference (p<0.05) comparing control and Erm-EnR infected cultures (asterisks).
Table 2. Erm is not required for neuronal proliferation.

Dominant-negative Erm was overexpressed in neural crest cells incubated in the presence of BMP2. The number of neurons within neuronal-only clones was analyzed in EnR-Erm- and control-infected cultures. The clones were classified into three categories according to their size. In all categories, a $\chi^2$ square analysis of two independent experiments revealed no significant differences between the sizes of control- and EnR-Erm-infected clones. The number of control- and EnR-Erm-infected clones analyzed was 63 and 57, respectively, in the first experiment, and 64 and 65 in the second experiment.

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Table 2. Erm is not required for neuronal proliferation.

6.3.4 Blocking Erm function in neural crest stem cells decreases proliferation rate in peripheral glial lineage without affecting glial fate decision

To assess the role of dominant-negative Erm in peripheral glia development, the infection experiments described above were repeated and the infected NCSCs in clonal cultures were exposed to glia-inducing signals. NRG1 instructs multipotent neural crest cells to adopt a glial fate (Hagedorn et al., 1999; Shah et al., 1994). p75-positive NCSCs infected by control virus extensively proliferated in the presence of NRG1 and within 6 days of incubation formed clones expressing Sox10 (not shown) as well as the glial differentiation marker S100 (Fig.5 A-F). Forced expression of EnR-Erm did not impair glial differentiation of NCSCs and infected cells were able to upregulate S100 expression similar to cells in control cultures (Fig.5 G-L). However, EnR-Erm-infected cells hardly proliferated, often forming clones of only one or two cells (Fig.5 H, J, L). To quantify this phenomenon, infected glial clones obtained after 6 days of NRG1 treatment were classified according to the cell number per clone. In control cultures, 29% of all glial clones comprised more than 20 cells, 53% were composed of 10 to 20 cells, and only 18% of the colonies had less than 10 cells per clone (Fig.6). In contrast, clones with more than 20 cells were rare (4% of all glial clones) and only 27% of the colonies were in the category of 10 to 20 cells upon EnR-Erm infection. Rather, most of the EnR-Erm-infected clones (69%) remained small, containing less than 10 cells (Fig.6). The reduction of the clone size upon infection of dominant-negative Erm was not simply due to increased cell death. Loss of clones occurred at a similarly low frequency in EnR-Erm infected and in control cultures (10% and 6%,
respectively). Moreover, daily observation of clones in culture did not reveal increased cell death within the infected clones, excluding selective elimination of cells as an explanation for the reduced clone size in EnR-Erm-infected cultures (data not shown).

Figure 5. Dominant-negative Erm reduces the proliferation rate in glial cells without affecting glial differentiation.
Infected NCSCs plated at clonal density expressed EGFP and p75 at one day (1d) after infection (A, C, G, I). Single infected NCSCs were mapped and grown in standard medium supplemented with NRG1 for 6 days (6d). Glial differentiation as assayed by anti-S100 staining occurred both in control cultures (B, D) and in EnR-Erm-infected cultures (H, J). However, the number of cells was reduced within glial clones infected with EnR-Erm as compared to control-infected clones. (E, F, K, L) phase contrast pictures.
Part III, Cellular function of Erm

Results

Figure 6. Quantitative analysis of the clone sizes obtained in NRG1-treated cultures. NCSCs were infected and treated as described in the legend to Fig.5. Quantification of the number of cells within glia-containing clones indicated that the amount of big clones (>20 cells) was reduced in EnR-Erm-infected cultures when compared to control-infected, NRG1-treated cultures. In contrast, the number of small clones (<10 clones) was highly increased upon EnR-Erm infection. The numbers are given as the mean ± s.d. of three independent experiments. In each experiment, 50 clones were analyzed (p<0.05, Student’s t-test; asterisks). Note that glial fate acquisition of NRG1-treated NCSCs was not affected by dominant-negative Erm (not shown).

We have recently shown that Erm expression is regulated by NRG1 in neural crest-derived cells (Hagedorn et al., 2000). Therefore, the effect of dominant-negative Erm on proliferation in NRG1-treated neural crest cells might be dependent on signaling by NRG1. Alternatively, the effect of dominant-negative Erm on proliferation might be lineage-specific independently of the lineage-inducing stimulus. In order to address this issue, gliogenesis in infected cells was induced by exposure to a signal different from NRG1. A soluble form of Delta (Delta-Fc) has previously been shown to promote gliogenesis from NCSCs isolated from sciatic nerve and from neural crest explant cultures (Morrison et al., 2000). Likewise, the majority of NCSCs that have been derived from rat neural crest explants, submitted to three rounds of infection with retrovirus, and replated at clonal density, adopted a glial fate when exposed to soluble Delta (data not shown). Thereby, promotion of gliogenesis by Notch activation was independent from the retroviral construct used for infection, since forced expression of EnR-Erm did not alter the frequency of glial clones generated in these cultures as compared to control-infected cultures (data not shown). Of the glial clones infected with control virus, 30% contained more than 20 cells per clone, 50% had 10 to 20 cells per clone, and merely 20% of all glial clones were smaller than 10 cells (Fig.7). In contrast, similar to treatment with NRG1, forced expression of dominant-negative Erm decreased the size of glial clones in the presence of Delta. In particular, only 5% of the EnR-Erm-expressing glial clones were bigger than 20 cells and 16% consisted of 10-20 cells. Most clones (73% of all glial colonies), however, remained smaller than 10 cells per clone (Fig.7). Cell death of founder cells and within developing clones was not affected by overexpression of EnR-Erm in Delta-treated cultures, as
assayed by daily observation of the cultures (data not shown). Thus, the equivalent cell numbers found in glial clones treated with NRG1 (Fig.6) and with Delta (Fig.7) demonstrate that dominant-negative Erm decreased the proliferation rate in developing peripheral glia independently of the glia-inducing signal.

![Figure 7. Reduced proliferation rate of EnR-Erm-infected glial cells induced by Notch activation.](image)

Single infected EGFP-positive NCSCs were mapped and grown for 6 days in standard medium supplemented with Delta-Fc (Materials and Methods). Clonal analysis revealed that EnR-Erm expression reduced the number of cells per glial clone while glial fate decision by NCSCs was not affected (not shown). Unlike control-infected clones, few EnR-Erm-infected clones consisted of more than 20 cells, while the number of clones that were less than 10 cells was highly increased upon inhibition of Erm function. The data are expressed as the mean ± s.d. of three independent experiments. In each experiment, 50 clones were scored. Asterisks indicate significant differences (p<0.005, Student’s t-test).

The above data indicate that glial fate acquisition and cell cycle progression are independently regulated. Accordingly, Erm might not only regulate proliferation in neural crest cells exposed to glia-promoting cues but also in glial cells that have already adopted their specific fate. In order to test this hypothesis, we treated neural crest cells with NRG1 for several days, which promotes gliogenesis and maintains endogenous Erm expression in glial cells (Hagedorn et al., 2000; Shah et al., 1994). Thereafter, infection with control retrovirus or with the EnR-Erm-expressing virus was performed and proliferation of infected glia was assayed as described before. Similar to the results obtained with neural crest cells cultured in gliogenic conditions, dominant-negative Erm also reduced the proliferation rate of differentiating glia (Table 3). In particular, in two independent experiments the number of glial clones containing more than 10 cells was significantly decreased upon EnR-Erm infection, while the number of clones with fewer than 10 glia increased as compared to control-infected cultures (Table 3). Thus, the role of Erm in regulating glial proliferation is independent of the process of glial fate decision.
Table 3. Erm regulates cellular proliferation in glial cells independently of glial fate acquisition.

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Table 3. Erm regulates cellular proliferation in glial cells independently of glial fate acquisition.

Neural crest cells were first treated for 3.5 days with NRG1 and, thereafter, infected with EnR-Erm and control virus. Single infected cells were mapped and subsequently maintained in NRG1 for another 5.5 days. The cell number within individual control- and EnR-Erm-infected glial clones was analyzed, scoring 58 and 61 clones, respectively, in the first experiment, and 51 and 59 clones in the second experiment. In general, the clone sizes were reduced in EnR-Erm-infected as compared to control-infected clones. A $\chi^2$ square analysis revealed a highly significant difference for both experiments. P values < 0.001.

6.4 Discussion

In the vertebrate nervous system, members of the Pea3 subgroup of Ets domain transcription factors have been implicated in establishing neuronal connections (Arber et al., 2000; Lin et al., 1998). In the present study, we provide evidence that the Pea3 factor Erm already plays a role at earlier stages of neural development. Erm is expressed in postmigratory multipotent neural crest cells, and its expression is maintained during neurogenesis and gliogenesis but downregulated when NCSCs adopt a non-neural fate (Hagedorn et al., 2000; this study). Loss-of-function experiments using a dominant-negative form indicate differential roles of Erm in neural crest development, depending on the cell lineage to be generated (Fig.8). Erm appears to be required for NCSCs to adopt a neuronal fate, while in neural crest cells promoted to become glia, Erm regulates proliferation without affecting glial fate acquisition. We cannot exclude that Ets domain transcription factors other than Erm are affected by overexpression of dominant-negative Erm. However, no Ets domain transcription factor other than Erm is known to be expressed in multipotent neural crest cells as well as in neural lineages derived from them. In particular, Pea3 and ER81, the Ets factors most closely related to Erm, are in contrast to Erm not expressed in early neural crest development but, in the PNS, are only found in differentiated sensory neurons (Lin et al., 1998; Paratore et al., 1999). Therefore, we assume that the observed effects in NCSCs expressing dominant-negative Erm are specific for Erm and reveal its multiple lineage-specific requirements.
Figure 8. Differential roles of the Ets factor Erm in neural crest cells. While early migratory neural crest cells do not express Erm, its expression is upregulated in multipotent neural crest cells aggregating in peripheral ganglia. Erm expression is maintained both in neurons and satellite glia generated from these neural crest cells (Hagedorn et al., 2000; this study). Loss-of-function (lof) experiments reveal differential roles of Erm, depending on the cell lineage. Erm is required for multipotent neural crest cells to adopt a neuronal fate, while it regulates proliferation without affecting fate decision in neural crest cells promoted to become glia.

6.4.1 Role of the Ets domain transcription factor Erm in neuronal fate decision by neural crest stem cells

Clonal cultures in which the fate of individual progenitor cells can be followed upon genetic manipulation allow distinguishing between roles of regulatory molecules in survival, proliferation, fate decision, or differentiation processes (Anderson et al., 1997; Sommer, 2001). Forced expression of a dominant-negative form of Erm did neither affect overall survival of NCSCs in clonal cultures nor did it prevent the expression of differentiation markers in neuronal, glial and smooth muscle-like lineages. However, blocking Erm function decreased the frequency of neuronal clones generated from NCSCs, leading to the concomitant increase of non-neuronal clones. Thereby, the type of non-neuronal clones preferentially generated was dependent on the culture conditions chosen. In standard medium permissive for neurogenesis, gliogenesis as well as the formation of smooth muscle-like cells, glial clones were produced at the expense of neuronal clones; in the presence of BMP2 that on single NCSCs induces neurogenesis and the formation of non-neural cells but not glia (Shah et al., 1996), non-neural clones were predominantly found in the cultures. Moreover, the frequency of glial clones was not affected by
perturbing Erm function in glia-promoting conditions, independent of whether NRG1 or Notch activation was used as glia-instructive signal. These data suggest that although expressed in developing glia, Erm is specifically required for efficient neuronal fate decision by NCSCs. Erm appears not to be unique among Ets domain transcription factors to regulate early developmental stages of a particular cell lineage (Remy and Baltzinger, 2000; Sharrocks, 2001). Overexpression experiments revealed a role of Pea3 in myogenic differentiation (Taylor et al., 1997), while ablation of the \textit{Pu.1} gene causes specific loss of lymphoid and myeloid lineages (Scott et al., 1994). Similarly to Erm in neurogenesis, \textit{Pu.1} is not absolutely required for T cell development but plays a role in efficient specification of progenitors to the T cell lineage (Spain et al., 1999).

Since Erm expression is maintained both in glial as well as neuronal lineages derived from multipotent cells, Erm is unlikely to act as a determinant of a neuronal fate on its own. In agreement with this idea, forced expression of full size Erm in NCSCs cultured in the absence of instructive growth factors yielded variable results with no indication of Erm inducing neurogenesis (data not shown). However, when NCSCs in clonal cultures were treated with BMP2, overexpression of full size Erm biased NCSCs to adopt a neuronal rather than a non-neural fate (data not shown). Thus, it is conceivable that Erm synergizes with another transcriptional regulator to accomplish its function in neuronal development, and BMP2 might induce expression of this co-factor. Modulation of both DNA-binding and transcriptional activity by protein-protein interactions is a characteristic property of Ets factors (Li et al., 2000; Sharrocks, 2001; Wasylyk et al., 1998). In particular, functional and physical interactions of basic helix-loop-helix (bHLH) transcription factors with Ets domain proteins have been observed. Several Ets factors synergistically interact with ubiquitously expressed bHLH proteins (Dang et al., 1998; Rivera et al., 1993). Members of the ternary complex factor (TCF) subfamily of Ets proteins associate with the bHLH factor E47, which increases DNA binding (Maira et al., 1996), and with Id HLH factors, which results in the dissociation of the transcription factor complex (Yates et al., 1999). Moreover, cooperative interactions of leucine zipper-containing bHLH proteins occurs with Ets-1, Pu.1, and Pea3, and enhances DNA-binding and transcriptional activation (Greenall et al., 2001; Sieweke et al., 1998; Tian et al., 1999). Given the widely acknowledged roles of bHLH factors in neural development (Anderson, 1999; Brunet and Ghysen, 1999; Lee, 1997) it will be interesting to investigate whether Erm interacts and cooperates with bHLH proteins to regulate neuronal fate decisions in the developing neural crest.

