Specific Diagnosis for Effective Treatment of Neuroblastomas and Pulmonary Neuroendocrine Tumors through Transcriptional Profiling-based Interpretation of Cellular Development

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Abstract

Induction of tumor differentiation through manipulation of regulatory genes is a relatively unexplored area of cancer therapy, however, it might suggest and additional approach for tumor-specific treatment. Therefore, in view of this idea, the present study hypothesized that neuroblastomas (NBs) and pulmonary neuroendocrine tumors (NETs), which are neoplasms of neural origin, are molecularly different based on their expression of regulatory genes and that exposure to extrinsic factors will alter their growth potential. Using reverse-transcription polymerase chain reaction (RT-PCR) analysis, the present study compared the expression of about 50 regulatory genes that are expressed at early stages of neurogenesis in five human NBs and NETs, also upon GDNF/TGF-β1 and retinoic acid (RA) treatment. According to our results, individual NBs and NETs reflected a certain stage of neural development characterized by their expression of discrete sets of regulatory genes. Upon monitoring and statistically analyzing changes in TF expression, following GDNF/TGF-β1 and RA treatment of NBs and NETs, two major adaptations were observed: The first adaptation included genes that exhibited cell-autonomous regulation and showed no change in their expression upon treatment. The second adaptation constituted genes that exhibited signal-dependent regulation and their expression was affected by GDNF/TGF-β1 and RA treatment. Upon determining the statistical significance of the number of genes expressed in each NB and NET, we observed a significant difference in the number of neurogenesis-involved regulatory genes expressed among both NBs (P < 0.0001) and NETs (P=0.02). The changes in number of genes expressed upon GDNF/TGF-β1 and RA treatment was not significantly different among NBs (P=0.13) however, significant differences were observed among NETs (P=0.01). Therefore, we could accept our first hypothesis for both NBs and NETs in that each of these tumors is molecularly unique within the diagnostic category (NB or NET), and has a characteristic profile of gene expression implicated in neurogenesis. Our second hypothesis could only be accepted for NETs, since only this group of tumors revealed a significant altered growth potential upon exposure to GDNF/TGF-β1 and RA.

Results obtained from the present study suggest a basic strategy for NB and NET diagnostics as well as therapeutics whereby a relatively small number of TFs, which are followed as identifiers of development and cell function, could be used to classify tumors based on their cellular potential for differentiation.
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Abbreviations

ART    artemin
ATCC  American type culture collection
BMP    bone morphogenetic proteins
BDNF   brain-derived neurotrophic factor
bHLH   basic helix-loop-helix
ChAT   choline acetyl transferase
CNPase 2’3’-cyclic nucleotide 3’-phosphodiesterase
CNS    central nervous system
DBH    dopamine beta-hydroxylase
DDC    dopamine-decarboxylase
DOPA   dihydroxyphenylalanine
EAT1/2 extracellular anti-TAPA-1/2
GABA   γ-amino butyric acid
GDNF   glial cell line-derived neurotrophic factor
GF     growth factors
GFAP   glial fibrillary acidic protein
GFNF   GDNF family of neurotrophic factors
GFR    GDNF receptor
Gln    glutamine
Glu    glutamate
Gly    glycine
GSH    glutathione
HD     homeo-domain
HMG    high mobility group
ICC    immunocytochemistry
IGF-1  insulin-like growth factor-1
IL     interleukin
LIF    leukemia inhibitory factor
LNGFR (p75) low-affinity nerve growth factor receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MAP-2</td>
<td>microtubule associated protein-2</td>
</tr>
<tr>
<td>MASH-1</td>
<td>mouse achaete-scute homolog-1</td>
</tr>
<tr>
<td>MDR1</td>
<td>multi-drug resistance gene-1</td>
</tr>
<tr>
<td>MEN</td>
<td>multiple endocrine neoplasia</td>
</tr>
<tr>
<td>NB</td>
<td>neuroblastoma</td>
</tr>
<tr>
<td>NC</td>
<td>neural crest</td>
</tr>
<tr>
<td>NCSC</td>
<td>neural crest stem cell</td>
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<tr>
<td>NE</td>
<td>neuroendocrine</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
</tr>
<tr>
<td>NET</td>
<td>pulmonary neuroendocrine tumor</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NGN</td>
<td>neurogenin</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron-specific enolase</td>
</tr>
<tr>
<td>NT-3/4</td>
<td>neurotrophin-3/4</td>
</tr>
<tr>
<td>NTN</td>
<td>neurturin</td>
</tr>
<tr>
<td>NTR</td>
<td>network of transcriptional regulators</td>
</tr>
<tr>
<td>O4</td>
<td>oligodendrocytic marker 4</td>
</tr>
<tr>
<td>Olf-1</td>
<td>olfactory neuron-specific transcription factor</td>
</tr>
<tr>
<td>Olf-1H</td>
<td>olfactory neuron-specific transcription factor homolog</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PSP</td>
<td>persephin</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RTKs</td>
<td>receptor tyrosine kinases</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SA</td>
<td>sympathoadrenal</td>
</tr>
<tr>
<td>SCLC</td>
<td>small cell lung cancer</td>
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<tr>
<td>SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-beta1</td>
</tr>
<tr>
<td>TβR</td>
<td>TGF-β receptor</td>
</tr>
<tr>
<td>TFs</td>
<td>transcription factors</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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<tr>
<td>ZF</td>
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Chapter 1: Introduction

Rationale

Neuroblastomas (NBs) and pulmonary neuroendocrine tumors (NETs) are neoplasms of neural origin [Langley and Grant, 1999; Anbazhagan et al., 1999]. NBs are one of the most lethal solid cancers of childhood while NETs continue to be a difficult nosologic and diagnostic problem [Hoehner et al., 1996; Cerilli et al., 2001]. Currently, about half of these cancers are not curable using traditional treatments of surgery, radiotherapy or chemotherapy. Therefore it is clinically important to find alternative therapies that are more specific, less toxic and targeted towards individual types of cancer. Since different regulatory networks are altered in diverse cancers, each individual type of tumor has a unique molecular signature that will distinguish one cancer type from another.

Lately, gene therapy has emerged as a potential alternative to standard treatments, but unfortunately the clinical benefits are only marginal due to low gene transfer efficiency in vivo. Differentiation therapy, a relatively unexplored area of gene therapy, suggests significant efficacy in the treatment of advanced or aggressive malignancies [Scott, 1997]. To appreciate the basis of differentiation therapy, one needs to understand the origin of the cancer cells, which evolved through a process of carcinogenesis. Tumor cells are essentially normal cells that through a series of environmental and/or genetic alterations have regressed to a more immature or less differentiated state. Due to this transformation, these cells have lost the ability to control their own growth – a control mechanism that mature cells possess. The application of differentiation therapy seeks to reverse the loss of the differentiated state and forces the tumor cell to resume a more mature phenotype. Therefore, in order to differentiate tumors into less aggressive/more mature forms, it is important to identify different molecular signatures/regulatory genes for each individual neoplasm that can be monitored for changes upon exposure to treatment with various agents/extrinsic factors.

Objectives

For the reasons outlined above, the primary incentive for the current study was to show that different types of NBs and NETs have characteristic expression profiles of
regulatory genes – molecular signature. Moreover, we also investigated whether and how these profiles change upon treatment with certain extrinsic factors/agents. Although the induction of tumor differentiation through manipulation of regulatory genes is a relatively unexplored area of cancer therapy, it might suggest an additional approach for tumor-specific treatment [Tallman et al., 1997]. Therefore, in view of this idea, we have investigated the following hypotheses:

- Individual NBs and NETs are molecularly different based on their expression of regulatory genes.
- Exposure of NBs and NETs to extrinsic factors such as GDNF/TGF-β and retinoic acid (RA) will alter their growth potential.

The following paragraph will emphasize our motivation for selecting the protocols that we applied in order to test our hypotheses. The present study took advantage of the human NB and NET cell lines, one of which has previously been used successfully as research model by a colleague in our laboratory [Palm et al., 1999]. Our motivation for choosing this model was prompted by the fact that it is a widely used research model especially for early stages of the biomedical research and developmental processes. Therefore data obtained through this model are beneficial as a preliminary indicator of a possible new approach to cancer diagnostics and can be applied to guide follow-up studies or other researchers in more effective therapeutics of NBs and NETs. We are also aware of potential shortcomings of the continuous cell line model, especially when compared to primary cultures. Two of the major concerns when cell lines are cultured for extended periods of time are genetic instability and phenotypic drift, which might lead to discrepancies when comparing results from different laboratories using the same cell line. Yet, we used this model since the main goal of our study was only to find a correlation between expression patterns of regulatory genes and a treatment response of NBs and NETs – making the use of primary cultures unnecessary.

In order to determine the gene expression profiles of these NBs and NETs, we analyzed the mRNA expression of several regulatory genes. Since one of the aims of our study was to propose a way to improve the diagnostics of NBs and NETs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary
for therapeutics, however, not critical for our purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart and Winzeler, 2000]. However, to identify whether the pathways that connect transcriptional regulators to neural differentiation, are active/functional in these NBs and NETs, we did determine the protein level for signaling pathways using immunocytochemical techniques. Protein determination in this case, seemed like the most favorable approach due to the availability of very good antibodies against receptors and members of these signaling pathways.

**Background**

**1.1 Molecular basis of tumorigenesis**

Significant progress has been made during the past two decades in identifying genetic changes in human neoplasia and how they contribute to the neoplastic state [Leon et al., 1994; Weinberg, 1996; Lander and Weinberg, 2000]. It is known that cells in a tumor originate from a common ancestral cell that at some point initiated an inappropriate reproduction program (non-lethal DNA mutation). The malignant transformation of the cell occurs through the accumulation of mutations in specific classes of the genes within the cell [Trichopoulos et al., 1996]. Current evidence suggests that around five to seven successive mutations are required before cancer develops; this is known as the multi-step theory of carcinogenesis [Caldas, 1998]. Three classes of genes are primary targets of DNA damage that contributes to tumorigenesis: proto-oncogenes, anti-oncogenes (tumor suppressor genes) and apoptosis regulating genes. Their protein products participate in the orchestration of the cell cycle: Proto-oncogenes code for proteins that promote cell growth and differentiation as well as controlling DNA synthesis and cellular metabolism. The products of tumor suppressor genes (e.g. growth inhibitors, nuclear transcription regulators) control cell proliferation, while apoptosis genes (e.g. bcl-2, bcl-x, p53) regulate cell survival and trigger apoptosis when DNA damage is irreparable. A wide set of mutations transforms proto-oncogenes into carcinogenic-oncogenes, causing excessive cell proliferation, while inactivation of tumor
suppressor genes deprives the cells of needed proliferation inhibiting factors. Simultaneous malignant transformation of two different cells is considered unlikely, implying that in most neoplasms an initial mutation arises in a single cell and that additional mutations accumulate during successive cell divisions of the cell progeny. Although these genetic changes are similar, the tumor often contains a mixture of different cells. This is true for embryonic neural tumors (NBs, NETs, etc.), mixed gliomas (oligodendroglioma/astrocytoma, ependymoastrocytoma, oligoependymoma) [Lantos et al., 1997] and carcinoid-adenocarcinoma (e.g. tumors that show a carcinoid pattern in some areas and an adenocarcinomatous pattern in others [WHO, 1980]. The presence of mixed cell types is a strong indication that these tumors might develop due to mutations that arise in mitotically active cells that are pluripotent (stem/progenitor cells), or from developmentally restricted cells that have acquired pluripotency through cumulative genetic alterations which have occurred during tumor progression.

1.2 Mechanism of gene regulation

In the late 1950's a study by Monod and Jacob proposed that cascades of differential gene expression are responsible for cell growth, differentiation and development [Monod and Jacob, 1961].

A major mechanism for regulating the expression of genes is at the level of transcription. Transcription of eukaryotic protein encoding genes is regulated by several protein factors: RNA polymerase II, general transcription factors (such as TFIIB, TFIID, TFIIE, TFIIF, TFIIF, TFIJ) and gene specific transcriptional regulators (activators and repressors) (Fig 1.1) [Reinberg et al., 1998; Drapkin et al., 1993; Weinmann, 1992]. These protein factors together form a network of transcriptional regulators (NTRs) characteristic to each individual cell phase, stage and type. Therefore, we can define a NTR as the molecular mechanism within the cell nucleus that responds by activating a set of genes that usher the cell through its growth cycle, as well as responding to external and internal changes. Each cell stage/phase/type is characterized by a single NTR, and therefore development along a cell lineage can be defined as a succession of different and typical NTRs. This leads to the conclusion that mixed developmental programs (fault NTRs), for example, muscle-nerve or epithelial-bone chimeras, are
prevented in normal development. It has been suggested that in developmental pathologies (including tumors), fault NTRs are prevalent and form the basis for disease [Cillo, 1994; MacLeod, 1996; Shah, 1995; Matise and Joyner, 1999].

Figure 1.1 In eukaryotes, genes encoding proteins are transcribed by RNA Polymerase II, whose activity is regulated by a core promoter upstream of the gene, and enhancer regions which may be very distant and either upstream or downstream of the gene. The core promoter determines the start of transcription and assembles a Pre-Initiation Complex containing the polymerase and a set of General Transcription Factors (from Department of Biochemistry and Molecular Biology Website, University College London, UK).
The following section provides information about the developmental, clinical and molecular aspects of the tumors analyzed in the present study:

1.3 Neuroblastomas

1.3.1 Development

The World Health Organization (WHO) classifies NBs as a group of embryonic tumors of the nervous system that are derived from 1) central neuroepithelium, 2) olfactory epithelium or 3) neural crest (NC) cells. Central NBs are tumors of the brain and spinal cord and peripheral NBs are tumors of cranial and spinal nerves as well as nerve roots. In contrast to the prevalence of peripheral neuroblastomas, their CNS counterparts are rare and account for less than 1% of primary tumors [Lantos et al., 1997]. Furthermore, peripheral NBs exhibit a much wider spectrum of cellular/phenotypic differentiation than the NBs of the CNS, referring to the diverse progeny of NC as a target for neoplastic transformation. During normal development, NC cells are a transient population of multipotent precursor cells named for their origin at the crest of the closing neural folds in vertebrate embryos [LaBonner and Bronner-Fraser, 1999]. Following neural tube closure, cells forming the NC become migratory while proliferating and/or differentiating in a highly controlled fashion. They respond to local signals, populate diverse regions throughout the embryo and give rise to neurons and glial cells of the peripheral nervous system (PNS), pigment cells, smooth muscle, cranio-facial cartilage and bone.

1.3.2 Clinical aspects

NBs are the most common extra cranial solid tumor of childhood, with two thirds of the cases presenting in children younger than five years of age [Hoehner et al., 1996; Miller et al., 1995]. These tumors arise within the sympathetic nervous system and can present as pelvic, mediastinal, abdominal, or neck neoplasms [Hayes et al., 1989]. Approximately 70% of all patients with neuroblastoma (NB) have disseminated disease at diagnosis. The prognosis for patients with NB is related to their age at diagnosis. NBs exhibit extremely malignant behavior in older children (>1 year of age) and adolescents, as they have a significantly decreased chance for cure despite intensive therapy [McCowage et al., 1995; Matthay et al., 1994; Philip et al., 1991]. In the last 30 years, only a nominal improvement has occurred in the treatment of older children with
metastatic disease at diagnosis [Shah and Ravindranath, 1998]. However, additional prognostic variables include age at diagnosis, gene status of the N-myc oncogene, histopathology, tumor DNA ploidy, 1p36 deletion and several serum markers [Seeger et al., 1985; Christiansen and Lampert, 1988; Hann et al., 1985]. According to numerous clinical studies, patients with hyperdiploid tumor DNA had a better prognosis than those with a diploid DNA content [Christiansen and Lampert, 1988; Look et al., 1984]. N-myc amplification, independent of disease stage or age at diagnosis, is associated with a poor prognosis [Oppedal et al., 1989; Christiansen and Lampert, 1988]. Expression of trkA proto-oncogene, low affinity nerve growth factor receptor (LNGFR), and HA-ras p21 is associated with a positive outcome and is inversely related to amplification of the N-myc oncogene [Tanaka et al., 1998; Suzuki et al., 1993]. An increased ratio of the excreted catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), lack of expression of glycoprotein CD44, elevated serum ferritin (> 142 ng/ml), serum neuron-specific enolase (> 100ng/ml), and serum lactate dehydrogenase, are all associated with a poor outcome [Liu et al., 2000; Comito et al., 1997; Hann et al., 1985; Zeltzer et al., 1986; Berthold et al., 1992]. High levels of p-glycoprotein (a product of MDR1 – multi drug resistance gene 1), has been suggested to correspond directly with a poorer outcome following chemotherapy of NBs [Nooter and Stoter, 1996].

1.3.3 Molecular pathogenesis

NBs are highly proliferative tumors, composed of primitive lineages and possessing a number of neurotransmitter and neuropeptide metabolic pathways [Lantos et al., 1997]. In addition to a spectrum of neuronal and ganglionic differentiation, the NBs of the PNS may express differentiation markers typical of Schwann cells. NBs are identifiable by the expression of both neuroblastic and neuro-secretory/neuro-endocrine proteins, including synaptophysin, MAP-2, neurofilaments (NF-H/M), βIII-tubulin, chromogranin A and neuron specific-enolase (NSE). On the other hand, it has been reported that glial-fibrillary associated protein (GFAP) immunoreactivity is rarely detected in NBs [Lantos et al., 1997].

NBs exhibit diverse molecular features that in part reflect their heterogeneous cellular origin. Majority of NBs may develop as a result of impaired differentiation of migratory
neural crest stem and progenitor cells which are arrested in their development along the sympathoadrenal (SA) lineages [Langley and Grant, 1999]. Neurons constituting the SA cell lineage acquire catecholaminergic fate following migration to the dorsal aorta while they are exposed to the signals of the bone morphogenetic (BMP) family of proteins [Reissmann et al., 1996]. Immaturity of catecholamine metabolism correlates to the poor prognosis of NB [Nakagawara et al., 1988] as blocked induction of dopamine decarboxylase (DDC) and high accumulation and/or secretion of DOPA associate with the unfavorable outcome of NB tumors [Ikeda et al., 1995; Ikeda et al., 1996]. These findings accentuate the fact that during development, the induction of the synthesis of DDC is more delayed than that of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) and that tumors with impaired catecholaminergic metabolism (lack of DDC synthesis) are arrested at early stages of development and thus possess highly aggressive capacity to proliferate and phenotypically are multipotent.

In the past few years, a great deal has been learned about the mechanisms underlying NC induction during normal development. In particular, a number of growth and transcription factors have been identified that are implicated in the development of NC. Recent studies have demonstrated that similar mechanisms are still operational in NBs – cells where developmental pathways have gone awry. For example, mouseachaete-scute homolog-1 (MASH-1), a basic helix-loop-helix (bHLH) transcription factor (TF), is essential for proper development of olfactory and most peripheral autonomic neurons, and for the formation of distinct neuronal circuits within the central nervous system [Anderson et al., 1997; Kageyama and Nakanishi, 1997]. Recently it was found that the hASH-1, the human homolog of MASH-1, is expressed in many human NBs and that downregulation of hASH-1, upon induced differentiation of these cells, results in concomitant induction of neurite outgrowth and expression of neural marker genes GAP-43 and neuropeptide Y (NPY) [Soderholm et al., 1998]. Another bHLH TF, dHAND, a marker gene for the developing human sympathetic nervous system, has recently shown to be expressed in both high- and low-stage neuroblastomas [Gestblom et al., 1999]. Olfactory neuron-specific transcription factor (Olf-1) is a bHLH TF that is proposed to play a role in neuronal differentiation of CNS and has been reported to exhibit DNA binding and transactivation activity in mouse NBs [Devireddy and Jones,
2000]. Its downstream genes encode a variety of regulatory proteins, including transcription factors and proteins involved in retinoid signaling, as well as adhesion/guidance molecules [Garel et al., 1999]. Homeo-domain (HD) transcription factor Arix/Phox2a contributes to the catecholamine synthesis activity in human NBs by regulating the DBH gene transcription in SHSY-5Y NB cells [Swanson et al., 2000]. Neuron-specific splicing of a neural repressor, zinc finger TF REST/NRSF/XBR, has been identified as predominant in certain NBs [Palm et al., 1999]. Similar findings in small cell lung cancer (SCLC) have suggested that the neuronal REST/NRSF/XBR isoform could potentially derepress a number of neuroendocrine genes, contributing to the neuroendocrine pathology [Coulson et al., 2000].

1.4 Pulmonary neuroendocrine tumors

1.4.1 Development

Tumors of the NE system represent a heterogeneous group of carcinomas including carcinoids. Carcinoid tumors were first identified as a specific, distinct type of growth in the mid 1800's, and the name “Karzinoide” (Carcinoid) was coined in 1907 by Oberndorfer in an attempt to designate these tumors as midway between carcinomas (cancers) and adenomas (benign tumors). Approximately 75% of pulmonary carcinoids are located centrally (lobar bronchi) and the remainder peripherally (segmental bronchi). The etiology of pulmonary carcinoid tumors is not completely known. However, some studies claim they are from endodermal origin, arising from stem cells of the bronchial epithelium known as Kulchinsky cells. These Kulchinsky cells, of the bronchi, are considered derived, possibly, from the neural crest [Cutz, 1982]. Data from a recent study support a concept that pulmonary carcinoid tumors are derived from these Kulchinsky cells [Colby et al., 1994] and thus ultimately from the neural crest [Anbazhagan et al., 1999].

1.4.2 Clinical aspects

In general, carcinoid tumors are rare but they are the most common gastrointestinal NE tumors [Godwin, 1975; Martensson et al., 1983; Kulke and Mayer, 1999]. The occurrence of carcinoid tumors is of clinical relevance and they are also widely
distributed. According to the location of origin they can be localized as follows: foregut: 10% in bronchial system of the lungs, 2-4% stomach; midgut: 30% to 50% appendix, 15% to 35% small intestine; hindgut: 15% rectum, 5-7% colon [Moesta and Schlag, 1990; Olney et al., 1985; Roggo et al., 1993].

Lung cancer is the leading cause of cancer death among both men and women [Silverberg et al., 1990; Hammar, 1994]. The incidence is 1 out of 1,000 people. Current clinical diagnostics divide broncho-pulmonary tumors into small cell (SCLC), non-small cell lung cancers (NSCLC) and carcinoid tumors. The latter include typical and atypical carcinoids according to their histological and clinical features, where atypical carcinoids constitute approximately 10-25% of carcinoid tumors [Gould et al., 1998]. Typical carcinoid tumor of the lung represents the most well differentiated and least biologically aggressive type of pulmonary neuroendocrine tumors. These tumors grow slowly and tend to metastasize infrequently. Atypical carcinoids have a more aggressive histologic and clinical picture. They tend to have less predictable biologic behavior and are associated with a worse prognosis than typical carcinoids, since they show a tendency towards increased proliferative activity and are more likely to spread to other organs [Laitinen et al., 2000].

1.4.3 Molecular pathogenesis

NE tumors arise from organs known to secrete NE messengers, e.g. hormones or substances with paracrine activity, and/or to contain cells displaying neuroendocrine characteristics or activity [Solcia et al., 1984].

