Neural Crest-Derived Sympathoadrenergic-like Progenitors of the Postnatal Murine Adrenal Gland

Dissertation submitted in fulfillment of the requirements for the degree of “Doctor of Human Biology” (Dr. biol. hum.) of the Faculty of Medicine of Ulm University

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<tbody>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxy-uridine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CHGA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine beta-hydroxylase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor2</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>NB</td>
<td>Neuroblastoma</td>
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<tr>
<td>NC</td>
<td>Neural crest</td>
</tr>
<tr>
<td>NCC</td>
<td>Neural crest cell</td>
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<tr>
<td>NCSC</td>
<td>Neural crest stem cell</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SAP</td>
<td>Sympathoadrenergic progenitor</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor-1</td>
</tr>
<tr>
<td>SN</td>
<td>Sympathetic neuron</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
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<td>μm</td>
<td>Micrometer</td>
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1. INTRODUCTION

The aim of this study was to identify and characterize a novel experimental source of neural crest-derived stem and progenitor cells, which are not only essential for development of the sympathetic peripheral nervous system, but also play a key role in the genesis of neuroblastoma, the most common and frequently lethal extracranial solid tumor in childhood. Therefore, experimental access to these stem cells is crucial, however, the derivation of neural crest-derived stem and progenitor cells from sources, such as embryonic stem cells and peripheral tissue, is complicated by cellular heterogeneity and low yield. Thus, isolating sufficient numbers of these progenitor cells for investigations had so far remained elusive. The studies in this thesis focused on describing the isolation and characterization of a rare and novel population of neural crest-derived sympathoadrenergic progenitors from postnatal murine adrenal glands. These advances may facilitate investigations about the cell of origin of neuroblastoma.

1.1 The neural crest

The neural crest (NC), often referred to as the fourth germ layer, is fundamental in the evolution of unique craniate and vertebrate tissues and sets vertebrates apart from all others metazoans (Hall, 2000). Neural crest cells (NCCs) have been used as a system to study embryonic induction, cellular and tissue specification, elucidation of cellular migratory pathways and analysis of cell-fate. The multipotent nature of NCCs enables them to differentiate towards several different cell types that can differ in their location, structure and function. Such variety of cells can range from neurons and glia of sensory, autonomic and enteric ganglia to medullary secretory cells, smooth muscle cells, melanocytes, bone and cartilage cells (Sauka-Spengler and Bronner-Fraser, 2008).
1. Introduction

1.1.1 Formation of the neural crest

The formation of NC cells is regulated by cell-intrinsic as well as extrinsic factors during their emergence and development ranging from embryonic gastrulation phase to late organogenesis. Induction of neural crest at the neural plate border between neuroectoderm and non-neural ectoderm is regulated by complex signaling events where several genes influence neural crest development through a gene regulatory network. The specification, emergence and migration of neural crest precursors progresses with the development of the neural tube, where these precursors start to accumulate close to the elevating neural fold, lying dorsally to the neural tube. Under the influence of NC specifiers, NC cells undergo epithelial to mesenchymal transition and by undergoing cytoskeletal rearrangements and losing cell-cell adhesions, they delaminate and migrate away from neuroepithelium. Influenced by the complex environmental cues and cellular interactions, neural crest cells acquire special cell-surface receptors and adhesion molecules, which help the NC cells to migrate to their prospective destinations (Sauka-Spengler and Bronner-Fraser, 2008).

Diverse vertebrate species, ranging from amphibians to teleosts, avians, marsupials, and mammals, have conserved pathways that control neural crest migration as a precisely controlled migration event (Kulesa et al., 2004). Neural crest can be further divided into four distinct axial populations - cranial, cardiac, vagal, and trunk. All of these populations follow their unique migration pathways and give rise to unique sets of cell and tissue types (Crane and Trainor, 2006).

Out of the four axial populations, cranial NCCs give rise to cartilage or bone, and in addition to pigment cells, connective tissue, sensory and parasympathetic ganglia (Crane and Trainor, 2006). Cardiac neural crest cells form the aorticopulmonary septum and conotruncal cushions and contribute to the formation of aortic arch smooth muscle (Kirby and Stewart, 1983) and parasympathetic cardiac ganglia. Vagal neural crest cells contribute to gut and form the majority of the neurons and glia that constitute the enteric nervous
Trunk neural crest cells differentiate primarily into neurons and glia, which constitute the peripheral nervous system, and pigment cells of the skin. A subset of trunk crest cells, belonging to the peripheral nervous system (PNS), is known as the sympathoadrenal lineage, which forms the sympathetic ganglia and neuroendocrine medullary cells of the adrenal gland (Anderson and Axel, 1986). These sympathoadrenal progenitors are the putative cells which lead to neuroblastoma (NB), a frequent childhood tumor with diverse clinical manifestation (Brodeur, 2003). Another subpopulation of posterior trunk neural crest is known as the sacral neural crest, it constitutes the enteric nervous system within the caudal hindgut (Burns and Douarin, 1998).

1.1.2 Neural crest cell migration

The migration of NC cells occurs dorsolaterally as well as ventrally. After completion of migratory events, the cells arrive at their final destination within the embryo and differentiate to different cell lineages, depending upon the location of destination tissue, Fig. 1. Intrinsic and extrinsic factors from within the neural tube, migratory pathways or peripheral targets are responsible to regulate the final fate of NC cells (Le Douarin et al., 2004; Trainor and Krumlauf, 2000).

Concerning trunk NC cells, the migrating trunk NC cells can be identified by the expression of a carbohydrate epitope HNK-1 (Bronner-Fraser, 1986). Derivatives of trunk neural crest cells form PNS components such as sympathetic neurons, glia and pigment cells of the skin, posterior trunk NCCs form the enteric nervous system (Crane and Trainor, 2006). Trunk NC cells continue to migrate ventrally until they aggregate close to the dorsal aorta, where they coalesce to form primary sympathetic chain. Dorsal migration of a subpopulation in primary sympathetic chain results in formation of definitive sympathetic chains composed of paravertebral sympathetic ganglia. Another subpopulation of cells migrate and aggregate into the adrenal gland to form adrenal medulla (Francis and Landis, 1999).
1. Introduction

Figure 1. Migratory pathways of NC cells and their main derivatives. A. Neural crest cells migrate dorsolaterally and ventrally within the embryo, forming the craniofacial area (red and green), the post-otic region (blue) constituting the cardiovascular and enteric neural crest, and the trunk migratory streams (yellow) forming the peripheral nervous system and pigment cells. B. Neural crest cells differentiate to diverse derivatives, a subset of which is shown. Image modified from (Kasemeier-Kulesa et al., 2008).

1.1.3 Role of MYCN in the migration of neural crest cells

MYCN regulates proliferation and differentiation of progenitor cells in peripheral nervous system (Grimmer and Weiss, 2006; Kobayashi et al., 2006) and is down-regulated when tissues terminally differentiate and become post-mitotic (Thomas et al., 2004). Migrating NC cells express MYCN before and during migration, however in the later migratory stages its expression is restricted to cells undergoing neuronal differentiation (Wakamatsu et al., 1997). Thus, MYCN regulates fate determination of NC cells, regulates their migratory potential, promotes their ventral migration and regulates the differentiation of NC cells towards neuronal lineage, in cooperation with other intracellular and extracellular factors (Wakamatsu et al., 1997).

In mouse, MYCN overexpression enhances cell proliferation in developing dorsal root ganglion and induces apoptosis of sensory progenitors in vivo (Kobayashi et al., 2006).
1.1.4 Transcriptional regulation in the neural crest

Gene expression in cells is regulated by transcription factors, which are downstream targets of various extracellular and intracellular signals. During the development of neural crest, several transcription factors play crucial roles, by regulating neural crest induction and migration in a temporal and spatial manner. Multiple regulatory networks regulate specific properties of NC cells, like their migratory potential and differentiation capacity. These regulatory modules consist of signaling molecules and transcription factors that regulate the induction of NC, specification of neural plate border, regulating the formation of bonafide NC and the regulation of downstream target genes (Sauka-Spengler and Bronner-Fraser, 2008).

During neural crest induction, the neuroepithelial cells undergo cytoskeletal rearrangements and epithelial-to-mesenchymal conversion (Crane and Trainor, 2006). The expression pattern of several key transcription factors, growth factors, and their targets that define NCC development and further SAP-specification are depicted in Fig. 2 and described below and in section 1.1.5.

Signals from surrounding cells such as the BMPs (bone morphogenetic proteins), FGF (fibroblast growth factor) and Wnts (wingless-type proteins) induce definite neural crest markers including SNAIL/SLUG (vertebrate homologs of Drosophila snail gene), PAX3 (paired box 3), SOX9/10 (sex determining region Y-box) (Betancur et al., 2010). Snail gene family members mark cells undergoing epithelial-to-mesenchymal transformations (Crane and Trainor, 2006; Meulemans and Bronner-Fraser, 2004). Sox9 and Sox10 are turned on in the premigratory neural crest of the trunk (Wegner and Stolt, 2005). Pax3 is required for development of multiple neural crest lineages and for activation of lineage-specific programs (Wu et al., 2008). p75 is the low affinity nerve growth factor receptor (LNGFR) and is responsible for neuronal survival and differentiation (Lee et al., 1992), Fig. 2.
1. Introduction

Figure 2. Specification of neural crest cells to NCSCs and SAPs. Neural crest induction begins at the neural plate border and is mediated by signals (green), from mesoderm and non-neural ectoderm. These signals along with BMP2 (from dorsal aorta) induce specific genes (red), which specify neural crest cells towards NCSCs, SAPs or sympathetic neurons.

1.1.5 The sympathoadrenergic progenitor cells (SAPs)

The sympathoadrenal cell lineage is derived as a sublineage of neural crest, which gives rise to sympathetic neurons, adrenal chromaffin cells, extra-adrenal chromaffin cells and the small intensely fluorescent cells of sympathetic ganglia and paraganglia (Huber et al., 2002). Sympathoadrenergic cells develop in the region of trunk NC that coalesce at the dorsal aorta to form sympathetic primordia. There are two views explaining SAP-specification towards chromaffin and neuronal cells. The first view indicates towards a bipotential SAP that can give rise to chromaffin and neuronal cells, the second view indicates the presence of separate committed NC-derived precursors for either cell types (Huber, 2006). Development of
SAPs from NC cells is initiated under the influence of BMP-induced transcriptional networks, when the NC cells reach the proximity of dorsal aorta (Achilleos and Trainor, 2012; Shah et al., 1996). Nestin and Musashi1 are expressed on the NCCs and progenitor cells and define the stem cell properties of NCCs and more recently of NCSCs (Okano et al., 2002; Shi et al., 2008). A cross-regulatory gene network regulates distinct gene expression profile of SAPs, their migratory pathways and their final differentiation to committed cell types. Within this network of SAP specification, PHOX2b (paired-like homeobox 2b) is pivotal (Pattyn et al., 1999) and transcription factor MASH1 (mammalian achate schute homolog 1) is important (Guillemot et al., 1993), both playing crucial role in the early specification of SAPs. GATA3 is specifically required to induce noradrenergic traits (Lim et al., 2000). Hand2 promotes differentiation both in neural crest cultures and in vivo (Howard et al., 1999). The two key catecholaminergic biosynthesis enzymes TH (Black, 1982) and DBH (Thomas et al., 1995) catalyze steps in the biosynthesis of epinephrine and norepinephrine and are expressed in both the neuronal and endocrine derivatives of the sympathoadrenal lineage. Both of these enzymes are under the regulatory control of Phox2b, dHand and GATA3. Subsequently, the cells migrate to their final destination organ, i.e., the sympathetic ganglia or the adrenal medulla, and to the locations of extra adrenal chromaffin tissue (Huber et al., 2002). Additional factors that differentiate SAPs in vitro towards mature sympathetic neurons and chromaffin cells include glial cell line-derived nerve growth factor (GDNF), neurturin (NTN), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT4), activating tyrosine kinase receptor B (TRKB), nerve growth factor (NGF), neurotrophin 3 (NT3) and activating tyrosine kinase receptor A (TRKA) (Escurat et al., 1990; Meulemans and Bronner-Fraser, 2004; Nakagawara, 2001; Sauka-Spengler and Bronner-Fraser, 2008).