6.4.2 Lineage-specific regulation of cell cycle progression by Erm

In contrast to neuronal fate decision, glial fate acquisition does not depend on Erm function since the frequency of glial clones generated from NCSCs was not decreased by expression of dominant-negative Erm. Furthermore, blocking Erm
function did not affect the expression of glial differentiation markers in neural crest-derived glia. Nevertheless, our clonal culture experiments suggest a specific role of Erm in the peripheral glial lineage. Clones expressing dominant-negative Erm contained considerably less glial cells than clones in control cultures. This was not due to selective elimination of glial cells, since cell death was neither increased in EnR-Erm-infected founder cells nor within infected clones. Therefore, our data indicate a role of Erm in regulating proliferation of glial cells.

Based on our data, activation of Erm in the peripheral glial lineage is required for mitogenesis. Interestingly, the mitogenic function of Erm appears to be specific for the glial lineage since, although fewer neuronal clones were generated upon EnR-Erm expression, these were not smaller than in control cultures. It is not clear whether Erm mediates the mitogenic activity of an extracellular signal in the glial lineage or whether Erm is part of an intrinsic program that ensures proliferation of neural crest cells adopting a glial fate, independent of an external stimulus. NRG1 type II (the NRG1 isoform also used in the present study) regulates Erm expression in presumptive satellite glia (Hagedorn et al., 2000) and might thus seem to represent a good candidate molecule to elicit Erm-dependent mitogenesis. However, NRG1 type II does apparently not increase the proliferation rate in cultured NCSCs (Shah et al., 1994). Rather, it instructs NCSCs to adopt a satellite glial fate in vitro (Hagedorn et al., 2000; Shah et al., 1994) and plays a role in survival of at least a subset of multipotent neural crest cells (Paratore et al., 2001). Intriguingly, both glial fate and survival are not affected by loss of Erm function. Furthermore, forced expression of dominant-negative Erm also decreased the proliferation rate in glial cells induced from NCSCs upon Notch activation. Hence, Erm regulates proliferation in a cell lineage-specific manner independent of the stimulus that promoted the generation of this lineage. This is also supported by our finding that the regulation of glial cell cycle progression by Erm is independent from the process of glial fate acquisition, since EnR-Erm not only impaired proliferation in neural crest cells undergoing gliogenesis but also in differentiating glia that have already adopted their fate.

That Erm is involved in regulating proliferation is interesting in light of the oncogenic potential attributed to several Ets domain transcription factors (reviewed in Dittmer and Nordheim, 1998). In particular, the Pea3 subfamily members Erm, Pea3, and ER81 are upregulated in mammary tumors overexpressing Neu/ErbB-2, a co-receptor of NRG1. Recent experiments using dominant-negative Pea3 revealed that these Ets factors are required for the generation of Neu/ErbB-2-positive mammary tumors (Shepherd et al., 2001). Likewise, activation of the Wnt/β-catenin pathway coincides with Erm and Pea3 upregulation in intestinal and mammary tumors (Crawford et al., 2001; Howe et al., 2001). Thereby, Pea3 factors might contribute to tumorigenesis by activation of target genes involved in enhanced invasiveness, suppression of apoptosis, and stimulation of proliferation (Dittmer and Nordheim, 1998). Evidence for a role of Ets factors in proliferation was provided by expression of dominant-negative mutants that suppressed mitogenic signaling by Colony Stimulating Factor-1 and Ras (Langer et al., 1992; Wasylyk et al., 1994). Furthermore, Ets-2 was shown to activate cdc2 and cyclin that are implicated in regulating progression through the cell cycle (Albanese et al., 1995; Wen et al., 1995).
Part III, Cellular function of Erm

Discussion

Finally, the Ets factor Fli regulates Erythropoietin-induced proliferation of erythroblasts, presumably by repressing transcription of the retinoblastoma (Rb) gene (Pereira et al., 1999; Tamir et al., 1999). Unlike Erm, which we showed to control proliferation without affecting survival or differentiation of glial cells, the capacity of Fli to induce proliferation was coupled with inhibition of both cell death and erythroblast differentiation. In sum, the combined data are consistent with a model in which Erm regulates cell cycle progression in normal development of specific cell lineages and in oncogenic growth of particular tumor subclasses.

6.4.3 Functional diversity of Erm in neural crest cell lineages

Although neural lineages derived from NCSCs share Erm expression as a common feature, our study indicates that Erm does not display equivalent cellular functions in these lineages. Rather, as mentioned above, Erm appears to be differentially required in a lineage-specific manner and plays a role in neuronal fate acquisition and glial proliferation. Moreover, the continuous expression of Erm in differentiated neurons (Hagedorn et al., 2000) is consistent with a function also at later stages of neuronal development that might be comparable to the role of the related factor ER81 in the formation of sensory-motor circuitry (Arber et al., 2000). The mechanisms by which Erm is able to acquire this functional diversity remain to be elucidated, but combinatorial interactions with regulatory factors are likely involved in modulating the biological activity of Erm (Li et al., 2000; Sharrocks, 2001; Sommer, 2001). Such interactions together with differential posttranslational modifications allow Ets domain transcription factors to act as downstream effectors of multiple signaling pathways and to control transcription of diverse sets of target genes (Wasylyk et al., 1998; Yordy and Muise-Helmericks, 2000). Ets-1 is able to interact with different leucine zipper-containing bHLH factors and selectively activates target gene expression in a tissue-specific manner, dependent on the combination of interacting partner molecules (Tian et al., 1999). In B-cells, functional synergy between a tissue-restricted and a ubiquitous bHLH protein is mediated by cooperation with an intermediate Ets domain transcription factor (Dang et al., 1998). Further functional diversity of Ets proteins is achieved by tissue-specific expression of splice variants or by expression of varying concentrations of the Ets factor (DeKoter and Singh, 2000; Iwamoto et al., 2000). In particular, similar to Erm in neural lineages, the Ets factor Pu.1 is differentially required for proliferation, specification, or differentiation in distinct myeloid lineages (DeKoter et al., 1998). In the neural crest, functional diversity of a given transcription factor is not without precedence, since we have recently demonstrated that survival and glial fate acquisition of NCSCs are regulated by an interplay of the transcription factor Sox10 with combinatorial signaling by the extracellular environment (Paratore et al., 2001). Similarly, we propose that Erm activity elicits distinct biological responses in neural crest cells dependent on the intracellular context of other regulatory molecules and the combination of extracellular signals acting on the cells.
7. Outlook Part I-III

7.1 In vivo fate mapping of Erm-positive progenitors

The presented data on Erm expression during neural crest development and its regulation by NRG1 is consistent with the idea that sensory neurons, presumptive satellite glia and Schwann cells might derive from a common Erm-positive neural crest precursor. Further, based on our data and data from others one might speculate that Erm-positive progenitors emigrate from the forming DRG and differentiate into Schwann cells along the peripheral nerves. So far, in vivo evidence for this hypothesis is still missing. However, the striking downregulation of Erm expression in Schwann cells allows us to investigate whether, in vivo, Schwann cells are derived from emigrated Erm-positive DRG progenitors by using the Cre/lox system. Thereby, depending on the promoter activity driving the Cre recombinase, gene sequences flanked by loxP sites can be deleted in a temporal and spatial fashion. After isolation of the Erm promoter (JA Hassell, in progress), mice should be generated that drive Cre recombinase under Erm regulatory sequences. Crossing this mice strain with reporter mice (R26R), persistent expression of Lac Z activated by the Cre-mediated recombination event should allow identifying descendants of Erm-positive progenitor cells in vivo (Soriano, 1999; Zinyk et al., 1998). This fate mapping approach might finally prove whether in vivo Schwann cells along the peripheral nerves are DRG-derived descendents of Erm-expressing cells or whether they arise from a different source.

7.2 The function of Erm during neural crest development in vivo

As already mentioned, it is possible that the Erm-EnR construct also represses the function of additional, yet unknown Ets domain transcription factors that might play a significant role in neural crest development. Therefore, it is reasonable to confirm and expand our in vitro results by additional in vivo experiments using mouse mutants. The early embryonic lethality of mice carrying a targeted deletion of Erm makes it a prerequisite to create conditional lineage-specific mouse mutants. The above mentioned Cre/lox system allows, by using different promoters, to address the biological function of Erm in different lineages and at different developmental stages. A promoter that is active in migrating neural crest would delete Erm function already at the onset of Erm expression. Therefore, the initial function of Erm during neural crest development can be investigated. For instance, mice expressing Cre recombinase under the control of the PLP promoter would provide a suitable tool to address possible roles of Erm in early fate decisions. Based on the activity of the PLP promoter in migrating neural crest, Erm would be deleted in multipotent progenitors and in presumptive satellite glia of the DRG from the beginning of Erm expression. Therefore, this system would allow us to answer
the question of whether Erm function is needed in order to establish an Erm-positive satellite glia state before becoming a Schwann cell and whether Erm is involved in neuronal fate acquisition.

7.3 Erm regulating proliferation

The reduced proliferation capacity that we observed within the glial lineage when suppressing Erm function is very interesting in respect to cancer progression where several Ets genes have been shown to be involved. Further, Shepherd et al. (2001) recently demonstrated that Pea3 subfamily genes are required for HER2/Neu mediated mammary oncogenesis. Our results indicate that Erm is required for proliferation of glial cells suggesting that Erm is involved in cell cycle regulation. Therefore, it would be very interesting to further address whether gain of function experiments affect cellular proliferation. However, our transgenic mice overexpressing Erm and in vitro gain of function experiments did not reveal any indication pointing towards a function of Erm in neural crest cell proliferation control. Furthermore, it is not very likely that glia and neurons of the PNS form tumors. Nevertheless, it remains to be investigated whether Erm function is involved in cell cycle control in other tissues than the neural crest where cancer is more likely to occur. Erm as well as the other two members of the Pea3 subfamily are expressed in a wide variety of different tissues such as mammary gland, kidney, thymus, thyroid gland, lung and many others.

7.4 Intrinsic differences between satellite glia and Schwann cells

The differential response of DRG-derived progenitors and Schwann cells to NRG1-treatment reveals intrinsic differences between presumptive satellite glia and early Schwann cells. At no developmental stage analyzed were Schwann cells competent to induce Erm expression upon NRG1-treatment. This is surprising regarding the fact that Schwann cells express the appropriate ErbB receptors for NRG1 signaling. Therefore, during development, Schwann cells seem to undergo an irreversible intrinsic switch, which further corroborates that neural crest derivatives change their sensitivity to specific extrinsic signals over developmental time. On the molecular basis, it remains a key question how this switch is accompanied. This issue might be addressed by gene chip experiments using microarrays. The isolation of DRG progenitor cells and early Schwann cells by staining for cell surface markers und subsequent FACS analysis should allow comparing the gene profiles of these two cell populations. Ideally, this analysis might lead to the identification of different signaling pathway components that are active in these two cell types. If not, candidate genes involved in early steps of gliogenesis can be identified. It is conceivable
that other yet unidentified Ets genes are involved in this glial maturation process.

7.5 Cooperative function of Erm with other transcription factors

It is a common feature of Ets transcription factors to modulate both DNA-binding and transcriptional activity by protein-protein interactions (Li et al., 2000; Sharrocks, 2001; Wasylyk et al., 1998). Therefore, Erm might synergizes with another transcriptional regulator to perform its function within the neuronal lineage. Particularly, ubiquitously expressed HLH proteins synergistically interact with Ets factors (Dang et al., 1998; Rivera et al., 1993). Our data suggest that BMP2 might induce expression of this yet unknown co-factor. Furthermore, Mash1, a prominent member of the bHLH transcription factor family, is induced by BMP2 signaling. Therefore, gain of function studies of Erm and Mash1 would indicate whether these two factors together are sufficient to promote neurogenesis in undifferentiated multipotent progenitors. However, such experiments are difficult to perform by either using double transfection experiments or double viral infection. Nevertheless, the cooperate action of different transcription factors would explain why Erm gain of function experiments did not reveal any phenotype. Additionally, data from the microarray experiments (described above) might be used for the identification of novel bHLH factors that are expressed in satellite glia. Otherwise, a more focused screen for Id HLH proteins or bHLH factors on satellite glia might be performed by RT-PCR.
8. Part IV

The cellular function of Sox10 in neural crest development

8.1 Introduction

8.1.1 Transcription factors regulating gliogenesis

A number of transcription factors involved in Schwann cell development have been characterized. The development of Schwann cells involves at least three major steps: the engagement of the neural crest cell into the Schwann cell lineage, which leads to the formation of Schwann cell precursors; the maturation of these precursors into embryonic Schwann cells; and finally the differentiation of the latter into the two Schwann cell types, myelinating and non-myelinating (Jessen and Mirsky, 1992). Putative genetic cascades controlling these developmental steps are not yet completely elucidated. The zinc finger transcription factor Krox-20 is expressed in Schwann cells and is required for the myelination of peripheral nerves. The differentiation of Schwann cells into myelin-forming cells depends on continuous signaling from the axon. Krox-20 appears to be a key component of the transduction cascade linking axonal signaling to myelination. In sensory ganglia, the microenvironment is capable of negatively regulating Krox-20, presumably by preventing the conversion of satellite glial cells toward a Schwann cell-like phenotype (Murphy et al., 1996). These data and data from the avian PNS supported the idea that different types of glia are derived from a common lineage and differentiate according to microenvironmental influences and that the conversion between the alternative cell types is possible when environmental signaling is modified. Besides Krox-20, the closely related Krox-24 (Egr-1) that binds to the same DNA target sequences as Krox-20 seems to be involved in gliogenesis. During embryogenesis, the two genes are expressed in a successive and mutually exclusive manner, Krox-24 being restricted to Schwann cell precursors and Krox-20 to mature Schwann cells. Both genes seem to play antagonistic roles during the development of the Schwann cell lineage. Their balance of expression might participate in the choice between myelinating and non-myelinating pathways (Topilko et al., 1994).

