Diss. ETH No. 15694

The Effect of Wnt/β-Catenin- and BMP- Signaling in Early Neural Crest Stem Cell Development

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

presented by
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September 2004

die Ehrlichen haben sich als korrupt erwiesen
die Biedermänner haben sich als Angeber erwiesen
die Vitalität erweist sich als Impotenz
Keuschheit erweist sich als triebübersteuert
Die Nüchternen haben sich als süchtig erwiesen
Die Verantwortung besassen erwiesen sich als verantwortungsscheu
Grossmut erweist sich als Kleinlichkeit
Disziplin erweist sich als Konfusion
Wahrheitsliebe hat sich als verlogen erwiesen
Furchtlosigkeit erweist sich als Feigheit
Und Gerechtigkeit als Grausamkeit

(Aus Helmut Heissenbüttel, das Textbuch, 1970)

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1. SUMMARY

The regulation of cell diversification is a central issue in developmental biology. The neural crest is an attractive system for investigating the mechanisms underlying such cell lineage specification in higher vertebrates. Neural crest stem cells (NCSCs) are defined as multipotent, self-renewing undifferentiated cells that can give rise to one or more classes of neurons as well as nonneuronal cell types (Stemple and Anderson, 1992). The fact that neural crest cells are initially multipotent and gradually undergo restrictions in their developmental potential implies influences of environmental signals during migration and at sites of differentiation. Understanding the regulatory interactions between extracellular signals and intracellular programs that control determination and differentiation processes represent a challenging question in developmental biology. A great deal of interest has focused on the potential therapeutic applications of neural stem cells which requires however, a better understanding of fundamental biological properties of these cells.

In the first part of my thesis, I describe a new role of the signaling protein Wnt, which has recently emerged as a key factor in controling stem cell expansion. In embryonic stem cells, haematopoietic stem cells and neural stem cells Wnts act mitotically on progenitor cells, which leads to amplification and self-renewal of the stem cell pool. To test whether Wnts are general stem cell growth factors we addressed the role of Wnt signaling in NCSCs. In contrast to previous studies, we were able to show that Wnt signaling in early NCSCs has little effect on the population size and instead regulates fate decisions. Sustained β-catenin activity in NCSCs promotes the formation of sensory neural cells *in vivo* as *in vitro* at the expense of all other neural crest derivatives. Thus, manipulation of a single signaling pathway in NCSCs is sufficient to influence the fate of virtually the entire population. Moreover, Wnt1 is able to instruct early NCSCs (eNCSCs) to adopt a sensory neuronal fate in a β-catenin-dependent manner, emphasizing a cell-type dependent role of Wnt/β-catenin in stem cells (Lee et al., 2004).

Further, I review the context-dependent role of canonical Wnt signaling in stem cells and discuss the different biological respones of Wnts in stem cells, which I subsequently summarize in three models (Kleber and Sommer, 2004).

In the second part of my thesis, I demonstrate a new role of the bone morphogenic protein (BMP) signaling factor in early neural crest development. The BMPs mediate a diverse array of developmental processes including survival, proliferation, morphogenesis, lineage commitment, inhibition of alternate lineages, differentiation and apoptosis (Hogan, 1996b; Mehler et al., 1997). In neural crest development BMPs have been shown to induce neural

crest in combination with Wnts (Bronner-Fraser, 1998; Bronner-Fraser, 2002; Garcia-Castro et al., 2002). In NCSCs, BMPs are modulated by community effects and induce autonomic neurogenesis *in vitro* (Anderson, 1993; Anderson, 1997; Anderson, 2000; Hagedorn et al., 2000a; Shah et al., 1996; White and Anderson, 1999; White et al., 2001) as well *in vivo* (Reissmann et al., 1996; Schneider et al., 1999). We now demonstrated that *in vitro* BMPs in combination with Wnts influence early NCSCs already as they emigrate of the neural tube. The combinatorial activity of BMPs and Wnts regulate the maintenance and early lineage segregation in NCSCs. Those eNCSCs, which do not encounter BMPs, aggregate and form sensory ganglia, while those who encounter BMPs and Wnts at the same time point remain multipotent. We show for the first time maintenance of a pure neural stem cell population over prolonged time. Moreover over time, maintained NCSCs loose their reponse to the sensory neuron-inducing activity of Wnt. Our findings answer the debated question of when and how lineage segregation between sensory and other neural crest derivatives in NCSCs occurs and is regulated. Our data also explain why autonomic neurons are not generated when NCSCs migrate out of the neural tube and encounter BMPs (Anderson, 2000). We further demonstrate that canonical Wnt activity decreases at sites of postmigratory NCSCs *in vivo* and demonstrate *in vitro* that postmigratory NCSCs are not Wnt-responsive anymore.

2. ZUSAMMENFASSUNG

Die Regulierung der zellulären Verschiedenartigkeit beinhaltet eine zentrale Frage in der Entwicklungsbiologie. Die Neuralleiste bietet ein attraktives System um jene Mechanismen der Zelllinienspezifizierung in höheren Organismen zu untersuchen. Neuralleisten-stammzellen sind als multipotente, selbsterneuernde und undifferenzierte Zellen definiert und können mehr als eine Klasse an Neuronen sowie Nicht-neuronale Zellarten generieren. Die Tatsache, dass Neuralleistenzellen anfangs multipotent sind und sukzessiv ihr Potential verlieren, lässt auf den Einfluss von Faktoren aus der näheren Umgebung schliessen, denen die Stammzellen während der Wanderung zu den Zielorganen ausgesetzt sind. Das Verständnis über extrazelluläre Faktoren, die intrazelluläre Programme zur Zell-differenzierung beeinflussen, ist eine herausfordernde Fragestellung in der Entwicklungs-biologie. Ein breites Interesse konzentriert sich mehr auf mögliche therapeutische Anwendungen von Neuralleistenstammzellen, welches jedoch ein besseres Verständnis von den grundlegenden biologischen Eigenschaften solcher Zellen voraussetzt.

Im ersten Teil meiner Doktorarbeit beschreibe ich eine neue Rolle des Signalfaktors Wnt, welcher unlängst eine Schlüsselrolle in der Stammzellentwicklung eingenommen hat. In embryonalen, hämatopoetischen sowie Stammzellen des Zentralnervensystems stimuliert Wnt die Zellteilung der Vorläuferzellen, welches zur Ausdehnung und Selbsterneuerung des Stammzellpools führt. Um herauszufinden, ob es sich bei Wnt um einen generellen Wachstumfaktor für Stammzellen handelt, analysierten wir die Funktion des Wnt Signals in Neuralleistenstammzellen. In früheren Studien konnten wir zeigen, dass das Wnt Signal in sogenannten frühen Neuralleistenstammzellen keinen Einfluss auf deren Vermehrung hat, sondern stattdessen entscheidend für die Entstehung bestimmter Nervenzellen ist. Die aktivierende Mutation in *β-Catenin* fördert die Entwicklung sensorischer Neuronen *in vivo* als auch *in vitro* auf Kosten der anderen auszubildenden Neuralleistenderivate. Auf diese Weise konnten wir zeigen, dass die Manipulation eines einzigen Signaltransduktionsweges in den Neuralleistenstammzellen ausreicht, um das Schicksal einer nahezu gesamten Zellpopulation zu beeinflussen.

Des weiteren, fasse ich in einem rückblickenden Artikel die zusammenhängende Rolle des kanonischen Wnt Signalweges in Stammzellen zusammen und zeige dessen verschiedenartige biologische Reaktionen in drei Modellen.

Im zweiten Teil meiner Doktorarbeit, demonstriere ich eine neue Rolle des Signalfaktors Bone Morphogenic Protein (BMP) in der frühen Neuralleistenentwicklung. Die BMPs steuern eine Vielzahl von Entwicklungsprozessen, wie zum Beispiel das Überleben, die Zellteilung,

die Morphogenese, die Regulierung der Zelllinien, sowie die Hemmung zur Entstehung alternativer Zelllinien, Differenzierung und Zelltod. In der Neuralleistenentwicklung konnte gezeigt werden, dass die BMPs in Kombination mit den Wnt Molekülen eine Induktion von Neuralleistenstammzellen auslösen. Hingegen generieren die Neuralleistenstammzellen, sowohl *in vivo* als auch *in vitro*, unter Einfluss der BMPs autonome Neuronen und zeigen die sogenannten community effects. Zusätzlich konnten wir jedoch mittels *in vitro* Experimenten zeigen, dass die BMPs, in Kombination mit den Wnt Molekülen, schon zum Zeitpunkt der Auswanderung aus dem Neuralrohr die Neuralleistenstammzellen beeinflussen. Dieses Zusammenspiel von BMP und Wnt reguliert so die frühe Aufspaltung von Neuralleistenstammzellen in diverse Zelllinien. Diejenigen frühen Neuralleistenstammzellen die nicht auf BMP Moleküle während der Wanderungsphase stossen, aggregieren und bilden sensorische Ganglien aus, während unter Einfluss von BMP und Wnt die frühen Neuralleistenstammzellen ihren Stammzellcharakter beibehalten. Zum ersten Mal konnten wir zeigen, wie man Neurale Stammzellen homogen und undifferenziert über längere Zeit beibehalten kann. Zudem verliern die beibehaltenen Neuralleistenstammzellen über Zeit die Fähigkeit mit Wnt sensorische Neuronen zu bilden. Unsere Resultate geben Klarheit über die Fragen und Diskussionen zu welchem Zeitpunkt und unter welchen Umständen die Aufspaltung in die sensorischen und anderen Neuralleistenderivate stattfindet und wie sie reguliert wird. Unsere Daten klären sogar, warum keine autonomen Neuronen schon bei der Emigration der Neuralleistenstammzellen aus dem Neuralrohr, trotz BMP Präsenz, gebildet werden. Des weiteren konnten wir zeigen, dass die kanonische Wnt Aktiviät in Regionen der postmigratorischen Neuralleistenstammzellen abnimmt und auch keine Reaktivität auf Wnt vorhanden ist.

3. INTRODUCTION

The vertebrate peripheral nervous system (PNS) arises from multipotent embryonic stem cells, the so-called neural crest stem cells (NCSCs), which are capable of producing diverse cell types upon differentiation. The neural crest is a transient population of multipotent precursor cells named for its sites of origin at the crest of the closing neural folds in the vertebrate embryos (LaBonne and Bronner-Fraser, 1999). Following neural tube closure, these cells become migratory and populate diverse regions throughout the embryo where they give rise to most of the neurons, glia cells, pigments cells, smooth muscle, craniofacial cartilage and bones. Interestingly, the neural crest is regionalized such that cells derived from different axial levels follow distinct migratory pathways and give rise to a stereotyped set of derivatives (Le Douarin and Kalcheim, 1999). The specification of fate in multipotent progenitor cells is a critical process in the development of an organism. Stem cells from the mammalian neural crest provide an ideal model system for understanding the control of self-renewal and differentiation because of their broad potential and accessibility to manipulation in clonogenic culture. It has been shown that the fate of NCSCs can be challenged *in vitro* by instructive extracellular signals such as bone morphogenic proteins (BMPs), which promotes autonomic neurons and neuregulin (NRG), which promotes glial differentiation. The factors that control the plasticity and sensitivity of NCSCs to their local environment originate from cellular sources within the embryo. At present, a few candidates are identified, but understanding the mechanisms responsible for the specification, guidance and differentiation of the neural crest remains a challenging question in developmental biology.

3.1 The Origin of Neural Crest

In the vertebrate embryos neural crest cells emerge from the dorsal neural tube and migrate along pathways that are characteristic of their axial level of origin, with cells from different axial levels populating different derivatives (Bronner-Fraser and Fraser, 1991; Le Douarin, 1986). Prior to the formation of the vertebrate nervous system three main cell layers have been generated. The *endoderm*, the innermost layer, give rise to the gut, lungs and liver; the *mesoderm*, the middle layer, gives rise to connective tissues, muscles and vascular system; and the *ectoderm*, the outermost layer, gives rise to the major tissues of the central and the peripheral nervous system. During neurulation the ectoderm becomes subdivided into three embryonic tissue types: the neural tube, the prospective neural crest cells and the epidermis (Figure 1).

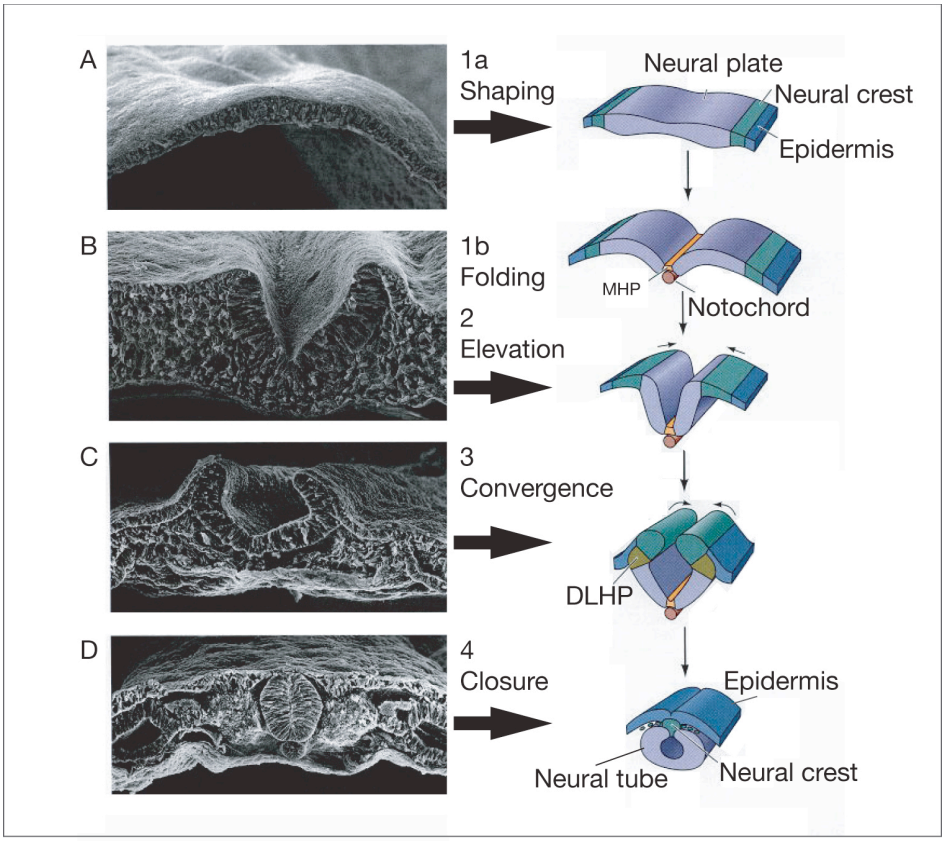

Figure 1: Formation of the neural tube.

Neural tube formation in the chick embryo. **(A, 1a/b)** Cells of the neural plate can be distinguished as elongated cells in the dorsal region of the ectoderm. Folding begins as the medial neural hinge point (MHP) cells anchor to notochord and change their shape, while the presumptive epidermal cells move towards the center. **(B, 2)** The neural folds are elevated as presumptive epidermis continues to move toward the dorsal midline. **(C, 3)**. Convergence of the neural folds occurs as the dorsolateral hinge point (DLHP) cells become wedge-shaped and epidermal cells push toward the center. **(D, 4)** The neural folds are brought into contact with one another, and the neural crest cells link the neural tube with the epidermis. The neural crest cells then disperse, leaving the neural tube separate from the epidermis (adapted from Gilbert Scott F., Developmental Biology; 2003).

The epidermis will eventually cover the entire surface of the embryo and contribute to the skin, while the neural tube and neural crest together give rise to most of the nervous system. Cells of the neural tube develop into neurons and glia of the central nervous system (CNS), whereas neural crest cells differentiate into the sensory neurons, postganglionic autonomic neurons and Schwann cells of the peripheral nervous system (PNS), as well as pigment cells, catecholamine-secreting cells of the adrenal medulla and some cranial cartilage (Le Douarin, 1982). As neurulation proceeds the edges of the neural plate approximate and fuse, and the neural tube separates from the overlying epidermis. Neural crest cells have been shown to arise from the neural folds and subsequently exit from the dorsal neural tube (Bronner-Fraser

and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Serbedzija et al., 1989), from which they migrate along characteristic pathways towards their target sites. The caudal region of the neural tube gives rise to the spinal cord, and the rostral region expands to form the brain. Initiation of neural crest cell migration proceeds in a head-to-tailward (rostrocaudal) wave in a highly patterned fashion through neighboring tissues. They localize in diverse but characteristic sites within the embryo and give rise to various derivatives of the PNS (Figure2).

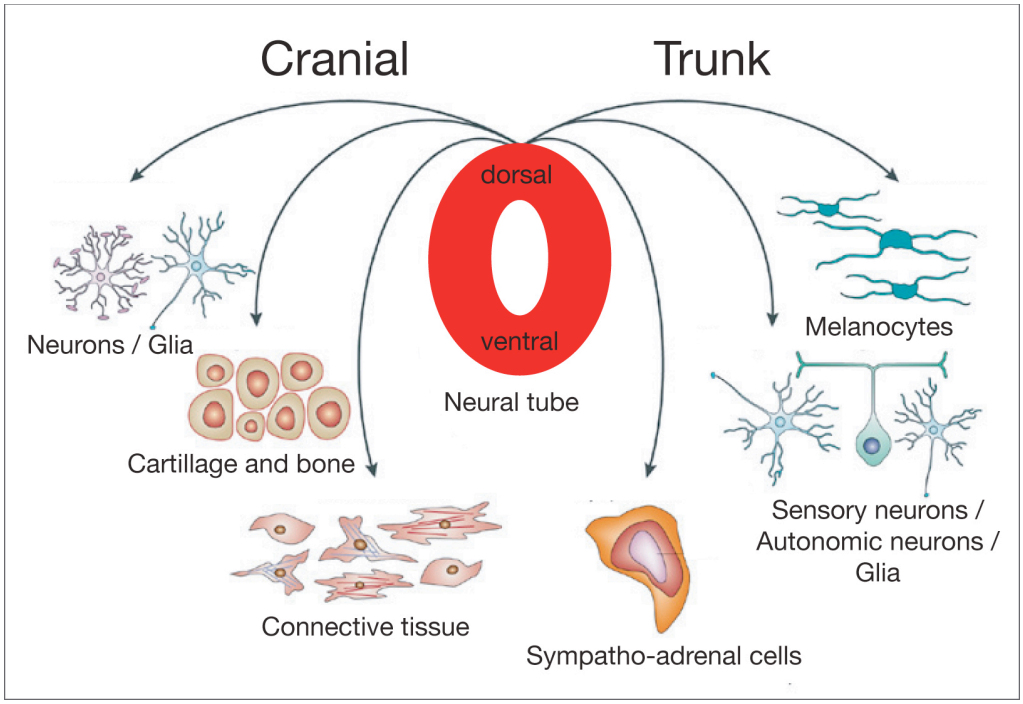

Figure 2: Derivatives of neural crest cells

Neural crest cells migrate throughout the body and differentiate into many different cell types. Although neural crest cells are pluripotent, differences exist between cells that are generated from different anteroposterior levels: neural crest cells in the trunk form melanocytes and several neuron and glia cell types, whereas neural crest cells in the cranial (the embryonic head region) also have the potential to form mesenchymal derivatives, such as cartilage, bone and connective tissue (adapted from Knecht and Bronner-Fraser, Nature Reviews, 2002).

What are the mechanisms underlying the generation of neural crest? When do the ectodermal lineages segregate? Fate mapping and grafting experiments revealed that juxtaposition of non-neural ectoderm and presumptive neural plate induces the formation of neural crest cells. The commitment of ectodermal cells to a neural/neural crest fate is still in progress at relatively late stages of development, at levels of open neural plate. The segregation of the epidermal lineage from the neural tube/neural crest lineages occurs around the time of neural tube closure. However, the finding that both neural crest and neural tube derivatives often arise

from a single precursor in the dorsal neural tube supports the idea that the neural crest cells are not a segregated population within the neural tube. Rather, there appears to be a common lineage for neural crest and CNS cells (Selleck and Bronner-Fraser, 1995).

3.2 Neural Crest Induction

Induction of the neural crest appears to be a multiphasic process and involves a combination of several instructive signals. The observation that during normal embryogenesis neural crest cells originate only at the most lateral edges of the neural plate, bordering the prospective epidermis, suggests that signals derived from the epidermal ectoderm might be involved in neural crest specification. *In vivo* grafts and *in vitro* coculture experiments have shown that induction of the neural crest in both amphibian and avian embryo can occur by interaction between the neural plate and ectoderm (Dickinson et al., 1995; Liem et al., 1995; Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995). Members of the bone morphogenic proteins (BMP) family are strong candidates as mediators of the epidermal signals. *In vitro* experiments have shown that BMP4 and BMP7 are expressed in the epidermal ectoderm and both proteins are sufficient to substitute the non-neural ectoderm in inducing neural crest (Liem et al., 1995). A common feature appears to be a requirement for down-regulation of BMP signaling in the neural plate, together with an increase in BMP signaling in the surface ectoderm, helping to define non-neural and neural fates. The question remains whether intermediate levels of BMP signaling at the interface between the surface ectoderm and neural plate mediate a neural crest fate. *In vitro* studies showed that proteins that inhibit BMP4/7 activity block the ability to induce neural crest cells (Liem et al., 1995). Furthermore grafting experiments using notochords, or cells secreting Sonic Hedgehog (Shh) (ventralizing factors) or Noggin (a BMP antagonist) reveal the requirement of BMP signaling in the initiation of neural crest specification and suggest three phases of neurulation that relate to neural crest formation:

(1) an initial BMP-independent phase that can be prevented by Shh-mediated signals from the notochord at a stage of open neural plate within the ectoderm;
(2) an intermediate BMP-dependent phase around the time of neural tube closure, when BMP4 is expressed in the dorsal neural tube, and
(3) a later premigratory phase which is BMP-insensitive and refractory to exogenous Shh and Noggin (Selleck et al., 1998).

However, in cultures of chick embryo neural plate tissue, intermediate levels of BMP signaling do not seem to be sufficient to induce neural crest markers, and therefore other factors in addition to BMPs must be required (Garcia-Castro et al., 2002). Most assays for neural crest induction are based on activation of the early marker Slug. This protein, a zinc finger transcription factor belonging to the Snail superfamily, have been shown to be essential for multiple steps in neural crest development (Manzanares et al., 2001; Nieto et al., 1994; Sefton et al., 1998). Current experimental evidence suggests that neural crest induction may involve a two-step process, with intermediate levels of BMP signaling initiating the first steps, and fibroblast growth factors (FGFs), retinoic acid (RA), or Wnts providing signals for the second step (Knecht and Bronner-Fraser, 2002; LaBonne and Bronner-Fraser, 1998) A role for Wnt proteins in neural crest formation was first suggested by experiments in *Xenopus laevis* in which coexpression of Wnt1 or Wnt3a with either neural inducers Noggin and Chordin in ectodermal explants caused ectopic activation of the neural crest markers Slug, Ap2 and Krox20 (Saint-Jeannet et al., 1997). Gain-of-function and loss-of-function studies of downstream targets of the Wnt signaling pathway revealed a requirement of Wnt in neural crest induction (Deardorff et al., 2001; Tamai et al., 2000). Garcia-Castro and colleagues showed that Wnt6 is localized in the ectoderm and is able to induce Slug and other neural crest markers in chick explant cultures, whereas BMPs could not unless the explants were supplemented with a richer culture medium containing additional factors (Garcia-Castro et al., 2002). Furthermore it has been shown that FGF signaling can induce neural crest formation when BMP signaling is blocked. This induction is probably mediated by Wnts because dominant-negative Wnts that antagonize normal Wnt signaling block induction. These findings are consistent with the neural crest phenotypes obtained in frog embryo experiments in which Wnt pathway components were either overexpressed or blocked. They also tie in with observations in the frog embryo that Wnt signaling components, such as Lef and β-catenin, directly bind to the Slug protein (Vallin et al., 2001). These data put Wnt signaling at the very top of the cascade that induces neural crest formation although it is likely that an interplay of multiple signaling pathways is essential in this complex process of early neural crest development (Figure 3).

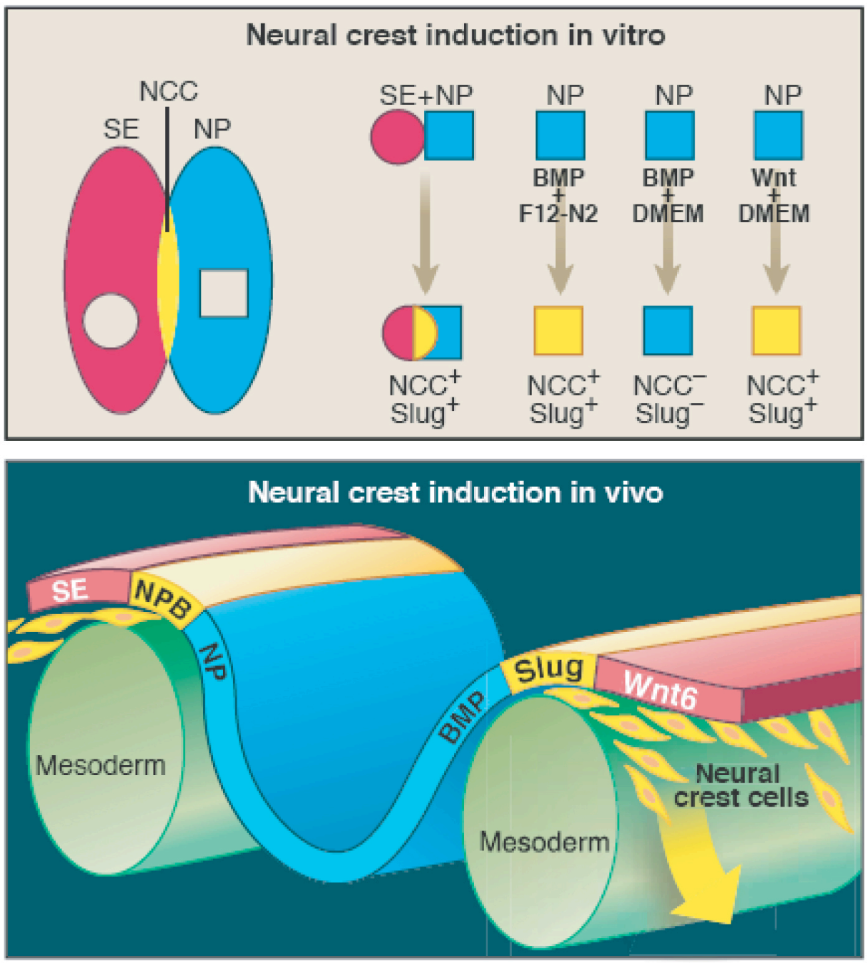

Figure 3: Seductive induction

Induction of neural crest cells in avian embryos. (Bottom) The induction of neural crest cells (NCC; yellow) at the border of the neural plate (NP; blue) and the surface ectoderm (SE; red) can be followed by expression of the Slug protein (yellow). Neural crest induction depends on contact-mediated interactions between the SE and NP. (Top) Explants of chick embryo NP tissue, cultured in rich medium (F12-N2) together with BMPs, express the Slug neural crest marker. In chemically defined medium without additives (DMEM), BMPs cannot induce expression of Slug, whereas Wnts can. *In vitro*, Wnt6 is expressed in the SE, which is adjacent to the NP. (Bottom) In response to Wnt signals from the SE, neural crest cells generated at the NP border (NPB) detach from the neural plate and migrate underneath the ectoderm and over the surface of the mesoderm (green) to their new location in the branchial arches. Here, they give rise to the peripheral nervous system, facial skeleton and melanocytes (adapted from Trainor and Krumlauf, Science, 2002)

3.3 Neural Crest Migration

After neurulation, neural crest cells emigrate from the dorsal neural tube, migrate extensively along defined pathways, and subsequently differentiate into diverse cell types, including most of the peripheral nervous system, cranial cartilage, and melanocytes (Le Douarin and

Kalcheim, 1999). In order to delaminate from the dorsal part of the neural tube, the cells have to undergo an epithelial to mesenchymal transition (EMT). The delamination of neural crest cells is a complex process of extensive changes within the developing embryo involving disruption of the basal lamina, dissociation of cell adhesions and changes in the extracellular matrix (ECM) (Erickson and Perris, 1993; Hay, 1995; Savagner, 2001). The influence of environmental signals is therefore crucial to ensure that migratory neural crest cells reach their final targets and adapt a suitable fate at a defined location. Recent evidence suggests that, in addition to being involved in the induction of neural crest, BMPs are required for the initiation of migration of trunk neural crest (Sela-Donenfeld and Kalcheim, 1999). In the chick embryo, BMP4 is expressed uniformly along the dorsal neural tube, and one of its antagonists, Noggin, is expressed in the dorsal neural tube in a high caudal to low rostral gradient. Ectopic expression of BMP4 causes premature migration of neural crest cells, whereas ectopic application of Noggin results in a delay in migration (Sela-Donenfeld and Kalcheim, 1999). As neural crest emigration occurs in a wave, from rostral to caudal, these findings suggest that BMP4 and Noggin act antagonistically to regulate delamination and migration of neural crest cells. A key issue is the identity of the targets of BMP signaling that underlie the EMT and migratory behavior of neural crest cells. Several studies have supported the idea that the expression of the Slug/Snail family members of transcription factors not only play a role in neural crest formation but also triggers neural crest delamination at its earliest stage (LaBonne and Bronner-Fraser, 2000; Nieto et al., 1994). Recent work has demonstrated that mouse Snail binds to the promoter and represses expression of the *E-cadherin* gene, which encodes a cell adhesion molecule expressed in a number of epithelial tissues, including the epidermis, but not mesenchymal cells. Following expression of *Snail* in the epithelial cell lines, *E-cadherin* is downregulated and the cells become mesenchymal and migratory (Cano et al., 2000). These data suggest that Snail-mediated repression is an important mechanism for the induction of an EMT in cells expressing *E-cadherin*. As *E-cadherin* is not expressed in the neural plate or premigratory neural crest, it is likely that other cadherins may be a target of Snail in this tissue. The expression of cadherin-6B and N-cadherin in chick embryo is detected at the sites of premigratory neural crest cell generation. Both genes get downregulated at the onset of neural crest migration. Instead cadherin-7 starts to get expressed (Nakagawa and Takeichi, 1995) and its expression persists during migration until homing of the neural crest cells to appropriate sites. High levels of ectopic cadherins have been shown to promote cessation of neural crest emergence and, importantly, such cells were unable to migrate dorsolaterally past the somite (Nakagawa and Takeichi, 1998).

Once the trunk neural crest cells have detached from the neural tube, they start migrating via two main pathways to their final targets where they finally differentiate into their specific fates:

(1) the ventrolateral path between the neural tube and somite, where neural crest cells give rise to neurons and glia cells of the PNS, e.g. dorsal root ganglia, sympathetic ganglia

(2) the dorsolateral path between the ectoderm and dermamyotome of the somite, where they differentiate into pigment cells of the skin (Erickson et al., 1992) (Figure 4)

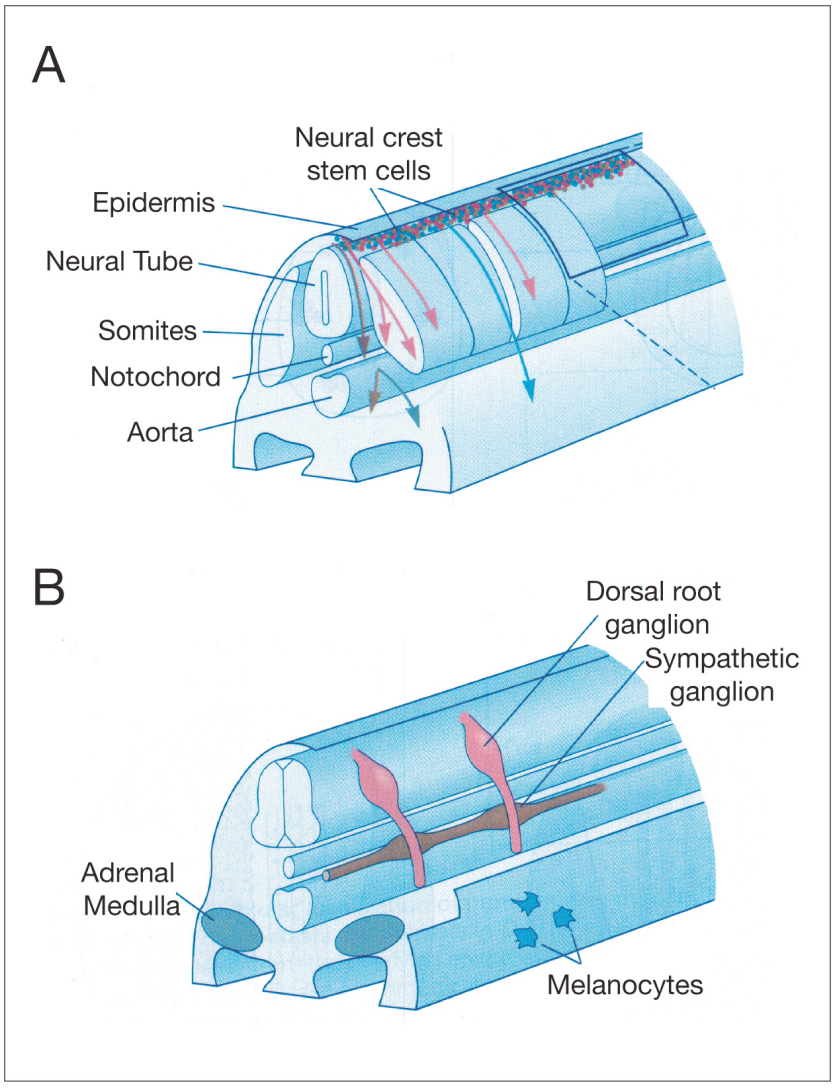

Figure 4: Neural crest migration

Neural Crest Cells migrate from the dorsal neural tube along different paths to their targets **(A)**, where they aggregate and differentiate into the structures of the peripheral nervous system **(B)** (adapted from Kandel, 2001).

During craniofacial development, cranial neural crest (CNC) cells migrate along a subectodermal pathway that extends ventrolaterally from the dorsal portion of the neural tube

between the ectoderm and the underlying mesodermal mesenchyme. After migration, CNC cells give rise to a variety of craniofacial tissue (Johnston, 1966; Le Douarin, 1982; Noden, 1975) including the sensory and parasympathetic ganglia, cartilage, bone muscles and connective tissue of the face (Le Douarin, 1982; Noden, 1983). Cardiac neural crest arises from neural tube at levels of the caudal hindbrain and the first two to three somites of the trunk. These neural crest cells migrate ventrally into the caudal pharyngeal arches and into the cardiac outflow tract where they are involved in forming the septum that divides the aorta and pulmonary artery (Waldo et al., 1998). Vagal neural crest cells emerge from the caudal hindbrain and migrate into and along the rostrocaudal extent of the gut where they form the enteric nervous system (Le Douarin and Teillet, 1973). Little is known about the molecular mechanisms underlying these differences in the migratory pathways. How are neural crest cells guided along these specific pathways? Several permissive or inhibitory molecules have been shown to play important roles in controlling neural crest migration. For example, a variety of ECM (extracellular matrix) components have been implicated, including chondroitin sulfate proteoglycans (Oakley et al., 1994) and peanut agglutinin (PNA) binding molecules (Oakley and Tosney, 1991) as inhibitory factors, and laminin (Perris et al., 1996) and fibronectin (Hocking et al., 2000) as permissive factors. EphrinB family members prevent neural crest entry into the caudal portion of each somite, resulting in segmental migration in the trunk region (Krull et al., 1997; Wang and Anderson, 1997). Other molecules implicated in active guidance of migrating neural crest cells include Semaphorin family members (Brown et al., 2001; Eickholt et al., 1999) and chemorepellents, like Slit2, which specifically repels trunk, not vagal, neural crest at the entrance of the gut mesenchyme (De Bellard et al., 2003). Although numerous important molecular players have been discovered so far, the mechanisms by which neural crest cells go through dramatic morphologic changes underlying the EMT and then navigate through the embryo to their final locations are still not fully understood (Figure 5).

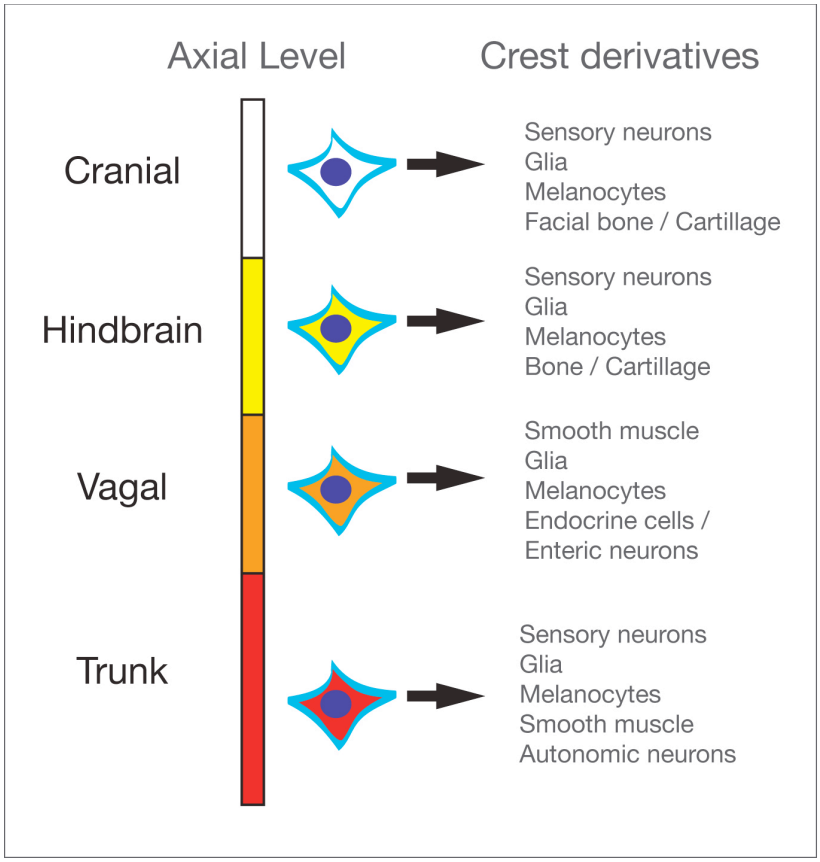

Figure 5: Variations in crest derivatives produced at different levels along the rostrocaudal extent of the neuraxis.

Only major subdivisions of the neuraxis and a simplified subset of crest derivatives are shown. The results are based on fate mapping experiments in avian embryos (adapted from Anderson, 1997).

3.4 Neural Crest Lineage Specification

Lineage restriction is the process that accompanies the differentiation of various cell types from a pool of multipotent stem or progenitor cells (Morrison et al., 1997). The key feature is the generation of a series of proliferating progenitor cells that exhibit gradual restriction in their developmental potential and fates. Since NCSCs are exposed to multiple signals, *in vivo*, the question arises of how stem cells would integrate such combined influences. One of the main goals in developmental biology is to understand the interplay between cell-intrinsic factors and environmental influences, and their mechanisms controlling the generation of different cell types. Moreover, cell-intrinsic changes affect fate decisions during neural crest development by changing the sensitivity of neural crest to specific extracellular signals (Kubu et al., 2002; Paratore et al., 2001; Paratore et al., 2002b; White et al., 2001). The fact that neural crest cells are plastic and sensitive to their local environment asks for the identity of

the environmental factors that influence the crest cell fate. Furthermore, it would be interesting to elucidate how these factors affect the fate of multipotent cells. Two alternative hypotheses can be set up for neural crest determination: at one extreme, the choice of fate could be made by a cell-autonomous mechanisms (either stochastic or deterministic) and the factors would support the survival or proliferation of lineage-restricted cells- this is referred to as „selective"; at the other extreme, the factors could cause multipotent cells to choose one fate (or a subset of fates) at the expense of others- this is referred to as „instructive" (Anderson, 1997). Several transplantation experiments have shown that neural crest cells gradually undergo restrictions in their developmental potential and their fate is influenced by cell-intrinsic changes in sensitivity to instructive differentiation signals (Le Douarin, 1986; White et al., 2001). However, it still remains unclear when and how restricted sublineages emerge, and why some derivatives are only produced from certain positions along the neuraxis.

Stemple and Anderson (1992) established an *in vitro* clonogenic culture system for rodent neural crest cells to assess their developmental potential on a cellular and molecular level. This analysis showed that, first, single mammalian neural crest cells are multipotent; second, multipotent neural crest cells generate multipotent progeny, indicating that they are capable of self-renewal and therefore are stem cells (Stemple and Anderson, 1992). Furthermore, *in vitro* experiments have identified several growth factors which regulate neural crest lineage determination (Figure 6). In rodents, three growth factors have been identified that bias the differentiation of neural crest cells along distinct lineages: glial growth factor (GGF, a neuregulin isoform) which promotes glial differentiation (Shah et al., 1994); transforming growth factor (TGFβ), which promotes smooth muscle differentiation; and bone morphogenic proteins (BMP), which promote the differentiation of autonomic neurons and to a lesser extent, smooth muscle (Shah et al., 1996). Recently, Morrison and colleagues described Delta, a Notch ligand, as another instructive factor, which promotes gliogenesis *in vitro* (Morrison et al., 2000b). Thus, the neural crest system is one of the few systems in which growth factors have been demonstrated to influence instructively the fate of multipotent stem cells. By contrast, in haematopoiesis there is clear evidence that at least some growth factors act selectively (Wu et al., 1995). This does not mean that growth factors do not exert selective effects on crest development as well. Indeed, there is evidence that GGF can also act as a survival factor for Schwann cell precursors (Dong et al., 1995) and neurotrophins were originally identified by their survival promoting effects on crest-derived peripheral neurons (Thoenen, 1991). This suggests that stem cell fates are not solely determined by what factors

are present in the environment but implicates a combinatorial function of instructive and selective signals, provided by extracellular growth factors and cell-intrinsic differences during development. However, it still remains unclear how a neural crest cell integrates the combined influence of opposing signals? Are neural crest cells equally competent to respond to all signals at the same time, or are there time frames for the cells to respond to different signals, which change with development? Understanding the interplay between intercellular signals and the context in which they are interpreted is one of the main goals in developmental biology, however the molecular basis of these processes still remains to be clarified.

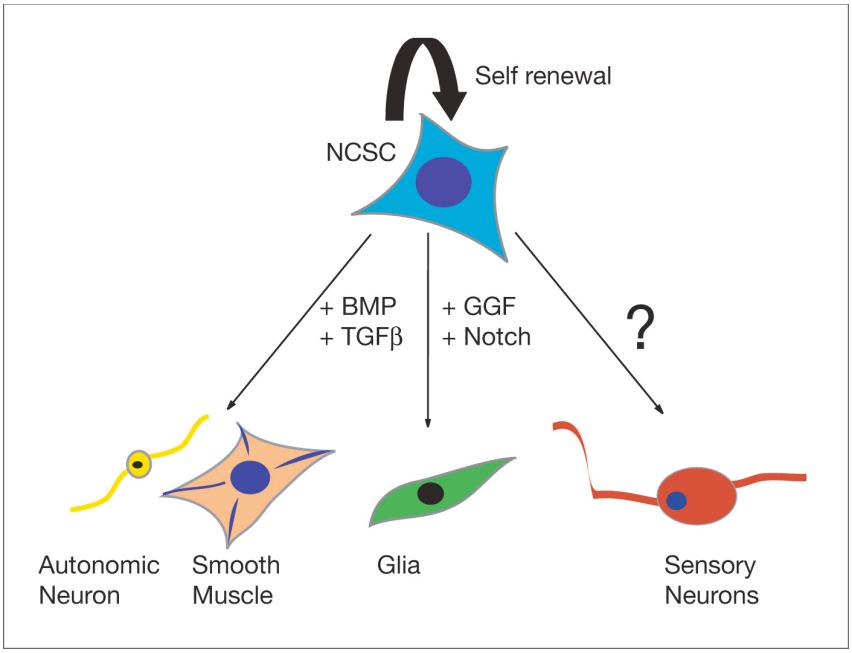

Figure 6: Specific neural crest fates are promoted by extracellular signaling molecules
The model shows the spectrum of fates available to a hypothetical pluripotent neural crest progenitor. While members of the TGFβ and BMP family act instructively on NCSCs to adopt an autonomic- or a smooth muscle fate, Neuregulin and Notch1 promote a glia fate in NCSCs. How a NCSC is able to adopt a sensory neuronal fate still has previously been unknown and was addressed in my thesis.

3.5 Homogeneity of Neural Crest Cells

An important issue in neural crest stem cell development is the elucidation of the individual neural crest lineages, because it constrains the possibilities for when and how cell fate decisions are made. So far, it has been assumed that multipotent neural crest cells are composed of a heterogeneous population with restricted developmental potential in proliferation and differentiation (Dupin et al., 1998; Ito et al., 1993; Le Douarin and

Kalcheim, 1999; Sieber-Blum, 1990). Several *in vivo* and *in vitro* studies revealed an early segregation of neural crest stem cells, which are already restricted in fate, however though, some cells maintain their multipotency and can be challenged to produce different fates under diverse environmental conditions. Especially, clonogenic cultures showed that while some neural crest cells give rise to multiple derivatives, other cells produce only one cell type under the same permissive conditions (Henion and Weston, 1997), which led to the assumption that the neural crest cells significantly differ from each other. Thus, the model was favored that the development of neural crest progressively generates restricted intermediates. However, it remained unsolved when this restriction occurred, pre- or postmigratory. Indeed, the time point of emigration seemed to be a critical factor defining the derivatives into which the neural crest cells would differentiate (Figure 7).

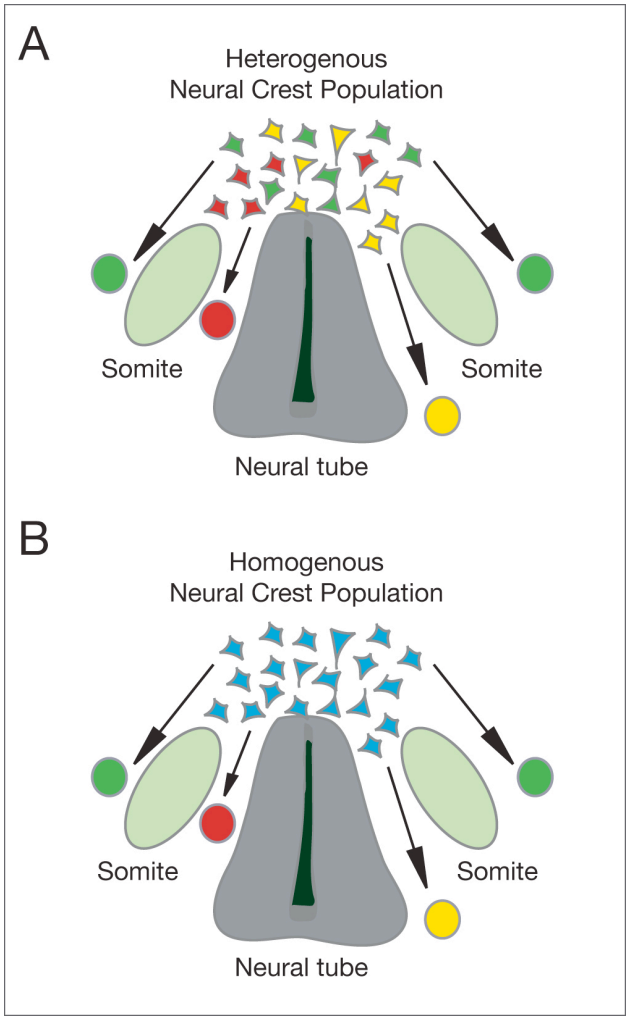

Figure 7: Alternative models of neural crest determination

The model A supports an early segregation of neural crest stem cells which are already fate restricted at the time point of migration, while model B predicts a homogeneous neural crest stem cell population at the time point of migration (adapted from Wolpert, 1999).

In avian trunk it was shown that the cells populating future sympathetic ganglia migrate before those that form the dorsal root ganglia (Serbedzija et al., 1989). Henion und Weston (1997) further determined that *in vitro* the initial neural crest population is already composed of heterogenic cells with distinct restricted fates with regard to the neurogenic and melanogenic sublineages. The only rigorous test of developmental restriction, however, is to challenge cells with instructive signals that promote various crest fates (Shah et al., 1996; Shah et al., 1994) and to determine whether the cells are resistant to the effects of such signals (Lo and Anderson, 1995). So far many signals have been described that promote the formation of particular fates in migratory and postmigratory neural crest stem cells. However, such instructive signals have only been identified for *in vitro* culture conditions. Moreover signals specifying sensory neuron formation of neural crest cells have not yet been reported.
In the first part of my thesis, I describe how the growth factor Wnt induces sensory lineage formation in neural crest stem cells. Using the *cre/loxP* system in the mouse model, we generated a gain-of-function mutation, in which *β-catenin*, a downstream target of the Wnt signaling pathway is constitutively overexpressed specifically in neural crest stem cells. This manipulation resulted in ectopic formation of sensory ganglia at the expense of virtually all other fates. We also analyzed these mutant cells *in vitro* and the outcome was a complete fate switch towards the sensory lineage. Moreover, *in vitro* we discovered a new type of neural crest stem cell, which unexpectedly displays, controversial to all published data, a homogenous character without any restriction in its developmental potential or fate. The isolation of these neural crest stem cells occurred at a very early time point (20 hours after migration) and we showed that these early neural crest stem cells (eNCSCs) are multipotent and can be challenged by various extracellular growth factors into different fates. Moreover, we demonstrate that upon Wnt1 signaling these emigrating eNCSCs are able to adopt a sensory neuronal fate in a β-catenin-dependent manner. Thus, for the first time we described with Wnt1 a growth factor, which actively promotes a sensory fate of neural crest stem cells *in vivo* as well as *in vitro*. This work was done in collaboration with Hye-Youn Lee.

In the second part of my thesis, I describe how Wnt and BMP cooperatively regulate the maintenance and early lineage segregation in NCSCs.
As described in part I of my thesis, Wnt signaling has recently been demonstrated to instruct early neural crest stem cells (eNCSCs) to adopt a sensory neuronal fate in a β-catenin-dependent manner. Although all eNCSCs are Wnt responsive not all adopt a sensory neuronal fate during development. Therefore, factors that counteract Wnt signaling when eNCSCs

emigrate from the dorsal neural tube must exist *in vivo*. Here we show that combined Wnt- and BMP-signaling instructively maintains multipotency in neural crest stem cells (NCSCs). This is the first time that a pure neural stem cell population can be maintained over prolonged time. Such cells remain multipotent, self-renew and respond to instructive growth factors. Moreover, over time NCSCs as well as postmigratory NCSCs isolated from different regions of the peripheral nervous system loose their responsiveness to the sensory neuron-inducing activity of Wnt. This establishes a new role of combined Wnt- and BMP-signaling in regulating multipotency and early lineage segregation in NCSCs.

This work was done in collaboration with Hye-Youn Lee.

Part I

Instructive Role of Wnt/β-Catenin in Sensory Fate Specification in Neural Crest Stem Cells

4. INTRODUCTION

Wnts are secreted small cysteine rich lipid-modified (Willert et al., 2003) proteins that play major roles during development such as proliferation, cell fate determination, apoptosis, tissue induction, morphogenesis and differentiation (Cadigan and Nusse, 1997). In humans, 19 *Wnt* genes have been identified so far and the chromosomal locations of each is known (Miller, 2002). *Wnt* genes have been found to perform an astonishing array of functions, from establishment of the basic body plan, such as early axis specification (Huelsken et al., 2000), neural crest specification (Garcia-Castro et al., 2002), neuron specification in the dorsal spinal cord (Muroyama et al., 2002), generation of various organs and tissues, like head induction (Mukhopadhyay et al., 2001) and brain patterning (Heisenberg et al., 2001), axon guidance (Yoshikawa et al., 2003) and synapse formation (Packard et al., 2002). The biological function of Wnts is highly conserved among species. Wnt/β-catenin signaling has been even found in embryos of the sea anemone *Nematostella vectensis*, an ancestor that existed before the evolution of the mesodermal germ layer (Wikramanayake et al., 2003). Further, in *Caenorhabditis elegans* Wnts regulate the expression of several homeobox genes (Korswagen, 2002), while the *Drosophila* homologue wingless (Wg) is the best-understood Wnt family member. The first insight into understanding the mechanism of Wnt signal transduction came from the existence of several fly genes with mutant phenotypes consistent with defects in Wg signaling (Cadigan and Nusse, 1997). Recently, Wnt signaling has also gained a substantial interest in stem cell development, since it turned out that the members of the Wnt family function as a key regulator in several stem cell systems. In embryonic stem (ES) cells Wnt signal activation is sufficient to keep them in a pluripotent state characterized by the markers Oct-4 and Nanog (Nakashima et al., 2004). Wnt signaling is endogenously activated in ES cells and is downregulated upon differentiation (Sato et al., 2004). Moreover, Wnt signaling in ES cells inhibits their neuronal differentiation by controlling BMP expression (Haegele et al., 2003). In haematopoetic stem cells (HSCs), it has been shown that overexpression of β-catenin a downstream component of the Wnt pathway induces proliferation of purified bone marrow-derived HSCs, while significantly inhibiting their differentiation. The finding that Wnt3a causes expansion of HSCs supports the interpretation that the effect of β-catenin reflects upstream Wnt activity. Moreover, Wnt signal activation maintains HSCs in long-term culture and leads to sustained reconstitution of haematopoetic lineages *in vivo* (Reya et al., 2003). Intestinal stem cells in the intestinal epithelium proliferate in response to Wnt ligands, which suggests that Wnt signals are essential for homeostasis of

the intestinal epithelium (Pinto et al., 2003). Studies on the hair follicle point to a delicate role of genes regulated by Wnt signaling (e.g. TCF/LEF transcription factors) in skin epithelial stem cell maintenance (DasGupta and Fuchs, 1999; Zhou et al., 1995). In the central nervous system Wnts act mitogenically on progenitor cells, and activation of ß-catenin (Cadigan and Nusse, 1997) leads to amplification of the neural progenitor pool (Chenn and Walsh, 2002; Megason and McMahon, 2002). In the developing spinal cord, β-catenin signals are essential for the maintenance of proliferation of neuronal progenitors, controlling the size of the progenitor pool, and for the decision of neuronal progenitors to proliferate or to differentiate (Zechner et al., 2003).