Taken together, these transcription factors and their downstream targets are required for induction, migration and differentiation of NC derivatives, resulting into the formation of SAPs (Fig. 3).
1. Introduction

Figure 3. Regulatory network controlling SAP development. BMPs from the dorsal aorta induce the expression of Mash1 and Phox2b. Mash1 and Phox2b precede dHand, Gata3 and Phox2a. Expression of the catecholaminergic enzymes TH and DBH depends on Mash1, Phox2b and Gata3. Solid arrows indicate that the regulation has been shown by both loss- and gain-of-function experiments; dashed arrows indicate gain-of-function experiments and might signify maintenance function, image adapted from (Goridis and Rohrer, 2002).

1.2 The adrenal gland

1.2.1 Anatomy and neural crest-origin of the adrenal medulla

The adrenal glands, also known as supra-renal glands are located at the top of the kidneys in mammals. Adrenal glands are endocrine glands important in the organism’s response to environmental stress and are the most common site of development of NB (Brodeur, 2003). The adrenal gland is composed of an outer cortex (divided into three zones which secrete glucocorticoids, mineralocorticoids and sex hormones), and an inner medulla. Adrenal medullary cells are neural crest-derived, in contrast to the mesoderm-derived adrenal cortex. A subset of migrating trunk NCCs, i.e. the sympathoadrenal lineage (detailed in the previous section), contributes to the formation of adrenal primordia, which is the initial site of medullary chromaffin cell development. The sympathoadrenal lineage is thought to be the origin of NB, as a majority of NB develop in the adrenal medulla (Brodeur, 2003). Thus elucidating the properties of SAPs is essential to understand sympathetic
PNS development or its aberrant transformation leading to NB.

Medullary cells are modified postganglionic cells of the PNS but they are devoid of axons and dendrites, thus receiving innervation directly from corresponding preganglionic fibers. The cells are arranged as clusters around large blood vessels. The secretory cells of the adrenal medulla are known as chromaffin cells as they form colored polymers of catecholamines after reacting with oxidizing agents, such as chromate. Chromaffin cells release catecholamines epinephrine or norepinephrine into the bloodstream in response to acetylcholine or calcium ion. The medullary cells synthesize other peptides apart from epinephrine and norepinephrine, such as neuropeptide Y, met-enkephalin, neurotensin, substance P, and chromogranin A (Rosol et al., 2001).

1.2.2 Catecholamine synthesis and secretion

The pre-ganglionic (cholinergic) sympathetic nerves originating in the spinal cord stimulate the chromaffin cells to synthesize and secrete epinephrine and lesser amounts of norepinephrine. Thus, as modified post-ganglionic nerve cells, chromaffin cells are neurosecretory cells (Nussey and Whitehead, 2001). Synthesis of catecholamines starts with the conversion of tyrosine to dihydroxy-phenylalanine (DOPA), which is a rate-limiting step catalyzed by tyrosine hydroxylase. DOPA is converted to dopamine, catalyzed by the enzyme aromatic l-amino acid decarboxylase. Further hydroxylation step catalyzed by dopamine β-hydroxylase converts dopamine to norepinephrine in the secretory granules. In most of the adrenal medullary cells, phenylethanolamine N-methyltransferase (PNMT) converts norepinephrine to epinephrine. The medullary activity of PNMT is influenced and enhanced by corticosteroids secreted from the cortical cells (Nussey and Whitehead, 2001). Overall, the catecholamines prepare us for ‘flight or fight’ actions with other actions such as increasing heart rate and stroke volume, increasing blood pressure, dilating the bronchi, mobilizing glucose and stimulation of lipolysis, mediated by β-adrenergic receptors (Nussey and Whitehead, 2001).
1.3 Neuroblastoma

1.3.1 Occurrence

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood (Brodeur, 2003) and is an embryonal malignancy of neural crest origin with the primary tumor arising at sites within the peripheral sympathetic nervous system (Brodeur, 2003). NB probably derives from primitive sympathetic neural precursors. Approximately 65% tumors are abdominal with the adrenal medulla as the most common site of tumor development (Brodeur, 2003). Other sites for NB development include neural crest-derived paraspinal sympathetic ganglia in the chest or abdomen, or in pelvic ganglia (Fung et al., 2008; Hansford et al., 2004).

1.3.2 MYCN in neuroblastoma

Advanced and rapidly progressing NB is often associated with amplification of the proto-oncogene MYCN, which reliably predicts poor prognosis, Fig. 4, (Brodeur, 2003; Brodeur et al., 1987; Brodeur et al., 1985; Chan et al., 1997; Hansford et al., 2004; Look et al., 1991; Schwab, 1993; Schwab et al., 1985; Seeger et al., 1985; Weiss et al., 1997). Mostly, MYCN copy number is proportional to N-myc expression (Nisen et al., 1988; Wada et al., 1993). It remains controversial if N-myc overexpression in the absence of MYCN amplification predicts poor outcome (Matthay, 2000). The divergent effects of N-myc observed in patients with NB are also reflected at the cellular level. Overexpression of N-myc in single copy NB cells results in cytokine-independent proliferation in vitro and increases tumorigenicity in the mouse (Schweigerer et al., 1990). Apart from its oncogenic role, MYCN also influences cellular processes such as proliferation, metabolism and cell differentiation (Adhikary and Eilers, 2005). MYCN is also known to induce apoptosis (Hogarty, 2003; Ushmorov et al., 2008). In cell lines derived from NB, multiple cell phenotypes with heterogeneous MYCN expression are present (Spengler et al., 1997).
1.3.3 Target cells of MYCN

As most of the neuroblasts are undifferentiated or poorly differentiated, their cellular origin is unknown, thus the target cells of MYCN in NB development remain unidentified. Initiation of neuroblastoma due to MYCN overexpression was first reported by Weiss et al., where MYCN transgene was targeted to neural crest cells of mice resulting in neuroblastoma (Weiss et al., 1997). Reports show that hyperplastic lesions in early postnatal sympathetic ganglia of these TH-MYCN transgenic mice (Alam et al., 2009; Weiss et al., 1997) are composed predominantly of Phox2b+ neuronal progenitors. Interestingly, MYCN induces expansion of these progenitors by promoting their proliferation and at the same time arresting differentiation (Alam et al., 2009). Expression of MYCN in sympathetic superior cervical ganglia of rat at levels similar to those in NB causes sympathetic neurons to re-enter S-phase while rescuing them from apoptosis, thus contributing to their transformation to NBs (Wartiovaara et al., 2002). These reports describe neuroblast hyperplasia in murine paravertebral sympathetic ganglion cells (Alam et al., 2009; Hansford et al., 2004), however, the study of NB development at the most common site of NB development, i.e. adrenal gland remains elusive as the MYCN

Figure 4. MYCN expression and survival of NB patients. Kaplan-Meier survival curve showing survival probabilities of NB subsets defined by MYCN expression levels. The log-rank test was used to assess difference in survival of the NB subsets indicated (Tang et al., 2006).
transgenic mouse model do not develop hyperplasia in the adrenal gland (Hansford et al., 2004).

The TH-MYCN transgenic mouse model, where N-myc expression is driven by rat TH gene promoter, which is expressed specifically in NC-lineage cells, is now a widely used murine model of NB. Tumor penetrance in this transgenic NB model depends on the strain used as well as on MYCN gene dosage (Weiss et al., 1997). Murine tumors initiated by the TH-MYCN transgene display a wide range of genetic aberrations with similarity to those found in human neuroblastoma (Hackett et al., 2003). NB tumors arise in the TH-MYCN transgenic model with ~33% of the heterozygous transgenic mice and 100% of the homozygous mice developing tumors. In tumor-prone paravertebral ganglia of TH-MYCN transgenic mice, neuroblast hyperplasia precedes NB development in the first weeks after birth, (Fig. 5), with cellular phenotypes and gene expression patterns recapitulating those of human NB (Teitz et al., 2011; Weiss et al., 1997).

High MYCN expression in the paravertebral ganglions of TH-MYCN mouse model might be preventing complete neuroblast deletion in the perinatal period, leading to delayed regression of neuroblast hyperplasia and NB formation, these findings closely mirror descriptions of human neuroblast hyperplasia in the adrenal medulla of infants (Hansford et al., 2004). Although adrenal gland is the most common site of NB development, there is no neuroblast hyperplasia in the adrenal medulla of these transgenic mice, despite a low level of MYCN expression. This might be due to the promoter-specific influences on the timing and amplitude of MYCN expression in tumor-prone tissues in this transgenic mouse model (Hansford et al., 2004).

As SAPs play an important role in development of sympathetic PNS and are the putative cells of origin of neuroblastoma, experimental access to SAPs is central for investigating NB development.
Figure 5. TH-MYCN transgenic mice develop heterogeneous tumors. Histopathology of NB tumors in MYCN transgenic mice is shown. **A.** An anaplastic tumor, where the arrows denote location of the normal paraspinous ganglion. **B.** A tumor showing broad range of differentiation, the short arrow denotes primitive small round blue cells, long arrow denotes malignant cells which have a more ganglionic appearance (Weiss *et al.*, 1997).
2. AIM OF THE STUDY

Experimental access to NCSCs and SAPs is crucial for investigating normal as well as aberrant development of the sympathetic PNS. However, derivation of NCSCs and SAPs from ES cells and peripheral tissue is complicated by cellular heterogeneity and low yield. Alternative primary sources of neural crest or neural crest-derived cells are thus required to understand the biology of endogenous neural crest cells.

With increasing evidence that the deregulated development of early sympathetic progenitors plays an important role in NB development, in this study, the aim was to isolate, identify and characterize a population of post-migratory prospective sympathoadrenergic progenitors of neural crest-origin from early post-natal mouse adrenal gland.
3. MATERIALS AND METHODS

3.1 Isolation and culture of adrenal-derived spheres

Spheres were derived in serum free suspension conditions from adrenal gland of 2-d-old mice (C57BL/6 strain). All animal experiments were performed by following the institutional guidelines for animal studies. The adrenal glands were surgically removed from mice, washed twice in Dulbecco’s Modified Eagle Medium (DMEM)/F-12 (1:1, Life Technologies, Carlsbad, CA) and incubated with Liberase Blendzyme (0.62 U/ml, Roche Diagnostics, Mannheim, Germany) for 45 min at 37°C. After mechanically dissociating the tissue to a single cell suspension, the enzyme activity was neutralized by 10% FBS. The cell suspension was washed twice in DMEM/F-12 and passed through a 70 µm cell strainer (BD Biosciences, San Jose, CA), single cells were collected and seeded at a density of $12 \times 10^4$ cells per ml in DMEM/F-12 (1:1, Life Technologies) containing basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) (20 ng/ml each; Miltenyi Biotec, Bergisch-Gladbach, Germany), 20 ng/ml leukemia inhibitory factor (LIF, Millipore, Temecula, CA), 10 U/ml Heparin (Ratiopharm GMBH, Ulm, Germany), 2% B-27 supplement, 100 U/ml penicillin and 100 µg/ml streptomycin, all from Life Technologies. The cells were cultured in 100 mm low-attachment plastic dishes (Greiner Bio-one GMBH, Frickenhausen, Germany) at 37°C in 5% CO$_2$ in a humidified incubator. Differential plating was employed (Chung et al., 2009), where freshly plated cells were transferred after 2 h, 8 h and 3 d to a new culture dish in order to eliminate adrenocortical and endothelial cells. Cultures were supplemented with fresh media, bFGF and EGF (20 ng/ml) twice per week. Leukemia inhibitory factor and heparin were added only during the first plating.