There are several markers characteristic of NE cells: 1) presence of voltage-depant Na+ and/or Ca+ channels in the cell membrane, 2) receptors for specific ligands (e.g. nerve-growth factor), 3) specific cytoskeletal proteins (e.g. intermediate filaments, neurofilaments), 4) NSE, 5) granule matrix constituents (e.g. chromogranins and Leu-7), 6) granule membrane constituents (e.g. cytochrome B561, synaptophysin, 7) specific amine biosynthetic enzymes [Heitz, 1986].

βIII tubulin, a known marker for neuronal cells, has also been shown to be a marker for NETs with wider distribution in poorly differentiated than well-differentiated tumors [Katsetos et al., 2000]. Moreover, human NETs, including carcinoids, have been
identified to share common characteristics with neuronal cells. Developmental studies have contributed evidence that similar transcriptional networks, including active and repressive bHLH factors, function in the differentiation of both NETs and neuronal cells [Ito, 1999]. A recent study of high-density cDNA array profiling of pulmonary carcinoids suggested that they are related to neural crest-derived brain tumors and that they show a similarity to oligodendroglioma and high-grade astrocytoma [Anbazhagan et al., 1999].

In general, the molecular basis of tumor formation is the disruption of signals that block proliferation and initiate differentiation. A variety of cytokines, growth factors and low molecular weight substances, that affect proliferation and differentiation of normal tissues, are considered targets for cancer therapy. RA derivatives, for example, are utilized in clinical trials as chemotherapeutic agents in the treatment of certain leukemias [Sanz et al., 2000], breast, cervical, lung, pancreas and other types of cancer [Decensi and Costa, 2000]. However, the clinical outcome of these trials is often poor, reflecting the lack of an appropriate classification of tumors. Currently, applied clinical diagnostic criteria do not consider molecular and developmental mechanisms underlying tumor formation. Understanding these mechanisms is essential to predict the response of individual tumors to potential treatments.

Given that cancers arise from proliferating cells, there are two major hypotheses of tumor origin: 1) tumors develop from stem or progenitor cells due to maturation arrest or 2) they originate from dedifferentiated mature cells that retain the ability to proliferate [Sell, 1993]. However, testing of these hypotheses has been limited because of the lack of phenotypic markers that identify specific populations in the ontogeny of a stem cell. Recent discovery of CNS stem cells in the adult brain [Pagano et al., 2000] have warranted the assumption that these cells and also their dividing progeny (various types of progenitors) could be the source of tumors as well [Shoshan et al., 1999]. Using specific cell surface markers (NG2 chondroitin sulfate proteoglycan and platelet derived growth factor (PDGF) receptor) it has been shown recently that oligodendroma, once assumed to originate from a mature oligodendrocyte, arises from the O-2A progenitor cells [Shoshan et al., 1999]. Similarly, NBs are suggested to develop from neural crest stem cells (NCSCs) [Israel, 1993].
Successful application of the stem cell based concept to cancer diagnostics and therapeutics, largely depends on the understanding of the mechanism that regulate the proliferation, differentiation, survival and migration of stem cell progeny during normal development. The analysis of gene expression is currently actively pursued using DNA microarray technology that allows whole-genome scale analysis of gene expression [Ross et al., 2000; Perou et al., 1999; Iyer et al., 1999]. However, application of these full-genome screening results requires development of the concept of regulatory mechanisms that establish and maintain the “identity” of a cell during development as well as in pathology.

Growth/neurotrophic factors, cytokines bind to cell surface receptors with the primary result of activating transcriptional regulation of nuclear target genes for the control of cellular proliferation and/or differentiation. Therefore growth factors and retinoids represent good therapeutic targets for neoplasia and disease. The following section will focus on activation of three of the major signaling pathways [receptor tyrosine kinase (RTK), TGF-β, and nuclear hormone receptor (NHR)] by the extrinsic factors utilized in this study (GDNF, TGF-β1 and RA) and its effect on transcriptional activation:

1.5 Transcriptional activation through receptor tyrosine kinase, transforming growth factor-beta, and nuclear hormone receptor signaling

The regulation of gene expression is a fundamental process within every cell that often allows for exquisite control over a gene’s activity [Emerson, 2002]. The identification of transcription factors as the key regulatory molecules in eukaryotic gene expression, have revealed that these proteins are potential targets for therapeutic interventions. It is known that extracellular signals regulate gene expression by triggering signal transduction cascades that result in the modulation of transcription factor activity. Several signaling pathways – Wnt [an amalgam of wingless (Wg) and int], TGF-β, Hedgehog (Hh), receptor tyrosine kinase (RTK), nuclear receptor, Jak/STAT (Janus kinases/Signal Transducers and Activators of Transcription) and Notch – are responsible for controlling a great majority of patterning and cell fate specification.
events during bilaterian development [Gerhart, 1999]. These pathways differ remarkably from each other as seen in their varied functions, for example, direct transcriptional regulation by the nuclear receptor proteins and extended protein phosphorylation cascades characteristic of RTK pathways. Despite their diverse functions, one thing that remains the same in all these pathways is the primary result of signaling: namely activation of specific target genes by signal-regulated transcription factors. Recent studies in this field revealed remarkable commonalities in the transcriptional mechanisms by which these signaling pathways control the expression of their target genes. Therefore, a central issue in mammalian biology is the elucidation of the precise molecular mechanisms that integrate signaling and transcriptional mechanisms to generate distinct cell types in complex organs.

Transcriptional control of each pathway is a function of signal-regulated transcription factors that bind to specific signaling pathway response elements (SPREs) in promoters and enhancers of target genes. Some of these pathways use transcriptional switch mechanisms for activation of target genes in the presence of signaling or repression in its absence. However, most of the major signaling pathways, act by switching their target genes from an “off” (transcriptional repression) to an “on” state (transcriptional activation), frequently using the same signal-regulated transcription factor and SPREs to do so. Nevertheless, the “switch mechanism” has not been shown directly in vivo. On the other hand, results from a recent study postulated that regulatory networks of competing transcription factors, prevalent in cells and organisms, are crucial for establishing true molecular on/off switches [Rossi et al., 2000].

Ligand binding to the pathway’s receptor is one of the functions of all major cell-signaling pathways for transcription activation of specific target genes. Upon termination of the signaling process, the receptors are inactivated and it is the breakdown of the deactivation mechanisms that often leads to cancer [Oved and Yarden, 2002].

1.5.1 Receptor tyrosine kinase pathway: Mechanism of GDNF signaling
Tyrosine kinase receptors (e.g. RET, Trk) are transmembrane proteins and are activated by ligand-induced (e.g. binding of neurotrophic factor) dimerization (Fig 1.2).
Dimerization stimulates tyrosine kinase activity leading to autophosphorylation of the receptor at its tyrosine domains causing a conformational change in the receptor. These phosphorylated tyrosine domains then serve as docking sites for the non-catalytic domains, called Src-homology 2 and 3 (SH2 and SH3). It contains target molecules that recognize the phosphorylated receptor and therefore activates a signal transduction mechanism in the cytoplasm via a G-protein (e.g. Ras) [Gilbert, 2000]. The function of G-proteins is to relay signals from the RTKs to the nucleus in order to stimulate cell proliferation and differentiation. G-proteins, like Ras, are found in an inactive state in the cytoplasm. Stimulation of the adapter (e.g. Grb2) and docking (e.g. Shc) proteins recruits the guanine nucleotide exchange factor (GEF), which activates the G-protein (e.g. Ras) by stimulating it to give up its guanine-diphosphate (GDP) (G-proteins cycle between two conformational states – active state when guanine triphosphate (GTP) is
bound and inactive state when GDP is bound) [Gilbert, 2000]. The activated G-protein induces a sequence of intracellular kinase phosphorylations. This leads to the phosphorylation of a protein called mitogen-activated protein (MAP) kinase/extracellular-signal-regulated kinase (ERK), which enters the nucleus and activates specific transcription factors that will be responsible for gene regulation and ultimately protein expression [Worby et al., 1996]. If the G-protein remains in an active state (GTP bound), it may lead to oncogenesis, so the GTPase activating protein (GAP) hydrolysates the G-protein back to an inactive state (GDP bound).

1.5.2 Transforming growth factor-beta pathway: Mechanism of signaling

The signaling mechanism of TGF-β consists of two receptor serine/threonine protein kinases, receptor type I (TβR-I) and type II (TβR-II) (Fig 1.3) [ten Dijke et al., 1994; Lin et al., 1992]. TβR-II is able to bind ligands in the absence of TβR-I, but cannot signal without the latter. TβR-I, on the other hand, does not bind ligands in the absence of TβR-II. When no ligands are present, TβR-II and TβR-I are homodimers on the cell surface, but after ligand binding (e.g. TGF-β), TβR-I and TβR-II form a hetero-tetrameric complex, consisting of two molecules of each of the receptor proteins [Yamashita et al., 1994].

TβR-II and TβR-I have similar molecular structures, consisting of short extracellular domains with cysteine-rich regions and intracellular domains with serine/threonine kinase regions. TβR-I, but not TβR-II, contains a glycine-and serine-rich region (GS domain) which is conserved in all type I receptors for members of the TGF-β superfamily [ten Dijke et al., 1994].
The most important feature of these receptors is their kinase activities that are essential for the signaling of these receptors, however the C-terminal tail of T\(\beta\)R-II is not required for signal transduction [Wrana et al., 1992; Wrana et al., 1994]. The serine/threonine kinase of T\(\beta\)R-II is constitutively active therefore, after ligand binding and heteromeric receptor complex formation T\(\beta\)R-II phosphorylates the GS domain as well as other regions on T\(\beta\)R-I, resulting in T\(\beta\)R-I kinase activation [Wrana et al., 1994; Souchelnytskyi et al., 1996]. The latter will then be responsible for the transmission of intracellular signaling by phosphorylating Smad proteins (Smad2 and Smad3) - due to simplification of nomenclature, the designation Smad has been suggested for vertebrate homologues of Sma and Mad. Sma and Mad are genes that have been identified as components in signal transduction pathways downstream of serine/threonine kinase receptors [Massaque et al., 1997]. Smad3 will then oligomerize with Smad4 [Lagna et al., 1996; Zhang et al., 1997], enters the nucleus and act as components of transcription factor complexes [Liu et al., 1997]. To date, the Smads are the only direct substrates of...
type I receptor kinases and play a central role in the transduction of receptor signals for
gene targeting in the nucleus [Massague and Chen, 2000]. After performing their
nuclear functions, Smads exit the nucleus and are degraded in the cytoplasm and thus end their life cycle. The activated gene responses, induced by Smads, underlie many
developmental and proliferative events in vertebrates, however when disrupted due to
mutations in Smad2 or Smad4, cancer may occur [Takaku et al., 1998].

According to previous studies, Smads have been identified as members of a
signaling pathway functioning downstream of the serine/threonine kinase receptors. At
present, three classes of Smads have been identified: a) receptor-regulated Smads (R-
Smads)/BMP targets - Smad1, Smad5, Smad8, TGF-β/activin targets - Smad2, Smad3,
b) common mediator Smads (Co-Smads), Smad4 and c) inhibitory Smads (I-Smads),
Smad6 and Smad7 (Fig 1.3) [Heldin et al., 1997; Massague, 1998; Attisano and Wrana,
1998].

1.5.3 Nuclear hormone receptor pathway: Mechanism of retinoid signaling
Members of the NHR superfamily of sequence-specific transcription factors stimulate or
repress the transcription of target genes in a ligand-dependant manner. The ligands for
NHRs are usually small lipophilic molecules such as retinoids (e.g. RA), steroids,
thyroxin and Vitamin D₃ [Mangelsdorf and Evans, 1995]. These receptors contain
distinct functional domains, which include a central DNA binding domain (DBD)
responsible for targeting the receptor to highly specific DNA sequences called hormone
response elements (HREs) (Fig 1.4) [Bourguet et al., 2000]. The ligand-binding domain
(LBD) is contained in the C-terminal part of the receptor and recognizes specific hormonal and non-hormonal ligands directing specificity to the biologic response. The
COOH-terminus of the LBD contains an AF2-motif that binds to accessory proteins, co-
activators, which in turn are believed to mediate contacts with the basal transcriptional
machinery and thereby trigger transcriptional activation [Wurtz et al., 1996].
Figure 1.4  The nuclear hormone-signaling pathway. Nuclear hormones regulate the expression of complex gene programs by binding to members of the nuclear receptor superfamily of ligand-activated transcription factors. The basic concept of the nuclear receptor signaling pathway involves production of the signal, its transport to peripheral organs and activation of the receptor by ligand binding (from Carsten Carlberg, Ph.D.).

Genetic studies have shown that transcription coregulators with no specific DNA-binding activity are essential components of transcriptional regulation, leading to the identification of a series of nuclear receptor-interacting coregulatory proteins. These include SRC1, TIF2/GRIP1, AIB1/ACTR/pCIP, the bridging protein factor CBP/p300, TIF1, SRA, ARA70, RIP140 and many others [Kamei et al., 1996; Hong et al., 1997; Anzick et al., 1997; Chakravarti et al., 1996; Glass and Rosenfeld, 2000; Robyr et al., 2000]. These proteins exhibit three characteristic features: firstly, they bind to target transcription factors in a ligand-dependant manner; secondly, many of them can directly interact with the basal transcriptional machinery and thirdly, some of them exhibit enzymatic function intrinsically linked to gene regulation, such as nucleosome-modifying histone acetyl transferase (HAT) or deacetylase (HDAC) activities. Additionally, they often harbor transferable transactivation or repression domains. Consequently, these coregulatory proteins seem to function by either remodeling of the chromatin structures and/or acting as adapter molecules between nuclear receptors and the components of the basal transcriptional apparatus.
Transcriptional repression mediated by unliganded nuclear receptors, has been ascribed to the binding of corepressors N-COR or SMRT [Chen and Evans, 1995; Horlein et al., 1995]. Both of these corepressors, N-COR and SMRT, recruit mSin3a, c-Ski and histone deacetylases, therefore forming multisubunit repressor complexes [Nagy et al., 1997; Nomura et al., 1999].

Therefore, to summarize nuclear hormone receptor signaling: Upon entering the cell, a nuclear/steroid hormone will bind to its complementary receptor, initiating a complex cascade of events. The hormone-receptor complex forms dimers that bind to the HRE. This binding will activate or in some cases inhibit transcription of the appropriate gene. The resulting mRNA from gene activation can then be translated into the respective protein to produce a physiological response.

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic molecule for a variety of peripheral and CNS neuron populations, whereas transforming growth factor-beta (TGF-β), according to previous studies, is essential for permitting neurotrophic actions of GDNF in vitro [Kriegelstein et al., 1998] and in vivo [Schober et al., 1999]. The decision to include these factors for treatment of NB and NET cells in the present study, were decided due to previous (non-published) research in our laboratory investigating the effects of different growth-, neurotrophic factors and cytokines on proliferation and differentiation of these specific tumors. According to these results, the GDNF/ TGF-β1 combination was favorable for the present study. The following section will focus on the extrinsic factors utilized in this study for activation of the pathways discussed in section 1.5:

1.6 Neurotrophic/growth factors and retinoids

1.6.1 Glial cell line-derived neurotrophic factor (GDNF)

Glial cell line-derived neurotrophic factor (GDNF) is a potent factor for the development of distinct neuronal populations [Airaksinen et al., 1999], for example, spinal cord motorneurons [Oppenheim et al., 1995], central and peripheral neurons [Tomac et al., 1995], sensory and autonomic neurons [Buj-Bello et al., 1995], cerebellar Purkinje cells [Mount et al., 1995], and the kidneys. According to the latter, GDNF was thought to only play a role in nephrogenesis, however recently Seguier-Lipszye et al. observed that GDNF is expressed in nephroblastoma (Wilms tumor – most frequent solid tumors of
childhood) of human kidneys [Moore et al., 1996; Durbec et al., 1996]. The results reveal that tissue strongly expressing GDNF is positively proliferative and has less apoptotic activity, indicating the possibility that GDNF may not be limited to normal nephrogenesis but may also be involved in the proliferation and apoptosis involved in nephroblastoma tumorigenesis [Seguier-Lipszye et al., 2001]. Therefore it is possible to speculate that GDNF may also be involved in tumorigenesis of the nervous system (e.g. NB and NE tumors), lending support to the present study that GDNF is mediating differentiation and proliferation of NBs and NETs.

GDNF is not only important for the development of certain neuronal populations, but also plays a crucial role in rescuing neural crest cells from apoptosis during their migration in the foregut [Taraviras et al., 1999]. In a recent study researchers demonstrated, using a human neuroectodermic cell line (NB: SK-N-MC), that RET tyrosine kinase activity is essential for GDNF to induce phosphatidylinositol 3-kinase (PI3K)/Akt and ERK pathways as well as neuroectodermic cell survival [Mograbi et al., 2001]. This data also supports the inclusion of GDNF in the present study.

Currently there are three GDNF-related neurotrophic factors, neurturin (NTN) [Kotzbauer et al., 1996], artemin (ART) [Balogh et al., 1998] and persephin (PSP) [Milbrandt et al., 1998]. The GDNF family of neurotrophic factors (GFNFs) denotes a structurally related subfamily of proteins within the transforming growth factor beta (TGF-β) superfamly, because it has a set of cysteines that are characteristic of the TGF-β family [Lin et al., 1993]. Like neurotrophins, GFNFs are synthesized as precursor polypeptides and processed to mature disulfide-bonded dimers [Rosenthal, 1998]. These neurotrophic factors, including GDNF, orchestrate several aspects of the maintenance and development of central and peripheral nervous systems, such as promoting the survival of numerous neuronal populations [Heuckeroth et al., 1997] including those of the peripheral ganglia [Balogh et al., 1998] and exhibiting neurotrophic activity on mesencephalic dopaminergic- and motor neurons [Milbrandt et al., 1998].

1.6.1.1 Signaling of "GDNF family of neurotrophic factors"
Each GFNF binds a specific GDNF receptor-α in order to activate c-Ret/RET (REarranged during Transfection). c-Ret/RET is a proto-oncogene that provides the
code (sequence) that cells in the body use to produce membrane receptors, in this case tyrosine kinase receptors (see also (b) below). Due to the importance of c-Ret/RET activation, GFNFs play important roles in GDNF signaling.

The biological action of the GFNFs is mediated via a unique two-component receptor complex, consisting of: 1) a high affinity glycosylphosphatidylinositol (GPI)-anchored cell surface binding protein (GFRα1-4) and 2) the receptor protein tyrosine kinase c-Ret/RET [Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Durbec et al., 1996]:

a) **GPI-anchored binding proteins (GFRα1-GFRα4)**: *In vivo* and *in vitro* research supported the rationale that for each GFNF there is a GFRα receptor to which it binds with the highest affinity in order to activate c-Ret/RET (Fig 1.5). The preferred combinations are GDNF-GFRα1 [Balah et al., 1998], NTN-GFRα2 [Sanicola et al., 1997], ART-GFRα3 [Balah et al., 1998] and PSP-GFRα4 [Masure et al., 2000; Lindahl et al., 2001]. Receptor-binding, cross-linking and receptor activation studies have indicated various interactions between the different ligand-receptor pairs, for example, NTN-GFRα1, ART-GFRα1 and GDNF-GFRα2 (Fig 1.5) [Balah et al., 1998; Baloh et al., 1997; Sanicola et al., 1997]. Until recently the believe was that GFRα cannot signal in the absence of c-Ret/RET, however Schober et al. demonstrated that GDNF may activate certain neural populations of cells through GFRα receptor in the absence of c-Ret/RET [Schober et al., 2000]. Interestingly, another study revealed that in contrast to GFRα1, GFRα2 fails to signal in the absence of c-Ret/RET, therefore indicating that c-Ret/RET-independent signaling of GFRα1 is ligand-specific and occurs only with GDNF [Pezeshki et al., 2001].

b) **c-Ret/RET receptor tyrosine kinase**: The c-Ret/RET proto-oncogene encodes a transmembrane receptor tyrosine kinase [Takahashi and Cooper, 1987; Ullrich and Schlessinger, 1990] that is expressed during embryogenesis on various neuronal subsets of the central and peripheral nervous system [Pachnis et al., 1993]. The catalytic and signaling activities of c-Ret/RET are controlled by autophosphorylation of several tyrosine residues, Tyr(905), Tyr(1015), Tyr(1062) and Tyr(1096), in the c-Ret/RET cytoplasmic domain. Stimulation with GDNF induced coordinated
phosphorylation of these four residues in neuronal cell lines. A recent study on coordinated activation of autophosphorylation sites in c-RET receptor, demonstrated the synchronized utilization of individual c-RET tyrosine residues in neurons in vivo and also reveal the important role of c-RET Tyr(1062) for GDNF mediated neuronal differentiation and survival [Coulpier et al., 2002].

![Figure 1.5](image)

**Figure 1.5** Binding of GDNF to c-Ret/RET. (A) GDNF dimer binds to two molecules of GFRα-1 and this complex binds to c-Ret/RET that dimerizes. (B) The dimerization of c-Ret/RET causes autophosphorylation of the tyrosine residues of the two subunits (from Kirmo Wartiovaara, Ph.D.).

### 1.6.1.2 Role of GDNF/c-Ret signaling in human cancer

As mentioned above, the c-Ret/RET proto-oncogene encodes a receptor tyrosine kinase for neurotrophic molecules. c-Ret/RET is a prime example of how point mutations, deletions and recombination of a single gene may cause different human diseases. Specific mutations in c-Ret/RET have been associated with the development of familial medullar thyroid carcinoma (FMTC) and multiple endocrine neoplasia (MEN) syndromes, types 2A and 2B. c-Ret/RET is also involved in Hirschsprung’s disease (HSCR) which occasionally occurs in association with MEN2A or FMTC [Donis-Keller et al., 1993; Mulligan et al., 1993]. The expression of c-Ret/RET is restricted to normal and malignant cells of neural crest origin, for example, NB, as observed in c-Ret/RET
transgenic mice [Groves and Anderson, 1996; Pisano and Birren, 1999]. According to literature, abnormalities in c-Ret/RET signaling pathway may play a role in NB tumorigenesis, rather than oncogenesis, such as MEN2-type c-Ret/RET activation by mutation [Peaston et al., 1998].

1.6.2 Transforming growth factor-beta (TGF-β)
Transforming growth factors (TGFs) beta (β) are homodimeric, disulfide-bonded proteins that belong to a superfamily of secreted polypeptide trophic factors that regulate cellular growth and differentiation, angiogenesis, morphogenesis, attract cells by chemotaxis and cause extracellular matrix remodeling [Moses et al., 1990; Roberts and Sporn, 1993; Massague et al., 2000].

Three subgroupings within the TGF-β superfamily have been identified: TGFs-β (TGF-β1 – TGF-β5), activins/inhibins and bone morphogenetic proteins (BMPs). TGF-β1 is the most abundant form and was identified as a regulator of mesenchymal growth and separately as an anti-mitogen in epithelial cells [Massague, 1990; Alexandrow and Moses, 1995]. Activins were identified as endocrine regulators of the pituitary function [Harland, 1994; Gaddy-Kurten et al., 1995]. The role of bone morphogenetic proteins (BMPs) is in bone repair and, independently, in the dorsalization of Drosophila [Mehler et al., 1997].

TGFs-β is synthesized as large precursors and is secreted as inactive complexes that are later activated by proteolytic processing. A significant feature of the action of the TGF-β family is that its biological effects often most clearly manifest in the presence of other trophic factors (e.g. GDNF in the present study), either by enhancing the action of the other trophic factors or by inhibiting-suppressing it.