4.1 Wnt/β-Catenin Signaling

Wnts activate receptor-mediated signal transduction pathways resulting in gene expression, cell behavior, cell adhesion and cell polarity (Cadigan and Nusse, 1997; Gonzalez et al., 1991; Jue et al., 1992).

Wnt proteins bind to frizzled seven-transmembrane span receptors using low-density lipoprotein receptor-related proteins (LRPs) as co-receptors (Cadigan and Nusse, 1997; Huelsken and Behrens, 2002). Once secreted, Wnt proteins associate with glycosaminoglycans in the extracellular matrix and are tightly bound to the cell surface (Miller, 2002). Attempts to characterize Wnt proteins, or collect them in a soluble active form have been hampered by their high degree of insolubility. There are only a few studies, which describe an effect of soluble, active Wnt collected from conditioned media (Krylova et al., 2002). Recently, however, Willert and colleagues were able to isolate active Wnt molecules. By mass spectrometry, they found that Wnts are palmitoylated on a conserved cysteine and that enzymatic removal result in loss of activity (Willert et al., 2003).

Three pathways are activated by Wnt:

(1) the Wnt/Ca^{2+} pathway
(2) the planar cell polarity (PCP) pathway
(3) the canonical Wnt pathway

The Wnt/Ca^{2+} pathway involves the activation of phospholipase C and leads to elevated Ca^{2+} levels and the Ca^{2+}-Calmodulin-dependent protein kinase II, which regulate cell adhesion and

motility (Kuhl et al., 2000; Saneyoshi et al., 2002). The PCP pathway acts through small GTPases such as RhoA and Rac and activates c-Jun amino-terminal kinase (JNK), which results in planar cell polarity and cytoskeletal rearrangements (Peifer and McEwen, 2002; Sokol, 2000). The canonical Wnt pathway that uses the complex of β-catenin/T-cell transcription factors (TCF) or lymphoid enhancer factor1 (Lef1) to activate Wnt specific target genes in the nucleus, is the best studied of the Wnt pathways (Peifer and Polakis, 2000). In the absence of Wnt intracellular levels of β-catenin are quite low. β-catenin is constitutively phosphorylated first by casein kinase Iα (CKIα) and then by the glycogen sythase kinase 3β (GSK-3β). The phoshorylation of β-catenin occurs in the Axin adenomatous polyposis coli (APC) multiprotein complex. Phoshorylation of β-catenin leads to ubiquitination and ensuing degradation in the proteasome (Eastman and Grosschedl, 1999; Huelsken and Behrens, 2002). Upon Wnt signaling *via* frizzled-and LRP-receptors, the protein dishevelled (Dsh) get activated and recruits GBP/Frat-1, which displaces the GSK-3β from the Axin/APC complex and releases β-catenin in the cytomplasm. β-catenin is stabilized and enters the nucleus where it associates with TCF/Lef transcription factors and induce transcription of Wnt target genes (Eastman and Grosschedl, 1999; Huelsken and Behrens, 2002) (Figure 8).

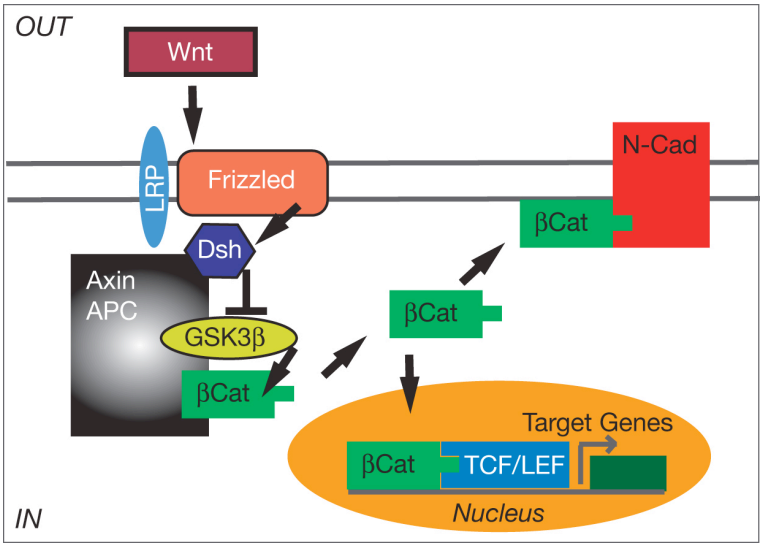

Figure 8: The canonical Wnt signaling pathway

Wnt proteins signal *via* Frizzled receptors using LRP proteins as co-receptors. Upon binding Dishevelled (Dsh) gets activated and displaces the GSK3β-kinase from the Axin/APC complex. β-catenin is released from the complex and translocated to the nucleus where it associates with TCF/LEF transcription factors to induce transcription of target genes. Moreover, β-catenin is also involved in Cadherin-dependent cell-cell interactions.

Wnts also play a major role in cancer. Molecular studies pinpointed activating mutations of the Wnt signaling pathway as the cause of 90% of colorectal cancer (CRC) and less frequently at other sites, such as hepatocellular carcinoma. However, Wnts themselves are only rarely involved in the activation of the pathway during carcinogenesis. Mutations mimicking persisting Wnt stimulation, generally inactivating *APC* mutations or activating *β-catenin* mutations, leads to uncontrolled transcription of genes that promote cellular proliferation and results in tumor formation (Giles et al., 2003). Germ-line mutations of *APC* in humans result in familial adenomatous polyposis and other tumor syndromes. In brain tumors it is noteworthy that medulloblastomas, highly malignant cerebellar tumors occur in a considerable proportion of patients with *APC* mutations (Haegele et al., 2003). Alterations in components of the destruction complex, which disturbs the normal regulation of β-catenin or altered β-catenin can lead to the formation of tumors by mimicking persisting Wnt signaling.

4.2 Canonical Wnt in Neural Crest Development

More recent work in several species has implicated Wnts as important key regulator in neural crest development. Mice lacking *Wnt1* and *Wnt3a* genes show deficiencies in expression of the neural crest markers CRABP-1 and AP2 within migrating neural crest cells and in neural crest derivatives, including partial loss of dorsal root ganglia, reduction of pigment cell precursors and cranial structures (Ikeya et al., 1997). The fact that some but not all neural crest derivatives are affected in these embryos suggests that Wnt1 and Wnt3a regulate the development of a subset of neural crest derivatives, implying the existence of additional regulatory factors, including other Wnts. In *Zebrafish* embryos, overexpression of β-catenin through mRNA injection in individual cranial neural crest cells promotes melanocyte formation at the expense of neurons and glia, whereas inhibition of Wnt signaling with a dominant-negative Wnt sustains neuronal fates at the expense of pigment cells (Dorsky et al., 1998). *In vivo* inhibition of Wnt6, which is expressed in the ectoderm adjacent to the neural folds, perturbs neural crest formation. Furthermore, Wnts trigger neural crest induction from naive neuroepithelium *in vitro* in conditioned medium even in the absence of factors (Garcia-Castro et al., 2002). Environmental signals acting prior to, during, or after migration of neural crest cells are believed to be involved in establishing lineage diversity from multipotent neural crest progenitors (Le Douarin et al., 1994). *In vitro* studies have identified several growth factors that can direct the differentiation of neural crest precursors along distinct lineages (Anderson, 1997). While these classes of signaling molecules appear to be involved

in neural crest cell diversification, until recently, a role for Wnt signaling in this process has been more difficult to establish. Wnt proteins are able to induce proliferation in different types of stem cells (Reya et al., 2003; van de Wetering et al., 2002; Willert et al., 2003). The question arises whether canonical Wnt signaling regulates stem cell self-renewal in general or whether this function is cell type-dependent. At the time of neural crest emigration Wnt proteins, namely Wnt1 and Wnt3a, are expressed in the dorsal neural tube, making them good candidates to be involved in neural crest development.

5. LINEAGE-SPECIFIC REQUIREMENTS OF *ß-CATENIN* IN NEURAL CREST DEVELOPMENT

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J. Cell Biology, Vol. **159**, Issue **5**, Pages **867-80**

5.1 Introduction

Multipotent stem cells have to generate various differentiated cell types in correct number and sequence during neural development (Sommer and Rao, 2002). The neural crest has turned out to be a valuable model system to study the mechanisms controlling this process. Neural crest cells in vertebrates give rise to neuronal and glial cells of the peripheral nervous system (PNS) and generate non-neural cells such as pigment and smooth muscle-like cells. Many signals have been described that promote the formation of particular cell fates in migratory and postmigratory neural crest stem cells (Anderson, 1997; Dorsky et al., 2000a; Sommer, 2001). Melanocyte formation is induced by Wnt signaling (Dorsky et al., 1998; Jin et al., 2001), whereas TGFß promotes the development of smooth muscle-like cells (Shah et al., 1996). Notch signaling and NRG1 isoforms promote the generation of satellite glia and Schwann cells (Hagedorn et al., 2000b; Leimeroth et al., 2002; Morrison et al., 2000b; Shah et al., 1994), while members of the TGFß family promote the differentiation of autonomic neurons (Hagedorn et al., 2000a; Reissmann et al., 1996; Shah et al., 1996). Signals specifying sensory neurons from neural crest cells, however, have not yet been reported. There is increasing evidence that the fate of multipotent neural crest stem cells is not only dependent on the action of individual signals, but is also influenced by the synergistic activity of multiple signals (Sommer and Rao, 2002). Hitherto unknown signals provided by cell-cell

interactions alter the biological activity of instructive growth factors such as TGFß, indicating that neural crest cells are able to integrate multiple cues (Hagedorn et al., 2000a; Hagedorn et al., 1999). Moreover, cell-intrinsic changes affect fate decisions during neural crest development by changing the sensitivity of neural crest cells to specific extracellular signals (Kubu et al., 2002; Paratore et al., 2001; Paratore et al., 2002b; White et al., 2001).

ß-Catenin is a good candidate to be involved in lineage decisions in neural crest development, given its dual function in Cadherin-dependent cell-cell interactions and in mediating Wnt signaling and given that both Cadherins and Wnt molecules have been implicated in neural development (Cadigan and Nusse, 1997; Yagi and Takeichi, 2000). By binding to the cytoplasmic domain of Cadherins and to a-Catenin, ß-Catenin links the Cadherin-dependent adhesion complex to the cytoskeleton and thereby strengthens cellular interactions (Gumbiner, 2000; Vleminckx and Kemler, 1999). Wnt signaling has been implicated in the regulation of cell proliferation, apoptosis, and cell fate decision (Cadigan and Nusse, 1997; Uusitalo et al., 1999). Activation of this pathway leads to stabilization of ß-Catenin and to its translocation into the nucleus, where it associates with other nuclear effectors to form a transcriptional activator complex. In various biological systems, Wnt/ß-Catenin signaling has been identified as part of a signaling network that involves interactions with other signal transduction pathways such as TGFß and Notch signaling (De Strooper and Annaert, 2001; Hecht and Kemler, 2000). A putative role of ß-Catenin in neural crest development is thus likely to reflect functions of Cadherin-mediated adhesion and Wnt signaling. Cadherins influence neural crest specification and emigration from the neural tube (Borchers et al., 2001; Nakagawa and Takeichi, 1998). Moreover, the expression of Cadherins in aggregating dorsal root ganglia (DRG) and in Schwann cells is consistent with a role at later stages of neural crest development (Pla et al., 2001). Wnt signaling is involved early in neural crest development and regulates neural crest induction and expansion (Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997), apart from the generation of melanocytes mentioned above.

To investigate the role of ß-Catenin in neural crest stem cells and their derivatives, we performed conditional gene ablation of *ß-catenin* in premigratory neural crest. Such an approach allows to circumvent the early embryonic lethality of mutants generated by gene deletion in the germ line (Haegel et al., 1995; Huelsken et al., 2001), and has been successfully applied before to reveal a requirement of *ß-catenin* in brain and craniofacial development, in skin stem cell differentiation, and in fate decisions between endoderm and

precardiac mesoderm (Brault et al., 2001; Huelsken et al., 2001; Lickert et al., 2002). The present study identifies ß-Catenin as a crucial regulator of sensory neuron specification and melanocyte formation.

5.2 Results

5.2.1 Inactivation of ß-catenin in neural crest

The role of *ß-catenin* in neural crest development was addressed by conditional gene inactivation in neural crest stem cells using the *Cre/loxP* recombination system (Gu et al., 1994). Cre-mediated recombination of a floxed allele in which essential sequences of the *ß-catenin* gene are flanked by *loxP* sites generates the *ß-catenin* floxdel allele, from which no functional ß-Catenin protein is expressed (Brault et al., 2001). In *wnt1-Cre* mice, Cre recombinase is active in the entire neural crest population (Danielian et al., 1998). To generate neural crest-specific *ß-catenin* mutant embryos, we crossed *wnt1-Cre* animals heterozygous for the *ß-catenin* floxdel allele with animals homozygous for the *ß-catenin* floxed allele. In *wnt1-Cre/ß-catenin*$^{floxed/floxdel}$ embryos derived from such breeding, ß-Catenin expression is efficiently eliminated in virtually all neural crest stem cells (Brault et al., 2001). In contrast, littermates lacking the *wnt1-Cre* transgene or carrying a wild-type *ß-catenin* allele express ß-Catenin normally and serve as control animals.

5.2.2 Loss of the melanocyte lineage in ß-catenin mutant embryos

We first investigated the role of *ß-catenin* in melanocyte development. Neural crest cells give rise to melanocytes of the skin, inner ear and part of the iris (Wehrle-Haller and Weston, 1997). Wnt signaling promotes melanocyte formation from neural crest cells and absence of *wnt1* and *wnt3a* in compound mutant mice results in loss of the melanocyte differentiation marker tyrosinase-related protein 2 (trp2) (Dorsky et al., 1998; Dunn et al., 2000; Ikeya et al., 1997; Jin et al., 2001). Similarly, we observed a complete absence of trp2 expression in *ß-catenin* mutant embryos at embryonic day (E) 10.5 and at E12.5 (Fig. 9; and data not shown). This phenotype was apparent at all sites of neural crest-derived melanocyte formation, such as underneath the surface ectoderm (Fig. 9A,B), around the retinal pigment epithelium (Fig. 9C,E), and around the otic vesicle (Fig. 9D,F). In contrast, trp2-positive cells in the retinal pigment epithelium, which are not generated from neural crest, were not affected in the mutant.

In order to address whether the lack of trp2 expression reflects a requirement for *ß-catenin* in early or late melanocyte differentiation, the expression of microphtalmia-associated transcription factor (mitf) was analyzed. Mitf activates pigment cell-specific genes such as *trp2* and is required for promoting melanoblast formation from precursor cells (Opdecamp et al., 1997; Yasumoto et al., 1997). In contrast to control embryos (Fig. 9G,H), mitf-positive melanoblasts were not detectable in *ß-catenin* mutant embryos (Fig. 9I,J), indicating that melanoblasts never formed in the mutant.

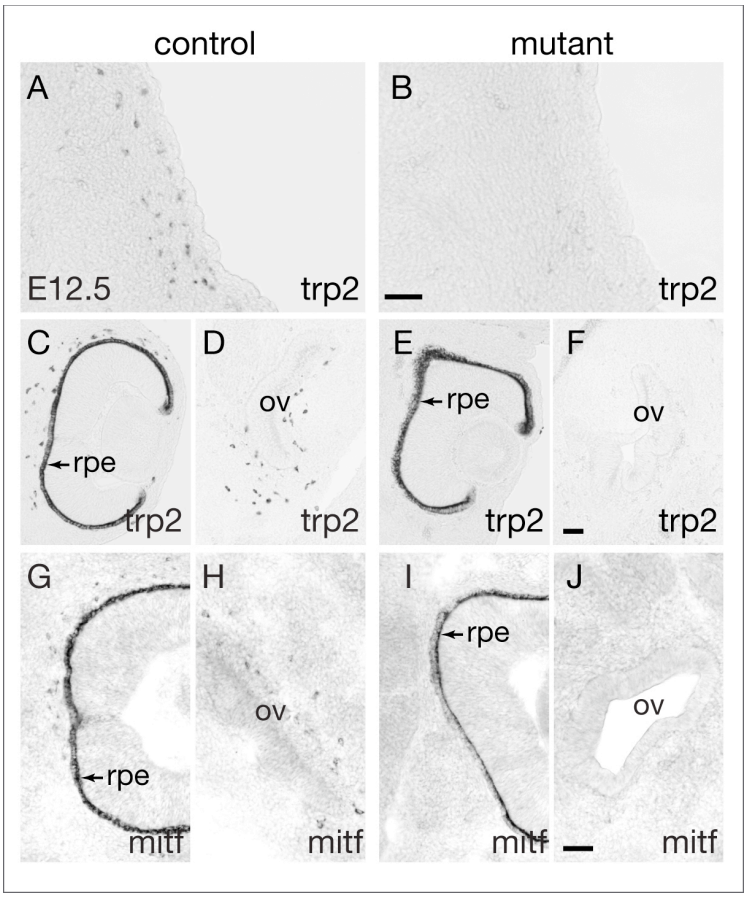

Figure 9. Absence of melanocytes in *ß-catenin* mutant embryos. Melanocytes and their precursors were marked by *in situ* hybridization analysis on transverse sections of E12.5 control (**A, C, D, G, H**) and mutant (**B, E, F, I, J**) embryos using trp2 (**A-F**) and mitf (**G-J**) riboprobes. In mutant embryos, trp2-positive melanocytes were absent underneath the surface ectoderm (**B**), in areas surrounding the retinal pigment epithelium (rpe**, E**) and around the otic vesicle (ov, **F**), whereas many melanocytes were present in control embryos (**A, C** and **D**). Many mitf-expressing melanoblasts were found around the eye (**G**) and the otic vesicle (**H**) of control but not of mutant embryos (**I, J**). Scale bars: 50 μm.

5.2.3 Analysis of peripheral neural structures

Our initial analysis of embryos in which *ß-catenin* had been eliminated by *wnt1-Cre* mediated gene ablation previously revealed a reduction of neuronal cells in cranial and dorsal root ganglia at E10.5 (Brault et al., 2001) suggesting a role of *ß-catenin* in the formation of

peripheral neurons. To elucidate this further, we first analyzed the PNS of mutant animals at a late embryonic stage. Only few occasional neurons marked by neurofilament 160 (nf) were detectable in the mutant as compared to the control in DRG of E16.5 embryos (Fig. 10A,B). In contrast, sympathetic ganglia and the enteric nervous system were not affected (Fig. 10E-H). To address whether the phenotype in mutant DRG was due to a specific requirement for *ß-catenin* in neuronal differentiation, we analyzed the expression of the transcription factor sox10. In the PNS, sox10 is a marker for multipotent progenitors and glial cells whose expression is downregulated as progenitors adopt a neuronal or non-neural fate (Paratore et al., 2001). While control DRG and peripheral nerves were composed of many sox10-positive cells, we were unable to detect any progenitor cells or presumptive satellite glia lateral to the neural tube where DRG normally form (Fig. 10C,D). Thus, only residual DRG were present in E16.5 mutant embryos, consisting of few neuronal cells without associated progenitor cells or glia. As expected given the near absence of sensory axons, mutant peripheral nerves were marked by sox10 but reduced in size (Fig. 10C,D). Absence of *ß-catenin* did apparently not impair early Schwann cell differentiation as Schwann cells in the mutant expressed P0 and MBP with a temporal expression profile comparable to the control (data not shown).

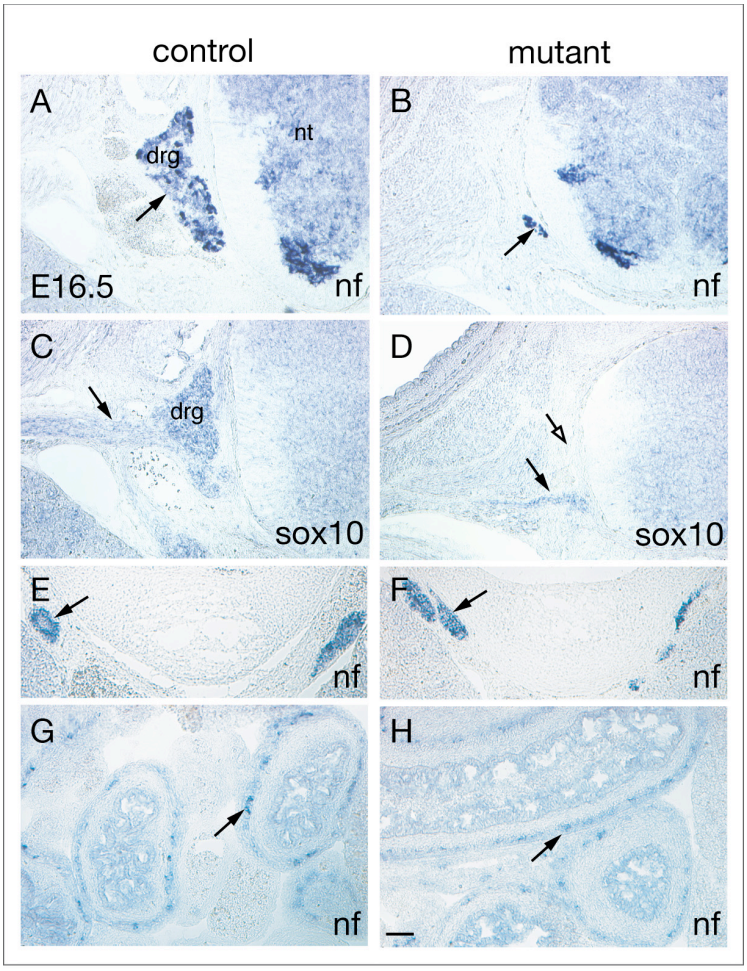

Figure 10. Analysis of control and mutant PNS. *In situ* hybridization experiments on transverse sections of E16.5 embryos with nf (**A, B; E-H**) and sox10 (**C, D**) riboprobes revealed reduction of neuronal (**A, B**, arrows) and complete absence of glial (**C, D,** open arrow) lineages in dorsal root ganglia (drg). Peripheral nerves marked by sox10 (**C, D,** arrows) were reduced in diameter, whereas other crest derivatives such as sympathetic ganglia (**E, F,** arrows) and the enteric nervous system (**G, H,** arrows) appeared to develop normally. nt: neural tube. Scale bar: 50 μm.

5.2.4 Specification of ngn2-dependent sensory progenitor cells requires ß-catenin

The neuronal subtypes in DRG are generated in two waves (Anderson, 1999). An early wave is dependent on the bHLH transcription factor neurogenin2 (ngn2) and gives rise to trkB- and trkC-positive neurons (Ma et al., 1999). In contrast, a somewhat later wave of neurogenesis produces mostly trkA-positive neurons and is dependent on the bHLH factor ngn1. Ngn2 is expressed already in migratory crest cells, while ngn1 expression is induced only upon cellular association in the forming DRG (Gradwohl et al., 1996; Sommer et al., 1996). To analyze whether the residual neurons found in the mutant at later embryonic stages (Fig. 10) belonged to a particular sensory subtype, we performed *in situ* hybridization experiments with trkA, trkB, and trkC riboprobes. Although these markers were readily detectable in control DRG at E16.5, none of these neurotrophin receptors appeared to be expressed by the few nf-positive cells present in the mutant (data not shown). Thus, these data left open whether the early ngn2-dependent or the later ngn1-dependent wave of neurogenesis, or both of these, were affected by *wnt1-Cre* mediated *ß-catenin* ablation. To address this issue, we examined the generation of ngn2-expressing sensory progenitor cells in *ß-catenin* mutants. Whole mount *in situ* hybridization analysis performed at E9.5 revealed ngn2 expression in the neural tube, in placodes, and in the neural crest of control embryos (Fig. 11A,B). In contrast, ngn2-positive cells were virtually absent in the neural crest along the rostro-caudal axis of mutant embryos, whereas neural tube and placodal ngn2 expression was readily detectable (Fig. 11C,D). Likewise, on transverse sections at E9.0, ngn2-expressing neural crest cells were found in control but not in mutant embryos (Fig. 11E,F). On adjacent sections, sox10-positive neural crest cells were detected emigrating from the neural tube of both control and *ß-catenin* mutant embryos suggesting that neural crest emigration was not generally affected (Fig. 11G,H). To monitor whether this phenotype reflected a delay in the generation of ngn2-positive cells, the expression of ngn2 was assayed at later developmental stages up to E12. Ngn2 expression in the PNS was also abolished in the mutant at these later stages (Fig.

11K,L; and data not shown) indicating that the ngn2-dependent sensory sublineage was never generated in the absence of ß-Catenin activity.

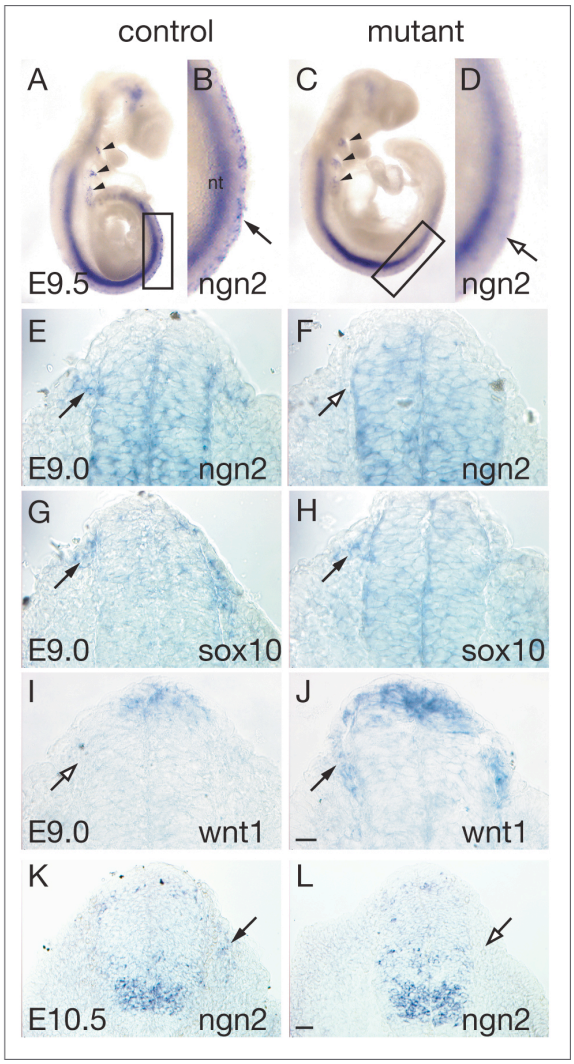

Figure 11. Absence of the ngn2-expressing sensory sublineage in the emigrating crest of mutant embryos. Whole mount *in situ* hybridization experiments showed ngn2 expression in the neural tube and in placodes (arrowheads) of control and mutant embryos at E9.5 (**A-D**). In migratory crest, ngn2 mRNA was only detectable in control (**A, B,** arrow) but not in mutant embryos (**C, D,** open arrow). The boxes in **A, C** indicate the areas enlarged in **B, D,** respectively. On transverse sections at E9.0, ngn2-positive neural crest cells were found in control (**E,** arrow) but not in mutant (**F,** open arrow) embryos. In contrast, on adjacent sections, neural crest emigrating from the dorsal neural tube was marked by sox10 mRNA both in control and mutant embryos (**G, H,** arrows). As at earlier stages, ngn2 mRNA was present in the DRG anlage of control (**K,** arrow) but not of mutant embryos (**L,** open arrow) at E10.5. Hybridization with a wnt1 riboprobe showed maintained wnt1 expression in the emerging crest of mutant embryos (**J,** arrow), whereas it was downregulated in neural crest of control embryos (**I,** open arrow) at E9.0. Scale bar: (**E-J**) 10 mm; (**K, L**) 20 μm.

The loss of ngn2-dependent sensory progenitors might be explained by a lineage-specific requirement of *ß-catenin* in survival, proliferation, or fate specification of precursor cells. Both in mutant and control embryos at E8.5 and E9, TUNEL labeling performed to assess cell death revealed no or only minimal apoptosis in the dorsal trunk neural tube or in the early migratory trunk neural crest (Brault et al. 2001; data not shown). These data rule out the possibility that lineage-specific cell death could lead to the elimination of ngn2-expressing cells in the *ß-catenin* mutant. Wnt signaling is involved in cell cycle regulation of neural tube cells and premigratory neural crest (Ikeya et al., 1997; Megason and McMahon, 2002). The drastic reduction of ngn2-positive cell numbers might thus be due to decreased proliferation. Given the apparently normal emigration of Sox10-positive cells in mutant embryos (Fig. 11H), the *ß-catenin* mutation would have to specifically affect cell cycle progression of the ngn2-expressing neural crest lineage. A putative lineage-specific change in cell cycle progression might be difficult to detect *in vivo.* Immunostaining of mutant embryos with anti-phospho-histone H3 as well as BrdU labeling at E9.5 did not provide evidence for reduced proliferation in the dorsal neural tube or in premigratory neural crest in the mutant (data not shown). To further address this issue, we performed cell culture experiments in which the extent of neural crest outgrowth can be assessed. Neural tubes of control and mutant embryos were isolated at E9 and neural crest cells were allowed to emigrate in defined culture conditions permissive for the generation of ngn2-dependent sensory neurons from Sox10-positive progenitor cells (Greenwood et al., 1999). In such a system, a reduction of ngn2-positive cell numbers by death or decreased proliferation rates would result in reduced outgrowth of neural crest cells with sensory neuronal potential. Twenty hours after plating the neural tubes, control and mutant explants formed by emigrating cells were highly similar and mainly composed of cells co-expressing the low-affinity neurotrophin receptor p75 and Sox10 (Fig. 12A-F), which are markers for neural crest stem cells (Paratore et al., 2001; Stemple and Anderson, 1992). Importantly, quantification of the outgrowth area (Huang et al., 1998) did not reveal any significant difference between control and *ß-catenin*-mutant neural crest explants (Fig. 12G), demonstrating that emigration of mutant neural crest cells was normal, and the size of mutant and control explants was similar. Taken together, our data suggest that the early loss of ngn2-expressing cells was not due to a lineage-specific elimination of premigratory or migratory neural crest cells by increased cell death or decreased proliferation.

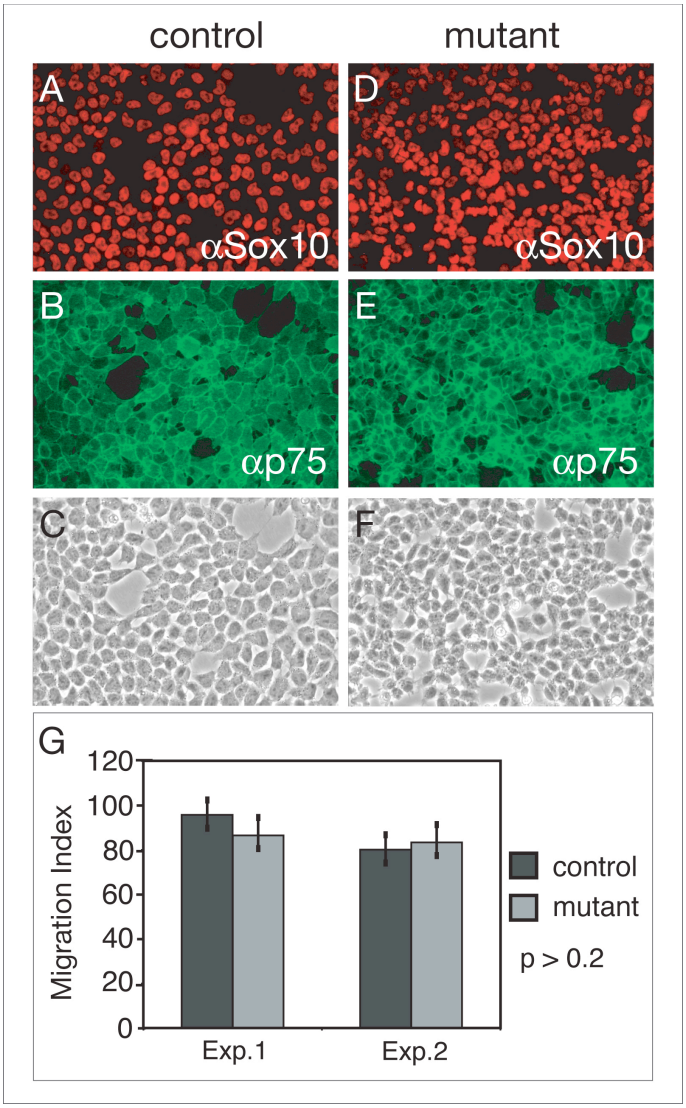

Figure 12. Normal emigration of mutant neural crest cells. Neural crest explants were obtained from neural tubes that had been isolated from control and *ß-catenin* mutant mice at E9 and cultured for 20 h to allow emigration of neural crest cells. After emigration, neural crest cells were fixed and immunolabeled with anti-Sox10 antibody (visualized by Cy3 fluorescence) (**A, B**) and anti-p75 antibody (visualized by FITC fluorescence) (**C, D**). Note that virtually all neural crest cells were double-positive for the neural crest stem cell markers p75 and Sox10. (**E, F**) Corresponding phase contrast pictures. (**G**) To compare and quantify the extent of control and mutant neural crest outgrowth after 20 h, the migration index was calculated using the NIH-Image 1.62 software (Materials and methods). Two independent experiments using non-sibling embryos were performed, scoring 3 explants of control and mutant embryos per experiment. Each bar represents the migration index (mean ±s.d.) of 3 different explants. Note that mutant explants were not significantly reduced in size and density.

A potential role of *ß-catenin* in lineage specification might be reflected by altered gene expression in mutant cells. Among various markers tested, we found expression of wnt1 to be

transiently maintained in emigrating neural crest cells, whereas usually wnt1 is rapidly downregulated in wild-type neural crest cells as they emerge from the neural tube (Fig. 11I,J). At later stages, this ectopic wnt1 expression was abolished (data not shown). These data might be interpreted that neural crest cells that would normally express ngn2 are unable to do so in *ß-catenin* mutants and aberrantly express wnt1. To further support the hypothesis that *ß-catenin* is involved in the specification of the early sensory neuronal lineage, a cell culture system was applied that allows monitoring of neuronal fate acquisition by neural crest cells on the cellular level. Neural crest stem cells were cultured in conditions that promote the generation of ngn2-dependent (but not ngn1-dependent) sensory neurons, as described before (Greenwood et al., 1999). As mentioned above, mutant cells emigrated normally from the neural tube in these conditions (Fig. 12). Upon continued culturing of such neural crest explants, many cells in control cultures adopted a sensory neuronal fate characterized by downregulation of Sox10 expression and induced expression of the POU domain transcription factor Brn-3A (Gerrero et al., 1993; Greenwood et al., 1999) (Fig. 13A).

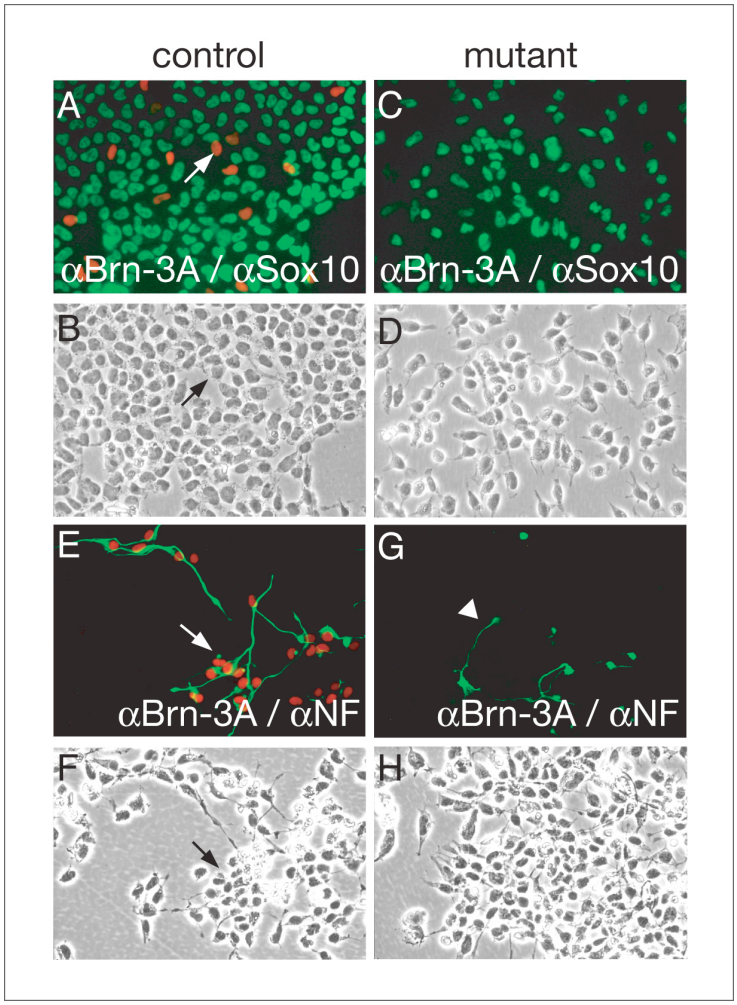

Figure 13. Mutant neural crest cells are unable to generate sensory neurons. Neural crest explants from control and mutant mice were prepared as described in the legend to Fig. 4. The cells were allowed to differentiate and were fixed after 36 h (**A-D**) or 48 h (**E-H**) in culture. The cultures were immunolabeled using anti-Brn-3A antibody (visualized by Cy3 fluorescence) (**A, B, E, F**), and double stained either with anti-Sox10 antibody (visualized by FITC fluorescence) (**A, B**) or with anti-neurofilament 160 (NF) (visualized by FITC fluorescence) (**E, F**). Sensory neuron precursors defined by the expression of Brn-3A (arrow in **A**) and sensory neurons defined by co-expression of Brn-3A and NF (arrow in **E**) were completely absent in mutant explants (**B, F**). Note that a few Brn-3A-negative non-sensory neurons were found in mutant explants (arrowhead in **F**). (**C, D, G, H**) Corresponding phase contrast pictures.

In contrast, mutant neural crest cells maintained their Sox10 expression and were unable to generate any Brn-3A-positive sensory neuronal precursors (Fig. 13B). At a later time point, control explants had generated multiple sensory neurons identified by co-expression of Brn-3A and NF, while in mutant cultures we never observed sensory neuron formation (Fig. 13E-H). Occasionally NF-positive cells were found in the mutant cultures (arrowhead in Fig. 13F). These were, however, Brn-3A negative, suggesting that some non-sensory neurons formed from mutant neural crest. Thus, signaling mediated by ß-Catenin is required for the specification of ngn2-dependent sensory neurons.

5.2.5 Impaired de novo neurogenesis in the DRG anlage

In a next set of experiments, we investigated whether *ß-catenin* might play a role in the generation of the second, *ngn1*-dependent wave of sensory neurons. To this end, *in situ* hybridization experiments were performed at stages when neural crest-derived cells in aggregating DRG upregulate ngn1 expression and contribute to extensive *de novo* neurogenesis. Sox10 and notch1 were used as markers for undifferentiated progenitor cells, while ngn1, neuroD and nf were used to mark the neuronal lineage. At E10.5, sox10-positive neural crest cells were found concomitantly with many ngn1-expressing progenitors in control DRG (Fig. 14A,E). In control animals, neurogenesis was apparent by the expression of neuroD, a bHLH factor acting downstream of ngn's (Ma et al., 1996), and by the presence of differentiated nf-positive neurons (Fig. 14G,I). In the mutant, however, sox10-expressing cells were present in the area in which ganglia formation occurs in the wild-type, but these cells did not appear to form proper ganglia (Fig. 14B). Moreover, mutant neural crest cells did not express notch1, unlike their control counterparts (Fig. 14C,D). Neurogenesis was drastically reduced in the mutant, with virtually no detectable ngn1-expressing cells and little

neuroD expression (Fig. 14F,H). Consequently, few differentiated neurons marked by nf were detectable in the mutant in the region of normal DRG formation (Fig. 14J).

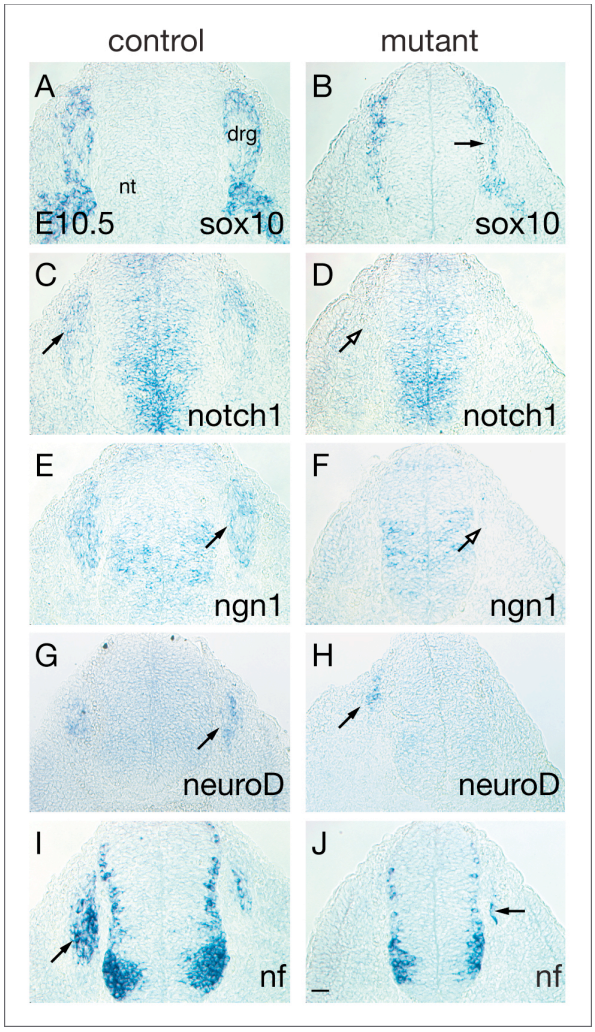

Figure 14. Failure of DRG formation from sox10-expressing neural crest-derived progenitor cells. Sox10-expressing cells were present in condensing DRG of control embryos (**A**) and lateral to the neural tube (nt) of mutant embryos (**B**, arrow) at E10.5. Near-adjacent sections hybridized with notch1 riboprobes demonstrate lack of notch1 expression in sox10-positive progenitors of mutant embryos (**D**, open arrow) whereas notch1 is expressed in progenitors of control embryos (**C**, arrow). Furthermore, ngn1-expressing cells were virtually absent lateral to the neural tube of mutant embryos (**F**, open arrow) whereas extensive ngn1 expression was found in the forming DRG of control embryos (**E**, arrow). In contrast to control embryos (arrows in **G, I**), only few neuroD- and nf-expressing cells were present in mutant embryos (arrows in **H, J**). Scale bar: 20 μm.

Sensory neurogenesis was even more affected at E11.5 and E12.5. In control animals at these stages, sox10 expression in progenitors and presumptive satellite glia outlined the DRG, in which multiple neuroD-positive neuronal precursors co-existed with differentiated neurons (Fig. 15). In contrast, neuroD-expressing cells were missing in the mutant (Fig. 15D, J) indicating that no new neurons were added to the few nf-positive cells that had been born at

earlier stages (Fig. 15F, L). Intriguingly, sox10 expression was also absent lateral to the neural tube and around the few sensory neurons of *ß-catenin* mutant embryos (Fig. 15B,H). Thus, progenitor cells that had been manifest at earlier stages in the area of normal DRG formation (Fig. 15) had disappeared in the mutant by E11.5.

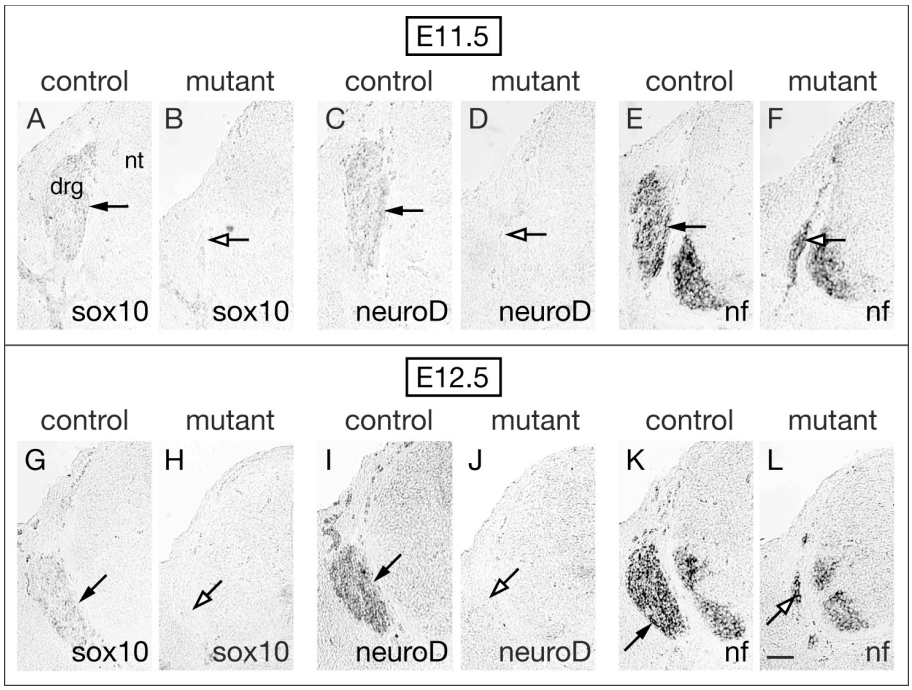

Figure 15. Absence of *de novo* neurogenesis in the DRG anlage of mutant embryos at later stages. At E11.5 (**A-F**) and E12.5 (**G-L**), progenitors and glial cells detected by hybridization with a sox10 riboprobe (**A, G,** arrows) as well as neurons and their precursors detected by expression of nf (**E, K,** arrows) and neuroD (**C, I,** arrows) mRNA, respectively, constituted the DRG in control embryos. In contrast, sox10- (**B, H,** open arrows) and neuroD- (**D, J,** open arrows) positive cells were completely missing in the mutant. Moreover, only few nf-expressing neurons were present in mutant embryos (**F, L,** open arrows). Scale bar: 50μm.

In vivo fate mapping of neural crest cells in control and mutant embryos

Cell death might be an explanation for the loss of neural crest cells that fail to form DRG in mutant embryos. To address this, staining for activated Caspase3 or TUNEL assays were performed at E10.5, E11.5 and E12.5 (Fig. 16C; and data not shown). However, we were unable to detect increased cell death in the mutant embryos in locations where DRG cells normally aggregate in the wild-type. Apart from cell death, there are alternative explanations for the disappearance of progenitor cells in the area of normal DRG formation. Mutant neural crest-derived cells might be able to localize to the site of normal DRG formation but might adopt an aberrant fate not detected by the neural markers tested so far. To investigate this

issue, we performed *in vivo* fate mapping experiments using the ROSA26 Cre-reporter allele *(R26R)* (Soriano, 1999). Upon Cre-mediated recombination, ß-galactosidase is expressed from this allele in Cre-expressing cells and their progeny. Thus, the fate of control and *ß-catenin* mutant neural crest cells can be followed in compound transgenic animals expressing *Cre* from the *wnt1* promoter. In animals wild-type for *ß-catenin*, ß-galactosidase expression delineated all neural crest-derived structures at E10.5 and at E12.5 (Fig. 16A,D; (Jiang et al., 2000)). In *ß-catenin* mutant embryos at E10.5, mutant cells expressing ß-galactosidase were localized in streams lateral to the neural tube without forming proper DRG (Fig. 16B).

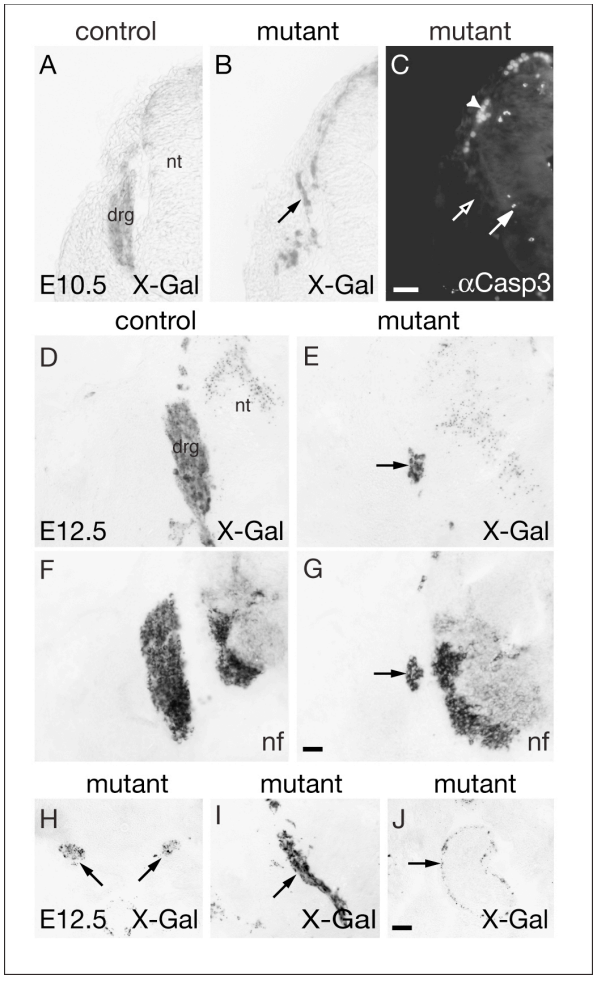

Figure 16. Survival and *in vivo* fate mapping of mutant cells. Control embryos carrying *wnt1-Cre* and the *R26R* allele displayed ß-galactosidase activity in the developing DRG at E10.5 (**A**) and E12.5 (**D**). At E12.5, the control DRG was outlined by nf expression (**F**). Cre-expressing neural crest cells and their derivatives were also detectable by ß-galactosidase expression in mutant embryos carrying *wnt1-Cre* and the *R26R* allele. At E10.5, mutant cells were localized lateral to the neural tube without forming proper DRG (**B**). No increased cell death was found in this area as shown by immunostaining for the activated form of Caspase3 (Casp3) (**C**, open arrow). (**B**) and (**C**) are adjacent sections. Note Casp3-positive cells within the neural tube (arrow) and autofluorescent blood cells present at the dorsal margins of the embryo (arrowhead). At E12.5, neural crest-derived mutant cells

expressing ß-galactosidase (**E**, arrow) were confined to the domain of few nf-positive cells present in the mutant (**G**, arrow). (**E**) and (**G**) represent adjacent sections. Mutant cells were able to normally colonize other neural crest-derived structures, such as sympathetic ganglia (**H**, arrows), peripheral nerves (**I**, arrow) and the enteric nervous system (**J**, arrow). Scale bars: (**A-C**) 50 mm; (**D-J**) 100 μm.

This expression pattern correlated with domains of Sox10 expression on adjacent sections (data not shown; see also Fig. 14). Most importantly, at E12.5, comparison of ß-galactosidase and nf expression revealed that the only neural crest-derived cells that localized lateral to the neural tube were the few sensory neurons present in *ß-catenin* mutants (Fig. 16E,G). In contrast, similar to control cells, mutant ß-galactosidase-positive cells localized to sympathetic ganglia (Fig. 16H), nerves (Fig. 16I), and the enteric nervous system (Fig. 16J). Hence, *in vivo* fate mapping did not reveal aberrant generation of non-neural cells from mutant progenitors in the area of normal DRG formation. Rather, mutant cells gave rise to multiple structures of the PNS while they failed to form proper DRG.

5.2 Discussion

In this study, we identify ß-Catenin as a crucial signal in neural crest development. Conditional *ß-catenin* gene ablation in the dorsal neural tube and in neural crest stem cells prevents the generation of melanoblasts and ngn2-dependent sensory neurons, presumably reflecting a role of *ß-catenin* in premigratory crest or at early stages of emigration (Fig. 17). Moreover, *ß-catenin*-mutant neural crest cells fail to aggregate in DRG and to produce ngn1-dependent sensory neurons while other neural crest derivatives such as the enteric nervous system and sympathetic ganglia form normally (Fig. 17). Analysis of mutant and control embryos together with *in vivo* fate mapping and cell culture experiments indicate a lineage-specific requirement of *ß-catenin* for the specification of both melanocytes and sensory neuronal lineages from neural crest cells.

5.2.1 Role of ß-catenin in melanocyte formation

Apart from its role in neural crest induction and expansion at early developmental stages (Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997), Wnt signaling has also been directly associated with the formation of the melanocyte lineage from neural crest. Both in avian cell cultures and in zebrafish *in vivo*, activation of the Wnt signaling pathway in neural crest cells promoted the formation of pigment cells (Dorsky et al.,

1998; Jin et al., 2001). In agreement with a role of Wnt signaling in melanocyte formation, ablation of *ß-catenin* in mouse neural crest cells not only leads to loss of the melanocyte differentiation marker trp2 but also of Mitf, a bHLH-zipper transcription factor regulating melanoblast specification (Opdecamp et al., 1997; Yasumoto et al., 1997). This finding might reflect a direct relationship between ß-Catenin-dependent signaling and induction of *Mitf* gene expression, as the *Mitf* promoter harbors a binding site for a ß-Catenin-containing transcription factor complex and can be activated by Wnt (Dorsky et al., 2000b; Takeda et al., 2000). It has been reported that in compound mutant mice lacking both *wnt1* and *wnt3a*, late-emigrating neural crest lineages are missing because of a general reduction of the neural crest cell population, which would primarily affect later- rather than earlier-forming neural crest derivatives (Ikeya et al., 1997). This is an unlikely explanation for the lack of melanocytes in *ß-catenin* mutant embryos because, unlike in chicken, melanocyte-forming neural crest cells in mouse embryos migrate along the dorsolateral pathway throughout the period of migration, including at early stages (Serbedzija et al., 1990). Taken together, the data indicate that ß-Catenin plays a role in specifying the melanocyte lineage from neural crest cells, most likely due to its function in mediating Wnt signaling (Fig. 17). Specification and early differentiation of melanocytes occurs in the so-called migration staging area (MSA), a space rich in extracellular matrix that is localized lateral to the dorsal neural tube (Dorsky et al., 2000a; Wehrle-Haller and Weston, 1997). In some but not all mutant embryos analyzed, we observed increased apoptosis in this area at E10.5 (but not at other stages) (data not shown), consistent with the idea that at least some cells that fail to be specified as melanocytes are subsequently eliminated by cell death. If so, this effect might be lineage-specific, since cell death in other neural crest derivatives such as in the DRG anlage was apparently not increased in the mutant.