The spheres were dissociated by treating with Accutase (PAA, Pasching, Austria) at 37°C for 5 min, followed by gentle trituration to obtain single cells. Single cells were replated as described above.
3.2 BrdU incorporation assay

To estimate the percentage of proliferating cells within the spheres, adrenal spheres cultured for 7 d in serum-free DMEM/F12 (1:1) supplemented with bFGF and EGF (20 ng/ml), were pulsed with 10 µM bromodeoxyuridine (BrdU, supplied with 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II, Roche Applied Science, Indianapolis, USA) for 24 h. Similar BrdU pulses were given to spheres from the same isolation up to four weeks (every seventh day).

BrdU-treated spheres were dissociated into single cells and were plated on poly-D-lysine/fibronectin-coated coverslips in a 24-well plate for 3 h. The cells were fixed and stained with anti-BrdU antibody as per the manufacturer’s instructions. Nuclei were counter-stained with DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride, 10 µg/ml, Sigma-Aldrich), number of BrdU-labeled nuclei in 15 visual fields per time point were counted (n=2) and the percentage of proliferating cells was calculated by dividing the number of BrdU-positive nuclei by total nuclei.

3.3 Reverse transcription PCR

Total RNA was isolated using TRIzol® Reagent (Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized from total RNA using SuperScript® III First-Strand Synthesis System and random hexamers (Life Technologies). PCR was carried out using 2.5% cDNA product from 1 µg input total RNA in a reaction volume of 25 µl using Taq DNA Polymerase (Sigma-Aldrich). β-actin was used as the housekeeping control.

PCR consisted of an initial denaturation at 94°C for 4 min followed by 29 amplification cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 45 s. Amplified products were electrophorased on 1.5% agarose gels containing ethidium bromide (0.5 mg/ml). Primer sequences and conditions are listed in Table 1.
### Table 1. List of primers

<table>
<thead>
<tr>
<th>Gene / Accession number</th>
<th>Primer sequences (F = forward, R = reverse)</th>
<th>Amplicon, annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI1 NM_007552</td>
<td>F GAT TGA CGT CAT GTA TGA AGA GG R ACC AGA TGA AGT TGC TGA TGA C</td>
<td>410 bp, 64°C</td>
</tr>
<tr>
<td>MYCN NM_008709</td>
<td>F GAG AGG ATA CCT TGA GGC AGC ACT C R CTC GCT GTC CTC CGA GTC TGA G</td>
<td>375 bp, 64°C</td>
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<tr>
<td>SOX2 NM_011443</td>
<td>F CAC AAC TCG GAG ATC AGC AA R CTC CGG GAA GGC GTG ACT TA</td>
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<tr>
<td>OCT4 NM_013633</td>
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<tr>
<td>DBH NM_138942</td>
<td>F ACC CGG GGG AGC TAC TCA TCA C R CAA AGG CTG CAG CTA CCA CCT TAC</td>
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<tr>
<td>TH NM_009377</td>
<td>F CTG TGG AGT TTG GGC TGT GTA A R CGC CGG ATG GTG TGA GGA GTG AT</td>
<td>313 bp, 62°C</td>
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<tr>
<td>HAND2 NM_010402</td>
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<tr>
<td>PHOX2B Y14493</td>
<td>F TCA ACC CCA CTC CTA CCC CTT TCC R TCA AGT TGG TTG TGG TGC CCC CGT</td>
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<td>GATA3 NM_008091</td>
<td>F CAG CCC ACC ACC CCA TTA CCA CTT G R GTC CTC CAG CCG GTC ATG CAC CTT</td>
<td>515 bp, 60°C</td>
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<td>NESTIN NM_016701</td>
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<td>MUSASHI1 NM_008629</td>
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<td>SOX9 NM_011448</td>
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<td>SOX10 XM_128139</td>
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<td>MASH1 NM_008553</td>
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<td>SLUG U97059</td>
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<tr>
<td>SNAIL X67253</td>
<td>F GCC TGG GGG CTC TGA AAG R AGG CCT GCC ACT GTG ATC TCT</td>
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<td>PAX3 NM_008781</td>
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<td>P75 NM_033217</td>
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<td>316 bp, 59°C</td>
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<td>TRKA NM_0010331 24</td>
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<td>TRKB NM_0010250 74</td>
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<td>PERIPHERIN NM_013639</td>
<td>F ACC CGG GAT GGG GAG AAG GTG R TGG ATC AGG CTG GGC CCT GC</td>
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3. Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Size</th>
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<td>AJ810516.1</td>
<td>F GTT TCA GCA GAT GCT GGA GC R GCA TGG AGG GCA TTT CTG TC</td>
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<td>SCN3A</td>
<td>NM_018732</td>
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<tr>
<td>SCN4A</td>
<td>NM_133199</td>
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<tr>
<td>SCN5A</td>
<td>NM_021544</td>
<td>F CAG GAG GCC ATG GAG ATG CTC R GAT TCC AGC ATG GTG GAC AC</td>
<td>763 bp, 55°C</td>
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<tr>
<td>SCN7A</td>
<td>NM_009135</td>
<td>F GTG AAA GAA CTC GAC GAA AG R GGG CAT TTC TTC CTG GAT GT</td>
<td>161 bp, 50°C</td>
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<tr>
<td>SCN8A</td>
<td>NM_0010774 99</td>
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<td>SCN9A</td>
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<td>865 bp, 58°C</td>
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<tr>
<td>SCN10A</td>
<td>NM_0012053 21</td>
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<td>828 bp, 58°C</td>
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<tr>
<td>SCN11A</td>
<td>NM_011887</td>
<td>F CAG AAA TGT CGC TGC CGA GAC AG R CCA GGT ACT TGG ATG CCA AG</td>
<td>446 bp, 55°C</td>
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</table>

3.4 Flow cytometry

Adrenal spheres were dissociated with Accutase and cell count was adjusted to a concentration of 0.6 \( \times 10^6 \) cells/ml in phosphate buffered saline (PBS)/3% bovine serum albumin (BSA). In case of intracellular proteins, the cells were fixed with 1% paraformaldehyde in PBS for 15 min on ice followed by permeabilization with 0.3% IGEPAL. After blocking with PBS/3%BSA for 30 min on ice, the cells were incubated with appropriate dilutions of the primary antibodies (Table 2) in PBS/3%BSA for 30 min at 4°C. In case of unconjugated antibodies, additional incubation with fluorophore conjugated secondary antibodies was carried out at 4°C for 30 min. Appropriate isotype controls were included to detect any nonspecific fluorescence. The cells were washed twice with PBS/3% BSA and \( 10^4 \) events were acquired with BD FACScan; data was analyzed using Cell Quest (both from Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com).
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3.5 Immunocytochemistry

Cells cultured on poly-D-lysine/fibronectin (150 µg/ml each, Sigma-Aldrich) coated glass coverslips were fixed with cold 95% ethanol / 5% glacial acetic acid for 20 min at -20°C, permeabilized for 5 min with 0.3% IGEPAL (Sigma-Aldrich), blocked for 30 min with 0.4% BSA and 4% goat serum in PBS, and were incubated overnight at 4°C with primary antibodies, listed in Table 2. For detection, the cells were washed thrice with PBS and incubated for 30 min at room temperature with appropriate fluorochrome-conjugated secondary antibodies (Table 2) for 30 min at room temperature. To detect horseradish peroxidase (HRP)-conjugated secondary antibodies or EnVision™ Dual Link polymer HRP (Dako, Denmark), diaminobenzidine solution (Sigma-Aldrich) was added for 10 min at room temperature. DAPI was used to counterstain nucleus. The cells mounted in fluorescent mounting medium (Dako, Glostrup, Denmark) were viewed using Olympus Provis inverted fluorescent microscope (Olympus) and images were acquired using analySIS (Soft Imaging System, Münster, Germany).

Table 2. List of antibodies

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Host / Isotype</th>
<th>Source</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Anti-Peripherin</td>
<td>Rabbit poly</td>
<td>Millipore</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-Peripherin (clone 8G2)</td>
<td>Mouse IgG1</td>
<td>Sigma-Aldrich</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-NF160</td>
<td>Rabbit poly</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-Nestin (clone rat-401)</td>
<td>Mouse IgG1</td>
<td>Developmental Hybridoma Bank, Iowa, USA</td>
<td>1:200</td>
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<tr>
<td>Anti-GFAP (clone GA5)</td>
<td>Mouse IgG1</td>
<td>Sigma-Aldrich</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-TH</td>
<td>Rabbit poly</td>
<td>Pel-Freez, Rogers, AR, USA</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-DBH</td>
<td>Rabbit poly</td>
<td>ImmunoStar, Inc. Hudson, WI, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-BMI1 (clone F6)</td>
<td>Mouse IgG1</td>
<td>Millipore</td>
<td>1:400</td>
</tr>
<tr>
<td>Anti-Musashi1</td>
<td>Rabbit poly</td>
<td>Millipore</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-Chromogranin A</td>
<td>Rabbit poly</td>
<td>Origene, USA</td>
<td>1:250</td>
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<tr>
<td>Anti-β3-Tubulin</td>
<td>Rabbit poly</td>
<td>Covance, Munich</td>
<td>1:1000</td>
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<tr>
<td>Anti-SF1</td>
<td>Rabbit poly</td>
<td>Sigma-Aldrich</td>
<td>1:50</td>
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<tr>
<td>Anti-CYP11A1</td>
<td>Rabbit poly</td>
<td>Santa Cruz</td>
<td>1:25</td>
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<tr>
<td>Anti-CYP11B2</td>
<td>Mouse IgG1</td>
<td>Millipore</td>
<td>1:50</td>
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</tbody>
</table>
### 3. Materials and Methods

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Anti-CD31</td>
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<td>1:20</td>
<td>Dianova, Hamburg</td>
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<tr>
<td><strong>Secondary antibodies</strong></td>
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</tr>
<tr>
<td>Anti-Rabbit IgG&lt;sub&gt;1&lt;/sub&gt; HRP</td>
<td>Goat</td>
<td>1:500</td>
<td>Life Technologies</td>
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<tr>
<td>Anti-Rabbit IgG A488</td>
<td>Goat</td>
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<td>Anti-Rabbit IgG A594</td>
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<td>Anti-Mouse IgG Alexa 488</td>
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<td>Anti-Mouse IgG Alexa 594</td>
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<td>1:500</td>
<td>Santa Cruz</td>
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<td>Southern Biotech, Alabama, USA</td>
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<td>EnVision&lt;sup&gt;+&lt;/sup&gt; Dual Link polymer-HRP</td>
<td>Goat</td>
<td>30 µl per section</td>
<td>Dako, Glostrup, Denmark</td>
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</table>

#### 3.6 Alkaline phosphatase activity

Cultured cells were fixed for 1 min in fixative solution (3% formaldehyde solution containing 6.75 mM citrate solution in 65% acetone). Diluted diazonium salt solution was prepared in water by mixing final concentrations of 0.002 M sodium nitrite to 0.1 mg/ml fast red violet alkaline, Sigma). After rinsing the fixed cells in deionized water for 2 min, 0.08 mg/ml naphthol AS-BI alkaline solution (Sigma) mixed with diluted diazonium salt solution was added to the cells. The cells were incubated in dark for 15 min at room temperature. After two washes with deionized water, cells were mounted in aqueous mounting media (Dako) and were evaluated microscopically.