1.6.2.1 Transforming growth factor-beta signaling – see section 1.5.2
1.6.2.2 Role of transforming growth factor-beta in human cancer
TGF-β plays two different and opposite roles in malignant neoplastic development. Firstly, it is a potent tumor suppressor that executes several forms of negative regulation of cell proliferation on target cells, for example, induction of G1 arrest, promotion of terminal differentiation, or activation of cell death mechanisms [Massague
and Weis-Garcia, 1996; Alexandrow and Moses, 1995] as well as mediating actions of chemopreventive agents (e.g. retinoids). Secondly, TGF-β has oncogenic activities that cause cells to escape from TGF-β-dependant growth arrest due to 1) inactivating mutations/dysregulated expression of components in the pathway [de Caestecker et al., 2000], and 2) Smad proteins [Fynan and Reiss, 1993]. Although it seems obvious that TGF-β should be downregulated in cancer cells, the contrary has been identified, showing that there is a substantial increase in the mRNA and protein expression of TGF-β in human cancers. In some cancers, a high increase in TGF-β expression correlates with advanced stages of malignancy and a poor prognosis. Two ways in which TGF-β augments malignant progression are: first it stimulates non-transformed cells in a tumor mass in order to suppress anti-tumor immune responses and to augment angiogenesis, and second it promotes invasion and formation of metastases in cell-autonomous manner – requiring TGF-β signaling activity.

In a study by Nagpal and Chandraratna, the signaling mechanism of RA and other retinoids were uncovered [Nagpal and Chandraratna, 1998]. However, a recent study, using microarray technology, revealed that RA-treatment affected the expression of hundreds of molecular markers including immediate early genes (signal transduction modulators and effectors; transcription factors) as well as those regulated relatively late in the differentiation process and that encode proteins with clear roles in neuronal function (growth factors and their receptors, cytoskeletal and extracellular matrix proteins, cell surface antigens) [Kelly and Rizzino, 2000].

1.6.3 Retinoic acid

Retinoids (vitamin A analogs) have been reported to regulate a wide range of biological processes, including vertebrate development, growth and differentiation [Gudas, 1994; Lotan et al., 1981]. The biological/anti-cancer effects of retinoids are mainly mediated by receptors of the nuclear receptor family, which function as ligand-dependant transcription factors. There are two types of retinoic acid (RA) receptors; retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each having three subtypes: alpha, beta and gamma encoded on separate genes [Chambon, 1994; Giguere, 1994; Kastner et al., 1994; Mangelsdorf et al., 1995]. Multiple isoforms can be generated using
alternative promoters or differential splicing [LeRoy et al., 1991; Kastner, 1990]. These receptors usually bind as heterodimers to specific DNA sequences and/or interact with other transcriptional regulators, including "orphan receptors" (group of receptors lacking identified ligands) COUP-TFs (chicken ovalbumin upstream promoter transcription factors) to regulate gene transcription [Leng et al., 1996]. According to another study, COUP receptors are a novel class of RAR and RXR regulators that is responsible for restricting RA signaling to certain elements. Therefore, the COUP orphan receptors may play an important role in cell- or tissue-specific repression of subsets of RA-sensitive programs during development [Tran et al., 1992; Lu et al., 1994].

NBs retain some of the features of neural crest progenitors therefore they can be induced to neuronal differentiation in the presence of appropriate signals [Abemayor and Sidell, 1989; Seeger et al., 1982]. Accordingly, when treated with RA, NBs have been used as a model to study differentiation along a neuronal lineage [Sidell et al., 1983; Sidell, 1982].

1.6.3.1 Retinoic acid (RA) signaling
RAR and RXR receptors mainly function as RAR-RXR heterodimers, modulating the expression of their target genes by binding to a variety of RA response elements (RAREs) (Fig 1.6) [Kastner et al., 1995; Mangelsdorf and Evans, 1995; Zhang and Pfahl, 1993; Umesono et al., 1991]. Ultimately, it is the changes in gene expression that mediate biological effects. It has been shown that a specific RARE, located in the RARβ2 promoter region, mediates RARβ2 induction in response to RA in a variety of cell lines [Hoffmann et al., 1990; Sucov et al., 1990], therefore, autoregulation of the RARβ gene might play an important role in amplifying the RA response. The latter notion has been supported by several studies indicating a correlation between an altered receptor expression and carcinogenesis – e.g. certain malignant cancers (including pulmonary and breast carcinomas) do not express RARβ [Gebert et al., 1991; Zhang et al., 1996; Zhang et al., 1994].
Figure 1.6   RXR and RAR are nuclear receptors that bind either all trans retinoic (tRA) or 9-cis retinoic acid (9cisRA). In the absence of ligand, corepressors with histone deacetylase activity are bound to the RXR/RAR heterodimer and suppress transcription. Once they bind retinoic acid, a conformational change in the receptors cause the dissociation of the corepressors and the binding of coactivators with histone acetylase activity. Following ligand binding by the heterodimer, the receptors and proteins in the basal transcription machinery (like TBP and TAF135) are degraded by the proteosome (from Kopf Eliezer, Ph.D.).

1.6.3.2    Effects of retinoic acid on human cancer
Retinoic acid plays an important role in regulating the growth and differentiation of normal, premalignant and malignant cells, especially with epithelial origin, mainly through interaction with two types of nuclear receptors, RARα, -β, -γ and RXRα, -β, -γ. The idea that RA directly controls gene transcription has attracted much attention and interest from scientists interested in altering the behavior of cancer cells through induction of differentiation. Treatment that induces differentiation of cancer cells and therefore preventing further proliferation, are known as differentiation therapy. One of
the single most successful examples of differentiation therapy is all-trans retinoic acid (ATRA), which causes frequent remission of acute promyelocytic leukemia (APL) by inducing differentiation of promyelocytes. ATRA treatment resulted in a dramatically improved prognosis, leading to complete remission in 95% of the patients and long-term survival in about 75% [Lo Coco et al., 1998]. The mechanism by which ATRA inhibits proliferation is largely unknown but one possibility is that ATRA acts by inhibiting the activity of the TF, AP-1 [DiSepio et al., 1999].

In the mouse skin carcinogenesis model, malignant progression is associated with downregulation of RARα and -γ expression at the mRNA level [Darwiche et al., 1995; Darwiche et al., 1996]. Therefore, it is possible that pharmacological retinoic acid exerts its chemopreventative action through upregulation of RARα expression [Hansen et al., 2000]. On the other hand, tumor promoters have been shown to directly downregulate these receptors [Kumar et al., 1994].

In most lung cancer cell lines and tumor biopsy specimens, RARβ mRNA is not detectable and RA treatment does not stimulate RARβ gene transcription [Geradts et al., 1993; Xu et al., 1997; Zhang et al., 1994]. However, a recent study that investigated the loss of the RARβ mechanism in lung cancer, found that loss of histone H3 acetylation on chromatin of the RARβ promoter consistently correlated with RA refractoriness in lung cancer cell lines [Suh et al., 2002].

When treated with RA, human NBs have been used as a model to study differentiation along the neuronal lineage [Sidell et al., 1983; Sidell, 1982]. In a recent study by Takada et al., a very interesting finding was observed in regard to the effects of RA on NBs. In this study RA induced moderate neurite outgrowth followed by massive death in NBs. However, upon addition of ligands, GDNF and NTN, apoptosis as well as nuclear accumulation of p53 in the cell were inhibited. According to their results RA-induced apoptosis of NBs, is associated with activation of both the caspase cascade and the p53-mediated pathway. However, neurotrophic signaling through GDNF-RET system may prevent neuronal cell death [Takada et al., 2001].

To date, RA has been praised to be remarkably effective at treating cancer during several stages of the tumorigenesis process, for example, as an inhibitor of squamous metaplasia, as a chemopreventative agent against epithelial carcinogenesis
and finally as a differentiation agent in APL [Chen et al., 1995; Lo Coco et al., 1998; Moon and Constantinou, 1997; Wang et al., 2000]. Nevertheless, the challenge will be to discover the therapeutic effects of this morphogen on solid tumors.
Chapter 2: Materials and Methods

2.1 Materials
Media, chemicals, kits, oligonucleotides and suppliers of these products are detailed in Appendix A.

2.2 Methods
2.2.1 Cell Culture
NB and NET cell lines were obtained from the American Type Culture Collection (ATCC) and propagated using media and culture conditions recommended by the ATCC (see Appendix). In all experiments the cells were adapted to DMEM/F12 media supplemented with B27 for analysis of growth factor effects in the absence of fetal bovine serum.
Details of cell lines used are shown in Tables 2.1 and 2.2.

Table 2.1. Description of neuroblastoma cell lines.

<table>
<thead>
<tr>
<th>ATCC #</th>
<th>Designation</th>
<th>Tissue</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTB-11</td>
<td>SK-N-SH</td>
<td>Neuroblastoma; brain</td>
<td>Epithelial</td>
</tr>
<tr>
<td>HTB-10</td>
<td>SK-N-MC</td>
<td>Neuroepithelioma; brain</td>
<td>Epithelial</td>
</tr>
<tr>
<td>CRL-2137</td>
<td>SK-N-AS</td>
<td>Neuroblastoma; brain</td>
<td>Epithelial</td>
</tr>
<tr>
<td>CRL-2271</td>
<td>SK-N-BE2</td>
<td>Neuroblastoma; brain</td>
<td>Neuroblast</td>
</tr>
<tr>
<td>CRL-2270</td>
<td>MC-1XC</td>
<td>Neuroblastoma; brain</td>
<td>Fibroblast</td>
</tr>
</tbody>
</table>
Table 2.2. Description of pulmonary neuroendocrine tumor cell lines.

<table>
<thead>
<tr>
<th>ATCC #</th>
<th>Designation</th>
<th>Tissue</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRL-5815</td>
<td>NCI-H727 (H727)</td>
<td>Carcinoid; lung; bronchus</td>
<td>Epithelial</td>
</tr>
<tr>
<td>CRL-5838</td>
<td>NCI-H720 (H720)</td>
<td>Atypical carcinoid; lung</td>
<td></td>
</tr>
<tr>
<td>CRL-5975</td>
<td>UMC-11 (MC-11)</td>
<td>Carcinoid; lung</td>
<td>Epithelial</td>
</tr>
<tr>
<td>CRL-5843</td>
<td>NCI-H835 (H835)</td>
<td>Carcinoid; lung</td>
<td></td>
</tr>
<tr>
<td>CRL-5893</td>
<td>NCI-H1770 (H1770)</td>
<td>Non-small cell lung cancer; lung</td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Proliferation assays

All cell lines were cultured at a medium density of $2 \times 10^4$ cells per well in a 24-well plate, using DMEM/F12 media supplemented with B27. After 24 hours, for proliferation of growth factor (GF)-treated cells, individual growth factors or their combinations with transforming growth factor-beta1 (TGF-β1) were added to the media and cells were incubated for a total of six days. The different types of growth factors used for this assay were: 1) nerve growth factor (NGF), 2) brain-derived growth factor (BDNF), 3) neurotrophin-3 (NT-3), 4) neurotrophin-4 (NT-4), 5) glial cell line derived neurotrophic factor (GDNF), 6) leukemia inhibitory factor (LIF), 7) bone morphogenetic protein 4 (BMP4), 8) transforming growth factor-beta1 (TGF-β1), 9) interleukin-1/ interleukin-2 (IL-1/IL-2), 10) insulin-like growth factor-1 (IGF-1) (see Appendix for growth factor concentrations). For proliferation of RA-treated cells, following incubation of 24 hours in DMEM/F12/B27 media, 1μM all-trans retinoic acid (RA) was added to the media and cells were again incubated in this media for the next five days. After six days cells were dissociated and the number of cells counted using a hemocytometer. The effect of GDNF/TGF-β1 or RA on cell proliferation was measured using a $[^{3}\text{H}]$thymidine incorporation assay (see 3.2.7 for details). Proliferation experiments were repeated twice independently.
2.2.3 Differentiation assays

NB and NET cell lines were plated on laminin-coated, 24 well plates at a medium density of $2 \times 10^4$ cells per well. The cells were cultured in DMEM/F12/B27 media containing GDNF (100ng/ml)/TGF-β1 (50pg/ml) or RA (1μM). After six days of incubation the cells were fixed with either ice-cold 4% paraformaldehyde (PFA) or 2% PFA containing 0.5% glutaraldehyde (for staining of neurotransmitters/amino acids) and expression analysis was performed using immunocytochemical techniques (see 3.2.4 for details). The following expression markers were used: 1) microtubule associated protein (MAP-2), 2) βIII-tubulin, 3) neurofilaments, 4) glial-fibrillary acidic protein (GFAP), 5) tyrosine hydroxylase (TH), 6) dopamine decarboxylase (DDC), 7) extracellular anti-TAPA (EAT1/EAT2), 8) 2'-3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and 9) O4. All the cells were counterstained with 4'-6'-diamidino 2'-phenylindole (DAPI) to reveal the nuclei in order to determine the total cell number. The number of cells positive for neuronal (MAP-2, βIII-tubulin, neurofilaments), astrocytic (GFAP, EAT1/EAT2), oligodendrocytes (O4, CNPase) and dopaminergic neuronal (DDC, TH) markers were counted in four fields of 200 cells, each. The percentages of either NB or NET cells that exhibited expression for these markers were presented as numbers.

2.2.4 Immunocytochemistry

Immunocytochemistry (ICC) was performed to determine whether the pathways that connect the transcriptional regulators (analyzed in the present study) to neural differentiation are truly functional in these NBs and NETs.

After six days of culturing, cells were fixed with ice-cold 4% paraformaldehyde (PFA) solution [4% PFA in 1xphosphate buffered saline (PBS), pH 7.4] for 20 minutes followed by two washes with 1xPBS. Plates were then either kept at 4°C (cells covered with 1xPBS and 0.1% sodium azide, to prevent bacterial growth) or used immediately. Cells stained for neurotransmitters or amino acids were fixed with ice-cold 2% PFA/0.5% glutaraldehyde (2% PFA in 1xPBS, pH 7.4 and 0.5% glutaraldehyde) for 10 minutes at room temperature. During fixation, 0.1% sodium borohydride (NaBH₄) in 1xPBS was prepared. This was used to quench the unreacted glutaraldehyde, which is very fluorescent if not reduced. After fixation glutaraldehyde was quenched for seven
minutes at room temperature, followed by two washes with 1xPBS. Plates were then either kept at 4°C (cells covered with 1xPBS and 0.1% sodium azide, to prevent bacterial growth) or used immediately. After blocking with 4% normal donkey serum (4% normal donkey serum in 1xPBS, 0.2% Triton X-100 and 1% Bovine serum albumin), the cells were incubated with the primary antibody, in blocking buffer, overnight at 4°C.

The primary antibodies used in this study: trkA, trkB, trkC, RXRα, RXRβ, RARβ, Nurr1, DDC, DBH, TH, p75 nerve growth factor receptor (NGFR), EAT1, EAT2, GFRα1, GFRα2, c-Ret, neurofilaments, internexin-α, neuronal nuclei (NeuN), CNPase, smooth muscle actin, GFAP, βIII tubulin, desmin, NSE, chromogranin A, O4, MAP-2, A2B5, NCAM and a kit of rabbit polyclonal antisera targeting L-alanine, gamma-amino butyric acid (GABA), L-arginine, L-aspartate, L-glutamate, L-glutamine, glutathione and glycine (see Appendix for dilutions and company information).

Following the primary antibody incubation (primary antibodies were diluted in blocking solution) and washing with 1xPBS (three times, five minutes each), a secondary antibody reaction was performed for one hour at room temperature. Three washes with 1xPBS, five to ten minutes each, were performed between the primary and secondary antibody incubation and after the secondary antibody reaction. Cy3-conjugated anti-Goat-, anti-mouse- and anti-rabbit were used as secondary antibodies (see Appendix for dilutions and company information). All the cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for staining. The percentage of NB or NET cells exhibiting expression after a six day-period of either RA or growth factor treatment, are presented as numbers. Blocking solution (without primary antibody) was used as negative control for primary antibodies.

The cells were viewed using an Olympus microscope. The images were collected with a camera device using Adobe Photoshop software.

2.2.5 Total RNA isolation

Total RNA was isolated from cells, as described in Timmusk et al., 1993, grown in culture media (specific for that cell line) or a differentiation media (see 3.2.3):

Following the six-day differentiation assay in T-75 flasks, culture medium was discarded and a volume of 4ml RNAwiz isolation reagent (see Appendix) was added to each flask.
After addition of RNA isolation reagent to cells, vigorous pipetting was necessary to homogenize the cells. The homogenate was either stored at -20°C or incubated at room temperature for five minutes, to dissociate the nucleoproteins from the nucleic acids, before RNA extraction. After adding 0.8ml chloroform to the homogenate and vigorously shaking it for about 20 seconds, it was incubated at room temperature for 10 minutes. The chloroform-homogenate mixture was then centrifuged at 3000rpm for 15 minutes at 4°C (the mixture separated into three phases: an upper colorless aqueous phase containing the RNA, a semi-solid inter phase containing most of the DNA and the lower organic phase). Following the centrifugation, the colorless upper phase was transferred into clean, RNase-free, glass centrifugation tubes together with 2ml RNase-free water and 4ml isopropanol. Sample was mixed well and allowed to precipitate at –20°C overnight.

The following morning the mixture was centrifuged at 5000rpm for 15 minutes at 4°C to pellet the RNA. After discarding the supernatant, the pellet was resuspended in 200μl of RNase-free water and transferred into RNase-free, 1.5μl eppendorf tubes. 0.5M sodium chloride and 100% ethanol were added and the mixture was then allowed to precipitate at –20°C for two to four hours. Following precipitation the mixture was centrifuged at 14 000rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet washed with 80% ethanol for 3 minutes. After removal of ethanol, the pellet was allowed to air dry before adding an appropriate volume (final volume – FV) of RNA-free water.

Isolated total RNA samples used for reverse transcriptase polymerase chain reaction (RT-PCR), were treated with RNase-free DNase-free (see Appendix) to eliminate any genomic DNA contamination that could interfere with the RT-PCR analysis when using the primer sets which do not discriminate between DNA and mRNA, namely hASH-1, neurogenin-1 (NN-1), Noggin, Chordin, Sox-2 and Brain-4 (Brn-4).

**DNAse I treatment:** This treatment was conducted according to the manufacturer's instructions. Briefly, it was performed as follows:

10% of the 10xDNAse I buffer and 1μl of DNase I enzyme were added to the FV of extracted RNA. The mixture was vortexed, incubated for 15 to 20 minutes at 37°C and the reaction terminated by adding 10% DNase I inactivation buffer to the volume of RNA-buffer-enzyme mixture.
Hereafter, the DNase I-treated RNA was ready to be used for complementary DNA (cDNA) synthesis.

2.2.6 RT-PCR (Two-step protocol)

In this study we followed the method previously outlined by Palm et al., 2000. The protocol is briefly described below:

2.2.6.1 Reverse Transcription (cDNA preparation) (Step 1)

(See Appendix for information on products used in cDNA preparation)

Total mRNA (5-10μg), from different cell lines, was used as template for transcription into first strand cDNA with 500μg/ml Oligo (dT) as a primer, reverse transcriptase (Superscript\textsuperscript{II}) and RNase-free water in a total volume of 20μl. The mixture was denatured at 70°C for 10 minutes and then chilled on ice for five minutes. After the denaturation process, the following components were added: 4μl 5 x first strand buffer, 2μl 0.1M DDT, 1μl 10mM dNTP-mix and 1μl (200 Units) of Superscript\textsuperscript{II} RT and incubated at 42°C for one hour. The reaction was terminated by incubation at 70°C for 15 minutes.

2.2.6.2 Polymerase chain reaction (PCR) (Step 2)

GC-Rich PCR system or the Expand\textsuperscript{TM} Long Distance PCR system kit was used for PCR reactions, according to the manufacturer's instructions.

Semi-quantitative analysis was performed in a 25μl volume PCR reaction containing 0.8μl of undiluted first strand cDNA, 1.25μl 10mM dNTP-mix, 1μl Primer 1 (sense), 1μl Primer 2 (anti-sense), 2.5μl Buffer 1, 2.5μl Buffer 2, 16.38μl RNA-free water and 0.38μl high-fidelity enzyme. DNA was amplified using in most cases the following conditions: 94°C (2min); 35-40 cycles of 94°C (30s), 56°C (40s), 72°C (150s). For all combinations of primers the annealing temperature and the number of cycles were optimized beforehand. All primer sets were designed so that sense and anti-sense recognized different exons - to allow discrimination between RT-PCR amplification products of genomic DNA and mRNA. All amplified PCR products were sequenced to rule out false positives using fmol\textsuperscript{®} DNA Cycle sequencing system (Promega). The amplified RT-PCR products were resolved on a 1.0-1.2% agarose gel.
Primer sequences that have been submitted to Genbank: GATA-2, GATA-3, Olf-1, Olf-1 homolog, Pbx-1, LMO4, Id3, Xanf1, Dlx-2, Sox-2, Sox-11, BMP-2, BMP-6, Noggin, Chordin, Smad-6, Smad-7, Msx-1, Msx-2, Hes-1, Hes-5, Hey-1, Hey-2, HeyL, MyT-1, MyT-2, MyT-3, Shh, Gli-1, Gli-2, Gli-3, Zic-1, Zic-2, Zic-3, Inx-2a, Inx-3, NN-1, NN-3, hASH-1, NeuroD1, NeuroD2, NeuroD3, NSCL-1, NSCL-2, Otx-1, Otx-2, Pax-6, Oct-2, Brn-2, Brn-4, Dlx-7 (see Appendix for Genbank IDs of primers).

**Statistical Analyses:** To determine whether the RT-PCR expression data of the five NBs and NETs were significantly different from each other, a two-way analyses of variance (ANOVA) test was performed. A significant difference was observed when $P \leq 0.05$.

Since the ANOVA's function is to reveal whether the null hypotheses should be accepted or rejected (in other words, that the means of all the tumors were the same or different), a Bonferroni post hoc test was necessary to determine which tumors were statistically different. Statistical analyses were performed using GraphPad Prism version 3.00 and Microsoft Excel 2000.

### 2.2.7 $[^3]H$Thymidine incorporation assay

Cells were plated on 12-well plates (in their conditioning media) at a density of $2-3 \times 10^4$ cells/well and treated with GDNF (100ng/ml)/TGF-β1 (50pg/ml) OR 1μM all-trans retinoic acid (RA) for 24 hours. The cells were labeled for the last five hours with 4μCi of $[^3]H$thymidine (ICN Radiochemicals Inc., Irvine CA, USA) and then removed from the plates by washing with 1xPBS. Cell suspensions were centrifuged for five minutes at 5000 rpm, supernatant removed and cell pellets were either stored at -80°C or immediately used in a thymidine incorporation assessment assay. For lysis of the cells, 350μl of 0.5M NaOH was added to the cell pellets and incubated at 37°C for 30 minutes (lysates were vortexed every 10 minutes). Following the incubation, 100μl of the lysate was added to an eppendorf tube containing 350μl of distilled water. For precipitation, 300μl of 25% TCA was added to each tube and incubated at 37°C for 10 minutes. This lysate was then filtered through a two-inch (in diameter) piece of filter paper, using a filtering device connected to a vacuum apparatus/pump. The filter paper was then washed three times with ice cold 10% TCA. After removing paper from the filtering apparatus, it was allowed to dry and then added to a vial containing scintillation liquid.
The retained radioactivity was counted for two minutes per vial in a liquid scintillation counter. Each data point was performed in quadruplicates and the mean was calculated for each point (experiments were repeated twice independently). Wells without GDNF/TGF-β1 or RA-treatment were used as untreated controls.