5.2.2 Early specification of ngn2-dependent sensory neuronal cells by ß-catenin

Neurogenic and melanogenic lineages segregate during earliest stages of neural crest development (Henion and Weston, 1997). Furthermore, promotion of melanocyte formation by Wnt signaling has been reported to occur at the expense of neural lineages (Dorsky et al., 1998; Jin et al., 2001). However, our analysis of neural structures in *ß-catenin* mutant embryos showed that, similar to the melanocyte lineage, particular neural structures are reduced in size rather than increased. Thus, our data do not support a model in which ß-Catenin regulates a fate switch between neurogenic and melanogenic lineages. The

discrepancy in results (Dorsky et al., 1998; Jin et al., 2001); this study) might be explained by distinct timing of interference with the Wnt signaling pathway, differential roles of Wnt and ß-Catenin, and species differences.

While the reduction of peripheral nerves observed in *ß-catenin* mutants is presumably secondary to the absence of sensory neurons, the lack of ngn2-dependent sensory neurons and their progenitors most likely reflects a primary role of ß-Catenin in the formation of this lineage. Normally, ngn2 expression is detectable in a subset of Sox10-positive neural crest cells as they emerge from the neural tube (Lo et al., 2002; Sommer et al., 1996). In *ß-catenin* mutants, Sox10-positive cells emigrate *in vivo* but ngn2 expression is absent or restricted to very few neural crest cells (Fig. 17). Similarly, Sox10-positive neural crest cells emigrate in neural tube explants *in vitro* but these cells are unable to adopt a sensory neuronal fate, even in conditions promoting ngn2-dependent sensory neurogenesis. These data indicate that neural crest cells that would normally express ngn2 emigrate but fail to acquire a sensory neuronal fate in the absence of *ß-catenin*. Alternatively, ngn2-positive cells or their progenitors might be selectively eliminated in the mutant neural tube before or at emigration. However, at E8.5 and E9 we did not observe increased cell death in premigratory and migratory neural crest in *ß-catenin*-mutant embryos as compared to control embryos (this study; (Brault et al., 2001)). Moreover, the normal outgrowth of mutant neural crest explants in culture also speaks against a lineage-specific elimination of ngn2-positive cells.

In vivo, the increased expression of wnt1 in neural crest cells lacking *ß-catenin* suggests that wnt1 expression is involved in a negative regulatory feedback loop with ß-Catenin. Moreover, this finding also allows the assumption that ß-Catenin controls the early specification of ngn2-dependent sensory neurons by mediating Wnt signaling rather than Cadherin-dependent cell adhesion. Thus, ß-Catenin, presumably as a component of the Wnt signal transduction pathway, can be added to the list of signals such as TGFß family members, Notch, and NRG that regulate cell fates in neural crest development (Anderson, 1997; Sommer, 2001). It remains to be determined whether this function is exerted by direct regulation of gene expression of the *ngn's* that encode bHLH factors specifying sensory neuron identity (Ma et al., 1999; Perez et al., 1999)

The absence of neural crest cells marked by ngn2 not only points to a role of *ß-catenin* at earliest stages of neural crest development but also suggests that the ngn2-positive lineage

might segregate from other neural crest lineages already before or shortly after delamination from the neural tube (Fig. 17). In agreement with this, *in vivo* fate mapping by dye injection revealed that most (but not all) neural crest cells were restricted to generate either sensory or autonomic neurons but not both neuronal subtypes (Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Similarly, sensory precursors in neural crest explants turned out to be determined, unable to respond to factors inducing autonomic neurogenesis (Greenwood et al., 1999). Moreover, lineage-tracing experiments have recently demonstrated that ngn2-expressing cells preferentially contribute to sensory rather than autonomic ganglia (Zirlinger et al., 2002). Given the early function of ß-Catenin in sensory neurogenesis and given the expression of Wnt's before neural crest emigration, the question arises of how undetermined cells with competence to generate derivatives other than sensory neurons are being maintained in the neural crest. ß-Catenin signaling can be modulated by the activity of other signaling molecules, such as components of the Notch pathway and TGFß-family members (De Strooper and Annaert, 2001; Hecht and Kemler, 2000). Thus, the biological response of a neural crest cell to ß-Catenin might depend on its cellular context that determines signal combination and concentration as well as timing of signaling the cell is exposed to.

5.2.3 ß-catenin and neurogenesis during peripheral ganglia formation

Although the number of sensory neurons is drastically reduced in the DRG anlagen of *ß-catenin*-mutant embryos, a few sensory neurons are born at early developmental stages. From E11.5 onwards, however, sensory neurogenesis, as assayed by neuroD expression, was completely abolished in the mutant. The early generation of the residual neurons might suggest that they are derived from the early, ngn2-dependent rather than from the later, ngn1-dependent wave of neurogenesis. This issue was, however, difficult to assess because of the low or absent expression of markers that would distinguish sensory neuronal sublineages, such as ngn1, ngn2, and trkA, B, and C (Anderson, 1999). The absence of neurotrophin receptor expression in the few neurons present in the mutant suggests that proper differentiation of sensory neurons either requires ß-Catenin activity in a cell-autonomous manner or depends on cellular interactions with neural cells missing in the mutant. In any case, however, our data clearly demonstrate a requirement of *ß-catenin* in the formation of both ngn2- as well as ngn1-dependent sensory neurons (Fig. 17).

It is unclear whether the virtual absence of ngn1 expression in the mutant reflects a role of ß-Catenin in Wnt signaling or in mediating Cadherin-dependent cellular adhesion. Interference

with Wnt signaling in the neural crest does not just lead to a phenocopy of *ß-catenin* mutants (Brault et al., 2001; Ikeya et al., 1997) suggesting Wnt-independent roles of *ß-catenin* in neural crest development. However, the differences in phenotypes could reflect different stages at which the Wnt signaling pathway has been perturbed or could be due to the fact that *ß-catenin* gene deletion affects signaling by different Wnt family members. Hence, we cannot exclude that, as proposed above for the ngn2-dependent early wave of sensory neurons, later-forming sensory lineages would also be specified by Wnt signaling. Alternatively, interference with Cadherin-dependent cell adhesion rather than Wnt signaling might lead to the absence of DRG formation in *ß-catenin* mutants. In support of this idea, cell-cell adhesion is defective in *ß-catenin* mutant embryos (Brault et al., 2001; Haegel et al., 1995). Moreover, there is circumstantial evidence that cellular interactions might be involved in sensory neurogenesis. Normally, ngn1 is induced as neural crest-derived cells aggregate to form DRG (Sommer et al., 1996) but it is not known whether there is a causal relationship between cellular association and ngn1 expression.

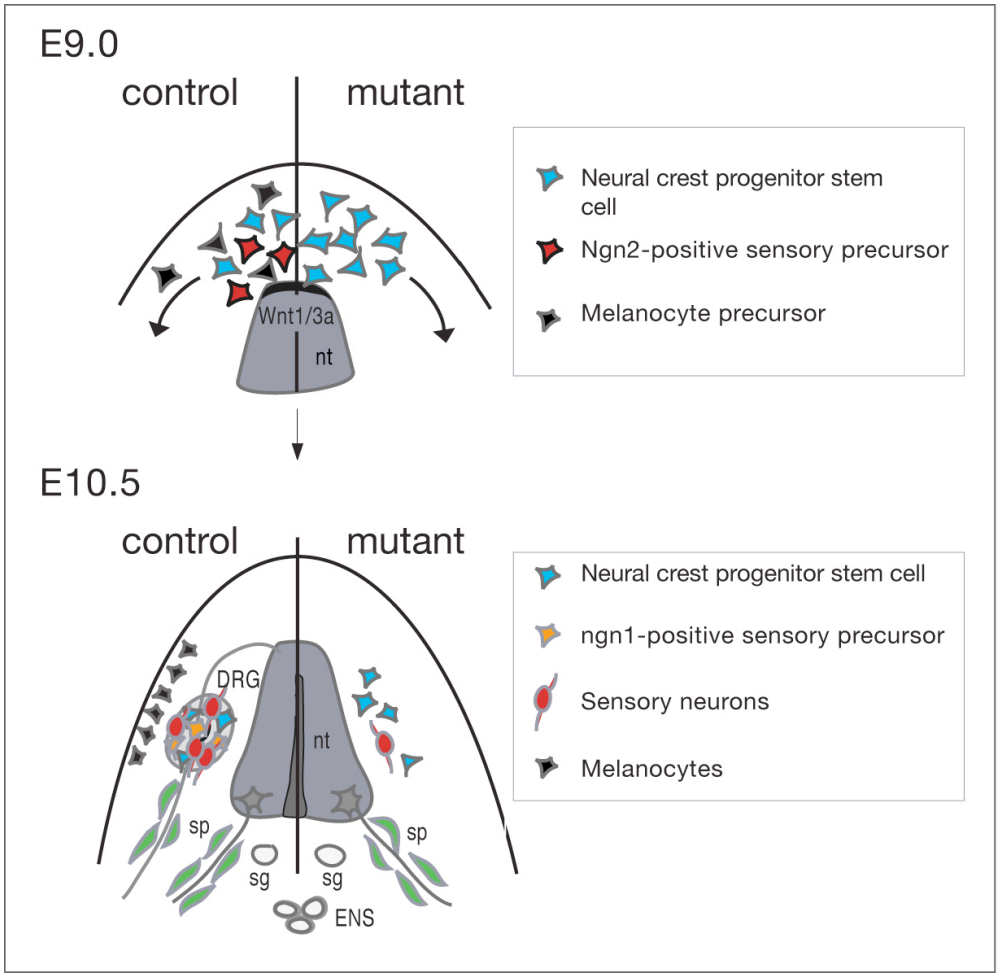

Figure 17. Lineage-specific requirement of *ß-catenin* in neural crest development. E9.0, control: emigrating neural crest appears to be heterogeneous, consisting of multipotent neural crest progenitor cells, early sensory

precursors (marked by ngn2 expression), and possibly melanoblasts. E9.0, *ß-catenin* mutant: neural crest fails to generate sensory precursors and melanoblasts. The specification of sensory and melanocytic fates in premigratory or early migratory neural crest might depend on signaling by Wnt1/Wnt3a, which are expressed in the dorsal neural tube (nt) at the stage of crest emigration. E10.5, control: neural crest-derived progenitor cells aggregate in DRG and produce ngn1-dependent sensory precursors. E10.5, mutant: progenitors lateral to the neural tube fail to aggregate and to form proper DRG; virtually no ngn1-expressing sensory precursors and only very few differentiated sensory neurons are detectable. This might point to a role of ß-Catenin in mediating cell-cell interactions possibly involved in sensory neurogenesis, although other ß-Catenin functions cannot be excluded. Other crest derivatives such as sympathetic ganglia (sg), the enteric nervous system (ENS) and Schwann cell precursors along peripheral nerves (sp) form independently of ß-Catenin activity.

Short-range cell-cell interactions termed community effects influence the response of multipotent progenitor cells to extracellular factors, thereby promoting neurogenesis at the expense of a non-neural fate (Hagedorn et al., 2000a; Hagedorn et al., 1999; Paratore et al., 2001). Furthermore, it has been suggested that early differentiating neurons might interact with DRG progenitor cells and serve as a "scaffold" for later-born sensory neurons (Anderson, 1999). According to this idea, the strong reduction of early-born sensory neurons in *ß-catenin* mutant embryos would impede ngn1-dependent sensory neurogenesis. Analysis of Sox10 expression reveals that *ß-catenin* gene ablation not only affects the induction of neuronal traits in undifferentiated progenitor cells. Rather, ganglionic structures delineated by Sox10-positive cells are not even formed, suggesting that undifferentiated progenitor cells fail to aggregate in DRG at any developmental stage (Fig. 17).

Absence of proper ganglia expressing Sox10 is not just due to aberrant downregulation of this marker, as demonstrated by *in vivo* fate mapping of mutant cells. *ß-catenin*-mutant neural crest cells expressing ß-galactosidase from the recombined *R26R* allele emigrate normally, are then found for a transient period lateral to the neural tube without forming overt DRG, and are later confined to the few sensory neurons present in the mutant. Thus, mutant cells do not adopt an alternative, non-neural fate in the area of normal DRG formation. Moreover, the phenotype is unlikely to be explained by mutant cells that would aggregate but then die, as we found no evidence for increased cell death in the mutant DRG anlage at any stage analyzed. Hence, the most easily conceivable interpretation of our data is that mutant neural crest cells fail to aggregate in DRG and to generate sensory neurons and satellite glia, and instead populate other neural crest-derived structures. However, we did not observe an increase in

non-sensory neural crest derivatives, suggesting that secondary mechanisms might regulate the generation of correct cell numbers in these tissues. Whether our data reflect the existence of migratory progenitors that in the wild type are able to generate both sensory as well as other neural crest-derived cell types remains to be addressed. In any case, however, the results are consistent with the hypothesis that cell-cell interactions are required for the promotion of neural fates in developing DRG. Signaling by Notch1, which is absent in the DRG anlage of *ß-catenin* mutants, might be involved in this process. Moreover, such cellular interactions are likely to be mediated by N-Cadherin-containing adhesion complexes, as progenitor cells aggregating in DRG express N-Cadherin (L. Hari and L. Sommer, unpublished; (Pla et al., 2001). Further experiments are required to elucidate the role of cell-cell interactions in promoting sensory neurogenesis.

6. INSTRUCTIVE ROLE OF WNT/β-CATENIN IN SENSORY FATE SPECIFICATION IN NEURAL CREST STEM CELLS

Lee, H.-Y.*, **Kléber, M.***, Hari, L., Brault, V., Suter, U., Taketo, M. M., Kemler, R. and Sommer, L. (2004).
Science, Vol. **303**, Issue **5660**, Pages **1020-3**

In NCSCs, specific ablation of the *ß-catenin* gene results in lack of melanocytes and sensory neural cells in dorsal root ganglia (DRG) (Hari et al., 2002). NCSCs without ß-catenin emigrate and proliferate normally but are unable to acquire a sensory neuronal fate. These results are consistent with a role of ß-catenin signaling in inducing a sensory fate. In order to further elucidate the function of Wnt/ß-catenin, we performed a gain-of-function approach, by constitutively overexpressing ß-catenin specifically in the neural crest stem cells.

6.1 Results

6.1.1 Targeting Construct and Nuclear Accumulation of β-Catenin

We used the *Cre/loxP* system to generate mice expressing a constitutively active form of ß-catenin specifically in neural crest cells (Harada et al., 1999). Certain serine and threonine residues near the ß-catenin N-terminus are required for phosphorylation by GSK3ß and ensuing ubiquitin-mediated proteolysis (van Es et al., 2003). The protein domain encompassing these phosphorylation sites is encoded by exon3. Deletion or mutation of these phosphorylation sites results in stabilization of ß-catenin and, thus, its enhanced nuclear activity. Stabilized ß-catenin can be locally expressed by tissue-specific Cre-mediated recombination in animals carrying a *ß-catenin* allele in which exon3 is flanked by *loxP* sites (flox(ex3)) (Harada et al., 1999). To achieve this, *wnt1-Cre* mice (displaying Cre activity in virtually all neural crest cells (Brault et al., 2001; Danielian et al., 1998)) were mated with *ß-catenin*$^{flox(ex3)/+}$ animals (Figure 18, A). In those embryos that inherited both *Cre* and a *ß-catenin*$^{flox(ex3)}$ allele, exon3 of *ß-catenin* was successfully deleted in NCSCs, as shown by PCR analysis of genomic DNA isolated from cultured control and mutant NCSCs. In culture, nuclear accumulation of β-catenin was found in virtually all mutant NCSCs (Figure 18, B). While none of the mutant embryos survived to term, they were recovered alive at normal frequency (i.e. 50%) at embryonic day (E) 9.5 and at slightly lower frequency at E10.5 and E11.5. Thereafter, their frequency was markedly reduced. The sole mutant embryo found at

E17 displayed severe craniofacial defects consistent with a developmental defect of cranial neural crest cells (Lee et al., 2003), as shown in Figure 18.

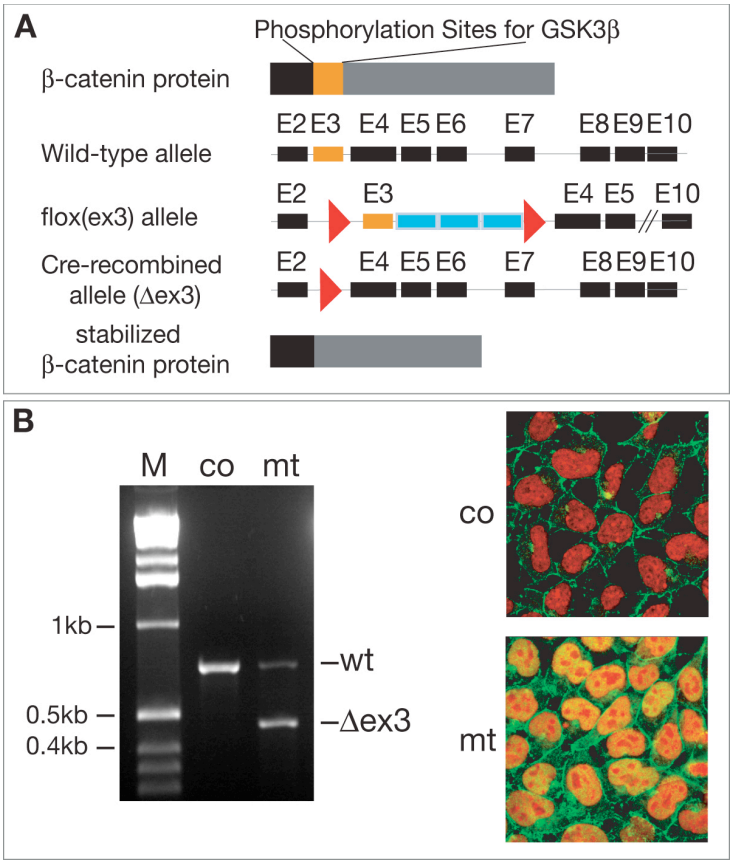

Figure 18: Generation of mice expressing a stabilized form of β-catenin in the neural crest

(**A**) Targeting strategy. The protein domain encompassing the phosphorylation sites is encoded by exon3 (orange box). Other exons are shown as filled boxes and the neo selection cassette in blue. Triangles indicate loxP sites. (**B**), PCR analysis of genomic DNA isolated from cultured control (co) and mutant (mt) NCSCs. The wild-type (wt) allele yields a product of 930bp, the deleted allele (Δex3) a product of 490bp. M, DNA size marker. Anti-β-Catenin immunostaining (green colour) combined with DRAQ5 nuclear labelling (red) show nuclear localization (yellow) of stabilized β-Catenin in virtually all mutant NCSCs in culture. Single confocal planes are shown.

6.1.2 In vivo Fate Mapping Using ACZL Reporter Mice

We first assessed the developmental potential of control and mutant neural crest cells by performing in vivo fate mapping experiments. In the control (Fig. 19A), neural crest cells emanating from the anterior neural tube populated the nasofrontal and periocular region, where they become mesenchyme that later generates bones, connective components, and vascular structures of the head (Chai et al., 2000; Etchevers et al., 2001; Noden, 1993). Neural crest cells also migrated into branchial arches that contribute to craniofacial skeletal tissue

and the major arteries (Jiang et al., 2000; Kirby and Waldo, 1995; Noden, 1993). In the mutant (Fig. 19B), however, cranial neural crest-derived cells hardly spread over the forebrain area and were more restricted to ventral head structures.

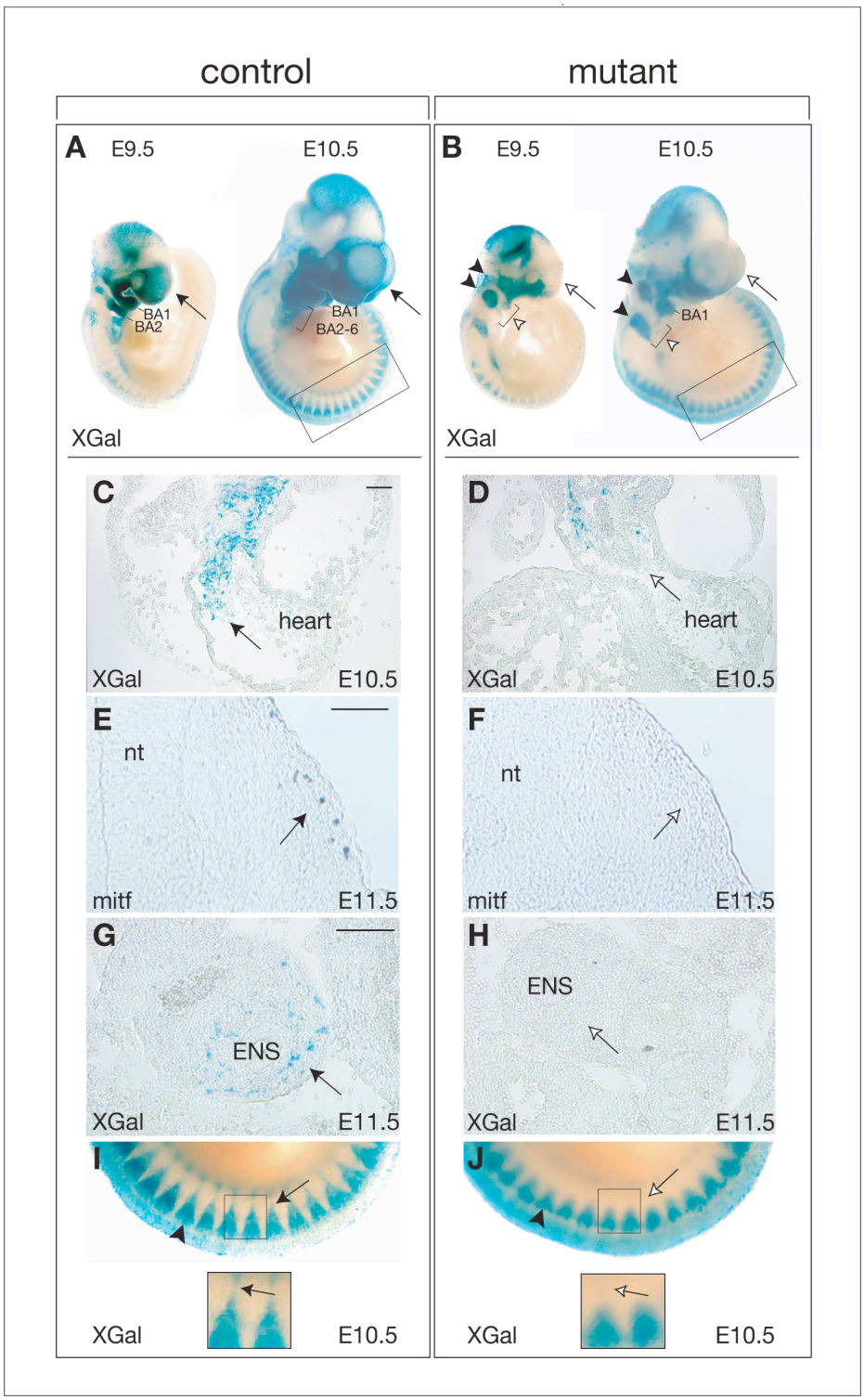

Figure 19: *In vivo* fate mapping of NCSCs expressing stabilized ß-catenin

(**A, B**) Neural crest cells and their progeny expressing ß-galactosidase were revealed by whole mount XGal stainings. Mesenchymal neural crest in the nasofrontal region is present in control (arrows) and absent in mutant embryos (open arrows). Arrowheads point to prominent ganglion-like structures, open arrowheads indicate missing branchial arches (BA) in mutants. (**C-J**) XGal stainings and in situ hybridization on transverse sections show absence or reduction of cardiac neural crest, mitf-positive melanoblasts, and the enteric nervous system (ENS) in the mutant. Peripheral nerves (**I, J**; enlarged areas marked by boxes in **A, B**, respectively) were also missing. Arrows illustrate presence, open arrows absence of neural crest derivatives. Note presence of DRG in control and mutants (arrowheads in **I, J**). nt, neural tube. Scale bars, 50µm.

Only a vestigial first branchial arch was present and all other branchial arches were absent. Instead, mutant neural crest cells aggregated in prominent cranial ganglion-like structures dorsal to the normal site of branchial arch formation.

Transverse sections revealed strongly reduced numbers of both neural crest cells in the cardiac outflow tract and neural crest-derived melanocytes (Fig. 19C-F). *In vivo* fate mapping also revealed a complete absence of the enteric nervous system (Fig. 19G, H). Moreover, mutant cells were unable to associate with peripheral axons and thus to contribute to the Schwann cell lineage in peripheral nerves (Fig. 19I, J). The markers sox10 and erbB3 were barely detectable in mutant peripheral nerves, and cad6-positive cranial nerves were missing (Fig. 19A, B), confirming the lack of presumptive Schwann cells. Absence of neural crest-derivatives in the mutant is not attributable to cell death, as no increased apoptosis was observed in regions normally comprising neural crest targets (Figure 19).

6.1.3 Sensory Neurogenesis at Ectopic Locations in the Mutant Embryo

Normally, cad6 expression at E10.5 is confined to neural structures and absent in mesenchymal neural crest derivatives (Fig. 20A, C, E). In the mutant, however, the entire area of ß-galactosidase-positive cranial neural crest cells also displayed cad6 expression (Fig. 20B, D, F). Within the ectopic domain of cad6 expression, neurofilament (nf) was readily detectable along with several transcription factors characteristic of the sensory lineage (Fig. 20H, K, L). Thus, upon sustained activation of ß-catenin, sensory neurons are generated in anterior regions of the embryo that are usually devoid of neural derivatives of the neural crest (Figure 20).

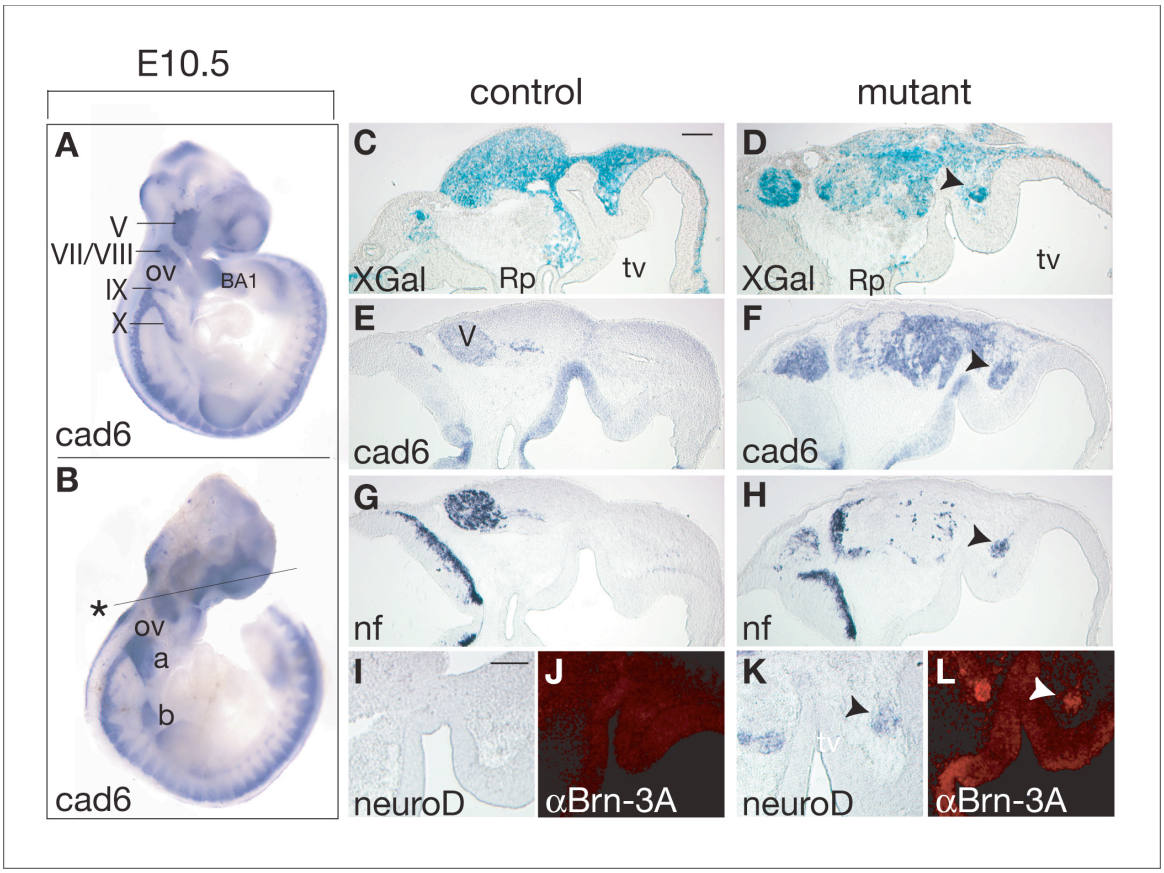

Fig. 20. Sensory neurogenesis at ectopic locations in mutant embryo.

(**A, B**) cad6 expression at E10.5 reveals ganglia with sensory neuronal features in the mutant (**B**) formed instead of cranial nerves IX and X (a) and at the location of normal superior cervical ganglia (b) (see also figure 14). V, VII/VIII, XI, X, cranial ganglia and nerves in the control (**A**). Ov, otic vesicle, BA, branchial arch. (**C-L**) Transverse sections cut at level (*) in (**B**). Note persistent expression of cad6 (**F**) in area of XGal-positive neural crest cells (**D**) and ectopic expression of nf in mutant (**H**). Ectopic neuronal cells (arrowheads) express the sensory markers neuroD (**K**) and Brn-3A (**L**). Rp, Rathke's pouch, tv, telencephalic vesicle. Scale bars, 100µm.

Similarly, the prominent cranial ganglia (Fig. 19B, arrowheads) contained many cells positive for ngn2, neuroD and Brn-3A, three transcription factors characteristic of the sensory lineage (Figure 21). Evidently, neural crest cells populating these structures have adopted a sensory neural fate (Figure 21).

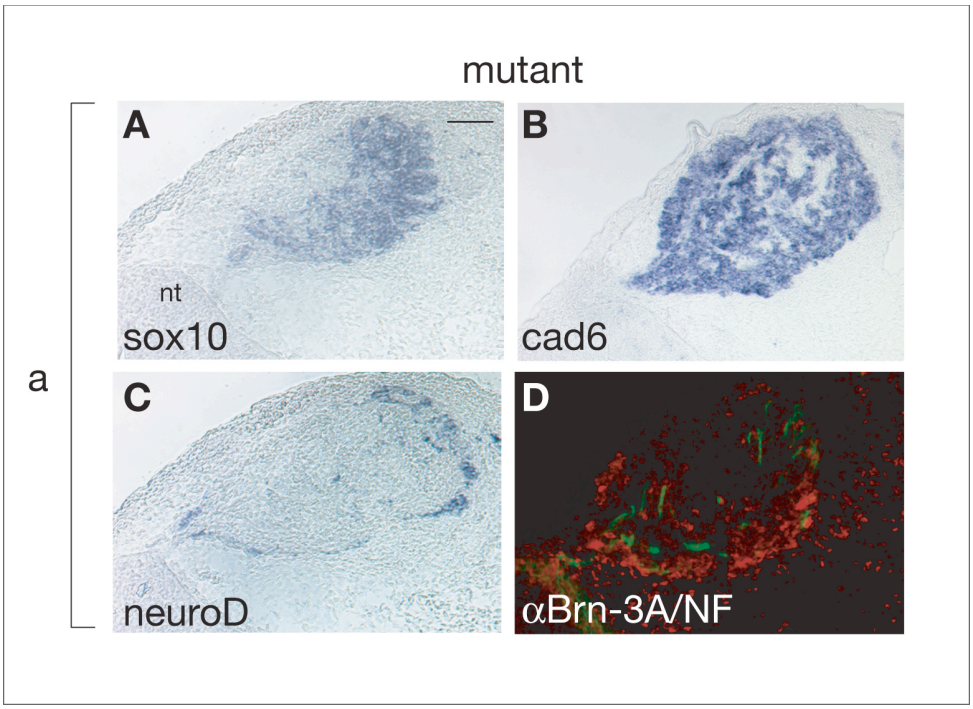

Figure 21: Formation of ganglionic structures with sensory features in mutant embryos.
(**A-D**) The prominent cranial ganglia that formed in the mutant instead of cranial nerves IX and X (see Fig. 19**B**; Fig. 20**B** (a)) contained many cells positive for sox10, cad6, neuroD mRNA, and Brn-3A (red) and NF (green) protein, as shown by staining on transverse sections at E10.5. Scale bar, 50µm.

6.1.4 Sensory Neurogenesis at the Expense of Autonomic Neurons

In the trunk, ngn2 is normally expressed in emigrating neural crest cells and marks cells fated for sensory neural lineages (Zirlinger et al., 2002). Its transient expression in cells aggregating in early DRG is followed by expression of the sensory markers ngn1 and neuroD. While lack of ß-catenin abolishes ngn2-expression (Hari et al., 2002), sustained ß-catenin activity resulted in increased numbers of ngn2-positive cells, both in the trunk and at ectopic cranial locations (Fig. 22A-D). Moreover, ngn2 expression was not restricted to the DRG anlage. Rather, ngn2-positive cells appeared to migrate on ventral routes and accumulated lateral to the dorsal aorta at sites of normal sympathetic ganglion formation (Fig. 22D, F). Ectopic expression of ngn1 and neuroD was also found in these structures (Fig. 22G, H; Fig. 23). In contrast, the autonomic neuronal markers mash1 and ehand were virtually absent in the mutant PNS (Fig. 22I, J, Fig. 23), indicating that sensory neurons were forming at the expense of sympathetic neurons. The data also reveal that, unlike some other neural crest target structures (Fig. 19C-J), sites of normal sympathetic ganglia development can be populated by sensory neurons (Figure 22, Figure 23).

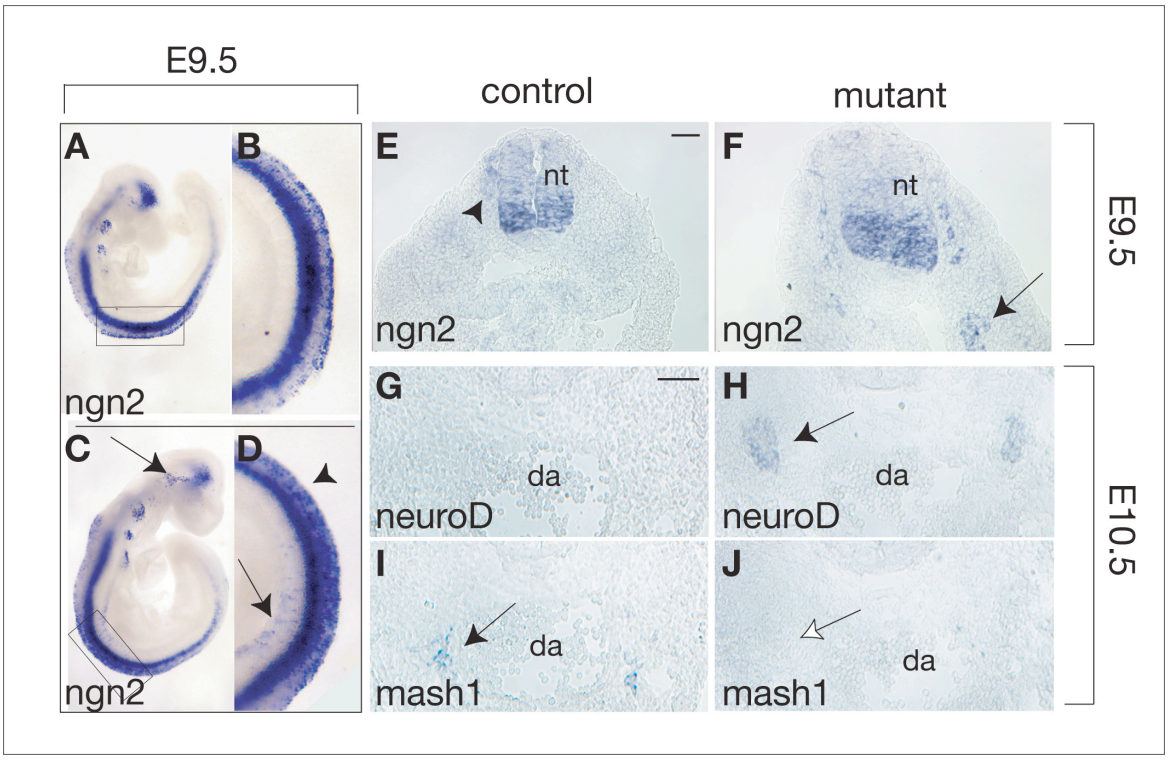

Figure 22: Sensory neurogenesis at autonomic locations in mutant embryos

(**A-J**) In situ hybridization experiments show ectopic ngn2 expression in cranial (arrow in **C**) and ventral (arrow in **D**) regions in the mutant, and increased expression in mutant migratory crest (arrowhead in **D**). (**A,B**), control. (**E,F**) On transverse sections at E9.5, ngn2-positive neural crest cells are restricted to the DRG anlage in control embryos (**E**, arrowhead) while they spread ventrally in the mutant (**F**, arrow). (**G-J**) At E10.5 in the mutant, neuroD-positive sensory neural cells (**H**, arrow) are found at the expense of mash1-positive autonomic neuronal cells (**I, J**, arrows). da, dorsal aorta. Scale bars, 50 µm.

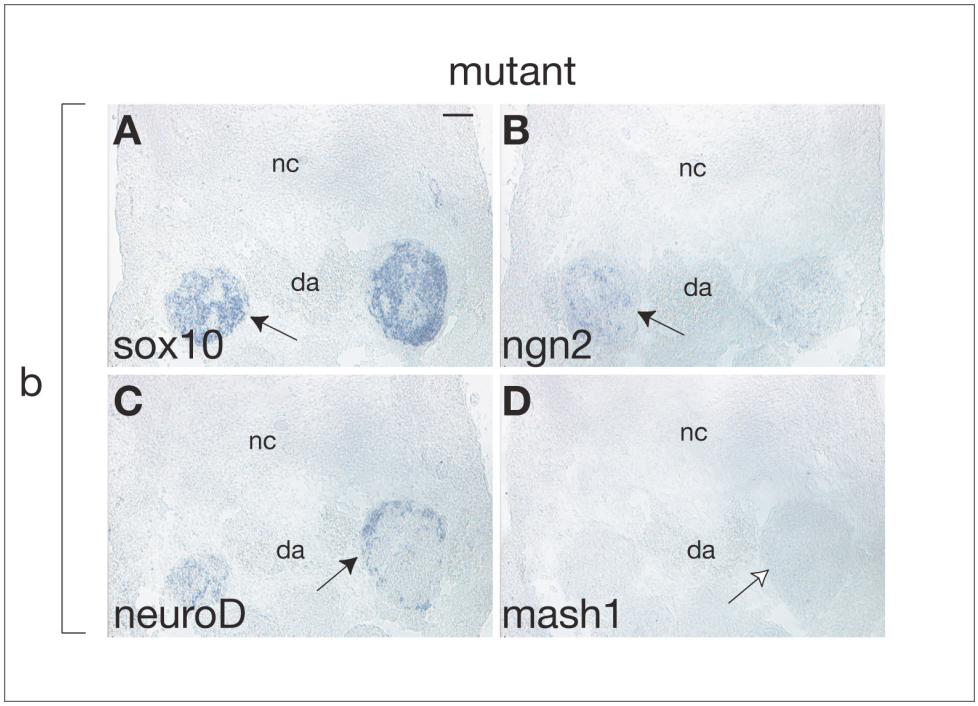

Figure 23: Formation of sensory ganglia at locations of normal superior cervical ganglia

(**A-D**) Ganglia with sensory features formed at the location of normal superior cervical ganglia (see (b) in Fig. 20B) and expressed the sensory lineage markers ngn1, ngn2, and neuroD. In contrast, the autonomic neuronal markers mash1 and ehand were not expressed. nc, notochord. Scale bar, 50 µm.

6.1.5 Stabilized β-Catenin Does not Affect Emigration and Proliferation of Neural Crest Cells

Thus, ß-catenin signaling appears not only to be required for sensory neurogenesis (Hari et al., 2002) but also to promote this fate. To evaluate this further, we investigated the developmental potential of isolated NCSCs expressing stabilized ß-catenin. In culture, both control and mutant migratory neural crest (20 h) expressed the NCSC-markers p75 and Sox10 (Fig. 24A, B) (Paratore et al., 2001), but were negative for the sensory differentiation marker Brn-3A. Proliferation and cell death during migration and differentiation (10 h; 30 h) were comparable in control and mutant explants. (Fig. 24A-F; table 1). Thus, the mutant phenotype appears to be due to an altered developmental potential of neural crest cells rather than to selective mechanisms or a defect before emigration. In agreement with this, we did not detect neural tube deficiencies at the time of neural crest delamination (Figure 24).

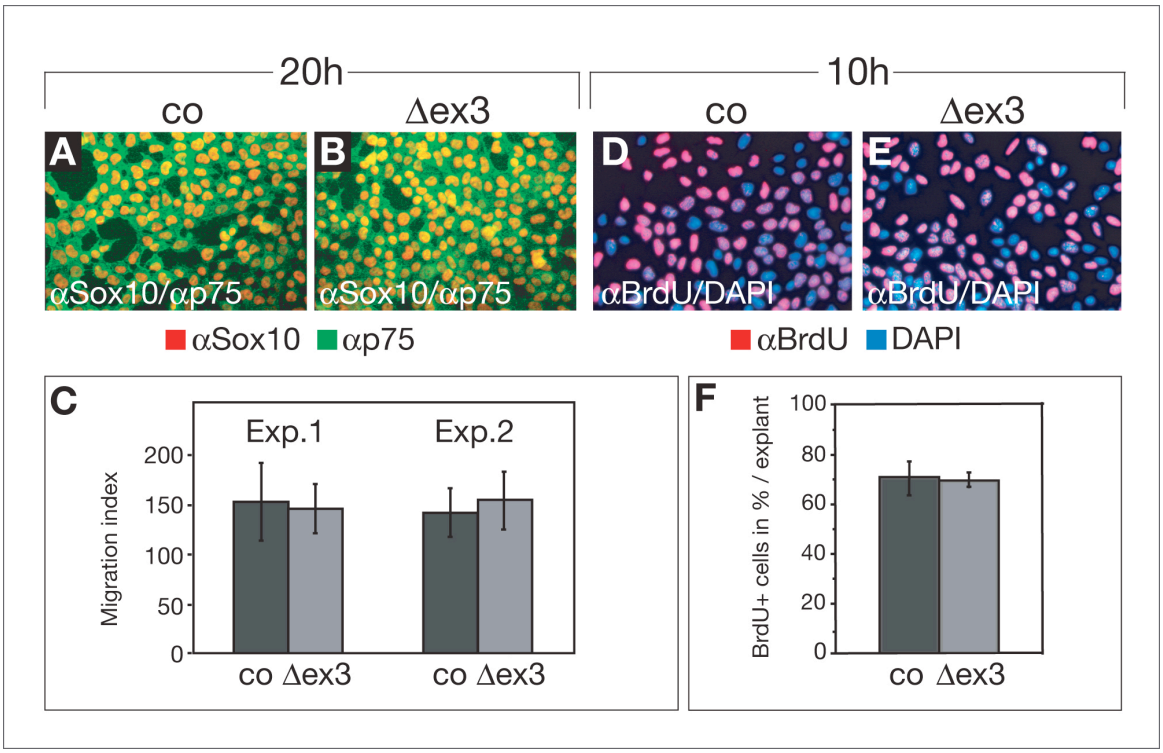

Figure 24: Stabilized ß-catenin does not affect emigration and proliferation of neural crest cells

(**A-C**) Quantification of the migration index did not reveal differences in migration of p75/Sox10-positive neural crest cells in control (co) and mutant (Δex3) explant cultures ($p > 0.2$). (**C**) Each bar represents the migration index (mean ±s.d.) of 3 different explants. (**D-F**) The number of proliferating, BrdU-incorporating cells was not

significantly different between control and mutant cultures (p >0.75). Three explants per genotype were analyzed, scoring 500-1000 cells per explant (**F**). Each bar represents the mean±s.d.

6.1.6 Stabilized β-Catenin Promotes Sensory Neurogenesis in Culture

In conditions permissive for sensory neurogenesis, control neural crest cells produced few Brn-3A-positive sensory neurons, while many cells maintained Sox10 expression (Fig. 25A; table 1). In contrast, virtually all mutant cells lost Sox10 immunoreactivity and the majority became Brn-3A positive, demonstrating that ß-catenin signaling is able to specify a sensory neuronal fate in most neural crest cells (Fig. 25B). As Sox10 has been associated with NCSC maintenance (Kim et al., 2003; Paratore et al., 2002a), the data also suggest that overexpression of ß-catenin promotes the loss of multipotency in NCSCs. In control cultures most Brn-3A-positive cells displayed features of fully differentiated neurons, while many mutant Brn-3A cells were not associated with NF-positive neurites (Fig.25 E, F), indicating that sustained β-catenin activity delays or interferes with neuronal differentiation. Likewise, *in vivo* many Brn-3A-positive sensory neuronal cells were NF-negative, and expression levels of trk neurotrophin receptors, differentiation markers for sensory neurons, were reduced in neural crest derivatives of mutant embryos. However, prolonged incubation in the presence of neurotrophins allowed mutant sensory cells to differentiate in culture, indicating that sustained ß-catenin activity delays rather than impedes neuronal differentiation (Fig. 25G-H; table 1).

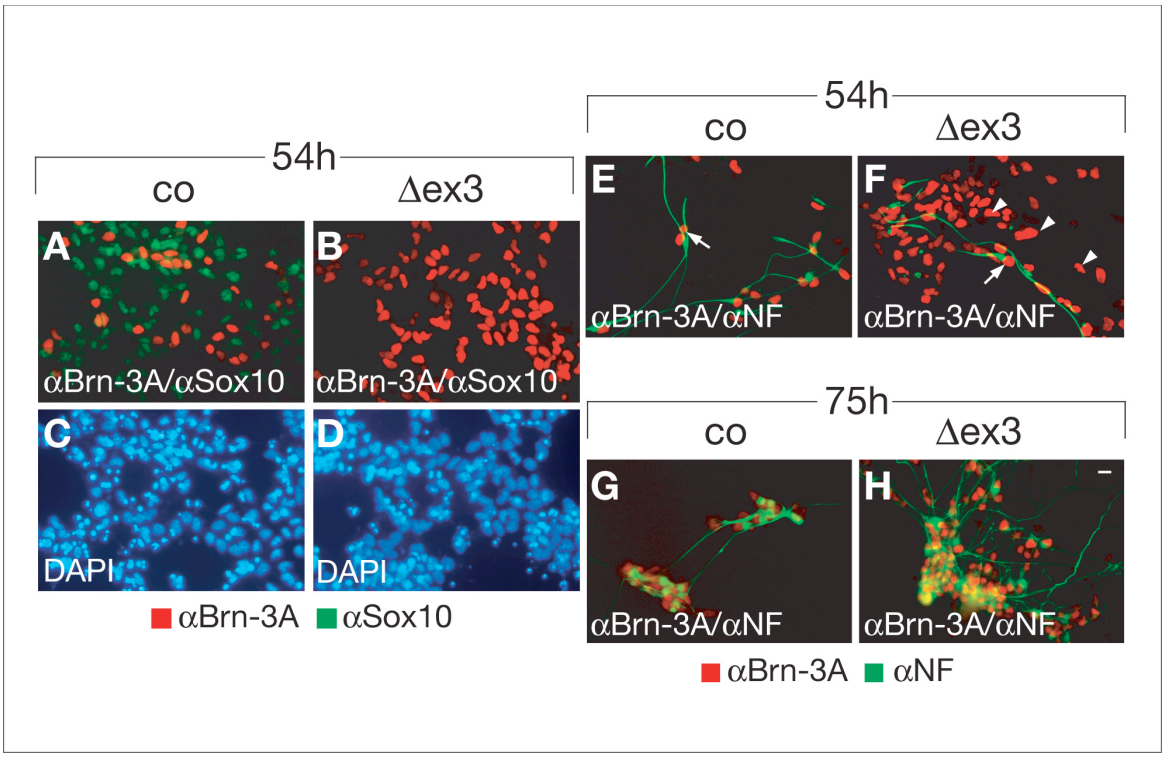

Figure 25: Stabilized β-catenin promotes sensory neurogenesis

(**A-D**) Upon differentiation, mutant neural crest cells lose Sox10 expression and stain for the sensory marker Brn-3A, while many control NCSC maintain Sox10 expression (table 1). (**E-H**) Differentiation is delayed in the mutant, since many Brn-3A-positive sensory neuronal cells (arrowheads) do not express NF (arrows) in the mutant at 54 h (table 1). After prolonged incubation for 75 h, however, most mutant cells are able to undergo full differentiation. Scale bar, 10 μm.

	% of all p75+ cells				% of all Brn-3A+ cells
	(20h)		(54h)		(54h)
	Sox10	Brn-3A	Sox10	Brn-3A	NF
co	100	0	70.3±6.3	29.7±6.3	100
Δex3	98.7±1.2	1.3±1.1	0	82.2±7.8*	18.7±9.9

	% of all cells		
	(10h)	(30h)	(30h)
	BrdU		TUNEL
co	70.9±6.4	36.6±3.1	1.23±0.4
Δex3	69.5±2.8	32.9±3.5	1.31±0.7

Table1: Marker expression, proliferation, and cell death of neural crest cells expressing activated ß-catenin.

Marker expression was assayed at 20 h and at 54 h. Note that 20 h after emigration, virtually all cells displayed features of NCSCs being positive for p75 and Sox10 but negative for the sensory differentiation marker Brn-3A. In conditions permissive for sensory neurogenesis, most cells in the control maintain Sox10 expression, while in the mutant, all cells lose NCSCs features and the majority adopts a sensory fate marked by Brn-3A (54 h). At 54 hours, 100% of all control Brn-3A-positive cells displayed features of fully differentiated neurons, while many mutant Brn-3A cells were not expressing NF. Proliferation and cell death during migration and differentiation (10 h; 30 h) are comparable in control and mutant explants. All figures represent the mean±s.d. of three experiments. At least 200 cells were counted per experiment.

6.1.7 Wnt/β-Catenin Regulates Sensory Neurogenesis

We next investigated whether the effect of activated β-catenin in neural crest cells reflects a role of Wnt signaling. Unlike in control conditions, wild-type NCSCs exposed to Wnt1 efficiently generated Brn-3A-positive sensory neurons (Fig. 26). When we similarly

challenged ß-catenin-deficient NCSCs with Wnt1, we were unable to detect any sensory neurons (Fig. 26D), in agreement with our previous finding that β-catenin is required for sensory neurogenesis (Brault et al., 2001; Hari et al., 2002). Thus, Wnt1 promotes sensory neurogenesis in a β-catenin-dependent manner. These results unambiguously establish a role of the canonical Wnt/β-catenin pathway in promoting fate decisions in NCSCs (Figure 26).

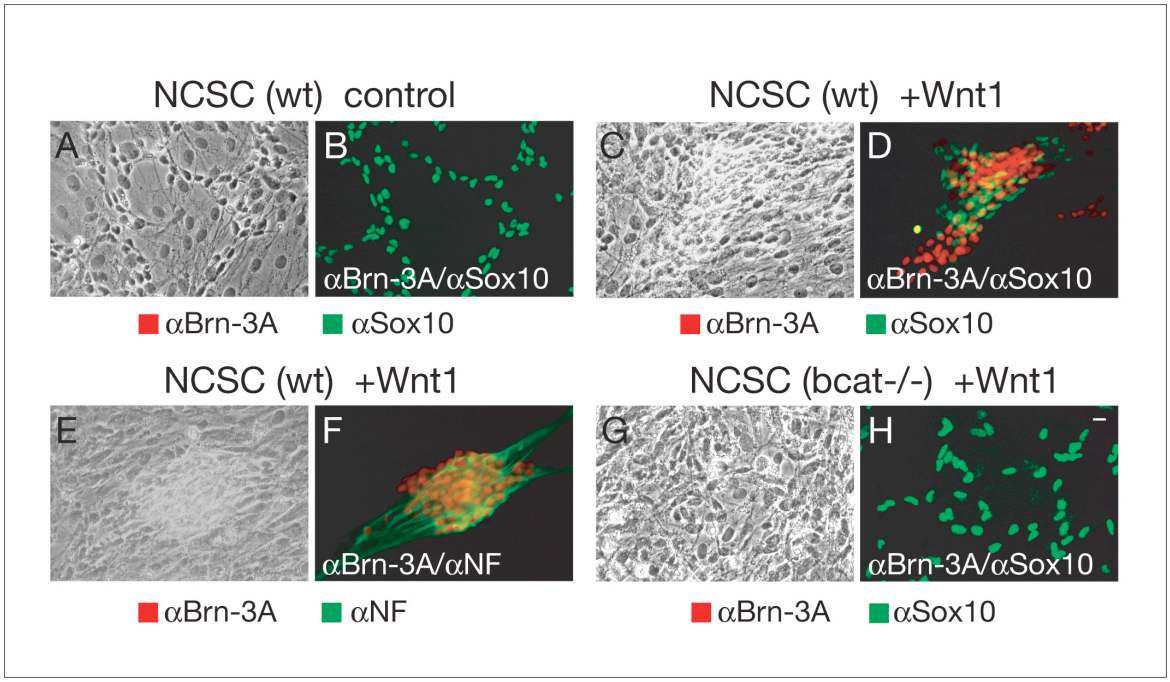

Figure 26: Canonical Wnt/β-catenin signaling instructs NCSCs to adopt a sensory fate

(**A-H**) The effect of Wnt1 is β-catenin dependent. Wild-type (wt) NCSCs exposed to control monolayers fail to generate Brn-3A-positive sensory neurons (**A,B**), while wt NCSCs exposed to Wnt1 monolayers form ganglion-like cell aggregates containing Brn-3A/NF-positive sensory neurons (**C-F**). β-catenin-deficient (β-cat$^{-/-}$) NCSCs are unable to generate sensory neurons, despite the presence of Wnt1 (**G-H**). Corresponding phase contrast pictures (**A, C, E, G**). Scale bar, 10 µm.