#### 3.7 Self-renewal assay

Clonal colony-formation assay was done to assess the proportion of cells capable of giving rise to new spheres. Enzymatically dissociated single cells from adrenal spheres were plated in 96-well culture plates at cell densities ranging from 0.5 cells/µl – 4 cells/µl per well, in the same serum-free mitogen-containing media that was used to initially derive the spheres. After 7 d, new spheres in each well were counted under the microscope. Percentage of self-renewing cells was calculated by dividing the number of new spheres generated by the number of initially seeded cells.
3.8 In vitro differentiation assay

For in vitro differentiation of adrenal-derived spheres, a serum-free basal differentiation media was used that consisted of DMEM/F-12 supplemented with 1% B27, 30 mM glucose (Sigma-Aldrich), 1 mM glutamine and 50 ng/ml BSA (Sigma-Aldrich). Spheres were plated on poly-D-lysine/fibronectin coated coverslips in 1% FCS containing media to promote attachment for 6 h and were further differentiated in serum-free conditions for 6 d in differentiation media containing a combination of 10 µM all-trans retinoic acid (ATRA, Sigma-Aldrich) and 100 µM ascorbic acid (Sigma-Aldrich) for neural differentiation, and a combination of 10 µM dexamethasone (Sigma-Aldrich) and 100 nM Phorbol 12-myristate 13-acetate (PMA, Millipore) for chromaffin differentiation of spheres. Immunocytochemistry was performed to analyze the extent of differentiation by detecting specific neural (peripherin, B3-Tubulin) and chromaffin markers (TH, DBH, CHGA) and the numbers of positive cells were quantified.

For differentiation towards adipocyte, chondrocyte and osteocyte lineages, adrenal-derived spheres were plated on coated glass coverslips and were differentiated for 14 d in case of adipocytes and osteocytes and 21 d for chondrocytes. StemPro® Differentiation kits (Life Technologies) were used for these three specific in vitro differentiations.

3.9 Intra-adrenal orthotopic transplantation

All animal experiments were performed by following the institutional guidelines for animal studies. Nude rat (RNU rat, Charles River, Sulzfeld, Germany), aged 8-12 weeks were used to create an orthotopic model of cell transplantation. The animals were anesthetized and an incision was made in the skin, just above the spleen. This skin/muscle/peritoneum incision via the dorso-lateral approach opened up the retroperitoneal space to expose the left adrenal gland. Adrenal-derived sphere cells were dissociated, labeled with 5 µM CFSE (Carboxyfluorescein diacetate succinimidyl ester, Life technologies) for 10 min at 37°C in serum-free media and resuspended in saline containing
3. Materials and Methods

8 mg/ml Fibrinogen (Sigma-Aldrich). Thrombin (8 U/ml, Sigma-Aldrich) was added to this cell suspension just before the injection to promote immobilization of cells via clot formation and to help the intra-adrenal retention of injected cells (Cardoso et al., 2010). Cell clots were microsurgically positioned within the adrenal glands. After transplantation, the incision was closed with polyester sutures (Ethibond Inc., West Somerville, NJ). Post-transplantation, the animals were sacrificed at specific time intervals to analyze the transplanted adrenals. The adrenal microsurgery for intra-adrenal orthotopic transplantation was performed with the help of Dr. Markus S. Huber-Lang from the Department of Traumatology, Hand- and Reconstructive Surgery, Ulm University.

3.10 Cryosectioning and immunohistochemistry

The rats were sacrificed after anesthesia by cervical dislocation and the adrenal glands were excised. Adrenal glands were frozen in Tissue-Tek® O.C.T.™ compound (Sakura Finetek Inc., Torrance, CA) in stainless on with liquid nitrogen bath). Cryosections (3 µm) were made with a Leica CM1850 cryostat (Leica, Wetzlar, Germany) maintained at -25°C and the sections were placed on Superfrost® Plus glass slides (Thermo Scientific, Franklin, MA).

For immunohistochemistry, after three washes with PBS, cryosections were fixed with paraformaldehyde (4%) for 15 min at room temp. After fixation, the sections were washed thrice with PBS, permeabilized with 0.3% IGEPAL (5 min), followed by blocking with 5% goat serum / 3% BSA in PBS (30 min). Primary antibodies diluted in blocking buffer were added to the sections followed by overnight incubation at 4°C. After three washes with PBS, appropriate HRP-conjugated secondary antibodies (Table 2) or EnVision™ Dual Link polymer-HRP (for nestin detection) were applied for 30 min. DAPI was used to counterstain nuclei and the sections were mounted in DAKO aqueous mounting medium for microscopic observation.
3.11 Electron microscopy

Adrenal-derived spheres were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer with 1% sucrose (pH 7.3) and were post fixed in 2% Osmium tetroxide for 2 h. After dehydrating through a series of graded ethanol to propylene oxide, sample was embedded and polymerized in epoxy resin. Sections (0.5 µm) were examined to analyze cells containing dense core vesicles in the cytoplasm, with the help of Mr. Schmidt at the Electron Microscopy core facility of Ulm University, using a Zeiss EM-10 electron microscope (Carl Zeiss, Oberkochen, Germany).

3.12 Electrophysiology

Adrenal-derived spheres were incubated with 10 µM of a potentiometric indicator dye di-8-amino-naphthylethenylpyridinium (di-8-ANEPPS) at 37°C for 30 min, followed by washing with Krebs-Ringer solution. Emission was filtered at 501 nm and detected by a photomultiplier. Action potentials were triggered by rectangular pulses (10 V, 0.5 ms) through two stainless steel electrodes in the bath solution directly adjacent to the cells of interest. Double pulses at decreasing intervals were used to determine the refractory period of the compound action potentials (CAP). Four traces were averaged and filtered with a 101-point central rolling mean. Baseline correction and bleaching correction were performed before normalizing to the peak of the initial CAP using macros written in R (R Development Core Team, 2010). For whole-cell voltage-clamp recordings (Axopatch-200B, Axon instruments, Berkeley, CA, USA), the bath solution contained (in mM) 150 NaCl, 2 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4; 291 mOsm), patch pipette (tip resistance 2.5-4 MΩ) contained (in mM) 105 CsF, 35 NaCl, 10 EGTA, 10 HEPES (pH 7.4; 287 mOsm). Pipette resistances were 2.5-4 MΩ and seal resistances 1-5 GΩ.

The methodology (section 3.12), patch clamp and action potential experiments, RT-PCR of sodium channels, related results (section 4.5.2, Fig. 19, Fig. 20) were conducted by Dr. Karin-Jurkat Rott and her group, at her laboratory at the Division of Neurophysiology, Ulm University.
3.13 HPLC analysis for catecholamine detection

Cells were homogenized in 250 µl sonication buffer consisting of 0.4 N perchloric acid, 0.5 mM sodium metabisulfite and 2% EDTA (all from Sigma-Aldrich). Sonication was done on ice with 3 bursts (5 sec each) at 50% amplitude, with 25 sec intervals on ice, using a Qsonica sonicator (Newtown, CT, USA). Sonicated lysate was centrifuged for 5 min at 10,000 rpm and the supernatant was used to analyze catecholamines by high performance liquid chromatography-electrochemical detection (HPLC-ECD) with the help of Dr. Max Kurlbaum at the Department of Clinical Chemistry, Ulm University. 250 µl sonicated cell lysate with 150 pg internal standard 3,4-Dihydroxybenzylamine (Chromsystems, Gräfelfing, Germany) was used. After neutralization with 6 ml neutralization buffer (Chromsystems), the solid-phase extraction column (Chromsystems) was filled with this solution and eluate was discarded. The column was washed with HPLC-grade water (Merck, Darmstadt, Germany). Substances were eluted with 6 ml elution buffer (Chromsystems). 5 N Hydrochloric acid (30 µl, Chromsystems) was added per 1 ml eluate and 20 µl of the acidified eluate was used to separate and quantify catecholamines by HPLC-ECD using Agilent 1100 series HPLC system (Agilent Technologies, Böblingen, Germany) and electrochemical detector EC3000 (Recipe Chemicals, Munich, Germany).
4. RESULTS

4.1 Postnatal mouse adrenal gland contains proliferating cells

4.1.1 A rare population of adrenal cells forms spheres in culture

Adrenal medulla is a trunk neural crest derivative containing the putative cells, which are the target of malignant development in NB. To isolate and enrich trunk NC-derived SAPs from mouse adrenal glands, sphere formation assay was employed, which is an assay used to isolate neural stem cells and cancer stem cells in serum-free suspension cultures. The serum-free non-adherent culture conditions used for sphere generation do not support the propagation of non-stem cell types, however, these conditions support the survival and expansion of sphere-forming, cytokine-responsive cells, which includes both stem and progenitor cells (Reynolds and Weiss, 1992). We were successful in isolating spheres in serum-free suspension cultures from postnatal mouse adrenal gland (Fig. 6A, B). The dissociated adrenal cells, seeded at low density in serum-free conditions, formed spheres after 2-3 d of plating on non-adherent plates. After the initial 3 d, spheres were transferred to a new culture dish to eliminate any attached cells. The spheres were compact (Fig. 6C), were mitogen responsive, and could be derived and maintained using bFGF, EGF, LIF, B-27 supplement and heparin. LIF and heparin were crucial for sphere derivation. Omitting these mitogens from the culture resulted in inefficient derivation and maintenance of spheres. Average size of adrenal-derived spheres was 128±2.46 µm (Mean±S.E.M., diameter of 80 spheres, 7 d old). The spheres could be cultured for up to two passages, with decreasing number and size of spheres in the next generation (as the number of secondary spheres generated from dissociated primary spheres was too less for further passaging).

Thus, this methodology yielded a rare population of sphere-forming cells from postnatal murine adrenal glands.
Figure 6. Postnatal mouse adrenal gland contains proliferating cells. Adrenal glands from newborn mouse were dissociated to single cells and seeded at low density in non-adherent serum-free conditions to form spheres.

A. Location of adrenal gland in postnatal mice (2 d old). Dissected mouse with adrenal gland (left, arrow) and an isolated adrenal gland (right), ruler = cm. B. Scheme of sphere derivation from adrenal glands. C. Dissociated adrenal cells form spheres in serum-free suspension cultures. Phase contrast image of adrenal-derived spheres (7 d in culture), scale bar equals 200 µm.
4. Results

4.1.2 Sphere formation is age-dependent

We asked whether adrenal glands from older mice form spheres in culture? Trials to isolate spheres from other ages (d15 or d30) yielded no spheres, indicative of a rare progenitor population that could be isolated and cultured as spheres only within this short early postnatal time from postnatal d2-3 adrenal glands (Fig. 7). The efficiency of sphere derivation from postnatal d2 mouse adrenal gland total cells was low, ranging from 0.02% - 0.03% (average 0.02±0.0015, n=3, histogram in Fig. 7). The spheres could be cultured for up to two passages, with decreasing number and size of spheres in the next generation (as the number of secondary spheres generated from dissociated primary spheres was too less for further passaging). Thus, it was evident that sphere formation was age-dependent as isolation from early postnatal mouse was possible, but not from the later time points.

![Figure 7. Sphere formation from adrenal gland depends on the age of postnatal mouse. Phase-contrast images of spheres derived from adrenal glands of mice (various postnatal age) are shown, scale bar equals 200 µm. 1.2 x 10^5 cells/ml were seeded in non-adherent serum-free condition, sphere-forming frequency is shown in the histogram. The percentage was calculated as: (number of spheres formed/number of seeded cells) x 100. Values represent mean±S.E.M. (n=3). Statistical analysis was performed using t-test. **, p<0.01; ***, p<0.001.](image-url)
4. Results

4.1.3 Spheres express alkaline phosphatase

Endogenous alkaline phosphatase (AP) expression is a property of undifferentiated cells. Adrenal-derived spheres expressed alkaline phosphatase (Fig. 8A). To analyze the interior structure of spheres, Haematoxylin and Eosin staining of cryosectioned spheres was done, which showed that the spheres were composed of densely packed cells, without lumen (Fig. 8B).

![Figure 8. Spheres express alkaline phosphatase. A. Spheres express alkaline phosphatase. Detection of endogenous alkaline phosphatase in a 7d old sphere, scale bar equals 50 µm. B. Spheres are composed of densely packed cells. Haematoxylin and Eosin staining of cryosectioned adrenal-derived sphere (3 µm thickness), 2 spheres are shown, scale bar equals 100 µm.](image)

4.1.4 Sphere cells have limited proliferative capacity

As there was no increase in sphere numbers in continuous culture and no increase in sphere numbers after passaging, the number of proliferating cells in spheres derived from 2 d old mice was estimated by BrdU assay. BrdU can incorporate into the newly synthesized DNA of replicating cells, and can thus detect proliferating cells.