**Statistical Analyses:** To determine whether the proliferation of untreated (control) tumors (using a $[^3]$H]thymidine incorporation assay) was significantly different from treated tumors, a paired Student's t-test was performed which compared the means of each untreated (control) and comparable GDNF/TGF-β1 or RA-treated data point. The t-test was performed using GraphPad Prism version 3.00.
Chapter 3: Results and Discussion

A. NEUROBLASTOMAS

Prior to discussing the results of our research, it is appropriate to accentuate once again the primary incentive for the current study, which was to show that different types of NBs have characteristic expression profiles of regulatory genes. Moreover, our study also investigated whether and how these profiles change upon treatment with certain extrinsic factors (GDNF/TGF-β1 and RA).

3.1 Molecular criteria commonly used in the prognostic evaluation of neuroblastomas

The following characteristics are known to be important in evaluating aggressiveness of NBs: 1) N-myc amplification; 2) hyperploidy; 3) VMA/HVA ratio in the urine; 4) NSE; 5) serum ferritin; 6) LDH (lactate dehydrogenase); 7) Gd2 (a sialic acid-containing glycosphingolipid); 8) CD44; 9) trkA; 10) 1p deletion [Evans et al., 1987; Mora et al., 2000; Sariola et al., 1991; Combaret et al., 1995]. These features are often indicators of treatment success. Therefore, in view of these published characteristics, we compared the prognostic impact of several molecular variables on five different human NBs with the aim to predict susceptibilities of these tumors to anticancer agents.

Myc amplification.

SK-N-BE2, SK-N-SH and SK-N-AS exhibit distinct levels of N-myc amplification with SK-N-SH having one copy of N-myc and SK-N-BE2 over 150 copies of N-myc [Taguchi et al., 1997]. MC-IXC contains 21 copies of the c-myc oncogene (Table 3.1). However some data argue that the expression of N-myc, but not the amplification state of the myc gene, in either of the cells, correlate with tumorigenecity [Iman and Shay, 1989].
**Table 3.1.** trk (full-length) receptor profile and expression of neural-restricted REST/NRSF/XBR mRNAs, in relation to myc amplification in different human neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc</td>
<td>no data</td>
<td>21</td>
<td>no data</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>trkA</td>
<td>10%</td>
<td>10%</td>
<td>100%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>trkB</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>10%</td>
</tr>
<tr>
<td>N-REST</td>
<td>0%</td>
<td>0%</td>
<td>&lt;50%</td>
<td>100%</td>
<td>&lt;50%</td>
</tr>
</tbody>
</table>

**Table legend:** The table above represents data for myc, trkA, trkB and N-REST. myc results were obtained from literature and represent the number of copies of the myc gene. TrkA and trkB results were generated in the present study using immunocytochemical techniques and represent the % of cells that expressed trkA or trkB. A colleague in our lab, also using immunocytochemical techniques, obtained results from N-REST expression.

**trkA/trkB expression.**

Alterations in the expression of the neurotrophins and their receptors are often examined in regard to N-myc amplification in NBs [Brodeur et al., 1997; Yamashiro et al., 1997]. Our data showed that SK-N-AS expressed both receptors (trkA and trkB), whereas SK-N-BE2 cells, which also showed high myc amplification, expressed trkB only (Table 3.1). 10% of SK-N-MC and MC-IXC cells exhibited strong trkA (full-length) immunoreactivity, while 10% of SK-N-SH cells were also trkB-full immunoreactive. Based on our findings, NBs can be classified into three different groups: trkB immunoreactive (SK-N-BE2), trkA and trkB co-immunoreactive (SK-N-AS) and predominantly one trk-reactive (trkB, SK-N-MC, MC-IXC; trkA, SK-N-SH).

**Expression of neural isoforms of REST/NRSF/XBR.**

A study by a colleague in our lab observed that neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in NBs [Palm et al., 1999]. Given that expression of neuronal src mRNA (src-N) has been established to be a favorable marker and inversely correlated with N-myc gene amplification in human NBs [Matsunaga et al., 1994], and that low levels of neuronal-src (src-N) and trkA mRNA expression characterize aggressive NB phenotypes [Matsunaga et al., 1998], we
examined the correlation between the expression of trkA and trkB receptors and neural REST/NRSF/XBR mRNAs in NBs. High expression of REST/NRSF/XBR transcripts with the insertion of exon N was observed in SK-N-BE2 cells, moderate in SK-N-AS and SK-N-SH cells, and no expression of neural REST/NRSF/XBR mRNAs was detected in SK-N-MC and MC-IJC cells (Table 3.1). Combined with myc amplification data, our results revealed no correlation between myc amplification and expression of neural REST/NRSF/XBR mRNAs. Although there was no clear association between trk-pattern of expression and N-REST mRNAs, our data revealed a true inclination to an association between trkA expression and the presence of N-REST mRNA in four out of five NBs (Table 3.1). Accordingly, NB clones can be grouped into two subsets in regard to their expression of neural-specific splice isoforms of REST/NRSF/XBR, for example, some NBs (SK-N-BE2, SK-N-AS, SK-N-SH) expressed these isoforms, and others (SK-N-MC and MC-IJC) did not.

**Multitransmitter-phenotype.**

According to previous studies, 93% (13 out of 14) of NB cell lines contain activities of both adrenergic and cholinergic neurons [Ross *et al.*, 1981] revealing their resemblance to neuroblasts of early developmental stages. This can be attributed to the fact that neuroblasts are pluripotent with respect to neurotransmitter phenotype during normal development. According to the ATCC data sheets, the choline acetyltransferase activity of MC-IJC is approximately four times higher than that of the parental line, SK-N-MC. Choline acetyltransferase (ChAT) expression in MC-IJC cells has also been previously confirmed [Casper and Davies, 1989]. Furthermore, ATCC data also revealed that SK-N-SH cells exhibit high and SK-N-BE2 cells moderate DBH activity, whereas SK-N-MC and MC-IJC are negative for DBH activity [ATCC data sheet] (Table 3.2 A). According to our results, all of the NBs expressed ChAT, with SK-N-AS and SK-N-BE2 cells expressing also TH (Table 3.2 B, Figure 3.1 N, O, P). However, the TH-positive cells usually appeared in clumps, rather than being equally distributed throughout the population (Figure 3.1 N, O, P). No expression of TH was observed in SK-N-MC, MC-IJC and SK-N-SH cells. We were not able to detect TH, DBH or DDC expression in SK-N-SH cells (Table 3.2 B), suggesting that our SK-N-SH clone might have lost its catecholaminergic activities. Given that these tumors had been passaged for years prior
to growing in culture, and that cell lines derived from tumors generally have very unstable karyotypes, the hundreds of clones isolated from the original will develop different characteristics. Therefore, when comparing our SK-N-SH clone to the original SK-N-SH clone, it is possible that ours could have been altered molecularly through handling/culturing [Sidell et al., 1986]. With regard to ChAT and catecholamine neurotransmitter synthesis, NB clones fell into two categories: purely cholinergic and mixed, cholinergic-adrenergic (Table 3.2A and B). No DDC expression was detected in any of the NBs (Table 3.2B), which is in good agreement with previous findings that in some NB cells there is a relative deficiency of DDC, resulting in accumulation and secretion of DOPA [Ikeda et al., 1994].

Table 3.2. Cholinergic and catecholaminergic properties of human neuroblastomas.

A. ChAT and DBH activity and expression of TH in human neuroblastomas based on published data.

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChAT activ</td>
<td>mod</td>
<td>high</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>TH</td>
<td>0.5-5%</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.5-5%</td>
</tr>
<tr>
<td>DBH activ</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

West et al., 1977

B. Expression of ChAT, TH and DDC in human neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH</td>
<td>0</td>
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<td>30</td>
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<tr>
<td>DDC</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table legend: To monitor expression of cholinergic and catecholaminergic markers in NBs, cells were plated on laminin-coated 24-well plates and cultured 24 to 72 hours prior to fixation. >10,000 cells were scored in each culture. The total number of cells per square centimeter was determined by counting the DAPI-stained nuclei. Following fixation, cells were stained with anti-TH, -ChAT, -DDC antibodies. Table B represents the mean ± SEM (highest value of 14%) for three independent experiments. ABBREVIATIONS: ChAT, choline acetyltransferase; TH, tyrosine hydroxylase; DBH, dopamine-beta-hydroxylase; DDC, 3,4-dihydroxyphenylalanine DOPA-decarboxylase; nd, no data; mod, moderate; +, expression; - no activity; 0, no expression; 30, 30% expression; "filled box", 100% expression; activ,
activity. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

Multiple neurotransmitter synthesis by human NB cell lines and clones is a well-established characteristic of NBs [Biedler et al., 1978]. Therefore, we analyzed the synthesis of the neurotransmitters GABA, glutamate (Glu), glutamine (Gln), Glycine (Gly) and Glutathione (GSH) using immunocytochemical techniques. Our results revealed that all NBs were Gln- and GSH- positive and most of them (with the exception of SK-N-AS) were also Gly-immunoreactive (Table 3.3). None of the NBs in our culturing system expressed the GABA neurotransmitter at detectable levels. Therefore, in regard to amino acid transmitter synthesis, the five NB clones fell into three subsets: cells (MC-IXC) that expressed four out of five transmitters (Glu, Gln, Gly, GSH), cells (SK-N-MC; SK-N-BE2 and SK-N-SH) that expressed three out of five transmitters (Glu, Gly, GSH or Gln, Gly, GSH) and cells (SK-N-AS) that expressed two out of five transmitters (Gln, GSH).

Table 3.3. Accumulation and synthesis of putative amino acid neurotransmitters in human neuroblastomas.

<table>
<thead>
<tr>
<th>Transmitter</th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glu</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gln</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gly</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GSH</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table legend: Cultured cells were plated on laminin-coated 24-well plates, fixed after 24 to 72 hours and then analyzed by immunocytochemistry using rabbit polyclonal antisera targeting gamma-amino butyric acid (GABA), L-glutamate (Glu), L-glutamine (Gln), glutathione (GSH), and glycine (Gly). Note that subsets of cells expressed multiple markers. ABBREVIATIONS: 0, no expression; "filled box", 100% expression; 90, 90% expression. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0% and 100%.

According to our results, no significant correlation was found for any of the amino acids and catecholamines, myc-amplification, trk patterns and presence of N-REST in the five
NBs analyzed. Therefore, as determined by multiple independent methods, our analyses demonstrated that these commonly used molecular criteria are a characteristic of individual NB cells rather than a basis for grouping that should underlie an effective therapy.

3.2 Identification of TF expression profile of human neuroblastomas
The two major hypotheses of the cellular origin of cancer are that tumors arise from: 1) stem (multipotent) or progenitor cells due to maturation arrest, and the 2) dedifferentiation of mature cells that retain the ability to proliferate [Sell, 1993]. Earlier studies have clearly demonstrated that the model of normal neural crest development is well applicable to NBs. Firstly, they showed that it is possible to isolate malignant neural crest stem cell-like cells from established human NB cell lines [Ciccarone et al., 1989]. Secondly, these cells typically to stem cells a) generated progeny that was capable of neural crest cell differentiation along either neural or non-neural lineages; and b) had the capacity for self-renewal [Ross et al., 1995]. While accepting the postulation that tumors recapitulate development, we hypothesized that individual NBs are molecularly different based on their expression of regulatory genes and that these expression profiles will change upon exposure to certain extrinsic factors. Thus, our study undertook this novel approach and analyzed human NBs through the prism of regulatory networks that are operational during neurogenesis. According to the model of Sasai, regulatory factors that connect neural induction and primary neurogenesis in vertebrates [Sasai, 1998] include secretory proteins (BMP-2, BMP-6, Noggin, Chordin) and transcription regulators that are downstream in the signaling cascade induced by these secretory proteins, namely bHLH, zinc-finger (ZF), high mobility group (HMG) and homeodomain-containing (HD) TFs.

In order to test our hypotheses (mentioned above), we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different neuroblastomas. Since one of the aims of our study was to propose a way to improve the diagnostics of NBs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our
purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

The group of regulatory genes analyzed, was subdivided into four gene groups, based on the function of the genes.

_Early negative regulators of neural differentiation (Gene Group 1)._ Genes that were analyzed included signals that can suppress neural differentiation and promote epidermogenesis, namely BMP-2 [Kanzler et al., 2000] and BMP-6 [Mehler et al., 2000] and also BMP signaling attenuating downstream factors Msx-1 [Monsoro-Burq et al., 1996] and Msx-2 [Monsoro-Burq et al., 1996; Marazzi et al., 1997]. We also examined the expression of factors involved in the inactivation of BMP signaling, such as neural inducers Noggin [Lim et al., 2000], Chordin [Sasai, 2001] and inhibitory Smads, Smad-6 [Hata et al., 1998] and Smad-7 [Kawabata et al., 1998; Casellas and Brivanlou, 1998; Bhushan et al., 1998]. Sublines MC-IXC, and SK-N-MC expressed both BMP-signaling supportive (BMP-2 and BMP-6, Msx-1, and Msx-2), and antagonistic (Smad-6 and Smad-7, and Noggin and Chordin) regulators (Table 3.4). In contrast, the rest of the NBs (SK-N-AS, SK-N-BE2, SK-N-SH) did not express any of these factors other than Smad-7. Since, the negative-feed back loop, formed by BMPs and Noggin in the SK-N-MC/IXC group, has been shown to be essential for the Indian Hedgehog (Ihh) expression and chondrocytic maturation [Kameda et al., 1999], it is possible to speculate that this autoregulatory pathway is kept active, not for the induction of neurogenesis in these NBs but rather to promote non-neural traits/lineages. This was further supported by the finding that TFs which are downstream targets of BMP signaling pathways in non-neural cells (such as Msx-1, Msx-2 and Smad-6) [Monsoro-Burq et al., 1996; Takase et al., 1998] were expressed, whereas hASH-1, the downstream target of BMP signaling in neural cells [Shou et al., 1999], was not expressed in the SK-N-MC/IXC group of NBs. Interestingly non-neural, specifically ectodermic and mesodermic traits as observed by the expression of Dlx-7 and Nkx-2.5, respectively, were identified in all NBs. Only MC-IXC, SK-N-AS and SK-N-BE2 cells
expressed Nkx-2.5, whereas all NBs expressed Dlx-7. In mouse embryos, Nkx-2.5 has been demonstrated to be primarily expressed in the mesendoderm and mesendoderm-derived organs, such as heart and gut [Pabst et al., 2000]. On the other hand, Dlx-7 is associated with the development of hair, teeth and bone [Price et al., 1998] however, is also expressed in normal hematopoietic cells [Shimamoto et al., 1997].

**Table 3.4.** mRNA expression profile of early negative regulators of neural differentiation in untreated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKNMC</td>
</tr>
<tr>
<td>BMP-2</td>
<td>0</td>
</tr>
<tr>
<td>BMP-6</td>
<td>0</td>
</tr>
<tr>
<td>Noggin</td>
<td>0</td>
</tr>
<tr>
<td>Chordin</td>
<td>0</td>
</tr>
<tr>
<td>Smad-6</td>
<td>0</td>
</tr>
<tr>
<td>Smad-7</td>
<td>0</td>
</tr>
<tr>
<td>Msx-1</td>
<td>0</td>
</tr>
<tr>
<td>Msx-2</td>
<td>0</td>
</tr>
<tr>
<td>Dlx-7</td>
<td>0</td>
</tr>
<tr>
<td>Nkx-2.5</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table legend:** mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated neuroblastomas. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

Therefore our data from early negative regulators of neural differentiation can conclude that patterns of Msx transcription factors and BMP inhibitors (Noggin, Chordin, Smad-6) are a strong indication of non-neural activities in NBs.
Transcriptional regulators linking neural inducers to activation of proneural genes (Gene Group 2).

On the other hand, suppression of BMP signaling, by neural inducers, leads to the activation of a number of genes, namely, genes in the Sox, Gli/Zic and Iroquois families that promote primary neurogenesis [Sasai, 1998]. Therefore, we decided to analyze the expression of Sox-2 [Zappone et al., 2000] from the Sox family; Gli-1, Gli-2, Gli-3, Zic-1, Zic-2, Zic-3 [Brewster et al., 1998] from the Gli/Zic family, and Irx-2a, Irx-3 [Bosse et al., 1997] from the Iroquois family of genes. SK-N-MC cells expressed all of the Sox, Gli/Zic and Iroquois family of genes studied (Table 3.5). Although, the expression pattern of MC-IXC cells resembled that of SK-N-MC cells, in so far as expressing Zic-2 and Gliis, the former did not express Zic-1, Zic-3 and Irx-3, revealing clear molecular differences between these two sublines. However, given that Zic-2 has been shown to counteract the neurogenic activity of Glis by inhibiting neurogenesis and inducing neural crest differentiation [Brewster et al., 1998] it is possible that the co-expression of Zic-2 with Glis was another indication that other than neural developmental programs are active in the SK-N-MC/MC-IXC group of NBs. The rest of the NBs showed similar expression of Gli-2 and Irx-2a, however different in their expression of Gli-3, Zic-1, and Irx-3. The expression of Gli-3 was detected in SK-N-AS cells, Zic-1 expression in SK-N-SH cells and Irx-3 in SK-N-BE2 cells.

Table 3.5. mRNA expression profile of transcriptional regulators linking neural differentiation to activation of proneural genes in untreated neuroblastomas.
Table legend: mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated neuroblastomas. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

According to the aforementioned results, we can conclude that one of the common features for all NBs was the expression of Gli-2 and Irx-2a mRNA. Given that for all stages of neural crest development, significant differences in the spatial expression of Zic, Gli and Irx, have been observed [Bosse et al., 1997; Nagai et al., 1997; Hui et al., 1994], it is appealing to speculate that the expression signature of Zic, Gli and Irx families of factors could be exploited as developmental stage designation markers for classification of NBs.

Transcriptional regulators linking neural determination to the specification of neuronal identity – e.g. proneural and neurogenic genes (Gene Group 3).

Another cascade of regulatory networks, that is operational during normal development and is well applicable for monitoring the activated developmental programs in NBs, involved proneural and neurogenic genes of the bHLH family. Our motivation for analyzing the proneural and neurogenic genes of the bHLH family was the fact that genes in the Sox, Gli/Zic and Iroquois families are capable of promoting the expression of proneural genes MASH-1 [Cohen et al., 2000] and neurogenins (NNs) [Sasai, 1998; Sasai, 2001]. Therefore, we pursued analyses of the expression of hASH-1, the human homologue of MASH-1, neurogenins-1 and -3 (NN-1, NN-3), and bHLH TFs acting downstream [Ma et al., 1998], mainly NeuroD (NeuroD1, NeuroD2, NeuroD3) and NSCL (NSCL-1, NSCL-2) family members. Proneural activity is also facilitated by the MyT (MyT-1, MyT-2, MyT-3) [Bellefroid et al., 1996] and Col/Olf-1/EBF (Olf-1, Olf-1Homolog) [Wang et al., 1993; Wang et al., 1997; Garel et al. 1999] families of TFs, and antagonized by the Hes (Hes-1, Hes-5) [Lyman and Yedvobnick, 1995; Bang et al., 1995] and Hey (Hey-1, Hey-2, HeyL) [Maier and Gessler, 2000; Leimeister et al., 2000]
families. Therefore, we also included the representatives of these families to the list of analyzed genes.

In the present study, we found that NBs expressing Hes-5, also expressed hASH-1, whereas NBs belonging to the SK-N-MC/MC-IIXC group, that did not express hASH-1, had no expression of Hes-5 mRNA either (Table 3.6). Interestingly, a recent study demonstrated that broad expression of Hes-1 in olfactory placodal domain is maintained in the absence of MASH-1, whereas, expression of Hes-5 is restricted to clusters of neural progenitor cells and requires MASH-1 function [Cau et al., 2000]. The same study also reports that olfactory placodes that are double mutant for Hes-1 and Hes-5 upregulate NN-1. In good agreement with the data of Cau et al., our results did not reveal the expression of NN-1 in any of NBs, given that all of them expressed Hes-1 mRNA. Accordingly, the pattern of NN-1, hASH-1, Hes-1 and Hes-5 expression could directly indicate a developmental origin (stage) of NB that corresponds to a certain stage of neurogenesis along olfactory sensory neuron lineage.

Our data also demonstrated that transcripts for members of three different families implicated in the Notch pathway, Hes, Hey, and MyT, are differentially expressed in human NB cells (Table 3.6). During normal development, Notch signaling regulates different aspects of cellular functioning. According to recent data, neuron-glial fate determination of crest cells is regulated, at least in part, by Notch-mediated lateral inhibition among crest-derived cells [Wakamatsu et al., 2000; Morrison et al., 2000]. Not only has Notch activation in neural crest-derived stem cells been shown to prevent neuronal differentiation and permit glial differentiation, but also abnormalities in the Notch pathway have been found to lead to premature neuronal differentiation and the lack of some cell types [Kageyama and Ohtsuka, 1999]. In the NBs under study, we detected the expression of the glial-specific marker, GFAP, only in SK-N-SH cells and not in any of the other NBs (Table 3.8). βIIIITubulin expression, that indicated neuronal differentiation traits in NBs, was identified in SK-N-BE2 and SK-N-SH cells. Given that data for the role of different Notch pathways (Hes, Hey, and MyT), leading to neuron-glial fate determination is missing, we could not identify any patterns of bHLH genes implicated in Notch pathway as aberrant and relate them to the expression of βIIIITubulin/GFAP in NBs.
Table 3.6. mRNA expression profile of transcriptional regulators linking neural determination to specification of neuronal identity in untreated neuroblastomas.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNB2</th>
<th>SKNAS</th>
<th>SKNSH</th>
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</thead>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
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</tr>
<tr>
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<td>HeyL</td>
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</tr>
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<td>MyT-1</td>
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</tr>
<tr>
<td>Off-1H</td>
<td></td>
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</table>

Table legend: mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated neuroblastomas. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

Transcription factors characterizing fate switches to specific neural lineages (Gene Group 4).