6.1.8 Wnt Acts Instructively on eNCSCs to Adopt a Sensory Neuronal Fate

The effect of Wnt/β-catenin signaling could, in principle, be explained by two distinct models: Wnt/β-catenin might promote the expansion of a sensory progenitor that has segregated from a neural crest cell with autonomic and other potentials (Greenwood et al., 1999; Sieber-Blum, 1989; Zirlinger et al., 2002). Alternatively, Wnt/β-catenin might have an instructive influence on fate decisions in an early NCSC able to generate sensory and autonomic neurons, glia, smooth muscle, and possibly other neural crest derivatives (Baroffio et al., 1991; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). The latter model is supported by the fact that *in vivo* sensory cells are not just

expanded, but rather generated at the expense of virtually all other neural crest lineages upon sustained β-catenin activation (Fig. 19, 20). To rigorously distinguish between these models, we challenged early neural crest cells at clonal density with Wnt1 and, on sister plates, with different growth factors previously shown to promote specific fates in NCSCs (Shah et al., 1996) (Fig. 27 A-F; table 2). In control cultures without instructive growth factors, 88±6.7% of all prospectively identified NCSCs generated mixed clones containing autonomic neurons and glia. In agreement with previous studies (Shah et al., 1996), BMP2 induced Mash-1-dependent autonomic neurogenesis in 90.5±3.7% of all NCSCs, while upon TGFβ treatment, about 70% of the cells adopted a smooth muscle-like fate. In the presence of Wnt1, 79.6±2.5% of all NCSCs generated clones containing Brn-3A-positive sensory neurons. Most of these (95.4±3.4%) were sensory neuron-only clones that were not associated with Sox10 staining. The clone size of Wnt1-treated NCSCs was small, with many NCSCs giving rise to a single sensory neuron (Fig. 27 B, C). Furthermore, cell death was minimal in all clonal experiments (table 2), excluding selective effects of the factors added. The combined data indicate that Wnt signaling does not induce proliferation of a restricted sensory progenitor but rather promotes sensory fate decision in multipotent early NCSCs (eNCSCs).

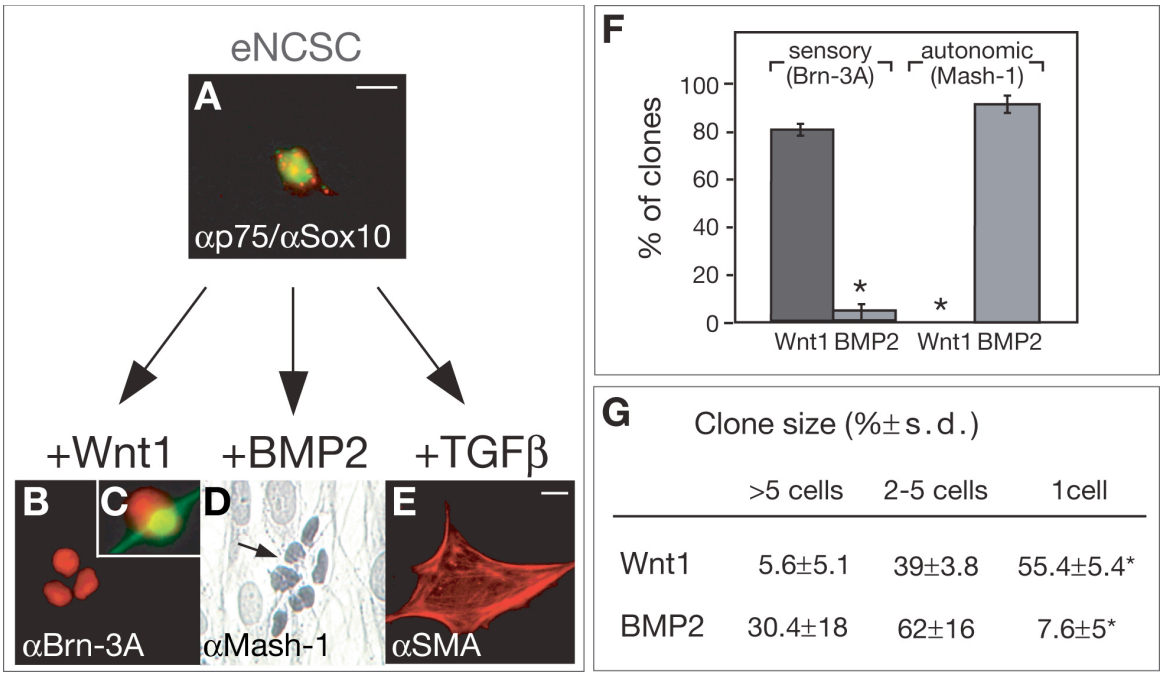

Figure 27: Wnt1/β-catenin signaling instructs eNCSCs to adopt a sensory neuronal fate

(**A-F**) Clonal analysis demonstrates responsiveness of wt early NCSCs (eNCSCs) to different instructive growth factors including Wnt1. 90.3±0.7% of the p75-positive (red) founder cells coexpressed Sox10 (green) (**A**). In the presence of Wnt1, founder cells generated clones of Brn-3A-positive sensory neurons (**B**) expressing NF (**C**). BMP2 instructed eNCSCs to generate Mash-1-expressing autonomic cells (arrow in **D**). TGFβ induced a smooth

muscle-like fate in most of the eNCSCs (**E**). Scale bars, 10 μm. (**F**, table 2) Quantification of clone composition. (*) p <0.001. The data are expressed as the mean±s.d. of three independent experiments. 50 to 150 clones were scored per experiment. (**G**) The cell number within individual Wnt1- and BMP2-treated clones was analyzed. Numbers (percentage of all clones per condition) are shown as the mean±s.d. of three independent experiments, scoring 50 to 150 clones per experiment. (*) p <0.01.

condition	death	sN	sN+G	G	aN
control	2±2	0	0	98±2	80.4±5.6
Wnt1	7.6±3.8	75.8±1	3.8±2.8	12.8±1.5	0
BMP2	9.5±3.7	4.5±3.9	0	15.3±2.9	90.5±3.7
TGFβ	34±21.4	0	0	0	0

Table 2: Clonal analysis of p75-positive NCSCs in the presence of different instructive growth factors. Wild-type eNCSCs at clonal density were treated as described in Materials and Methods, and the progeny of single p75-positive cells was characterized by immunocytochemistry for Brn-3A and Sox10 or, independently, for Mash-1. The numbers indicate the phenotype of the progeny in %±s.d. '†' designates lost clones. A colony was labeled 'sN' (sensory neuron), 'G' (presumptive glia), or 'aN' (autonomic neuron) when at least one cell was expressing the corresponding marker. Note that in control conditions, 85.5±2.0% of the 'G'-clones are associated with NF. This, together with the Mash-1 staining, indicates that most clones in the control are mixed 'aN+G'. In the presence of TGFβ, all surviving clones were composed of smooth muscle-like cells. The low numbers of lost clones demonstrate that Wnt1, BMP2, and TGFβ are not selectively eliminating certain cell lineages but are acting instructively on eNCSCs. The numbers represent the mean±s.d. of three experiments, scoring 50 to 100 clones per experiment.

In vivo, members of the Wnt family specify neural crest from early dorsal neuroepithelial cells (Garcia-Castro et al., 2002). Furthermore, ablation of *wnt1* and *wnt3* pointed to a role of Wnt signaling in expansion of dorsal neural tube cells including the premigratory neural crest (Ikeya et al., 1997). Our present data, together with the β-catenin loss-of-function analysis, indicate a subsequent function of Wnts in neural crest cells as they emigrate. Although the sensory lineage segregates early in PNS development (Greenwood et al., 1999; Sieber-Blum, 1989; Zirlinger et al., 2002), emigrating neural crest cells initially represent a population of stem cells (eNCSCs) that are homogeneous with respect to many developmental potentials,

including sensory, autonomic, glial, smooth muscle-like, and possibly mesenchymal and other lineage formation (Fig. 2; (Frank and Sanes, 1991)). Similar to other growth factors that promote alternative fates (Shah et al., 1996), Wnt/β-catenin induces sensory neurogenesis by acting instructively on these eNCSCs. The molecular context that allows Wnt signaling to regulate cell cycle progression in certain stem cells (Chenn and Walsh, 2002; Megason and McMahon, 2002; Reya et al., 2003; van de Wetering et al., 2002; Willert et al., 2003) and fate decision processes in NCSCs awaits investigation (Figure 28).

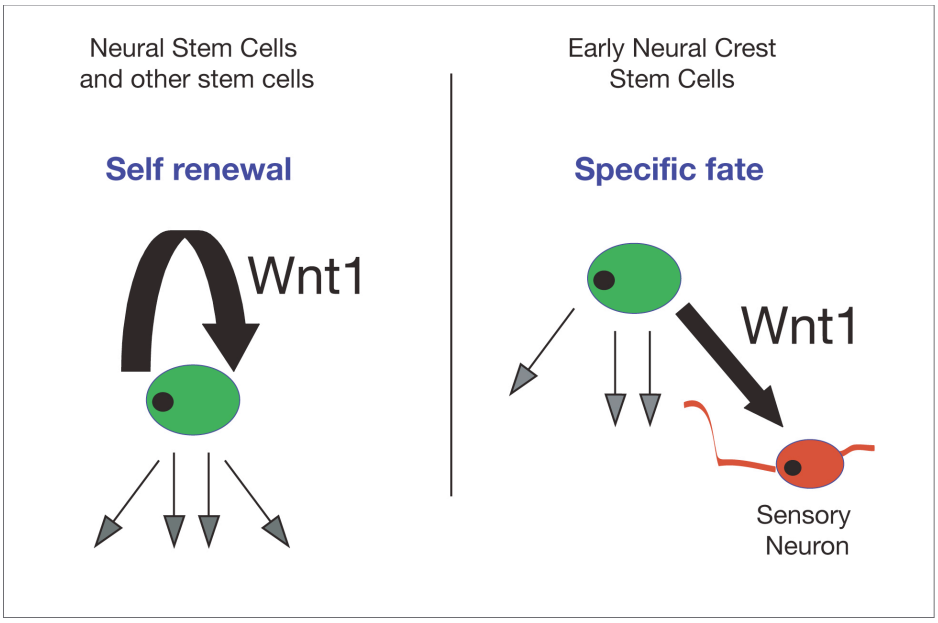

Figure 28: Wnt signaling in neural crest stem cells

While Wnt proteins control stem cell expansion in certain stem cells, Wnt/β-catenin signal activation in neural crest stem cells has little effect on the population size and instead regulates fate decisions.

6.2 Discussion

6.2.1 Making sense of the sensory lineage (Adapted from Marianne Bronner-Fraser; (Bronner-Fraser, 2004))

Due to the fact that NCSCs can give rise to so many different progeny the question arises whether they are true „stem cells"? The definition of a stem cell is, that it divides to form one multipotent daughter cell like itself and another that is biased toward a particular cell fate. In support of the idea that NCSCs are „true stem cells" with stem cell properties, individual NCSCs form multiple derivate tissues both in *vivo* (Bronner-Fraser and Fraser, 1988) and in *vitro* (Anderson, 1997). Furthermore, they have the ability to self-renew, consistent with the principal characteristic of a true stem cell. How is a neural progenitor cell driven to adopt one of its many possible fates? A variety of growth factors can bias neural crest cells grown at clonal density toward certain lineages. For example, bone morphogenic protein (BMP) causes cloned neural crest cells to form autonomic neurons; glia growth factor (GGF, know as neuregulin) drives them to become glia or Schwann cells (Leimeroth et al., 2002; Shah and Anderson, 1997). Endothelin as well as Wnts have shown to bias NCSCs to become melanocytes (Dorsky et al., 1998; Lahav et al., 1998). However, the factors responsible for driving NCSCs to become sensory neurons have remained elusive. One possibility is that NCSCs lose the ability to form sensory neurons as they emigrate from the dorsal neural tube. Support for this hypothesis comes from early studies showing that neural tube cultures (containing premigratory NCSCs within the neural tube) could form sensory neurons, but that this ability appears to be absent in NCSCs that are already migrating (Lo et al., 2002). Similarly, transplanted dorsal neural tubes, but not transplants of migrating NCSCs, give rise to sensory neurons (Le Douarin and Kalcheim, 1999). An alternative explanation is that the neural tube may contribute some factor required for the formation of sensory neurons.

One family of factors prominent in the dorsal neural tube in the region from which NCSCs emerge is the Wnt family of secreted ligands, particularly Wnt1 and Wnt3A. In the nervous system, Wnt proteins play a variety of roles in neural crest development. They are important for induction (Garcia-Castro et al., 2002), proliferation (Dickinson and McMahon, 1992) and specification (Dorsky et al., 1998) of the melanocyte lineage during various stages of neural crest development. In the neural tube they are important for the proliferation and amplification of the neural progenitor cell pool (Zechner et al., 2003). To adress the role of canonical Wnt signaling in the development of NCSCs, we engineered mice in which the canonical Wnt pathway is constitutively activated in neural crest progenitor cells of the

developing dorsal neural tube. The mutant neural crest cells migrated to many appropriate locations within the embryo but failed to differentiate properly. Even in the location where sympathetic ganglia should form, the NCSCs containing activated β-catenin formed sensory neurons rather than sympathetic neurons. In addition, there were reduced numbers of NCSCs in the peripheral nerve that is normally populated by Schwann cells, few cells were found in the cardiac outflow tract and under the skin where pigment cells differentiate. Instead, sensory neurons were generated in unexpected sites. These findings suggest that constitutive activation of the canonical Wnt pathway biases neural crest cells toward a sensory neuronal fate (Figure 29).

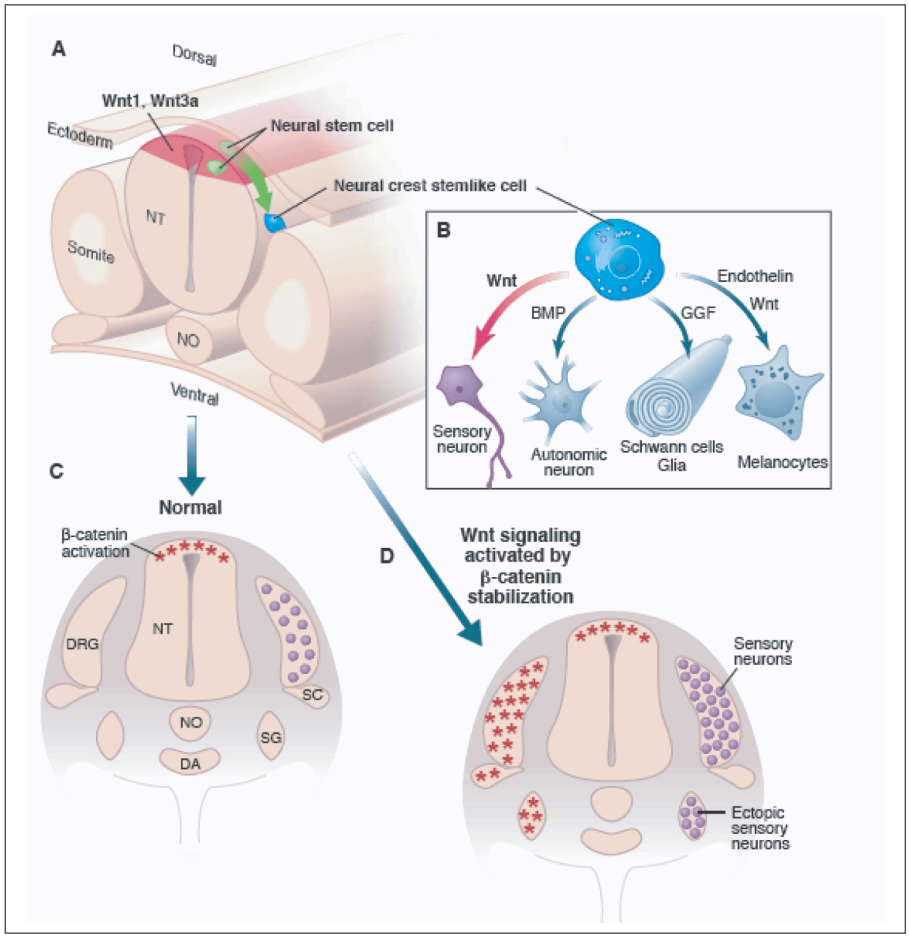

Figure 29: Making sense of the sensory lineage

(**A**) Neural crest precursor cells are contained within the dorsal neural tube (NT). They undergo an epithelial-mesenchymal transition, giving rise to migratory neural crest cells with stem cell-like properties. (**B**) Under the influence of growth factors encountered by the environment, neural crest stem-like cells are biased towards various fates. The work by Lee, Sommer, and colleagues shows that activation of Wnt signaling biases these cells toward a sensory lineage. In contrast, BMP signals influence neural crest cells to acquire properties of sympathetic neurons; GGF drives them toward a glial cell fate; and endothelin and later Wnt signals drive them toward a melanocyte fate. (**C**) During normal development, migrating neural crest cells settle in a variety of

locations to form dorsal root ganglia (DRG), sympathetic ganglia (SG) and Schwann cells (SC) along the ventral route. (**D**) In transgenic mice in which the canonical Wnt-signaling pathway activated in migrating neural crest cells, these cells express markers of sensory neurons even in ectopic locations like the sympathetic ganglia, suggesting that activation of Wnt signaling transform neural crest cells into sensory neurons. (NO, notochord; DA, dorsal aorta) (Bronner-Fraser, 2004).

One possible explanation for these results is that Wnt signaling may promote proliferation and expansion of sensory progenitor cells so that they greatly outnumber other neural crest derivatives. Therefore Wnt would act selective to promote the expansion of a sensory progenitor rather than act on a early homogenous neural crest population. Alternatively, canonical Wnt signaling may play an instructive role-biasing NCSCs to form sensory neurons at the expense of other neural crest derivatives. In that model Wnt would act instructively on a so far unidentified homogeneous population of early NCSCs (Figure30).

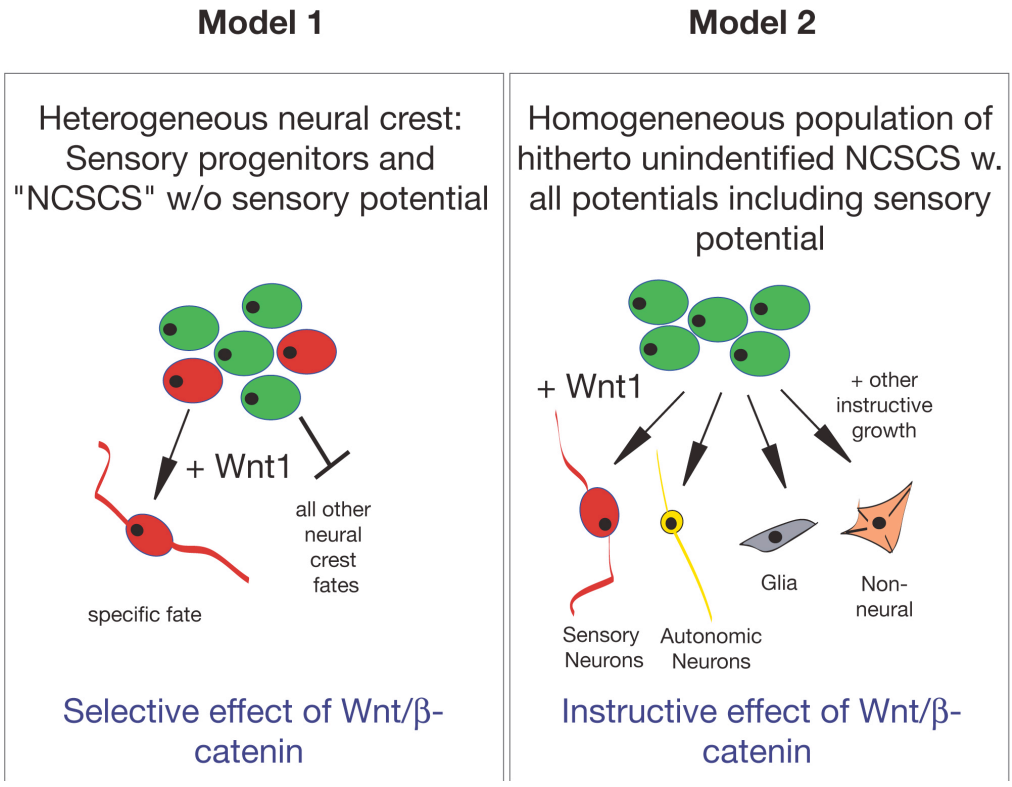

Figure 30: Selective versus instructive effects

(**A**) The first model favours a selective effect of Wnt/β-catenin signaling in the expansion of sensory progenitors, rather than acting on a multipotent neural crest stem cell population (**B**) This model favours an instructive effect of Wnt/β-catenin signaling on a homogenous early neural crest stem cell population with all potentials including a sensory potential, in which Wnt/β-catenin promotes sensory neurogenesis.

To distinguish between these possibilities at a cellular level, a single eNCSC had to be challenged with Wnt and other factors recently been shown to promote certain fates in NCSCs (Shah et al., 1996). In the presence of Wnt, a large majority (80%) of the clones were very small and only formed sensory neurons. This result implies that Wnts do not affect proliferation of multipotent neural crest progenitors, but rather bias their fate toward a sensory phenotype. Taken together, our data suggest that Wnt signaling acts early in the neural tube to promote sensory fate decisions in multipotent early NCSCs. But how do we reconcile this finding with other studies where activation of Wnt signaling drives neural crest cells to form melanocytes in the zebrafish (Dorsky et al., 1998) or causes overgrowth of the dorsal neural tube in the mouse (Zechner et al., 2003)? This issue is very likely to be one of timing. Perhaps activation of Wnt signaling in early NCSCs promotes a sensory fate, whereas later activation in combination with endothelin promotes melanocyte formation. This difference in timing could explain the differences in the gain-of-and loss-of-function phenotype. There is ample evidence that Wnts play multiple roles at multiple times, even in the same cell type. The challenge in the future will be to understand how the same ligand can elicit such different responses depending on the stage of development. In addition to stimulatory, migrating neural crest cells encounter environments rich in inhibitors that may counteract the influences of signaling molecules such as Wnts and BMPs. Perhaps these inhibitors titrate or delay responses as neural crest cells migrate along particular pathways. In this way, the lineage choices of neural crest progenitor cells may become progressively more limited as development proceeds.

6.3 Outlook

6.3.1 Convergence of Wnt/β-catenin by other Signaling Pathways

Given that all eNCSCs can give rise to sensory neurons in a clonal assay (Lee et al., 2004), and although in *vivo* all cells have seen Wnt shortly before they emigrate out of the neural tube (Brault et al., 2001; Soriano, 1999), the question arises why not all cells adopt a sensory fate *in vivo* during development. We therefore suggest the existence of factors, which would modulate Wnt activity as NCSCs emigrate out of the tube. Such factors therefore must be able to modulate canonical Wnt in different ways. They could either inhibit or they could cooperate with the canonical Wnt signaling pathway, leading to different biological responses. Such an interplay in combination with spatial- and cell intrinsic-changes could

allow a precise regulation of how a diverse array of cell types are generated. Such factors could act at the extracellular level or at every downstream molecule of Wnt, which might also result in different biological responses. First candidates might be the Dickkopf proteins (Dkks), which have been shown to directly interact with the Wnt coreceptor LRP. Binding of Dkks to LRPs competitively disrupts the binding complex of Wnt and frizzled and therefore inhibts canonical Wnt signaling already at the very first step (Brott and Sokol, 2002; Glinka et al., 1998; Semenov et al., 2001). However, when we added soluble Dkk-1 protein to eNCSCs, to eNCSCs in the presence of Wnt1-expressing feeder layers or to eNCSCs in the presence of control feeder layers, we did not see any biological effect compared to control plates. From these data we conclude that Dkk proteins play either not a significant role during neural crest development, the concentration of added soluble Dkk might be not enough to compete with secreted or endogenous Wnt or the time point at which NCSCs are challenged with Dkk proteins is too late to influence the effect of canonical Wnt. We therefore aim to investigate the role of other signaling molecules, namely Notch, FGFs and BMPs, which have already been shown to interact with the Wnt signaling pathway. Inhibitory cross-talk between Notch and Wnt signaling has been reported in various biological systems (De Strooper and Annaert, 2001), occurring at the level of the Notch extracellular domain binding to Wnt (Wesley, 1999) or at the level of the Notch intracellular domain binding to Disheveled (Axelrod et al., 1996). Cooperative functions between Notch and Wnt have also been reported. In haematopoetic stem cells canonical Wnt induces Notch1 expression (Reya et al., 2003). In neural crest development loss of Notch1 in β-catenin loss-of function mutation (Hari et al., 2002) strongly indicates that canonical Wnt signaling influences Notch1. Whether Notch1 expression, induced by Wnt signaling, acts inhibitory on Wnt via a negative feedback loop might be investigated in a double mutant, in which β-catenin is constitutively activated and Notch1 ablated. According to this working hypothesis sox10 expression should be completely lost within the ectopic ganglia of sensory neurons in such double mutants, since Notch1 might normally function to maintain sox10-positive cells. A careful analysis of such double mutants at embryonic day 10.5, however, indicated a similar phenotype as obtained in the single β-catenin gain-of-function mutants. Possibly, the time point of Notch1 expression at the stage of the forming dorsal root ganglia at embryonic day 10.5, might be too late for Notch signaling to influence early migrating neural crest stem cells. Thus, it is presently unclear whether Notch1 plays a role in NCSCs. An explanation for early lineage segregation might be that canonical Wnt is modulated at the very early time point of neural crest emigration. We therefore have to focus on growth factors expressed at the stage of neural crest emigration.

6.3.2 Signaling Pathways that could Interact with Canonical Wnt at the Stage of Neural Crest Emigration

An early lineage segregation of eNCSCs leads to the generation of sensory neurons and further migrating NCSCs remaining Sox10-positive (Anderson, 2000). When we ought to understand how Wnt-responsive eNCSCs segregate to sensory as well other neural crest derivatives, we have to focus on the molecules which are expressed in the dorsal part of the neural tube, the molecules that are expressed between the interface of the neural tube and the ectoderm as well as on the molecules that are expressed in the surface ectoderm. In parallel to Wnts, BMPs and FGFs might be good candidates, possibly involved in such a process. Evidence obtained primarily from studies in *Xenopus* models, strongly suggests that two independent signals are essential for neural crest induction. First, BMP signals, which must be partially modulated (Liem et al., 1997; Liem et al., 1995; Marchant et al., 1998), and a separate input, that can be either Wnt signals (Bronner-Fraser, 2002; Dorsky et al., 1998; Dorsky et al., 2000a; Garcia-Castro et al., 2002; Ikeya et al., 1997) or FGF signals (Holowacz and Sokol, 1999). Expression of early neural crest markers such as slug and AP2α are therefore dependent on Wnts, BMPs and FGFs (Luo et al., 2003). BMPs as well FGFs have recently been shown to interact with the canonical Wnt signaling pathway (Hussein et al., 2003; Israsena et al., 2004; Labbe et al., 2000). It is likely that BMPs and FGFs are still present as NCSCs emigrate from the neural tube and that an interplay between these factors might regulate early fate decisions processes in eNCSCs by modulating canonical Wnt. In order to address such a hypothesis eNCSCs should be exposed to all factors at the same time point and in parallel one should try to specifically inhibit one of the factors by adding pharmalogical inhibitors, such as noggin for BMPs (McMahon et al., 1998) and chicken embryo extract for Wnts (Kléber and Sommer, in press). Moreover, the medium in which we culture eNCSCs contains high doses of bFGF (Greenwood et al., 1999; Kléber and Sommer, in press; Monsoro-Burq et al., 2003). Changes in FGF dosage in the medium might clarify the question, how FGFs might be involved in that process.

6.3.3 Canonical Wnt Activity at Sites of Postmigratory NCSCs

Before testing the response of postmigratory NCSCs to Wnt, it would be interesting to check whether canonical Wnt signaling is still present at sites of postmigratory NCSCs. If not, this would suggest that postmigratory NCSCs do not need canonical Wnt signaling in order to elicit their biological function.

To address that question an analysis of whole mount embryos of mice harboring TOPGAL should be performed, a β-galactosidase gene under the control of a LEF/TCF and β-catenin inducible promoter. Such an analysis at different time points will reveal whether canonical Wnt signaling persists at locations of postmigratory NCSCs.

6.3.4 The Role of Canonical Wnt Signaling in Postmigratory NCSCs

Cell-intrinsic changes during neural crest development can affect fate decisions by changing the sensitivity of neural crest cells to integrate extracellular factors (Kruger et al., 2002; Paratore et al., 2001; Paratore et al., 2002b; White et al., 2001). Since the timing at which a certain stem cell integrates canonical Wnt can elicit different responses it would be interesting to know whether postmigratory NCSCs show similar effects like eNCSCs when they are exposed to Wnt. *In vivo* xenotransplantation studies have demonstrated that postmigratory NCSCs isolated from sciatic nerve compared to migratory NCSCs display quantitatively less neurogenic activity (White and Anderson, 1999; White et al., 2001). Parallel *in vitro* clonogenic assays indicated that this reduced neurogenic activity reflects a decreased sensitivity to BMP2 between sciatic nerve derived NCSCs and migratory NCSCs (White et al., 2001). Moreover, gut NCSCs were more responsive to neurogenic factors, while sciatic nerve NCSCs were more responsive to gliogenic factors. These data clearly demonstrate that cell-intrinsic differences between NCSCs isolated from different regions of the PNS regulate the generation of neural diversity (Bixby et al., 2002). The decreased sensitivity of sciatic nerve NCSCs to BMP2 is correlated with a small but statistically significant decrease in the expression levels of the type 1A BMP receptor (BMPR1a) mRNA between the neural crest populations. Likewise, in regard to canonical Wnt it would be interesting to check whether frizzled receptors are still expressed in postmigratory NCSCs. To address that question, one should isolate postmigratory NCSCs from DRG and sciatic nerve and expose them to Wnt. A careful clonal analysis should clarify of whether canonical Wnt is still able to instruct and promote sensory neurogenesis or elicit another response compared to controls.

6.4 Wnt Signaling and the Regulation of the Stem Cell Function

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Current Opinion Cell Biology, Vol. **16**, Issue **6**

Canonical Wnt signaling plays a crucial role in controlling cell expansion in many types of stem cells. Recent studies, however, demonstrated that Wnt is not only a general stem cell growth factor but can also influence cell lineage decisions in certain stem cell types by promoting specific fates at the expense of others. Thus, Wnt signaling elicits multiple functions in stem cells. Wnt activity appears to depend on cell-intrinsic properties that might change with time during development, thereby altering the cellular response to Wnt. Moreover, the spatial context of a stem cell also determines how the cell interprets Wnt signal activity, in that synergistic or antagonistic signaling pathways can modulate Wnt signaling. How a stem cell integrates Wnt and other signals and how such signaling networks regulate stem cell function on the molecular level remains to be elucidated.

6.4.1 Introduction: Canonical Wnt Signaling

Wnt proteins are cysteine-rich lipid-modified proteins (Willert et al., 2003) that play a major role in various processes during development including cell proliferation and differentiation, cell fate decisions, apoptosis, axial polarity, and axonal guidance (Cadigan and Nusse, 1997; Nelson and Nusse, 2004). The details of the Wnt signal transduction pathways have been covered elsewhere (see e.g. (Nelson and Nusse, 2004)), and we only briefly summarize here the main features of the pathway. Wnts tightly interact with ECM molecules and elicit their full biological activity on neighboring cells by binding to frizzled receptors. Canonical Wnt signaling leads to subsequent activation of Dsh, which prevents ubiquitin-mediated degradation of cytoplasmic β-catenin by displacing GSK3β from the Axin/APC complex. Upon activation, β-catenin is stabilized and translocated to the nucleus where it associates with TCF/Lef transcription factors and induces transcription of Wnt target genes. Besides the canonical pathway, Wnts can also induce the cell polarity pathway, which directs asymmetric cytoskeletal organization, and the Ca^{2+} pathway via G-proteins. Canonical Wnt activity can be altered at several steps of the signal transduction pathway by other signaling molecules, including Notch, TGFβ factors, FGFs, and Shh (De Strooper and Annaert, 2001; Nelson and Nusse, 2004). Moreover, due to the dual function of β-catenin in both Wnt signaling and Cadherin-dependent cell-cell interactions, canonical Wnt signal activation can directly

influence cell adhesion. Cell-cell interactions can furthermore be influenced by Wnt-induced upregulation of cell-adhesion markers, which in turn can negatively regulate canonical Wnt signaling (Nelson and Nusse, 2004).

In this review, we discuss recent progress of how the canonical Wnt signaling pathway influences the regulation of stem cells in regard to both expansion and differentiation. We focus on the cell-intrinsic properties of a stem cell and on the extracellular environment in which the stem cell encounters the Wnt signal.

6.4.2 Canonical Wnt Signaling Regulating Stem Cell Maintenance and Proliferation

Wnt/β–catenin signaling maintains "stemness" in stem cells from different tissues and prevents them from differentiating. In embryonic stem cells, overexpression of Wnt1 or of stabilized β-catenin and lack of APC results in the inhibition of neural differentiation and in activation of downstream targets of Wnt signaling including cyclins and c-myc (Aubert et al., 2002; Haegele et al., 2003). Moreover, treatment of embryonic stem cells with a synthetic pharmacological inhibitor of GSK3β activates the canonical Wnt pathway and sustains pluripotency and self-renewal (Sato et al., 2004). Finally, mutations in APC that are associated with increased tumor incidence and increased intracellular doses of β-catenin interfere with embryonic stem cell differentiation into the three germ layers (Kielman et al., 2002).

As in embryonic stem cells, Wnts regulate proliferation of intestinal stem cells (Pinto et al., 2003; van de Wetering et al., 2002), skin stem cells (Alonso and Fuchs, 2003), and haematopoietic stem cells (Austin et al., 1997; Reya et al., 2003). In particular, treatment of haematopoietic stem cells with Wnt proteins and sustained expression of β-catenin promotes self-renewal in long-term cultures and increases the reconstitution of haematopoietic lineages *in vivo* (Reya et al., 2003). In contrast, applying soluble inhibitors of the Wnt signaling pathway or ectopic expression of Axin reduces haematopoietic stem cell growth in culture and haematopoietic lineage reconstitution *in vivo*. Wnt signaling in haematopoietic stem cells might be mediated by Notch1 and the transcription factor HoxB4, both of which have also been implicated in self-renewal of haematopoietic stem cells (Antonchuk et al., 2002; Varnum-Finney et al., 2000).

Wnts play major roles during CNS development. Ablation of *wnt1* results in severe defects of the midbrain, the cerebellum, and the developing spinal cord (Ikeya et al., 1997; McMahon

and Bradley, 1990; McMahon et al., 1992), while ablation of *wnt3A* results in a total loss of the hippocampus (Lee et al., 2000). The defects observed in Wnt mutants are possibly explained by perturbed proliferation of stem or progenitor cells in the ventricular zone (Dickinson et al., 1994). In agreement with this view, overexpression of Wnts indicated their mitogenic role during spinal cord development (Megason and McMahon, 2002). Moreover, sustained expression of a stabilized form of β-catenin in neuroepithelial precursors in the ventricular zone led to enlarged brains with increased cerebral cortical size due to an increase of the progenitor pool (Chenn and Walsh, 2002). Similarly, in the developing spinal cord, β-catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation (Zechner et al., 2003). It remains to be shown whether the observed phenotype is due to changes in neural stem cells or in a subpopulation of amplifying progenitors. Supporting these *in vivo* experiments, infection of mouse forebrain explant cultures with a retrovirus expressing Wnt7a promoted proliferation and suppressed neural differentiation of neural precursor cells (Viti et al., 2003). Likewise, overexpression of β-catenin in neural stem cells isolated from mouse cortex generated significantly more secondary neurospheres in the presence of FGF2 than did control neural stem cells (Israsena et al., 2004).

The function of Wnt in stem cell expansion and in suppression of differentiation likely explains why deregulation of the Wnt signaling pathway is associated with the induction and progression of several forms of cancer. It also suggests that cancers may result from the dysregulation of stem cell programs. Accordingly, Wnt/β-catenin signaling is not only essential for the homeostasis of the intestinal epithelium (Pinto et al., 2003; van de Wetering et al., 2002) but sustained β-catenin activity has also been implicated in the formation of colon carcinoma (Harada et al., 1999; van de Wetering et al., 2002). Likewise, certain forms of medulloblastomas harbor mutations in genes encoding components of the Wnt signaling pathway (Oliver and Wechsler-Reya, 2004). Therefore, the knowledge of how canonical Wnt signaling regulates cell proliferation during normal development should yield important insights into regulatory mechanisms involved in cancer progression.

6.4.3 Canonical Wnt Signaling Promoting Fate Decisions in Stem Cells

In addition to the above mentioned examples of Wnt promoting proliferation, Wnt also influences lineage decisions in stem cells. Hirabayashi and colleagues recently reported that Wnts promote neuronal differentiation of neural stem cells in the neocortex at E11.5 at the expense of neural stem cell expansion (Hirabayashi et al., 2004). In another study, in which

neural stem cells derived from the telencephalon were cultured as neurospheres and challenged by Wnt signaling, it was reported that Wnt3A inhibits the maintenance of neural stem cells and promotes the differentiation into neuronal and astrocyte lineages (Muroyama et al., 2004). Moreover, although canonical Wnt signaling has been associated with controlling spinal cord progenitor proliferation (Zechner et al., 2003), Muroyama and colleagues have described a requirement for Wnt signaling in neuronal specification in the dorsal spinal cord (Muroyama et al., 2002). It is not yet clear how these results can be reconciled. Apart from different experimental approaches, region-specific or subtype-specific functions of Wnts might be involved. A role of canonical Wnt signaling in fate acquisition is also supported by studies on nonneural tissues. During muscle injury, Wnts promote myogenic specification and are involved in muscle regeneration (Polesskaya et al., 2003), while in the skin, *in vivo* manipulation of genes encoding Wnt signaling components indicates an essential role of Wnt in fate decision processes of epidermal stem cells (Huelsken et al., 2001; Merrill et al., 2001). In particular, β-catenin-deficient stem cells fail to differentiate into follicular keratinocytes and instead adopt an epidermal fate (Huelsken et al., 2001).

Likewise, Wnt has been shown to regulate lineage decisions rather than stem cell maintenance in neural crest stem cells (NCSCs). Neural crest cells generate most structures of the PNS and nonneural tissues such as cells in the outflow tract of the heart, craniofacial bone and cartilage, connective tissue, and melanocytes of the skin (Le Douarin and Dupin, 2003). Wnts have several functions during neural crest development. They induce neural crest from neural plates, and inhibition of Wnt signaling perturbs neural crest formation *in vivo* (Garcia-Castro et al., 2002). Wnt1 and Wnt3A are expressed in the dorsal part of the neural tube at the time of neural crest emigration, and deletion of both *wnt1* and *wnt3A* results in a marked reduction of neural crest derivatives (Ikeya et al., 1997). Furthermore, activation of Wnt signaling in neural crest has been associated with the formation of pigment cells (Dorsky et al., 1998; Dunn et al., 2000; Jin et al., 2001). To address the role of canonical Wnt signaling specifically in NCSC development, conditional loss- and gain-of function mutants have been generated, in which β-catenin was either ablated or stabilized in NCSCs. Upon loss of β-catenin, sensory ganglia and melanocytes fail to develop *in vivo*. In culture, NCSCs lacking β-catenin emigrate and proliferate normally, but are unable to acquire a sensory neuronal fate (Hari et al., 2002). In contrast, sustained β-catenin activity in NCSCs results in the formation of sensory neurons (including some at ectopic locations) at the expense of all other neural crest derivatives *in vivo* (Lee et al., 2004). As in the loss-of-function study (Hari et al., 2002), cell culture experiments revealed that NCSCs with sustained β-catenin activity migrate and

proliferate normally. However, virtually all mutant cells gave rise to sensory neurons, while the expression of NCSC markers was suppressed. Furthermore, clonal analysis of wild type cells demonstrated that an early population of NCSCs (eNCSCs) is able to give rise to sensory neurons in response to Wnt. Thus, unlike in other stem cells, canonical Wnt instructively promotes cell fate decisions rather than stem cell expansion in eNCSCs (Lee et al., 2004).

6.4.4 Cell-Intrinsic Differences Regulate the Output of Canonical Wnt Signaling

The question arises as to how canonical Wnts can fulfill diverse functions in stem cells. The fact that stem cells from different locations interpret Wnt in different ways obviously reflects an activation of distinct genetic programs in response to the same signal (Fig. 31A). While cyclin D1 and c-myc are direct target genes of β-catenin/Lef1 involved in cell expansion (see http://www.stanford.edu/~rnusse/pathways/targets.html), recent studies revealed the proneural genes neurogenins as targets of β-catenin/TCF/Lef during neurogenesis (Hirabayashi et al., 2004; Israsena et al., 2004). The activation of specific sets of Wnt target genes is likely to be mediated by cell type-specific intrinsic properties. The nature of these is unknown to date, but alterations in the combination of TCF/LEF transcription factors interacting with β-catenin may be involved (Merrill et al., 2001). In the future, transcriptome and, if possible, proteome comparisons between different types of stem cells might help to identify molecules involved in determining their Wnt responsiveness.

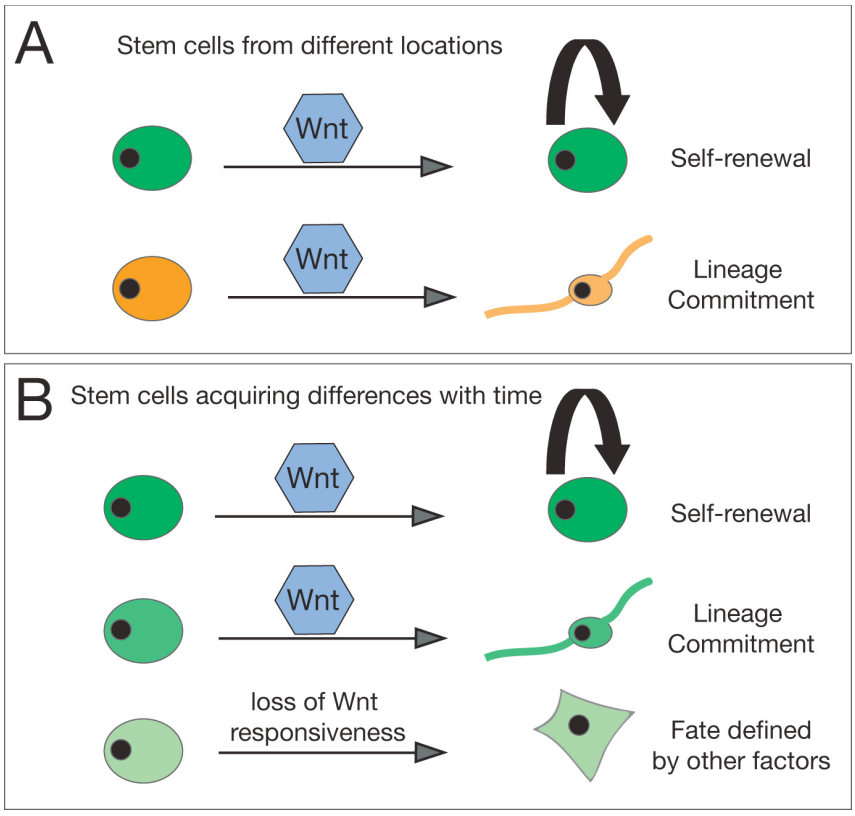

Figure 31: Cell-intrinsic differences among stem cells influence the biological function of Wnts. (**A**) Different stem cell types differentially respond to canonical Wnt signaling and undergo either self-renewal or lineage commitment. The differential responsiveness of stem cells is presumably due to distinct cell-intrinsic determinants (indicated in the figure by differently colored cells). (**B**) A stem cell type of a given cell lineage (indicated in various grades of green) can integrate canonical Wnt signaling in different ways, depending on its cell-intrinsic properties which change over time. At different stages of development, Wnt promotes stem cell self-renewal or lineage commitment, or the cell loses its ability to respond to Wnt.

Changes in intrinsic properties might also be acquired by a given stem cell over time. According to this model (Fig. 31B), the time point during development at which a stem cell is challenged by Wnt signals determines whether the cell responds by self-renewal or differentiation. There is precedence for this process to occur in neural crest development. Cell-intrinsic differences among stem cells have been demonstrated not only between postmigratory NCSCs from different regions of the PNS, but also between NCSCs isolated at different time points from a particular structure such as the gut (Bixby et al., 2002; Kruger et al., 2002). Thereby, cell-intrinsic changes affect fate decisions during neural crest development by changing the sensitivity of neural crest cells to specific extracellular signals (Kubu et al., 2002; Paratore et al., 2001; White et al., 2001). Conceivably, such changes might allow Wnt signaling to promote sensory neurogenesis in early NCSCs, whereas it induces pigment cell differentiation at later stages (Bronner-Fraser, 2004). Moreover, cell-intrinsic changes might lead to the loss of a cell's responsiveness to Wnt (Fig. 31B). Indeed, preliminary data suggest that postmigratory NCSCs, although multipotent, loose their responsiveness to Wnt signal activation while they are still receptive of other instructive growth factors (Kleber M. and Sommer L., unpublished). A well documented case in which Wnt signaling can elicit its effects on stem cells in a stage-specific manner has recently been provided by Hirabayashi et al. (Hirabayashi et al., 2004). As mentioned above, these authors have shown that Wnt acts as a neurogenic factor on neural stem cells from the neocortex at E11.5. Intriguingly, it was found that canonical Wnt signaling induces lineage commitment only at these late stages of cortical development, whereas at earlier stages it controls the expansion of neural stem cells. Likewise, Wnt7a enhances proliferation of cortical cells only at early but not at late developmental stages (Viti et al., 2003). While the role of Wnt on early neural progenitors is supported by the results obtained by manipulating Wnt signaling *in vivo* (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003), the stage-dependent requirements of canoncial Wnt signaling in neural stem cells should be confirmed

using inducible genetic systems that can be spatially and temporally regulated *in vivo* (Leone et al., 2003).

6.4.5 Modulation of Canonical Wnt by the Extracellular Environment

Besides the cell-intrinsic cues that influence the biological activity of Wnt in distinct stem and progenitor cell types, the same type of stem cell might respond in different ways to Wnts dependent on its extracellular microenvironment. In this model (Fig. 32), Wnt signaling interacts with signaling pathways induced by other cues, and the signaling output is determined by the combined activity of multiple factors (Nelson and Nusse, 2004). For instance, Notch signaling is involved in an inhibitory crosstalk with Wnt signaling at several levels of the signal transduction pathways (De Strooper and Annaert, 2001; Foltz et al., 2002). Furthermore, modulation of canonical Wnt signaling by members of the TGFβ family occurs on the transcriptional level, where Smad4 directly associates with β-catenin/TCF/Lef and cooperatively induces transcription of specific target genes (Hussein et al., 2003; Labbe et al., 2000).

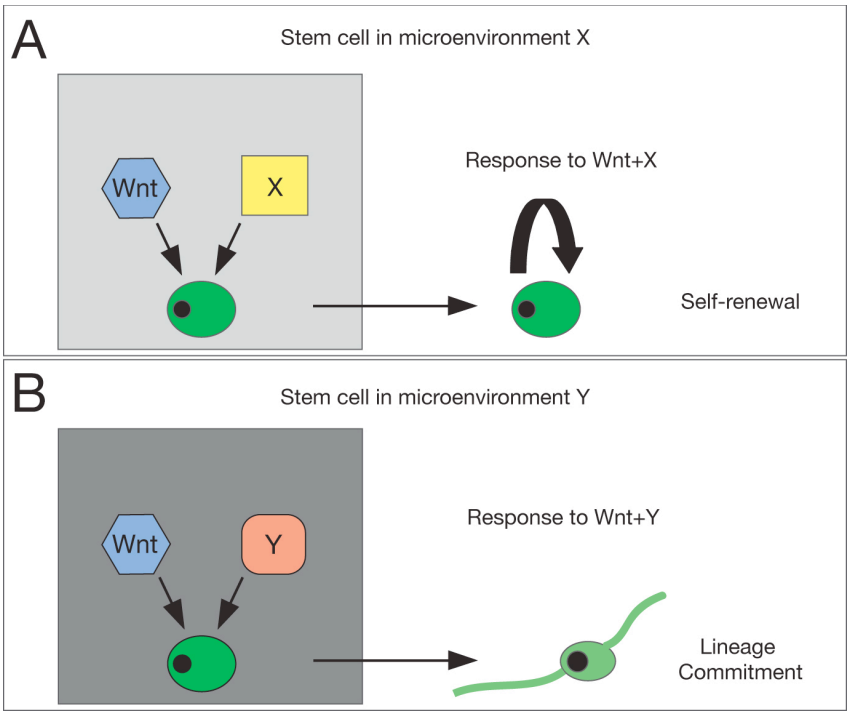

Figure 32: The effect of canonical Wnt on a particular type of stem cell is context-dependent. In a microenvironment X, Wnt activity is modulated by the factor(s) X. In this context, Wnt signaling elicits self-renewal. However, the very same stem cell in microenvironment Y, in which Wnts are modulated by the factor(s) Y, responds to Wnt signaling by adopting a specific cell fate rather than by self-renewing. Thus, the biological activity of Wnt in a particular microenvironment is influenced by the convergence of Wnt signaling with other signal transduction pathways.

In the nervous system, canonical Wnt and BMP signaling cooperatively regulate graded Emx2 expression in the dorsal telencephalon (Theil et al., 2002), whereas the two factors have antagonistic functions in establishing neuronal and glial lineages as opposed to melanocytes in neural crest (Jin et al., 2001). Recent studies also manifest a role for FGFs in modulating canonical Wnt signaling. FGF together with Wnt signaling regulate late features of the dorsal telencephalon (Gunhaga et al., 2003). Levels of active β-catenin are increased in neural stem cells from the ganglionic eminence when they are cultured in the presence of FGF2 (Israsena et al., 2004). Moreover, overexpression of β-catenin in the presence of FGF2 maintains neural progenitors in a proliferative state, while overexpression of β-catenin in the absence of FGF2 results in enhanced neuronal differentiation in adhesive cultures (Israsena et al., 2004). In cortical cells, however, Wnt supports both maturation and proliferation, but only the maturation effect has been reported to be FGF-dependent (Viti et al., 2003). Finally in haematopoetic stem cells, β-catenin activation upregulates Notch1 (Reya et al., 2003), while NCSCs that lack β-catenin fail to express Notch1 in the forming DRG (Hari et al., 2002), suggesting crosstalk between these signaling pathways.

It is conceivable that future experiments will bring to light further examples of signaling pathways interacting with canonical Wnt signaling during stem cell development. In eNCSCs, for instance, factors counteracting Wnts in inducing sensory neurogenesis most probably act at the time point of neural crest emigration from the dorsal neural tube (Fig. 33). This prediction is based on the fact that although virtually all eNCSCs appear to be responsive to Wnts present in the dorsal neural tube (Lee et al., 2004), obviously not all NCSCs adopt a sensory fate in normal development *in vivo*. Thus, while Wnt signaling induces a sensory fate in some NCSCs –presumably by activating the expression of proneural genes in these cells (Hirabayashi et al., 2004; Israsena et al., 2004)– others maintain their stem cell features. Whether this is achieved by sterical hindrance of Wnt to bind to some cells, by inhibition of Wnt activity through an antagonistic factor, or by the active maintenance of stemness in some neural crest cells due to the synergistic activity of Wnt and other factors(s) remains to be elucidated. An equally challenging question is how the initial heterogeneity of emigrating neural crest cells is established, since in the migratory crest the cells with a sensory fate are intermingled in a ‘salt and pepper’ fashion with multipotent neural crest cells fated for other lineages (Zirlinger et al., 2002).