Pax-3, a member of the paired box gene family is expressed in Schwann cell precursors and appears to play a role in the maturation or survival of embryonic Schwann cells (Moase and Trasler, 1990; Kioussi et al., 1995). Microinjection experiments have suggested that Pax-3 might be involved in maintaining a non-myelinating phenotype in postnatal Schwann cells (Kioussi et al., 1995). Besides these three transcription factors, the POU domain protein Tst-1/Oct6/SCIP is likely to control important stages of Schwann cell development, including the initiation of myelination around birth. The fact that Tst-1/Oct6/SCIP is much more active in glia than in several other cell types has been taken as
evidence for the existence of accessory proteins in glia that modulate the activity of Tst-1/Oct6/SCIP in a cell-type specific manner (Monuki et al., 1993; Fig. 1).

Figure 1. Transcription factors expressed in early neural crest precursors and myelinating Schwann cells.

The spatial and temporal expression pattern of distinct sets of transcription factors defines lineage commitment, precursor to Schwann cell transition and terminal differentiation by regulating different sets of target genes. NC: neural crest cell, Pre: Schwann cell precursor, Em: embryonic Schwann cell, ProM: promyelinating Schwann cell, mSC: myelinating Schwann cell. (Figure from Wegner M., 2000)

In sum, many findings started to elucidate the development of the Schwann cell lineage and glial fate specification in the PNS, but several processes and cascades/regulators of transcription factors still remain obscure. In contrast to the enormous number of transcription factors that help to specify neuronal fate as described in the first part of my thesis, very little is known about the regulatory mechanisms involved in early gliogenesis of the peripheral nervous system when fate specification occurs. It remains to be shown whether bHLH factors are required for gliogenesis in the peripheral nervous system. In Drosophila, transcription factors other than bHLH proteins are implicated in early glial development such as the earlier mentioned gcm (reviewed in Granderath and Klämbt, 1999). Additionally, development of midline glia requires, among other genes, the activity of Dichaete encoding a Sox protein (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. These and other analysis in embryonic stem cells suggested that Sox proteins might function as cell type-specific accessory proteins for POU domain proteins (Yuan et al., 1995).
8.1.2 The HMG group transcription factor Sox10

Sox proteins are characterized by a DNA-binding domain with similarity to the high-mobility group (HMG) domain of the sex-determining factor SRY. So far, more than 20 members of the Sox gene family have been identified in mammals. These are involved in diverse developmental processes such as chondrogenic differentiation, sex determination or hematopoiesis (reviewed in Wegner, 1999). The consensus sequence for Sox proteins has been defined as the heptameric sequence 5'-(A/T)(A/T)CAA(A/T)G-3' (Harley et al., 1994) which is bound by the highly conserved HMG DNA-binding domain. Upon DNA binding the overall protein structure remains unaltered whereas the DNA is subjected to a large conformational change. DNA bound by any Sox factor is bent 70-85° (Connor et al., 1994; Pontiggia et al., 1994). This interaction in the minor groove is relevant, because most other transcription factors mainly target the major groove. Therefore, binding of Sox proteins to DNA in close proximity to other transcription factors is sterically possible. This led to the hypothesis that Sox proteins function as architectural proteins by organizing local chromatin structure and assembling biologically active multiprotein complexes (Werner and Burley, 1997; Wolffe, 1994). In addition, a number of Sox proteins act as classical transcription factors holding a transactivation domain and thereby transactivate individual downstream genes.

Initially, Sox10 was identified in a screen using degenerate primers corresponding to the HMG domain of Sox proteins on cDNA prepared from primary Schwann cell cultures (Kuhlbrodt et al., 1998b). Sox10 contains an open reading frame of 466 amino acids with a molecular weight of 56 kDa. It exhibits a predominant expression in glial cells of the nervous system, but is not able to reveal autonomous transcriptional activity (Kuhlbrodt et al., 1998b). Instead, Sox10 functions synergistically with the POU domain protein Tst-1/Oct6/SCIP with which it is coexpressed during certain stages of Schwann cell development. Sox10 also modulates the function of Pax3 and Krox-20. Sox10 synergistically activates Pax3, while Krox-20 is partially repressed as shown by a synthetic promoter construct (Kuhlbrodt et al., 1998a). Furthermore, a role of Sox10 in peripheral gliogenesis is supported by its capacity to regulate P0 gene expression (Peirano et al., 2000). Based on the weak transcriptional activity of Sox10 itself, Kuhlbrodt and colleagues proposed that transcription factors such as Tst-1/Oct6/SCIP, Pax3 and Krox-20 are able to regulate specific target genes in glial cells because of the presence of transcriptional modulators such as Sox10. Different combinations of stage-specific transcription factors using the same glia-specific modulator would mediate spatially and temporally unique gene activation events that catalyze glial development. The same transcription factors allow different modulators to target other genes in different cells, thus tailoring transcription factor function to the need of the cell. It remains still unclear whether the modulation involves direct protein-protein interaction or whether the three dimensional assembly on DNA is sufficient for activation of the promoter. Nevertheless, deletion of the DNA binding site on the promoter for one of the cooperating transcription factors diminished the synergistic activity (Ryan and Rosenfeld, 1997). Additionally, truncated Sox10 consisting only of the HMG domain is not sufficient to mediate synergy with Tst-1/Oct6/SCIP (Giese et al., 1992). Thus, Sox10 exerts its function as an architectural protein.
and mediates assembly of nucleoprotein complexes on promoter DNA most likely not only by DNA bending but also through highly specific protein-protein interactions.

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 a variety of 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 region 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 in Wegner, 2000). These symptoms are also found in human patients 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. In the next part of this thesis, I addressed the cellular function of Sox10 by fate analysis of Sox10 mutant cells in different contexts in vitro and in vivo. I demonstrate that Sox10 is not only required for the survival of multipotent progenitor cells, but also for glial fate acquisition. Moreover, haploinsufficiency of Sox10 affects fate decisions of neural crest cells in a context-dependent manner by altering their responsiveness to complex extracellular cues.
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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Published 2001 in:
Development \textbf{128}, 3949-3961
8.2 Results

8.2.1 Expression of Sox10 protein in neural crest stem cells

Previous *in situ* hybridization experiments 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 only for 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 dorsal root ganglia (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; Paratore et al., 2002). 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).
Figure 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, and 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.

8.2.2 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−/− 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−/− 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). In contrast, cell death was considerable in DRG of Sox10\(^{-/-}\) embryos and was increased 13 to 17 fold in mutant as compared to 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.

Figure 2. Sox10-dependent survival of undifferentiated postmigratory neural crest cells.
(A, B, C) Apoptotic cell death of undifferentiated postmigratory Sox10\(^{-/-}\) neural crest cells. Transverse cryosections through DRG from E11 embryos were triplelabeled 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\(^{-/-}\) (-/-) embryos. Each bar represents the number (mean +/− 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\(^{-/-}\) (-/-) 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 due to cell death of the founder neural crest cell. The data were obtained from 4 independent experiments counting 60 clones each. Note that cell death of wild type cells but not of Sox10−/− mutant cells was significantly reduced in the presence of NRG1.

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). In 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 a) NRG1 can indeed act as a survival factor for undifferentiated postmigratory neural crest cells and b) 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 prior to 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−/− 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−/− mutant cells, despite the presence of NRG1 (33% of all clones died as compared to 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−/− 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−/− 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.

8.2.3 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/- 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/- 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 and G), but instead adopted the morphology of non-neural cells (Fig. 3I and 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; Paratore et al., submitted).

Figure 3. Loss of glial potential in Sox10/- undifferentiated neural crest cells.

(A-J) Cells isolated from DRG of wild type or Sox10/- 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/- 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 4 independent experiments. 60 clones were scored per experiment.
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\(^{-/-}\) 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.

8.2.4 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 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\textsuperscript{+/−} 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).

Figure 4. Haploinsufficiency of Sox10 affects glial fate acquisition but not survival of neural crest cells.
Undifferentiated p75-positive cells isolated from DRG of wild type and Sox10\textsuperscript{+/−} mutant E13 embryos (A-D) were incubated at clonal density either in differentiation medium ("no add") or in differentiation medium supplemented with NRG1 ("+NRG1"). Wild type cells produced mainly O4-positive glia-containing clones (E). No neurofilament-positive cells were observed neither in wild type nor in Sox10\textsuperscript{+/−} clones (H, I, J). Sox10\textsuperscript{+/−} clones contained no O4-positive cells (F, G).

(N, O) Quantitative analysis (see legend to Fig. 3K). ‘Death’ indicates loss of clones. Note that glial fate specification is impaired in both conditions while cell death in Sox10\textsuperscript{+/−} mutant cells is similar to wild type cells. NRG1 is acting as a survival factor for Sox10\textsuperscript{+/−} mutant cells and wild type cells, independently of fate decisions. In both conditions a glial to non-neural cell fate switch was observed in the Sox10\textsuperscript{+/−} experiments. (N) represents the data of 4, (O) the data of 3 independent experiments. 50 clones were scored per experiment.
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+/- 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. 4 N, O).

Surprisingly, however, while their survival capacity was comparable to 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+/- 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+/- 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. Since cell death was not significantly increased in Sox10+/- cell cultures as 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, even in conditions which are gliogenic for wild type multipotent cells.

To investigate whether different glia-inducing growth factors elicit similar responses in Sox10+/- 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 to 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+/- 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+/- neural crest cultures.
8.2.5 Fate decisions of Sox10 mutant cells are context dependent: community effects promote neurogenesis and suppress a non-neural fate in Sox10 heterozygous mutant neural crest cells

The generation of a non-neural cell type by single Sox10 heterozygous mutant neural crest cells (Fig. 4) and by the surviving Sox10 heterozygous 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 heterozygous mutant postmigratory neural crest cells were incubated in differentiation medium, exactly as described above 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 seven days in these conditions, wild type postmigratory neural crest cells proliferated and differentiated to form arrays of O4-positive glial cells (Fig. 5A and 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). In contrast, few cells with glial morphology developed from Sox10 heterozygous 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 heterozygous mutant progenitors started to aggregate in high-density cultures (Fig. 5H and J), forming clusters of NF160-positive cells (arrow in Fig. 5G; insets in Fig. 5G and 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 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 heterozygous 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 heterozygous glial progenitor cells to differentiate, since p75 staining marking undifferentiated neural crest cells and glial progenitors was also drastically reduced (data not shown). Thus, a frequent, though not exclusive, fate adopted by Sox10 heterozygous mutant neural crest
cells in cultures allowing short range cell-cell interactions is neuronal while their wild type counterparts mostly generate glia.

Figure 5. Neurogenesis of Sox10$^{+/−}$ mutant cells in high-density cultures. Undifferentiated p75-positive cells isolated from DRG of wild type and Sox10$^{+/−}$ 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) points to a NF-positive sensory neuron present at time of isolation. Only few O4-positive glia were generated in Sox10$^{+/−}$ mutant cultures (arrow in E). Many mutant cells formed aggregates of NF-positive neuronal cells (arrow in G, H). Insets in G and 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, J).
8.2.6 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 spatio-temporal 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 in Adlkofer and Lai, 2000). Since 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 were 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).

Figure 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: 20µm.

(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).
8.3 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., 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 since, even when challenged with various
gliogenic conditions, surviving Sox10\textsuperscript{+/−} 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\textsuperscript{+/-} 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.

Figure 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. In
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 sum, extracellular signals influencing cell fate decisions are differentially interpreted
by postmigratory neural crest cells, depending on the cellular context and the level of Sox10.
8.3.1 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 spatio-temporal 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 in 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(-/-) 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-positivemenatal 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(-/-) 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, since clonogenic cultures of Sox10(+/-) 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.
8.3.2 Multipotent neural crest cells require Sox10 to adopt a glial fate

The lack of multiple differentiated neural crest-derived lineages in Sox10\(^{-/-}\) 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 in regulating fate specification processes, which would also lead to disturbed appearance of neural crest-derived lineages in Sox10\(^{+/+}\) 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\(^{-/-}\) 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\(^{+/+}\) 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\(^{-/-}\) and Sox10\(^{+/+}\) mutant cells indicate 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, since lack of the NRG1 receptor ErbB3 does not interfere with glial fate acquisition (Riehmacher 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
Part IV, Cellular function of Sox10

Discussion

crest development is apparently not affected by overexpression of Sox10, either in cultured rat neural crest stem cells (Paratore C. and Sommer L., unpublished) or in chicken embryos (Rohrer H., 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 glial cell missing (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 that 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.