During normal development, cells that are selected to become neurons begin to express NeuroD1 [Korzh et al., 1998; Lee et al., 1995]. However, numerous other regulators are needed to provide fine positional and specification information to maturing neurons.
Therefore, we decided to examine the expression of selected transcriptional regulators that have previously been identified to specify various neuronal phenotypes, namely, Brain-4 (Brn-4), Brain-5 (Brn-5), Dlx-2, Gata-2, Gata-3, LMO4, Otx-1, Otx-2, Pax-6, Pbx-1, and Sox-11. Brn-4 mediates, at least in part, the actions of epigenetic signals that induce striatal neuron-precursor differentiation [Shimazaki et al., 1999] and controls cellular phenotypes in the neuroendocrine system [Rosenfeld et al., 1996]. The expression of Brn-5 is suggested to be an early event in the process of terminal neuronal differentiation, whereas its expression occurs in postmitotic neurons (in many CNS neuron populations) but not in proliferating neuronal progenitors [Cui and Bullett, 1998]. Dlx-2 that is expressed in M-phase cells of the ventricular zone is required for differentiation of basal forebrain neurons and craniofacial morphogenesis [Eisenstat et al., 1999]. Gata-2 is required for the generation of V2 interneurons [Nardelli et al., 1999] and loss of Gata-3 leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system [Lim et al., 2000]. In addition, both, Gata-2 and Gata-3 are characterized as abundant in cells constituting the sub-ventricular zone (SVZ) [Luskin, 1993]. Regionalized expression of LMO4 during brain development has suggested a role for this gene in autonomic, motor and neuroendocrine regulation [Hermanson et al., 1999]. Otx genes, Otx-1 and Otx-2 have a crucial role in specification, regionalization and terminal differentiation of rostral central nervous system [Lim et al., 2000]. Pax-6 encodes a transcription factor that plays a pivotal role in brain patterning, neuronal specification, neuronal migration and axonal extension [Osumi, 2001]. The homeobox gene Pbx-1 is expressed in the postnatal sub-ventricular zone (SVZ) and interneurons of the olfactory bulb [Redmond et al., 1996]. Chicken Sox-11 is transiently upregulated in maturing neurons after they leave the neural epithelium of the CNS and exhibits a lineage restricted pattern of expression in the peripheral nervous system [Uwanogho et al., 1995], whereas the expression of human Sox-11 supports its role in the developing nervous system [Jay et al., 1995].

Expression of TFs that control different neural phenotypes contrasted the two groups of NBs, SK-N-MC/MC-IXC and SK-N-SH(SK-N-AS/SK-N-BE2, even more. Dlx-2, Otx-1, and Pax-6 were only expressed in the SK-N-MC/MC-IXC group of NBs and not in any of the other three cell lines (Table 3.7). Expression of Otx-2 was characteristic of
SK-N-MC cells only. Given that the expression of Otx-2 precedes that of Otx-1 during normal mouse gastrulation [Acampora et al., 1999], the presence of Otx-2 expression in SK-N-MC cells may accentuate their more primordial nature when compared to MC-1XC cells. Our results also showed that NBs expressed a variety of TFs that have been previously identified as specifiers of various neuronal phenotypes. However, expression of many of these selected TFs has been mapped to a mitotically active and immature region of the nervous system – the SVZ, suggesting that cell fate determination is ongoing already at that stage. Given that SVZ of the lateral ventricle houses developmentally immature neural stem and progenitor cells in the adult mammalian CNS [Alvarez-Buylla et al., 2000], the detection of TFs that are characteristic to SVZ-housed neural stem or progenitor cells (GATA-2, GATA-3, LMO4, Pbx-1, and others) in NBs would, thus, immediately associate these tumors with highly proliferative nature and phenotypic multipotency. Another interesting piece of evidence that came out from studying the expression of this set of TFs was the identification of LMO4 and Brn-4 expression in all NBs. Both of these genes are implicated in neuroendocrine (NE) regulation [Rosenfeld et al., 1996; Hermanson et al., 1999]. Intermediate filament proteins, chromogranins, synaptophysin, CD56, CD57, CD99, NSE, protein gene product 9.5, and specific neuropeptide products are common markers used for diagnostic evaluation of NE differentiation of NBs [Wick, 2000]. However, it remained to be established whether LMO4 and Brn-4 are associated with or are specific for any of these NE characteristics of NBs.

Based on the expression profiles of the four gene groups, NBs could be easily assorted into two different groups. As expected MC-1XC and SK-N-MC grouped closely together, reflecting their common origin, since MC-1XC is a twice cloned subline of the neuroepithelioma SK-N-MC cells [ATCC data sheet]. The other three NBs (SK-N-AS, SK-N-BE2, SK-N-SH), despite their different origin [ATCC data sheet] were found to be more similar in their expression patterns to each other than to the SK-N-MC/MC-1XC group of NBs.

Our data also concluded that these five NBs reflected characteristics of immature neural stem or progenitor cells and emphasized that these NBs provided a permissive environment for the expression of certain phenotypic traits while repressing others.
Table 3.7. mRNA expression profile of transcription factors characterizing fate switches to specific neural lineages in untreated neuroblastomas.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNBE2</th>
<th>SKNAS</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pbx-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMO4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dlx-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox-11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Otx-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Otx-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brn-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brn-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table legend: mRNA expression data are show in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated neuroblastomas. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

It was now crucial to determine whether the pathways that connect these transcriptional regulators to neural differentiation, are truly functional. Therefore we examined the expression of general immature neuronal (internexin-α), mature neuronal (βIII tubulin, phosphorylated neurofilaments, N-CAM, MAP-2), and glial (GFAP) markers in the five NBs. The following data confirmed the differences between the two groups of NBs (SK-N-MC/MC-IXC and SK-N-BE2/SK-N-AS/SK-N-SH) even further. All NBs expressed internexin-α (75-90%) and phosphorylated neurofilaments (75-90%) (Table 3.8; Figure 3.1E, G, H). Overlapping populations of internexin-α-positive (+) SK-N-BE2 and SK-N-SH cells expressed also βIII tubulin (<10%) (Table 3.8). In addition, small (<5%), non-overlapping populations of SK-N-SH cells expressed also MAP-2 and GFAP (Table 3A;
Figure 3.1C). Weak N-CAM expression was observed in a minor population of SK-N-BE2 cells (<1%) (Table 3.8; Figure 3.1A). A possible explanation for the weak N-CAM staining observed here might be due to the fact that this N-CAM monoclonal antibody (from DAKO) recognizes the less polysialated form of N-CAM and is mostly used to stain neuron-like cells that survived transplantation [Baker et al., 2000]. Therefore this antibody might just stain more mature cells and not embryonic-type of cells, meaning that if our study did not reveal staining of N-CAM in treated (or untreated) NBs, that those cells were just not mature enough to express this form of N-CAM that is recognized by the DAKO antibody.

According to our data of neuronal and glial marker expression, NBs shared the potential for neural commitment/differentiation (Table 3.8). However, the mechanisms underlying the realization of this potential are not operational and the apparent developmental pathways are not functional in these cells.

Table 3.8. Percentage of neuronal and glial marker expression in untreated neuroblastomas.

<table>
<thead>
<tr>
<th>marker</th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Internexin-α</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>N-CAM</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>NF-L-phos</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;5</td>
<td>0</td>
</tr>
<tr>
<td>MAP-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F-12 media supplemented with B27. Following culturing, cells were fixed and stained with antibodies against human-reactive βIII-tubulin, GFAP, internexin-α, NF-L-phos, N-CAM and MAP-2. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The percentage of NBs that exhibited GFAP, internexin-α, N-CAM, NF-L-phos, MAP-2 and βIII-tubulin, over a six-day period, are presented as numbers. ABBREVIATIONS: 0, no expression; "filled box", 100% expression; 90, 90% expression; 80, 80% expression; <1, <1% expression; <5, <5% expression. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.
3.3 The effect of GDNF/TGF-β1 treatment on human neuroblastomas

Many primary NBs express c-Ret, GFRα1, GFRα2 as well as their ligands, GDNF and NTN, suggesting that c-Ret signaling may play an important role in regulating the growth, differentiation, and cell death of NBs [Hishiki et al., 1998]. However, GDNF alone induces a rather weak differentiation independent of the NB stages [Hishiki et al., 1998]. Given that during neurogenesis TGF-β1 acts synergistically with various growth factors to enhance certain intracellular signaling pathways [Unsicker, 2000], we proceeded to study the effect of GDNF/TGF-β1 on human NBs in a six-day culture system, as well as their expression of c-Ret, GFRα1, GFRα2. These results would ultimately determine if our hypothesis, which stated that NBs would alter their growth potential upon exposure to GDNF/TGF-β1, is accepted or not.

3.3.1 Effects of GDNF/TGF-β1 on proliferation of neuroblastomas

First, we examined the expression of c-Ret, GFRα1 and GFRα2 in NBs using immunocytochemical techniques. The reason for this approach was due to the fact that the presence/absence of these growth factor receptors in the NBs could possibly explain their cytostatic/non-cytostatic response to growth factor treatment.

Expression of full-length c-Ret, GFRα1 and GFRα2 were detected in all NB cells, however, GFRα2 immunoreactivity was fairly weak in SK-N-MC and SK-N-SH cells (Table 3.9).
Table 3.9. Percentage of receptor expression in neuroblastomas, before and after GDNF/TGF-β1 treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
<th>GDNF/TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SKNMC</td>
<td>MCIXC</td>
</tr>
<tr>
<td>c-Ret</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFRα1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFRα2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 medium supplemented with B27, in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). Following six days of culturing, cells were fixed and processed for c-Ret, GFRα1 and GFRα2 using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The percentage of NBs that exhibited c-Ret, GFRα1 and GFRα2 expression over a six-day period of GDNF/TGF-β1 treatment, are presented as numbers. ABBREVIATIONS: 0, no expression; “filled box”, 100% expression.

Following six days of culturing, several differences were observed in the proliferation of NBs grown in serum-free medium supplemented with GDNF (100ng/ml) and TGF-β1 (50pg/ml), compared to NB cells grown in the absence of these factors (untreated controls). Proliferation assays of moderate-density cultures (2 x 10^4 cells per well) in a 24-well plate, demonstrated that GDNF/TGF-β1 treatment led to a significantly decreased (a. 3.4±0.3-fold decrease) number of cells in MC-IXC cultures (Table 3.10). In contrast, GDNF/TGF-β1 treatment resulted in a significantly enhanced proliferation of SK-N-MC and SK-N-BE2 cells, with a 3.6±0.4- and 2.2±0.2-fold increase, respectively, whereas no proliferation effect was observed in SK-N-AS and SK-N-SH cells (Table 3.10).
Table 3.10. Effect of GDNF/TGF-β1 on proliferation of neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MCIXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDNF/TGF-β1</td>
<td>3.6±0.4↑</td>
<td>3.4±0.3↓</td>
<td>NE</td>
<td>2.2±0.2↑</td>
<td>NE</td>
</tr>
</tbody>
</table>

**Table legend:** Cells were grown for six days on laminin-coated 24-well plate in DMEM/F12/B-27 media in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). After six days cells were dissociated, an aliquot of cells counted using a hemocytometer. The effect of GDNF/TGF-β1 on cell proliferation was measured using a [³H]thymidine incorporation assay (see materials and methods). Cells were plated on 12-well plates at a density of 2-3 × 10⁴ cells/well and treated with the mix of GDNF/TGF-β1 for 24hours. Cells were labeled for the last five hours with 4μCi of [³H]thymidine, and lysed in 0.2 N NaOH. [³H]thymidine incorporation into the DNA was quantified using a β-counter. Each data point was performed in quadruplicates. Values represent the relative change (in folds) in cell number normalized to the relative change in cell number of untreated cultures. Three independent experiments were performed to assess the changes in cell number. ABBREVIATIONS: NE, -no effect in comparison with control; ↑ - enhanced proliferation in comparison with control; ↓ - reduced proliferation in comparison with control.

We are aware however, that the cell death of MC-1XC cells upon GDNF/TGF-β1 might reflect both cytostatic as well as cytotoxic effects due to the administered drug/growth factors and therefore a cell viability assay, like trypan blue, would have been a highly accurate approach to distinguish between these two outcomes (cytostatic and cytotoxic). Yet, one of the aims of our study was to determine a combination of growth factors/neurotrophins that exhibited suppressive activity on the proliferation of NBs. For this reason numerous growth factor/neurotrophin matrices were examined and the effects of these drug/s or combinations of factors on cell death of different NBs, were estimated by morphological criteria only (see materials and methods, section 2.2.2). Given that in most cases administered factors or combination of factors had no apparent effect on tumor cell growth or on cell death. When considering that NB control cultures were grown for six days in serum-free, growth factor-free medium, there was no significant cell death and the majority of cells, about 80%, of cells survived. Therefore, we considered it unnecessary to examine the cytotoxic or cytostatic nature of these growth factors, any further. Although we do agree that estimates of total cell viability would be necessary to exclude cell death as a cause of the reduction of overall cell number, it should be mentioned that thorough analyses of the mechanisms of the
effects of the drugs (growth factors) were planned as a continuation of the studies presented in the present thesis. Therefore, the identification of possible regulatory routes and respective involvement of TFs following application of certain growth factors/neurotrophins to NBs is only discussed and not subjected to experimental analyses.

3.3.2 Effects of GDNF/TGF-β1 on differentiation of neuroblastomas along neural lineages
To identify whether the pathways that connect transcriptional regulators to neural differentiation, are active/functional in these NBs, we determined the protein level for signaling pathways using immunocytochemical techniques. Protein determination in this case, seemed like the most favorable approach due to the availability of very good antibodies against receptors and members of these signaling pathways. Therefore, to define the effects of GDNF/TGF-β1 on differentiation of NBs, cells were cultured for six days on laminin-coated plates and treated with serum-free medium supplemented with GDNF (100ng/ml) and TGF-β1 (50pg/ml). Post-treatment, NBs were subjected to immunocytochemical analyses using an astrocytic (GFAP) and a neuronal marker (βIII-tubulin). According to our results, GDNF/TGF-β1 treatment induced a significant increase in the number of βIII-tubulin-immunoreactive cells in the SK-N-MC/MC-IXC group of NBs and a marked increase in GFAP-positive cell populations in all NBs (Table 3.11).
Therefore, in medium-density cultures, GDNF/TGF-β1 treatment increased the net production of NB cells with neuronal and glia-like properties (Table 3.11).
Table 3.11. Effects of GDNF/TGF-β1 on the expression of βIII-tubulin and GFAP in neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (untreated)</td>
<td>GDNF/TGF-β1</td>
</tr>
<tr>
<td>SKNMC</td>
<td>0</td>
<td>10-25</td>
</tr>
<tr>
<td>MCIXC</td>
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<td>10-25</td>
</tr>
<tr>
<td>SKNAS</td>
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<td>10-25</td>
</tr>
<tr>
<td>SKNBE2</td>
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<td>10-25</td>
</tr>
<tr>
<td>SKNSH</td>
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<td>10-25</td>
</tr>
</tbody>
</table>

Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 media supplemented with B27 in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). Following culturing for six days, cells were fixed and stained with antibodies against human-reactive βIII-tubulin and GFAP. ABBREVIATIONS: 0, no expression; 10-25, 10-25% expression; “filled box”, 100% expression. The reason for adding “red” blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

3.3.3 Changes in the TF expression profile of neuroblastomas upon GDNF/TGF-β1 treatment

Given that cooperative effects of GDNF and TGF-β1 resulted in either enhanced or inhibited proliferation of NBs (Table 3.10) and elevated expression of certain neural markers (Table 3.11), we next tested our second hypothesis, which stated that extrinsic factors, GDNF/TGF-β1, alter the expression of regulatory genes in NBs (as described in section 3.2). To test our hypotheses, we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different neuroblastomas upon GDNF/TGF-β1 treatment. Since one of the aims of our study was to propose a way to improve the diagnostics of NBs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

Two major adaptations in the TF networks of NBs were observed upon GDNF/TGF-β1 treatment: The first adaptation constituted a group of genes that
exhibited cell-autonomous regulation and showed no change in their expression upon GDNF/TGF-β1 treatment (Table 3.12). Our data were in good agreement with earlier findings showing that differential expression patterns of region-specific TFs observed in vivo were maintained even when neuroepithelial cells were isolated and cultured in vitro [Nakagawa et al., 1996]. This group of genes that showed no change upon growth factor treatment, comprised of 1) early negative regulators of neural differentiation, such as BMP-6, Noggin, Smad-7, Dlx-7; 2) transcriptional regulators linking neural inducers to activation of proneural genes, such as Gli-1, Gli-2, Gli-3, Zic-2, Zic-3; 3) transcriptional regulators linking neural determination to specification of neuronal identity — e.g. proneural and neurogenic genes, such as NN-1, NN-3, NeuroD3, NSCL-1, Hes-1, MyT-1, MyT-3, Olf-1, Olf-1H; 4) expression of factors characterizing fate switches to specific neural lineages, such as GATA-2, GATA-3, Pbx-1, LMO4, Sox-11.

The second adaptation constituted a group of genes that exhibited signal-dependent regulation and revealed expression that was affected by GDNF/TGF-β1 treatment (Table 3.12). Specifically, 1) early negative regulators of neural differentiation in which MC-IXC showed distinctive TF regulation patterns. The expression of BMP-2, Chordin, and Smad-6 was undetectable in MC-IXC cells after treatment (Table 3.12). A recent study reports that the expression of the Smad-6 is dramatically induced by BMP-2 in various cells [Takase et al., 1998], suggesting that the downregulation of Smad-6 in MC-IXC cells, is a direct result of the downregulation of BMP-2 mRNA in these cells upon GDNF/TGF-β1 treatment. Another interesting finding involved TFs of the Msx family. The expression of Msx-1 was upregulated in the SK-N-AS/SK-N-BE2/SK-N-SH group of NBs while expression of Msx-2 was only induced in SK-N-BE2 and SK-N-SH; 2) transcriptional regulators linking neural inducers to activation of proneural genes, in which SK-N-SH showed no expression of Zic-1 mRNA, upon GDNF/TGF-β1 treatment, when compared to untreated cells (Table 3.12). No expression of Irx-2a was detected in MC-IXC and SK-N-BE2 cells. GDNF/TGF-β1 treatment caused a downregulation in the expression of Irx-3 mRNA in SK-N-MC and SK-N-BE2 and an upregulation in Sox-2 expression of SK-N-SH cells; 3) transcriptional regulators linking neural determination to specification of neuronal identity, in which GDNF/TGF-β1 treatment induced the expression of hASH-1, NeuroD1, Hes-5 and Hey-1 in SK-N-MC and MC-IXC cells, while
downregulating the expression of NeuroD2 and NSCL-2 mRNAs in all but SK-N-MC cells (Table 3.12). The expression of HeyL was downregulated and that of MyT-2 mRNA upregulated in MC-IXC cells, whereas HeyL mRNA was upregulated and MyT2 mRNA downregulated in SK-N-BE2 cells. According to these results, the two genes (HeyL and MyT-2) were interdependently regulated in these two NBs (MC-IXC and SK-N-BE2). In a way this regulatory knot is anticipated, given that HeyL is positively [Maier and Gessler, 2000; Leimeister et al., 2000] and MyT (X-Myt1) negatively regulated [Bellefroid et al., 1996] by the Notch/Delta signal transduction pathway. These results indicate that the GDNF/TGF-β1 signaling pathways activate the expression of one or more proneural or neurogenic bHLH genes in NBs; 4) expression of factors characterizing fate switches to specific neural lineages, in which expression of Pax-6 was lost in MC-IXC cells, while that of Dlx-2 upregulated and Brn-5 downregulated in the SK-N-AS/SK-N-BE2/SK-N-SH group of NBs, upon GDNF/TGF-β1 treatment (Table 3.12). Expression of both, Otx-1 and Otx-2 mRNAs was downregulated in SK-N-MC cells, in contrast to SK-N-SH cells, where both genes were upregulated in response to GDNF/TGF-β1 treatment. Otx-1 induction was also observed in SK-N-AS and SK-N-BE2 cells.
Table 3.12. mRNA expression profile of regulatory genes in untreated and GDNF/TGF-β1-treated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
<th>GDNF/TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-MC</td>
<td>IXC</td>
</tr>
<tr>
<td>Early negative regulators of neural differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-2</td>
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<td>BMP-6</td>
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<td>Noggin</td>
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<tr>
<td>Chordin</td>
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<td></td>
</tr>
<tr>
<td>Smad-6</td>
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<td></td>
</tr>
<tr>
<td>Smad-7</td>
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</tr>
<tr>
<td>Msx-1</td>
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<td>Msx-2</td>
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<td>0</td>
</tr>
<tr>
<td>Dlx-7</td>
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<td></td>
</tr>
<tr>
<td>Nkx-2.5</td>
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</tr>
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<td>Gli-1</td>
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</tr>
<tr>
<td>Gli-2</td>
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</tr>
<tr>
<td>Gli-3</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Zic-3</td>
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</tr>
<tr>
<td>Irlx-2a</td>
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<td></td>
</tr>
<tr>
<td>Irlx-3</td>
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<tr>
<td>Sox-2</td>
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</tr>
<tr>
<td>Transcriptional regulators linking neural differentiation to activation of proneural genes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN-1</td>
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<tr>
<td>NN-3</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>HeyL</td>
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</tr>
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<td>MyT-1</td>
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</tr>
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<tr>
<td>MyT-3</td>
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<td>Olf-1</td>
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</tr>
<tr>
<td>Olf-1H</td>
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<tr>
<td>Transcriptional regulators linking neural determination — e.g., proneural and neurogenic genes.</td>
<td></td>
<td></td>
</tr>
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<td>Pbx-1</td>
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<td>Pax-6</td>
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<td>0</td>
</tr>
<tr>
<td>Brn-4</td>
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<td>0</td>
</tr>
<tr>
<td>Brn-5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Table legend:** mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated and GDNF/TGF-β1 treated neuroblastomas. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

**ABBREVIATIONS:** N-MC, SK-N-MC; IXC, MC-IXC; BE2, SK-N-BE2; AS, SK-N-AS; SH, SK-N-SH.

These results identified two adaptations in the regulatory gene expression profile of NBs upon GDNF/TGF-β1 treatment. The first adaptation constituted a group of genes that exhibited cell-autonomous regulation and showed no change in their expression upon GDNF/TGF-β1 treatment (Table 3.12). This group of genes comprised mostly of transcriptional regulators linking neural inducers to activation of proneural genes as well as TFs associated with specification of neuronal identity, e.g. proneural and neurogenic genes. The second group included genes that exhibited signal-dependent regulation and in which the expression was affected upon GDNF/TGF-β1 treatment. This group comprised mostly of TFs associated with specification of neuronal identity, e.g. proneural and neurogenic genes.

Another important outcome of this study comes from the comparison of the responses of the two sibling NB cells (SK-N-MC and MC-IXC) to drug treatment. Exposure of these two sublines of cells to GF treatment resulted in SK-N-MC cells becoming hyper-responsive (responded with enhanced proliferation) to ambient levels of GFs, whereas activating neural differentiation-associated signaling pathways in MC-IXC cells. Yet in cancers, considering that tumor progression accompanies with the increased genomic instability and failed maintenance of genomic integrity [Duesberg and Rasnick, 2000; Duesberg et al., 1999], the situation where neighboring cells are different types of multipotent cells, is very common. By identification of common targets for treatment in these sibling NBs (Table 3.8, Table 3.9A), our approach enabled elaboration of effective therapeutic strategies for these NB tumors and tumors in general.
3.4 The effect of retinoic acid treatment on human neuroblastomas

Good progress has been made in uncovering the steps in the receptor-dependant signaling mechanism by which RA and other retinoids work [Nagpal and Chandraratna, 1998]. Using microarray technology, it was recently established that RA treatment affected the expression of hundreds of molecular markers including immediate early genes (signal transduction modulators and effectors; transcription factors) as well as those that are regulated relatively late in the differentiation process and that encode proteins with clear roles in neuronal function (growth factors and their receptors, cytoskeletal and extracellular matrix proteins, cell surface antigens) [Kelly and Rizzino, 2000]. Beyond the chromatin-remodeling activity, it has been observed that RA promotes its actions by means of transcription-dependant mechanisms [Borriello et al., 2000].