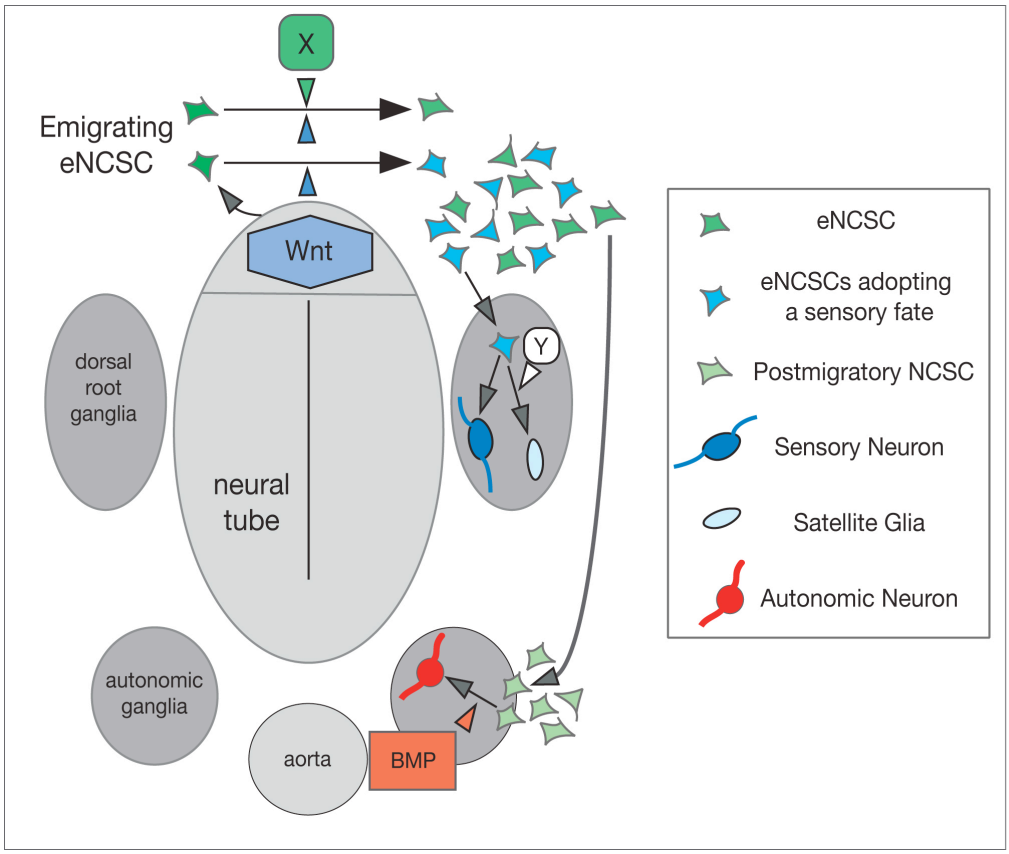

Figure 33: Model of Wnt-dependent lineage segregation in early neural crest stem cells. Wnt expressed in the dorsal neural tube induces the formation of sensory neural cells (blue) from eNCSCs (green) as the latter emigrate from the neural tube. In principle, all eNCSCs are responsive to Wnt signaling. However, because normally not all eNCSCs adopt a sensory fate *in vivo*, we propose that a factor X counteracts Wnt activity early in neural crest development, preventing sensory neurogenesis in some of the emigrating neural crest cells. These cells remain multipotent and encounter other instructive growth factors as postmigratory neural crest cells (light green). For simplicity, of all possible neural crest-derived structures only forming autonomic ganglia are shown, in which BMP induces an autonomic fate. In the dorsal root ganglia, a factor Y prevents some sensory progenitor cells from differentiating into sensory neurons, allowing the formation of satellite glia.

6.4.6 Conclusions

Taken together, we propose that the biological function of canonical Wnts depends on the cell-intrinsic 'status' of a stem cell as well as on its extracellular microenvironment, which may alter Wnt activity during development. This implies that Wnt has to be viewed as part of a signaling network that changes with time and location, and that a stem cell has to integrate Wnt and other signals in a stage- and location-specific manner in order to ensure the generation of appropriate numbers of new stem cells and of differentiating progeny. Future studies will have to focus on how such signaling networks are established and how they elicit multiple Wnt-dependent responses in stem cells.

Part II

Interplay between Wnt and BMP Signaling in Early Neural Crest Stem Cells

7. INTRODUCTION

The building of an organism from a single cell to a multicellular three-dimensional structure of characteristic shape and size is the result of coordinated gene activation that directs the developmental fate of individual cells. The acquisition of different cell fates manages an intricate interplay of cell proliferation, migration, growth, differentiation and death, elaborating and bringing together cellular ensembles in a precise manner. Intrinsic, cell-autonomous factors as well as nonautonomous, short-range and long-range signals guide cells through distinct developmental paths.

The Bone Morphogenic Proteins (BMPs) with more than 20 members represent the largest and most rapidly expanding subclass of the transforming growth factor-β (TGFβ) superfamily of ligands that transduce intracellular signals through serine-threonine kinase receptor subunits (Bonini and Choi, 1995; Hogan, 1996a). BMPs mediate a diverse array of developmental processes including survival, proliferation, morphogenesis, lineage commitment, inhibition of alternate lineages, differentiation and apoptosis (Hogan, 1996b; Mehler et al., 1997).

BMP was first discoverd because of its ability to induce endochondral bone formation when injected subcutaneously in mice. An important role for BMP signaling in formation of mesenchymal condensations is supported by the suppression of formation of condensations when the BMP antagonist, Noggin is expressed in early chick limbs (Pizette and Niswander, 2000) and by the enlarged cartilage primordial in Noggin knock out mice (Brunet et al., 1998). BMPs have multiple important roles during later stages of cartilage development. For instance, addition of BMPs to bone explants increases proliferation of chondrocytes and Noggin blocks chondrocyte proliferation (Minina et al., 2002; Minina et al., 2001). BMP signalling increases the expression of indian hedgehoc (Ihh) by prehypertrophic chondrocytes. In both of these actions BMPs oppose the effects of FGF signaling (Minina et al., 2001). Moreover, BMP2, BMP6 and BMP9 are potent factors to osteogenic differentiation of mesenchymal stem cells (Peng et al., 2004). Further BMPs play a major role during kidney development. BMP7 has been shown to be absolutely crucial for proper kidney formation by acting as an anti-differentiation factor for the mesenchyme cell population (Godin et al., 1999). During intestine development BMP signaling delimits ectopic crypt induction once the orthotropic structures have been specified (Haramis et al., 2004). In *Drosophila* BMPs regulate the development of the larval neuromuscular junction (Keshishian and Kim, 2004). During limb development BMPs regulate the dorsoventral patterning of the apical ectodermal ridge (Wang et al., 2004). In embryonic stem cells biology BMPs seem to play different roles.

Studies of mouse embryonic stem (ES) cell differentiation *in vitro* provided evidence for a critical role of BMP2 in the differentiation of extra-embryonic endoderm, and in the cavitation of the embryo, the process whereby programmed cell death in a subpopulation of the pluripotent stem cells of the inner cell mass leads to the formation of the egg cylinder (Coucouvanis and Martin, 1999). Recently, it has been shown that noggin can block this form of differentiation and further induces the appearance of a novel cell type that can give rise to neural progenitors (Pera et al., 2004). On the other hand BMP4 play a different role in supporting self-renewal of ES cells by inhibiting mitogen-activated protein kinase pathways (Qi et al., 2004). BMP4 signaling induces expression of inhibitor of differentiation (Id) genes and sustains ES cell self-renewal in collaboration with leukemia inhibitory factor (LIF) over STAT3 (Ying et al., 2003).

7.1 BMP Signaling in the Nervous System

BMP ligands and receptor subunits are present throughout neural development within discrete regions of the embryonic brain and within neural crest-derived pre- and postmigratory zones. The vertebrate organizer in the dorsal blast pore lip contains a number of BMP related factors and BMP regulatory proteins that are involved in neurulation and dorsoventral patterning. The organizer contains mesoderm and endoderm and orchestrates a correctly patternend nervous system in the neighbouring dorsal ecotderm. BMP4 and BMP7 are expressed in the ectoderm and are epidermis inducers with concurrent inhibition of neurulation (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b; Tanabe and Jessell, 1996).

BMPs have also been implicated in dorsoventral patterning of the neural tube. In the chick, BMP4 and BMP7 are expressed in non-neural ectoderm, and these BMPs induce dorsal cell types in neural plate explants (Hogan, 1996b). In the dorsal neural tube, BMPs exert their effects through local and long-range signaling. Long-range signaling patterns are propagated by specific BMP-binding proteins that establish diffusible gradients and signaling cascades necessary for the differential induction of dorsal cell types (Hogan, 1996a; Piccolo et al., 1996; Tanabe and Jessell, 1996). Moreover, manipulation of BMP signaling in the chicken neural tube showed that BMPs provide patterning information to both dorsal and intermediate cells. BMPs regulate the expression boundaries of the homeobox proteins Pax6, Dbx2 and Msx1 that generates precursor populations with distinct developmental potentials. These data strongly suggests that BMPs are the key regulators of dorsal cell identity in the developing neural tube (Timmer et al., 2002). Recently it has been shown that the LIM homeodomain

transcription factor Lmx1 which regulates the extend of roof plate induction is induced by BMP signaling from the epidermal ectoderm (Chizhikov and Millen, 2004; Millen et al., 2004).

In the developing spinal cord BMP4 present in dorsal regions actively inhibits the differentiation of oligodendrocyte precursors and in combination with ventrally derived sonic hedgehoc (shh), defines the region in which the founders of the oligodendrocyte lineage arise (Zhou and Anderson, 2002). Implantation of noggin-producing cells early in development promoted the subsequent appearance of oligodendrocyte precursors in dorsal neural tube, suggesting that endogenous dorsally expressed BMPs inhibits oligodendrogenesis (Mekki-Dauriac et al., 2002). Other studies have shown that the inhibition of early oligodendrogenesis by BMPs is not restricted to chick spinal cord. Implantation of BMP-coated beads into developing *Xenopus* is sufficient to suppress ventral oligodendrogenesis, whereas implantation of either Shh-coated beads or anti-BMP coated beads is sufficient to induce dorsal oligodendrogenesis (Hall and Miller, 2004; Miller et al., 2004). BMPs appear to inhibit oligodendrogenesis at several stages of oligodendrocyte differentiation, becoming a more effective inhibitor at later stages. In the cortex the BMPs have been shown to exhibit different actions on different time points. Early in development BMP stimulation increases neuronal differentiation in cortical cultures (Mehler et al., 2000). Likewise later in development, BMP stimulation enhances the appearance of astrocytes in cortical cultures (Mehler et al., 2000). The induction of astrocytes from cultured mouse neuroepithelial cells by BMPs reflects an inhibition of neuronal development and suggests that this cytokine may regulate the appearance of astrocytes (Nakashima et al., 2001; Yanagisawa et al., 2001). In cultures of postnatal cortex and cerebellum treatment with BMP enhances astrogenesis (Angley et al., 2003). However, the development of astrocytes in coculture experiments with neural progenitor cells and neurons could be abolished by the BMP inhibitor noggin (Gomes et al., 2003). Taken together, BMPs act as major regulators of neural fate determination in the vertebrate CNS.

In the chick embryo, BMP4 is expressed selectively in the dorsal regions of the odd-numbered rhombomeres and induces segmental apoptosis of hindbrain neural crest by upregulating its own expression and the expression of Msx2 (Graham et al., 1996). The same group also showed that BMP4 mediated apoptosis is restricted to some rhombomeres while in other rhombomeres hindbrain neural crest cells are prevented from apoptosis by expression of the BMP inhibitor noggin (Smith and Graham, 2001). During neural crest development BMPs in combination with Wnts and FGFs induce neural crest by activating the early neural crest

marker slug (Trainor and Krumlauf, 2002). Further, BMP2 and BMP4 have been shown to play an instructive role on NCSCs to promote an autonomic fate (Anderson et al., 1997; Shah et al., 1996). *In vitro* studies revealed that BMPs exerts community effects on rat NCSCs (Hagedorn et al., 2000a; Hagedorn et al., 1999) cultured according to (Stemple and Anderson, 1992). Communities of NCSCs generate exclusively autonomic neurons while a single NCSC exposed to BMP2 generate mixed clones of autonomic neurons and non-neural smooth-muscle cells (Hagedorn et al., 2000a). Furthermore, BMP4 and BMP7 are expressed by the dorsal aorta at a crucial time during post-migratory neural crest-derived sympathetic differentiation (Anderson, 1997; Reissmann et al., 1996; Schneider et al., 1999).

7.2 The BMP Signaling Pathway

BMPs elicit their effects through activation of combinations of two type I and two type II serine/threonine kinase receptors. Type I receptors within such complexes act downstream of type II receptors and determine the specificity of the signal (Massague, 1998). There are three distinct type II receptors, BMP type II (BMPRII) and activin type IIA and IIB receptors (ActRIIA and ActRIIB) and three distinct type I receptors (activin receptor-like kinases (Alks) ALK2, ALK3/BMPR1a and ALK6/BMPR1b that have been identified for ligands of the BMP subgroup. ALK3 and ALK6 are activated by BMP2, BMP4 and BMP7 while ALK2 bind BMP6 and BMP7. The type I receptors within the tetrameric receptor complex initiate signal propagation by phosphorylation of the receptor activated Smads. The name Smads originates from a fusion between *Drosophila mothers against dpp (Mad)* and *C. elegans Sma* (Derynck et al., 1996). The Smad family can be divided into three distinct subfamilies: receptor-regulated Smads (R-Smads) common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads). BMP receptors activate Smad1, Smad5 and Smad8 (R-Smads) and initiate a phosphorylation cascade. The activated R-Smads assemble into heterotrimeric complexes with Smad4 (Co-Smad) in the cytoplasm and the whole complex is then translocated to the nucleus where the expression of BMP target genes is induced (Figure34).

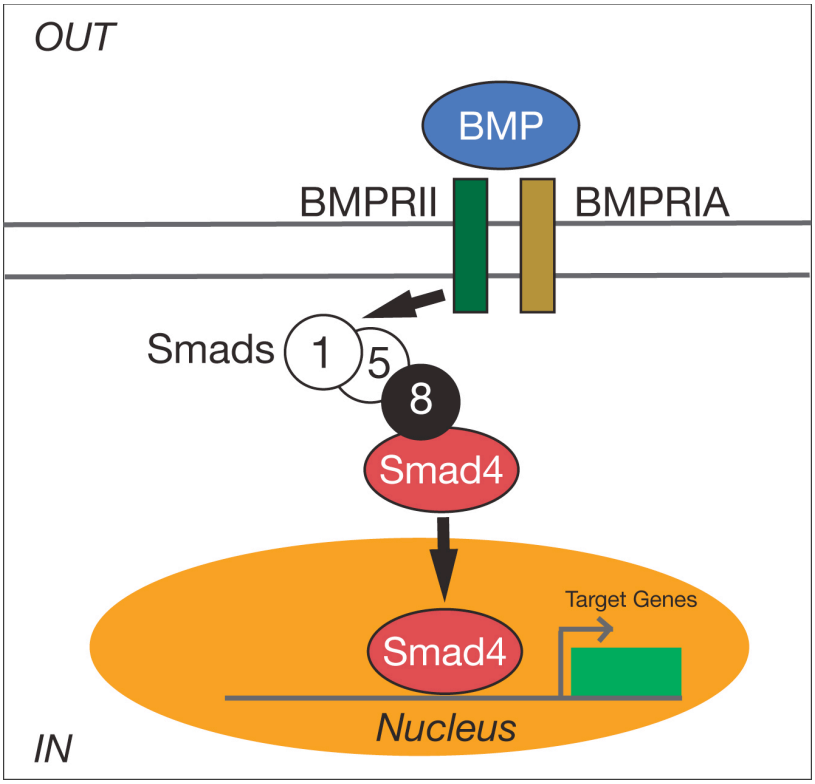

Figure 34: The BMP signaling pathway

BMP proteins signal *via* BMPR typeII- and BMPR typeI-kinase receptors. Upon binding a phosphorylation cascade is induced in which Smad1, Smad5 and Smad8 bind to Smad4. The whole complex is then translocated to the nucleus where transcription of BMP target genes is induced.

Moreover, the complex can also recruit co-repressors or co-activators or even directly interact with other signal transduction pathways (Derynck, 1998; Inman et al., 2002; Theil et al., 2002). BMPs also initiate other non-Smad intracellular pathways like the Jun N-terminal kinase (JNK) pathway and the caspase pathway (Ghosh-Choudhury et al., 2002; Hay et al., 2001; Heldin et al., 1997).

Several studies in which the BMPR1a was specifically ablated revealed different roles of BMP signaling during development. A null mutation of the BMPR gene results in early embryonic lethality around the time of gastrulation (Mishina et al., 1995). Ablation of the BMPR1a in the limb ectoderm results in gross malformations of the limb with complete agenesis of the hindlimbs (Ahn et al., 2001). During hair follicle development BMPR1a is essential for the differentiation of progenitor cells of the inner root sheath and hair shaft (Kobielak et al., 2003). Specific ablation of BMPR1a in NCSCs however, did not result in a disturbance of neural crest development but rather established a role of neural crest cells

involved in the development of the cardiac outflow tract of the heart (Stottmann et al., 2004). Mice that lack BMPR1b (Alk2) in NCSCs display craniofacial defects (Dudas et al., 2004). Mutations in the BMP signaling pathway lead to the formation of various tumors. The key player Smad4 is frequently mutated in human carcinomas, leading to loss of growth inhibition (Schutte, 1999). In prostate cancerogenesis the BMP signal pathway has been implicated at a number of steps in the signal transduction cascade. Transcript profiling data demonstrated downregulation of BMP2 and Smad8 at the mRNA level in prostate cancer tissue compared to normal prostate tissue (Sakou et al., 1999). BMPs are also present in a number of bone tumors. Osteosarcomas producing BMPs contain less-differentiated mesenchymal cells. BMPs are also expressed in malignant fibrous histocytomas of bone and dedifferentiated chondrosarcomas exhibiting undifferentiated features. Clinically, BMPs have utility as diagnostic and prognostic markers for characterizing the stage of differentiation of mesenchymal cells and mesenchymal tumors (Yoshikawa et al., 2004).

8. Combinatorial Wnt and BMP Signaling Regulate Maintenance and Early Lineage Segregation of NCSCs.

Maurice Kléber, Hye-Youn Lee, Heiko Wurdak, Johanna Buchstaller, Ueli Suter, Doug Epstein and Lukas Sommer

Manuscript in preperation

8.1 Introduction

Factors that maintain stem cells in an undifferentiated state are only poorly described. The promise for future cell replacement therapies lies in the ability of stem cells to self-renew indefinitely *in vitro* without losing their ability to differentiate. In embryonic stem (ES) cells activation of the canonical Wnt pathway is sufficient to maintain self-renewal (Sato et al., 2004). Recently, it was reported that BMPs in collaboration with LIF sustains ES cell self-renewal (Ying et al., 2003). Similar to ES cells, the self-renewal of haematopoetic stem cells (HSCs) is controlled by canonical Wnt signaling (Reya et al., 2003). In the nervous system, factors that maintain neural stem cells in an undifferentiated state have not been described so far. In neural crest biology it is not yet known whether it is possible to maintain a pure population of NCSCs. NCSCs are a transient population of stem cells that detach from the dorsal part of the neural tube, migrate along different pathways, where they aggregate and form the structures of the peripheral nervous system (Le Douarin and Kalcheim, 1999). NCSCs from the rostral part of the neural tube also give rise to connective tissue of the head, chondrocytes, which contribute to cranialfacial skeletal, osteoblasts and odontoblasts of the teeth (Bronner-Fraser, 1995). NCSCs also migrate into the cardiac outflow tract of the heart where they participate in the septation of the cardiac outflow tract into aorta and pulmonary (Sieber-Blum, 2004). Although, various factors including Wnts have been described to instructively promote certain fates in eNCSCs (Lee et al., 2004), mechanisms that regulate the maintenance and early lineage segregation of NCSCs have not been identified (Kleber and Sommer, 2004). Although it has been shown that NCSCs behave *in vitro* as multipotent neural progenitors it was not possible to maintain these cells over prolonged time in a fully defined medium (Stemple and Anderson, 1992). Due to the fact that NCSCs were cultured in the presence of 10% chicken embryo extract (CEE), factors that might regulate neural crest self-renewal were hard to identify (Stemple and Anderson, 1992).

Due to the finding that all eNCSCs are Wnt responsive (Lee et al., 2004) factors that might modulate canonical Wnt signaling in either an inhibiting or in a cooperative manner as

eNCSCs emigrate from the neural tube have not been described so far (Kleber and Sommer, 2004). There is increasing evidence that the fate of multipotent NCSCs is not only dependent on the action of individual signals but is also influenced by the synergistic activity of multiple signals (Sommer and Rao, 2002). Apart from the role of BMPs to induce autonomic fates in eNCSCs (Lee et al., 2004) the question of whether BMPs might be involved in modulating canonical Wnt at the time point of early neural crest emigration remains elusive. In many aspects, BMPs are good candidates. First of all, BMPs, namely BMP4 and BMP7 are expressed in the anterior dorsal midline region at the time point of neural crest emigration (Hogan, 1996a; Hogan, 1996b; Tanabe and Jessell, 1996). Second, convergence between BMP signaling and canonical Wnt signaling has been reported in several studies. In embryonic stem cells Smad4 functionally interact with TCF/Lef transcription factors to induce expression of Msx2 (Hussein et al., 2003) and in the dorsal telencephalon Wnt and BMP signaling cooperatively regulate Emx2 expression (Theil et al., 2002) (Figure35).

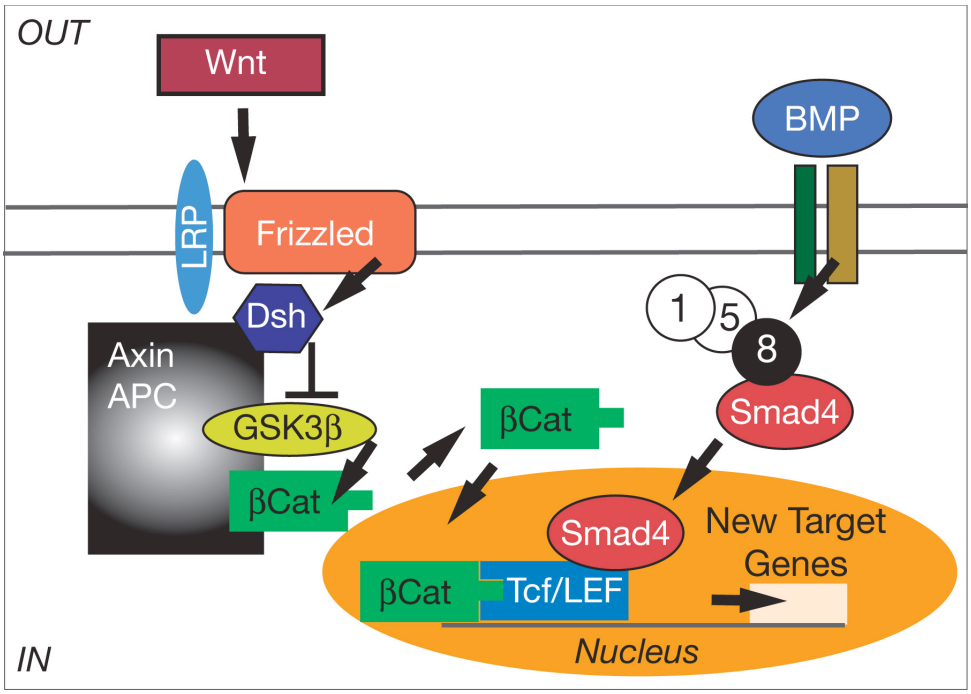

Figure 35: Convergence between BMP and canonical Wnt signaling

Convergence between BMP and canonical Wnt signaling has been reported to occur at the transcriptional level where Smad4 can directly bind to TCF/Lef transcription factors. Instead of BMP- or Wnt-target genes the combination of both can now induce the transcription of new target genes.

Further, Lo and collegues reported that levels of BMPs appear to be the primary determinants that modulate the neurogenins to promote either sensory or autonomic neurogenesis in neural tube derived cultures (Lo et al., 2002). A recent study revealed that target-derived BMP signaling limits sensory neuron number and the extent of peripheral innervation *in vivo* (Guha et al., 2004). Overexpression of noggin resulted in a significant increase in the number of neurons in the trigeminal and dorsal root ganglia. Conversely, overexpression of BMP4 resulted in a significant decrease in the number of dorsal root ganglion neurons (Guha et al., 2004). Interplay between Wnt and BMP would also resolve the paradox of sensory and autonomic lineage segregation and explain why autonomic neurons are not generated shortly after NCSCs emigrate from the dorsal neural tube, despite dorsal BMP expression (Anderson, 2000). Although lineage segregation occurs already in migratory NCSCs it still remains unclear why some NCSCs remain multipotent while others differentiate into transient progenitors (Anderson, 2000). Our results further extend the role of Wnts and BMPs by showing, that, in addition to their role in neurogenesis, these molecules synergistically cooperate together to regulate the maintenance and early lineage segregation in NCSCs.

8.2 Results

8.2.1 BMP suppresses Sensory Neurogenesis in Early Neural Crest Stem Cells

Previously, conditions have been established that are permissive for the generation of sensory neurons from neural crest explant cultures (Greenwood et al., 1999). At early stages, a high percentage of all neural crest cells in these cultures are so-called early neural crest stem cells (eNCSCs) that in clonal cell cultures can be challenged by various instructive growth factors to give rise to sensory or autonomic neurons, or non-neural smooth muscle-like cells (Lee et al., 2004). These data indicated that with respect to their potentials, explants of eNCSCs represent a highly homogeneous cell population. Consistent with these findings, the cells in such explants expressed the NCSC-markers p75 and Sox10 after 20 hours in culture (Paratore et al., 2001; Stemple and Anderson, 1992), while no sensory neuronal precursors marked by Brn-3A (Fedtsova and Turner, 1995) or neurofilament (NF) 160 were detectable at this stage (Fig. 36A-D) (Lee et al., 2004). However, upon incubation for an additional 24 hours, 9.4±1.6% of all cells per explant expressed Brn-3A (Fig. 36E, F, K), as described (Greenwood et al., 1999). Based on our previous clonal analyses (Lee et al., 2004), these cells likely arose from multipotent eNCSCs.

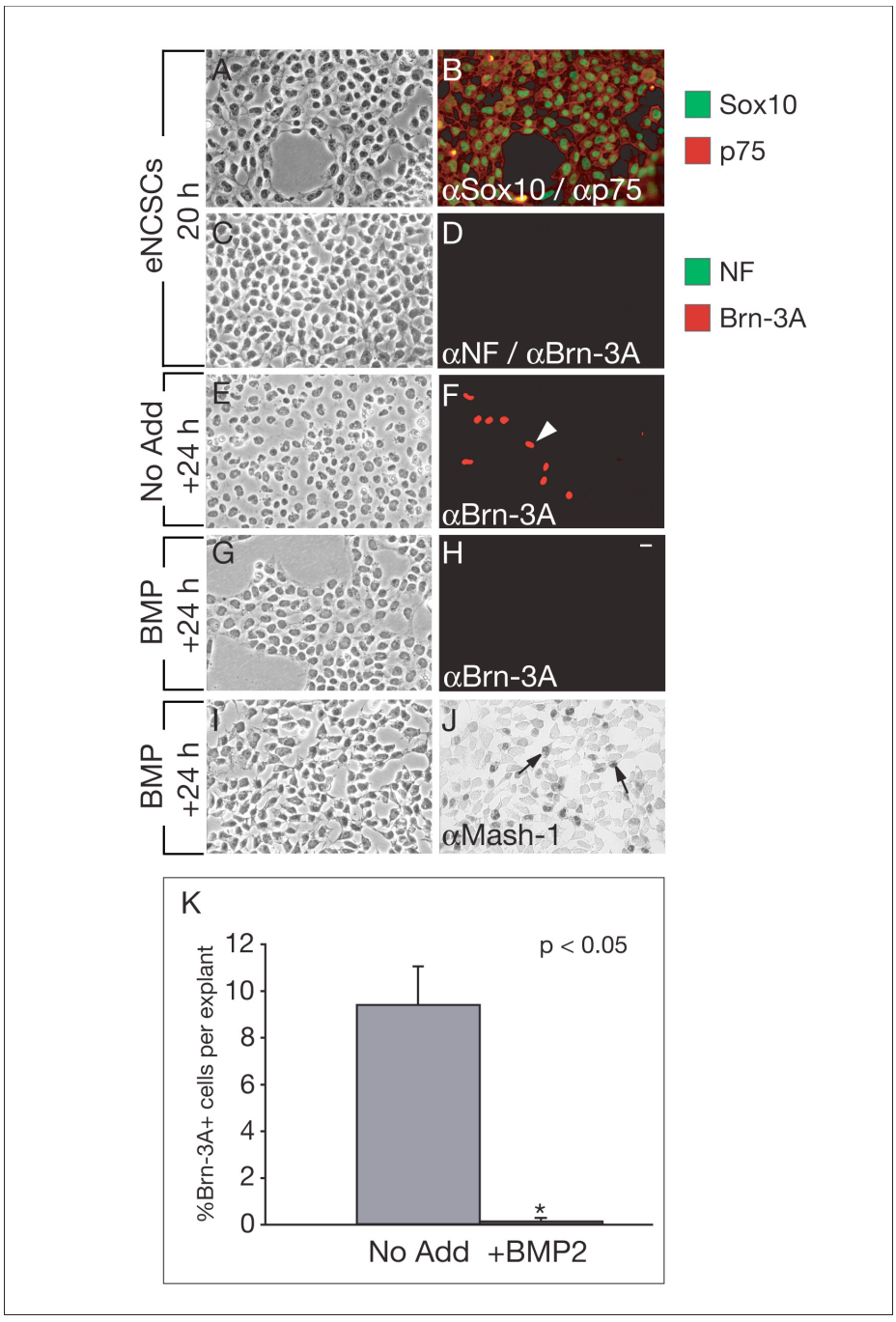

Figure 36: BMP suppresses sensory neurogenesis in early neural crest stem cells. Early neural crest explants were obtained from wild-type neural tubes that had been isolated from mice at E9 and cultured for 20 h to allow emigration of eNCSCs. After 20 h in culture all eNCSCs express the stem cell markers Sox10 and p75 (**B**) and do not express the sensory marker Brn-3A or NF (**C**). Early neural crest explants that were challenged with 50ng/ml BMP2 for additional 24 h fail to generate Brn-3A-positive sensory neurons (**H**) and instead start to express the autonomic marker Mash-1 (**J**, arrows), while Brn-3A-positive sensory neurons arised in control explants in the absence of BMP2 (**F**, arrowhead). Quantification of the average percentage of Brn-3A-positive cells per explant after 24 h growth in 'No add' or in 50ng/ml BMP2 reveal a statisticly significant decrease (mean ± SD) in the percentage of Brn-3A-positive cells by BMP2 ($p<0.05$, *t*-test) (**K**). Three explants per experiment in three different experiments were analyzed, scoring 1500 to 3000 cells per explant (**A**, **C**, **E**, **G**, and **I**) Corresponding phase contrast pictures. Scale bar 10μm.

Alternatively, they might have been generated from lineage-restricted sensory neuron precursors present at early stages of neural crest emigration (Greenwood et al., 1999). To distinguish between these possibilities, we challenged neural crest explant cultures with BMP2, a factor instructively promoting autonomic neurogenesis in NCSCs at the expense of other fates (Shah et al., 1996). BMP2-treated cultures gave only rise to 0.1±0.1% Brn-3A-positive sensory neurons whereas the generation of autonomic progenitors expressing the transcription factor Mash1-1 was promoted (Fig. 36G-K). Thus, BMP signaling prevents sensory neurogenesis in early neural crest explant cultures.

8.2.2 BMP Signaling Counteracts Wnt/β-catenin-dependent Sensory Neurogenesis

Canonical Wnt signaling is both required and sufficient for sensory neuronal fate specification in eNCSCs (Hari et al., 2002; Lee et al., 2004). Accordingly, as in BMP2-treated cultures, the formation of Brn-3A-positive sensory neurons was abolished in explants of *β-catenin*-deficient eNCSCs (Fig. 37A-C) (Hari et al., 2002). However, unlike in cultures exposed to BMP2, no NF^+/Brn-$3A^-$ autonomic neurons were formed in the absence of canonical Wnt signaling. This suggests that BMP signal activation does not only suppress Wnt/ß-catenin-dependent sensory neurogenesis, allowing autonomic neurogenesis as a default fate decision process, but also actively promotes autonomic neurogenesis.

In neural crest explants, sensory neurogenesis is presumably promoted by Wnts expressed in the neural tube that is present in these cultures (Dorsky et al., 1998). The restricted expression of Wnts and its short-range activity (Cadigan and Nusse, 1997; Miller, 2002) likely explains the relatively low percentage of sensory neurons found in these explants and their generation in close proximity to the neural tube (Figs. 37A, 38E) (Greenwood et al., 1999). Therefore, the suppression of sensory neurogenesis in neural crest explants by BMP2 conceivably reflects BMP activity counteracting neural tube-derived Wnt signaling. To address whether BMP2 would also counteract more pronounced Wnt/ß-catenin activity, we exposed neural crest cells expressing a constitutively active form of ß-catenin to BMP2 (Harada et al., 1999). In the absence of BMP2, sustained ß-catenin activity promoted sensory neurogenesis in eNCSCs, as described (Lee, et al., 2004) (Fig. 37D). In the presence of BMP2, sensory neurogenesis was completely inhibited, even upon constitutive ß-catenin signal activation in eNCSCs. Surprisingly, however, unlike in cultures of wild type neural crest cells, BMP2 was unable to promote the formation of NF^+/Brn-$3A^-$ autonomic neurons (Fig. 37E). Thus,

persistent ß-catenin signaling appears to suppress BMP2-induced autonomic neurogenesis, while BMP2 prevents sensory neurogenesis promoted by ß-catenin signal activation. Therefore, Wnt and BMP signaling are possibly involved in a cross talk to regulate neurogenesis in neural crest cells.

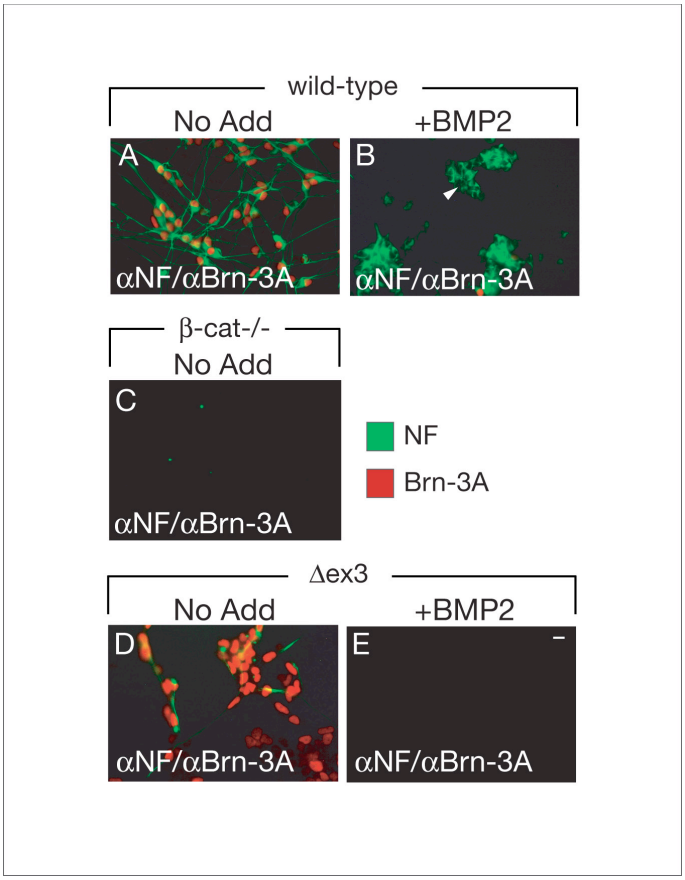

Figure 37: BMP signaling counteracts Wnt/β-catenin dependent sensory neurogenesis. Wild-type (wt) eNCSCs cultured in conditions permissive for sensory neurogenesis for 60 h generate fully differentiated sensory neurons (**A**) expressing Brn-3A and NF, while eNCSCs challenged with 50ng/ml BMP2 fail to generate sensory neurons and instead generate clusters of autonomic neurons expressing NF, but not the sensory marker Brn-3A (**B**, arrowhead). β-catenin deficient NCSCs (β-cat-/-) are unable to generate Brn-3A- and NF-positive sensory neurons (**C**) due to disrupted Wnt signaling. Stabilized β-catenin (Δex3) increases sensory neurogenesis as shown by Brn-3A stainig in virtually all mutant cells (**D**). Early neural crest explants expressing stabilized β-catenin challenged with 50ng/ml BMP2 fail to generate sensory neuronal cells expressing Brn-3A and NF, and also fail to generate autonomic neurons expressing NF (**E**). Scale bar 10μm.

8.2.3 Maintenance of Neural Crest Stem Cell Markers and Suppression of Differentiation by Combined Wnt and BMP Signaling

To further address the interaction between canonical Wnt and BMP signaling, we exposed explant cultures of wild type neural crest cells to either Wnt1, BMP2, or both of these factors.

While BMP2 alone induced the generation of ganglia-like structures consisting of NF^+/Brn-$3A^-$ neurons, exposure of neural crest explants to Wnt1 led to the formation of ganglia containing NF^+/Brn-$3A^+$ sensory neurons (Fig. 38A-E). Thereby, Wnt1-induced sensory neurons were located close to the neural tube, while BMP2-induced autonomic neurons developed distal to the neural tube, reflecting the localization of sensory and autonomic ganglia *in vivo*. Intriguingly, the simultaneous application of BMP2 and Wnt1 to neural crest cells prevented the development of NF-expressing neurons, and no ganglia-like structures were present in these cultures (Fig. 38C, F). This was not simply due to the loss of neural crest cells or impaired migration, because many Sox10-positive neural crest cells were detectable in Wnt1 plus BMP2-treated explant cultures. These data indicate that the combined activity of Wnt1 and BMP2 suppresses both autonomic and sensory neuron formation.

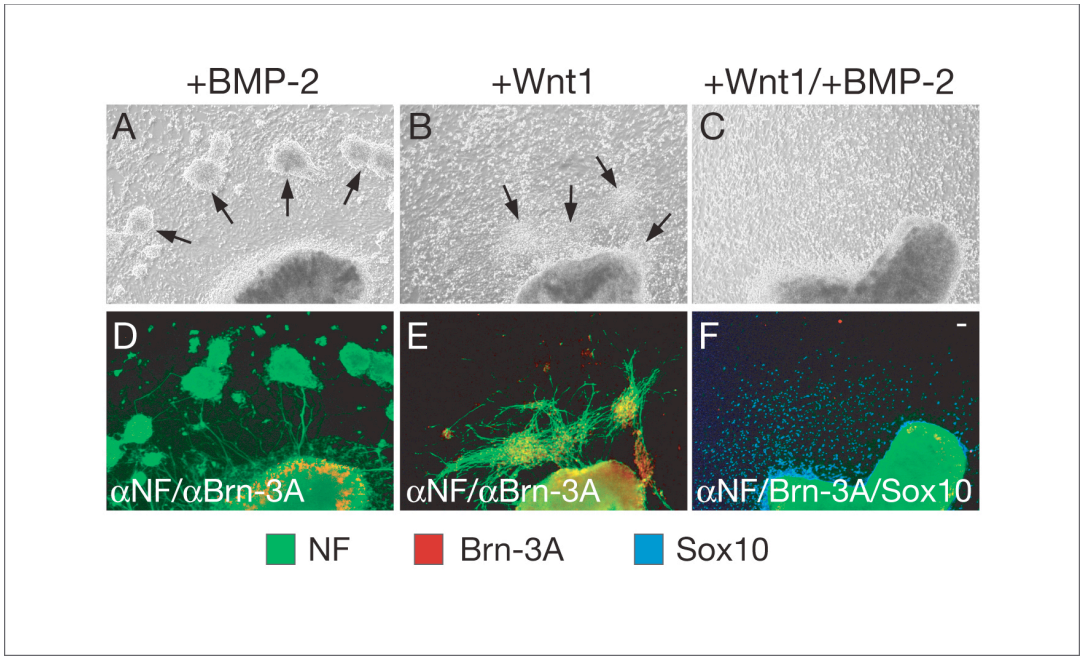

Figure 38: Combined activity of Wnt1 and BMP2 suppresses both autonomic and sensory neuron formation. Wild-type (wt) eNCSCs exposed to fibroblast monolayers supplemented with 50ng/ml BMP2 for 5 d generate autonomic ganglion-like structures that are symmetrically located around the neural tube (**A**, arrows), and express NF (**D**). Wt eNCSCs exposed to Wnt1 monolayers form sensory ganglion-like cell aggregates that are located close to the neural tube (**B**, arrows), and express both Brn-3A and NF (**E**). In the presence of Wnt1 and 50ng/ml BMP2, eNCSCs fail to generate autonomic as well sensory ganglia (**C**) and instead remain Sox10-positive (**F**). To make the cells visible, the Sox10 staining was artificially marked blue (**F**). Scale bar 10μm.

Early stages of neurogenesis were also suppressed in Wnt1/BMP2-treated neural crest explant cultures. While BMP2 alone induced Mash1 expression (Fig. 39C), which labels autonomic progenitor cells (Sommer et al., 1995), combined Wnt1 plus BMP2 did not allow the appearance of Mash-1-positive cells in neural crest explants (Fig. 39I).

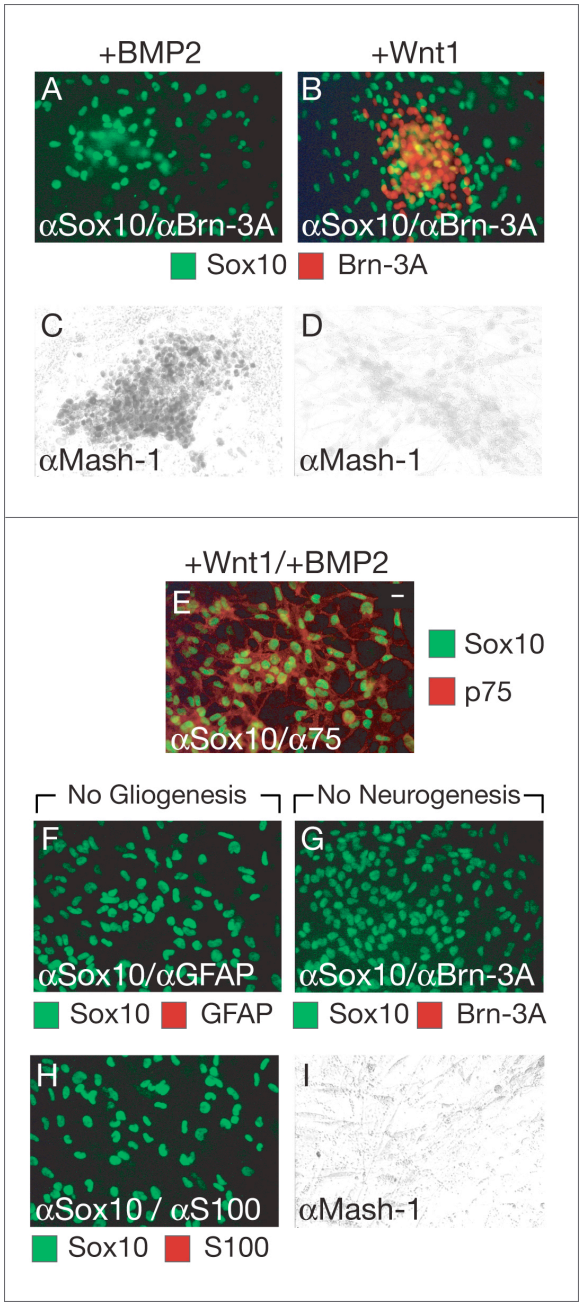

Figure 39: Maintenance of Neural Crest Stem Cell Markers and Suppression of Differentiation by Combined Wnt and BMP Signaling. Wild-type (wt) eNCSCs exposed to fibroblast monolayers supplemented with 50ng/ml BMP2 generate Mash-1-and Sox10-expressing ganglion-like structures (**A**/**C**), that do not contain Brn-3A-positive cells (**A**), while eNCSCs exposed to Wnt1-expressing fibroblasts generate sensory ganglia, expressing Brn-3A and Sox10 (**B**), but not Mash-1 (**D**). eNCSCs exposed to both Wnt1 and 50ng/ml BMP2 for 5

d maintain the neural crest stem cell markers Sox10 and p75 (**E**). Moreover, they neither express the glial markers GFAP and S100 (**F**/**H**), the sensory marker Brn-3A (**G**) nor the autonomic marker Mash-1 (**I**). Scale bar 10mm.

Likewise, Wnt1 promoted Brn-3A-positive sensory precursor cells only in the absence of BMP2 (Fig 39B, D). Instead virtually all cells in Wnt1/BMP2-treated neural crest explants expressed p75 and Sox10 (Fig. 39E). Both of these markers are found in NCSCs but are also expressed during gliogenesis (Paratore et al., 2001; Stemple and Anderson, 1992). To address whether combinatorial Wnt and BMP signaling would suppress neurogenesis by promoting gliogenesis, we stained neural crest cultures with the glial differentiation markers GFAP and S100. However, GFAP and S100 expression was not detectable in cultures simultaneously exposed to Wnt1 and BMP2, even after prolonged incubation of 7 days (Fig. 39F, H). Thus, Wnt1/BMP2-treated neural crest explants consisted of many cells expressing the NCSC-markers p75 and Sox10, while no sign of either neuronal or glial differentiation was observed.

8.2.4 Combined Wnt and BMP Signaling Instructively Promote Neural Crest Stem Cell Maintenance and Factor Responsiveness

The lack of apparent neurogenesis and gliogenesis upon treatment of neural crest cells with Wnt1 plus BMP2 could indicate a selective elimination of neural crest cells as they undergo differentiation. Alternatively, our data could reflect an instructive role of combined Wnt and BMP signaling on individual eNCSCs to maintain expression of stem cell markers while preventing their differentiation. To distinguish between these possibilities, explants of neural crest cells were replated at clonal density, individual eNCSCs were prospectively identified by virtue of their p75 expression (Stemple and Anderson, 1992), and their developmental potential was assessed by challenging them with either Wnt1, BMP2, or a combination of both factors. On sister dishes it was determined that 92.1±1.9% of all p75-positive cells also expressed Sox10 (Fig. 40A). In agreement with our previous findings (Lee et al., 2004), exposure of p75-labelled cells to Wnt1 alone induced the formation of clones containing Brn-3A-positive sensory neurons in 83.1±5.8% of all eNCSCs (Fig. 40B, E), while upon BMP2 treatment 85.4±5.7% of all eNCSCs adopted an autonomic fate marked by Mash-1 (Fig. 40C, F). In contrast, combined application of Wnt1 and BMP2 suppressed both sensory and autonomic neurogenesis in virtually all eNCSCs, so that only very few sensory and autonomic cells were produced in these conditions (0.8±1.4% and 0±0%, respectively). Instead,

84.9±5.9% of all eNCSCs challenged with Wnt1 plus BMP2 continued to express Sox10 (Fig. 40D, E, F).

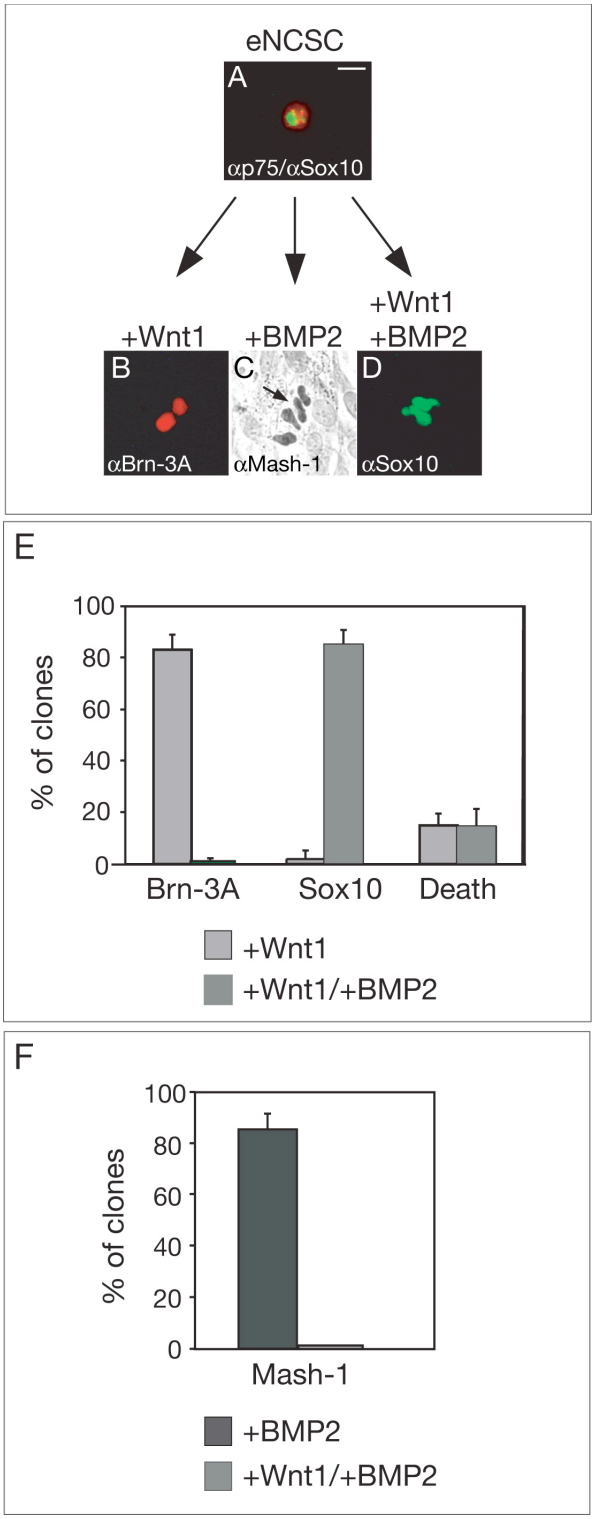

Figure 40: Wnt and BMP instructively promote stem cell features in eNCSCs. Clonal analysis demonstrates responsiveness of wt eNCSCs to the instructive growth factors Wnt1 and BMP2 and the combination of both. Of the p75-positive (red) founder cells, 92.1±1.9% coexpressed Sox10 (green) (**A**). Founder cells exposed to Wnt1 generated clones of Brn-3A-positive sensory neurons (**B**). BMP2 instructed eNCSCs to generate Mash-1-

expressing autonomic cells (**C**, arrow). In the presence of both Wnt1 and BMP2, founder cells generated Sox10-positive cell clones (**D**). Scale bar 10μm. (**E**/**F**) Quantification of clone composition. (*) P< 0.001. The data are expressed as the mean ± SD of three independent experiments. 50 to 80 clones were scored per experiment.

Furthermore, cell death was minimal in all clonal experiments, excluding selective effects of Wnt and BMP signaling. Thus, the combined activities of Wnt1 and BMP2 do not selectively eliminate neuronal progenitors but rather instructively promote maintenance of the NCSC-marker Sox10 in the vast majority of all NCSCs.

The clone size of Wnt1 plus BMP2 treated NCSCs was small, as only 47.6±7.7% gave rise to clones between 2-5 cells and 4.3±3.9% to clones bigger than 5 cells (table 3). We suggest that the slow self-renewal might be explained by slow cell cycle progression of stem cells as opposed to fast cycling transient amplifying progenitors, when NCSCs are treated with BMP2 alone. Such Mash-1 expressing progenitor clones gave rise to 76.4±4.4% of clones between 2-5 cells and 19.2±7.9% of clones bigger than 5 cells (table 3).

	Clone size (%± s.d.)		
	>5 cells	2-5 cells	1cell
+Wnt1/+BMP2	4.3±3.9	47.6±7.7	48.1±11.2
+Wnt1	2.1±1.9	26.2±12.3	71.7±14.2
+BMP2	19.2±7.9	76.4±4.4	4.4±3.9

Table 3: Slow self-renewal upon Wnt and BMP. The cell number within individual Wnt1-, BMP2- and Wnt1+BMP2-treated clones was analyzed. Numbers (% of all clones per condition) are shown as the mean ± SD of three independent experiments, scoring 50 to 80 clones per experiment. (*) P < 0.01.

To address whether Wnt and BMP exerts its effects only when they are added at the same time point eNCSCs were exposed first to Wnt1 for 2 days and then subsequently one day to Wnt1/BMP2 (Fig. 41). The addition of BMP2 on day 3 however, had no influence on the generation of sensory neuron clones, which demonstrates that Wnt1 and BMP2 had to be added concomitantly (Fig.41).

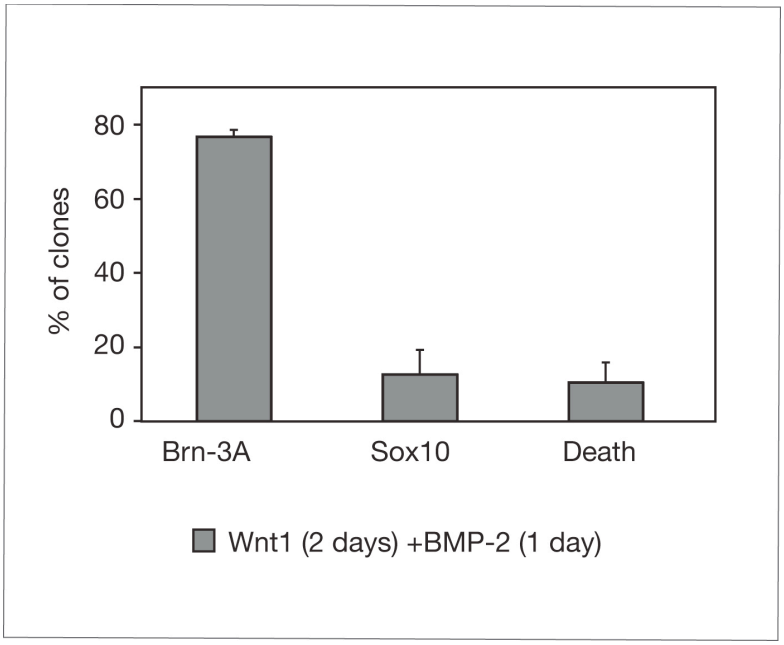

Figure 41: Wnt and BMP had to be added concomitantly.

Clonal analysis of eNCSCs exposed to Wnt1 for 2 d followed by 1 d Wnt1 plus BMP2 treatment. The addition of BMP2 on day3 has no effect on eNCSCs, as similar to the effect of Wnt1 alone.