8.3.3 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\textsuperscript{+/-} 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) that 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\textsuperscript{+/-} 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\textsuperscript{+/-} 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\textsuperscript{+/-} 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+/− embryos. There is ample evidence that the regulation of the correct neuronal cell number involves programmed cell death both in a target-dependent (reviewed in 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+/− 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 sum, 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 cross talk 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 dosage 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 patients heterozygous for a Sox10 mutation.
9. Part V

The cellular function of Sox10 in the enteric nervous system and in Hirschsprung disease

9.1 Introduction

The investigation on the cellular function of Sox10 in early fate specification and survival of neural crest cells encouraged us to address these topics in the development of the enteric nervous system. Furthermore, the Dom mouse that carries a spontaneous mutation in the Sox10 gene serves as a mouse model system for Hirschsprung disease characterized by enteric aganglionosis. Therefore, we wanted to investigate whether mice carrying a Sox10 targeted deletion are a suitable model for Hirschsprung disease. If so, what are the cellular mechanisms that result in Hirschsprung disease and the onset of a megacolon? Is it due to a defect in migration, proliferation, differentiation, or survival? These are the main questions that will be addressed in the following part of my thesis.

9.1.1 The enteric nervous system (ENS)

The enteric nervous system (ENS) is a very peculiar structure of the peripheral nervous system since the majority of the enteric neurons is not directly innervated by the brain or the spinal cord. Furthermore, the ENS consists of approximately 100 million neurons in higher mammals. These numbers roughly equals to the number of neurons in the spinal cord. Therefore, the ENS is also called ‘the second brain’. The ENS consists of a collection of interconnected ganglia (enteric ganglia) that are arranged throughout the gut wall in concentric rings (Furness et al., 1987). The main functions of the ENS are the peristaltic movements performed by contraction of intrinsic smooth muscle and the regulation of secretory glands. These activities are all performed without input from the central nervous system. A network of intrinsic sensory neurons and interneurons as well as exciting and inhibiting motor neurons form the peristaltic reflex circuit.

Neurons and glia that form the ENS arise from neural crest cells that emigrate from the neural tube. Neural crest cells from three axial levels of the neuraxis give rise to the derivatives that contribute to the ENS: vagal, rostro-truncal, and lumbo-sacral crest. The vagal crest emigrating from the hindbrain region is the main source for enteric neurons. After a long-lasting debate about the contribution of the sacral crest, chick-quail grafting experiments by Burns and Le Douarin (1998) unquestionably showed that sacral cells also contribute to the hindgut. However, these cells give rise to only 17% of enteric neurons in the distal hindgut, with their contribution decreasing rostrally to 0.3% in the rostral hindgut. The colonization of the gut by neural crest cells has been intensively investigated. These studies showed a unidirectional, rostral-to-caudal
colonization of the developing gut (Kapur et al., 1992; Young et al., 1998). During colonization, the most caudal cells are undifferentiated, whereas cells rostral to the wavefront are at various stages of differentiation. It has been suggested that differentiation of migrating cells into neurons and glia does not take place until they have reached their final destination. Therefore, it is a prerequisite that at least a subpopulation of cells remains undifferentiated until they reach their final position in the hindgut. Considering that these cells migrate from the hindbrain region to the hindgut along almost the entire length of the developing embryo, it is a key question how the process of remaining undifferentiated and producing the right number of differentiated cells, is regulated given that during migration the cells are exposed to many different signals provided by the microenvironment.

The microenvironment consists of neural crest-derived cells, other cells in the mesenchyme, and the extracellular matrix. The extracellular matrix components provide directional signals to migrating cells and, together with neighboring cells, supply signals for neural crest cell differentiation (reviewed in Camilleri, 2001). However, it remains to be investigated whether the crest cells are attracted by specific chemoattractants. So far, several molecules and signaling pathways that control proliferation and differentiation in the gut have been characterized. Most of these were identified by loss of function mutants where normal development of the ENS was impaired. Specifically, glial cell line derived neurotrophic factor (GDNF) and its receptor Ret are involved in ENS maturation. It belongs to a small family of closely related neurotrophic factors that also include persephin, artemin and neurturin (Lin et al., 1993; Kotzbauer et al., 1996; Milbrandt et al., 1998; Baloh et al., 1998). GDNF function is mediated by the GPI-anchored molecule GFRα1 and the receptor tyrosine kinase Ret whereas neurturin function is mediated by GFRα2 and Ret (Jing et al., 1996; Treanor et al., 1996). During colonization of the gut, stimulation of the vagal and sacral crest by GDNF is absolutely necessary for survival. If either GDNF (Pichel et al., 1996; Sanchez et al., 1996), Ret (Schuchardt et al., 1994), or GFRα1 (Enomoto et al., 1998) are deleted in developing mice, all mutants display almost identical phenotypes characterized by the lack of enteric neurons caudal to the stomach. Only a small region in the gut that is colonized by trunk crest is normally developed. In vitro experiments suggested that GDNF promotes survival, proliferation and differentiation of ENS precursors (Chalazonitis et al., 1997; Hearn et al., 1998). At later stages in development, GDNF loses its ability to promote proliferation, and acts only as a growth-differentiation factor for enteric neurons, not for glia. At this stage, other neurotrophic factors such as NT-3 exhibit mitogenic activity (Chalazonitis et al., 2001). Neurturin and GFRα2 null mutations only affect specific subpopulations of neurons in the intestine by showing reduced myenteric plexus innervation and abnormal gastrointestinal motility (Heuckeroth et al., 1999; Rossi et al., 1999). Therefore, neurturin seems to be required for the maintenance of enteric ganglia and acts as an important trophic factor for postmitotic enteric neurons (Heuckeroth et al., 1999; Rossi et al., 1999). This is consistent with the model that GDNF is crucial for the survival, proliferation and differentiation early in ENS development and that neurturin takes over as a survival factor at later stages. Therefore, neurotrophic factors seem to act in a stage-specific manner with non-overlapping functions in mammalian development.
Endothelin (ET)-3 and its ligand ETB represent an additional ligand/receptor combination with an important function in the development of the ENS. Initially, they were identified in two naturally occurring mutants: the piebald lethal mouse and the lethal spotted mouse, respectively (Lane, 1966). These mice lack their enteric neurons in the colon and rectum. Again, the effect of ET-3 on neural crest cells and their derivatives are stage-dependent. On early emigrating neural crest, ET-3 increases the proliferation rate and the generation of melanocytes. However, later in development the action on enteric cells is different. It does not promote but inhibits enteric neuronal development (Chalazonitis et al., 1997; Wu et al., 1999). Instead, development into smooth muscle is promoted, and muscle secretion of laminin-1, which is a powerful promoter of enteric neuronal development, is downregulated (Chalazonitis et al., 1997; Wu et al., 1999). It is likely that in vivo ET-3 that is produced by non-neural crest cells in the gut has multiple cellular targets such as the mesenchymal smooth muscle cells and the enteric crest.

9.1.2 Hirschsprung disease

As mentioned before, Hirschsprung disease (congenital megacolon or congenital aganglionosis) is characterized by absence of enteric ganglia from varying lengths of the terminal colon. The aganglionic segment of the bowel acts as an obstructive lesion so that the gut proximal to the aganglionic zone dilates because the peristaltic movements of the distal gut are impaired. This disease occurs with an incidence of 1 in 5000 births in humans. Many mouse models have been described, which are suitable models for Hirschsprung disease including mutations in the above-mentioned GDNF/ret/GFRα1-, neurturin/ret/GFRα2- and ET-3/ETB-signaling pathways. Additionally, many transcription factors were identified that play a key role in ENS development and maturation such as, among others: Mash1 (Lo et al., 1991; Lo and Anderson, 1995), Phox2a (Tiveron et al., 1996), Phox2b (Pattyn et al., 1997) and Sox10 (Kuhlbrodt et al., 1998b; Herbarth et al., 1998; Southard-Smith et al., 1998; Fig. 2).
Yet another mouse model for Hirschsprung disease, the Dom mouse, arose spontaneously at the Jackson Laboratory and was identified due to a white fur spotting (Lane and Liu, 1984). Often, Hirschsprung disease is accompanied by neural crest-derived melanocyte deficiencies (Hirschsprung/Waardenburg syndrome) indicating a multigenic neurocristopathy. Southard-Smith et al (1998) and Herbarth et al (1998) established that a spontaneous mutation leading to premature termination of the transcription factor Sox10 is responsible for the absence of neural crest derivatives in the Dom mice. The Sox10 open reading frame C-terminal to the HMG box is shifted by a single base insertion resulting in a protein in which the N-terminal DNA-binding domain is present, but the putative activation domain is replaced by a novel domain of 99 amino acids. It was suggested that the phenotype is based on a dominant-negative action of the Dom protein. Recently, Britsch et al. (2001) observed that heterozygous mice carrying a targeted Sox10 null allele reproduce those phenotypes observed in heterozygous Dom mice. Haploinsufficiency of Sox10 can cause pigmentation and megacolon defects (Waardenburg/Hirschsprung disease). However, the mechanism by which Sox10 haploinsufficient animals establish this disease is still unknown. In any case, all the above-mentioned mutations result in an absence of enteric ganglia at variable length of the gut. Some investigators claim that defective environmental factors such as the extracellular matrix, diffusible substances, or transmembrane factors initiate aganglionosis while others suggest a survival or migratory effect. Dom mice display a defective colonization of the gut by vagal neural crest. During embryonic stages E11-E13.5, a retarded colonization and a reduced amount of neuroblasts was observed (Kapur et al., 1996). First, Kapur et al (1996) concluded from their experiments using a chimera transplantation approach that the Dom mutation alters the microenvironment surrounding enteric neural crest such that the milieu is not conducive to complete intestinal colonization. Further, they claim that enteric neural precursors never colonize the Sox10(Dom)/Sox10(Dom) gut in vivo (Kapur, 1996). Nevertheless, transplantation of wild type enteric neural precursors into Sox10(Dom)/Sox10(Dom) mice revealed a normal and completely populated gut. In contrast, neurons were never observed when Sox10(Dom)/Sox10(Dom) mid- or hindgut grafts were transplanted without wild type gut cells. These latter grafting experiments supported the hypothesis that the microenvironment of Sox10(Dom)/Sox10(Dom) is not defective –as first suggested- but that Sox10(Dom)/Sox10(Dom) cells display a cell-autonomous phenotype. Furthermore, excessive cell death in early migrating vagal neural crest explains the absence of distal ganglia in homozygous mutants (Kapur, 1999). In summary, cellular mechanisms resulting in Hirschsprung disease still remain to be elucidated, especially focusing onto heterozygous mouse models. So far, the function of Sox10 in homozygous mutants is established as a survival factor for
the enteric neural precursors as well as for postmigratory undifferentiated progenitors (see previous part of this thesis). However, insight into Sox10 function in Hirschsprung disease has to be gained. Furthermore, the targeted mutant Sox10 mouse might be one of the first mouse models for Hirschsprung disease that displays the phenotype in the heterozygous state, as is the case in human patients.

9.1.3 ENS consists of multipotent neural crest stem cells

Enteric neural precursors are a migratory population of cells that remains to a certain extent its multipotency. *In vitro*, c-Ret-positive enteric cells from E14 rat embryos were plated clonally and their fate was analyzed. Unlike neural crest stem cells, these cells divided only sparetly and the developmental potential was highly restricted (Lo and Anderson, 1995). This favored a model of neural crest diversification by progressive restriction of the developmental potential involving the production of committed precursors. Other studies, however, have suggested that enteric neural crest cells maintain their multipotency during a considerable period of time after gut invasion. Back-transplantation experiments confirmed that the enteric neural crest cells maintain their multipotency and their migratory capability (Rothman et al., 1990; Rothman et al., 1993). Additionally, single enteric neural crest cells were transplanted into gut wall in organ culture and revealed multipotency, self-renewal capability and a highly proliferative behavior (Natarajan et al., 1999). Therefore, possible scenarios in Sox10<sup>+/−</sup> animals resulting in Hirschsprung disease are: the potential of the enteric neural crest is reduced/ altered, or the self-renewal capacity is impaired and the amount of undifferentiated progenitors is reduced, or the survival capacity of the cells is affected, or finally, the cells lose their migratory potential. I addressed these questions in this project using *in vitro* and *in vivo* approaches.

9.2 Results

9.2.1 Agangionosis in the hindgut of Sox10 heterozygous mutant animals

The phenotypes of Sox10(Dom)/Sox10(Dom) mice appear to be identical to the phenotypes observed in Sox10<sup>+/−</sup> mice. Additionally, Sox10(Dom)/Sox10(Dom) mice serve as a model system for Hirschsprung disease displaying aganglionosis or hypoganglionosis in the ENS. In order to investigate the ENS of Sox10<sup>+/−</sup>, we first performed *in situ* hybridization experiments using neuronal markers on cross sections of heterozygous Sox10 mutant mice at E17. This
analysis revealed that the ENS of Sox10\(^{+/−}\) mice is affected and that the neuronal markers c-Ret (Fig. 1A, B) and neurofilament (Fig. 1C, D) are absent in the hindgut. In contrast, the number of enteric ganglion cells appeared to be comparable in more proximal parts of wild type and heterozygous gut. Therefore, we suggest that these mice represent another valuable mouse model for Hirschsprung disease.

**Figure 1.** Sox10\(^{+/−}\) mice display aganglionosis in the hindgut and develop megacolon. In situ hybridization analysis on E17 cross-sections revealed that the gut of Sox10\(^{+/+}\) animals (+/+ ) is entirely innervated by neurons as assessed by c-Ret (A; arrows) and neurofilament (NF) (C; arrows) expression. In contrast, the neuronal markers c-Ret (B; open arrows) and NF (D) are absent in distal gut of Sox10\(^{+/−}\) animals (+/−) indicating that these animals completely lack enteric neurons in the hindgut. More proximal parts of the gut are normally innervated. Scale bars: 100 \(\mu\)m.