Drug resistance in cancer is a major obstacle to successful chemotherapy. Vitamin A and its derivatives (retinoids) are involved in a diverse array of developmental and physiological regulatory processes having profound effects on the proliferation and differentiation of many cell types and, including those responsible for the development of the mature nervous system. NBs are derived from the neural crest and retain some features of neural progenitors, e.g. the ability to undergo neuronal differentiation in the presence of appropriate signals [Abemayor and Sidell, 1989; Seeger et al., 1982]. Therefore, when treated with RA, human NBs have been used to study differentiation along neuronal lineage [Sidell et al., 1983; Sidell, 1982]. Since RA has shown to have profound effects on the proliferation and differentiation of NBs, it has initiated a series of clinical trials in which RA and its derivatives were used. However, quite disappointingly, the outcome of trials using RA in the battle against NBs is vague. Apparently, cancer cells exposed to RA may be directly induced to express a subset of genes that confers resistance or allows some cells to escape killing and form the relapsed resistant tumor. Indeed, recent studies have demonstrated that in some NBs, RA activates the BDNF-trkB signal transduction pathways that stimulate cell survival, disaggregation, and invasion, i.e. all the characteristics of metastatic cells [Matsumoto et al., 1995], and thus contributes to a more chemoresistant phenotype [Scala et al., 1996]. Therefore, to gain further insight into RA-therapy-related NB progression, changes in the proliferation,
synthesis of neurotransmitters, general neural markers and expression of transcriptional regulators that occurred following RA-treatment of NBs, were monitored.

3.4.1 Effects of RA on proliferation of neuroblastomas

RA and RA- analogues have been identified to induce differentiation and inhibit proliferation of NB cells \textit{in vitro} and \textit{in vivo} [Ponthan et al., 2001; Reynolds, 2000]. We observed that six days of 1\mu M RA treatment resulted in the growth arrest of two out of five NBs, namely SK-N-BE2 and MC-IXC cells. MC-IXC cells exhibited a 3-fold decrease and SK-N-BE2 a 2.2-fold decrease in their mitogenic activity as identified by counts of cell numbers (Table 3.13) (see section 3.3.1 for explanation of cytostatic and cytotoxic effects upon drug treatment). However, six days of RA-treatment did not significantly affect the proliferation rate of SK-N-MC, SK-N-AS and SK-N-SH cells. Our data were in good agreement with previous studies, reporting that SK-N-MC cells are relatively resistant to the anti-proliferative effects of 1\mu M RA in monolayer culture [Haussler et al., 1983]. According to another finding, RA inhibited proliferation and induced accumulation of SK-N-SH cells in the G0/G1 phase of the cell cycle [Goplen et al., 1994]. RA also partially inhibited the proliferation of SK-N-AS cells, although did not induce them to differentiate (ATCC data sheet). In our culture system (media and duration of treatment), the proliferation of SK-N-AS and SK-N-SH cells remained largely unaffected upon six days of RA treatment.

\textbf{Table 3.13.} Effect of RA on proliferation of neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>SK-N-MC</th>
<th>MC-IXC</th>
<th>SK-N-AS</th>
<th>SK-N-BE2</th>
<th>SK-N-SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>+RA treatment</td>
<td>1.3±0.2↑</td>
<td>3.0±0.5↓</td>
<td>1.3±0.2↑</td>
<td>2.2±0.1↓</td>
<td>NE</td>
</tr>
</tbody>
</table>

\textbf{Table legend:} Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 media supplemented with B27 in the presence of 1\mu M RA. After six days cells were dissociated, an aliquot of cells counted using a hemocytometer. The effect of RA on NB cell proliferation was measured using a \textsuperscript{3}Hthymidine incorporation assay. Cells were plated on 12-well plates at a density of 2-3 x 10\textsuperscript{4} cells/well and treated with 4\muCi of \textsuperscript{3}Hthymidine and lysed in 0.2N NaOH. \textsuperscript{3}Hthymidine incorporation into the DNA was quantified using a \textbeta-counter. Each data point was performed in quadruplicates. Values represent the relative change (in folds) in cell number normalized to the cell number of untreated cultures. Three
independent experiments were performed to assess the changes in cell number. ABBREVIATIONS: ↑ - enhanced proliferation; ↓ - reduced proliferation; NE, -no effect compared with control.

As mentioned for growth factor treated NBs, we are aware however, that the cell death of certain NBs (MC-IXC and SK-N-BE2) upon RA-treatment might reflect both cytostatic as well as cytotoxic effects due to the administered drug and therefore a cell viability assay, like tryphan blue, would have been a highly accurate approach to distinguish between these two outcomes (cytostatic and cytotoxic). When considering that NB control cultures were grown for six days in serum-free, RA-free medium, there was no significant cell death and the majority of cells, about 80%, of cells survived. Therefore, we considered it unnecessary to examine the cytotoxic or cytostatic nature of these growth factors, any further. Although we do agree that estimates of total cell viability would be necessary to exclude cell death as a cause of the reduction of overall cell number, it should be mentioned that thorough analyses of the mechanisms of the effects of the drugs (RA) were planned as a continuation of the studies presented in the present thesis. Therefore, the identification of possible regulatory routes and respective involvement of TFs following application of RA to NBs is only discussed and not subjected to experimental analyses.

RA treatment has also been shown to affect the morphology of different cell types that are characteristic of NB cell cultures. N (neuronal)-type cells exhibit prolonged neuronal processes, whereas S (epithelial, substrate-adherent, Schwann cell-like)-type cells lose their adherence to substratum and become apoptotic upon exposure to RA [Voigt et al., 2000]. In our NB cultures we observed a subpopulation of SK-N-MC cells that remained attached on the tissue culture substrata, while another subpopulation became rounded and grew as multi-cellular aggregates. For SK-N-BE2 cells a similar phenomenon was observed except that there was less aggregation (a few spheres) and the attached cells exhibited long neurite-like extensions. SK-N-AS and MC-IXC cells grew as monolayers and their extensions were short and thick (Fig. 3.1,O). SK-N-SH cells exhibited contact-mediated growth inhibition, both in untreated and RA-treated conditions, and revealed long, thin neurite-like extensions (Fig. 3.1B).
Therefore, based the morphology of the five human NBs, SK-N-BE2 and SK-N-SH resembled neural cell morphology the most.

**Figure 3.1.** Expression of various neural markers in five different human neuroblastomas (see next page for figure).

**Figure Legend:** Neural marker expression identified by immunofluorescence in untreated and RA-treated NB cells. NBs were plated on laminin-coated 24-well plates in DMEM/F12 medium (with or without 1μM RA) at a density of 2-3 × 10⁴ cells/well and incubated for 24 hours. Following this incubation, cells were fixed and analyzed for several neural markers, using immunocytochemical techniques. ABBREVIATIONS: c, control (untreated); I-α, internexin-α; TH, tyrosine hydroxylase; +RA, RA-treated.

### 3.4.2 Effects of RA on differentiation of neuroblastomas along neural lineages

To identify whether the pathways that connect transcriptional regulators to neural differentiation, are active/functional in these NBs, we determined the protein level for signaling pathways using immunocytochemical techniques. Protein determination in this case, seemed like the most favorable approach due to the availability of very good antibodies against receptors and members of these signaling pathways. Therefore, to define the effects of RA on differentiation of NBs, cells were cultured for six days on laminin-coated plates and treated with serum-free medium supplemented with 1μM RA. Following six days of exposure to RA, the number of βIIIITubulin-expressing cells was significantly increased in SK-N-SH and MC-IXC cultures (Table 3.14, Fig 3.1B). The expression of GFAP, however, was weak in RA treated SK-N-SH cultures (approximately 1% of cells remained GFAP-immunoreactive) and not present at all in any of the other NBs (Figure 3.1C and D). A significant loss of internexin-α expression was observed in SK-N-AS (approximately 70% of the cells lost internexin-α immunoreactivity) and SK-N-SH (approximately 60% of the cells lost internexin-α immunoreactivity) cells, but not in the other three NBs (Figure 3.1E, F, G, H).

Our data revealed that RA promoted βIIIITubulin expression in a majority of the NBs (MC-IXC, SK-N-BE2, and SK-N-SH), whereas it suppressed differentiation along glial lineages (inhibited expression of GFAP) in all NBs. It is interesting to note here that the expression of βIIIITubulin has been found in most tumors of neuronal origin (ganglioneuroblastoma, ganglieneuroma, medulloblastoma, neuroblastoma,
sympathoblastoma), in contrast to its non-detectable expression in tumors of non-neuronal origin [Draberova et al., 1998]. In view of this background, one may conclude that RA treatment revealed the more neuronal nature of MC-IXC, SK-N-BE2 and SK-N-SH cells, while SK-N-MC and SK-N-AS are most likely of non-neuronal origin (which is in good agreement with their morphology). Our data also revealed that "more neuronal" NBs also tended to preferentially expressed internexin-α, a marker expressed in neural precursor cells at the onset of neuronal differentiation during the development of the nervous system [Fliegner et al., 1994]. In addition to that, another interesting finding from our study revealed the differential effects of RA on the synthesis of glutathione (GSH) in various NBs (see Table 3.18). In normal development, all cells of the embryonic nervous system express high levels of GSH, whereas adulthood neurons (except the dorsal root ganglia and the cerebellar granule cells) become GSH-negative and glia, ependyma, choroid plexus and neurovascular cells become rich in GSH and variously express other GSTs [Lowndes et al., 1994]. Accordingly, reduced levels of GSH in NB cells, in response to RA treatment, might be an indication of initiated neuronal and suppressed glial differentiation in these tumors. As Schwann cell stroma has been reported to greatly increase NB differentiation [Kwiatkowski et al., 1998], it becomes quite evident from these and our findings that drugs which do not support glial differentiation are likely to remain highly ineffective in relation to differentiating NB tumors.

**Table 3.14.** Expression of βIII-tubulin, GFAP and internexin-α in untreated and RA-treated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MC-IXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>βIII-tubulin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>GFAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Internex</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>SKNMC</th>
<th>MC-IXC</th>
<th>SKNAS</th>
<th>SKNBE2</th>
<th>SKNSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>βIII-tubulin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>GFAP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Internex</td>
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<td>0</td>
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<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

**Table legend:** Cells were grown for six days on laminin-coated 24-well plates in DMEM/F-12 media supplemented with B27 in the presence of 1μM RA. After six days of culturing, cells were fixed and processed for βIII-tubulin, GFAP, internexin-α. All the cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for neurons (βIII-tubulin-positive) and astrocytes (GFAP-
positive). The percentages of NBs that exhibited expression for βIII-tubulin, GFAP, and internexin-α over a six-day period of RA treatment are presented as numbers. ABBREVIATIONS: 0, no expression; 1, 1% expression; 10, 10% expression; 20, 20% expression; 30, 30% expression; 50, 50% expression; 90, 90% expression; “filled box”, 100% expression; βIII-tub, βIII-tubulin; internex, internexin-α. The reason for adding “red” blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

Combined our data revealed that NBs exhibited high variability in response to RA treatment based on the rate of tumor growth, changes in their cellular morphology and expression of general neural markers. However, in view of these results one may conclude that RA treatment revealed neuronalness of MC-IXC, SK-N-BE2 and SK-N-SH cells, while SK-N-MC and SK-N-AS are most likely of non-neuronal origin.

3.4.3 Effects of RA on expression of molecular markers commonly used in the prognostic evaluation of NBs

According to our hypotheses, five NB tumors belong to five individual groups and showed individual responses to RA treatment. In a recent study it was shown that about 50% of NBs detected through mass screening had factor(s) indicating an unfavorable biological nature [Tanaka et al., 2000]. In the light of these findings, our data although collected from a limited source of material, showed that several of the currently used prognostic molecular markers do not allow reliable evaluation of the treatment outcome already at in vitro settings. In another mass screening study where biological features, such as N-myc status, DNA ploidy, Shimada histology, NSE and ferritin were investigated, some cases with recurring disease had no unfavorable factors, whereas the factors causing this remained unclear [Tajiri et al., 2001].

trkA/trkB expression.

It has been shown that RA treatment of NBs induced the expression of trkB, the receptor for the neurotrophins, BDNF, NT-3, and NT-4/5 [Kaplan et al., 1993]. In response to six days of RA treatment, we observed upregulation in trkB expression of SK-N-BE2 and SK-N-SH cells, and modest changes in the expression pattern of full-length trkA in SK-N-MC and MC-IXC cells (Table 3.15, Fig. 3.1R, S, T, U).
Table 3.15. Effect of RA on receptor expression in untreated and RA-treated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
<th>RA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>trkB</td>
<td>SKNMC 10%</td>
<td>SKNMC 0%</td>
</tr>
<tr>
<td>trkA</td>
<td>SKNAS 0%</td>
<td>SKNAS 0%</td>
</tr>
</tbody>
</table>

Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 media supplemented with B27 in the presence of 1μM RA. Following culturing, cells were fixed and processed for trkA and trkB using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The percentage of NBs that exhibited trkA and trkB expression over a six-day period, are presented as numbers. ABBREVIATIONS: 0, no expression; 10, 10% expression; 20, 20% expression; 50, 50% expression; “filled box”, 100% expression. The reason for adding “red” blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

Multi-transmitter phenotype.

In adult-derived neural stem cell cultures, RA has been established to sustain or upregulate trkA, trkB, trkC, and p75NGFR expression, whereas the sequential application of RA followed by BDNF or NT-3 led to a significant increase in neurons displaying mature GABA, ChAT, TH, or calbindin phenotypes [Takahashi et al., 1999]. Regarding that, we investigated the possibility that RA could affect the expression of various transmitters in NBs.

Expression of ChAT and TH: It has been reported that some NBs change their neurotransmitter phenotype from noradrenergic to cholinergic under RA treatment as a consequence of changes in the expression and activity of the biosynthetic machinery for both neurotransmitters [Handler et al., 2000]. In cultures of rat post-mitotic sympathetic neurons, RA has been observed to induce cholinergic differentiation of these neurons by reducing the activities of TH and DBH, and decreasing the levels of NE [Berrard et al., 1993]. In good agreement with these findings, in our culture conditions, we did not observe changes in the expression of ChAT in NBs upon RA treatment (Table 3.16). In this context, it is of interest to note that stimulation of ChAT activity has been described for MC-IXC cells upon RA treatment [Casper and Davies, 1989]. We observed that RA treatment significantly reduced the expression of TH in SK-N-AS cells (less than 10% of
cells remained TH-immunoreactive upon treatment) (Figure 3.10, P), whereas it had no effect on the expression of TH in SK-N-BE2 cultures (Table 3.16, Fig. 3.1N). Moreover, we observed that a few SK-N-AS cells (less than 1%) started to express DDC upon RA treatment.

**Table 3.16.** Expression of ChAT, TH and DDC in untreated and RA-treated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>A (Control untreated)</th>
<th>B (RA treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKNMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKCIX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKNAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKNBE2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKNSH</td>
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</tbody>
</table>

**Table legend:** Cells were grown for six days on laminin-coated 24-well plates in DMEM/F-12 media supplemented with B27 in the presence of 1μM RA. Then fixed and stained with antibodies against human ChAT, TH, and DDC. All NBs expressed ChAT, while failed to express detectable levels of DDC. The percentages of NBs that exhibited expression of ChAT, TH and DDC over a six-day period of RA treatment are presented as numbers. ABBREVIATIONS: 0, no expression; 1, 1% expression; 10, 10% expression; 30, 30% expression; "filled box", 100% expression. The reason for adding “red” blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

Changes in expression and nuclear localization of an orphan receptor Nurr1 and a homeodomain transcription factor, a pair of transcription factors that are associated with catecholaminergic phenotype, in NBs upon RA treatment: A pair of transcription factors that is associated with the dopaminergic phenotype in the ventral midbrain during normal development, consists of an orphan receptor Nurr1 and a homeodomain transcription factor Ptx-3 [Hynes and Rosenthal, 1999]. Our data revealed that RA treatment significantly changed the cellular localization of Nurr1 in SK-N-MC, SK-N-BE2 and SK-N-SH cells, by supporting its preferential nuclear translocation (Fig. 3.11, J, K, L, M), whereas no change in the nuclear localization of Nurr1 was observed in MC-IXC and SK-N-AS cells both, before and after treatment. On the other hand, sustained expression of TH was observed only in SK-N-BE2 cells before and after treatment, whereas other NBs exhibited either reduced or lack of TH expression upon treatment, suggesting that expression of TH in NBs could be affected by Ptx-3/Nurr1 interactions. Indeed, we
observed that RA treatment resulted in the loss of Ptx-3 in all but SK-N-BE2 cells (Table 3.17).

**Table 3.17.** Nuclear expression of an orphan receptor, Nurr1, and a homeodomain transcription factor, PITX3, in untreated and RA-treated neuroblastomas.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
<th>RA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nurr1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PITX3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table legend:** Effects of RA-treatment on the expression of Nurr1 protein and PITX3 mRNA in different NBs, as detected by immunofluorescence and RT-PCR, respectively. Cells were cultured for six days with and without RA and then part of the cells was fixed and stained for Nurr1 (shown in Figure 3.1) and the rest of the cells was subjected to total RNA isolation and RT-PCR analysis using PITX3-specific primers. Numbers represent the proportion of Nurr1-positive cells showing Nurr1 nuclear localization out of the total number of cells in the culture. ABBREVIATIONS: 0, no expression; 10, 10% expression; 30, 30% expression; 50, 50% expression; 80, 80% expression; "filled box", 100% expression. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

**Changes in expression of other transmitters:** Next, we examined the changes in the regulation of the expression of other amino acid transmitters in NB cells in response to RA-treatment. Upon RA treatment, the synthesis of an inhibitory amino acid, GABA, was not subject to regulation in any of the NBs (Table 3.18). Whereas the synthesis of another inhibitory amino acid Gly, was substantially downregulated in SK-N-MC, MC-IXC, and SK-N-BE2 cells and not affected in SK-N-AS and SK-N-SH cells. There were outstanding differences observed also in the levels of Glu and Gln between NBs upon RA treatment. Notably, the synthesis of Glu was not detectable in any of the NBs upon treatment, whereas the synthesis of Gln was affected in MC-IXC, SK-N-BE2 and SK-N-SH cells and remained unaltered in SK-N-MC and SK-N-AS (Table 3.18). Accordingly, RA had selective effects on glycinergic, glutamatergic and glutaminergic pathways and did not affect GABAergic pathways in RA-differentiated NB cells. Cells have developed a number of defensive mechanisms to maintain intracellular redox homeostasis.
including the glutathione (GSH) system. Intracellular levels of GSH have been shown to affect the sensitivity of cells to cell death-inducing stimuli, as well as the mode of cell death [Fernandes and Cotter, 1994]. Redox disequilibria induced by GSH depletion may serve as a general trigger for apoptosis in neuronal cells [Nicole et al., 1998]. Recent studies have demonstrated that various tumors express enhanced levels of GSH. Moreover, there are grounds for claiming that GSH plays a crucial role in cell proliferation and tumor resistance [Locigno and Castronovo, 2001]. Our data showed that even though under the RA treatment, GSH levels were significantly reduced in most of NBs with the exception of SK-N-AS cells, significant decrease in cell number was observed only in RA-treated MC-IXC and SK-N-BE2 cultures (Table 3.18).

Table 3.18. Differentiated neuroblastomas showed heterogeneous responses to transmitter synthesis.

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<th>A Control (untreated)</th>
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<th>SKNAS</th>
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Table legend: Differentiation of NBs with RA revealed differences in the percentage of cells that expressed transmitters before versus after treatment. The above table shows the percentages of cells synthesizing/accumulating GABA, L-Glutamate (Glu), L-Glutamine (Gln), Glycine (Gly) and Glutathione (GSH) before and after treatment with 1μM RA for six days. ABBREVIATIONS: 0, no expression; 10, 10% expression; 30, 30% expression; 40, 40% expression; 90, 90% expression; “filled box”, 100% expression. The reason for adding “red” blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

To summarize, in most of the untreated cells, concentrations of the amino acid neurotransmitters, GABA, glutamate (Glu), glutamine (Gln), glycine (Gly) and glutathione (GSH) were low in some they were markedly high. This made it quite easy to identify NB cells by the combination of synthesized amino acid neurotransmitters. All cell lines contained Gly and GSH and in many NBs they coexisted with other amino acids such as Glu, Gln, but in none with GABA. The patterns of the amino acid
neurotransmitter-content suggested that there are no purely mono-transmitter cell populations in NBs. Given that NBs are highly proliferative tumors, due in part by a number of active neurotransmitter and neuropeptide metabolic pathways supporting their proliferation [Lantos et al., 1997], the combination of synthesized amino acid neurotransmitters may allow to predict their grade of aggressiveness. Accordingly, mechanisms regulating transmitter synthesis during NB development, such as soluble factors, cell-cell contact and others, are of crucial importance in tumor responses to drug treatments. Our study showed that RA treatment, which had different effects on the proliferation and differentiation of NB cells, caused changes in the intracellular concentrations of some neurochemicals. Although all NBs expressed ChAT and some expressed also TH, one evident finding revealed that upon RA treatment all NBs continued to express ChAT, while no initiation of TH expression was present. On the contrary, a majority of NBs showed no or little TH expression upon treatment. During normal development, the transmitter properties of both developing and mature sympathetic neurons have been found to be plastic where an initial, immature state is adrenergic which is then converted to a cholinergic state [Potter et al., 1980]. Here, we showed that although RA induced cholinergic neurochemical differentiation of NBs, in the majority of cases, it is not accompanied by the mitotic inhibition and maturation of cells composing the tumor to neurons and glia.