As revealed from the BrdU incorporation assay in a time-chase experiment of 4 weeks, it was evident that spheres contained BrdU positive cells (left panel, Fig. 9), and the percentage of BrdU-incorporating proliferative cells in the spheres significantly decreased gradually over a period of four weeks (week1, 41.7±4.4 to week4, 8.16±2.5), right panel, Fig. 9. These observations indicate that sphere cells in culture had limited proliferative capacity.
4. Results

Figure 9. Early spheres contain proliferating cells. BrdU (10 µM) was added to the sphere cultures (from d2 mice) at indicated time followed by further incubation for 24 h before immunodetection. Fluorescent micrograph of a 7d old sphere stained with anti-BrdU (green nuclei) and nuclei counterstained with DAPI (blue), left panel, scale bar equals 50 µm. Quantitative analysis of BrdU-incorporating nuclei in spheres in 4 weeks of continuous culture (right panel). BrdU-labeled nuclei in 15 visual fields per time point were counted and the percentage of proliferating cells was calculated as: (number of BrdU-positive nuclei / total nuclei) x 100. Values represent mean±S.E.M. (n=2). Statistical analysis was performed using student’s t-test. ***, p<0.001.

4.1.5 Spheres show restricted self-renewal capacity

The clonal propagation of secondary spheres from single cells obtained from primary spheres indicates the self-renewal capacity. In order to assess the self-renewal capacity of adrenal-derived spheres, one-week-old spheres were dissociated to single cells and were replated in suspension in 96-well non-adherent plates at various clonal densities (0.12 cells/µl, 0.25 cells/µl, 0.5 cells/µl, 1 cell/µl, 2 cells/µl and 4 cells/µl). After 10 d of initial seeding, there were no spheres in low plating densities (0.12 – 1 cell/µl). At a seeding density of 2 cells/µl or 4 cells/µl, only few secondary spheres, small in size (diameter 58±5.90 µm, Mean±S.E.M. of 15 secondary spheres) as compared to the parent spheres (diameter 128±2.46 µm) were observed and the sphere formation capacity was less than 1% (~ 0.6% in both cases), indicating low proliferation and sphere forming rate, thus supporting the BrdU incorporation data. The size of these newly formed spheres did not increase even after 3-4 weeks in culture (Fig. 10).
4. Results

Thus, the limited self-renewal capacity of adrenal derived spheres indicated a finite life span of these cells in culture, in line with their rarity and limited proliferation rate.

4.2 Spheres express genes associated with NCSCs and SAPs

The adrenal derived spheres were characterized through RT-PCR for expression analysis of genes associated with regulation of cell-type specification and lineage restriction in the development of neural crest stem cells and sympathoadrenal lineage. Most of the genes investigated were expressed, with the exception of Oct4, Pax3, TrkA and TrkB.

Sox2 and Bmi1 (associated with neural stem cell self-renewal) and MYCN as well as genes associated with the specification of neural crest were expressed, such as Slug, Snail, Sox9, Sox10 and p75 (LNGFR, low affinity nerve growth factor receptor) Fig. 11. Adrenal-derived spheres expressed all the transcription factor genes involved in regulation and lineage-specification of noradrenergic neurons from sympathetic precursors, namely Musashi1, Mash1, Phox2b, GATA3, Hand2, TH and DBH. Among the genes associated with sympathetic neurons, peripherin was detected at low level; TrkA and TrkB were not expressed, Fig. 11.

Figure 10. Spheres have limited self-renewal potential. Bright field microscopic images of adrenal-derived spheres formed after 8 d and 21 d of clonal seeding, scale bar equals 200 µm.
4. Results

Figure 11. Adrenal-derived spheres express NC and SAP-related genes and transcription factors. RT-PCR analysis of 7 d old spheres derived from postnatal d2 mouse adrenal glands. β-Actin was used as the housekeeping control.

Taken together, adrenal-derived sphere cells expressed gene profile characteristic of NCSCs and SAPs, thus confirming the neural crest-origin of these cells and confirming the SAP-like gene expression pattern indicated by the expression of characteristic SAP regulatory transcription factors like MASH1, Phox2b, Hand2 and GATA3.

4.2.1 The majority of cells constituting the spheres are neural progenitors

Nestin expression is associated with the progenitor state of lineage-restricted sympathetic progenitor cells (Shi et al., 2008). To estimate the percentage of nestin expressing cells, sphere cells were characterized for nestin expression by flow cytometry (Fig. 12). A majority of sphere cells were nestin+ (61%), thus indicating towards the presence of a mitogen responsive population of stem/progenitor cells within the spheres. CD57 (HNK1) is a surface marker.
described to be expressed on migratory neural crest cells and as we could detect the genes associated with neural crest and subsequent sympathoadrenal development, we analyzed expression of CD57 and p75 (Low Affinity Nerve Growth Factor receptor, LNGFR, also expressed on early migratory NCSCs) on sphere cells by flow cytometry. Expression of both CD57 and p75 on sphere cells was not detected (Fig. 12).

This data indicated the majority of sphere cells to be in a progenitor state (nestin positive). The absence of CD57 and p75 (markers of migratory NCSCs) indicated that the sphere cells were post-migratory.

![Fluorescence intensity graph](image)

**Figure 12. Nestin-expressing cells constitute the majority of adrenal spheres.** Adrenal-derived sphere cells were stained with anti-nestin, anti-CD57 and anti-p75. For flow cytometry, appropriate isotype controls (dashed line) were used for each experiment; grey tinted peaks define the specific antigen expression. Staining of cells, flow cytometry and analysis were performed as described in materials and methods.

**4.3 Spheres contain many progenitor cells and few neurons or glial cells**

As majority of the sphere cells were nestin⁺, and expressed NCSC and SAP-related genes, we sought to address the cellular composition of spheres by analyzing them for neuronal, glial and catecholaminergic markers.
4. Results

4.3.1 Spheres are composed mainly of nestin$^+$ cells and few neurons

Double-immunostaining with anti-nestin and anti-peripherin revealed that the majority of cells in a sphere were nestin$^+$, along with a cluster of peripherin$^+$ cells with long processes (Fig. 13). This confirmed the flow cytometry data on nestin and showed the location of peripherin$^+$ cell cluster in the spheres.

**Figure 13. The majority of sphere cells express nestin.** Co-staining of spheres (cultured overnight on poly-D-lysine/fibronectin-coated coverslips) with nestin (red) and peripherin (green). A single sphere is shown. Nuclei were counterstained with DAPI. Scale bar equals 100 µm.

4.3.2 Spheres co-express Bmi1 and Musashi1

Bmi1 is a marker for stem and progenitor cells in neural stem cells, and Musashi1 is a neural RNA-binding protein expressed in NPCs and NSCs. As the sphere cells expressed the transcripts for Bmi1 and Musashi1, the expression of Bmi1 and musashi1 was analyzed on sphere cells by immunofluorescence.
The co-expression of Bmi1 and Musashi1 in the nucleus of sphere cells indicated that this cell type was indeed related to cells with neural stem cell-like features (Fig. 14).

**Figure 14.** Many sphere cells co-express Bmi1 and Musashi1. Co-staining of spheres with anti-Bmi1 (green) and anti-Musashi1 (red). Merged images with DAPI counterstained nuclei and antigens are shown. Scale bar equals 100 µm.

### 4.3.3 The expression of GFAP is mutually exclusive of peripherin

To assess the presence of glial cells in spheres, the spheres were co-stained with anti-peripherin and anti-GFAP (glial fibrillar acidic protein). Only a few cells in a sphere were GFAP\(^+\), close to the peripherin\(^+\) cluster, the expression of GFAP and peripherin was mutually exclusive (Fig. 15). Quantification of cells positive for nestin (80.96±1.39%), peripherin (9.69±0.43%) and GFAP (5.62±0.33%) is shown as histogram in lower panel, Fig. 15.
4. Results

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Figure 15. Peripherin expression is mutually exclusive of GFAP. Co-staining of adrenal-derived spheres (cultured overnight on poly-D-lysine/fibronectin-coated coverslips) with anti-peripherin (red) and anti-GFAP (green). Nuclei were counterstained with DAPI. Scale bar equals 100 µm. Quantification (8000 cells) is shown in the histogram.

4.3.4 Peripherin co-localizes with key catecholaminergic markers

As the presence of peripherin (Class III intermediate filament) positive cells indicated the cellular identity of these cells to be lineage-restricted neurons of the peripheral sympathetic nervous system, the expression patterns of catecholaminergic neuron markers TH and DBH were analyzed. In co-staining experiments of anti-peripherin with anti-TH (Fig. 16A) or anti-peripherin with anti-DBH (Fig. 16B), it became evident that these two key catecholaminergic
markers co-localize with peripherin. As the expression of peripherin was mutually exclusive of nestin or GFAP, the co-expression of peripherin with catecholaminergic markers proved these double-positive cells were lineage-restricted neurons, containing the key catecholamine synthesis enzymes.

Figure 16. Peripherin co-localizes with catecholaminergic markers. A. Peripherin$^+$ cells co-express TH. Co-staining of spheres with anti-tyrosine hydroxylase (TH, red) and anti-peripherin (green) is shown. B. Peripherin$^+$ cells co-express DBH. Co-staining with anti-dopamine β-hydroxylase (DBH, red) and with anti-peripherin (green) is shown. Merged images with DAPI counterstained nuclei and antigens are shown. Scale bar equals 100 µm.
Thus, immunofluorescence characterization revealed the cellular composition and lineage specification of adrenal sphere cells; with a majority of cells being in stem cell state (nestin⁺) along with few lineage-restricted cells (peripherin⁺ or GFAP⁺) and few catecholaminergic neurons (expressing peripherin with TH and DBH).

**4.4 Spheres do not contain cortical and endothelial cells**

The isolation of spheres from 2 d old mouse adrenal gland was done by dissociating total adrenal gland. The adrenal gland is comprised of 2 different structures: outer cortex (mesodermal origin) and inner medulla (trunk neural crest-derived). To confirm that adrenal-derived spheres did not contain adrenocortical cells and endothelial cells, the spheres were compared with freshly dissociated 2 d old adrenal gland cells.

Expression of adrenocortical markers such as SF-1 (Steroidogenic factor 1), CYP11A1 and CYP11B2 was analyzed, along with the expression of endothelial marker CD31. SF-1 (NR5A1, nuclear receptor subfamily 5, group A, member 1) plays important role in early specification and development of adrenal cortex (Parker and Schimmer, 1997). CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) catalyzes the first step of steroid biosynthesis by converting cholesterol to pregnenolone and CYP11B2 (aldosterone synthase) is primarily expressed in zona glomerulosa of adrenal cortex where it regulates the generation of the mineralocorticoid aldosterone (Parker and Schimmer, 1997). CD31 is expressed on endothelial cells.

The expression of adrenocortical and endothelial markers was compared between mouse adrenal gland cells and 7 d old spheres. Immunohistochemistry showed that dissociated adrenal gland cells were positive for SF-1, CYP11A1, CYP11B2 and CD31 (Fig. 17, left panel); on the other hand, adrenal-derived spheres were negative for these markers (Fig. 17, right panel), thus confirming that spheres were devoid of adrenocortical and endothelial cells.
Figure 17. Adrenal-derived spheres do not contain cortical and endothelial cells. Analysis of 2 d old adrenal gland cells (cytospin, left panel) for the expression of adrenocortical markers and endothelial marker CD31. 7 d old spheres (cryocuts) stained for the same markers are shown (right panel). Nuclei were counterstained with hematoxylin. Scale bar = 100 µm.
4. Results

4.5 Few sphere cells contain dense core vesicles and electrical activity

4.5.1 Dense core vesicles are present in few sphere cells

To determine the ultra-structural composition of adrenal-derived spheres, electron microscopy was performed on sections of spheres. Ultra-structural analysis revealed the heterogeneous composition of sphere cells. A majority of cells contained large nuclei surrounded by little cytoplasm (Fig. 18, left panel), while a minority of sphere cells (9%) contained typical dense core vesicles (site of catecholamine synthesis and release) in the cytoplasm (Fig. 18, right panel, arrows).