**9.2.2 Reduction of the progenitor cell number at early stages of ENS development in Sox10 heterozygous mutants**

Sox10 is expressed in multipotent neural crest cells but is downregulated as these cells adopt a neuronal fate. Therefore, the loss of neuronal cells in Sox10\(^{+/−}\) mice might be secondary to a defect in multipotent enteric progenitors. Therefore, the number of undifferentiated progenitor cells in the developing enteric nervous system was analyzed using erbB3 and Sox10 as progenitor markers. Sox10 transcripts are readily detectable by in situ hybridization experiments in migratory neural crest cells and in progenitors present in peripheral ganglia and nerves of Sox10\(^{+/−}\) embryos (Fig. 2A, B). In contrast, the hindgut of Sox10\(^{+/−}\) mutants at E13 was devoid of Sox10 (Fig. 2D) and erbB3 (data not shown) expression, suggesting that the distal part of the ENS is never colonized by any neural crest-derived progenitor cell. Thus, the absence of an ENS in the distal gut of Sox10-mutant animals correlates with the absence of progenitor markers in the hindgut of mutant animals at early developmental stages. Strikingly, although they were not completely absent as in the hindgut, progenitor cells were reduced in number in the duodenum and in the midgut of
mutant embryos, too. While normally the developing myenteric plexus of the proximal gut is outlined by multiple progenitor cells forming a continuous ring of Sox10 expression (arrow in Fig. 2C), Sox10-positive cells appeared sparsely distributed in the early ENS of the mutant midgut (Fig. 2D). Likewise, higher magnification of proximal gut sections hybridized with an ErbB3 riboprobe revealed that ErbB3 staining was confined to relatively few ENS progenitor cells in the mutant midgut, while many ErbB3-positive cells were detectable on wild type sections (arrows in Fig. 2E, F). Additionally, whole intestines of wild type or mutant embryos at different developmental stages ranging from E13 to E17 were dissociated and the number of multipotent enteric precursors marked by the low affinity neurotrophin receptor p75 and by Sox10 was quantified. Strikingly, the amount of progenitors was significantly reduced in heterozygous mutants at all stages examined. For instance at E17, the number was reduced by 50% (data not shown). Thus, these data confirm the results of our in vivo experiments and demonstrate that Sox10 haploinsufficiency leads to a reduced size of the multipotent progenitor pool in the gut.

Figure 2. The hindgut of Sox10+/− mutants is not colonized by enteric progenitor cells and the amount of progenitors in more proximal parts of the gut is reduced. In situ hybridization studies showed readily detectable Sox10 expression levels in dorsal root ganglia (DRG) and peripheral nerve of wild type (+/+ ) and mutant (+/-) embryos at E13 (A, B). nt, neural tube. In contrast, the hindgut (HG) of Sox10−/+ animals at E13 is devoid of Sox10 expression (D; open arrowhead). At this developmental stage, the wild type hindgut is colonized by Sox10-positive cells (C; arrowhead). At the level of duodenum and midgut (MG), the expression levels of the progenitor markers Sox10 and ErbB3 are reduced in the mutant (D, open arrow in F) as compared to the wild type (arrows in C, E). Note the unaltered expression levels of ErbB3 in the mutant gut epithelium (compare E and F). Scale bars: (B, D) 200µm; (F) 100µm.
The apparent aganglionosis in the hindgut and the reduced progenitor pool might be explained by a reduced survival capacity of Sox10<sup>+/−</sup> enteric cells. Such a phenotype was already observed in Sox10<sup>−/−</sup> and Sox10(Dom)/(Dom) animals. However, the analysis of TUNEL stainings on sections from E11 to E16 revealed no increased cell death in the enteric nervous system of Sox10<sup>+/−</sup> mutants (data not shown) suggesting that the observed phenotype in heterozygous animals cannot be explained simply by a reduced survival capacity of the migrating heterozygous precursor cells.

### 9.2.3 Enteric neural crest-derived cells lose their progenitor state and adopt a neuronal fate in Sox10-heterozygous mutant animals

Cell culture experiments had allowed us to demonstrate that, dependent on the extracellular context, Sox10<sup>+/−</sup> neural crest cells adopt different fates as do their wild type counterpart (Paratore et al., 2001). Thus, the phenotype in the ENS of Sox10<sup>+/−</sup> animals might also be explained by aberrant cell fate decisions. Therefore, fate mapping experiments should allow us to follow the fate of undifferentiated Sox10-positive cells. Sox10 mutant mice have been generated by insertion of lacZ reporter gene in frame of the Sox10 locus. Therefore, persistent expression of the β-galactosidase protein permits following the fate of former Sox10-positive cells. Comparing Sox10 expression of wild type and heterozygous animals at E13 (Fig. 3A, B) and E15 (Fig. 3D, E) reveals X-Gal staining at locations (Fig. 3C, F) where Sox10 expression as assessed by in situ hybridization has already been downregulated. Additionally, given the absence of cell death at all stages examined (data not shown), our data suggest that neither Sox10-expressing progenitor cells nor their derivatives were able to colonize the hindgut (Fig. 3C, inset).
Figure 3. *In vivo* fate mapping of mutant ENS progenitor cells.
In situ hybridization analysis on E13 and E15 midgut sections shows strong Sox10 staining in the wild type (A, D; arrows). In contrast, at E13 Sox10 mRNA expression is barely detectable in the heterozygous midgut (B; open arrow) and completely gone at E15 (E; open arrow). Note the Sox10 expression in more proximal regions of the gut (E, arrowhead). (C, F) Persistence of β-galactosidase expressed from the Sox10-locus allows fate mapping of mutant progenitor cells. Adjacent sections to (B) and (E) were stained for β-galactosidase and revealed prominent staining in the midgut (C; arrow) whereas the hindgut was devoid of β-galactosidase staining (inset in C). At E15, β-galactosidase staining was still present at sites (F; arrow) where Sox10 mRNA was not detectable (E, open arrow), indicating that cells derived from progenitors have survived but downregulated Sox10 expression. Scale bars: (C) 200µm; inset in (C) 100µm; (F) 400µm.

Based on our previously mentioned results obtained by analyzing the developmental potential of Sox10+/- cells, we hypothesized that the precursors of the ENS might differentiate into neurons instead of maintaining an undifferentiated multipotent state. To determine the nature of the enteric cells derived from mutant progenitor cells, we performed double labeling experiments, staining midgut sections of E15 embryos for β-galactosidase and neuronal markers by immunohistochemistry. During ENS development, the tyrosine kinase receptor c-Ret is first expressed by postmigratory enteric progenitor cells derived from p75/Sox10/ErbB3-positive neural crest cells and is later confined to the neuronal lineage (Lo et al., 1995). Confocal microscopy analysis of midgut sections at E15 revealed that virtually all mutant neural crest-derived cells expressing β-galactosidase also expressed c-Ret (Fig. 4A-C). In addition, enteric neuronal cells are marked by the neuron-specific protein PGP9.5 (Young and Newgreen, 2001). Strikingly, the vast majority of β-galactosidase-positive cells found in Sox10+/- mutant midgut coexpressed this neuronal trait (Fig. 4D-F). In contrast, cells expressing late neuronal differentiation markers such as neurofilament160 and peripherin were detectable much less frequently than β-galactosidase-expressing cells, both at E15 and E17 (data not shown). Thus, without leading to precocious appearance of fully differentiated neurons, Sox10 haploinsufficiency promotes mutant neural crest cells in the gut to lose their progenitor state and to preferentially adopt a neuronal fate.

Figure 4. β-galactosidase-positive cells adopt a neuronal fate in the Sox10+/- mutant ENS.
Confocal microscopy analysis of midgut sections at E15 revealed that many cells that are c-Ret positive as assessed by fluorescence in situ hybridization (A) are also positive for anti-β-galactosidase immunostaining (B). Moreover, the anti-β-galactosidase staining (E) co-localizes with the neuronal marker PGP9.5 in many cells (D). (C) and (F) represent the corresponding confocal overlays; the yellow color indicates double-positive cells. Single confocal planes are shown.
9.3 Discussion

To our knowledge, Sox10+/− mice represent the first animal model system for Hirschsprung disease that display enteric aganglionosis in the heterozygous state. Hence, this model is closest to the human patient situation and, therefore, it is a very valuable tool to address disease-associated questions. Past investigations focused mainly onto homozygous animals as, for instance, the Dom mice or c-Ret mutant mice. Sox10+/− mutant enteric cells are able to migrate into proximal portions of the intestine during early development. However, in contrast to wild type cells, mutant cells progressively lose their progenitor state and Sox10 haploinsufficiency promotes mutant neural crest cells in the gut to adopt a neuronal fate instead. This results in a depletion of the overall progenitor pool. As a consequence, the hindgut in the mutant cannot be colonized by progenitor cells, leading to aganglionosis of the terminal bowel.

In vivo fate mapping of Sox10-heterozygous mutant cells that express β-galactosidase from the Sox10 locus showed that the extent of β-galactosidase expression in the mutant is comparable to the expression domain of neural markers in the wild type, confirming our above-mentioned data that the progeny of Sox10-expressing neural crest cells are not eliminated by cell death. However, β-galactosidase-positive neural crest-derived cells fail to express the progenitor markers Sox10 and ErbB3, and the total number of undifferentiated enteric progenitor cells is substantially decreased in the mutant relative to the wild type gut. Instead, we observed expression of neuronal markers in the vast majority of mutant cells in the midgut. It is worth mentioning, though, that the total number of neurons in this area seems not to be altered as compared to wild type embryos, indicating that neuronal cell numbers are regulated by secondary, Sox10-independent mechanisms. In conclusion, this is consistent with our previously reported analysis of multipotent neural crest stem cells,
which -dependent on the cellular context- adopt an aberrant neuronal fate. The environment encountered by mutant progenitor cells in the gut is apparently not able to promote all fates appropriate for early ENS development, namely progenitor maintenance and neurogenesis (Kapur, 1999, Paratore et al., 2001). Based on these results, we propose a model wherein the progenitor pool that is necessary for complete colonization of the gut cannot be maintained in the Sox10+/− ENS. Further, it has been proposed that the migratory ability of enteric crest derived cells decreases with their progressive differentiation, and neuroblasts and neurons are believed to be more sessile than undifferentiated progenitor cells. Therefore, the generation of neuronal cells with the concomitant loss of the progenitor state in Sox10+/− neural crest cells would result in a depletion of cells with migratory potential. As a consequence, the hindgut becomes aganglionic because it is the last part of the gut to be colonized by neural crest-derived cells.

In sum, we further elucidated the cellular mechanism leading to the establishment of Hirschsprung disease and provide more insight into this complex disease. It would be very interesting to see whether transplants of wild type cells are able to rescue the phenotype and re-colonize the aganglionic part of the gut. At least in the Dom mice, wild type cells were able to colonize the Sox10(Dom)/Sox10(Dom) gut and to differentiate into neurons.
10. Outlook Part IV & V

10.1 Molecular basis of the community effect

Fate decisions in wild type and Sox10 mutant neural crest cells are influenced by short range cell-cell interactions. The molecular basis of signal integration from single cells versus a cellular community is still unknown and remains one of the most challenging questions in this field. One possibility is that this community effect might be mediated by short-range interactions through direct cell-cell contact. Further, we hypothesize that gap junctions might play a role in this cell-cell communication. There are indications from previous publications that gap junctions play different roles in neural crest development (Xu et al., 2001). Connexin 43 in gap junctions, for instance, has been shown to have an essential role in mediating functional coupling of neural crest cells (Radice et al., 2001). It is expressed abundantly in migrating neural crest cells and modulates the migratory behavior of these cells. In general, gap junctions can artificially be uncoupled by adding synthetic uncoupling reagents to the cell culture medium. Therefore, neural crest cells should be grown in cell communities and their gap junctions be uncoupled in order to see whether signal interpretation is altered. It might be that uncoupled cell communities interpret signals similarly to single cells. In vivo, mice carrying a targeted deletion of Connexin 43 might be analyzed. The advantage of an in vivo approach is that no synthetic uncoupling reagents, which often are toxic to the cells, have to be applied.

Although the experiments using Sox10+/− DRG progenitor cells were performed in high density cultures revealing a community effect, the cell density was by far not high enough for direct cell-cell contact. Therefore, I think it is very unlikely that only direct cell-cell interactions, if at all, mediate the community effect. I suggest that factors secreted by the cells themselves influence their further development. The concentration of this unknown soluble factor(s) might be too low in single cell cultures. Thus, I recommend to test whether conditioned medium from high-density cell cultures is able to mimic the community effect on single cell cultures. If so, the identification of this factor(s) by biochemical means would be very interesting.