3.4.4 Changes in the TF expression profile of NBs upon RA treatment
According to our second hypothesis the sensitivity of NBs to RA, is determined by their level of expression of regulatory genes. To test our hypotheses, we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different neuroblastomas upon RA treatment. Since one of the aims of our study was to propose a way to improve the diagnostics of NBs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that
quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

As observed upon GDNF/TGF-β1 treatment, our results revealed two major adaptations in the TF networks of NBs upon RA treatment: The first adaptation constituted a group of genes that exhibited cell-autonomous regulation and showed no changes in their expression upon RA treatment (Table 3.25C; Table 3.19). This group of genes comprised mostly of 1) *early negative regulators of neural differentiation*, such as BMP-2, BMP-6, Noggin, Chordin, Smad-7, Msx-1; 2) *transcriptional regulators linking neural inducers to activation of proneural genes*, such as Gli-1, Gli-2, Zic-2, Zic-3, Sox-2; 3) *transcriptional regulators linking neural determination to the specification of neuronal identity - proneural and neurogenic genes*, such as NN-1, Olf-1, Olf-1H, NeuroD3, NSCL-1, Hes-1, Hey-2, HeyL, MyT-2, MyT-3; 4) *transcription factors characterizing the fate switches to specific neural lineages*, such as GATA-2, GATA-3, LMO4, Pax-6 and Sox-11. The second adaptation constituted a group of genes that exhibited signal-dependent regulation and revealed expression that was affected by RA treatment (Table 3.21). Specifically, 1) *early negative regulators of neural differentiation*, such as Smad-6 mRNA was upregulated in SK-N-BE2 and SK-N-SH cells and downregulated in MC-IXC cells, whereas expression of Msx-2 was upregulated in SK-N-AS, and SK-N-BE2 and SK-N-SH cells upon RA treatment. Dlx-7 expression was downregulated in SK-N-MC/MC-IXC group and SK-N-SH, whereas Nkx-2.5 was upregulated in SK-N-MC and SK-N-SH upon RA treatment; 2) *transcriptional regulators linking neural inducers to activation of proneural genes*, as RA affected the expression of Gli-3 mRNA in MC-IXC and SK-N-AS cells, the expression of Zic-1 mRNA in SK-N-MC and SK-N-SH cells, and the expression of Irlx-2a mRNA in MC-IXC cells, and Irlx-3 mRNA in SK-N-MC and SK-N-BE2 cells; 3) *transcriptional regulators linking neural determination to the specification of neuronal identity, e.g. proneural and neurogenic genes*, as upregulation of hASH-1, NeuroD1 and Hey-1 mRNAs and downregulation of MyT-1 mRNA was observed in SK-N-MC and MC-IXC cells, whereas downregulation of NeuroD2 mRNAs was detected in all NBs, and that of NSCL-2 mRNA in SK-N-MC and SK-N-SH cells, however, upregulated expression of NSCL-2 mRNA was detected in SK-N-SH cells upon treatment; changes were also observed in the expression of NN3
and Hes-5 mRNAs upon RA treatment; 4) transcription factors characterizing the fate switches to specific neural lineages, as the expression of Brn-4 mRNA was decreased in all of NBs and Brn-5 mRNA was downregulated in SK-N-AS, and SK-N-BE2 and SK-N-SH cells, and that of Dlx-7 and Pbx-1 mRNAs in SK-N-AS and SK-N-SH cells, respectively, whereas induction of Dlx-2 mRNAs was observed in SK-N-AS, and SK-N-BE2 cells. Otx-1 and Otx-2 mRNAs were subject to regulation in SK-N-SH and SK-N-MC cells, respectively.
Table 3.19. mRNA expression profile of regulatory genes in untreated and RA-treated neuroblastomas.

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**Table legend:** mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated and RA-treated neuroblastomas. To
visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same neuroblastoma, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

ABBREVIATIONS: N-MC, SK-N-MC; IXC, MC-IXC; BE2, SK-N-BE2; AS, SK-N-AS; SH, SK-N-SH.

Taken together, the addition of RA switched transmitter phenotypes in all five the NBs and caused specific changes in the regulation of trks, general neural markers and transcriptional regulators in these tumors.

Interestingly, many of the changes in the expression of some of these transcription factors have been evidenced in normal cells/development to occur in response to RA. It is worthwhile noting here that the BMP-2 promoter has been shown to be regulated by RA [Helvering et al., 2000], as well as a novel cis-acting RA-responsive element being implicated in the regulation of Hes-1 gene [Wakabayashi et al., 2000]. During Xenopus neurogenesis RA has been identified to expand the X-MyT-1, X-NN-1 and Gli-3 domains and inhibit the expression of Zic-2 and Shh in the neural ectoderm [Franco et al., 1999]. Exposure of adult rat hippocampus-derived neural stem cell cultures to RA has been shown to cause an immediate up-regulation of NeuroD, increased p21 expression, and concurrent exit from the cell cycle [Takahashi et al., 1999]. Similar to that, RA-treatment induced and greatly increased MASH-1, NeuroD and NSCL-2 mRNA in mouse teratocarcinoma P19 cells [Itoh et al., 1997]. Specific changes that have been described in association with RA-mediated neuronal differentiation of NBs include transient activation of Hes-1 and down-regulation of hASH-1 expression [Grynfeld et al., 2000; Ichimiya et al., 2001]. Our study is unique in the sense that we demonstrated distinct sets of transcription regulators that are implicated in neurogenesis and are affected upon RA treatment, and that some changes lead to induced proliferation of NBs, whereas others result in the initiation of differentiation and cell death. Combined our data revealed that NBs exhibited variability in their regulation of TF expression upon RA treatment, as do they respond to RA with great variability in their mitotic activity, neurotransmitter profile, and expression of neural phenotypic markers.
Finally, our study determined whether the different gene expression profiles of these tumors as well as gene expression variability observed in untreated and treated (GDNF/TGF-β1 and RA) NBs, was statistically significant. To accomplish this, we firstly performed the analyses in all five treated (GDNF/TGF-β1 and RA) and untreated NBs in the four different gene groups by counting the number of genes expressed in each gene group for each tumor, also upon treatment. Secondly, the expression data of all four groups of genes were combined in order to finally determine the statistical significance of total gene expression differences between the five human NBs as well as between treatments applied. The analyses were performed using histograms, to reveal number of genes expressed, and two-way analysis of variance (ANOVA)/F-test (95% confidence level), to reveal the statistical significant difference between tumors and treatments in a specific gene group. Upon identification of a significant difference in a specific gene group, a Bonferroni post hoc test was performed to identify between which tumors or treatments the significant differences were observed. Given that every gene, belonging to a gene group, has a specific individual function apart from the main function of the group, therefore, in order for us to perform statistical analyses of the data, we have to assume that all genes in a given gene group perform the same function which is the main function of that specific gene group.

**Gene Group 1: Early negative regulators of neural differentiation.**

As revealed previously, the group of early negative regulators of neural differentiation consisted of 10 genes that included signals that can suppress neural differentiation and promote epidermogenesis. This group included the genes BMP-2, BMP-6, Noggin, Chordin, Smad-6, Smad-7, Msx-1, Msx-2, Dlx-7 and Nkx-2.5. Our histogram (Figure 3.2), revealed the total of group 1 genes expressed in the five untreated, GDNF/TGF-β1 and RA-treated NBs. According to the structure/results of this histogram, the SK-N-MC/MC-IHC group, revealed the highest expression out of 10 genes, also upon treatment. Upon performing a two-way analysis of variance, it was clear that there was a significant difference ($P = 0.0002$) between the five NBs based on the different number of group 1 genes expressed in each tumor. However, no significant difference ($P = 0.57$) was found in the gene expression patterns between any of the treatments.
Figure 3.2. Histogram of untreated, GDNF/TGF-β1 and RA-treated NBs revealing expression of early negative regulators of neural differentiation (Gene Group 1).

Figure legend: mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents early negative regulators of neural differentiation (Gene Group 1). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars); GDNF/TGF-β1 (red bars) and RA (blue bars) treated neuroblastomas. To visualize results, the total number of genes expressed in Gene Group 1, by a single NB sample, is represented by a bar. Data are presented as mean ± SD (n = 10), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. SK-N-BE2, SK-N-AS, SK-N-SH were considered significantly different from SK-N-MC and MC-IXC when analysis of variance showed an F value with \( P < 0.02 \).

Since our data revealed that tumors, expressing group1 genes, are significantly different from each other, a Bonferroni post hoc test was performed to determine between which tumors were the significant variation observed. According to our data, the gene expression of SK-N-MC and MC-IXC is significantly different from SK-N-BE2 (\( P = 0.015 \)), SK-N-AS (\( P = 0.004 \)) and SK-N-SH (\( P = 0.014 \)).

Therefore, according to our analyses, there is a significant difference between these tumors in regard to their expression of early negative regulators of neural differentiation. Since the high expression of group 1 genes in the SK-N-MC/MC-IXC group indicated suppression of neural differentiation and promotion of epidermogenesis, we can conclude from our results that other than neural developmental programs are also active in the SK-N-MC/MC-IXC group of NBs compared to the other three tumors.

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Therefore, according to our analyses, there is a significant difference between these tumors in regard to their expression of early negative regulators of neural differentiation. Since the high expression of group 1 genes in the SK-N-MC/MC-IXC group indicated suppression of neural differentiation and promotion of epidermogenesis, we can conclude from our results that other than neural developmental programs are also active in the SK-N-MC/MC-IXC group of NBs compared to the other three tumors.
**Gene Group 2:** Transcriptional regulators linking neural differentiation to activation of proneural genes.

The nine genes constituting transcriptional regulators linking neural differentiation to activation of proneural genes are responsible for promoting primary neurogenesis. These genes are activated upon suppression of BMP signaling by neural inducers [Sasai, 1998]. Gene group 2 consisted of Gli-1, Gli-2, Gli-3, Zic-1, Zic-2, Zic-3, Irx-2a, Irx-3 and Sox-2. Results from this gene group showed that SK-N-MC had the highest expression out of nine genes, also upon treatment. Upon performing a two-way analysis of variance, it was clear that there was an extremely significant difference ($P < 0.0001$) between the five NBs in regard to the number of group 2 genes expressed. However, we observed that the gene expression for each treatment was not significantly different ($P = 0.749$) from each other.

**Figure 3.3.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NBs revealing expression of transcriptional regulators linking neural differentiation to activation of proneural genes (Gene Group 2).

![Histogram of gene expression](image)

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents transcriptional regulators linking neural differentiation to activation of proneural genes (Gene Group 2). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated neuroblastomas. To visualize results, the total number of genes expressed in Gene Group 2, by a single NB sample, is represented by a bar. The number of genes was expressed as mean ± SD ($n = 9$), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. MC-IXC, SK-N-BE2, SK-N-AS and SK-N-SH were
considered significantly different from SK-N-MC and MC-IXC significantly different from SK-N-BE2 when analysis of variance showed an $F$ value with $P < 0.02$.

Since the variation between the tumors was significant, it allowed us to perform a Bonferroni post hoc test to reveal which tumors differed from each other. Our data revealed that the gene expression of group 2 genes in SK-N-MC is significantly different from MC-IXC ($P = 0.008$), SK-N-BE2 ($P = 0.003$), SK-N-AS ($P = 0.003$) and SK-N-SH ($P = 0.003$). Also was there a significant difference observed between MC-IXC and SK-N-BE2 ($P = 0.015$). As observed in gene group 1 (Figure 3.2), the fact that the SK-N-MC showed the highest gene expression is an indication that apart from neural-, non-neural programs are also active in this tumor. We can therefore also conclude, that the other four tumors, especially SK-N-BE2, SK-N-AS and SK-N-SH had less active non-neural programs than the other two NBs.

**Gene Group 3: Transcriptional regulators linking neural determination to specification of neurogenic genes – e.g. pronuclear and neurogenic genes.**

The genes, constituting transcriptional regulators linking neural determination to specification of neurogenic genes is operational during normal development and is well applicable for monitoring the activated developmental programs in NBs. This group of genes involved pronuclear and neurogenic genes of the bHLH family. Gene group 3 consisted of 18 regulatory genes namely, NN-1, NN-3, hASH-1, NeuroD1, NeuroD2, NeuroD3, NSCL-1, NSCL-2, Hes-1, Hes-5, Hey-1, Hey-2, HesL, MyT-1, MyT-2, MyT-3, Olf-1 and Olf-1H. According to the histogram for gene group 3 (Figure 3.4), the expression patterns for the genes of this group were very similar. The highest number of genes expressed was observed in untreated and GDNF/TGF-β1 treated SK-N-BE2 and SK-N-MC, respectively. Our two-way analysis of variance data revealed that there was no significant difference ($P = 0.432$) between the five NBs in regard to the amount of group 3 genes expressed. Also was there no significant difference ($P = 0.246$) observed in the gene expression patterns for each treatment.

These results are in good agreement with our findings of neuronal marker expression in these NBs, revealing that all NBs exhibited some level of neuronal expression, with the SK-N-MC/MC-IXC group revealing a lower expression of neuronal
markers compared to the other three tumors. Therefore, we can speculate that all five
NB tumors, exhibited expression of neuronal markers/activity. We can also speculate
that the SK-N-MC/MC-IXC group has more active non-neural programs and less active
neuronal programs when compared to the other three tumors (SK-N-BE2, SK-N-AS and
SK-N-SH), which were just the opposite.

Figure 3.4. Histogram of untreated, GDNF/TGF-β1 and RA-treated NBs revealing expression of
transcriptional regulators linking neural determination to specification of neurogenic genes (Gene Group 3).

Figure legend: mRNA expression data are shown in histogram-format with each bar representing the
number of genes expressed in the group of genes that represents transcriptional regulators linking neural
determination to specification of neurogenic genes (Group Group 3). Three bars grouped together
represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars),
GDNF/TGF-β1 (red bars) and RA (blue bars) treated neuroblastomas. To visualize results, the total
number of genes expressed in Gene Group 3, by a single NB sample, is represented by a bar. The
number of genes was expressed as mean ± SD (n = 18), calculated for three independent experiments.
SD values were calculated between tumors of the same treatment. No significant P values could be
derived from the analysis of variance for the five tumors, as well as the different treatments.

Gene Group 4: Transcription factors characterizing fate switches to specific neural
lineages.
The 11 genes constituting transcription factors that characterize fate switches to specific
neural lineages are Brn-4, Brn-5, Dlx-2, Gata-2, Gata-3, LMO4, Otx-1, Otx-2, Pax-6,
Pbx-1, and Sox-11. During normal development, cells that are selected to become
neurons begin to express NeuroD1 [Korzh et al., 1998; Lee et al., 1995]. However, numerous other regulators are needed to provide fine positional and specification information to maturing neurons. Therefore, we can assume that gene group 4 consists of transcriptional regulators that have previously been identified to specify various neuronal phenotypes. Our histogram (Figure 3.5) revealed that, as seen in gene groups 1 and 2, the SK-N-MC/MC-IXC group again has the highest expression of all untreated NBs. However, the highest gene expression for growth factor treated NBs were observed in SK-N-SH. According to our analysis of variance there was almost a significant difference ($P = 0.0517$) observed between the five NBs in regard to the amount of group 4 genes expressed. However, there was definitely no significant difference ($P = 0.229$) observed in the gene expression patterns for each treatment.

**Figure 3.5.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NBs revealing expression of transcription factors characterizing fate switches to specific neural lineages (Gene Group 4).

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents transcription factors characterizing fate switches to specific neural lineages (Gene Group 4). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated neuroblastomas. To visualize results, the total number of genes expressed in Gene Group 4, by a single NB sample, is represented by a bar. The number of genes was expressed as mean ± SD ($n = 11$), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. No significant $P$ values could be derived from the analysis of variance for the five tumors, as well as the different treatments.
Upon combining the number of genes expressed in all four groups of genes, for each tumor (now assuming all 48 neurogenesis-involved regulatory genes having the same function), we observed that (Figure 3.6) the SK-N-MC/MC-IXC group of NBs showed the highest number of gene expression, when compared to the other three tumors (which seemed to group together). No drastic changes were observed when comparing the different treatments to each other, in all of the tumors. According to our two-way analysis of variance results, the five NBs were significantly different ($P < 0.0001$) from each other in regard to their expression of the 48 neurogenesis-involved regulatory genes, analyzed in our study. However, the gene expression patterns for each treatment, was not significantly different ($P = 0.128$) from each other. In order to determine which NBs are significantly different from each other based on their expression of the 48 regulatory genes, we performed a Bonferroni post hoc test. The data of this test revealed that gene expression of SK-N-MC was significantly different from SK-N-BE2 ($P = 0.008$), SK-N-AS ($P = 0.0005$) and SK-N-SH ($P = 0.007$). We also observed a significant difference between the gene expression of MC-IXC and SK-N-BE2 ($P = 0.008$).

**Figure 3.6.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NBs revealing the number of genes expressed in all four groups of genes, combined.

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in all four groups of genes, combined. Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated neuroblastomas. To visualize results, the number of
genes expressed in all four groups of genes combined, by a single NB sample, is represented by a bar. The number of genes was expressed as mean ± SD (n = 48), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. SK-N-BE2, SK-N-AS and SK-N-SH were considered significantly different from SK-N-MC and MC-IXC significantly different from SK-N-BE2 when analysis of variance showed an F value with P < 0.01.

The aforementioned results of the five human NBs analyzed in this study as well as previous discussion about our data confirmed by the literature, allowed us to reject the null hypotheses and accept our first hypotheses in that each of the five analyzed, NB tumors is molecularly different from the other based on their expression of the four groups of neurogenesis-involved regulatory genes. Based on our statistical analyses, we accepted the null hypotheses and therefore rejected our second hypotheses in that the five NBs did not exhibit significantly different responses to drug (GDNF/TGF-β1 and RA) treatment. The results of our second hypothesis could therefore suggest that 1) NBs just do not respond significantly to the extrinsic factors that were administered in the present study, or 2) the drug treatments (extrinsic factors) were not sufficient enough for the differentiation of these five NBs in regard to altering their untreated regulatory gene expression profile or that 3) the approach we undertook in statistically identifying differences between treatments in these NBs, was just not sensitive enough to reveal the changes/differences.
B. PULMONARY NEUROENDOCRINE TUMORS

Before discussing the results obtained from NETs, it is appropriate to accentuate again the primary motivation for the current study, which was to show that different types of NETs have characteristic expression profiles of regulatory genes. Moreover, our study also investigated whether and how these profiles change upon treatment with certain extrinsic factors/agents.

3.5 Molecular criteria commonly used in the prognostic evaluation of pulmonary neuroendocrine tumors

There are several markers characteristic of NETs: 1) presence of voltage-dependant Na+ and/or Ca+ channels in the cell membrane; 2) receptors for specific ligands (e.g. nerve-growth factor); 3) specific cytoskeletal proteins (e.g. intermediate filaments, neurofilaments); 4) NSE; 5) granule matrix constituents (e.g. chromogranins and Leu-7); 6) granule membrane constituents (e.g. cytochrome B561, synaptophysin, N-CAM); 7) specific amine biosynthetic enzymes; 8) expression of growth factors and nuclear antigens [Heitz, 1986; Kloppel and Heitz, 1994; Wiedenmann and Huttner, 1989; Chaudhry et al., 1992; Langley, 1994]. These markers are generally referred to as "broad-spectrum markers" and they are very valuable in the diagnosis of tumor producing messengers as well as in the diagnosis of inactive or poorly differentiated tumors. Therefore, in view of these published characteristics of NETs, our study compared the prognostic impact of several molecular variables on five different human NETs with the aim to predict susceptibilities of these tumors to anticancer agents.

trkA/trkB/trkC expression

Initially we determined the expression of specialized receptors for neurotrophins, such as the trk family members (trkA, trkB, trkC) in NETs using immunocytochemical techniques. Our data revealed no full-length trkC expression in any of the NETs, whereas immunoreactivity of full-length trkA and trkB was detected in H835, MC-11 and H1770 cells (Table 3.20; Figure 3.7B and C).
Table 3.20. trk (full-length) receptor profile in different human pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th></th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC-11</th>
<th>H1770</th>
</tr>
</thead>
<tbody>
<tr>
<td>trkA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>trkB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>trkC</td>
<td>0</td>
<td>0</td>
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</table>

Table legend: Cells were cultured for six days on laminin-coated 24-well plates in serum-free DMEM/F12 medium supplemented with B27 and in the presence of 1uM RA. After six days, cells were fixed and processed for trkA, trkB and trkC using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei and four fields of 200 cells were counted for evaluation. The percentage of NETs exhibiting expression for trkA, trkB and trkC over a six-day period, upon RA treatment is presented as numbers. ABBREVIATIONS: 0, no expression; “filled box”, 100% expression.

Figure 3.7. Expression of receptors in five different human pulmonary neuroendocrine tumors (see next page for figure).

Figure Legend: Trk receptor expression identified by immunofluorescence in untreated NETs. NETs were plated on laminin-coated 24-well plates in DMEM/F12 medium at a density of 2-3 × 10^4 cells/well and incubated for 24 hours. Following this incubation, cells were fixed and analyzed for several neural markers, using immunocytochemical techniques.

3.6 Identification of TF expression profile of human pulmonary neuroendocrine tumors

It has become clear that some of the same genetic pathways that lead to normal development are altered in cells that have gone awry in cancer [Zhang et al., 1997; Holzman, 1996]. According to Monod and Jacob, cell differentiation is achieved by the activation of specific sets of genes, with each distinct cell type expressing a different subset of genes [Monod and Jacob, 1961]. The activation of specific genes in each stage of cell development is controlled by transcriptional regulators, which altogether, consolidate a Network of active Transcriptional Regulators (NTR). NTR, thus, can be defined as the molecular machinery that controls all the important aspects of a cell’s life, including responses to external as well as internal changes.
Therefore, our study asked the question whether the transcriptional regulator network approach is applicable to tumors (NETs), in other words, are tumors molecularly different based on their expression of regulatory genes? In order to test this hypothesis, we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different NETs. Since one of the aims of our study was to propose a way to improve the diagnostics of NETs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

The analyzed transcriptional regulators were all related to the regulation of neurogenesis and the expression of some of them (NeuroDs, for example) has been shown to accompany with cellular decisions of exiting cell cycle at different stages of development. The set of analyzed genes included secretory proteins (e.g. BMP-2, BMP-6, Noggin, Chordin and Shh) and transcription regulators that are downstream in the signaling cascade induced by secretory proteins, namely bHLH, zinc-finger (ZF), high mobility group (HMG) and homeodomain-containing (HD) transcription factors (TFs).

**Gene Group 1: Early negative regulators of neural differentiation.**

All NET cell lines expressed regulators for both BMP-supportive and antagonistic signaling (Table 3.21). According to others [Schneider et al., 1999], BMPs induce autonomic neurogenesis in neural crest cultures and stimulate sympathetic neuron development when overexpressed *in vivo*. Of all the regulatory factors implicated in BMP signaling, the homeodomain TFs, Msx-1 and Msx-2, clearly indicated the differences between these NETs, for it was only expressed in H727 and H720 compared to MC-11 and H1770, where it was not detected. H835 expressed only Msx1. In addition, other differences were also observed among the different tumors, such as the expression of Noggin (not identified in H720, MC-11 and H1770) and Smad-6 (not expressed in H835 and MC-11) (Table 3.21). The presence of BMP
antagonists, Noggin and Chordin, as well as inhibitory Smads, reveal a strong auto-inhibitory signaling in most of these NETs that support differentiation along neural lineages. Simultaneous expression of the Msx family of TFs, in some of the NET cell lines, suggests divergent differentiation traits for these lung tumors.

Surprisingly, H727 and H720 expressed the transcription factor, Nkx-2.5 (involved in myogenic pathways) (Table 3.21). Nkx-2.5 marks the earliest recognizable cardiac progenitor cells and is activated in response to inductive signals involved in lineage specification. This TF is also expressed in the developing foregut, thyroid, spleen, stomach and tongue [Reecy et al., 1999] and is also required for the determination of developmental asymmetry of the heart [Biben and Harvey, 1997] and spleen [Patterson et al., 2000]. Furthermore, some previous studies have demonstrated that the expression of region-specific genes is regulated in a lineage-dependant cell-autonomous manner, compared to an environment-dependant non-cell autonomous manner [Lumsden et al., 1994; Robel et al., 1995; Nakagawa et al., 1996]. All NETs expressed Dlx-7, except H835 cells.