Figure 18. A minority of sphere cells contain dense core vesicles. Electron microscopic images of adrenal-derived sphere cells, showing cells without dense core vesicles (left) and with several cytoplasmic dense core vesicles (right, arrows). Scale bar equals 2 µm in left and 1 µm in right panel.

The dense core vesicles had an eccentric core and contained irregular lucent spaces between the eccentric core and granular membrane (Fig. 18). Thus, ultrastructural analysis of adrenal-derived sphere cells indicated towards the identity of a majority of cells being in a progenitor cell-like state due to absence of dense core vesicles, and the identity of a minority of cells to be similar to chromaffin-like, as mature chromaffin cells from adrenal gland also contain dense core vesicles.
4.5.2 Few cells in the spheres are functional neurons

In excitable cells such as neurons, sodium channels are present and they are responsible for generating action potentials. Sodium channels are membrane proteins that form ion channels to conduct sodium ions through a cell’s plasma membrane. On analyzing the voltage-gated sodium channels by RT-PCR, SCN7A encoding NaV2.1 channel was most abundantly expressed. Additionally, SCN2A, SCN3A, and SCN9A encoding voltage-gated Nav1.2, Nav1.3, and Nav1.9 respectively were expressed in significant amounts (Fig. 19), suggesting that these few cell clusters in the spheres may be able to generate action potentials. RT-PCR for sodium channels in adrenal-derived spheres (Fig. 19) was performed by Dr. Karin-Jurkat Rott and her laboratory at the Division of Neurophysiology, Ulm University.

![RT-PCR Image]

Figure 19. Adrenal-derived spheres contain sodium channels. RT-PCR of adrenal-derived spheres (lane1) showing the presence of indicated voltage-gated sodium channels. Mouse tissue lysate mix (pancreas, heart, muscle, brain, liver, kidney) was used as a positive control for RT-PCR.

In adrenal-derived spheres, few cell clusters exhibiting neuronal morphology were observed. As the analyzed sphere cells expressed the transcripts of various sodium channels and functional maturation of neurons can be assessed by their ability to generate action potential through voltage-gated sodium channels; electrophysiological analysis was performed to define the electrophysiological properties of these neuronal-like cell clusters. Electrophysiological analysis of adrenal-derived sphere cells (Fig. 20) was
performed by Dr. Karin-Jurkat Rott and her laboratory at the Division of Neurophysiology, Ulm University.

The recordings were done on cells having a well-defined neuronal morphology. Compound action potential (CAP) recordings were performed using the fast potentiometric indicator Di-8-ANEPPS. Neurite containing cell clusters (5%) on the edge of attached spheres (Fig. 20A, *) exhibited responses after external electrical stimulation (Fig. 20B, *). A brief initial depolarisation was followed by a transient hyperpolarisation. On the other hand, the flat non-neuronal cells (95%), which were not a part of the neurite containing cell clusters, showed no response upon electrical stimulation (Fig. 20A, B, **). The signals were sensitive to application of Tetrodotoxin (TTX). TTX is known to block action potentials by binding to the voltage-gated sodium channels. With 100 nM TTX, signal amplitude was decreased and fully suppressed at 1 µM TTX (Fig. 20C), thus indicating the signals being generated specifically from tetrodotoxin-sensitive sodium channels. Double pulse experiments determining the refractory period proved the signals to be compound action potentials (Fig. 20D). In accordance, whole-cell patch-clamp measurements showed typical voltage-gated sodium inward currents (peak current amplitudes ranging from a few hundred pA up to 7 nA), which are required to generate the action potential (Fig. 20E).

Taken together, a minority of the sphere cells had characteristic dense core vesicles as site of catecholamine storage and release, indicating towards their similarity to adrenal chromaffin cells. The presence of voltage-gated sodium channels indicated the presence of required physiological characteristics of functional mature neurons, available in the few neurite containing cell clusters of adrenal-derived spheres. Thus, electrophysiological analysis showed functional maturation of a few cells in adrenal-derived spheres, as they could fire action potentials and could generate typical Na⁺-mediated inward currents.
4. Results

4.6 Sphere cells can be differentiated to neuronal and chromaffin cell types

One of the hallmark of progenitor cells is their capacity to differentiate, thus to demonstrate the differentiation potential of adrenal-derived sphere cells, *in vitro* differentiation of sphere cells was carried out.

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**Figure 20.** Adrenal-derived spheres contain few mature-appearing neurons exhibiting electrical activity. **A.** Plated spheres contain few neurite containing cell clusters. An image showing a neurite containing cell cluster (*) and area outside of the cluster (**). **B.** Only the neurite containing cells respond to electrical stimulation. Presence of compound action potentials (CAP) in the cell cluster (*) and lack of CAP in area outside of it (**). **C.** Tetrodotoxin specifically blocks action potential. High concentration of TTX inhibited the signal amplitude. **D.** Short-interval impulses decrease the signal amplitude of second CAP. Representative traces of double pulse stimulation, whereby the second CAP was elicited at $\Delta t$ of 100, 50, 40, 30, 20, 10, and 5 ms. **E.** Patch-clamp measurements show typical sodium inward currents. Representative voltage-activated inward currents from a cell depolarized in 5 mV steps from –70 to +35 mV for 60 ms.
4.6.1 Neuronal markers are upregulated post-differentiation

Extracellular and intracellular cues regulate fate decision of progenitor cells. To analyze the state of commitment and to confirm the SAP-like status of adrenal-derived sphere cells, an adherent differentiation assay was used to differentiate them towards neural and chromaffin cells, the two cellular subtypes derived from SAPs. Adrenal-derived spheres could not attach firmly to several coatings tested, like 1% gelatin, collagen IV, poly-L-ornithine or laminin. Spheres could attach on a combination of matrix coating consisting of poly-D-lysine and fibronectin, i.e., two extracellular matrix components sequentially added to form a layer on the surface of culture vessel. The spheres were seeded in 1% FCS containing basal media and were allowed to attach for 6 h. Further, in serum-free differentiating media, the attached spheres were allowed to differentiate for 6 d at 37°C. The differentiation capacity was evaluated by analyzing the expression of neuronal and chromaffin markers by immunocytochemical staining.

*In vitro* differentiation induced a significant increase of neurites in sphere cells as compared to the undifferentiated controls, as evident from Fig. 21. Neuronal differentiation with a combination of ATRA and ascorbic acid was assessed by using β3-Tubulin as a generic neural marker and peripherin as sympathetic neuron marker. Analysis of β3-Tubulin expression in spheres treated with a combination of retinoic acid and ascorbic acid revealed the increase of β3-Tubulin+ cells and neurites post-differentiation (right panel, Fig. 21). In undifferentiated controls, the neurites were short and few, as compared to the differentiated spheres (left panel, Fig. 21). Similarly, peripherin+ cells and neurites were increased in spheres treated with the combination of retinoic acid and ascorbic acid (right panel, Fig. 21).

Taken together, this combinatorial approach efficiently increased neuronal properties, i.e., neurite formation and increase in neuronal cells, thus indicating neuronal differentiation capacity of sphere cells.
21. Adrenal-derived spheres can be induced in vitro towards neuronal lineage. 7 d old spheres adhering to PDL/FN-coated coverslips were differentiated for 6 d in medium containing ATRA and ascorbic acid. Spheres cultured with or without specific factors and stained for β3-Tubulin and peripherin are shown. Nuclei were counterstained with DAPI. Scale bars equal 100 µm. Quantification of positive cells is shown in histograms (1000 cells of two independent experiments were analyzed). Statistical analysis was performed using the t-test. ***, p<0.001.
4.6.2 Chromaffin markers are upregulated post-differentiation

To analyze the capacity of sphere cells to differentiate into chromaffin cells, a combination of the synthetic glucocorticoid dexamethasone and PMA was used. Glucocorticoid-mediated chromaffin induction has been described for chromaffin upregulation in bovine chromospheres (Chung et al., 2009). PMA-mediated protein kinase C activation, leading to chromaffin upregulation has been described for bovine and human fetal chromaffin cells (Jozan et al., 2007; Wan et al., 1989). Chromaffin induction was assessed by analyzing TH, DBH and CHGA as specific chromaffin markers. The combination of dexamethasone and PMA significantly increased the chromaffin markers as compared to the undifferentiated controls (Fig. 22).
4. Results

Figure 22. Chromaffin markers are upregulated post-differentiation. 7 d old spheres adhering to PDL/FN-coated coverslips were differentiated for 6 d in medium containing dexamethasone and PMA. Spheres were cultured with or without specific factors and stained for TH, DBH and CHGA. Nuclei were counterstained with DAPI. Scale bars = 100 µm. Quantification of positive cells is shown in histograms (1100 cells of 2 independent experiments). Statistical analysis was performed using the t-test. *, p < 0.05; ***, p < 0.001. This data showed the capacity of chromaffin cell induction in sphere cells under the influence of specific chromaffin-specification factors.

4.6.3 Chromaffin cell induction upregulates catecholamine levels

Chromaffin cells produce catecholamines dopamine, norepinephrine and epinephrine, which are the major neuroendocrine hormones of adrenal medulla. In vitro, we observed that dexamethasone and PMA upregulated the chromaffin markers TH, DBH and CHGA. To directly assess catecholamine production, the sonicated cell lysates of spheres treated with or without dexamethasone and PMA were subjected to HPLC-ECD for total catecholamine content analysis (with the help of Dr. Max Kurlbaum, Dept. of Clinical Chemistry, Ulm University). In line with in vitro chromaffin differentiation, HPLC analysis of sonicated cell lysates of sphere cells differentiated with dexamethasone and PMA showed upregulation of catecholamine levels. The levels of norepinephrine and epinephrine were significantly upregulated after treatment with dexamethasone and PMA (Fig. 23).
4. Results

Figure 23. Chromaffin cell inducing conditions upregulate catecholamines in spheres. HPLC analysis of catecholamine content in lysates of spheres differentiated in adherence for 6 d in differentiation medium supplemented with or without dexamethasone and PMA. The means of three independent experiments are shown. Statistical analysis was performed using the t-test; n.s., not significant; *, p<0.05.

Thus, the in vitro differentiation data confirmed the differentiation capacity of adrenal-derived spheres towards neuronal and chromaffin cell types and upregulation of catecholamine content in differentiated sphere cells further confirmed their chromaffin differentiation capacity.

4.6.4 Spheres partially differentiate and survive in vivo

Differentiation of stem cells and progenitor cells in vivo is spatio-temporally regulated and is cell-type and niche specific. To assess whether adrenal-derived sphere cells have differentiation capacity in vivo, CFSE-labeled sphere cells were implanted surgically into adrenal gland of immunodeficient rat. Rat adrenal gland was chosen, being comparatively bigger to small-sized mouse adrenal glands. Post-transplantation analysis of rat adrenal gland after 3 weeks showed that the sphere cells had survived and integrated into the cortico-medullary border of the adrenal glands. At this time point, the progenitor marker nestin was partially downregulated, where few nestin-positive transplanted sphere cells could be detected only at the cortical periphery of host adrenal gland (Fig. 24, upper panel). Bmi1 was undetectable
in the transplanted cells (Fig. 24, middle and right panel). The implanted sphere cells showed no increase in the expression of markers specific for peripheral neurons (peripherin) and for catecholaminergic cells (TH), in comparison to spheres (Fig. 24). As downregulation of progenitor markers was observed, with no concomitant upregulation of committed neuronal or catecholaminergic markers, this data supports the limited partial differentiation of adrenal-derived sphere cells in vivo.