10.2 Comparing the gene expression profiles of Sox10+/+ and Sox10+/− cells

Yet another approach for identifying intrinsic differences between heterozygous and wild type cells is gene profiling by microarray techniques. This new technique is, by choosing the right experimental setup, a very powerful tool for analyzing a large number of genes (Simon et al., 2002). Combined with an analyzing tool/software it might be capable to find even small differences of expression levels in components of signaling pathways and transcription factors.
when comparing the gene profiles of Sox10 heterozygous and wild type cells. Additionally, this might lead to the identification of yet unknown genes, which play an important role during development. Control and Sox10\(^{-/-}\) mice might be bred into reporter mice carrying GFP under the PLP or the nestin promoter, both of which are expressed in neural crest and its derivatives. Thereby, neural crest-derived migratory, pre- and postmigratory progenitors can be isolated by FACS analysis that allows the isolation of a relatively pure population of neural crest cells. Alterations in expression of downstream genes caused by gene ablation of Sox10 might explain how Sox10 haploinsufficiency leads to the phenotype of the mutant ENS. Furthermore, sets of genes (gene clusters) which are similarly regulated allow identifying signal transduction pathways and possible transcription factors modulated by Sox10 (see also below). Thereby, the Sox10 function in survival and glial fate acquisition of neural crest progenitors can be further clarified.

10.3 Identification of transcription factors modulated by Sox10 early in neural crest development

It has been reported that Sox10 modulates several different transcription factors during gliogenesis and melanocyte development (Peirano et al., 2000; Kuhlbrodt et al., 1998a). However, no partner of Sox10 has been described that could explain its early survival effect. We hypothesize that Sox10 might modulate the activity of different transcription factors early in neural crest survival and glial fate acquisition. Based on results from Drosophila where Sox proteins together with POU factors are involved in glial fate acquisition we think that Sox10 might also interact with a yet unknown POU factor. Therefore, we performed a RT-PCR based screen using degenerate primers directed against the highly conserved POU domain on cDNA derived from neural crest cells treated with NRG1. This treatment instructs undifferentiated cells to adopt a glial fate and thereby, they upregulate genes important for glial fate acquisition. The vast majority of the sequenced clones represented the POU factor Oct1. Oct1 has already been identified and described earlier (Dick et al., 1991; Zwilling et al., 1995) and is claimed to be an ubiquitously expressed transcription factor. However, its expression pattern has not been extensively analyzed on a cellular level. A control screen on cDNA generated from brain RNA resulted in the identification of a different POU factor, which demonstrates the specificity of our screening approach. Therefore, gain of function experiments overexpressing both Sox10 and Oct1 might be performed in undifferentiated neural progenitors in order to see whether these two factors are sufficient to induce a glial fate. If this is the case, Oct1 can conditionally be ablated in the peripheral nervous system and any role in gliogenesis addressed \textit{in vivo} and \textit{in vitro} (ongoing collaboration with Patrick Mathias). In parallel, an additional screen with newly designed primers should be performed for the identification of a POU factor with a more specific expression pattern in neural crest. A yeast two-hybrid screen might be an alternative for identifying partner proteins directly interacting with Sox10.
10.4 Mice carrying a conditional targeted deletion of the Sox10 gene

Mice that carry a conditional targeted deletion of the Sox10 gene might allow separating the early survival effect of Sox10 from later functions in neural crest development by controlling the cell type or the timing of recombination using Cre recombinase. Promoter regions from Oct6, P0 or Krox20 that are active during myelination are optimal to investigate whether Sox10 plays an additional, yet unknown role during myelination or in myelin maintenance. It was reported that Sox10 modulates transcription factors involved in the development of Schwann cells and in myelination such as Krox20, Krox24 and P0 (Peirano et al., 2000; Kuhlbrodt et al., 1998a). Therefore, it is very interesting to investigate the role of Sox10 after glial fate acquisition. Additionally, experiments involving in vitro myelination assays might complement this in vivo analysis. In sum, the different roles of Sox10 during neural crest development might possibly be dissected using distinct temporal and spatial targeted deletions. This is especially significant for a modulator protein such as Sox10, which seems to mediate different functions in combination with diverse partners at different developmental time points.

10.5 Clonal analysis of multipotent enteric precursor cells

In vitro, clonal analysis of dissociated enteric progenitor cells would further elucidate the defect in maintaining the progenitor pool or any fate specification problem of Sox10+/− mutant enteric cells. This kind of experiments allow investigating whether in the ENS, NCC proliferate via asymmetrical or symmetrical cell divisions and how or at which point in development the Sox10 mutation affects ENS development. So far, a cell culture system that allows maintaining mouse ENS progenitors at clonal density and that is permissive for multiple fates, has not yet been established. Therefore, I think it would be very important to set up such a culture system in order to address the above mentioned questions. Additionally, in vivo transplantation experiments might further clarify the developmental potential of Sox10-mutant cells.
11. Material and Methods

An important question studying gene function during development is the cellular expression of certain candidate genes. So far, this was a difficult task because radioactive and non-radioactive \textit{in situ} hybridization techniques may not allow resolution of mRNA expression at the cellular level. Furthermore, the assessment of cellular colocalization of different mRNA markers is hampered by the precipitate-forming chromogen that is used to visualize the transcripts in traditional \textit{in situ} hybridization techniques. To overcome these problems, we have developed a fluorescence \textit{in situ} hybridization method that allows to detect cellular coexpression of a given mRNA with cell type-specific mRNA or protein markers on embryonic sections as described below.

Embryonic Gene Expression Resolved at the Cellular Level by Fluorescence \textit{in situ} Hybridization

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Published 1999 in:
11.1 Abstract

Tyramide signal amplification has successfully been applied to enhance detection limits of both immunological reactions and in situ hybridization methods. The technique uses short-range deposition of activated tyramide mediated by horseradish peroxidase. We have adapted this method to fluorescence in situ hybridization on embryonic tissue sections using fluorophore-labeled tyramide. The sensitivity of the procedure was sufficient to analyze the embryonic expression of mRNAs encoding both transcription factors and structural proteins. Combining fluorescence in situ hybridization and immunofluorescence with confocal microscopy allows the simultaneous detection of distinct mRNA species or of mRNAs together with proteins on the cellular level. Thus, the cell types expressing a particular gene at a given developmental stage can be studied even if no antibody to the gene product of interest is available. Moreover, the technique allows to study in situ the combinatorial marker expression that characterizes progenitor stages of a given cell lineage.

11.2 Introduction

Databases of sequenced genes are currently expanding at high rates but the functions of most of these novel genes remain to be resolved. A first indication of potential gene function is given by the characterization of its expression pattern, in particular by the identification of the cell types expressing the gene. This task poses often considerable challenges to developmental biologists because different cell types cannot easily be distinguished by their morphology in early embryonic development. In the developing peripheral nervous system, for instance, different types of neuronal progenitors are intermingled with presumptive glial cells (Le Douarin and Dupin, 1993; Weston, 1991). Cell types expressing a given gene can be characterized by studying the coexpression of cell type-specific markers on dissociated cells. This approach can be limited, however, by the small size or the accessibility of the developing organs to be analyzed. For instance, forming dorsal root ganglia or sympathetic ganglia can barely be dissected and dissociated from very young embryos. If protein markers for the different cell types and developmental stages are established, coexpression with the gene product of interest can be addressed in situ by immunofluorescence using appropriate antibodies. Is this not the case, however, studying cellular expression is difficult because radioactive and non-radioactive in situ hybridization techniques may not allow resolution of mRNA expression at the cellular level. Furthermore, the assessment of cellular colocalization of different mRNA markers is hampered by the precipitate-forming chromogen that is used to visualize the transcripts in traditional in situ hybridization techniques.

To overcome these problems, we have developed a fluorescence in situ hybridization method that allows to detect cellular coexpression of a given mRNA with cell type-specific mRNA or protein markers on embryonic sections. This method is based on tyramide signal amplification (TSA; also called...
catalyzed reporter deposition (CARD)) that applies the short-range deposition of tyramide activated by horseradish peroxidase (HRP) conjugated to an antibody or to streptavidin (Adams, 1992; Bobrow et al., 1989). TSA has been successfully used to amplify antibody-mediated stainings on tissue sections and in cell culture (Ligterink et al., 1997; Sharp et al., 1995; Xu et al., 1996; Zou et al., 1994). In addition, the detection of faint immunoreactivity by electron microscopy (Sharp et al., 1995) and double immunofluorescence labeling using two antibodies raised in the same host species (Hunyady et al., 1996; Shindler and Roth, 1996) have been achieved using TSA. Ligand-HRP conjugates have been visualized by TSA to measure cellular uptake of transferrin (Strous et al., 1996), and TSA was used to detect DNA targets in interphase and metaphase chromosomes by in situ hybridization with DNA probes (Kerstens et al., 1995; Speel et al., 1997). TSA in situ hybridization has also been applied to localize mRNA in dissociated cells (de Haas et al., 1996) and to enhance the detection limits of mRNA expression in embryonic sections using a chromogen (Komminoth and Werner, 1997; Landry and Hokfelt, 1998). Moreover, TSA was adapted to analyze the ultrastructural localization of mRNAs by electron microscopy (Schöfer et al., 1997).

The method described in this report employs in situ hybridization in combination with the fluorescent reporters Cy3-tyramide and FITC-tyramide as substrates for HRP-mediated signal amplification. The procedure is fast, highly sensitive and reliable, and allows detecting the simultaneous expression of two distinct mRNAs or of a mRNA and a protein on a single tissue section.

11.3 Materials and Methods

11.3.1 cDNA and riboprobes

Partial mouse Erm cDNA was a gift from Amgen (Thousand Oaks, CA); murine Pea3 cDNA (Xin et al., 1992) was cloned by RT-PCR from four-week mouse brain cDNA using gene-specific primers: Pea3-3 (5’ primer: gcacgaattccTCAGAAGATCTCTTCCAGG) containing an EcoRI site and Pea3-4 (3’ primer: gcactctagaCGAGCGGCTCAGCTTGGTC) including an XbaI site. PCR amplification was performed (40 cycles; 1 min 94°C, 1 min 55°C, 1 min 72°C), and the PCR products were digested with the appropriate restriction enzymes and cloned into the Bluescript (pBS) SK- vector (Stratagene Cloning Systems). The neurofilament (NF160) probe was as described (Sommer et al., 1995). Riboprobes were fluorescein (Flu)- or digoxigenin (Dig)-labeled according to the manufacturer’s instruction (Boehringer-Mannheim). Riboprobes (800-2000bp) were hydrolyzed to fragment lengths of 300-400bp that was optimal to perform the in situ hybridization procedures described in this report.
11.3.2 Non-radioactive in situ hybridization

Non-radioactive in situ hybridization with Dig-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse embryos (Birren et al., 1993; Sommer et al., 1996). Briefly, mouse embryos were fixed in 4% paraformaldehyde/PBS for two hours, incubated in 30% sucrose and embedded in Tissue Tek OCT compound (Plano, Wetzlar, Germany). Twenty µm cryosections were collected on SuperFrost Plus slides (Merck) and warmed to room temperature. The sections were dried at 50°C for 15min and fixed in 4% paraformaldehyde in diethyl pyrocarbonate (DEPC)-treated PBS for 20min. After washing twice in DEPC-treated PBS for 5min each, sections were treated with Proteinase K (Boehringer Mannheim; 25µg/ml in 50mM Tris-Cl pH7.5, 5mM EDTA) for 5min. The slides were washed twice in DEPC-treated PBS for 7min each and refixed in 4% paraformaldehyde/DEPC-treated PBS for 15min. After washing with DEPC-treated PBS, the slides were incubated for 10min in 0.25% (v/v) acetic anhydride dispersed in 0.1M triethanolamine-Cl pH8.0, rinsed briefly and washed in DEPC-treated PBS for another 5min. The prehybridization was performed in hybridization buffer (50% formamide, 5xSSC, 100 g/ml heparin, 1x Denhardt’s solution, 0.1% Tween 20, 0.1% CHAPS, 5mM EDTA, 1mg/ml yeast tRNA) for 3 hours at 60°C followed by hybridization overnight at 60°C using a probe concentration of 1-2µg/ml. The washing procedure was as follows: 1xSSC at 60°C for 10min, 1.5xSSC at 60 C for 10min, twice in 2xSSC at 37°C for 20min. To reduce background due to non-hybridized RNA, sections were RNase-treated (0.2µg RNaseA in 2xSSC at 37°C for 30min), followed by washing twice in 0.2xSSC for 30min each at 60°C and twice in PBS/0.1% Tween20 for 10min at 60°C. Finally, the sections were washed in PBT (PBS, 0.1% Triton X-100, 2mg/ml bovine serum albumin) for 15min at room temperature. The sections were incubated in 20% heat-inactivated sheep serum in PBT for 1-5 hours followed by incubation with alkaline phosphatase (AP)-coupled sheep anti-Dig antibody (1:2000 dilution; Boehringer Mannheim) at 4°C overnight. The sections were washed three times in PBT for 30min each, followed by two washes in AP buffer (100mM Tris pH9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween20, 5mM levamisole (Sigma)). The chromogens NBT (330μg/ml) and BCIP (175μg/ml) (Boehringer Mannheim) were added as AP-substrates and the color reaction was performed in the dark for 12-20 hours. Before mounting in glycerol, sections were washed in PBS and fixed in 4% formaldehyde for 10min.