Sox10 has been implicated in maintaining multipotency of NCSCs both in culture and in the enteric nervous system *in vivo* (Kim et al., 2003; Paratore et al., 2002a). Therefore, we addressed the question of whether sustained Sox10 expression in Wnt1/BMP2-treated eNCSCs also reflects maintenance of NCSC multipotency. In a first experiment, explants of eNCSCs (Fig. 42A) were incubated for 5 days with Wnt1 plus BMP2. Thereafter, some dishes were fixed and analyzed for p75/Sox10 expression, while sets of sister dishes received either of the following treatments (Fig. 42): 1) some dishes were kept in Wnt1/BMP2 to assess whether maintenance of NCSC-marker expression can occur over prolonged time periods and to exclude differentiation events arising by default over time (Fig. 42C); 2) to investigate, whether the cells were still responsive to the sensory fate-inducing activity of Wnt1, Wnt1 treatment was continued while BMP activity was suppressed by the addition of the BMP-inhibitor noggin (Zimmerman et al., 1996) (Fig. 42D, E) ; 3) to assess the potential to generate non-neural smooth muscle-like cells, Wnt1 activity was suppressed by the addition of chicken embryo extract (which inhibits Wnt/ß-catenin-dependent sensory neurogenesis; Fig. 43) and TGFß was applied to the cultures (Fig. 42F); 4) to test the gliogenic potential of the cells, Wnt activity was suppressed and NRG1 was added (Fig. 42G); and 5) to monitor autonomic neurogenesis, Wnt signaling was suppressed and BMP2

was continuously administered (Fig. 42H, I). While the uninterrupted exposure of NCSCs to both Wnt1 and BMP2 sustained expression of Sox10 and p75 in all cells without apparent differentiation (Fig. 42C), TGFß-, NRG1-, and BMP2-treatment of cells that have been maintained in Wnt1 plus BMP2 for 5 days promoted the efficient generation of non-neural smooth muscle actin-positive cells, glia, and autonomic neurons, respectively (Fig. 42F-I) However, when neural crest cells that have been maintained in culture for 5 days were treated with Wnt1, only relatively few sensory neurons were formed and Wnt1 appeared to have no effect on most of the cells (Fig. 42D, E) In all conditions, virtually no cell death was observed (not shown). Thus, the combined application of Wnt1 and BMP2 not only allows to maintain Sox10/p75 expression in neural crest cell populations, but also preserves their responsiveness to various growth factors including TGFß, NRG1, and BMP2. In contrast, neural crest cells appear to loose with time their responsiveness to the sensory neuron-inducing activity of Wnt signaling.

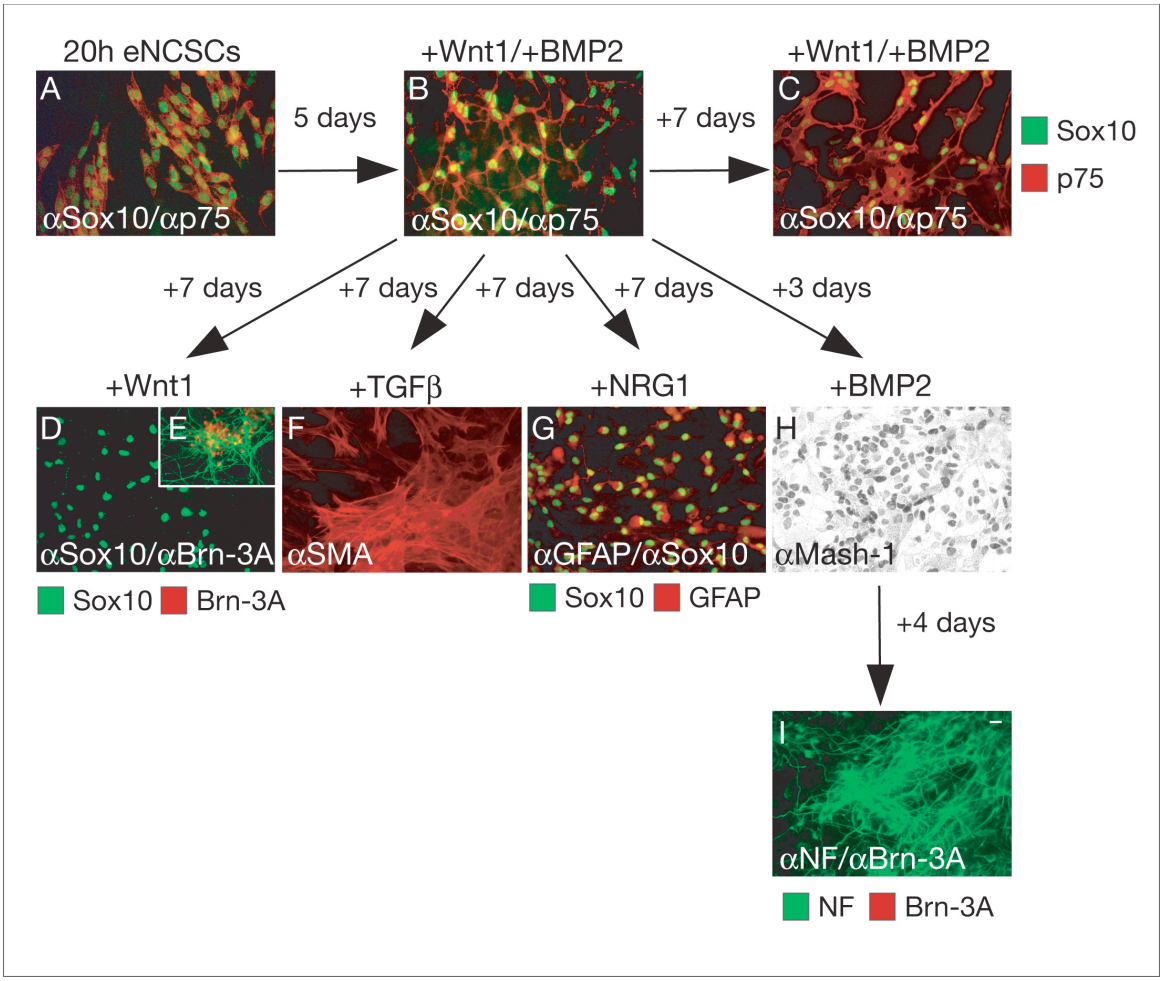

Figure 42: Maintenance of multipotency in NCSCs by combinatorial Wnt and BMP signaling. After emigration, eNCSCs (**A**) were exposed to Wnt1 and BMP2 and maintained for 5d (**B**). To test multipotency, NCSCs were maintained for an additional 7 days in the presence of Wnt1 and BMP2 as shown by the expression of Sox10 and p75 (**C**). On sister plates, in the absence of BMP2, NCSCs loose the responsiveness to Wnt1 as most of the cells maintain Sox10 expression (**D**) and sensory neurons expressing Brn-3A and NF were only rarely found (**E**). In the absence of Wnt1 and BMP2, NCSCs adopt smooth-muscle-like fates marked by expression of SMA in the presence of TGFβ (**F**) and glial fates marked by expression of Sox10 and GFAP in the presence of the neuregulin NRG1 (**G**). In the absence of Wnt1, many NCSCs express the autonomic marker Mash-1 in the presence of BMP2 (**H**) after 3 days, and differentiate into autonomic neurons after an additional 4 d (**I**). Scale bar 10μm.

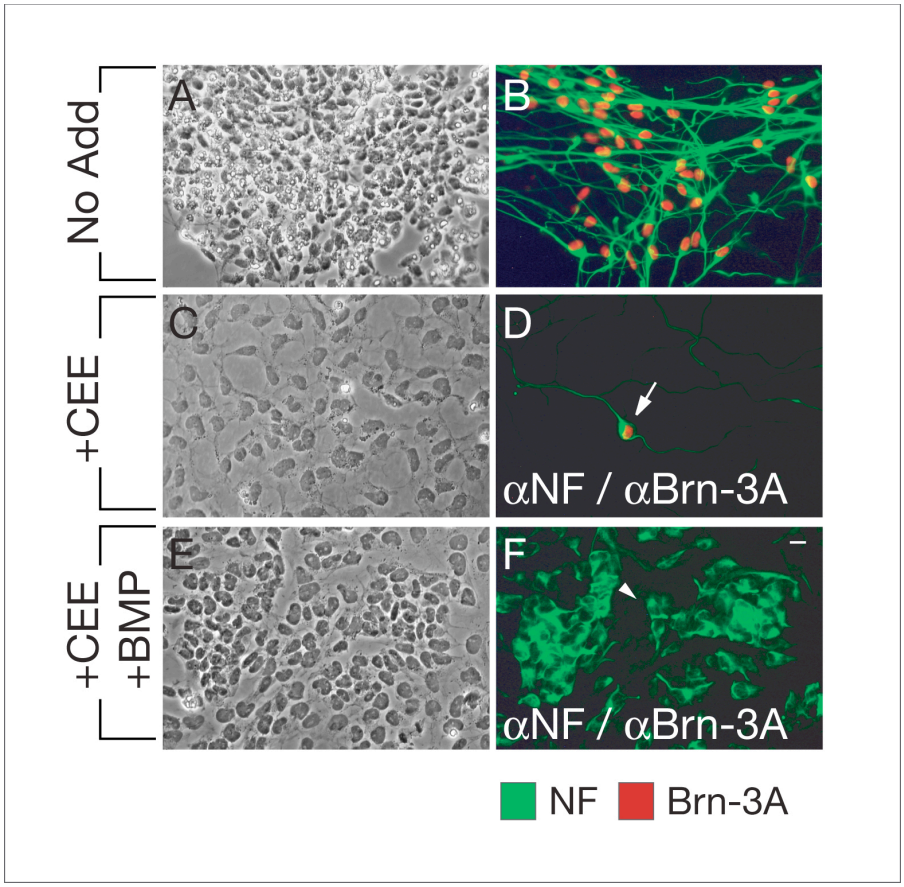

Figure 43: Chicken embryo extract (CEE) suppresses sensory neurogenesis in early neural crest explants. After 60 h in culture conditions permissive for sensory neurogenesis, early neural crest explants generate Brn-3A-and NF-expressing sensory neurons (**B**). Upon addition of 10% CEE to 20 h early neural crest explants sensory neurogenesis is almost completely blocked as only very few sensory neurons arise after 60 h in culture (**D**, arrow). Upon addition of 10% CEE and 50ng/ml BMP2, early neural crest explants generate clusters of autonomic neurons expressing NF and Mash-1 (**F**, arrowhead; not shown). Corresponding phase contrast pictures (**A**, **C**, **E**). Scale bar 10μm.

To address whether maintained responsiveness to instructive growth factors is a feature of the vast majority of Wnt1/BMP2-treated neural crest cells or only of subpopulations of the cells, NCSCs that have been incubated with Wnt1 plus BMP2 for prolonged time periods (11 days) (Fig. 44A) were replated at clonal density and prospectively identified by virtue of their p75 expression. 96.4±1.9% of all p75-positive cells were co-expressing Sox10 (Fig. 44B, table 4). Subsequently, single p75-positive cells were challenged with either TGFß, NRG1, or BMP2 (Fig. 44C, D, E). Consistent with the data obtained in mass cultures, the majority of all NCSCs that have been maintained in culture were responsive to these instructive growth factors (table 4). However, while TGFß promoted the generation of smooth muscle-like cells and NRG1 the formation of glia, as expected, BMP2-treated founder cells predominantly gave rise to clones of smooth muscle-like cells and only a minority of the clones contained autonomic neurons (table 4). Thus, similar to eNCSCs present in the emigrating neural crest (Fig. 40), NCSCs maintained in culture were sensitive to various instructive growth factors but the quality of their response changed with time (Fig. 44). This suggests that during culture intrinsic properties have changed in NCSCs, similar to the intrinsic changes observed in NCSCs isolated from the gut at different developmental (Kruger et al., 2002).

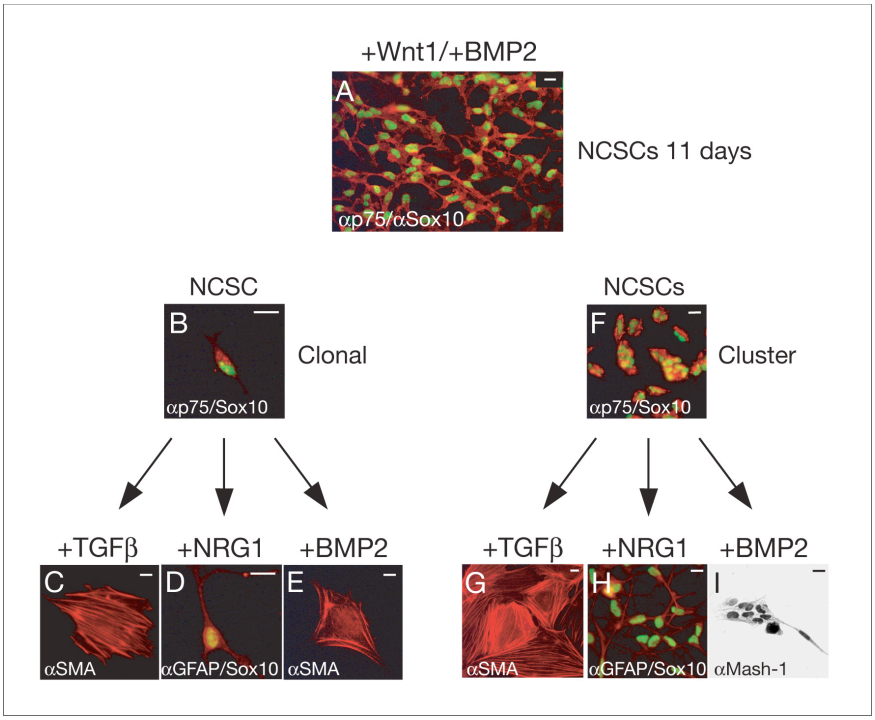

Figure 44: Wnt and BMP regulate maintenance and early lineage segregation of NCSCs. NCSCs were cultured in the presence of Wnt1 and BMP2 for 11 d (**A**) and either replated at clonal density (**B**) or on sister plates as clusters (**F**). At clonal density 96.4±1.9% of the p75-positive (red) founder cells coexpressed Sox10 (green) (**B**). Clonal analysis demonstrates responsiveness of maintained NCSCs to different instructive growth

factors (**C-E**). In the presence of TGFβ, founder cells generated clones of smooth-muscle-like cells (**C**), NRG1 instructed NCSCs to generate Sox10- and GFAP-expressing glia cells (**D**), while BMP2 at clonal density instructed maintained NCSCs to adopt a smooth-muscle-like fate (**E**). However, in cell clusters of maintained NCSCs BMP2 promoted the generation of Mash-1 expressing autonomic neurons, suggesting that BMP activity is modulated by community effects (**I**). At high density TGFβ induced clusters of smooth-muscle-like cells expressing SMA (**G**), while NRG1 generated clusters of glia cells (**H**). Scale bar 10μm.

% of all cells

Plating efficiency 65-70

condition	death	NCSC	sN	G	aN	Non NR
TGFβ	26.4±3.7	12.4±3.6	0	0	1.7±1.6	59.5±4.4
NRG1	17.7±5.6	0	0	82.3±5.6	0	0
BMP2	27.4±2.9	8.4±1.6	0	0	9.0±3.2	57.9±4.2
Wnt1	15.4±4.0	71.1±3.4	13.5±3.7	0	0	0

% of all cells in an autonomic cell cluster

	death	Mash-1
BMP2	1.4±1.3	98.6±1.3

Table 4: Clonal analysis of p75-positive NCSCs, maintained for 12 days in the presence of Wnt1 and BMP2, before exposed to different instructive growth factors. After 11 days in culture in the presence of Wnt1 and BMP2, wild-type NCSCs were replated at clonal density, labeled and exposed to different instructive growth factors as described in Materials and Methods. The progeny of single p75-positive cells was characterized by immunocytochemistry for Brn-3A, Sox10 and GFAP, for GFAP and Sox10, for Sox10, NF and SMA or, independently for Mash-1. The numbers indicate the phenotype of the progeny in % ± SD. 'death' designates lost clones. A colony was labeled 'NCSC' (neural crest stem cell), 'sN' (sensory neuron), 'G' (presumptive glia), 'aN' (autonomic neuron) or 'Non NR' (non neural) when at least one cell was expressing the corresponding marker. Note that in the presence of TGFβ and BMP2 some clones still expressed Sox10. The low numbers of lost clones demonstrate that BMP2, GGF2 and TGFβ are not selectively eliminating certain cell lineages but are acting instructively on maintained NCSCs. Note that NCSCs exposed to Wnt1 have lost their responsiveness, as only very few clones were composed of sensory neurons. The numbers represent the mean ± SD. of three experiments, scoring 40 to 80 clones per experiment. To quantify the community-dependend effect of BMP2 on maintained NCSCs Mash-1 expression and cell death was assessed in clusters. Note that all cells expressed Mash-1 and cell death was minimal, excluding selective effects.

Previously, community effects provided by short range cell-cell interactions have been shown to influence fate decisions in NCSCs (Hagedorn et al., 2000a; Hagedorn et al., 1999). In particular, community effects modulate signaling of TGFß family members to promote autonomic neurogenesis rather than smooth muscle cell formation in NCSCs. Such context-dependent signal modulation might explain the discrepancy between the strong neurogenic effect of BMP2 seen in mass cultures (Fig. 42H) and the predominant smooth muscle-inducing activity of BMP2 in clonal cell cultures (Fig. 44E). To test this idea, NCSCs that have been maintained in the presence of Wnt1/BMP2 were replated as cell clusters (Fig. 44F) allowing short-range cell-cell interactions rather than as single cells (Fig. 44G-I). Subsequent treatment of BMP2 induced autonomic neurogenesis in virtually all cells within NCSC clusters (Fig. 44I). In fact, 98.6±1.3 of all cells within BMP2-treated NCSC-clusters expressed the autonomic marker Mash1 while cell death was minimal (1.4±1.3% of all cells). Thus, depending on the context, BMP2 efficiently instructed NCSCs after prolonged Wnt1/BMP2 exposure to become autonomic neurons, while TGFß induced clusters of smooth-musle cells and NRG1 clusters of glia (Fig. 44G, H). In sum, the combined activities of Wnt1 plus BMP2 support maintenance of multipotency and responsiveness to TGFß, NRG1, and BMP2 in NCSCs.

8.2.5 Loss of Wnt Activity and Wnt Responsiveness during Neural Crest Development

Although NCSCs that have been maintained in culture were sensitive to various instructive growth factors, their response to the sensory neuron-inducing activity of Wnt1 was clearly reduced when compared to the response of eNCSCs (compare Figs. 38, 39. with Fig. 42). This suggests that maintenance of NCSCs in culture leads to loss of their Wnt responsiveness with time. To address this issue, Wnt1/BMP2-treated NCSCs were replated at clonal density, as described above, and exposed to Wnt1 alone. In contrast to single eNCSCs (Fig. 40), only 13.5±3.7% of all maintained NCSCs generated sensory neuron-containing clones while the remaining cells displayed no obvious response to Wnt1 (Fig. 45A, B, table 4). This finding might represent a cell culture artefact with no relevance to neural crest development *in vivo*. Alternatively, the change in Wnt1-responsiveness of NCSCs might reflect processes also occuring *in vivo*. Cells with NCSC features persist during development in various postmigratory targets of the neural crest, including the sciatic nerve and the DRG (Bixby et al., 2002; Hagedorn et al., 1999; Morrison et al., 1999). To assess whether Wnt1 could induce sensory neurogenesis in postmigratory NCSCs present at later developmental stages, NCSCs

were isolated either from the sciatic nerve or from the DRG at embryonic day (E) 12, plated at clonal density, prospectively identified by p75 labeling, and incubated with or without Wnt1. Previous studies have shown that 80 to 90% p75-positive neural crest cells present in the sciatic nerve and in the DRG at these developmental stages are sensitive to the instructive activities of TGFß, NRG1, and BMP2 (Hagedorn et al., 1999; Morrison et al., 1999). However, in contrast to migratory eNCSCs, neither sciatic nerve- nor DRG-derived NCSCs were able to undergo increased sensory neurogenesis in response to Wnt1 (Fig. 45C, D), indicating changes of Wnt signal interpretation that have been acquired over time *in vivo*. To address whether Wnt/β-catenin activity persists at sites of postmigratory NCSCs, whole mounts of mice harboring TOPGAL, in which a β-galactosidase gene is under the control of a TCF/Lef and β-catenin inducible promoter, were analyzed (Fig. 45E-L). While Wnt activity is observed in migrating eNCSCs at E9.5 (Fig. 45E, F, arrow), Wnt activity decreases over time as observed at E10.5 (Fig 45G, H, arrow). At E12.5 there is no observable Wnt activity at sites of postmigratory NCSCs (Fig 45I, J, open arrow), while β-Gal exrpression in neural crest derivates can be observed in Wnt1-cre ROSA R26 mice (Fig 45K, L). Thus, concomitant with the loss of Wnt responsiveness over time, Wnt activity decreases during neural crest development.

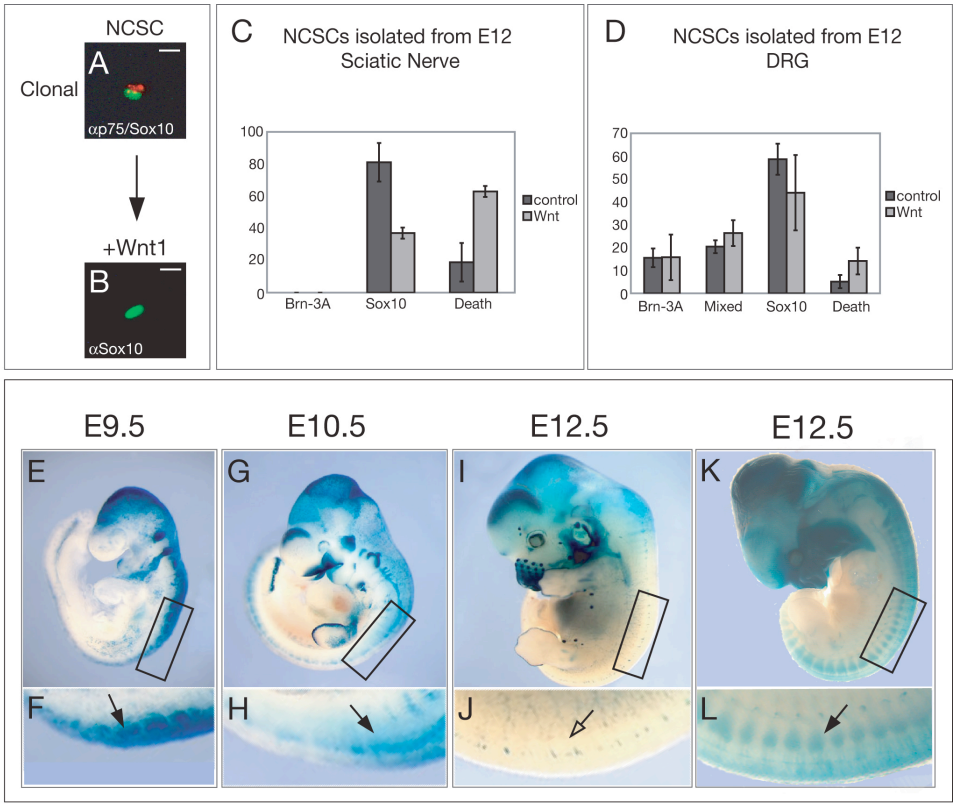

Figure 45: Loss of Wnt responsiveness and Wnt activity during neural crest development. NCSCs maintained for prolonged time replated at clonal density (**A**) and exposed to Wnt1 loose the responsiveness to Wnt1 and remain Sox10-positive (**B**), while they only rarely generate Brn-3A-positive sensory neurons and are negative for the glial marker GFAP (**table 4**, not shown). To test responsiveness of postmigratory NCSCs to the instructive growth factor Wnt1, NCSCs isolated from E12 sciatic nerve and DRG were isolated and exposed to Wnt1-expressing and control monolayers. Clonal analysis revealed that postmigratory NCSCs from sciatic nerve are not responsive to the sensory neuron-inducing activity of Wnt1, as no single Brn-3A expressing clone was generated from p75-positive founder cells. Note that 81.4±4.0% of all p75-positive founder cells coexpressed Sox10 (**C**). Clonal analysis demonstrates that postmigratory NCSCs isolated from DRG are not Wnt responsive anymore as there is no significant difference between Wnt1 and control. Of the p75-positive founder cells, 65±5.9% coexpressed Sox10. Note that some sensory neuronal progenitors also hasd been mapped, expressing p75, as about 20% of the clones gave rise to single sensory neurons in Wnt1- and control- conditions (**D**).Whole mount analysis of mice harboring TOPGAL, in which a β-galactosidase gene is under the control of a TCF/Lef and β-catenin inducible promoter (**E**, **G**, **I**). At E9.5 (**E**) Wnt/β-catenin activity is observed in regions of migrating neural crest (**F**, arrow). At E10.5 (**G**) Wnt/β-catenin activity decreases at sites of postmigratory NCSCs (**H**, arrow). At E12.5 (**I**), there is no Wnt/β-catenin activity at sites of postmigratory NCSCs (**J**, open arrow), while neural crest derivatives can be observed in Wnt1-cre ROSA R26 mice at E12.5 as control (**K**, **L**, arrow). Enlarged areas (**F**, **H**, **J**, and **K**) are marked by boxes in (**E**, **G**, **I**, and **K**), respectively.

8.3 Discussion

In this study we describe for the first time the maintenance of a pure neural stem cell population by combinatorial Wnt and BMP signaling. We identify Wnt and BMP signaling as the signals that instructively maintain NCSCs in an undifferentiated state in a fully defined medium. Although, the self-renewal is slow, NCSCs expressing the neural crest markers Sox10 and p75 can be maintained in culture in the presence of Wnt and BMP over prolonged time periods without differentiation. Further, maintained NCSCs differentiate into different fates in the presence of instructive growth factors, when Wnt and BMP are withdrawn. Moreover, with respect to sensory and autonomic neurogenesis, Wnt and BMP have antagonistic functions in eNCSCs. Over time maintained NCSCs loose the responsiveness to Wnt, while they are still responsive to other instructive growth factors including BMP. These cell culture data are highly consistent with processes occuring *in vivo*, because we find that postmigratory NCSCs to be non-responsive to the sensory fate inducing activity of Wnt. In addition, Wnt activity decreases at sites of postmigratory NCSCs. Taken together, combinatorial Wnt- and BMP-signaling regulates the maintenance and early lineage segregation in NCSCs.

A major question in neural crest biology is, when sensory progenitors segregate from the neural crest stem cell population. So far, it was neither possible to show at which time point such an early lineage segregation occurs during neural crest development, nor have the mechanisms underlying this process been described so far. Based on our finding that all eNCSCs are Wnt responsive (Lee et al., 2004), but not all generate sensory neurons *in vivo*, an unknown factor X must exist that counteracts Wnt signaling already as eNCSCs migrate out of the dorsal neural tube (Kleber and Sommer, 2004). Since BMP2 and BMP4 are produced by the ectoderm and the dorsal neural tube, they might be good candidates to be involved in such a process. BMPs are able to induce autonomic neurogenesis in NCSCs *in vitro* (Morrison et al., 1999; Shah et al., 1996; Shah and Anderson, 1997) and *in vivo* (Reissmann et al., 1996; Schneider et al., 1999). Therefore, the question arose why NCSCs that emerge from the neural tube do not immediately differentiate into autonomic neurons dorsally. Anderson already speculated that roof plate signals, such as Wnts could qualitatively change the effect of BMPs, on emigrating neural crest cells (Anderson, 2000).

8.3.1 BMP Suppresses Sensory Neurogenesis in Early Neural Crest Stem Cells

The first indication that BMP might be involved in counteracting Wnt signaling, was the finding that BMP2 significantly blocks sensory neurogenesis in early neural crest explants. This is in contrast to earlier studies, which have shown that BMP2 does not prevent either dividing or postmitotic precursors from executing a sensory fate (Greenwood et al., 1999). This discrepancy could be explained by the time point at which BMP2 is applied to cultures and by species differences. Greenwood and colleagues applied BMP2 to rat neural crest explants after 24 h, at a time point where Brn-3A is already expressed and lineage segregation already occurred in culture. From those data they conclude that lineage segregation between sensory and other neural crest derivates occurs at a very early stage, probably before, or shortly after emigration (Greenwood et al., 1999). In contrast, we applied BMP2 on eNCSCs after 20 h of emigration in mouse cultures and after 15 h in rat cultures. At that time point all eNCSCs represent a homogenous population with respect to the neural crest markers Sox10 and p75. When we applied BMP2 on early neural crest explants we were able to significantly reduce the percentage of Brn-3A-positive sensory progenitors per explant. Based on this data we conclude that after endogenous Wnt has induced the expression of the sensory marker Brn-3A, such cells are no longer challengeable by BMP2. When we similarly applied BMP2 to early neural crest explants expressing stabilized β-catenin we found that BMP2 completely

blocks sensory neurogenesis even in the presence of persisting Wnt signaling. Although BMP2 can block sensory neurogenesis in such explants, BMP induced autonomic neurogenesis is also inhibited. This shows that sustained β-catenin interferes with BMP-dependent autonomic neurogenesis and indicates that BMP2 interferes with canonical Wnt signaling downstream of β-catenin at a transcriptional level. Convergence between Wnt- and BMP-signaling has been reported in several studies in which Smad4 directly associates with TCF/Lef transcription factors (Hussein et al., 2003; Labbe et al., 1998). BMP2 therefore counteracts Wnt/β-catenin dependent sensory neurogenesis in eNCSCs.

8.3.2 Maintenance of Neural Crest Stem Cell Markers and Suppression of Differentiation by Combined Wnt and BMP Signaling

Besides the inhibitory effect of BMP2 on Wnt1-dependent sensory neurogenesis from eNCSCs, BMP2 efficiently generates autonomic ganglia when applied to eNCSCs grown on feeder cell monolayers, while in contrast Wnt1 induces the formation of sensory ganglia. However, the combination of both, namely Wnt1 and BMP2 applied on eNCSCs, inhibits autonomic as well as sensory neurogensis. Instead, all cells remain Sox10-positive. Studies in avian embryos suggested that Wnt- and BMP-signaling have antagonistic functions in the specification of trunk neural crest (Jin et al., 2001). Our data reveal that Wnt and BMP have antagonistic functions, by suppressing neurogenesis in general. Studies in the dorsal telencephalon also reported that canonical Wnt and BMP signaling cooperatively regulate the expression of certain genes (Theil et al., 2002). In neural crest development Wnt and BMP could synergistically cooperate together by maintaining Sox10-expression, while other fates are suppressed.

Recently, the transcription factor Sox10 has been demonstrated to be involved in maintaining multipotency in NCSCs by inhibiting neuronal differentiation (Kim et al., 2003). On the other hand Sox10 is also expressed in glial cells (Paratore et al., 2001). In the presence of Wnt1 and BMP2, cultured NCSCs are Sox10- as well p75-positive, while they did neither express gliogenic nor neuronal markers. These results suggests that Wnt and BMP play a crucial role in maintaining multipotency in NCSCs. Kim and colleagues could show that for neural crest maintenance a higher *Sox10* gene dosage is needed (Kim et al., 2003). In addition, recent studies demonstrate that Sox-proteins can interact with β-catenin and thus with Wnt signaling (Akiyama et al., 2004; Sinner et al., 2004). Whether Sox10 might be a direct target gene of combined β-catenin/TCF/Lef- and Smad4-transcription factors remains

to be elucidated. Further, we found that Wnt and BMP instructively promote stem cell features in eNCSCs, as only Sox10-expressing clones arose in these conditions. Analysis of the clone size demonstrated self-renewal in about 52% of all clones. Stemple and Anderson described that NCSCs self-renew in about 54% (Stemple and Anderson, 1992). We conclude that the self-renewal in these NCSCs is slow as opposed to fast cycling transient amplifying progenitors. When we exposed eNCSCs at clonal density to Wnt1 and on day 2 subsequently to BMP2, BMP2 had no effect anymore as most eNCSCs adopted a sensory fate. This shows that Wnt and BMP have to act concomitantly. Maintenance of the stem cell state involves both maintenance of stem cell markers and inhibition of overt differentiation as well as maintenance of multipotency. Indeed, NCSCs maintained in culture efficiently differentiated in response to different instructive growth factors that have been added after stem cell expansion. Interestingly, maintained NCSCs loose the responsiveness to Wnt, as only very few sensory ganglia expressing Brn-3A and NF were found, while most of the cells remained Sox10-positive. On the other hand NCSCs efficiently adopted smooth-muscle-like fates in the presence of TGFβ, glial fates in the presence of NRG1, and autonomic fates in the presence of BMP2 alone. This clearly demonstrates, that the maintained NCSCs are intrinsically different compared to an eNCSC. Intrinsic differences among NCSCs from different stages and lovations have been described in various studies (Bixby et al., 2002; Kubu et al., 2002; White et al., 2001). During development, NCSCs undergo cell-intrinsic changes to instructive growth factors, while maintaining their multipotency capacity (White et al., 2001). This suggests, that combined Wnt and BMP signaling maintains multipotency of NCSCs, but changes their ability to respond to Wnt signaling. These data show that maintained NCSCs intrinsically differ from eNCSCs, by having a constraint potential in regard to the generation of cell fates in response to instructive factors.

8.3.3 Wnt and BMP Regulate the Maintenance and Early Lineage Segregation of NCSCs

In order to exclude selective effects maintained NCSCs were challenged by different instructive growth factors including Wnt1. Our results demonstrated, that maintained NCSCs are still multipotent, as they adopt a smooth-muscle-like fate in the presence of TGFβ and a glial fate in the presence of NRG1. Surprisingly, maintained NCSCs in culture displayed community effects, as single NCSCs adopted a smooth-muscle-like fate in response to BMP2, while NCSCs replated at high-density generated clusters of autonomic neurons expressing Mash-1. However, maintained NCSCs are not Wnt responsive anymore, and remain Sox10-

positive. Taken together we propose, that Wnt and BMP not only regulate the maintenance but are also crucial for early lineage segregation of NCSCs. Lineage segregation is the process that accompanies the differentiation of various cell types from a pool of multipotent stem or progenitor cells (Morrison et al., 1997). One key feature of lineage restriction is the generation of various proliferating progenitor cells, which exhibit gradual restrictions in their developmental potential (Anderson, 2000). Our data further support the model of early lineage segregation between sensory and other neural crest derivatives (Figure 9). Wnts, namely Wnt3A and Wnt1 (Dorsky et al., 1998), that are expressed in the dorsal neural tube, induce the formation of sensory neural cells from a homogeneous pool of eNCSCs. Although all eNCSCs are responsive to Wnt (Lee et al., 2004), not all eNCSCs adopt a sensory fate during development. We propose that combined Wnt- and BMP-signaling instructively maintains multipotency in NCSCs by preventing sensory neurogenesis in some of the emigrating NCSCs. These cells remain multipotent migratory NCSCs (Figure 9). This model also explains why emigrating NCSCs do not differentiate into autonomic neurons dorsally and resolves the paradox of the sensory- and autonomic-decision, discussed by Anderson in a recent review (Anderson, 2000). Such a model also explains how the initial heterogeneity of neural crest cells is established at the time point of emigration when lineages are intermingled with multipotent NCSCs fated for other lineages (Zirlinger et al., 2002). Although maintained NCSCs can give rise to various crest derivatives, including non-neural, neural and glial fates, they are not Wnt responsive anymore. The combined data suggest that after delamination eNCSCs migrate further to become postmigratory NCSCs that encounter other instructive growth factors. Likewise, results from clonal experiments revealed that postmigratory NCSCs isolated from sciatic nerve and DRG are not Wnt responsive anymore. In parallel Wnt/β-catenin activity decreases at sites of postmigratory NCSCs, as shown by the analysis of mice harboring TOPGAL, in which Wnt/β-catenin activity can be directly followed. We propose that through such a mechanism the organism regulates the correct generation of cell types at distinct locations and prevents the ectopic formation of sensory neurons. Our data also clearly demonstrate that the second wave of sensory neurogenesis *via* Ngn1 in the forming DRG (Ma et al., 1999) is apparently not directly regulated by canonical Wnt signaling. Recent studies also support our model of early lineage segregation by combinatorial Wnt and BMP signaling. Guha and colleagues reported that overexpression of the BMP inhibitor noggin resulted in a significant increase of sensory neurons in the trigeminal and dorsal root ganglia, while in contrast overexpression of BMP4 resulted in a significant decrease in the number of sensory neurons in the DRG (Guha et al., 2004). Inhibition of BMP at the time point of neural

crest emigration therefore disturbs the regulation of combinatorial Wnt-and BMP-signaling in a dosage dependent manner and most of the cells adopt a sensory fate at the expense of other neural crest derivatives. In contrast overexpression of BMP disturbs the formation of sensory neurons as most of the cells remain multipotent and migrate further.

We consider that our findings give important insights of how early lineage segregation in NCSCs is established from multipotent eNCSCs as they emigrate from the dorsal neural tube. Moreover, our data resolve some remaining questions which were previously unknown. The question of how neural stem cells can be maintained in a pure multipotent state is important for possible tissue replacement therapies. Factors that maintain NCSCs in an undifferentiated state have not been previously described. Furthermore, it is important to understand and resolve the mechanisms of how lineage segregation and diversification is achieved during development. Early NCSCs, NCSCs and postmigratory NCSCs are transient populations of stem cells that intrinsically change over time by changing the sensitivity to specific extracellular signals. However, it still remains elusive of whether such maintained NCSCs would also give rise to the same derivatives *in vivo* upon transplantation in chick. Since *in vitro*, maintained NCSCs do not display a high proliferation rate at clonal density, unknown factors that would promote proliferation might exist. Future studies will also reveal of how the Sox-proteins are involved in the combinatorial Wnt and BMP signaling on the transcriptional level.

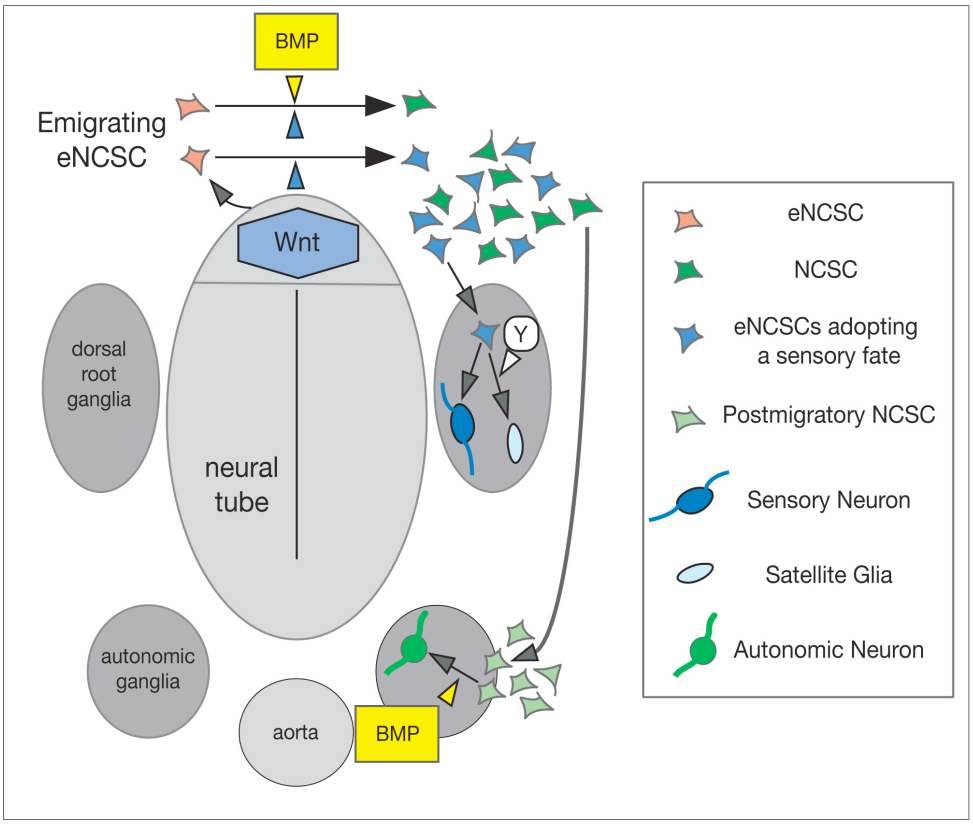

Figure 46: Model of combinatorial Wnt- and BMP-dependent lineage segregation in early neural crest stem cells. Wnt expressed in the dorsal neural tube induces the formation of sensory neural cells (blue) from eNCSCs (red) as the latter emigrate from the neural tube. In addition, BMPs (yellow) are expressed in the dorsal midline region at the time point of neural crest emigration. We propose that some eNCSCs that encounter Wnt and BMP are instructively promoted to maintain neural crest stem cell features instead of adopting a sensory fate. Such cells remain multipotent (green) and migrate further where they are exposed to other instructive growth factors as postmigratory NCSCs (light green). Over time, NCSCs and postmigratory NCSCs loose their responsiveness to Wnt. Furthermore, Wnt activity decreases at sites of postmigratory neural crest derivatives. For simplicity, of all possible neural crest-derived structures only forming autonomic ganglia are shown, in which BMP induces an autonomic fate. In the dorsal root ganglia, a factor Y prevents some sensory progenitor cells from differentiating into sensory neurons, allowing the formation of satelite glia.

8.4 Outlook

8.4.1 Transplantation of Maintained NCSCs

Heterotopic transplantation experiments employing the chick-chimera system have been already addressed by Le Douarin and co-workers. These highly informative experiments have indicated that, to a first approximation, axial differences in crest cell fate are environmentally rather than intrinsically determined (Le Douarin, 1980). The chick chimera system allowed the tracing of injected cells *in vivo*, and revealed whether an isolated stem cell, injected into the chick also functionally was able to give rise to different derivatives *in vivo*. Many of these experiments were performed to analyze how lineage segregation during neural crest development occurs (Bixby et al., 2002; Kruger et al., 2002; Le Douarin, 1980; Le Douarin, 1986; Morrison et al., 1999; White and Anderson, 1999; White et al., 2001). *In vivo* transplantation is a useful tool for assessing the differentiation capacities of isolated stem cell populations, because the host embryo should contain all of the relevant differentiation signals, which may be difficult to identify *in vitro*. White and colleagues showed that they could inject rat NCSCs from E10.5 neural tube explants into host chick embryos and that these rNCSCs gave rise to glia and neurons expressing appropriate markers in sensory and sympathetic ganglia (White and Anderson, 1999). In a further study the same authors nicely showed that migratory NCSCs compared to sciatic nerve NCSCs are intrinsically different when transplanted into chick embryos (White et al., 2001). Morrison and colleagues used the same tool to characterize sciatic nerve NCSCs (Morrison et al., 1999) and in further studies they revealed cell-intrinsic differences between stem cells from different regions of the PNS (Bixby et al., 2002). So in order to complete our study of early lineage segregation and maintenance of NCSCs by combinatorial Wnt and BMP signaling, it would very much

strengthen the study if we transplanted NCSCs that have been maintained in culture into the migratory neural crest stream of chick embryos to elucidate whether such NCSCs are able to give rise to various derivates in the host embryo. Moreover, we could also consider comparing eNCSCs with maintained NCSCs and postmigratory NCSCs isolated from sciatic nerve and DRG. Such experiments would manifest the role of combinatorial Wnt- and BMP-signaling in neural crest development.

8.4.2 A Factor for Self-Renewal

Although Wnt and BMP instructively promote NCSC maintenance from eNCSCs, clonal analysis revealed that the clone size of Sox10-containing clones was relatively small, suggesting that combined Wnt and BMP signaling does not induce proliferation. As about 50% of the clones divided we either suggest that those maintained NCSCs only proliferate slowly, or that a third factor is needed for effiecient self-renewal. Since NCSCs were cultured in a fully defined media (Kléber and Sommer, in press) in the presence of Wnt and BMP, we exclude that unidentified factors could counteract proliferation. In order to investigate possible inhibition of the proliferation rate I would rather suggest to specifically manipulate the media components. First, I would start to reduce the FGF levels, as our culture media contains high doses of FGF. Several studies have reported that FGFs can modulate canonical Wnt activity. For instance in the CNS, FGF2 increases the levels of active β-catenin in neural progenitors from the ganglionic eminence. β-catenin overexpression maintains these cells in a proliferate state when cultured as neurosperes in the presence of FGF2, while it increases the proportion of neurons in adhesive cultures in the absence of FGF2 (Israsena et al., 2004). It might be possible that modulation of FGF levels could influence proliferation of maintained NCSCs. Recently, we addressed the role of FGFs in eNCSCs. When eNCSCs emigrate in the absence of FGF, the explants are quite small and cell death within the explants is increased. However, it is so far not clear if the effect is due to perturbed proliferation in early neural crest explants or due to increased cell death. Although emigration of eNCSCs is perturbed in the absence of FGF, the explants can still give rise to sensory neurons when cultured in conditions permissive for sensory neurogenesis. Whether these preliminary data reflect a role of FGF in early neural crest development remains to be elucidated. Beside FGFs, I would expose maintained NCSCs to factors that are expressed as NCSCs emigrate out of the neural tube. In parallel, gene expression profiling data from multipotent NCSCs might unravel new, so far unidentified factors that are involved in that process (Buchstaller et al., 2004).

8.4.3 What is the Mechanisim Underlying NCSC Maintenance by Combinatorial Wnt and BMP Signaling

It would be very interesting to investigate the mechanism of how combinatorial Wnt and BMP signaling regulate neural crest stem cell maintenance and lineage segregation. I would start to investigate which target genes might be induced by the β-catenin/TCF/Lef and Smad4-complex. The fact that all cells remain Sox10-positive raises the question of whether the *Sox* genes might be targets of β-catenin/TCF/Lef and Smad4. I would search for possible TCF/Lef DNA binding sequences within the promoter region of Sox10. TCF/Lef transcription factors recognize the nucleotide sequence 5' CTTTGWW 3' (where W indicates A or T) (Eastman and Grosschedl, 1999). Should such sequences exist in the promoter region of Sox10, I would perform a luciferase assay, in which cells would be co-transfected with plasmids expressing β-catenin/TCF/Lef and Smad4 together with a plasmid, in which the *Sox10* promoter region including the important nucleotide sequence is subcloned to the *Renilla* luciferase gene. As a control I would mutate the TCF binding site and compare the luciferase activity to the unmutated TCF binding site. A similar experiment has been performed to show that ngn1 is a direct target gene of β-catenin /TCF/Lef (Hirabayashi et al., 2004). A possible interaction between the transcriptional β-catenin/TCF/Lef and Smad4-complex and Sox10 could also occur on a protein level. Indeed, the human Sox7 has recently been shown to interact with β-catenin in cell culture (Takash et al., 2001). Furthermore in other contexts, Sox proteins (Sox17, Sox3 and Sox7) can antagonize β-catenin /TCF/Lef-mediated transcription (Takash et al., 2001; Zhang et al., 2003; Zorn et al., 1999). Thus, the interaction between β-catenin/TCF/Lef-transcription factors with Sox proteins may explain, how the Wnt signaling pathway can elicit diverse transcriptional responses in different cellular contexts. A recent study demonstrated that the Sox17 protein contains a motif in the C terminus, which is conserved in all members of the SoxF subfamily, and that this motif is required for the ability of Sox17 to both transactivate genes and bind β-catenin (Sinner et al., 2004). During chondrocyte differentiation β-catenin physically interacts through its Armadillo repeats with the C-terminal transactivation domain of Sox9 (Akiyama et al., 2004). Whether such effects between the β-catenin/TCF/Lef and Smad4-complex and the Sox10 protein also occur during early lineage segregation in NCSCs remains to be elucidated.

Another interesting question to address would be whether combinatorial Wnt and BMP signaling induces the expression of Id proteins. Id proteins are one of the most crucial targets of BMP signaling. Id proteins were originally identified as negative targets of bHLH transcription factors. Four Id proteins, Id1 to Id4, have been identified in mammals.

Generally, Id proteins act as positive regulators of cell cycle and negative regulators of cell differentiation (Norton, 2000; Yokota and Mori, 2002). Id proteins positively regulate cell cycle progression as has been demonstrated by analyzing Id1-Id3 double knockout mice (Lyden et al., 1999). BMP signaling increases the synthesis of Id1, Id2, and Id3 (Hollnagel et al., 1999). Id promoter activation by BMPs occurs in a manner that requires Smad1, Smad5 and Smad4 (Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). In the nervous system, neurogenic bHLH transcription factors such as Mash-1, ngns and NeuroD induce neurogenesis. BMP2 induces the expression of Id1 and Id3 in neuroepithelial cells in a Smad-dependent manner. Both Id1 and Id3 repress the promoter activation induced by bHLH heterodimers containing neurogenic bHLH factors. Id1 and Id3 therefore inhibit neurogenesis (Lyden et al., 1999). In regard to neural crest development the question arises why on one hand BMPs induces autonomic neurogenesis by activation of the bHLH factor Mash-1 (Shah et al., 1996; Sommer et al., 1995) and on the other hand they have shown to repress neurogenesis. In embryonic stem cells, BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3 (Ying et al., 2003). One explanation could be that the BMPs elicit different responses in collaboration with other signaling pathways such as the Wnts. Since combinatorial Wnt- and BMP-signaling in eNCSCs completely blocks bHLH-dependent neurogenesis, the question arises whether combinatorial Wnt and BMP signaling induce the expression of Id proteins, which then inhibit sensory as well autonomic neurogenesis. To address that question I could imagine to perform western blots of maintained neural crest stem cell lysates and test whether they express Id proteins. If in parallel an inducible *Id1-3* knockout would be available, I would cross those loxP *Id*-mice together with *wnt1*-Cre mice. In Id mutants the combinatorial Wnt and BMP signaling would be disturbed in a way, that apart from sensory neurons, autonomic neurons also would arise dorsally at the expense of further migrating NCSCs.

8.4.4 The Role of Ngn1 and Notch1 in the Formation of the Early Dorsal Root Ganglion

In the early forming dorsal root ganglia we suggest that a factor Y prevents some sensory progenitor cells from differentiating into sensory neurons, allowing the formation of satellite glia (Kleber and Sommer, 2004) (Figure 46). At that stage, (i.e. at about E10.5), *in situ* hybridization experiments revealed that Notch1 as well as the proneural gene *ngn1* is expressed, while ngn2-expressing cells are rarely found (Hari et al., 2002; Sommer et al., 1996). Analysis of *wnt1*-Cre *loxNotch1* mutants *in vivo* at E10.5 revealed that Sox10 is

markly reduced and ngn1 is absent at sites of dorsal root ganglia formation (data not shown). Further, Brn-3A-expression is increased in such mutants. Quantification of dissociated DRG cells revealed that in the mutant significantly more NF-positive cells are found at the expense of Sox10-positive presumptive glial cells (Lee, Kleber and Sommer, unpublished). From these data we suggest that Notch1 might be involved in the regulation of early DRG formation by regulating the *bias* between sensory neurons and satellite glia cells. Complementary cell culture experiments, however, did not confirm these ideas, probably due to the fact that eNCSCs cultured on Wnt1-expressing monolayers generate sensory ganglia but might do not fully develop to a Ngn1 and Notch1 dependent stage. To address these questions, I suggest to first improve and further establish the cell culture system and evaluate, whether it might be possible to find Notch1- and Ngn1-expressing cells within such sensory ganglia. Antibodies for Notch1 as well as for Ngn1 are available in the laboratory. Another experiment would be to add soluble delta on forming sensory ganglia and to see whether there is a difference in the ratio between Brn-3A-and Sox10-positive cells. It is still possible that another factor is needed to mimic ongoing formation of the DRG in culture. A study in zebrafish revealed that sonic hedgehog signals are required for the development of ngn1-dependent dorsal root ganglia neurons (Ungos et al., 2003). Addition of sonic hedgehog (shh) proteins to sensory ganglia in culture however, did not result in an upregulation of Ngn1 or in an increase of ganglia size. Thus shh might not be involved in this process in the mouse. Again, a careful analysis of gene expression profiling studies might unravel new, so far unidentified factors that are involved in Ngn1-dependent neurogenesis and satelite glia formation (Buchstaller et al., 2004).

8.4.5 Early NCSCs and their Potential to Generate Non-Neural Derivatives

NCSCs migrate throughout the body and differentiate into many cell types. It has been reported that although NCSCs are pluripotent, differences exist between cells that are generated from different anterioposterior levels. NCSCs from the trunk form melanocytes, sensory neurons, autonomic neurons, glia and non-neural derivatives, whereas NCSCs in the cranial region also have the potential to form mesenchymal derivatives, such as cartilage, bone and connective tissue (Knecht and Bronner-Fraser, 2002). Trentin and colleagues recently reported from analyzing the self-renewal capacity of various types of neural crest precursor cells, that trunk neural crest did not generate cartilage. This provides additional evidence that trunk NCSCs lack skeletogenic potential. However, this is in contrast to an

earlier study, which showed that trunk neural crest has skeletogenic potential. In this study, avian trunk NCSCs form both cartilage and bone cells, and after placement in the head they contributed to cranial skeletal components (McGonnell and Graham, 2002). Early transplantation experiments by Le Douarin and colleagues also indicated that axial differences in crest cell fates are environmentally rather than intrinsically determined (Le Douarin, 1980). Cranial NCSCs have been show to differentiate into chondrocytes, osteoblasts, and also odontoblasts of the teeth (Bronner-Fraser, 1995; Hall, 1990). Chondrogenesis results in the formation of cartilages, initial skeletal elements, which can serve as templates for endochrondral bone formation. One initial step is the expression of the transcription factor Sox9 (Bi et al., 1999). Recently it has been shown that Sox9 is required for the determination of the chondrogenic cell lineage in the cranial neural crest. The ablation of Sox9 in cranial NCSCs causes the cells to lose their chondrogenic potential (Mori-Akiyama et al., 2003). Factors that are involved in chondrogenesis have been described in various studies. Those include TGFβ {Ionescu, 2003 #5118}, BMPs (Dudas et al., 2004; Sammons et al., 2004), IGFs (Canalis, 1993), FGFs (Amizuka et al., 2004), Wnts (Akiyama et al., 2004), and parathyroid hormone-related peptide (PTHrP) (Amizuka et al., 2004; Amizuka et al., 2002; Amizuka et al., 2003). The question now arises of whether eNCSCs are "the truly multipotent NCSC" that could also gives rise to cartilage, bone and connective tissue. When eNCSCs are cultured in the presence of TGFβ and IGF1, they rapidly differentiate into dividing SMA-positive cells. Our idea is now to test whether we are able to let these cells further differentiate in the presence of TGFβ, IGF1, BMP, FGF or PTHrP. Although in a preliminary experiment such cells lost the immunreactivity to SMA and were cultured for approximately a month we were not able to characterize these cells, since they did not express the chondrocyte markers Sox9 and collagen type II. An interesting finding was that PTHrP induced cell death when applied directly on eNCSCs or SMA-positive cells. We therefore added PTHrP after culturing the cells for approximately 2 weeks. However, so far we failed to induce chondrogenesis from eNCSCs. Further, it might be possible that not all factors are needed at the same time point. It might also be possible that fibronectin is not the appropriate substratum to drive the cells into a chondrogenic fate. To design new experiments I would replate eNCSCs at high density onto collagen coated plates or NIH3T3 feeder monolayers. In sum, it remains to be shown whether eNCSCs have skeletogenic potential. In regard to tissue replacement therapies, an appropriate stem cell system that allows the generation of a wide variety of homogeneous cell types might be of general interest and would open new doors to microarchitecture and tissue-engineering methods.