Taken together, in this in vivo setting, the sphere cells survived and preserved membrane integrity, without differentiating towards chromaffin cells.
Figure 24. Adrenal-derived spheres survive in situ where they downregulate expression of progenitor markers but do not differentiate to chromaffin cells. Pre-implantation adrenal-derived spheres attached to glass coverslips (left column) and three weeks post-implantation rat adrenal gland prior implanted with CFSE-labeled sphere cells (middle and right columns) were stained for nestin, BMI1, peripherin and TH. Stainings were visualized by immunohistochemistry. In middle and right columns, fluorescent images (CFSE, green) are overlaid on immunohistochemistry images. Nuclei were counterstained with hematoxylin. Images in right column are magnifications of images shown in middle column. Scale bars are 200 µm (left and middle columns) and 50 µm (right column).
5. DISCUSSION

The present study has addressed the isolation and characterization of neural crest-derived sympathoadrenergic progenitor-like cells from postnatal murine adrenal glands and demonstrates properties of these cells in *in vitro* and *in vivo* conditions. Controlled regulation of NC differentiation is essential for normal NC development. Developmental misregulation of NCCs, or in some cases the reversion of differentiated NCCs to their parental NC cells may result in cancers of NC origin such as neuroblastoma (Brodeur, 2003). Thus, the isolation strategies, characteristics and physiological traits of SAPs described in this study help to understand normal and aberrant development of peripheral sympathetic nervous system.

5.1 Published attempts to obtain NCSCs and SAPs from ES cells

Embryonic stem cells have been used to obtain NC-derivatives through various differentiation schemes. However, the cellular complexity of differentiated cells leads to challenges like heterogeneity and less control on the cellular fate.

To obtain NC and NC derivatives without the potentially confounding effects of experimental immortalization, NCSC-like and more committed cells have been differentiated from murine ESCs. These reports used various differentiation protocols on murine ESCs to obtain functional MAP2* neurons (yield 60%) (Okabe *et al.*, 1996), TH* cells generated with PA6 co culture (yield 75%) (Kawasaki *et al.*, 2000), TH* neurons (yield 40%) (Rolletschek *et al.*, 2001), neuroectodermal derivatives (Rathjen *et al.*, 2002; Ying *et al.*, 2003), TH*peripherin* neurons (yield 50%) (Mizuseki *et al.*, 2003), β3-Tubulin* cells (yield 45%) (Conti *et al.*, 2005), BMP-induced β3-Tubulin* cells (yield 25%) (Gossrau *et al.*, 2007), β3-Tubulin* cells (yield 4%) (Gossrau *et al.*, 2007; Motohashi *et al.*, 2007) and β3-Tubulin*peripherin* NC progenitors (Kawaguchi *et al.*, 2010). Although ESC differentiation is a convenient
approach to derive NC-derivatives, the protocols used often lead to cellular heterogeneity of differentiated cells, influence of unknown factors from underlying stromal cells and often-unmet requirements for defined media conditions. Moreover, these cells may differ from in situ cells, reflecting the differences of obtaining SAPs by in vitro differentiation of ESCs.

Efforts have been undertaken to differentiate human ESCs towards NC precursors by isolating cells expressing p75 and CD57 (Lee et al., 2007), where p75$^+$ cells also expressed markers of more differentiated NC derivatives like peripheral neurons (yield 55%) or catecholaminergic cells (yield 2% TH$^+$ neurons) (Lee et al., 2007). In another strategy, neurogenic induction by co-culturing hESCs with mouse PA6 stromal cells (Jiang et al., 2009) has allowed for p75 expression-based in vitro isolation of multipotent human NCSCs. Coculturing human induced pluripotent stem cells (iPSCs) with MS5 cells (Lee et al., 2010) or human ESCs with PA6 stromal cells (Pomp et al., 2005) generated NC-like intermediate cells with the characteristics of PNS and CNS neurons. PA6-induced human ES-derived neurospheres generated peripheral sensory neuron-like cells, however, the neurogenic potential and proliferation of PA6-induced NSP cells was reduced with extended passaging (Pomp et al., 2008). While these studies could generate PNS derivatives, isolation and analysis turn out to be complicated pertaining to lengthy differentiation protocols (1 week – 4 months) and FACS-associated cellular damage.

Taken together, these various findings provide important insight to obtain ES-derived neural crest cells using in vitro differentiation protocols, however, two central issues remain associated with ES-derived NCSCs. First, these studies report the derivation of NCSCs, not SAPs. Second, the unidentified factors from underlying stromal cells and serum lead to cellular heterogeneity of differentiated cells (Achilleos and Trainor, 2012). Thus, to eliminate these issues, further identification of new sources and well-defined strategies to derive NCSCs and SAPs are required.
5. Discussion

5.2 Published attempts to obtain NCSCs and SAPs from rodents

As SAPs remain elusive, it is crucial to obtain them in sufficient numbers, to elucidate the normal and malignant development of sympathetic PNS. Apart from the \textit{in vitro} strategies discussed in section 5.1, attempts have been undertaken to isolate NCSCs and SAP-like cells from \textit{in vivo} sources.

The transient \textit{in vivo} migratory nature of NCSCs leads to the assumption that NC-derivatives cease to exist postnatally, however, there have been reports on the existence of post migratory NCSCs from rodent PNS tissues such as sciatic nerve, gut or sympathetic ganglia (Bixby \textit{et al.}, 2002; Joseph \textit{et al.}, 2004; Kruger \textit{et al.}, 2002; Morrison \textit{et al.}, 2000a; Morrison \textit{et al.}, 2000b; Morrison \textit{et al.}, 1999; Taylor \textit{et al.}, 2007). Other reports have isolated NC-derivatives from murine trunk and cranium (Trentin \textit{et al.}, 2004) and dorsal root ganglion (Li \textit{et al.}, 2007). Post migratory NCSCs have also been derived from murine skin (Fernandes \textit{et al.}, 2004; Lang \textit{et al.}, 2005; Osawa \textit{et al.}, 2005; Toma \textit{et al.}, 2001; Wong \textit{et al.}, 2006), epidermis (Hu \textit{et al.}, 2006; Sieber-Blum \textit{et al.}, 2004), cardiac side population (Tomita \textit{et al.}, 2005) and corneal precursors (Yoshida \textit{et al.}, 2006). As isolation of NCSCs and SAPs is hampered by their small number and limited \textit{in vitro} life span, murine neural tube explant cells have been experimentally immortalized by oncogenes (Maurer \textit{et al.}, 2007; Rao and Anderson, 1997).

The above attempts to obtain NCSCs from \textit{in vivo} sources describe various approaches to obtain post-migratory NCSCs and their derivatives. Although these findings imply that multipotent neural crest-derived precursors can be generated from various tissues, these studies do not lead to access the true SAPs. These studies differ from each other either by the procedure or by outcome. Importantly, the cells reported in the above reports do not meet the hallmark property of SAPs, i.e. their bipotential capacity to differentiate towards chromaffin and neuronal cells. Thus experimental access to SAPs remains difficult, demanding exploration of new strategies, sources and strict differentiation regimes.
Recently, the efforts to identify and to isolate SAPs have led to studies reporting the derivation of SAP-like cells from human fetal adrenal and adult bovine adrenal. Fetal noradrenaline-secreting chromaffin cells isolated from human fetal adrenal gland have been reported to propagate as neurospheres (Jozan et al., 2007; Zhou et al., 2006). However, access to this source is limited for logistical and ethical reasons. In the recent years, isolation of chromaffin progenitors as ‘chromospheres’ from adult bovine adrenal glands has provided evidence that proliferation and differentiation competent nestin+ progenitor cells can be isolated from adult bovine adrenal medulla (Chung et al., 2009; Ehrhart-Bornstein et al., 2010; Vukicevic et al., 2012). These bovine chromaffin progenitors were enriched by differential plating and culturing as ‘chromospheres’ under non-adherent conditions. Using dexamethasone, or ATRA with ascorbic acid, chromosphere-derived cells could be differentiated to mature chromaffin cells and dopaminergic neurons, respectively, indicating the SAP-like differentiation capacity of these bovine chromospheres. However, the characterization of bovine chromospheres makes it unclear whether this sphere population derived from adult bovine adrenal glands represents a well-defined progenitor population. Thus, it remains undefined if bovine-derived chromospheres were distinctly homogeneous, or were a heterogeneous population composed of progenitor cells as well as more differentiated cell types.

The post-migratory NCSCs and bovine chromospheres described above do resemble neural crest-like cells and share features like multipotency and self-renewal. However, these reported cells fall short of their specification towards SAP-like cell type. Moreover, there are differences among these cell types, depending on the isolation strategy and responsiveness to different growth factors. Therefore, there is still a need to gain easy access to in vivo sources to obtain true SAPs using simple strategies, in order to define their properties and SAP-like differentiation capacity (Achilleos and Trainor, 2012).
5.3 Adrenal glands from young mice as a new *in vivo* source of SAPs

The *in vitro* and *in vivo* approaches discussed above demonstrate the isolation strategies of NCSC-like and SAP-like cells. However, these approaches do not reflect on how to procure the true SAPs, or SAP-specification. As mouse is a preeminent model for several human diseases and adrenal gland is the most common site of NB, we aimed to isolate SAP-like cells directly from the adrenal medulla of postnatal mice. To this end we used similar conditions recently described for generating chromaffin progenitors from adult bovine adrenal gland (Chung *et al.*, 2009; Ehrhart-Bornstein *et al.*, 2010; Vukicevic *et al.*, 2012). Using these selective conditions, we succeeded in selecting and expanding rare proliferating cells from adrenal glands of young mice with limited self-renewal capacity and having characteristics of bipotential SAP-like cells.

5.3.1 A rare progenitor pool exists in postnatal mouse adrenal glands

Our study shows that a rare population of potential NC-derived progenitors exists transiently, shortly after birth in mice and that these cells can be taken into *in vitro* culture to study the properties and characteristics of this rare population. In this study, sphere formation assay (Reynolds and Weiss, 1992) was used to isolate the spheres from postnatal mouse adrenal gland. Neurosphere formation is described for neural stem cells that form free-floating spheres in suspension cultures. The rationale of sphere-forming assay is that the stem cells get selective advantage to form small aggregates, while these culture conditions prevent non-stem cell types to grow. Due to the small size of mouse adrenal gland, whole adrenal gland was used, rather than the dissected medullae. Thus, to prevent adrenocortical and endothelial contamination of spheres, differential plating (Chung *et al.*, 2009) was employed, where plated cells were transferred to new dishes after short intervals. Differential plating of dissociated adrenal gland cells and their non-adherent growth as spheres in serum-free medium ensured that there was no contamination of adrenocortical or endothelial cells in the spheres. Using
these rationales of sphere formation and differential plating, we could isolate proliferating NC-derived progenitors as spheres from murine adrenal gland.

Unlike bovine chromospheres (Chung et al., 2009), murine adrenal-derived spheres could not be derived from adult mice. This might be explained by the presence of very low number of sphere-forming progenitors in the mouse adrenal or the specificity of mouse strain used. The frequency of adrenal cells able to generate spheres was very low, notwithstanding that many of the cells put into the sphere forming assays were cortical cells destined to die under the conditions of the assay. Fitting to accepted characteristics of progenitors (Crane and Trainor, 2006; Vankelecom and Gremeaux, 2009), i.e., rarity and limited self-renewal potential, this suggests that a minority of adrenal medullary cells in mice are putative NC-derived progenitors that mature rapidly after birth. The latter is in line with the rapid postnatal decrease of nestin+ proliferating cells in murine sympathetic superior cervical ganglia (Shi et al., 2008) and comparable to the description of tissue-derived stem and progenitor cells in various reports (Chen et al., 2005; Dontu et al., 2003; Fernandes et al., 2004; Sarig et al., 2006; Seaberg et al., 2004).