11.3.3 Direct tyramide signal amplification (TSA) to detect mRNA expression in situ

To analyze mRNA expression by TSA, the usual in situ hybridization protocol (see above) using either Dig-labeled or Flu-labeled riboprobes was applied with the following changes: Proteinase K treatment, refixation and acetylation were omitted; prehybridization and hybridization were performed at 65°C and the concentration of tRNA in pre- and hybridization buffer was 0.3mg/ml. Subsequent to hybridization, washing was carried out in 0.2xSSC for 15min at
65°C, followed by two consecutive washes (0.2xSSC) for 30 min at 65°C and two washing steps in PBT for 20min at room temperature. After the last washing step, the sections were treated for 15min with freshly prepared 1% H2O2 in methanol in order to block endogenous peroxidase activity and washed three times with TNT (0.1M Tris-HCl, pH7.5, 0.15M NaCl, 0.05% Tween 20) for 5min each. A blocking step with TNB (0.1M Tris-HCl, pH7.5, 0.15M NaCl, 0.5% blocking reagent; blocking reagent supplied by NEN Life Science Products) for 30min at room temperature was followed by a 30min incubation with HRP-coupled sheep anti-Dig antibody (Boehringer Mannheim) or with HRP-coupled sheep anti-Flu antibody (NEN Life Science Products), respectively, diluted at 1:100 in TNB. Thereafter, the sections were washed three times in TNT for 5min each. After rinsing the sections again twice in TNT, mRNA expression was detected with fluorophore-labeled tyramide prepared according to the manufacturer’s instructions (NEN Life Science Products). Incubation with fluorophore-labeled tyramide was for 5-12min, followed by three washing steps in PBS and mounting of the slides in AF1 (Citifluor).

11.3.4 Simultaneous detection of two distinct RNA transcripts

To visualize mRNA expression of two distinct genes, sections were hybridized simultaneously with a Flu-labeled riboprobe and a different Dig-labeled riboprobe (Fig.1A). The Flu-labeled probe was detected as described above. After the incubation with fluorophore-labeled tyramide (Cy3-coupled tyramide or FITC-coupled tyramide (NEN Life Science Products)) used to detect the Flu-labeled riboprobe, the sections were washed three times in TNT (5min each). To block the peroxidase activity of anti-Flu-HRP, the sections were incubated in freshly prepared 1% H2O2 in methanol for 20min and washed three times in TNT for 5min each. Visualization of the second, Dig-labeled probe was by a 30min incubation with HRP-coupled anti-Dig antibody diluted at 1:100 in TNB. After washing the sections three times in TNT for 5min each, the hybridized antisense RNA was detected by tyramide labeled with an appropriate fluorophore. Finally, the sections were washed three times in PBS and mounted in AF1. In some experiments, the Dig-labeled riboprobe was detected prior to visualization of the Flu-labeled probe. Samples were visualized by confocal microscopy (Leica) and image stacks were analyzed using Imaris and Selima image processing software (Bitplane AG, Technopark Zürich, Switzerland).
Figure 1: Schematic representation of double mRNA \textit{in situ} hybridization and mRNA hybridization combined with immunofluorescence using the TSA amplification system.

(A) Sections are hybridized with Flu- and Dig-labeled riboprobes. Anti-Flu antibody coupled to horseradish peroxidase (HRP) is used for detection of the first, in this example the Flu-labeled riboprobe. HRP activates Cy3-labeled tyramide that is deposited near to the enzyme site. Thereafter, HRP is blocked by incubation with H$_2$O$_2$ and the second, in this example the Dig-labeled riboprobe is detected by an anti-Dig antibody conjugated to HRP. After activation and deposition of FITC-labeled tyramide, the sections are mounted. It is also possible to first visualize the Dig-labeled riboprobe prior to the Flu-labeled riboprobe, or to first use FITC-labeled tyramide before applying Cy3-labeled tyramide (not shown). (B) Fluorescence \textit{in situ} hybridization, in this case using a Flu-labeled riboprobe, can also be followed by standard immunofluorescence to detect a protein marker. The stainings can be further amplified by using indirect TSA involving biotinylated tyramide and HRP-coupled streptavidin (SA). F = Fluorescein (Flu); D = Digoxigenin (Dig); T = tyramide; B = biotin.

11.3.5 \textit{Indirect amplification of hybridization signals using biotinylated tyramide}

The signal intensity obtained by the direct TSA \textit{in situ} hybridization method (see above) was further enhanced by the following modifications. Tissue sections were incubated with HRP-conjugated antibody (either anti-Dig or anti-Flu antibodies) and washed in TNT. Subsequently, the sections were treated with
biotin-labeled tyramide (prepared according to the manufacturer’s instructions; NEN Life Science Products) for 10min and washed three times in TNT for 5min each. The deposited biotin was detected by streptavidin-HRP (SA-HRP; NEN Life Science Products) used at a 1:100 dilution in TNB for 30min and washed again three times in TNT for 5min each. In cases of weak gene expression, a further level of amplification was achieved by an additional round of biotin-labeled tyramide/SA-HRP treatment. The SA-HRP was then used to deposit fluorophore-labeled tyramide for 5-10min. After washing in PBS, the sections were mounted in AF1.

11.3.6 Immunohistochemistry

To combine detection of mRNA with the analysis of protein expression (Fig.1B), immunofluorescence was carried out subsequent to the last washing step of the TSA in situ hybridization protocols. The sections were blocked for 30min in blocking solution (10% normal goat serum, 0.3% Triton X-100, 0.1% BSA in PBS) and then incubated for 2 hours with a mouse monoclonal anti-neurofilament (NF-160) antibody (1:100 dilution; Sigma). Detection was by Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) diluted at 1:100. After washing in PBS, the sections were mounted in AF1. Samples were analyzed by confocal microscopy as described above.

11.4 Results

The ets domain transcription factor erm is strongly expressed in the developing lung buds (Chotteau-Lelièvre et al., 1997). We compared erm expression in the lung buds of embryonic day (E) 12 mouse embryos by conventional non-radioactive in situ hybridization (Fig. 2A), by direct TSA in situ hybridization (Fig.2B) or by indirect TSA in situ hybridization (Fig.2C,D) using Dig-labeled riboprobes. In the direct amplification procedure, Cy3-tyramide (Schmidt et al., 1997) served as substrate (Fig.2B) while in the indirect method, two rounds of amplification using a combination of biotinylated-tyramide/SA-HRP followed by Cy3-tyramide were performed (Fig.2C). High expression levels of erm were detected both by the direct and by the indirect procedure although two rounds of amplifications (indirect TSA) enhanced the signal significantly. A ten-fold shorter exposure time was sufficient to achieve approximately equal signal intensity applying the indirect compared to the direct TSA method (data not shown). In situ hybridization with a sense erm probe did not reveal specific fluorescent signals neither by the direct procedure (data not shown) nor by the indirect TSA method (Fig.2D). However, the indirect TSA method resulted in the appearance of strong, dot-like signals (arrow in Fig.2C). Although they appeared to be specific, such signals masked single cell morphology on the microscopic level (compare Fig.2B and 2C) and hamper the interpretation when expression is studied at high magnification or by confocal microscopy (see below). In both,
the direct and the indirect amplification procedure extensive washing after incubation with conjugated HRP and after deposition of fluorophore-labeled tyramide was critical to minimize background labeling (data not shown).

Figure 2: Expression of the ets domain transcription factor erm revealed by different in situ hybridization protocols. Cryosection of mouse E12 lung buds were hybridized with a Dig-labeled antisense erm riboprobe and subjected to a conventional non-radioactive in situ hybridization protocol that visualizes mRNA expression by a chromogen (A). Alternatively, lung bud expression of erm was analyzed by the direct TSA amplification system using Cy3-coupled tyramide (B). Signal enhancement was achieved by the indirect TSA system using biotinylated tyramide and HRP-conjugated streptavidin (C). Two rounds of amplification yielded dot-like fluorescence stainings (arrow) that appear to be specific but mask to some extent the cellular morphology. Hybridization with the sense erm riboprobe followed by indirect signal amplification revealed no specific staining (D). Incubation with Cy3-labeled tyramide was for 10min. Scale bar: 25µm.

Our fluorescence in situ hybridization method is not restricted to the detection of erm mRNA but can be applied to various other riboprobes. The ets domain transcription factor pea3 is coordinately expressed in functionally related motor neuron pools and peripheral sensory neurons (Chotteau-Lelièvre et al., 1997; Lin et al., 1998). Pea3 mRNA expression was strong in subsets of motor neurons and relatively weak in subpopulations of sensory neurons by using conventional techniques (Fig.3A). Direct deposition of FITC-labeled tyramide revealed a similar pea3 expression pattern (Fig.3B). The relatively weak expression of pea3 in the dorsal root ganglia (drg) was amplified by one additional round of combined treatment with SA-HRP and biotinylated- and FITC-conjugated tyramide (Fig.3C). Further rounds of amplification were yielding stronger though specific signals but their dot-like appearance limited the gain of information (data not shown; see also Fig.2C). Signal strength was also influenced by the time during which activated tyramide was allowed to be deposited. To detect pea3 transcripts in motor neurons, incubation with FITC-
labeled tyramide for 5min was optimal. Extending this period to 10min enhanced the specific signal intensity but resulted again in strong, dot-like signals disturbing the delineation of cell morphology at the microscopic level. Transcripts expressed at lower levels than pea3 mRNA in motor neurons were best visualized by tyramide incubation for 10min. Incubation periods longer than 10min did not improve detection of hybridization signals (data not shown).

Figure 3: Detection of pea3 mRNA expressed in motor neurons and in dorsal root ganglia.
Staining of mouse E12 embryonic sections using a Dig-labeled riboprobe displays strong pea3 expression in a subset of motor neurons (mn) and a punctuate expression pattern in dorsal root ganglia (drg). (A) Non-radioactive *in situ* hybridization using a chromogen. The strong staining in motor neurons is reproduced by the direct TSA system (B). Incubation with FITC-labeled tyramide was for 5min. The weaker expression in drg is visualized by indirect TSA using one round of amplification by biotin-streptavidin complexes and incubation with FITC-labeled tyramide for 10min (C). Note the characteristic punctuate expression pattern of pea3 in drg. The control using a sense pea3 riboprobe is negative (D). The arrowheads in (B) and in (D) indicate autofluorescent blood cells. Scale bar: 75µm.

The availability of a sensitive *in situ* hybridization technique based on fluorescence allows to study cellular colocalization of distinct markers using confocal microscopy. Detection of a given mRNA is combined with the visualization of another mRNA or of a protein (Fig.1). The markers are distinguished by the use of distinct fluorophores in either fluorescence *in situ* hybridization or in immunofluorescence analysis. We established this experimental paradigm by investigating the cellular expression of pea3 mRNA in the developing neural tube of mouse embryos at E12. In agreement with Lin
et al. (1998), pea3 was expressed in a subset of neurofilament (NF160)-positive motor neurons while other neuronal cells were pea3 negative (Fig.4) as assayed by the direct TSA in situ hybridization technique. The filamentous staining as revealed by the anti-NF160 antibody (Fig.4B) colocalized with the cytoplasmic labeling of pea3 mRNA in pools of motor neurons (arrows in Fig.4A,B and C; Fig.4C is an overlay of A and B). Similar results were obtained when detection of pea3 mRNA (probe labeled with Dig-UTP and visualized by FITC-tyramide; Fig.4D) was combined with staining of neurofilament mRNA (probe labeled with Flu-UTP and visualized by Cy3-tyramide; Fig.4E) using the direct TSA in situ hybridization procedure. Overlay of the confocal micrographs revealed cellular coexpression of pea3 and neurofilament mRNAs in many motor neurons of the chosen field (arrows in Fig.4D,E and F). In double in situ hybridization, HRP is used to detect both transcripts (Fig.1). Thus, it is imperative to inactivate HRP visualizing the first mRNA before proceeding with the labeling of the second transcript. We controlled for efficient inactivation of HRP used to detect the first message, neurofilament, by omitting the incubation with anti-Dig antibody in the FITC-staining procedure detecting the second mRNA, pea3. No false-positive FITC-labeling was detectable (data not shown) indicating that the signals obtained by direct TSA double in situ hybridization (Fig.4D-F) were specific for neurofilament and pea3 mRNA, respectively.

Figure 4: Cellular colocalization of pea3 and neurofilament in motor neurons.
Sections through E12 ventral neural tube were stained for pea3 and neurofilament and subsequently analyzed by confocal microscopy. Single confocal planes are shown. The left column (A-C) represents mRNA hybridization of pea3 and immunofluorescence staining of neurofilament 160 protein (NF). Pea3 is detected by FITC-labeled tyramide (A) while NF-staining was carried out using a Cy3-conjugated secondary antibody (B). (C) represents the confocal overlay of (A) and (B). Yellow color indicates double positive cells (arrows in A-C) revealing individual motor neurons coexpressing pea3 and NF. The right column (D-F) displays a double in situ hybridization of pea3 and neurofilament (nf) mRNAs stained by the direct TSA system. Neurofilament transcripts were detected by Cy3-coupled tyramide that has been deposited for 10min (E), followed by labeling of pea3 mRNA with FITC-tyramide that has been incubated for 5min (D). The confocal overlay (F) demonstrates that in the chosen field, a pool of motor neurons is double positive for pea3 and neurofilament (arrows in D-F) while other neuronal cells are pea3 negative. Scale bar: 20µm.
11.5 Discussion

In this report, we describe a fluorescence *in situ* hybridization protocol that allows analyzing simultaneously the expression of distinct mRNA species on single embryonic tissue sections. In addition, the fluorescence *in situ* hybridization method can also be combined with immunofluorescence to study cellular colocalization of mRNA and protein *in situ*. Double non-radioactive *in situ* hybridization analysis has widely been used beforehand to investigate the embryonic expression of different genes. However, these studies were based on visualization of the mRNA species by different chromogens that result in distinctly colored precipitates at the site of expression. Although this method can be applied to reveal expression of distinct genes in complementary (i.e. non-overlapping) areas (Ma et al., 1997; Spassky et al., 1998), it hardly allows to elucidate cellular coexpression of markers since the colored precipitate representing one gene product masks the distinctly colored precipitate identifying another gene product if the genes are coexpressed in the same cell.