9 MATERIALS AND METHODS:

Primary Cultures from Embryonic and Newborn Tissues*:* Neural Crest Stem Cells

Maurice Kléber and Lukas Sommer

Cell Biology: A Laboratory Handbook 3ED, **Elsevier,** in press

9.1 Introduction

Cellular diversity in the vertebrate peripheral nervous system is achieved by differentiation of neural crest stem cells (NCSCs) in a spatially and temporally regulated fashion. During embryonic development, neural crest cells detach from the neuroepithelium of the dorsal neural tube and migrate to their sites of terminal differentiation (Le Douarin and Kalcheim, 1999). At least a subpopulation of these cells are multipotent and able to give rise to neuronal, glial and non-neural derivatives, as has been shown by grafting experiments and by clonal analysis in culture and *in vivo* (Anderson, 1997; Ziller et al., 1983). Moreover, some crest cells display features of stem cells that not only generate multiple cell types but also have the capacity to self-renew (Morrison et al., 1999; Stemple and Anderson, 1992). In migrating neural crest and in target tissues of the neural crest, multipotent crest cells coexist with cells that have more restricted developmental potential (Sommer, 2001). Cell intrinsic differences between crest cells from different regions of the peripheral nervous system are involved in the generation of neural diversity. In addition, the decision of a NCSC to survive, self renew or differentiate depends on the combinatorial activity of multiple environmental signals (Sommer, 2001). To identify these signals, NCSCs have to be challenged by altering both their extracellular environment and their intrinsic genetic programs. This is greatly facilitated by the availability of neural crest culture systems. Since signals required maintaining undifferentiated, multipotent NCSCs for an extended period of time in culture have not yet been identified, different laboratories have established various cell culture conditions. Here we describe methods for culturing rat and mouse neural crest stem cells, largely based on articles by Stemple and Anderson (1992) (rat NCSCs), Sommer et al. (1995) (mouse NCSCs), Greenwood et al. (1999) (culture conditions permissive for sensory neurogenesis), and Morrison et al. (1999) (postmigratory NCSCs).

9.2 Materials and Instrumentations

Instruments and plastic ware:

Modular Incubator Chamber (Billups-Rothenberg Inc., www.brincubator.com); Dumont #3 and #5 forceps (Fine Science Tools, Cat. No. 11231-30 and Cat. No. 11251-10); Vannas style iris spring scissors (Fine Science Tools, Cat. No. 15000-02); cell culture dishes 35mm x 10mm style (Corning Cat. No. 430165); 6 well cell culture dishes, (Corning Cat. No. 430166); tissue culture dishes 60mm x 15mm style (Nunclon DSI Cat. No. 064194); Omnifix 50 ml (Braun Cat. No. 459785OF); Millex syringe driven filter unit 0.22μm (Millipore Cat. No. SLGPO33RB); Steritop 500 GP express plus membrane 0.22μm (Milipore Cat. No. SCGPT05RE).

General buffers and reagents:

Hanks' balanced salts (HBSS) without Ca^{2+} and Mg^{2+} 95.18g (Amimed Cat. No. 3-02P30-M); Hanks' balanced salts without phenol red 97.5g (Amimed Cat. No. 3-02P32-M); PBS Dulbecco (D-PBS) 50l (Biochrom KG Cat. No. L-182-50); formaldehyde solution 250ml (Fluka Cat. No. 47608); potassium hydroxide (Fluka Cat. No. 60375).

Enzymes:

Dispase 1 (neutral protease) 10x 5mg (Roche Cat. No. 1 284 908); collagenase type 1, 3, and 4 (Worthington Biochemical Coorporation); trypsin-EDTA 0.25% 100ml, (Invitrogen-Gibco Cat. No. 25200-056); trypsin (10x) 2.5% 100ml, (Invitrogen-Gibco Cat. No. 25090-028); hyaluronidase type IV-S 50mg (Sigma H-4272); deoxyribonuclease type 1 (Sigma Cat. No. D-4263).

Substrates:

Fibronectin (0.1% solution) 5mg (Sigma Cat. No. F1141); poly-D-lysine (pDL) 5mg, (Sigma Cat. No. P-7280).

Media components:

Dulbecco's modified Eagle medium (DMEM)-low glucose 500ml (Invitrogen-Gibco Cat. No. 11880-028); DMEM 500ml (Invitrogen-Gibco Cat. No. 41966-029); minimum essential medium (MEM) 500ml (Invitrogen-Gibco Cat. No. 31095-029); Leibovitz's L15 medium powder (Invitrogen-Gibco Cat. No. 41300-021); dimethylsulfoxide (DMSO) 25l (Aldrich Cat. No. 27,043-1); N2-supplement (100x) 5ml (Invitrogen-Gibco Cat. No. 17502-048); 2-mercaptoethanol (Sigma Cat. No. M-7522); B-27 supplement (50x) 10ml (Invitrogen-Gibco Cat. No.17504-048); forskolin 10 mg (Sigma Cat. No. F-6886); fetal bovine serum (FBS) 500ml (different suppliers); water, cell culture tested 500ml (Sigma Cat. No. W-3500); phenol

red solution 100ml (Sigma Cat. No. P-0290); imidazole 1g (Sigma Cat. No. I-0250); hydrochloride acid solution 100ml (Sigma Cat. No. H-9892); sodium bicarbonate 500g (Sigma Cat. No. S-5761); dexamethasone 25mg (Sigma Cat. No. D-4902); bovine albumin crystalline 5g (Sigma Cat. No. A-4919); glycerol 99.5% 500ml (Invitrogen-Gibco Cat. No. 15514-011); transferrin, holo, bovin plasma 100mg (Calbiochem Cat. No. 616420); putrescine, (Sigma Cat. No. P-7505); (+/-)-a-tocopherol (Vitamin E) 5g (Sigma Cat. No. T-3251); insulin 100mg (Sigma Cat. No. I-6634); human epidermal growth factor, (hEGF) 200μg (R&D Systems Cat. No. 236-EG-200); human nerve growth factor (β-NGF) (R&D Systems Cat. No. 256-GF-100); selenious acid (Aldrich Cat. No. 22,985-7); basic fibroblast growth factor (bFGF) 25μg (R&D Systems Cat. No. 233-FB-025); progesterone 1g (Sigma Cat. No. P-8783); human neurotrophin 3 (NT3) 10μg (BioConcept Cat. No. 1 10 01862); human brain derived neurotrophic factor (BDNF) 10μg (BioConcept Cat. No. 1 10 11961); insulin-like growth factor (IGF1) 250μg (R&D Systems Cat. No. 291-G1-250); retinoic acid (Sigma Cat. No. R-2625).

Stable Vitamin Mix:

Aspartic acid 100g (Sigma Cat. No. A-4534); L-glutamic acid 100g (Sigma Cat. No. G-8415); L-proline 25g (Sigma Cat. No. P-4655); L-cystine (Sigma Cat. No. C-7602); p-aminobenzoic acid (Aldrich Cat. No. 42,976-7); 3-aminoproprionic acid 100g (Sigma Cat. No. A-9920); vitamin B-12 1g (Sigma Cat. No. V-6629); myo-inositol 50g (Sigma Cat. No. I-7508); choline chloride 100g (Sigma Cat. No. C-7527); fumaric acid 100g (Sigma Cat. No. F-8509); coenzyme A 100mg (Sigma Cat. No. C-4282); D-biotin (Sigma Cat. No. B-4639); DL-α-lipoic acid 5g (Sigma Cat. No. T-1395).

1:1:2:

Dextrose (D-(+)-glucose (Sigma Cat. No. G-7021); L-glutamine (Sigma Cat. No. G-6392); penicillin-streptomycin 100ml (Invitrogen-Gibco Cat. No. 15140-015).

Mix7:

DL-β-hydroxybutyric acid sodium salt (Sigma Cat. No. H-6501); cobalt chloride 25g (Sigma Cat. No. C-8661); oleic acid 250mg (Sigma Cat. No. O-7501); α-melanocyte stimulating hormone (α-MSH) 5mg (Sigma Cat. No. M-4135); prostaglandin$_{E1}$1mg (Sigma Cat. No. P-5515); 3,3',5- triiodo L-thyronine (T3) 100mg (Sigma Cat. No. T-6397).

FVM:

DMPH B Grade (Calbiochem Cat. No. 31636); L-glutathione (Sigma Cat. No. G-6013); L-ascorbic acid 25g (Sigma Cat. No. A-4544).

9.3 Procedures

9.3.1 Solutions and Stocks:

9.3.1.1 Chicken Embryo Extract (CEE)

Incubate white chicken eggs for 11 days at 38°C in a humidified atmosphere. Wash eggs with 70% ethanol, open the top of each shell, remove the embryos and place them into a petri dish containing MEM at 4°C. Macerate the embryos by pressing through a 50ml syringe into a 50ml centrifuge tube (Falcon) (approximately 25ml of homogenate per tube). Add 25ml of MEM per 25ml of chicken homogenate. Shake the tubes at 4°C for 1 hour. Add 100 µl (800U) sterile hyaluronidase to 50ml chicken homogenate and centrifuge the mixture for 6 hours at 30'000 x g at 4°C. Collect the supernatant, filtrate through a 0.22µm Steritop filter and distribute in 5ml aliquots. Store at –80°C until use.

9.3.1.2 Stable Vitamin Mix (SVM)

Collect 198ml water into a detergent-free beaker. To the 198ml water add 0.6g aspartic acid, 0.6g L-glutamic acid, 0.6g L-proline, 0.6g L-cystine, 0.2g p-aminobenzoic acid, 0.2g 3-aminoproprionic acid, 80mg vitamin B-12, 0.4g myo-inositol, 0.4g choline chloride, 1.0g fumaric acid and 16mg coenzyme A. Suspend 0.4mg D-biotin and 100mg DL-α-lipoic acid in 10ml water and add 2ml of this solution to the solution in the beaker. Mix the solutions, prepare 1,5ml aliquots and store at –20°C until use.

9.3.1.3 L-15CO2

Add 3.675g L-15 powder, 0.019g imidazole and 1.6ml SVM to 288ml water in a 500ml beaker. Mix the solution until dissolved and add 240µl of 1M HCl to adjust the pH between 7.35 and 7.40. Mix in a 100ml beaker 0.8g sodium bicarbonate, 120µl phenolred, and 59ml water. Apply CO_2 directly to this solution using a Pasteur pipette until it turns yellow and no further color change can be observed. Then mix the sodium bicarbonate solution with the L-15 solution and apply again CO_2 for a short time. Determine the pH, which should range

between 7.15 and 7.25. Filter the solution through a 0.22µm filter into a 500ml tissue culture bottle and store at 4°C until use.

9.3.1.4 1:1:2

Slowly dissolve 60g dextrose in 160ml water by stirring. Adjust the volume to 200ml after dextrose is dissolved. Add 100ml glutamine (200mM) and 100ml penicillin-streptomycin, filter the solution through a 0.22µm filter and distribute in 2ml aliquots. Store at –20°C until use.

9.3.1.5 Fresh Vitamin Mix (FVM)

Dissolve 5mg DMPH, 25mg glutathione, and 500mg L-ascorbic acid in 80ml water by stirring. After all chemicals are dissolved, raise the pH to 5-6 with 1M potassium hydroxide. Then adjust the volume to 100ml, filter the solution through a 0.22µm filter and store in 550µl aliquots at –20°C until use.

9.3.1.6 Mix7

Dissolve 630mg DL-β-hydroxybutyrate in 10ml water (1000x stock). Dissolve cobalt chloride to 10mg/ml in water and then add 25µl from that solution to 10ml of L15CO_2 to obtain a stock of 25µg/ml (1000x). Dissolve biotin to 10mg/ml in DMSO and then dilute to 1mg/ml in L15CO_2 (1000x). Dissolve oleic acid to 2.8mg/ml in water and then add 37.5µl of this to 10ml of L15CO_2 to 10µg/ml (1000x). Dissolve αMSH to 1mg/ml in water and dilute to 0.1mg/ml in L15CO_2 (1000x). Dissolve prostaglandin to 1mg/ml in 95% ethanol and dilute 1:100 in L15CO_2 to 10µg/ml (1000x). Dissolve T3 to 10mg/ml in DMSO and add 67.5µl of this to 10ml L15CO_2 to 67.5µg/ml (1000x). Add 5ml of each of the above solutions to 15ml of L15CO_2, filter the solution through a 0.22µm filter and store in 550µl aliquots at –20°C until use.

9.3.1.7 Additives

Dissolve most additives in H_2O, except for the following: dissolve retinoic acid to 17.5mg/ml in DMSO, and then dilute this solution 1:500 in equal volumes of 95% ethanol and L15CO_2 to 35µg/ml (1000x). Dissolve vitamin E to 50mg/ml in DMSO and dilute this 1:10 to 5mg/ml (1000x). Dissolve 3.93mg dexamethasone in 10ml 95% ethanol to 1mM stock and for use, dilute stock 1:100 in L15CO_2. Dissolve 50mg insulin in 10ml 5mM HCl solution. Dissolve 31.5mg progesterone in 10ml 95% ethanol to 10mM and dilute 1:100 in 95% ethanol to a

0.1mM stock. Dissolve 100mg transferrin in 2ml 1x D-PBS. Dissolve 100µg β-NGF in 2ml $L15CO_2$(+1mg/ml BSA) to 50µg/ml. Dissolve 200µg hEGF in 2ml $L15CO_2$(+1mg/ml BSA) to 100µg/ml. Dissolve 25µg bFGF in 1ml $L15CO_2$(+1mg/ml BSA) to 25µg/ml. Dissolve 10µg NT-3 in 400µl $L15CO_2$(+1mg/ml BSA) to 25µg/ml. Dissolve 10µg BDNF in 400µl $L15CO_2$(+1mg/ml BSA) to 25µg/ml. Dissolve 250µg IGF-1 in 2ml $L15CO_2$(+1mg/ml BSA) to 125µg/ml. Store these additives at –80°C until use. Dissolve 80mg putrescine in 10ml water to 8mg/ml. Dissolve 1.29g selenious acid in 10ml water and dilute to a stock of 0.1mM. Store these additives at 4°C until use.

9.3.2 Media

Standard Medium (SM)

To prepare 50ml of defined medium (DM) (Stemple and Anderson, 1992) take: 46.3ml $L15CO_2$, 50mg BSA (1mg/ml), 2ml 1:1:2, 500µl FVM, 315µl glycerol, 100µl putrescine (16µg/ml), 100µl transferrin (100µg/ml), 50µl vitamin E (5µg/ml), 50µl EGF (100ng/ml), 50µl insulin (5µg/ml), 20µl NGF (20ng/ml), 15µl selenious acid (30nM), 8µl bFGF (4ng/ml), 10µl progesterone (20nM), 0.5µl dexamethasone (100nM), 500µl Mix7. To prepare 50ml standard medium, add to 45ml DM 5ml CEE and 50µl retinoic acid (35ng/ml). Filter the medium through a 0.22µm filter and store at 4°C until use. (In brackets, final concentrations).

A simplified SM has been used by Morrison and colleagues (Bixby et al., 2002; Morrison et al., 1999). To prepare 50ml of Standard Medium take: 38.9ml DMEM low glucose, 2ml 1:1:2, 25µl retinoic acid (17.5ng/ml), 500µl N2 salt supplement (1%), 15µl selenious acid, 1ml B27 supplement (1:50), 2.5µl 2-mercaptoethanol (50µM), 8µl IGF1 (20ng/ml) , and 7.5ml CEE (15%). For SM1 add 40µl bFGF (20ng/ml). After 6 days of culture incubation, use SM2 to allow differentiation: reduce bFGF levels to 20µl (10ng/ml) and CEE to 500µl (1%), and fill up to 50ml with DMEM low glucose. Filter the medium through a 0.22µm filter and store at 4°C until use.

Comment:

In our hands, the simplified medium according to Morrison et al. (1999) was less efficient in supporting neural crest cultures than the more complex standard medium (Stemple and Anderson, 1992). To increase cell survival, IGF1 has been added in a more recent study by Morrison and colleagues (Bixby et al., 2002).

Medium Supporting Early Neural Crest Stem Cells and Sensory Neurogenesis (SN1+SN2)

To prepare 50ml of SN medium (Greenwood et al., 1999) take: 47.1ml L15CO_2, 50mg BSA (1mg/ml), 2ml 1:1:2, 50µl insulin (5µg/ml), 100µl putrescine (16µg/ml), 10µl progesterone (20nM), 15 µl selenious acid (30nM), 0.5µl dexamethasone, 143µl glycerol, 50µl vitamin E (5µg/ml) and 500µl Mix7.

SN1: to culture NCSCs for 2-3 days, add 20µl bFGF (10ng/ml) to SN. For further differentiation use SN2, which is prepared by adding the following reagents to SN: 8µl bFGF (4ng/ml), 25µl EGF (50ng/ml), 25µl retinoic acid (17.5ng/ml), 25µl NGF (25ng/ml), 25µl BDNF (12.5ng/ml), 25µl NT3 (12.5ng/ml), and 250µl CEE (0.05%). Filter the medium through a 0.22µm filter and store at 4°C until use. (In brackets, final concentrations).

9.3.3 Isolation

9.3.3.1 Migratory Neural Crest Stem Cells from Neural Tube Explant Cultures

Mouse NCSCs are isolated at embryonic day 9 (E9) (Sommer et al., 1995) while rat NCSCs are isolated at E10.5 (Stemple and Anderson, 1992) (Figure 1). The isolation of trunk neural crest is described here.

1. Sacrifice time-mated females by CO_2 asphyxiation in accordance with National Institutes of Health guidelines.
2. Remove the uterus into a 10cm petri dish containing sterile HBSS without phenol red.
3. With a pair of fine spring scissors cut an opening along the length of each uterus, being careful not to cut into embryo and yolk sac.
4. Under a dissecting microscope, remove the embryos by gently squeezing the uterus with a Dumont #3 forceps while cutting the surface of the decidua and amnionic sac with another #3 forceps. After every embryo has been removed from the uterus transfer the embryos to a new 10cm petri dish containing sterile HBSS without phenol red.
5. Use an L-shaped electrolytically sharpened tungsten needle and a Dumont #5 forceps to dissect a block of tissue from a region corresponding to the region caudal to the heart to the most caudal somite. Pool and place the trunks into a new 3cm petri dish. They can be stored for an hour at 4°C.

6. Prepare digestion mix using 12ml HBSS without Ca^{2+} and Mg^{2+} and 1 vial (5mg) dispase1. Distribute digestion mix to three 3cm petri dishes. Transfer the trunks with a Pasteur pipette to dispase mix and transfer them from the first to the second and then from the second to the third petri dish. Slowly triturate for 2 minutes at RT. Place the dish at 4°C for 6 minutes.
7. Triturate the trunks gently and patiently until the neural tubes are free of other tissues. Transfer every tube to DMEM+10%FBS to stop the digestion reaction. Then transfer the tubes to the appropriate media.
8. Coat 35mm corning tissue culture dishes with fibronectin (FN) as described below (3.4.1) and pre-incubate with appropriate media. Withdraw the media and plate 3-4 neural tubes directly onto the dish. Monitor each step carefully under the dissecting microscope.
9. Allow the tubes to attach for 30 minutes at 37°C in a 5%CO_2 atmosphere, and then gently flood the dish with 1ml medium. If the tubes do not attach, withdraw the media gently and repeat the step until the tube is properly attached to the dish. Incubate the dishes in medium appropriate to the experiment (9.3.4).

9.3.3.2 Isolation of Postmigratory Neural Crest Stem Cells

Postmigratory NCSCs from dorsal root ganglia (DRG) (Figure 1), from sciatic nerve, and from gut have been isolated at various stages of development, both from mouse and rat embryos (Bixby et al., 2002; Hagedorn et al., 1999; Lo and Anderson, 1995; Morrison et al., 1999; Paratore et al., 2002b; Pomeranz et al., 1993). Recent studies have also shown that NCSCs can be isolated from postnatal and adult gut (Kruger et al., 2002). Note that the plating efficiency is very low for adult gut NCSCs.

9.3.3.2.1 NCSCs from Embryonic DRG

1. Sacrifice time-mated females as described. After removal from the uterus transfer the embryos to a new 10cm petri dish containing sterile HBSS without phenol red.
2. Use an L-shaped electrolytically sharpened tungsten needle and a Dumont #5 forceps to dissect a block of tissue from a region rostral to the heart to the most caudal somite. Pool and place the trunks into a new 3cm petri dish. They can be stored for an hour at 4°C.
3. Gently drive the tungsten needle between the cartilage primordium of the vertebral bodies and the neural tube while stabilizing the trunk with the forceps. Take care

not to damage the neural tube. Dissect the cartilage primordium by pulling the needle ventrally. Tear apart the tissue lateral to the neural tube to display the ventral part of the neural tube.

4. Hold the neural tube with a Dumont #3 forceps and separate from dorsal muscle and epithelial tissue using another forceps.
5. Collect the DRGs, which remain attached to the neural tube, using the tungsten needle. Pool the DRGs in ice cold HBSS without phenolred.
6. Centrifuge the DRGs for 2 minutes at 2000 rpm and withdraw the HBSS. Digest the DRGs by incubation in 0.25% trypsin, 3.5mg collagenase type1 in HBSS without Ca^{2+} and Mg^{2+} for 20 minutes at 37°C.
7. Stop the reaction by adding FBS to 10%, centrifuge the cells for 2 minutes at 2000 rpm, resuspend the cells in the appropriate medium, and plate the cells onto culture dishes that have been precoated with either fibronectin or pDL/fibronectin (see below (3.4.1)).

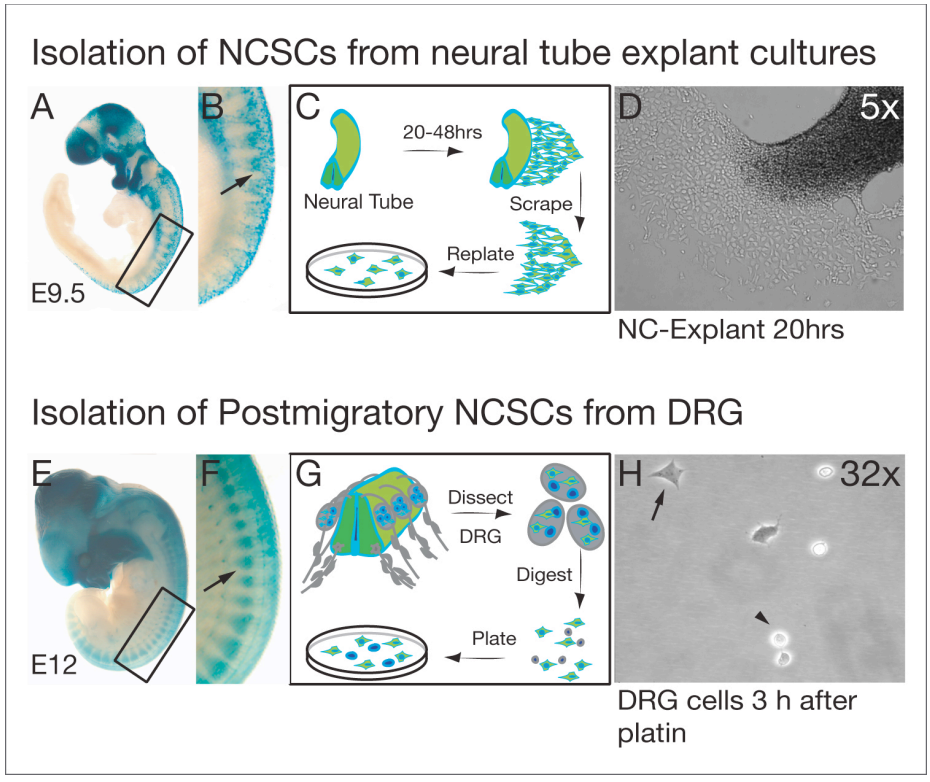

Figure 47. To illustrate the localization of migratory and postmigratory neural crest cells (arrows), X-gal staining was performed on mouse embryos at E9.5 (A) and at E12 (E), in which neural crest cells had been marked by Wnt1Cre-mediated recombination (Ikeya et al., 1997) of the ROSA26 reporter gene (Soriano, 1999). The boxes in A, E represents areas enlarged in B, F, respectively. (C, D) Scheme of the explant culture system of trunk neural crest and phase contrast picture of a neural crest explant at 5x magnification. (G, H) Isolation

scheme of postmigratory NCSCs from DRG and phase contrast picture of DRG-derived postmigratory NCSCs (arrow) and neuronal cells (arrowhead).

9.3.3.2.2 Sciatic Nerve NCSCs

1. Isolate embryos as described before.
2. Fix embryos on a wax support. Cut an opening dorsolateral to the hind limb, proximal to the spinal cord. Nerve and nerve plexus is revealed underneath muscle tissue.
3. Fix hind limb with a Dumont #5 forceps. With another #5 forceps pull out sciatic nerve running into hind limb. Dissect sciatic nerves into ice cold HBSS without Ca^{2+} and Mg^{2+}. Centrifuge cells at 2000 rpm for 2 minutes, withdraw HBSS and resuspend the pellet in a solution containing 0.025% trypsin and 1mg/ml type 3 collagenase.
4. Incubate for 4 minutes at 37°C, then quench the digestion with 2 vol of $L15CO_2$ containing 1mg/ml BSA, penicillin/streptomycin and 25μg/ml deoxyribonuclease type 1.
5. Centrifuge the cells at 2000 rpm for 2 minutes and slowly triturate them in medium.

9.3.3.2.3 NCSCs from the Enteric Nervous System

1a. To prepare enteric NCSCs from embryos, dissect the entire gut distal to the stomach and digest in 1mg/ml collagenase type I in HBSS without Ca^{2+} and Mg^{2+} for 20min at 37°C. Preparations from older embryos are digested for 45min in a solution that, in addition to the collagenase, contains 0.01% trypsin.

1b. The digestion is stopped by the addition of FBS to 10%. Subsequently, the cells are centrifuged for 2min at 1800rpm, triturated, and resuspended.

2a. To isolate and culture early postnatal gut NCSCs, separate the small intestine from the attached mesentry and place into ice cold HBSS without Ca^{2+} and Mg^{2+}. Peel free the outer muscle/plexus layers of the underlying epithelium, mince and dissociate in 0.025% trypsin/EDTA plus 1mg/ml type 4 collagenase in HBSS without Ca^{2+} and Mg^{2+} for 8 minutes at 37°C. Quench the digestion with two volumes medium, centrifuge the cells and triturate.

2b. Filter the cells through a nylon screen to remove clumps of cells and undigested tissue. Before plating, resuspend the cells in medium.

9.3.3.3 Flow Cytometry

Isolation of prospectively identified NCSCs by FACS avoids contamination by non-neural cells and allows enrichment of the NCSC population. Suspend dissociated cells in antibody binding buffer, add primary antibody (or mixture of antibodies) at appropriate concentration, and incubate for 20-25 minutes on ice. Wash 3x in antibody binding buffer and incubate with fluorophore-conjugated secondary antibody. Wash cells and resuspend in buffer containing 2μg/ml of the viability dye 7-aminoactinomycin D (7-AAD; Molecular Probes, Eugene, OR). This step allows exclusion of 7-AAD-positive dead cells during the FACS procedure. To isolate NCSCs from sciatic nerve, cells have been sorted that express the neurotrophin receptor p75 but not P0, a PNS myelin component (Morrison et al., 1999). For the isolation of gut NCSCs, a selection for p75 / α4 integrin doublepositive cells has been performed (Bixby et al., 2002; Kruger et al., 2002). Prior to and after sorting, it is recommended to keep tissue culture plates in sealed plastic bags gassed with 5% CO_2, to maintain the pH in the medium.

9.3.4 Culture of NCSCs

9.3.4.1 Substrate Preparation

Dishes coated with fibronectin: dilute 5ml (1 vial; 5mg) fibronectin in 20ml sterile 1x D-PBS. Apply 1ml fibronectin to 35mm Corning tissue culture dishes and withdraw it immediately and add the appropriate media. It is possible to reuse the fibronectin solution for several times. Dishes coated with poly-D-lysine/fibronectin: resuspend 5mg poly-D-lysine in 10ml cell culture water. Rinse each 35mm corning tissue culture dish with 1ml poly-D-lysine solution. Allow plates to air dry. Subsequently, wash 2x with tissue culture water and air dry plates again. Apply fibronection to poly-D-lysine-coated plates as described above.

9.3.4.2 Culturing NCSCs from Neural Tube Explants in SM

In the absence of instructive growth factors (see 3.4.5), the following conditions are permissive for the generation of autonomic neurons, peripheral glia, and non-neural smooth muscle-like cells. After neural tube isolation (3.3.1.), the culture dishes are incubated at 37°C, 5% CO_2 and 20% O_2 (from air) for 24 hours (rat neural crest) to 48 hours (mouse neural crest) in SM. At this stage, most of the emigrated neural crest cells co-express the transcription factor Sox10 and the low affinity neurotrophin receptor p75 as markers for undifferentiated NCSCs (Paratore et al., 2002b; Stemple and Anderson, 1992). For further incubation, it is possible to scrape away the neural tube from the neural crest cells that migrated onto the

substrate, using an L-shaped tungsten needle and an inverted phase-contrast microscope equipped with a 5x or 10x objective lens (Figure 1). Differentiated cell types become apparent after a few days of culture in SM (Stemple and Anderson, 1992). Differentiation is promoted by the addition of 10% FBS and 5µM forskolin (Sommer et al., 1995).

Comments:

In the presence of the neural tube and upon addition of NT3, BDNF and LIF, the generation of sensory neurons is observed proximal to the neural tube, in addition to the autonomic neurons that are found scattered throughout the outgrowth after 8 days in culture (Greenwood et al., 1999).

9.3.4.3 Culturing NCSCs from Neural Tube Explants in SN Medium

In the absence of instructive growth factors (see 3.4.5), neural tube explants in SN medium consist of early NCSCs that can generate sensory neurons. After plating, neural tube explants are incubated in SN1 medium for 20 hours. Outgrowth of NCSCs occurs during this period. To allow sensory neuronal differentiation, withdraw the SN1 medium from the plates after 48 hours of explant incubation and add SN2 medium for another 2 days.

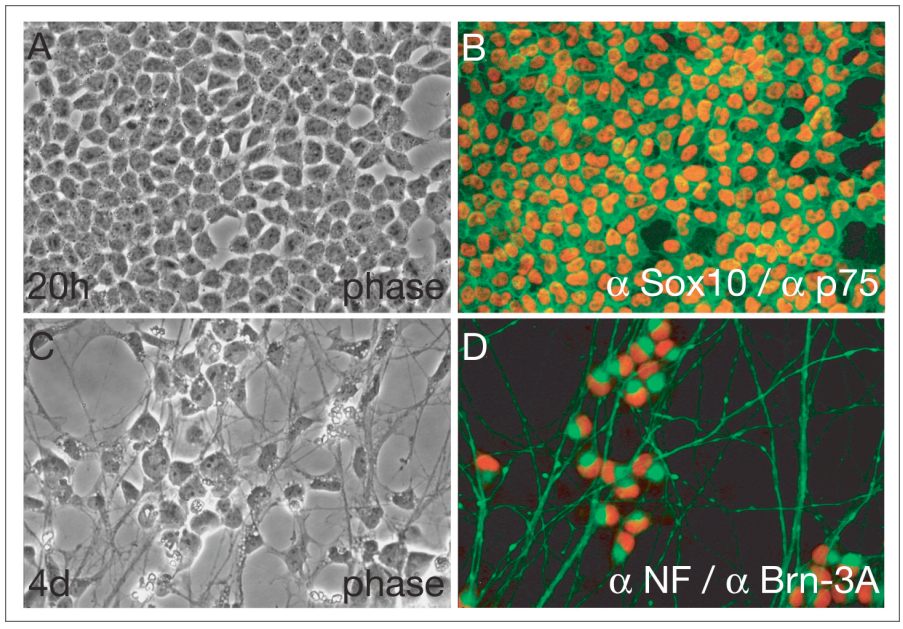

Figure 48. NCSCs are identified by co-expression of the transcription factor Sox10 and the low affinity neurotrophin receptor p75 (**B**). Neural tube explant cultures cultured in SN conditions for 20 hours were fixed with 3.7% formaldehyde in D-PBS for 10 minutes. The cells were treated for 10 minutes at RT with blocking buffer containing 10% goat serum, 0.3% Triton X-100, 0.1% BSA in D-PBS and stained with rabbit anti-mouse

p75 (1:300 dilution, Chemikon International) for 1 hour at RT and with monoclonal anti-Sox10 antibody (1:10 dilution; Paratore et al., 2001) for 2 hours at RT. Within 4 days in culture, neural crest cells differentiate into sensory neurons identified by co-expression of the POU transcription factor Brn-3A and NF160 (**D**). Immunocytochemistry with polyclonal rabbit anti-Brn-3A antibody (1:300 dilution (Fedtsova and Turner, 1995) and monoclonal anti-NF160 antibody NN18 (1:300 dilution, IgG, Sigma-Aldrich) were carried out at RT for 1 hour. Immunostainings were visualized by incubation for 1 hour at RT using the following secondary antibodies at 1:200 dilution: Cy3-conjugated goat anti-mouse IgG; Cy3 conjugated goat anti-rabbit IgG; FITC-coupled donkey anti-rabbit IgG (Jackson Immuno Research Laboratories); and FITC-coupled horse anti-mouse IgG (Vector Laboratories). (**A, C**) Corresponding phase contrast pictures.

Comments:

20 hours after having plated the isolated neural tubes, the early neural crest explants cultured in SN1 neither express the sensory marker Brn-3A nor NF160, while virtually all neural crest cells express p75 and Sox10 (Figure 2A/B) (Hari et al., 2002). Sensory neurons obtained after prolonged incubation are characterized by co-expression of the POU domain transcription factor Brn-3A and NF160 (Fedtsova and Turner, 1995) (Figure2C/D).

9.3.4.4 Replating NCSCs from Neural Tube Explants and Cloning Procedure

Allow rat NCSCs to emigrate for 24 hours, and mouse NCSCs for 48 hours. After scraping away the neural tubes, carefully wash plates once with DMEM. Detach the NCSCs by treatment with a 0.05% trypsin solution for 2 minutes at 37°C. Quickly resuspend the cells in DMEM+10%FBS to abolish the reaction. Centrifuge the cells at 2000 rpm for 2 minutes and resuspend the pellet in 1ml fresh medium. Count the cells in a Neubauer counting chamber. Plate the cells at low density (100-300 cells/35mm plate). Let the cells settle down for approximately 3 hours. Single NCSCs are mapped by labelling the surface antigen p75 on living cells (Stemple and Anderson, 1992). The staining is performed in SM for 30 minutes using a rabbit anti-mouse p75 antibody (1:300 dilution, Chemikon International). Wash the cells 3 times in DMEM and visualize the staining by using a Cy3-coupled goat anti-rabbit IgG (Jackson Laboratories) in SM for 30 minutes at RT. Wash the cells 3 times in DMEM and add 1ml fresh SM medium to each plate. Detect p75-expressing NCSCs with an inverted fluorescence microscope at 10x magnification. Mark single founder cells by inscribing them with a 3-4mm circle using a grease pencil on the bottom of the dish.

Comments:

For reasons not entirely clear, mouse NCSCs display a low survival capacity at clonal density. Clonal experiments with mouse NCSCs are therefore only possible under certain conditions (such as in the presence of fetal bovine serum) (Paratore et al., 2001). In the rat, clonogenic culture systems allowed assessment of the state of commitment of neural crest cells by exposing individual cells to changing environmental cues. By such experiments, instructive growth factors have been identified that are able to promote the differentiation of NCSCs to specific lineages in vitro (Morrison et al., 2000a; Morrison et al., 1999; Shah et al., 1996; Shah et al., 1994). Bone morphogenic protein 2 (BMP2) promotes a neuronal and, to a lesser extent, a smooth muscle-like fate, while single neural crest cells are instructed by transforming growth factor-ß (TGFß) to adopt a non-neural fate. Furthermore, individual neural crest cells choose a glial fate upon either Notch signal activation or treatment with GGF, an isoform of neuregulin1 (NRG1). However, the response of NCSCs to instructive growth factors is modulated by short range cell-cell interactions termed community effects and other signals (Hagedorn et al., 1999).

Moreover, serial subcloning experiments demonstrated the self-renewal capacity of NCSCs (Morrison et al., 1999; Stemple and Anderson, 1992). The signals promoting self-renewal and maintenance of NCSCs have not yet been discovered.

9.3.4.5 Culturing Postmigratory NCSCs

Cells are cultured either in 35mm or in 6-well plates that have been precoated with poly-D-lysine and fibronectin (see above). Both the traditional SM according to Stemple and Anderson (1992) and a simplified SM (Bixby et al., 2002; Morrison et al., 1999) have been successfully used to culture postmigratory NCSCs (Bixby et al., 2002; Hagedorn et al., 1999; Morrison et al., 1999). When using the simplified SM, incubate cells in SM1 for 6 days, and then add SM2 for another 8 days to favor differentiation. For clonal analysis, directly plate cells at low density after dissociation of postmigratory neural crest target tissues (100-300 cells/35mm plate; fewer than 30 cells per well of a 6-well plate).

Comments:

Although neural crest cells isolated both from neural tube explant cultures and from various neural crest-derived tissues have been shown to be multipotent and responsive to instructive growth factors, cell-intrinsic differences between NCSCs from different origins affect fate

decisions by changing the sensitivity of the cells to specific extracellular signals (Bixby et al., 2002; Kruger et al., 2002; Paratore et al., 2001; White et al., 2001).

9.3.4.6 Culturing NCSCs at Reduced Oxygen Levels

Reduced levels of oxygen have been shown to influence survival, proliferation and cell fate decision of neural stem cells (Morrison et al., 2000a). To culture neural tube explants at reduced oxygen levels, put all the dishes after neural tube isolation into a gas-tight modular incubator chamber and flush the chamber for 3-5 minutes with a custom gas mixture of 1% O_2, 6% CO_2 and balance N_2 to generate an actual O_2 level of 3-6%. The gas-tight chamber is housed inside a normal incubator. Once cultures are established in the reduced oxygen chamber minimize the opening to avoid reperfusion.

Comment:

We observed that in SN1 NCSCs appeared healthier after 20 hours when cultured at reduced oxygen levels (Kléber et al., unpublished). Moreover, culturing NCSCs at reduced oxygen levels in the presence of BMP2 and forskolin revealed that low oxygen levels can influence cell fate (Morrison et al., 2000a).

9.4. Pitfalls

1 In order to establish NCSC cultures follow carefully the instructions. Note that small differences in concentrations of media ingredients may influence outgrowth, proliferation, survival, and differentiation of NCSCs.

2 Different batches of CEE and FBS might have different effects on the cultures. When using a new batch of CEE or FBS always compare it to an older batch.

3 During isolation, triturate the neural tubes slowly and patiently. Rapid trituration can damage the neural tubes, which can impair efficient neural crest outgrowth.

4 Do not exceed the time of the digestion during isolation.

5 Always use fresh media. The media should not be stored for more than one week.

10. MATERIALS AND METHODS (continuation)

10.1 Mating Scheme and Genotyping

Time-mated control and mutant mice were performed with mice heterozygous for a floxed allele and positive for the transgene Wnt1-Cre and bred onto mice that were homozygous for a floxed allele over a C57BL/6N background. Embryos were produced by time mating, assigning the morning after pairing as E0.

Transgenic mice carrying the Cre-recombinase under the control of the *wnt1* promoter were provided by Andy McMahon, Cambridge (Danielian et al., 1998).

The Rosa26 Cre-reporter (R26R) mouse strain was obtained from Philippe Soriano, Seattle (Soriano, 1999).

10.1.1 β-Catenin Loss-of Function

Mice were provided from Rolf Kemler, Freiburg (Brault et al., 2001).

Embryos heterozygous for the *ß-catenin* floxed and floxdel alleles and carrying the *wnt1-Cre* transgene were referred to as mutant embryos, while littermates, which inherited the incomplete combination of the above alleles, served as control animals.

10.1.2 β-Catenin Gain-of Function

Mice were provided from Makoto M. Taketo, Japan (Harada et al., 1999).

wnt1-Cre mice were mated with *ß-catenin*$^{flox(ex3)/+}$ animals or, in order to perform in vivo fate mapping experiments, with *ß-catenin*$^{flox(ex3)/+}$*/ACZL* mice. Embryos that inherited *wnt1-Cre* and a *ß-catenin*$^{flox(ex3)}$ allele are referred to as mutant embryos, while littermates, which inherited the incomplete combination of the above alleles, served as control animals. The *wnt1-Cre* and the *ACZL* transgenes and the Δex3 allele were identified as described (Akagi et al., 1997; Brault et al., 2001; Harada et al., 1999) Genotyping for the *ß-catenin*$^{flox(ex3)}$ allele was performed by PCR using primers ßcatEx2s (GACACCGCTGCGTGGACAATG) and ßcatEx3as (GTGGCTGACAGCAGCTTTTCTG) for 30 cycles of 94°C for 1 min, 65°C for 1min, and 72°C for 1 min.

10.2 *In Vivo* Fate Mapping Experiments

The fate of control and mutant neural crest cells and their progeny was followed in compound transgenic *wnt1-Cre* animals bred to the ACZL mouse reporter line. In this reporter line, a floxed *lacZ* gene is restored and properly expressed upon Cre-mediated recombination. Recombined cells on frozen sections and in whole mount embryos were detected by histological staining with the chromogenic substrate X-Gal. For the X-gal staining, the sections and the embryos were incubated in a phosphate-buffered saline solution (5mM potassium ferrocyanid, 5mM potassium ferricyanid, 2mM $MgCl_2$ and 2mM X-gal) (AppliChem) at 37°C for at least 2 hours. The reaction was stopped by washing the slides or embryos twice in PBS and by a further fixation in paraformaldehyde (PFA) (Fluka) for 15 min.

10.3 *In Situ* Staining Procedures

Nonradioactive *in situ* hybridization with digoxigenin-labeled riboprobes was performed on frozen sections and on whole mount embryos 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). Cryosections (20 μm) were collected on SuperFrost Plus slides (Merck) and warmed up to RT. The sections were dried at 50°C for 15 min and fixed in 4% paraformaldehyde in diethyl pyrocarbonate (DEPC)- treated PBS for 20 min. After washing twice in DEPC-treated PBS for 5 min each, sections were treated with Proteinase K (Roche) for 5 min. The slides were washed twice in DEPC-treated PBS for 7 min each and refixed in 4% paraformaldehyde/DEPC-treated PBS for 15 min. After washing with DEPC-treated PBS, the slides were incubated for 10 min in 0.25% (V/V) acetic anhydride dispersed in 0.1M triethanolamine-HCl pH8.0, rinsed briefly and washed in DEPC-treated PBS for another 5 min. Prehybridization was performed in hybridization buffer containing 50% formamide, 5x SSC, 100mg/ml heparin, 1x Denhardt's solution, 0.1% Tween 20, 0.1% CHAPS and 5mM EDTA, 1mg/ml yeast tRNA; for 6 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: once 0.2% SSC at RT for 10 min in preheated SSC, twice in 0.2% SSC at 65°C for 30 min. Then the sections were washed twice in PBT (PBS, 0.1% Triton-X-100, 2mg/ml bovine serum albumin) for 20 min at RT. Afterwards the sections were blocked in PBT plus 20% sheep serum for 3-6 hours at RT followed by incubation with alkaline

phosphatase (AP)-coupled sheep anti-Dig antibody (1:2000 dilution; Roche) overnight at 4°C. The sections were washed three times in PBT for 30 min each, followed by a prewash in AP buffer (100mM Tris-pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20) for 5 min at RT. Then levamisole (Sigma Aldrich) was added to the AP buffer at a concentration of 5mM and sections were washed a second time for 5 min at RT. To develope the signal, the chromogens NBT (330 μg/ml) and BCIP (175 μg/ml) (Roche) were added as AP-substrates and the color reaction was performed in the dark for 3-6 hours. Before mounting in glycerol, the sections were washed in PBS and fixed in MEMFA (0.1M MOPS pH7.5, 2mM EGTA, 1mM $MgSO_4$ and 3.9% formaldehyde) for 20 min.

10.4 Cell Culture

Methods for culturing rat and mouse neural crest stem cells are largely based on articles by Lee et al., (2004); Stemple and Anderson (1992) (rat NCSCs), Sommer et al. (1995) (mouse NCSCs), Kléber and Sommer, in press; Hari et al., (2002); Greenwood et al. (1999) (culture conditions permissive for sensory neurogenesis); and Morrison et al., (2001).

10.4.1 Migratory Neural Crest Stem Cells

Time-mated control and mutant mice were performed with mice heterozygous for a floxed allele and positive for the transgene Wnt1-Cre and bred onto mice that were homozygous for a floxed allele over a C57BL/6N background.

Mouse neural crest explant cultures under conditions permissive for sensory neurogenesis were prepared as reported (Greenwood et al., 1999; Kléber and Sommer, in press), with the exception that the cultures were incubated for 20 h at reduced oxygen levels (Morrison et al., 2000a), which influences fate and survival of NCSCs. After 20 h of neural crest emigration, the neural tubes were removed from the cultures and used for genotyping by PCR (Sommer et al., 1995). To allow differentiation, the explants were cultured for 54 hours in conditions permissive for sensory neurogenesis without additional factors (Kléber and Sommer, in press). To allow full differentiation of mutant and control sensory neurons, the defined medium was modified by slightly changing the following factors after 50 h of culture: bFGF was reduced to 4ng/ml; neurotrophin 3 (NT3) was added at a concentration of 6.25ng/ml; brain-derived nerve growth factor (BDNF) was added at a concentration of 6.25 ng/ml, and retinoic acid was added to a final concentration of 17.5ng/ml.

To inhibit sensory neurogenesis, the neural tubes were removed after 20 h of neural crest emigration and the media was supplemented with either 50ng/ml BMP2, with 10% CEE or the combination of both, respectively. To exclusively generate non-neural fates expressing smooth muscle actin (SMA) the media was switched to standard media, according to Stemple and Anderson, and supplemented with 20ng/ml IGF-1 (R&Dsystems) and 0.1ng/ml TGFβ1 (R&Dsystems).

10.4.2 Culture of eNCSCs on Fibroblast Feeder Layers

Time-mated NMRI mice were obtained from Elevage Janvier (Le Genest St.Isle, France).
Wnt1-expressing and control NIH3T3 fibroblasts (Arnold et al., 2000) were irradiated (3000 rad, 3,5 min) to abolish proliferation and plated as monolayers onto 35mm corning culture dishes. To challenge neural crest explants with Wnt1, mouse neural tubes were isolated and plated onto the feeder cell-layer to allow neural crest emigration directly onto the monolayers. During emigration the eNCSCs were cultured in reduced oxygen levels as described. To allow differentiation eNCSCs were cultured for 5 days in a fully defined medium permissive for sensory neurogenesis (Greenwood et al., 1999; Kléber and Sommer, in press).
To perform signaling cross-talk experiments 50ng/ml BMP2 (R&D systems) was added to either Wnt1-expressing- or control NIH3T3 fibroblasts after 20 h and cultures were incubated for 5 days. To further challenge the NCSCs after 5 days in culture on Wnt1-expressing fibroblasts, the cells were washed three times in DMEM and cultured for 7 additional days in SN1 supplemented with 100ng/ml noggin (R&D systems) or 50ng/ml BMP2 (R&D systems), in standard media, according to Stemple and Anderson, containing either 20ng/ml IGF-1 (R&Dsystems) and 0.1ng/ml TGFβ1 (R&Dsystems), 1nM NRG1 (R&D systems), or 50ng/ml BMP2 (R&Dsystems).

10.4.3 Clonal Analysis of Rat eNCSCs

Time mated Wistar rats were obtained from Elevage Janvier (Le Genest St.Isle, France).
For clonal analysis, rat neural tubes were isolated at E10.5 and plated onto fibronectin-coated dishes as reported (Hagedorn et al., 1999). NCSCs were allowed to migrate for 15 h at low oxygen levels in defined medium according to (Greenwood et al., 1999). NCSCs were replated (Hagedorn et al., 1999) either onto Wnt1-expressing monolayers, Wnt1-expressing monolayers supplemented with 50ng/ml BMP2, control monolayers, or pdL/fibronectin-

coated dishes at clonal density. The plating efficiency was approx. 60 to 70%. 3 h after replating, living NCSCs were labeled for 30 min at RT using a rabbit anti-mouse p75 polyclonal antibody (1:300 dilution; Chemicon International), followed by a 30 min incubation with Cy3-conjugated goat anti-rabbit IgG (1:200 dilution; Jackson Immuno Research Laboratories), and single p75-positive clone founder cells were mapped (Hagedorn et al., 1999). Some culture dishes were fixed immediately after p75-staining and further processed for Sox10 immunostaining. Sister dishes with NCSCs on Wnt1-expressing layers were cultured in defined medium (Greenwood et al., 1999) and analyzed after 3 d in culture. NCSCs on control layers and pdL/fibronectin-coated dishes were incubated in standard medium according to (Stemple and Anderson, 1992). Some cultures on control monolayers were supplemented with 50ng/ml BMP2 (R&D Systems) 2 d after plating and analyzed after an additional 24 h. Cultures on substrate-coated dishes were treated with 0.1ng/ml TGFß (R&D Systems) for 4 d.

10.4.4 Maintenance of Rat and Mouse NCSCs

To evaluate maintenance of multipotency, rat E10.5 or mouse E9 neural tubes were directly plated onto Wnt1-expressing monolayers. After 15 h (rat) or 20 h (mouse) in culture in reduced oxygen levels 50ng/ml BMP2 was supplemented to SN1 and cells were cultured for 5 up to 11 days in the presence of BMP2 and Wnt1. After 11 days remaining fibroblasts and neural tubes were removed and the explants were replated onto new Wnt1-expressing feeder monolayers, or pdL/fibronectin coated plates at clonal density. Living single cells were mapped as described. Some culture dishes were fixed immediately after p75-staining and further processed for Sox10 immunostaining. Sister dishes with NCSCs on Wnt1-expressing layers were cultured in SN1 alone or SN1 supplemented with BMP2 and analyzed after 3 d in culture. NCSCs on pdL/fibronectin-coated dishes were incubated in standard medium according to Stemple and Anderson (Stemple and Anderson, 1992). Cultures on substrate-coated dishes were supplemented with 50ng/ml BMP2 (R&D Systems), or on sister plates with 20ng/ml IGF-1 (R&Dsystems) and 0.1ng/ml TGFß (R&D Systems), or supplemented with 1nM NRG1 (R&D systems) for 4, 5 and 7 days respectively.

10.4.5 Postmigratory Neural Crest Stem Cells

Time-mated control and mutant mice were performed with mice heterozygous for a floxed allele and positive for the transgene Wnt1-Cre and bred onto mice that were homozygous for a floxed allele over a C57BL/6N background.

To analyze control and mutant postmigratory NCSCs, DRG were dissected from single embryos at E12 and individually dissociated by incubation in 0.25% trypsin (Gibco-Invitrogen), 0.35mg/ml collagenase type1 (Worthington Biochemical) in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (Amimed) for 15 min, followed by addition of 1/10 volumes of FBS.

Postmigratory NCSCs from control and mutant embryos were isolated from Sciatic nerve at E12.5 (Jessen and Mirsky, 1991) and individually dissociated by incubation in 0.025% trypsin (Gibco-Invitrogen) and 1mg/ml collagenase type3 (Worthington Biochemical), followed by addition of 1/10 volumes of FBS.

The cells were centrifuged for 2 min at 2000 rpm, resuspended in standard medium according to Stemple and Anderson, (1992); and plated at clonal density on Wnt1-expressing- or control- fibroblasts and onto pdL/fibronecting-coated dishes. Living single cells were mapped as described. Some culture dishes were fixed immediately after p75-staining and further processed for Sox10 immunostaining. Sister dishes with postmigratory NCSCs on Wnt1-expressing layers were cultured in SN1 alone and analyzed after 3 d in culture. Postmigratory NCSCs on control layers and pdL/fibronectin-coated dishes were incubated in standard medium according to (Stemple and Anderson, 1992) and analyzed after 4 days in culture.

11.5 Migration and Proliferation of Cultured Cells

To quantify the neural crest outgrowth, digital images of explant cultures at 20 h were acquired and NIH image 1.62 software was used to measure the size of the explants. The outgrowth area (mm^2) was divided by the perimeter (mm) of the explant. This normalized number (in mm) is referred to as migration index and gives an estimate of the total outgrowth of a neural crest explant (Hari et al., 2002; Huang et al., 1998). To quantify the percentage of proliferating cells during emigration, neural crest cells were treated with BrdU at 8 h as they migrated, and BrdU incorporation was analyzed at 10 h. To quantify the percentage of proliferating cells during differentiation, neural crest cells were treated with BrdU at 24 h, and BrdU incorporation was analyzed at 30 h. BrdU incorporation was assessed according to the

manufacturer's instructions (Roche Diagnostics) and visualized using goat anti-mouse IgG conjugated to Cy3 (1:200 dilution; Jackson Immuno Research Laboratories).

11.6 TUNEL Assay and Immunohistochemistry

For TUNEL analysis cryosections were fixed in 2% PFA/PBS for 10 min and washed three times 5 min in PBS. Explants were fixed in 3.9% FA for 10 min and three times washed in PBS. Further they were blocked for 1 hour in 10% goat serum, 0.1% BSA, 1% Triton X-100 in PBS. 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).