Murine adrenal-derived spheres were responsive to mitogens such as bFGF, EGF and LIF; the growth factors known for their regulatory effects on stem cells (Chen et al., 2009; Chen et al., 2005; Fauquier et al., 2008; Reynolds and Weiss, 1992). Adrenal-derived spheres did not express the surface markers associated with migratory NCSCs (CD57 and p75), thus confirming their post-migratory nature. The isolated spheres expressed progenitor markers similar to bovine chromospheres (Chung et al., 2009). In particular, murine adrenal-derived spheres expressed NCSC and SAP-associated factors, as well as the key catecholaminergic markers TH and DBH. This expression profile is suggestive of adrenal-derived spheres to be a progenitor cell population of neural crest origin in postnatal mouse adrenal gland. In our study, nestin was expressed in a majority of adrenal-derived sphere cells, giving ex vivo evidence of the presence of progenitor cell population in postnatal mouse adrenal gland. In spite of expression of nestin, the proliferation of adrenal-derived spheres slowed down and finally stopped in
5. Discussion

culture, a similar observation related to sphere expansion with other sphere models from adult mouse pituitary gland (Chen et al., 2005) and adult mouse pancreas (Seaberg et al., 2004). Only a minority of murine adrenal-derived sphere cells contained catecholamine storage vesicles (indicating them to be differentiated chromaffin cells) and a few neuronal clusters in the spheres showed electrophysiological response in terms of electric excitability.

Taken together, the limited proliferation, limited self-renewal and presence of a minority of mature functional cells indicates the sphere population to be composed mainly of NC-derived progenitor cells.

5.3.2 Adrenal-derived spheres contain bipotential SAPs

SAPs isolated from adrenal glands can differentiate into chromaffin cells or sympathetic neurons, depending on the specific instructive signals (Anderson and Axel, 1986; Huber, 2006). Two hypotheses exist concerning the specification of chromaffin cells and sympathetic neurons (Fig. 25).

![Diagram of neural crest cell differentiation]

Fig. 25. Two hypotheses exist concerning the diversification of chromaffin cells and sympathetic neurons. A. The classic hypothesis indicates the existence of a common bipotential SAP that can differentiate towards chromaffin cells and sympathetic neurons. B. A second hypothesis suggests the development of sympathetic neurons and chromaffin cells from independent NC-derived precursors. Adapted from (Huber, 2006).
The first hypothesis indicates that the specification of chromaffin cells and sympathetic neurons is sourced from a common progenitor (the SAP), Fig. 25A. A second hypothesis (Fig. 25B) indicates towards the presence of separate NC-derived precursors for chromaffin cells and sympathetic neurons (Huber, 2006). Regardless of the common origin or independent development of these two cell types, the differentiation of progenitor cells is known to be a complex combinatorial event (Sommer, 2001).

We asked whether adrenal gland-derived sphere cells represented true SAPs, i.e. if they contained the capacity to differentiate into neuronal and chromaffin cells. In vitro differentiation of sphere cells under neuronal-inducing conditions led to upregulation of neuronal markers. Glucocorticoid-mediated chromaffin induction significantly upregulated the chromaffin-specific markers, along with an increase in the catecholamine content. These finding revealed the SAP-like properties of murine adrenal-derived sphere cells, via expression of progenitor markers and a bipotential-specification capacity, both together indicating towards the presence of a common bipotential progenitor for chromaffin and sympathetic cells. Thus, there were similarities in the outcome of in vitro differentiation between murine adrenal-derived spheres and bovine chromospheres (Chung et al., 2009). However the differentiation of murine sphere cells towards neuronal and chromaffin cells was more efficient as compared to bovine chromosphere cells. In particular, the in vitro upregulation of peripherin, a peripheral sympathetic neuron marker, and the upregulation of chromaffin markers TH, DBH and CHGA at cellular level in the sphere cells were more efficient, as compared to bovine chromospheres.

It has to be noted that our conclusions about lineage are based on forced differentiation in vitro using medium conditions not completely defined. Furthermore, as the cell populations are heterogeneous, additional steps will be required to generate pure SAP-lineage competent progenitors. These caveats notwithstanding, the ability to generate SAP lineage-competent progenitors with moderate heterogeneity from postnatal murine adrenal gland opens new avenues to probe the functions of these progenitors and the consequences of their possible malignant transformation.
5. Discussion

Taken together, similar to bovine chromospheres (Chung et al., 2009), mouse adrenal-derived spheres, that were predominantly nestin-positive, could be efficiently differentiated to chromaffin cells and sympathetic neurons, suggesting the existence of a common bipotential sympathoadrenergic progenitor.

5.3.3 In vivo differentiation leads to partial downregulation of progenitor markers in implanted spheres

A rodent model to demonstrate lineage differentiation capacity of NC-derived cells has not been described yet; most of the in vivo migration and differentiation studies related to the NCCs have been limited to avian models.

Neural crest plasticity was first reported in quail vagal and trunk NCCs (Bronner-Fraser et al., 1980; Cohen and Konigsberg, 1975; Le Douarin, 1973) and multipotent mouse NCCs were identified using neural tube explants (Stemple and Anderson, 1992). The lineage decision of migrating NC cells in vivo is known to be regulated spatiotemporally (Crane and Trainor, 2006; Sauka-Spengler and Bronner-Fraser, 2008; Schneider et al., 1999; Seladonenfeld and Kalcheim, 1999; Shah and Anderson, 1997). The studies described above and the fact that multiple signals control NCC fate (Ruhrberg and Schwarz, 2010), indicate that NC population is already a heterogeneous mix of cells at the onset of migration (Le Douarin and Dupin, 2003). Thus, the multifactorial regulation and heterogeneity of NC-derivatives contribute to the complexity of setting up in vivo experiments for the analysis of SAP differentiation potential.

Avian NCCs have been used to demonstrate the migration and differentiation of NC cells in vivo, where quail NCCs implanted in chicken embryo trunk in vivo were able to migrate and contribute to NC-derivatives after 3 d (Bronner-Fraser et al., 1980). To analyze the differentiation potential of murine adrenal-derived SAPs in vivo, we used nude rat adrenal gland. After implantation into rat adrenal gland, the bulk of transplanted murine adrenal-derived sphere cells exhibited normal morphology and retained CFSE dye, suggestive of an
intact cytoskeleton as well as membrane integrity, therefore confirming post-engraftment survival and integration *in situ*. Upon transplantation, the progenitor markers were downregulated, while there was no concomitant upregulation of neuronal or chromaffin markers.

In our *in vivo* rat model, the lack of differentiation *in situ* might have been caused by either a suboptimal niche (due to ultrastructural incompatibility between mouse and rat tissue) or surgery-related perturbations such as wound healing. Alternatively, the differentiation capacity of adrenal sphere-derived SAP-like cells might be limited. Thus, more sophisticated approaches might be required to address *in vivo* differentiation, for example by using mouse adrenal gland as orthotopic model (notwithstanding the smaller size of mouse adrenal gland). More detailed exploration needs to be done *in vivo*, to delineate the interactions of transplanted cells with adjacent niche and to understand the mechanism of lineage choice under the influence of niche-dependent intrinsic factors.

Taking together, the *in vitro* differentiation indicated towards the common bipotential progenitor nature of adrenal-derived sphere cells while the *in vivo* implantation data showed the capacity of adrenal-derived sphere cells to integrate into the adrenal gland, without differentiating towards chromaffin cells.

### 5.4 Implications of this study on NB development

The transgenic mouse model of NB indicates SAPs to be the putative cell of origin of NB (Alam *et al.*, 2009; Hansford *et al.*, 2004; Weiss *et al.*, 1997; Weiss *et al.*, 2000), a notion supported by recent findings of cooperation between MYCN and NB-associated ALK mutant in chick SAPs (Reiff *et al.*, 2011), zebrafish (Zhu *et al.*, 2012), and mice (Berry *et al.*, 2012). *In vitro*, c-myc-immortalized NC-progenitor-like cell line JoMa1 can be transformed to NB-like cells upon substitution of its c-myc activity by MYCN (Schulte *et al.*, 2013). Murine SAPs to study NB development remained elusive. This study demonstrates isolation of SAPs directly from early postnatal mouse adrenal gland, the most common site of NB development in humans, thus facilitating
experimental access to SAPs, the putative cells of origin of NB. Notwithstanding the small number, limited self-renewal, slow proliferation and hard-to-transfect nature of sphere cells, the oncogenic transformation of adrenal-derived SAPs might have the potential to elucidate the events and cellular transformation that occur during neuroblastoma development.
6. SUMMARY

Sympathoadrenergic progenitors (SAPs) of the peripheral nervous system (PNS) are important for normal development of the sympathetic PNS and for the genesis of neuroblastoma, the most common and often lethal extracranial solid tumor in childhood. However, it remains difficult to isolate sufficient numbers of SAPs for investigations. In this study, the aim was to improve the generation of sympathoadrenergic progenitors by isolating them from postnatal murine adrenal glands. Sphere-formation assay was used along with differential plating, in order to select and enrich progenitor cells from mouse adrenal gland. Using sphere-formation assay as the culture system, mitogen-responsive spheres in serum-free suspension culture conditions were isolated and enriched from early postnatal mouse adrenal gland. The sphere formation capacity from mouse adrenal glands was age-dependent; spheres could be obtained from early postnatal mouse. The isolated sphere cells were proliferative initially and gradually lost their proliferation capacity in continuous culture. In line with the gradually declining proliferation, the sphere cells were unable to self-renew in culture, thus indicating towards their progenitor-like status. The sphere cells were devoid of adrenocortical or endothelial cells, thus confirming their medullary origin, and expressed signature markers of NC and SAP-related molecules and transcription factors.

The sphere cells did not express neural crest-related surface markers CD57 and p75, thus confirming their post-migratory progenitor-like status. Detailed analysis of sphere composition revealed the presence of a majority of nestin-positive progenitor cells, along with few peripheral neurons co-expressing catecholaminergic key enzymes tyrosine hydroxylase and dopamine beta-hydroxylase. A few sphere cells expressed glial marker and a minority of sphere cells contained dense core vesicles, a characteristic property of mature chromaffin cells. The electrophysiological analysis of neuronal-like clusters from the spheres confirmed these few clusters to be mature functional neurons, as they were able to fire action potential.
To prove the SAP-like nature of adrenal-derived cells, the adrenal-derived progenitors were differentiated with specific factors \textit{in vitro}. The sphere cells could be differentiated towards chromaffin cells by using a combination of the synthetic glucocorticoid dexamethasone and PMA, and towards neuronal cells by using a combination of retinoic acid and ascorbic acid. Thus, the \textit{in vitro} differentiation capacity towards chromaffin cells and sympathetic neurons indicated the spheres to contain bipotential SAPs, which is an important step defining the hallmark SAP-like nature of these murine-derived sphere cells. In order to elucidate if the sphere cells were capable of showing similar differentiation capacity to chromaffin and neuronal cells \textit{in vivo}, nude rat adrenal gland was used as an orthotopic transplantation model. The \textit{in vivo} differentiation regime resulted in the integration of transplanted cells \textit{in situ}, where progenitor markers were downregulated and neuronal or chromaffin markers were not upregulated. Although the sphere cells survived and integrated in the rat adrenal post-implantation, a few transplanted sphere cells continued to express progenitor marker nestin at the cortical periphery, indicating towards their partial differentiation \textit{in situ}. This lack of differentiation towards chromaffin and neuronal cells \textit{in vivo} could be attributed to the ultrastructural incompatibility and species-related differences between rat and mouse, in addition to the indirect effects that might have resulted from surgical intervention.

Taken together, the isolation and characterization of murine adrenal gland-derived SAPs facilitates direct experimental access to SAPs of the sympathetic PNS in mice, the preeminent animal model for biomedical research. These advances may further facilitate investigations about the development and malignant transformation of the sympathetic PNS.
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Statutory declaration

I hereby declare that I wrote the present dissertation with the topic:

Neural Crest-Derived Sympathoadrenergic-like Progenitors of the Postnatal Murine Adrenal Gland

independently and used no other aid than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current rules of the University of Ulm for assuring good scientific practice.

Ulm

(Shobhit Saxena)