In addition, non-radioactive *in situ* hybridization methods using chromogens do not permit analysis by confocal microscopy that is, however, a prerequisite to attribute expression to a particular cell layer. We have carried out comparable experiments as presented in this study using conventional, chromogen-based *in situ* hybridization but were unable to assess whether or not a given neurofilament-positive cell indeed coexpresses a given ets domain transcription factor (data not shown).

Visualization by fluorescence permits to combine *in situ* hybridization analysis with confocal microscopy. To that end, it is necessary to amplify the fluorescent hybridization signal since the use of fluorescein labeled riboprobes alone or direct visualization of a riboprobe by a fluorophore-conjugated antibody is not sufficiently sensitive to detect mRNA species on embryonic cryosections (data not shown). We found that TSA using fluorophore-labeled tyramide is the method of choice to achieve the required sensitivity. Using this method, we have been able to reproduce the expression patterns of various mRNAs previously revealed by conventional non-radioactive *in situ* hybridization methods. Even after several rounds of amplification, the specificity of the hybridization signal was not significantly affected. However, in cases of mRNAs expressed at high levels, extensive amplification might yield very strong, dot-like signals that conceal single cell morphology at the microscopic level. Analysis of such highly amplified pea3 mRNA hybridization signals by confocal microscopy revealed fluorescence not only in the cytoplasm but also in the nucleus of some cells in the developing neural tube (data not shown). Whether these signals were specific due to exceptionally stable nuclear immature pea3 mRNA or caused by deposition of activated tyramide at a distance remains to be determined. Schöfer et al. (1997) reported that the distance of tyramide deposition increases with enhanced amplification of the tyramide reaction with a maximal signal scattering (as measured by electron microscopy) of 300nm. Diffusion of labeled tyramides by extensive signal amplification might thus affect the resolution of mRNA localization at the ultrastructural level but should still allow the interpretation of mRNA expression at cellular resolution.

There are several critical parameters affecting signal-to-noise ratio and signal intensity of fluorescence TSA *in situ* hybridization. Unspecifically bound HRP
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has to be thoroughly washed off before addition of tyramide in order to reduce background labeling. Likewise, extensive washing after deposition of fluorophore-labeled tyramide minimizes background staining. Amplification of specific signals can be achieved by either prolonged incubation time with activated tyramide, by one or several rounds of indirect amplification involving biotinylated tyramide, or by a combination of these methods. The optimal procedure has to be adapted for the specific riboprobe to be analyzed. It is conceivable that for a given riboprobe, areas with high transcript levels are best visualized by direct TSA using fluorophore-labeled tyramide (e.g. pea3 mRNA expression in motor neurons) while domains of weaker expression may require additional amplification steps (e.g. pea3 mRNA expression in dorsal root ganglia).

Another point to be considered is the level of autofluorescence of the tissue that is expressing the gene of interest. While the developing nervous system offers no obstacle in this respect, blood cells are highly autofluorescent rendering the analysis of blood cell markers by fluorescence in situ hybridization very difficult. Appropriate controls involving comparably labeled sense riboprobes are thus imperative. Furthermore, we recommend to determine first the overall expression pattern of a novel gene by conventional non-radioactive in situ hybridization followed by fluorescence in situ hybridization to study gene expression at the cellular level. Since the method is fast and reliable, controls and the appropriate TSA procedure are easily established.

In sum, fluorescence in situ hybridization analysis allows the characterization of cell types expressing a given gene at specific developmental stages. In the present study we revealed pea3 expression in a subset of neurofilament-positive motor neurons that could only be demonstrated previously by the availability of the corresponding antibodies (Lin et al., 1998). Such tools are often not available to analyze the initial expression pattern of novel genes. Fluorescence in situ hybridization thus opens new avenues to elucidate cellular gene expression during embryonic development. In addition, the characterization of combinatorial marker expression in progenitor cells will facilitate the analysis of cell lineage differentiation in vivo.
11.6 Materials and Methods (continuation)

11.6.1 Non-radioactive in situ hybridization using chromogens

Non-radioactive in situ hybridization with digoxigenin-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse embryos as described in Paratore et al. (1999; see above). Additional antisense riboprobes were as follows: partial mouse Erm cDNA was a gift from Amgen; ErbB3 riboprobe (a gift H. Werner, ZMBH, Heidelberg); 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); murine Ets-1 was cloned by RT-PCR from four-week mouse brain cDNA using gene-specific primers: Ets-5 (5’primer: cggtacctAGAAGCGGCCGCTGACATC) containing a BamH1 site (small letters); Ets-6 (3’primer: acgggtaccGGTGTATCCAGCAGGCT) containing a Kpn1 site. The cDNA was subjected to PCR (40 cycles; 1 min 94°C, 1 min 55°C, 1 min 72°C). The PCR products were digested with the appropriate restriction enzymes and cloned into the Bluescript (pBS) SK-vector (Stratagene Cloning Systems).

11.6.2 Cell culture

Rat:
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). 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 E14 or E16 embryos and dissociated by incubation in 0.25% trypsin (Gibco BRL), 0.3mg/ml collagenase type I (Worthington Biochemical) in Ca²⁺/Mg²⁺-free Hank’s balanced salt solution (Amimed) for 25min, followed by addition of 1/10 vol of FBS. The cells were centrifuged for 3min at 1800rpm, washed once in standard culture medium (Stemple and Anderson, 1992) and plated at approx. 300 cells (clonal density) or at 20’000 cells per 35 mm culture dishes (Corning) coated with 0.5mg/ml poly-D-lysine (pDL) (Roche Diagnostics) and 0.25mg/ml fibronectin (FN) (Roche Diagnostics). DRG cultures were maintained in standard culture medium. In some experiments, 1nM rhGGF2, a soluble NRG1 isoform (a gift from M.Marchionni, Cambridge NeuroScience) was added three hours after plating the cells; alternatively, cells were treated with standard culture medium supplemented...
with 10% FBS and 5µM forskolin (Sommer et al., 1995; Stemple and Anderson, 1992). In some experiments, 1.6 nM BMP2 (a gift from Genetics Institute, Cambridge MA) or 4 fM TGFβ1 (R&D Systems) were added three hours after plating the cells. Sciatic nerves from E14, E16 or E18 embryos were dissected and dissociated as in Morrison et al. (1999). Cells isolated from sciatic nerves were then centrifuged, washed and plated as described above for DRG cultures.

Mouse:
Time-matings were performed with mice heterozygous for a targeted deletion of Sox10 that have bred onto a C3HeB/FeJ background (Britsch et al., 2001). Neural crest cultures were performed from E9 embryos as previously reported (Stemple and Anderson, 1992) with modifications according to Hagedorn et al. (1999, 2000b). To assay for endogenous Sox10 expression, glial cells were generated by incubation of neural crest explants in differentiation medium (that contains 10% FBS and 5 µM forskolin) prepared as described in 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 in Hagedorn et al. (1999; 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 h after plating the cells.

11.6.3 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 TGFβ for 8 hours while control cells on sister dishes were maintained in standard medium. BrdU was added directly to the cultures (final concentration 10µM) 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
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(25µM) (Calbiochem) was directly added to the medium 30 minutes prior to the addition of TGFß. Cells were fixed after 24 hours.

11.6.4 Plasmids

EnR-Erm was constructed by fusing the engrailed repressor (EnR) domain sequence (a gift from A. Braendli, ETH Zurich, and R. Rupp, University of Munich) to the 5’ end of the sequence encoding the DNA-binding domain of Erm. The Erm Ets domain was PCR amplified from pSV-Erm (Defossez et al., 1997), introducing a stop codon at the 3’ end. This fragment was cloned together with EnR into pSE280 (Invitrogen) to generate pSE280-EnR-Erm. NLS-MT-EnR-Erm was subcloned into pBabe-EGFP (a gift from B. Amati, DNAX Research Institute, Palo Alto, CA) resulting in the retroviral construct EnR-Erm containing also the reporter gene EGFP. pSV-Erm and 3xTORU Luc were both gifts from J.-L. Baert and Y. de Launoit, Institut de Pasteur de Lille, and have been described in Defossez et al. (1997).

11.6.5 Transfection assays

HEK cells were maintained in DMEM supplemented with 10% fetal bovine serum. Sub-confluent cells were transfected in 6-well plates with SuperFect (Qiagen) according to the manufacturer’s instructions. Each dish was transfected with 0.1µg CMV-lacZ, 0.3µg 3xTORU Luc, 0.25µg pSV-Erm, and 2.75µg EnR-Erm or 2.75µg pBabe-EGFP. 48h post transfection the cells were harvested and the luciferase activity measured using LucLite Plus (Packard). To normalize for transfection efficiency, a CMV-LacZ plasmid was co-transfected and the β-galactosidase activity was determined using Tropix Galacto-Star kit according to the manufacturer's instructions (Perkin Elmer).

11.6.6 Cell cultures and retroviral infection

Neural crest cultures were performed from embryonic day (E) 10.5 embryos as reported above. One day after plating the neural tubes, the emigrated cells were replated onto 0.25mg/ml fibronectin (FN) (Roche Diagnostics) coated dishes. 14h later the cells were infected with control- or EnR-Erm-expressing retroviral supernatants. After 2.5h of incubation the medium was changed to standard medium (Stemple and Anderson, 1992) and the cells were allowed to recover for 30min. This infection procedure was repeated twice (Lo et al., 1997). After the last recovery step the cells were replated clonally onto 35-mm culture dishes (Corning) coated with 0.5mg/ml poly-D-lysine (Roche Diagnostics) and FN and cultured in SM. Another 18h later when transgene expression has taken place, infected and EGFP-positive single cells were mapped by inscribing a circle on the bottom of the cell culture dish. In some experiments, the standard
medium was supplemented with either 1nM rhGGF2, a soluble NRG1 isoform (a gift from M. Marchionni, Cambridge NeuroScience), 1.6nM BMP2 (a gift from Genetics Institute, Cambridge, MA) or Delta-Fc (a gift from G. Weinmaster, University of California Los Angeles) at the time point of labeling. Production and concentration of Delta-Fc was as described in Morrison et al. (2000).

11.6.7 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 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) (1:500 dilution; Sigma); rabbit polyclonal anti-NF160 (1:200 dilution); monoclonal anti-SMA (IgG) (1:400 dilution; Sigma); mouse anti-peripherin (IgG) (1:200 dilution; Chemicon International); monoclonal Sox10 antibody (1:3 dilution; Paratore et al., 2001); mouse monoclonal anti-human c-Myc antibody (9E10; 1:3 dilution; Developmental Studies Hybridoma Bank); rabbit anti-cow S100 (1:200 dilution, Dako). In order to perform GFAP staining a mixture of monoclonal anti-GFAP antibodies Ab-1 and Ab-2 (IgG) (NeoMarkers; each used at a 1:100 dilution) was applied for 1 hour at RT. Alternatively, the cells were fixed in 70% ethanol/50 mM glycine pH2.0 at -20°C for 30 min. Then they were washed with PBS and incubated in methanol for another 30 min.

After blocking, monoclonal anti-GFAP (1:200 dilution; Sigma) was applied for 2 h at RT. Rabbit anti-Oct-6/SCIP antibody (Zwart et al., 1996) (a gift from D. Meijer, Erasmus University, Rotterdam) was used at a 1:200 dilution in 10% goat serum, 1% Triton-X-100, 0.1% BSA in PBS for 45 min at RT. After primary antibody incubation, the labeling was visualized by incubation for 1h at RT with FITC-conjugated secondary antibodies (anti-mouse antibody, Vector Laboratories; anti-rabbit antibody, Jackson ImmunoResearch Laboratories) or Cy3-conjugated secondary antibodies (anti-mouse and anti-rabbit antibody, Jackson ImmunoResearch 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. For Mash-1 staining, 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 radish peroxidase (HRP)-coupled goat anti-mouse IgG (1:200 dilution; Pierce) and HRP development using diaminobenzidine (DAB) as substrate. Rabbit polyclonal anti-Erm antibodies (Janknecht et al., 1996) were affinity-purified using Reacti-Gel HW-65 according to the manufacturer’s
instructions (Pierce). Erm labeling (antibody dilution 1:200) was performed for 2 hours at RT, followed by incubation with biotin-SP-conjugated donkey anti-rabbit IgG antibody (Jackson Immuno Research Laboratories). Staining was visualized by the ABCComplex Kit (DAKO) with horseradish peroxidase (HRP) development using diaminobenzidine (DAB) as substrate.

11.6.8 TUNEL Assay and Immunohistochemistry

Cryosections were fixed in 2% PFA/PBS for 10min, washed three times 5min in PBS and blocked for 1h in 10% goat serum, 0.1% BSA, 1% Triton X-100 in PBS. The anti-p75 or anti-NF160 stainings were identical as described above. 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.
12. References


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13. Publications


14. Acknowledgements

I would like to thank Prof. Dr. Lukas Sommer for giving me the opportunity to carry out this work in his research group, for all his support and enthusiasm, and for teaching me how to think scientifically.

I am grateful to Prof. Dr. Ueli Suter for accepting me as a PhD student at his unit and for his continuous support over the years.

Further, I would like to thank Prof. Dr. Isabelle Mansuy for having accepted to be a co-referee of my PhD thesis.

Furthermore, I am very thankful to:

The entire Suter group and the other people from the IZB for the good working atmosphere and their support.

Many former and present lab mates from the E26 including all the diploma students.

My parents and my sister for their endless support.
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