11.7 Immunocytochemistry

Explant and cell clones were fixed with 3.7% formaldehyde in PBS for 10 min and washed three times in PBS. Labeling of the surface antigen LNGFR was performed on living cells without fixation or on fixed explants using rabbit anti-mouse p75 nerve growth factor (NGF) receptor polyclonal antibody (1:300 dilution; Chemicon International) visualized by Cy-3- or FITC- conjugated goat anti-rabbit IgG secondary antibody (1:200 dilution; Jackson Laboratories). To label intracellular antigens, cells were permeabilized for 10 min at room temperature (RT) in blocking buffer containing 10% goat serum and 0.3% Triton-X-100 in PBS. Stainings for the following antibodies was performed overnight at 4°C or alternatively for 1 hour at RT: rabbit polyclonal anti-Brn-3A antibody (1:1000 dilution; (Fedtsova and Turner, 1995)), monoclonal anti-NF160 antibody NN18 (IgG) (1:500 dilution; Sigma Aldrich), rabbit polyclonal anti-NF160 antibody (1:500 dilution; Chemicon International), monoclonal anti-smooth muscle actin (SMA) (IgG) (1:400 dilution; Sigma Aldrich), mouse anti-peripherin (IgG) (1:200 dilution; Chemicon International), monoclonal anti-Sox10 antibody (1:3 dilution; (Paratore et al., 2001)), rabbit anti-cow S100 (1:200 dilution; DAKO) and anti-rabbit GFAP (1:200 dilution; DAKO). After primary antibody incubation, the labeling was visualized by incubation for 1 hour at RT with Cy3-conjugated antibodies (anti-mouse IgG and rabbit IgG) (1:200 dilution; Jackson Laboratories) or FITC-conjugated antibodies (anti-mouse IgG and rabbit IgG) (1:200 dilution; Jackson Laboratories). For anti-ß-Catenin staining, cells were permeabilized 20 min in blocking buffer containing 10% goat serum, 0.3% Triton-X-100 in PBS and stained overnight at 4°C using a polyclonal antibody

(1:300 dilution; Sigma). DRAQ5 staining was performed according to the manufacturer's instructions (1:50 dilution; Biostatus Lt.). For Mash-1 staining, cells were incubated with a monoclonal antibody (1:100 dilution; BD Biosciences) overnight at 4°C. After washing three times in PBS, cells were incubated in HRP-buffer containing 2% goat serum and 1% NP40 for 20 min followed by incubation with a horse raddish peroxidase (HRP)-coupled goat anti-mouse IgG antibody (1:200 dilution; DAKO) for 1 hour at RT and by HRP development using diaminobenzidine (DAB) as substrate.

11.9 Immunohistochemistry

Paraformaldehyde-fixed embryo sections were blocked in blocking solution (1% goat serum, 0.3% Triton-X-100 in PBS) for 1 h. Expression of Proteins was performed on freshly sliced 20μm cryosections of mouse embryos using the following antibodies: polyclonal rabbit anti-Brn-3A antibody (1:1000 dilution; (Fedtsova and Turner, 1995)) and monoclonal mouse anti-Neurofilament160 (NF) antibody (1:500 dilution; Sigma). Unspecific antibody binding was reduced by incubating sections in blocking buffer for one hour at room temperature. Primary antibodies diluted in blocking buffer were added on all sections overnight at 4°C. After three washing steps of 10 minutes with PBS, the staining was detected with Cy3-conjugated antibodies (anti-mouse or anti-rabbit IgG) (1:200 dilution; Jackson Laboratories) or FITC-conjugated antibodies (anti-mouse IgG) (1:200 dilution; Jackson Laboratories). Before mounting, the sections were washed three times with PBS for 10 minutes. Sections were fixed in 4% PFA for 15 min or directly used without fixation.

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12 PUBLICATION LIST

12.1 Published

Lee, H.-Y.*, **Kleber, M.*,** Hari, L., Brault, V., Suter, U., Taketo, M. M., Kemler, R. and Sommer, L**.** (2004). Instructive role of Wnt/β-catenin in sensory fate specification in neural crest stem cells. *Science* **303**, 1020-3. * These authors contributed equally to this work.

Lee, H.-Y., **Kleber, M.,** Sommer, L. (2004). Neue Rolle des Signalfaktors Wnt in der Stammzellbiologie. *Laborwelt 5. Jahrgang, Nr. 1/2004.*

Kleber, M. and Sommer L. (2005) Cell Biology: A laboratory handbook 3ED, Section 1.2: Primary Cultures from Embryonic and Newborn Tissues: Neural Crest Stem Cells. *Elsevier*

Hari, L., Brault, V., **Kleber, M.,** Lee, H.-Y., Ille, F., Leimeroth, R., Paratore, C., Suter, U., Kemler, R. and Sommer, L. (2002). Lineage-specific requirements of β-catenin in neural crest development. *J Cell Biol* **159**, 867-80.

Paratore, C., Hagedorn, L., Floris, J., Hari, L., **Kleber, M.**, Suter, U., Sommer, L. (2002). Cell-intrinsic and cell-extrinsic cues regulating lineage decisions in multipotent neural crest-derived progenitor cells. *Int J Dev Biol*. **46**, 193-200

12.2 Submitted

Kleber, M. and Sommer L. (2004) Wnt Signaling and the Regulation of the Stem Cell Function
*Curr.Op.Cell Biol.**16**, 6*

Kleber, M., Lee, H.-Y., Wurdak H., Buchstaller, J., Mantei N., Suter U., Epstein D. and Sommer, L. Combinatorial Wnt and BMP signaling regulate maintenace and early lineage segregation in neural crest stem cells.
Submitted august

12.3 In preparation

Lee, H.-Y., Jahreiss, L., **Kleber, M.**, Bühlmann, S., Suter, U., Radtke, F. and Sommer, L. Requirement of Notch1 for Dorsal Interneuron Specification and Boundary Formation in the Dorsal Spinal Cord.

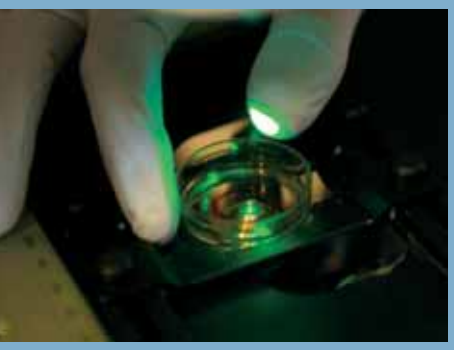
Dutt S., **Kleber M.**, Sommer L. and Zimmermann D.(2004) Versican V0 and V1 restricts the migration of neural crest stem cells and axons during the development of the peripheral nervous system

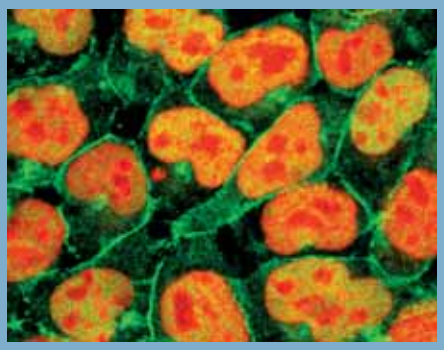
13 REPRINTS

ETH-RAT

2003





Signalprotein «Wnt»: Neue Aspekte in der Stammzellentwicklung (Titelbild)

Aufgrund ihres therapeutischen Potenzials bilden Stammzellen heute ein Schwerpunktthema biomedizinischer Forschung. Um aber dieses Potenzial verwirklichen zu können, müssen die Signalfaktoren bekannt sein, welche die Entwicklung einer Stammzelle in die verschiedenen Zelltypen eines Organismus steuern können. Ein wichtiger Faktor in der Stammzellbiologie ist das Signalprotein «Wnt». Bei etlichen Arten von Stammzellen, wie zum Beispiel embryonalen Stammzellen oder Stammzellen des Zentralnervensystems, führt «Wnt» zur Zellvermehrung, während gleichzeitig die Entstehung ausgereifter Zelltypen unterdrückt wird.

Wissenschaftler vom Institut für Zellbiologie der ETH Zürich haben eine neue Rolle von «Wnt» entdeckt. Sie konnten zeigen, dass «Wnt» in Stammzellen des peripheren Nervensystems keinen Einfluss auf die Vermehrung der Stammzellen hat, sondern im Gegenteil die Entstehung bestimmter Nervenzellen anregt. Stammzellen verschiedener Herkunft scheinen also recht unterschiedlich auf gleiche Signale ihrer Umgebung zu reagieren. Für eine (zurzeit noch visionäre) Stammzelltherapie könnte das bedeuten, dass ein bestimmter Stammzelltyp zum Beispiel für die Behandlung einer bestimmten Nervenkrankheit in Frage käme, für die Therapie einer anderen Krankheit oder Verletzung hingegen ungeeignet wäre.

cells, in contrast to control cells (Fig. 4E). The expression pattern of IκBβ correlated well with that of Foxj1, diminishing particularly in response to anti-CD3 or IL-2 stimulation (fig. S1B). In addition, transduction of primary Th cells by Foxj1 increased IκBβ expression (fig. S1C), and Foxj1 could transactivate the IκBβ promoter (fig. S1D). Finally, Foxj1$^{-/-}$ T cells contained diminished levels of IκBβ mRNA (Fig. 4F). These findings strongly suggest that Foxj1 antagonizes NF-κB activity at least in part by inducing and/or maintaining IκB activity. As a consequence of this, deficiency in Foxj1 leads to spontaneous NF-κB activation and subsequent immune dysregulation.

We suggest that Foxj1 regulates early Th activation, enforcing T cell quiescence by regulating NF-κB activity, in part via IκB (fig. S6). However, recent studies suggest that NF-κB may preferentially play a role in later, postcommitment phases of Th1 development and proliferation (*14*), suggesting that Foxj1 may also enforce quiescence by modulating the activity of another class(es) of transcriptional regulators and/or may have as-yet undefined direct effects on T cell differentiation genes, such as LKLF (*18*) or Tob (*19*). Regardless, the present findings are consistent with prior observations demonstrating dysregulated NF-κB activity in both human (*20*) and murine (*21*, *22*) lupus and inflammatory phenotypes of animals deficient in IκB activity (*23*) and furthermore are consistent with the significantly reduced expression of Foxj1 in murine lupus Th cells (fig. S1). Thus, studies to define further the target genes regulated by Foxj1 as well as other Fox transcription factors will undoubtedly shed insight into the regulation of Th differentiation and immune tolerance.

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24. We are grateful to T. Kitamura for the pMX/PlatE retroviral system; R. Schreiber for the use of the ABI PRISM (Applied Biosystems, Foster City, CA) sequence detection system; M. Oukka and L. Glimcher for the NF-κB-luc construct and the 293-T cell line; M. R. Marshall for technical assistance; and J. Green, K. Murphy, A. Shaw, and W. Yokoyama for critical commentary during the preparation of the manuscript. This work was supported in part by the Siteman Cancer, Diabetes Research and Training, and the Digestive Diseases Research Core (DK52574) Centers of the Washington University School of Medicine, as well as grants from NIH (AI01803 and AI057471 to S.L.P. and HL56244 and HL63988 to S.L.B.), the Lupus Research Institute, and the Broad Medical Research Foundation. S.L.P. is supported in part by an Arthritis Investigator award from the Arthritis Foundation. S.L.B. is a recipient of a Career Investigator award from the American Lung Association.

Supporting Online Material
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21 November 2003; accepted 19 December 2003

Instructive Role of Wnt/β-Catenin in Sensory Fate Specification in Neural Crest Stem Cells

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Wnt signaling has recently emerged as a key factor in controlling stem cell expansion. In contrast, we show here that Wnt/β-catenin signal activation in emigrating neural crest stem cells (NCSCs) has little effect on the population size and instead regulates fate decisions. Sustained β-catenin activity in neural crest cells promotes the formation of sensory neural cells in vivo at the expense of virtually all other neural crest derivatives. Moreover, Wnt1 is able to instruct early NCSCs (eNCSCs) to adopt a sensory neuronal fate in a β-catenin–dependent manner. Thus, the role of Wnt/β-catenin in stem cells is cell-type dependent.

Wnt proteins are able to induce proliferation in different types of stem cells (*1*–*3*). In the central nervous system, Wnts act mitogenically on progenitor cells, and activation of β-catenin, a component of the canonical Wnt signaling pathway (*4*), leads to amplification of the neural progenitor pool (*5*, *6*).

The question arises whether canonical Wnt signaling regulates stem cell self-renewal in general or whether this function is cell type–dependent. To address this issue, we analyzed the role of Wnt/β-catenin in NCSCs. Neural crest cells generate most of the vertebrate peripheral nervous system (PNS) and several non-neural derivatives (*7*). Wnt signaling has previously been implicated in early stages of neural crest development, such as neural crest induction and melanocyte formation (*8*–*10*). In NCSCs, specific ablation of the β-*catenin* gene results in lack of melanocytes and sensory neural cells in dorsal root ganglia (DRG) (*11*). NCSCs without β-catenin emigrate and proliferate normally but are unable to acquire a sensory neuronal fate.

These results are consistent with a role of β-catenin signaling in inducing a sensory fate. To address this hypothesis, we used the *cre/loxP* system to generate mice expressing a constitutively active form of β-catenin specifically in neural crest cells (fig. S1) (*12*). We first assessed the developmental potential of control and mutant neural crest cells by performing in vivo fate mapping experiments (*13*). In the control (Fig. 1A), neural crest cells emanating from the anterior neural tube populated the nasofrontal and periocular region, where they become mesenchyme that later generates bones, connective components, and vascular structures of the head (*14*–*16*). Neural crest cells also migrated into branchial arches that contribute to craniofacial skeletal tissue and the major arteries (*14*, *17*, *18*). In the mutant (Fig. 1B), however,

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cranial neural crest–derived cells hardly spread over the forebrain area and were more restricted to ventral head structures. Only a vestigial first branchial arch was present; all other branchial arches were absent. Instead, mutant neural crest cells aggregated in prominent cranial ganglion-like structures dorsal to the normal site of branchial arch formation.

Transverse sections revealed strongly reduced numbers of both neural crest cells in the cardiac outflow tract and neural crest–derived melanocytes (Fig. 1, C to F). In vivo fate mapping also revealed a complete absence of the enteric nervous system (Fig. 1, G and H). Moreover, mutant cells were unable to associate with peripheral axons and, thus, to contribute to the Schwann cell lineage in peripheral nerves (Fig. 1, I and J). The markers sox10 and erbB3 were barely detectable in mutant peripheral nerves (*19*), and cad6-positive cranial nerves were missing (Fig. 2, A and B), confirming the lack of presumptive Schwann cells. Absence of neural crest–derivatives in the mutant is not attributable to cell death, because no increased apoptosis was observed in regions normally composed of neural crest targets (*19*).

Normally, cad6 expression at embryonic day 10.5 (E10.5) is confined to neural structures and absent in mesenchymal neural crest derivatives (Fig. 2, A, C, and E). In the mutant, however, the entire area of β-galactosidase–positive cranial neural crest cells also displayed cad6 expression (Fig. 2, B, D, and F). Within the ectopic domain of cad6 expression, neurofilament (nf) was readily detectable, as were several transcription factors characteristic of the sensory lineage (Fig. 2, H, K, and L) (*19*). Thus, upon sustained activation of β-catenin, sensory neurons are generated in anterior regions of the embryo that are usually devoid of neural derivatives of the neural crest. Similarly, the prominent cranial ganglia (Fig. 1B, arrowheads) contained many cells positive for sensory markers (fig. S2). Evidently, neural crest cells populating these structures have adopted a sensory neural fate.

In the trunk, ngn2 is normally expressed in emigrating neural crest cells and marks cells fated for sensory neural lineages (*20*). Its transient expression in cells aggregating in early DRG is followed by expression of the sensory markers ngn1 and neuroD. Whereas lack of β-catenin abolishes ngn2-expression (*11*), sustained β-catenin activity resulted in increased numbers of ngn2-positive cells, both in the trunk and at ectopic cranial locations (Fig. 2, M to P). Moreover, ngn2 expression was not restricted to the DRG anlage. Rather, ngn2-positive cells appeared to migrate on ventral routes and accumulated lateral to the dorsal aorta at sites of normal sympathetic ganglion formation (Fig. 2, P and R). Ectopic expression of ngn1 and neuroD was also found in these structures (Fig. 2, S and T; fig. S2). In contrast, the autonomic neuronal markers mash1 and ehand were virtually absent in the mutant PNS (Fig. 2, U and V; fig. S2), indicating that sensory neurons were forming at the expense of sympathetic neurons. The data also reveal that, unlike some other neural crest target structures (Fig. 1, C to J), sites of normal sympathetic ganglia development can be populated by sensory neurons.

Thus, β-catenin signaling not only appears to be required for sensory neurogenesis (*11*) but also to promote this fate. To evaluate this further, we investigated the developmental potential of isolated NCSCs expressing stabilized β-catenin. In culture, both control and mutant migratory neural crest expressed the NCSC-markers p75 and Sox10 (Fig. 3, A and B) (*21*). Moreover, migration, proliferation, and cell death was not significantly altered between the explants (Fig. 3, A to F; table S1), indicating that the loss of nonsensory lineages in the mutant is not due to selective mechanisms before or during neural crest emigration. In conditions permissive for sensory neurogenesis, control neural crest cells produced few Brn-3A–positive sensory neurons, and many cells maintained Sox10 expression (Fig. 3; table S1). In contrast, virtually all mutant cells lost Sox10 immunoreactivity and the majority became Brn-3A–positive, demonstrating that β-catenin signaling is able to specify a sensory neuronal fate in most neural crest cells. Because Sox10 has been associated with NCSC maintenance (*22*, *23*), the data also suggest that overexpression of β-catenin promotes the loss of multipotency in NCSCs.

We next investigated whether the effect of activated β-catenin in neural crest cells reflects a role of Wnt signaling. Unlike in control conditions, wild-type NCSCs exposed to Wnt1 efficiently generated Brn-3A–positive sensory neurons (Fig. 4). When we similarly challenged β-catenin–deficient NCSCs with Wnt1, we were unable to detect any sensory neurons (Fig. 4D), in agreement with our previous finding that β-catenin is required for sensory neurogenesis (*11*, *24*). Thus, Wnt1 promotes sensory neurogenesis in a β-catenin–dependent manner.

The effect of Wnt/β-catenin signaling could, in principle, be explained by two distinct mod-

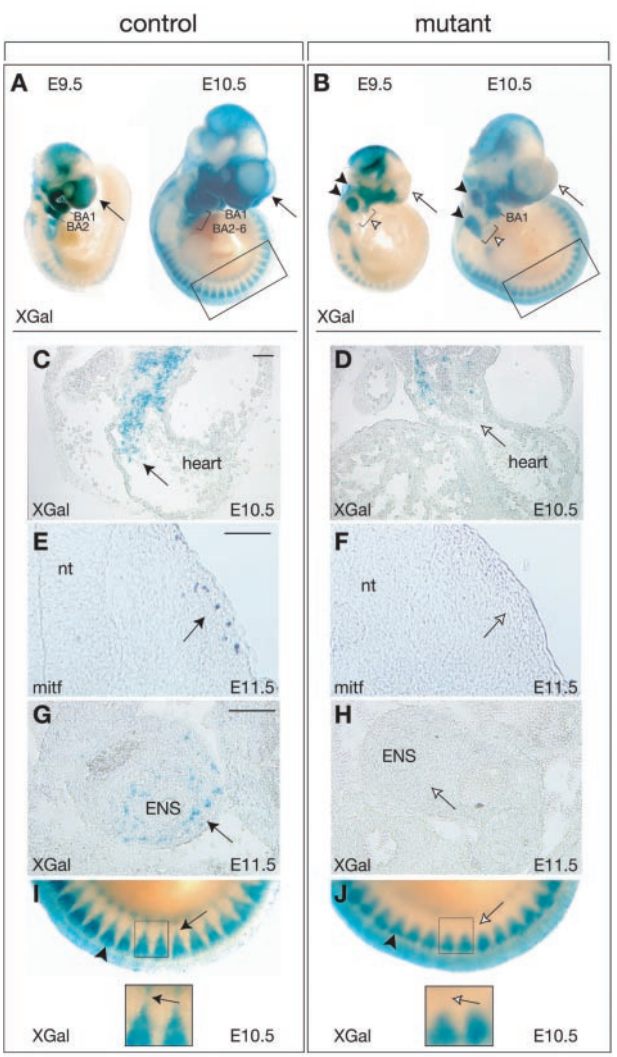

Fig. 1. In vivo fate mapping of NCSCs expressing stabilized β-catenin. (**A** and **B**) Neural crest cells and their progeny expressing β-galactosidase were revealed by whole mount XGal stainings (*13*). Mesenchymal neural crest in the nasofrontal region is present in control (arrows) and absent in mutant embryos (open arrows). Arrowheads point to prominent ganglion-like structures; open arrowheads indicate missing branchial arches (BA) in mutants. (**C** to **J**) XGal stainings and in situ hybridization on transverse sections show absence or reduction of cardiac neural crest, mitf-positive melanoblasts, and the enteric nervous system (ENS) in the mutant. Peripheral nerves [(I) and (J), enlarged areas marked by boxes in (A) and (B), respectively] were also missing. Arrows illustrate presence of and open arrows absence of neural crest derivatives. Arrowheads in (I) and (J) denote presence of DRG in control and mutant, respectively. nt, neural tube. Scale bars, 50 μm.

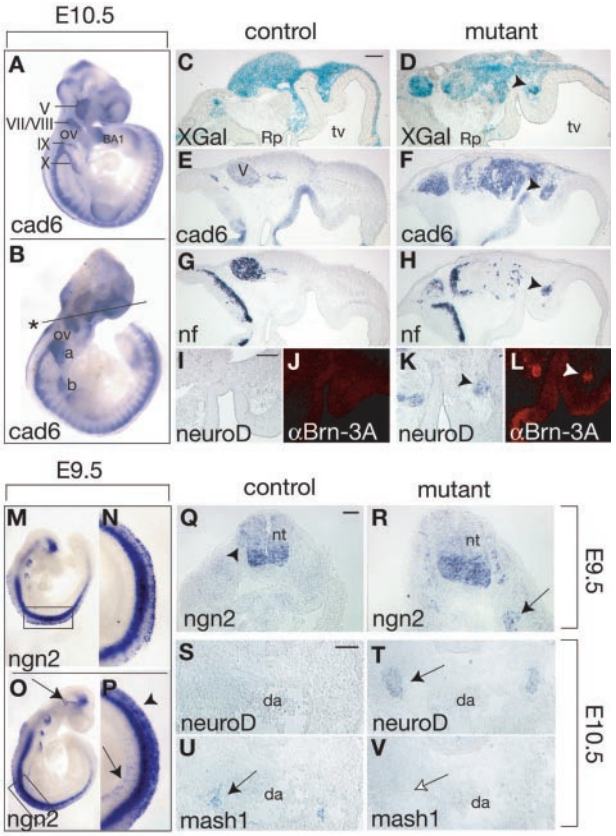

Fig. 2. Sensory neurogenesis at ectopic locations in mutant embryo. (**A** and **B**) cad6 expression at E10.5 reveals ganglia with sensory neuronal features in the mutant (B) formed instead of cranial nerves IX and X (a) and at the location of normal superior cervical ganglia (b) (see also fig. S2). V, VII/VIII, XI, and X designate cranial ganglia and nerves in the control (A). Ov, otic vesicle; BA, branchial arch. (**C** to **L**) Transverse sections cut at level (*) in (B). Note persistent expression of cad6 (F) in area of XGal-positive neural crest cells (D) and ectopic expression of nf in mutant (H). Ectopic neuronal cells (arrowheads) express the sensory markers neuroD (K) and Brn-3A (L). Rp, Rathke's pouch; tv, telencephalic vesicle. Scale bars, 100 μm. (**M** to **P**) In situ hybridization experiments show ectopic ngn2 expression in cranial [arrow in (O)] and ventral [arrow in (P)] regions in the mutant and increased expression in mutant migratory crest [arrowhead in (P)]. Control, (M) and (N); mutant, (O) and (P). (**Q** and **R**) On transverse sections at E9.5, ngn2-positive neural crest cells are restricted to the DRG anlage in control embryos [arrowhead in (Q)], whereas they spread ventrally in the mutant [arrow in (R)]. (**S** to **V**) At E10.5 in the mutant, neuroD-positive sensory neural cells [arrow in (T)] are found at the expense of mash1-positive autonomic neuronal cells [arrows in (U) and (V)]. da, dorsal aorta. Scale bars, 50 μm.

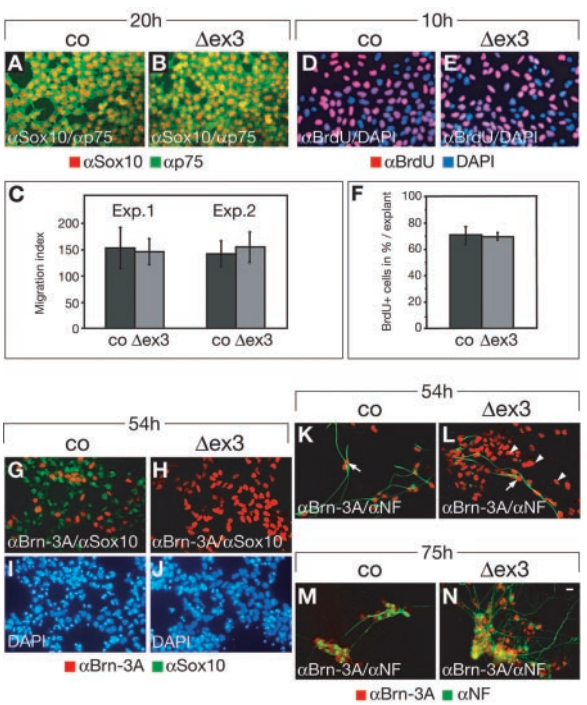

Fig. 3. Stabilized β-catenin does not affect emigration and proliferation of neural crest cells but promotes sensory neurogenesis. (**A** to **C**) Quantification of the migration index (*13*) did not reveal differences in migration of p75/Sox10-positive neural crest cells in control (co) and mutant (Δex3) explant cultures ($P > 0.2$). (C) Each bar represents the migration index (mean ± SD) of three different explants. (**D** to **F**) The number of proliferating, bromodeoxyuridine (BrdU)–incorporating cells was not significantly different between control and mutant cultures ($P > 0.75$). Three explants per genotype were analyzed, scoring 500 to 1000 cells per explant (F). Each bar represents the mean ± SD (**G** to **J**) Upon differentiation, mutant neural crest cells lose Sox10 expression and stain for the sensory marker Brn-3A, whereas many control NCSCs maintain Sox10 expression (table S1). (**K** to **N**) Differentiation is delayed in the mutant, because many Brn-3A–positive sensory neuronal cells (arrowheads) do not express NF (arrows) in the mutant at 54 hours (table S1). After prolonged incubation for 75 hours, however, most mutant cells are able to undergo full differentiation. Scale bar, 10 μm.

els: Wnt/β-catenin might promote the expansion of a sensory progenitor that has segregated from a neural crest cell with autonomic and other potentials (*20*, *25*, *26*). Alternatively, Wnt/β-catenin might have an instructive influence on fate decisions in an early NCSC able to generate sensory and autonomic neurons, glia, smooth muscle, and possibly other neural crest derivatives (*27*–*30*). The latter model is supported by the fact that in vivo sensory cells are not just expanded but rather generated at the expense of virtually all other neural crest lineages upon sustained β-catenin activation (Figs. 1 and 2). To rigorously distinguish between these models, we challenged early neural crest cells at clonal density with Wnt1 and, on sister plates, with different growth factors previously shown to promote specific fates in NCSCs (*31*) (Fig. 4, E to J; table S2). In control cultures without instructive growth factors, 88 ± 6.7% of all prospectively identified NCSCs generated mixed clones containing autonomic neurons and glia (*19*). In agreement with previous studies (*31*), BMP2 induced Mash-1–dependent autonomic neurogenesis in 90.5 ± 3.7% of all NCSCs, whereas upon transforming growth factor–β (TGF-β) treatment, about 70% of the cells adopted a smooth muscle–like fate. In the presence of Wnt1, 79.6 ± 2.5% of all NCSCs generated clones containing Brn-3A–positive sensory neurons. Most of these (95.4 ± 3.4%) were sensory neuron–only clones, which were not associated with Sox10 staining. The clone size of Wnt1-treated NCSCs was small, with many NCSCs giving rise to a single sensory neuron (Fig. 4, G and K). Furthermore, cell death was minimal in all clonal experiments (table S2), excluding selective effects of the factors added. The combined data indicate that Wnt signaling does not induce proliferation of a restricted sensory progenitor but rather promotes sensory fate decision in multipotent eNCSCs.

In vivo, members of the Wnt family specify neural crest from early dorsal neuroepithelial cells (*9*). Furthermore, the effects of ablation of *wnt1* and *wnt3* suggest a role of Wnt signaling in expansion of dorsal neural tube cells, including the premigratory neural crest (*10*). Our present data, together with the β-catenin loss-of-function analysis (*11*), indicate a subsequent function of Wnts in neural crest cells as they emigrate. Although the sensory lineage segregates early in PNS development (*20*, *25*, *26*), emigrating neural crest cells initially represent a population of stem cells (eNCSCs) that are homogeneous with respect to many developmental potentials, including sensory, autonomic, glial, smooth muscle–like, and possibly mesenchymal and other lineage formation (Fig. 2) (*27*). Similar to other growth factors that promote alternative fates (*31*), Wnt/β-catenin induces sensory neurogenesis by acting instructively on these eNCSCs. The molecular context that allows Wnt signaling to regulate cell cycle progression in certain stem

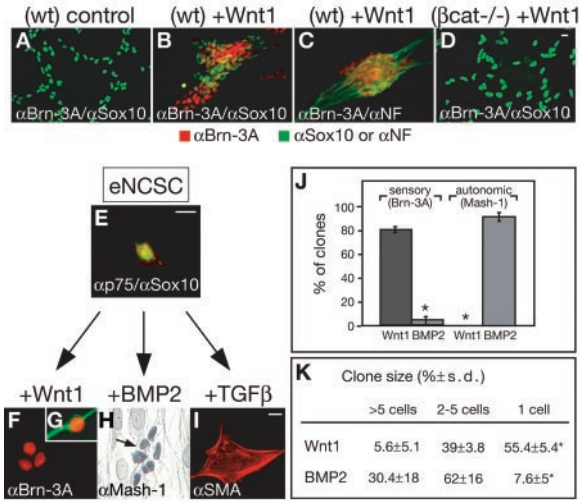

Fig. 4. Wnt1/β-catenin signaling instructs eNCSCs to adopt a sensory fate. (**A** to **D**) The effect of Wnt1 is β-catenin dependent. Wild-type (wt) NCSCs exposed to control monolayers fail to generate Brn-3A–positive sensory neurons (A), whereas wt NCSCs exposed to Wnt1 monolayers form ganglion-like cell aggregates containing Brn-3A/NF-positive sensory neurons [(B) and (C)]. β-catenin–deficient (β-cat$^{-/-}$) NCSCs are unable to generate sensory neurons, despite the presence of Wnt1 (D). Scale bar, 10 μm. (**E** to **J**) Clonal analysis demonstrates responsiveness of wt eNCSCs to different instructive growth factors including Wnt1. Of the p75-positive (red) founder cells, 90.3 ± 0.7% coexpressed Sox10 (green) (E). In the presence of Wnt1, founder cells generated clones of Brn-3A–positive sensory neurons (F) expressing NF (G). BMP2 instructed eNCSCs to generate Mash-1–expressing autonomic cells [arrow in (H)]. TGF-β induced a smooth muscle–like fate in most of the eNCSCs (I). Scale bars, 10 μm. (J) Quantification of clone composition (see also , fig. S2). (*) $P < 0.001$. The data are expressed as the mean ± SD of three independent experiments. Fifty to 150 clones were scored per experiment. (**K**) The cell number within individual Wnt1- and BMP2-treated clones was analyzed. Numbers (percentage of all clones per condition) are shown as the mean ± SD of three independent experiments, scoring 50 to 150 clones per experiment. (*) $P < 0.01$.

cells (*1*–*3*, *5*, *6*) and fate decision processes in NCSCs awaits investigation.

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32. Supported by grants of the Swiss National Science Foundation (to L.S. and U.S.) and by the National Center of Competence in Research "Neural Plasticity and Repair." We thank N. Mantei and R. Cassada for critical reading of the manuscript, A. McMahon and A. Berns for providing transgenic animals, and E. Ehler for advice with confocal microscopy. We acknowledge M. Wegner and J. Lee for riboprobes, and M. Wegner and E. Turner for antibodies.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1091611/DC1
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17 September 2003; accepted 1 December 2003
Published online 8 January 2004;
10.1126/science.1091611
Include this information when citing this paper.

Anterior Cingulate Conflict Monitoring and Adjustments in Control

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Conflict monitoring by the anterior cingulate cortex (ACC) has been posited to signal a need for greater cognitive control, producing neural and behavioral adjustments. However, the very occurrence of behavioral adjustments after conflict has been questioned, along with suggestions that there is no direct evidence of ACC conflict-related activity predicting subsequent neural or behavioral adjustments in control. Using the Stroop color-naming task and controlling for repetition effects, we demonstrate that ACC conflict-related activity predicts both greater prefrontal cortex activity and adjustments in behavior, supporting a role of ACC conflict monitoring in the engagement of cognitive control.

A major goal of cognitive neuroscience is to understand the precise neural mechanisms that underlie cognitive control (*1*). An important question about the nature of cognitive control is how do the processes involved in implementing control become engaged, or in other words, what controls control (*2*)? One partial answer comes from the conflict hypothesis, which posits that monitoring of response conflict acts as a signal that engages control processes that are needed to overcome conflict and to perform effectively (*3*, *4*). Two brain regions that have been associated with cognitive control processes are the ACC and the prefrontal cortex (PFC). Although it is commonly accepted that the PFC is involved in implementing control (*5*–*7*), there have been differing hypotheses regarding the contribution made by the ACC (*8*–*10*). One of these, the conflict hypothesis, contends that a function of ACC is the monitoring of processing conflict (*3*, *11*). However, this has recently been challenged on two grounds: first, failure to find behavioral evidence for trial-to-trial adjustments in control following conflict when stimulus repetitions

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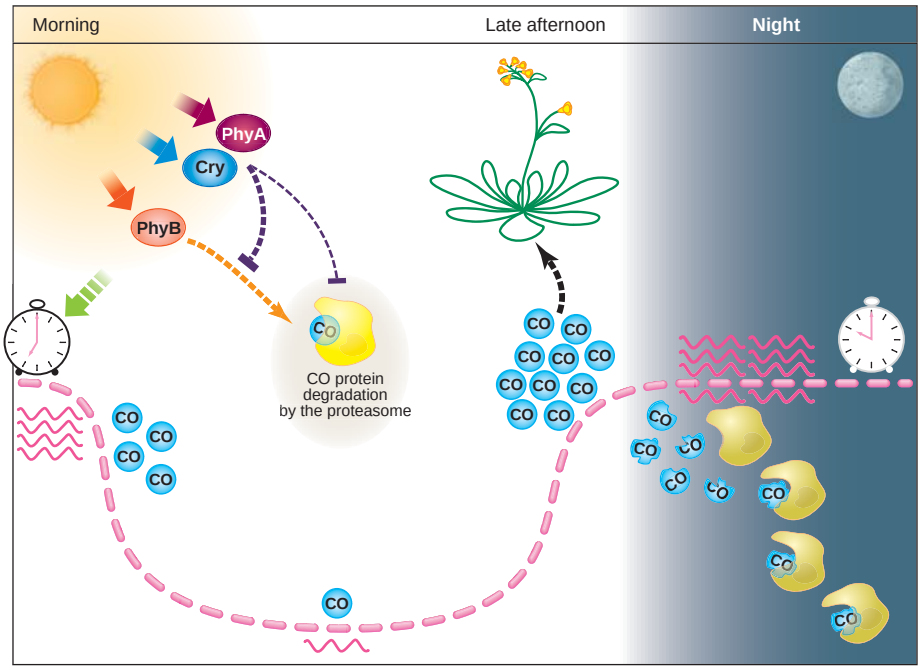

To flower or not to flower. The interplay of photoreceptors and the circadian clock in the regulation of CO expression. *CO* mRNA levels (wavy magenta lines) are regulated by the circadian clock, which is entrained through the action (green arrow) of phytochrome and cryptochrome (Cry) photoreceptors. The peak circadian rhythm of *CO* mRNA expression (dashed magenta curve) runs from the late afternoon to the early morning. CO protein levels (depicted by the number of blue spheres) are determined not only by *CO* mRNA expression, but also by protein degradation in the proteasome (yellow). CO degradation is promoted by phyB, but is inhibited by Cry and phyA during the day. During the night, the amount of CO mRNA remains high, but little CO protein accumulates because of CO proteolysis. During long days, phyA and Cry help to maintain the higher CO protein levels that promote flowering. (Arrows indicate stimulatory actions; lines with bar-head represent inhibitory actions; dashed lines suggest the involvement of additional proteins.)

photoreceptor regulation of CO degradation, coupled with the circadian clock that generates photoperiodic response rhythms of *CO* transcription, enables plants to have a lower amount of CO protein in short days and to gradually increase the level of CO protein as day length increases. Similar mechanisms are probably also at work in rice, a short-day plant, in which CO acts as a transcription suppressor of FT to inhibit flowering during long days (*12*).

It remains puzzling why CO abundance in wild-type plants peaks in the late afternoon or evening of a long day, but not in the early morning when its mRNA level is also relatively high. It is possible that the activity of phyA, cry2, or phyB may oscillate during the light phase of a long day, even though the amount of these photoreceptors remains relatively constant during long days (*11*, *13*). Alternatively, the abundance or activity of other proteins that are involved in CO degradation may be controlled by the circadian clock such that they fluctuate throughout the light phase of a long day. Identification of proteins fingering CO for destruction will help us to solve this puzzle. Some recent studies of *Arabidopsis COP1* and *ZTL* genes, which are involved in light responses or photoperiodic flowering, are noteworthy in this regard. *COP1* encodes a RING-finger protein with WD-40 repeats that is responsible for the proteasome-mediated degradation of the transcription factor HY5 in the dark (*14*); *ZTL* encodes a LOV-domain/F-box protein that is required for the proteasome and circadian clock–dependent degradation of the clock protein TOC1 (*15*). Could COP1, ZTL, or ZTL-related proteins also play a part in the CO degradation reported by Valverde and co-workers? Chances are we may not need to wait until next spring for an answer.

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DEVELOPMENT

Making Sense of the Sensory Lineage

Marianne Bronner-Fraser

Neural crest cells, a uniquely vertebrate cell type, are characterized by their ability to migrate throughout the developing embryo and to form many diverse tissues. These cells arise within the developing central nervous system and subsequently migrate away, sometimes moving extremely long distances to populate peripheral regions of the embryo (see the figure). Neural crest cells are multipotent progenitors that give rise to an impressive array of cell types, including neurons and glia of the peripheral nervous system, cartilage and bones of the face, and melanocytes (pigment cells) (*1*). Perhaps the best studied neural crest derivatives are the peripheral ganglia, which comprise neurons and support cells that form as aggregates outside the brain and spinal cord. These contain neurons of many different flavors, including sensory neurons (which relay touch and pain information to the brain) and autonomic neurons (which innervate various organs and modulate their activity). A report on page 1020 of this issue by Lee *et al.* (*2*) sheds light on the mechanism through which sensory neurons are generated from multipotent neural crest progenitor cells.

The fact that neural crest cells give rise to so many different progeny has raised the fascinating question of whether they are "stem cells." A stem cell divides to form one multipotent daughter cell like itself and another that is biased toward a particular cell fate. In support of the idea that neu-

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ral crest cells have stem cell properties, individual neural crest cells form multiple derivative tissues both in vivo (*3*) and in vitro (*4*). Furthermore, they have some ability to self-renew, consistent with the principal characteristic of a true stem cell.

How is a neural crest progenitor cell driven to adopt one of its many possible fates? A variety of growth factors can bias neural crest cells grown at clonal density (~ single cell density) toward certain lineages. For example, bone morphogenetic protein (BMP) causes cloned neural crest cells to form autonomic neurons; glia growth factor (GGF, also called neuregulin) drives them to become glia or Schwann cells (*5*); and endothelin as well as Wnts can bias them to become melanocytes (*6, 7*). However, the factors responsible for driving neural crest cells to become sensory neurons have remained elusive.

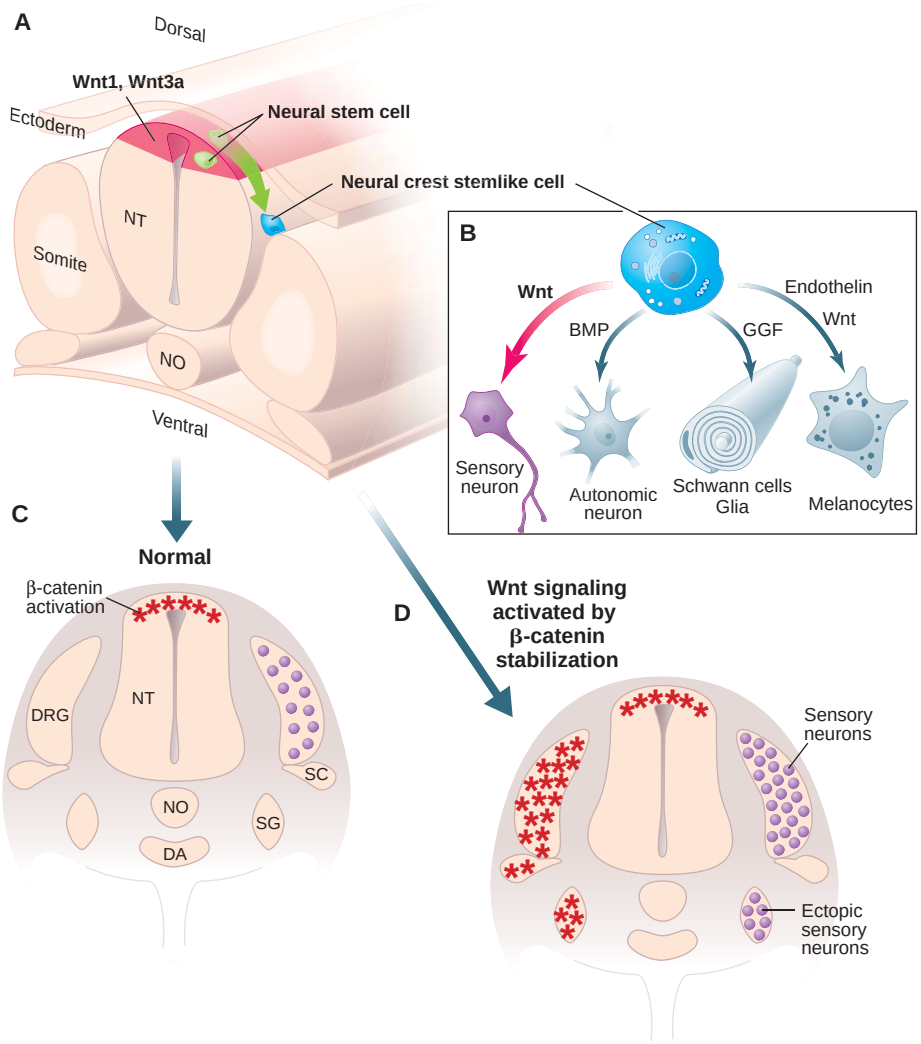
One possibility is that neural crest stem cells lose the ability to form sensory neurons as they emigrate from the neural tube. Support for this hypothesis comes from early studies showing that neural tube cultures (containing premigratory neural crest cells within the neural tube) could form sensory neurons, but that this ability appears to be absent in neural crest cells that are already migrating. Similarly, transplanted dorsal neural tubes, but not transplants of migrating neural crest cells, give rise to sensory neurons (*1*). An alternative explanation is that the neural tube may contribute some factor required for the formation of sensory neurons.

One family of factors prominent in the dorsal neural tube in the region from which neural crest cells emerge is the Wnt family of secreted ligands, particularly Wnt1 and Wnt3a. Wnt proteins play a variety of roles in neural crest development. They are important for induction (*8*), proliferation (*9*), and specification of the melanocyte lineage (*7*) during various stages of neural crest development. In addition, they are important for the proliferation and amplification of the neural progenitor cell pool.

In their new work, Lee, Sommer, and colleagues (*2*) examined the involvement of Wnt in the specification of neural crest lineages in the mouse. Activation of the "canonical" Wnt signaling pathway results in stabilization of β-catenin protein, which moves to the nucleus, pairs with a member of the TCF/LEF (T cell factor/lymphoid enhancer factor) family of transcription factors, and activates transcription. The investigators engineered mice in which the canonical Wnt pathway was constitutively activated in neural crest progenitor cells of the developing dorsal neural tube. The mutant neural crest cells migrated to many appropriate locations within the embryo but failed to differentiate properly. Even in the location where sympathetic ganglia should form, the neural crest cells containing activated β-catenin formed sensory rather than sympathetic neurons. In addition, there were reduced numbers of neural crest cells in the peripheral nerve that is normally populated by Schwann cells (a type of glia), and few cells were found in the cardiac outflow tract and under the skin where pigment cells differentiate. Instead, sensory neurons were generated in unexpected sites. These findings suggest that constitutive activation of the canonical Wnt pathway biases neural crest cells toward a sensory neuronal fate.

One possible explanation for these results is that Wnt signaling may promote proliferation and expansion of sensory progenitor cells so that they greatly outnumber other neural crest derivatives. In this model, Wnt might promote proliferation of a "sensory" progenitor cell. Alternatively, Wnt signaling may play an instructive role—biasing neural crest stem cells to form sensory neurons at the expense of other neural crest derivatives.

To distinguish between these possibilities at a cellular level, Lee *et al*. (*2*) added Wnt proteins to neural crest cells grown at clonal density in vitro. In the presence of



On the move. (**A**) Neural crest precursor cells are contained within the dorsal neural tube (NT). They undergo an epithelial-mesenchymal transition, giving rise to migratory neural crest cells with stem cell–like properties. (**B**) Under the influence of growth factors encountered in the environment, neural crest stem-like cells are biased toward various fates. The work by Lee, Sommer, and colleagues (*2*) shows that activation of Wnt signaling biases these cells toward a sensory lineage. In contrast, BMP signals influence neural crest cells to acquire properties of sympathetic neurons; GGF drives them toward a glial cell fate; and endothelin and later Wnt signals drive them toward a melanocyte fate. (**C**) During normal development, migrating neural crest cells settle in a variety of locations to form dorsal root ganglia (DRG), sympathetic ganglia (SG), and Schwann cells (SC) along the ventral root. (**D**) In transgenic mice in which the canonical Wnt signaling pathway is activated in migrating neural crest cells, these cells express markers of sensory neurons even in ectopic locations like the sympathetic ganglia, suggesting that activation of Wnt signaling transforms neural crest cells into sensory neurons. (NO, notochord; DA, dorsal aorta)

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Wnt, a large majority (~80%) of the clones were very small and only formed sensory neurons. This result implies that Wnts do not affect proliferation of multipotent neural crest progenitors, but rather bias their fate toward a sensory phenotype. In a complementary experiment, this same group found that loss of Wnt signaling in neural crest cells causes loss of both the sensory and melanocyte lineages (*10*). Taken together, these data suggest that Wnt signaling acts early in the neural tube to promote sensory fate decisions in multipotent neural crest progenitor cells.

The new work nicely shows that the Wnt pathway promotes sensory neurogenesis. But how do we reconcile this with other studies where activation of Wnt signaling drives neural crest cells to form melanocytes in the zebrafish or causes overgrowth of the dorsal neural tube in the mouse? The issue is very likely to be one of timing. Perhaps activation of Wnt signaling in early neural crest progenitors promotes a sensory fate, whereas later activation promotes pigment cell differentiation. This difference in timing could explain the differences in the gain-of-function (*2*) and loss-of-function (*10*) phenotypes. There is ample evidence that Wnts play multiple roles at multiple times, even in the same cell type. The challenge in the future will be to understand how the same ligand can elicit such different responses depending on the stage of development. In addition to stimulatory factors, migrating neural crest cells encounter environments rich in inhibitors that may counteract the influences of signaling molecules such as Wnts and BMPs. Perhaps these inhibitors titrate or delay responses as neural crest cells migrate along particular pathways. In this way, the lineage choices of neural crest progenitor cells may become progressively more limited as development proceeds.

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PHYSICS

Packing in the Spheres

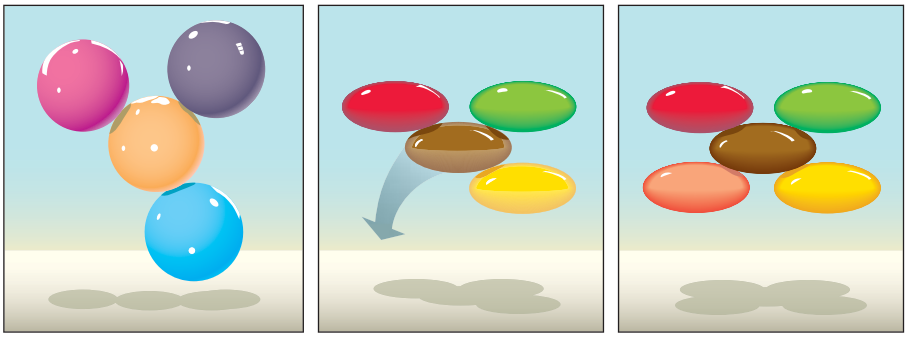
David A. Weitz

How many candies are in the bag you had for a snack with your coffee? How many grains of sugar are in the package you added to your coffee? And how many coffee beans came in the package that you used to brew the java? These may seem like idle questions, but they are, in fact, very important. The answers tell us how to pack materials efficiently, taking up the least volume possible, and are thus of critical interest to merchants, packagers, and shippers. Physicists, materials scientists, and mathematicians have also been studying these questions for centuries, and in recent years, it has seemed that the solution to these questions was becoming clear (*1*–*3*). Physicists have traditionally cast the problem in terms of that simplest approximation to all objects, the sphere. However, as reported by Donev *et al*. on page 990 in this issue (*4*), the behavior of spheres is apparently the exception rather than the rule. As soon as the shape of objects becomes nonspherical, the packing efficiency increases by a surprisingly large amount.

The question of packing at first seems very simple: How many marbles can you pack in a jar? To keep things simple, of course, you use perfectly spherical marbles, and to quantify the answer, you will measure the volume fraction, φ, occupied by the marbles. A simple experiment to do, but one that is tricky to understand. If you put the marbles in the jar very gently, a relatively small number will fit, with a volume fraction of around $\varphi \approx 0.6$. But then, if you very gently shake the marbles, so that they pack down as much as possible, but still remain completely disordered, their volume fraction increases to about $\varphi \approx 0.64$. This is the highest volume fraction of spheres packed to retain a random configuration, and is called random close packing, or φ_{RCP}. Virtually all the spheres are jammed in place, so none can move. Indeed, extensive experimental studies (*3*) of random packings give this value for the maximum volume fraction. Similarly, computer simulations (*5, 6*) with several different algorithms give the same value. Thus, it has long been thought to be a universal value, even though its actual magnitude cannot be predicted analytically.

There are two other important ways that our jar of marbles can be packed, however. The first occurs if you shake the jar very hard, allowing the marbles to jump up slightly and completely rearrange themselves. Then they begin to order, forming layers of spheres packed in a hexagonal lattice, with each layer nestled in the hollows formed by the layer beneath it. This structure is nearly crystalline, and forms the highest volume fraction packing of spheres, with $\varphi = 0.74$. Interestingly, although we are intuitively sure that this is the highest volume fraction packing of spheres, it has only been rigorously proven in the past few years (*7*).

The second important form of packing occurs if you pack the marbles even more gently than for random close packing; in fact, you must first put them in a fluid that provides neutral buoyancy, so there is no gravitational force whatsoever. Then, after the marbles settle, the packing seems even less dense and the volume fraction is only $\varphi \approx 0.56$ (*8*). This is called random loose packing, and represents the minimum pack-



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Shape is destiny. The shape of objects has a big effect on how densely they can be packed into a given volume. (**Left**) Spherical objects can only be pushed sideways and not rotated by neighbors, so they cannot experience torque. (**Middle**) Ellipsoidal objects can be rotated away by their neighbors and escape confinement. (**Right**) As a result, more neighbors (and denser packing) are required to balance the forces on an individual ellipsoid than on a sphere.

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14 ACKNOWLEDGMENTS

First of all I would like to thank my Angel and Evenstar Hye-Youn Lee for her love, bravery, loyalty, support and the great teamwork in the lab. I am proud that we succeded and won the battle.

I am very very thankful to my parents André and Barbara Kléber for insiting me to finish my PhD, and supporting me in any kind of matter. Without their help I would have never accepted the bad working conditions of a PhD student.

I am very thankful to Hai-Yul Lee and Sam-Joong Yun for the great support and ginseng tea

I further thank my brothers Philippe and Marc, my sisters Sie-Youn and Soo-Youn Lee

Further I would like to thank Daniel Molina, Reto Gafner, Andreas Marti and Alain Egli simply for beeing the Dudes

Many thanks to my lab colleages for loyalty and the great black E26-Spirit, namely Christof Eichenberger as Master Brau Samwise Gamschee, Heiko Wurdak as Hombre, Sebastian Fuchs as Sprengstoffmeister and Luca Jahreiss as Mr. Schweiz. One for all and all for one!

I am very thankful to Fabian Ille for professional computer support

I would like to thank Prof. Dr. Lukas Sommer for giving me the opportunity to work in his group and showing me real scientific business; for teaching me how to think scientifically and his never ending willingness for scientific discussions.

I am thankful to Prof. Dr. Ueli Suter for accepting me as a PhD in his unit. Many thanks for supporting the projects by sharing equipments and materials with our group and for advises over the years.

I am very thankful to Prof. Dr. Stephan Neuhauss for accepting the co-reference for my Ph.D.

Special thanks to Quentin Tarantino

15 CURRICULUM VITAE

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2004 / 02	SSN Symposium, Lausanne, Switzerland „Instructive Role of Wnt/β-catenin in sensory fate specification in neural crest stem cells“
2004 / 01	NCCR Mini-Symposium, Institute of Cell Biology „Instructive Role of Wnt/β-catenin in sensory fate specification in neural crest stem cells“