Table 3.21. mRNA expression profile of early negative regulators of neural differentiation in untreated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>BMP-2</th>
<th>BMP-6</th>
<th>Noggin</th>
<th>Chordin</th>
<th>Smad-6</th>
<th>Smad-7</th>
<th>Msx-1</th>
<th>Msx-2</th>
<th>Dlx-7</th>
<th>Nkx-2.5</th>
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</table>

Table legend: mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same
NET, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

**Gene Group 2: Transcriptional regulators linking neural inducers to activation of proneural genes.**

Consistent with others [Sasaki et al., 1999], the data of this study showed a co-expression of Gli-1, Gli-2 and Gli-3 in H720 and H727 cells, respectively (Table 3.22). In addition, the expression of Gli-1 was detected in MC-11 and Gli-2 in H835 and H1770. Expression analysis of the Zic family of TFs revealed that Zic-2 was expressed in all carcinoids (H720, H835, MC-11, H720) but not in the NSCLC (H1770) (Table 3.22). Zic-1 and Zic-3 could be useful markers to discriminate between different types of typical carcinoids, for Zic-1 expression was only detected in MC-11 and Zic-3 in H720 and H835. Given that during development significant differences in the spatial expression of Zic-1, Zic-2 and Zic-3 have been observed, at all stages [Nagai et al., 1997], it is possible to argue that the expression signature of the Zic family could be considered as a cell type-specific classification marker for certain types of NETs. Finally, our data indicated that Irx-2a was expressed in all NETs whereas no Irx-3 was observed in any of these cell lines (Table 3.22). Sox-2 was detected in all NETs except in H1770.

**Table 3.22.** mRNA expression profile of transcriptional regulators linking neural inducers to activation of proneural genes in untreated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>A. Control (untreated)</th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC11</th>
<th>H1770</th>
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<td>Gli-1</td>
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</table>
Table legend: mRNA expression data are shown in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same NET, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflects intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

Gene Group 3: Transcriptional regulators linking neural determination to the specification of neuronal identity, e.g. proneural and neurogenic genes.

The expression of hASH-1 was detected in all NETs except for H1770 (Table 3.23). This data were confirmed by another group [Ito et al., 2001], showing the expression of hASH-1 only in carcinoids and small cell lung carcinomas (SCLC) but not in non-small cell lung cancer cells (NSCLCs). A comparison of the expression between hASH-1 and NN-3 revealed their co-expression in H727, H835 and MC-11 cell lines (Table 3.23). During development, neurogenins and MASH-1 are expressed in complementary domains of the nervous system [Ma et al., 1997; Fode et al., 2000]; therefore it might be possible that different cell populations in individual NETs express these pro-neural genes. Alternatively, these two programs may operate in the same cell, revealing the deregulated (Notch/Delta) signaling. NN-1, NN-2 and Math-1 have recently been shown to regulate not only neurogenesis, but also neuronal subtype specification [Scardigli et al., 2001; Gowan et al., 2001]. Interestingly, the expression of downstream bHLH TFs, characteristic of neuronal post-mitotic differentiated cells including NeuroDs (NeuroD1, NeuroD2, NeuroD3) and NSCLs (NSCL-1 and NSCL-2), were observed in all NETs, however in variable patterns. H1770 expressed all these neuronal TFs. None of the NE tumor cell lines expressed NSCL-2, except H1770. H727 and H720 cells did not express NeuroD3, whereas MC-11 showed an expression for NeuroD3 but not for NeuroD2. The H835 cells showed no NeuroD expression (Table 3.23). All NETs, except MC-11, expressed Hes-1, Hes-5 and all three members of the Hey family of TFs, Hey-1, Hey-2 and HeyL, respectively (Table 3.23). Hes-5 expression was not detected in MC-
11 cells. All tumors expressed MyT-1 and MyT-3, however MyT-2 was detected only in H727, H835 and H1770.

Recent data have demonstrated that Notch signaling prevents premature differentiation of neuronal [Kageyama and Ohtsuka, 1999] and pancreatic endocrine cells [Jensen et al., 2000], confirming the notion that Notch signaling is important for differentiation processes within the groups of equivalent cells [Chitnis, 1995]. Consistent with this finding, the data of our study suggested that aberrant Notch signaling grounds the basis for high expression of NE and neuronal markers observed in proliferating NETs. Olf-1 expression was not detected in H720 and MC-11, whereas all NETs expressed Olf-1H.

**Table 3.23.** mRNA expression profile of transcriptional regulators linking neural determination to specification of neuronal identity in untreated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (untreated)</th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC11</th>
<th>H1770</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN-1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NN-3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hASH-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NeuroD1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NeuroD2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NeuroD3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NSCL-1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>NSCL-2</td>
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<td>Hes-1</td>
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<td>Hes-5</td>
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<td>Hey-1</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
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</tr>
<tr>
<td>Olf-1</td>
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<td>Olf-1H</td>
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<td>0</td>
</tr>
</tbody>
</table>

**Table legend:** mRNA expression data are show in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same NET, were analyzed three months apart and these samples were highly correlated in gene expression with each
other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

**Gene Group 4: Transcription factors characterizing the fate switches to specific neural lineages.**

Furthermore, all cell lines expressed GATA-2, LMO4, Dlx-2, whereas the expression of the rest of the regulators varied. GATA-3 expression was absent in H835, MC-11 and H1770 cell lines. In addition, MC-11 and H720 also lacked Olf-1 expression (Table 3.24). Pbx-1 was undetectable in H1770. Sox-11 was not identified in H727 and H720 cell lines (Table 3.24). These differences in the expression of regulators characteristic to immature neural progenitors may reflect that each of these tumors has a different developmental history (being derived from non-identical progenitors). Alternatively, environmental factors, such as cell culture conditions, were permissive (could elicit/support) for expression of certain factors and restrictive for others with regards to defective regulatory circuits/networks in tumors. Therefore, the identification of TF expression that distinguishes highly mitotic and immature regions of the nervous system in NETs during normal development, suggest that all these tumors have characteristics of neural stem or progenitor cells.

An interesting finding observed in this study, was the expression of the Otxs, where Otx-1 is expressed in all NETs and Otx-2 not in any of them. Pax-6 was only present in H720, MC-11 and H1770. Only H720 and H835 expressed Brn-2, whereas MC-11 and H1770 expressed Brn-4 (Table 3.24).
Table 3.24. mRNA expression profile of transcription factors characterizing the fate switches to specific neural lineages in untreated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC11</th>
<th>H1770</th>
</tr>
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<tbody>
<tr>
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<td></td>
<td></td>
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</tr>
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<td>Pbx-1</td>
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<td></td>
</tr>
<tr>
<td>LMO4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dlx-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sox-11</td>
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<td></td>
</tr>
<tr>
<td>Otx-1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Otx-2</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pax-6</td>
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</tr>
<tr>
<td>Brn-4</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brn-5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table legend: mRNA expression data are show in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression patterns of all genes were analyzed in untreated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same NET, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

In conclusion, solely on the expression of transcriptional regulators, these five NETs revealed their apparent origin from cells of different developmental background. Their neural stem cell-like expression patterns of transcriptional regulators, as distinguished by the specific expression of a set of genes characteristic to SVZ (such as GATA-2, GATA-3, Olf-1, Olf-1H, Pbx-1, LMO4, Dlx-2) as well as to normal human neural stem cells [Palm et al, 2000], related all these tumors to resemble a multipotent neural stem or progenitor cell [Luskin, 1993], with H727 expressing all these SVZ markers. Comparison of the patterns of regulatory factors in the five NETs, suggested that these tumors are all molecularly different and can be sub-classified on the bases of expression of regulators which are known to demarcate three earliest neurogenic developmental pathways, BMP, Notch and SHH, respectively. Our data showed that individual members of Zic family as well as of MyT-2, Hes-5, Smad-6, if to mention a
few, could be used as molecular signatures of NETs for cell-based classification. None of these regulators have been proposed/identified before in the definition of diagnostic subgroups of NETs, revealing that our approach of developmental transcriptional networks has led to the identification of putative novel diagnostic criteria.

Our data also established that on the basis of the prominence of neural features, the H1770 tumor would classify this as developmentally more advanced/mature than the rest of the NETs. Its characteristic set of expressed transcriptional regulators included many which are typical of postmitotic neural cells, including all NeuroDs, NSCL-1 and NSCL-2, and the structural proteins - NFs. It should be noted here that in these tumors, the existing neural differentiation pathways are kept active independently of their proliferative capacity, since all tumors under study with the exception of H835, are highly mitogenic irrespective of the expressed transcriptional regulators. The uncoupled regulation of proliferation from neurally terminal differentiation occurs also in normal development, since it was shown recently that if embryonic rat hippocampal neurons are cultured in Neurobasal/B27 with FGF2, nearly all neurons proliferate and fire action potentials and display immunoreactivity for neurofilament, MAP-2, tau, and glutamate [Brewer, 1999]. Thus, expression of post-mitotic neural regulators in NETs is uncoupled from their proliferative capacity, but it establishes another diagnostic and also therapeutic criteria for these tumors.

Based on our data, we can conclude that comparative expression analysis of transcriptional regulators using the developmental approach enabled to establish novel diagnostic criteria for the tumor classification, which is based on the developmental identity of the cell that resembles the tumor the most. The transcription regulatory network-approach, on the other hand, found the primary basis for the understanding of potential differentiation pathways/mechanisms applicable for tumor-specific therapy.

3.7 The effect of GDNF/TGF-β1 treatment on human pulmonary neuroendocrine tumors

Initially, this study determined the expression of specialized receptors for components of the GDNF complex (c-Ret, GFRα1, GFRα2) and neurotrophins (p75, trkA, trkB, trkC) in NETs. The expression analysis of these receptors enabled the determination of various
growth/neurotrophic factors to be included in this study, with the purpose of revealing a factor and/or combination of factors that might play an important role in the differentiation and cell death of the NETs under study. Therefore, the analyzed tumors were exposed to nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), GDNF, insulin-like growth factor-1 (IGF-1), interleukin-1α/β (IL-1α/β) and/or with combination of transforming growth factor-beta-1 (TGF-β1) (see materials and methods, section 2.2.1). No significant changes in proliferation rates were detected in either of the NETs exposed to the growth/neurotrophic factors, except for GDNF in combination with TGF-β1. Therefore, this study proceeded to examine whether the GDNF/TGF-β1 combination of factors will alter the growth potential of NETs.

3.7.1 Effects of GDNF/TGF-β1 on proliferation of pulmonary neuroendocrine tumors
First, we examined the expression of p75, c-Ret, GFRα1 and GFRα2 in NETs using immunocytochemical techniques. The reason for this approach was due to the fact that the presence/absence of these growth factor receptors in the NETs could possibly explain their cytostatic/non-cytostatic response to growth factor treatment.

All untreated NETs expressed neurotrophin receptor p75, however, in H727 cells p75-immunoreactivity was restricted to 80% of the cells (Table 3.25; Figure 3.7A). Upon growth factor treatment, only MC-11 cells showed a downregulation in p75 receptor (Table 3.25). Expression of full-length c-Ret was detected in all NETs, except for H727 cells (Figure 3.7D). GFRα1 immunoreactivity was only present in untreated H835 cells and GFRα2 in untreated H727 and H835 (Table 3.25). No GFRα1 expression was detected upon growth factor treatment, however GFRα2 was only detected in H727 cells.
Table 3.25. Receptor expression in untreated and GDNF/TGF-β1 treated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>A</th>
<th>Control (untreated)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H727</td>
<td>H720</td>
<td>H835</td>
<td>MC-11</td>
</tr>
<tr>
<td>c-Ret</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFRα1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFRα2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>GDNF/TGF-β1</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>H727</td>
<td>H720</td>
<td>H835</td>
<td>MC-11</td>
</tr>
<tr>
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<td>GFRα1</td>
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<tr>
<td>GFRα2</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Table legend: Cells were cultured for six days on laminin-coated 24-well plates in serum-free DMEM/F12 medium supplemented with B27 and in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). After six days, cells were fixed and processed for c-Ret, GFRα1 and GFRα2 using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei and four fields of 200 cells were counted for evaluation. The percentage of NETs that exhibited expression for c-Ret, GFRα1 and GFRα2, over a six-day period upon RA treatment is presented as numbers. ABBREVIATIONS: 0, no expression; 100, 100% expression.

Figure 3.7. Expression of receptors in five different human pulmonary neuroendocrine tumors (see opposite page for figure).

Figure Legend: p75 and c-Ret receptor expression identified by immunofluorescence in untreated NETs. NETs were plated on laminin-coated 24-well plates in DMEM/F12 medium at a density of 2-3 x 10^4 cells/well and incubated for 24 hours. Following this incubation, cells were fixed and analyzed for several neural markers, using immunocytochemical techniques.

Following six days of culturing, several differences were observed in NBs grown in serum-free medium supplemented with GDNF (100ng/ml) and TGF-β1 (50pg/ml), compared to NETs grown in absence of these factors (untreated controls). Proliferation assays of moderate-density NET cultures (2 x 10^4 cells per well in a 24-well plate) demonstrated that GDNF/TGF-β1 treatment did not have any effect on proliferation of H720, H835 and MC-11 cells (Table 3.26). Most surprisingly, GDNF/TGF-β1 suppressed proliferation of H727 cells (six fold) and stimulated proliferation of H1770 four fold.
Table 3.26. Effect of GDNF/TGF-β1 on proliferation of pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>GDNF/TGF-β1</th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC-11</th>
<th>H1770</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.0±1.0↓</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>3.9±0.9↑</td>
</tr>
</tbody>
</table>

**Table legend:** Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12/B27 media in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). Following six days, cells were dissociated and an aliquot of cells counted using a hemocytometer. The effect of GDNF/TGF-β1 on cell proliferation was measured using a [3H]thymidine incorporation assay (see materials and methods). Cells were plated on 12-well plates at a density of 2.3 x 10⁴ cells/well and treated with the mix of GDNF/TGF-β1 for 24hours. Cells were labeled for the last five hours with 4μCi of [3H]thymidine and lysed in 0.2 N NaOH. [3H]thymidine incorporation into the DNA was quantified using a β-counter. Each data point was performed in quadruplicates. Values represent the relative change (in folds) in cell number normalized to the relative change in cell number of untreated cultures. Three independent experiments were performed to assess the changes in cell number. Abbreviations: NE, -no effect in comparison with control; ↑ - enhanced proliferation in comparison with control; ↓ - reduced proliferation in comparison with control.

However, we are aware that the decreased proliferation of H727 cells upon GDNF/TGF-β1 might reflect both cytostatic as well as cytotoxic effects due to the administered drug/growth factors and therefore a cell viability assay, like tryphan blue, would have been a highly accurate approach to distinguish between these two outcomes (cytostatic and cytotoxic). Yet, one of the aims of our study was to determine a combination of growth factors/neurotrophins that exhibited suppressive activity on the proliferation of NETs. For this reason numerous growth factor/neurotrophin matrices were examined and the effects of these drug/s or combinations of factors on cell death of different NETs, were estimated by morphological criteria only (see materials and methods, section 2.2.2). Given that in most cases administered factors or combination of factors had no apparent effect on tumor cell growth or on cell death. When considering that NET control cultures were grown for six days in serum-free, growth factor-free medium, there was no significant cell death and the majority of cells, about 80%, of cells survived. Therefore, we considered it unnecessary to examine the cytotoxic or cytostatic nature of these growth factors, any further. Although we do agree that estimates of total cell viability would be necessary to exclude cell death as a cause of the reduction of overall cell number, it should be mentioned that thorough analyses of
the mechanisms of the effects of the drugs (growth factors) were planned as a continuation of the studies presented in the present thesis. Therefore, the identification of possible regulatory routes and respective involvement of TFs following application of certain growth factors/neurotrophins to NETs was only discussed and not subjected to experimental analyses.

To conclude, our results demonstrated that each NET responded differently to growth factor treatment in regard to the synergistic effects of GDNF and TGF-β1. Current evidence indicates that GDNF forms a tripartite complex with c-Ret and a member of a family of four extracellular, GPI-linked alpha-receptors (GFRalpha 1-4), to activate c-Ret signaling [Eketjall et al., 1999]. The only feature that stands out in our receptor expression studies is the varied expression of GFRα1 and GFRα2 receptors in different NETs. Although GDNF binds with high affinity to GFRα1, it could also bind to other members of GFRalpha GPI receptors. Thus, our observations of GDNF/TGF-β1 induced differentiation of H727 cells, suggested that GDNF may mediate c-Ret-derived signals in these cells by binding to GFRα2. H1770 cells responded with enhanced proliferation, when exposed to GDNF/TGF-β1, however, expressed neither GFRα1 nor GFRα2. Studies on the early organogenesis in the chick embryo, have suggested that possible combinations of novel unidentified receptors acting with c-Ret or with GFRalphas may mediate GDNF-derived signals [Homma et al., 2000], therefore, it is highly likely that H1770 cell expression applied GDNF signaling pathways that are currently unknown to the wide audience of neuroscientists.

3.7.2 Effects of GDNF/TGF-β1 on differentiation of pulmonary neuroendocrine tumors along neural lineages

To identify whether the pathways that connect transcriptional regulators to neural differentiation, are active/functional in these NETs, we determined the protein level for signaling pathways using immunocytochemical techniques. Protein determination in this case, seemed like the most favorable approach due to the availability of very good antibodies against receptors and members of these signaling pathways. Therefore, to define the effects of GDNF/TGF-β1 on differentiation of NETs, cells were cultured for six days on laminin-coated plates treated with serum-free medium supplemented with
GDNF (100ng/ml) and TGF-β1 (50pg/ml). Following the six-day treatment, NETs were subjected to immunocytochemical analyses using an astrocytic (GFAP) and a neuronal (βIII-tubulin) marker. According to our results no changes were observed in any of the NETs for the expression of βIII-tubulin upon GDNF/TGF-β1 treatment, when compared to untreated controls. GFAP-immunoreactivity was downregulated in H1770 cells upon GDNF/TGF-β1 treatment and remained unchanged in all the other NETs (Table 3.27).

Thus, our differentiation assay revealed that in medium-density cultures, GDNF/TGF-β1 treatment did not have a marked effect on the net production of neuronal and glia-like properties in NETs, therefore allowing all NETs to remain neuronal (βIII-tubulin expression).

Table 3.27. Effects of GDNF/TGF-β1 on the expression of βIII-tubulin and GFAP in pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th></th>
<th>control (untreated)</th>
<th>GDNF/TGF-β1</th>
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<tbody>
<tr>
<td></td>
<td>H727</td>
<td>H720</td>
</tr>
<tr>
<td>βIII-tub</td>
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<td></td>
</tr>
<tr>
<td>GFAP</td>
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Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 media supplemented with B27 in the presence of GDNF (100ng/ml) and TGF-β1 (50pg/ml). Following culturing, cells were fixed and stained with antibodies against human-reactive βIII-tubulin and GFAP. ABBREVIATIONS: 0, no expression; “filled box”, 100% expression.

Therefore, according to our results we can conclude that GDNF/TGF-β1 signaling resulted in the activation of mixed developmental programs (neurons/astrocytes/oligodendrocytes/smooth muscle cells) in different NETs. We examined O4, CNPase weak immunoreactivity in the same cells that were MAP-2, A2B5, and desmin-positive (Figure 3.7F). In the case of in H727 cells, it is possible that such activation/induction of mixed differentiation programs finalized in the execution of programmed cell death, and that this type of a suicidal mechanism (through activation of "too many" developmental programs), as such presented, to be an attractive target for cancer drug therapy.
3.7.3 Effects of GDNF/TGF-β1 on expression of molecular markers used in the prognostic evaluation of pulmonary neuroendocrine tumors

*trkA/trkB/trkC expression.*

Our data revealed that GDNF/TGF-β1 treatment, did not induce any changes in the expression of the trk family of receptors, in any of the five NETs analyzed (Table 3.28).

**Table 3.28.** Trk (full-length) receptor profile in different human NETs upon GDNF/TGF-β1 treatment.

<table>
<thead>
<tr>
<th></th>
<th>A Control (untreated)</th>
<th></th>
<th>B GDNF/TGF-β1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>H727</td>
<td>H720</td>
<td>H835</td>
</tr>
<tr>
<td>trkA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>trkB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>trkC</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table legend:** Cells were cultured for six days on laminin-coated 24-well plates in serum-free DMEM/F12 medium supplemented with B27 and in the presence of 1μM RA. Following six days of culturing, cells were fixed and processed for trkA, trkB and trkC using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei and four fields of 200 cells were counted for evaluation. The percentage of NETs exhibiting expression for trkA, trkB and trkC over a six-day period upon RA treatment, are presented as numbers. ABBREVIATIONS: 0, no expression; “filled box”, 100% expression.
Multi-transmitter phenotype.

Three of the five NETs have been reported to exhibit high levels of L-dopamine decarboxylase (DDC) activity (ATCC technical sheets) revealing that these cells synthesized noradrenaline as a neuroactive compound (neurotransmitter). Dopamine (DA) availability as a precursor for DDC activity is dependent, however, on the synthesis of tyrosine hydroxylase (TH). Therefore we decided to examine the expression of TH in growth factor-treated NET cells using ICC techniques. GDNF/TGF-β1 treatment ceased the expression of TH in H720, H835 and MC-11, and significantly decreased (18-fold, from 90% to 5%) the number of TH-positive cells in H727 cultures (Figure 3.7G and H). In contrast, growth factor treatment increased (from 5% to 80%) the TH-positive population of cells in H1770 cultures (Table 3.29).

Table 3.29. Changes in TF expression associated with dopaminergic/noradrenergic phenotype in untreated and GDNF/TGF-β1 treated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Table 3.29. Changes in TF expression associated with dopaminergic/noradrenergic phenotype in untreated and GDNF/TGF-β1 treated pulmonary neuroendocrine tumors.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Control (untreated)</td>
</tr>
<tr>
<td>TH</td>
</tr>
<tr>
<td>Phox2a</td>
</tr>
<tr>
<td>Phox2b</td>
</tr>
<tr>
<td>PITX3</td>
</tr>
<tr>
<td>Hsal1</td>
</tr>
<tr>
<td>Hsal2</td>
</tr>
<tr>
<td>Nurr1</td>
</tr>
</tbody>
</table>

**Table legend:** NET cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 medium supplemented with B27 in presence of GDNF (100ng/ml) and TGF-β1 (50ng/ml). Following six days of culturing, cells were fixed and processed for transmitters using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The table above shows the percentage of untreated and GDNF/TGF-β1-treated NETs expressing TH, Phox2a, Phox2b, PITX3, Hsal1, Hsal2 and Nurr1. **ABBREVIATIONS:** 0, no expression; "filled box", 100% expression; 5, 5% expression; 90, 90% expression; 80, 80% expression. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.
To explore the accompanying changes in the expression of TFs associated with dopaminergic/noradrenergic phenotype, we examined the expression of Phox2a and Phox2b, PITX3, Nurr1, Hsai1 and Hsal2 in GDNF/TGF-β1 treated cells. The expression of Phox2a and Phox2b was not affected, however, the patterns of PITX3, Hsal1, Hsal2 and Nurr1 were significantly altered in GDNF/TGF-β1 treated cells. First, the expression of PITX3 was lost in all cells except H1770. Second, MC-11 cells did not express any of these regulators other than Nurr1. The expression of Hsal1 was lost in H835 cells, and Hsal2 in H727 cells. Given that in Drosophila Shh requires functional FGF signaling to activate Spalt [Carl and Wittbrodt, 1999], it is possible that the downregulation of Hsal1 and Hsal2 expression in H835 and H727 cells is due to the diminished/weakened Shh signaling upon GDNF/TGF-β1 treatment. Furthermore, Nurr1 and PITX3, essential for the dopaminergic phenotype during CNS development [Sakurada et al., 1999], displayed certain patterns of regulation in H727 and H1770 cells. In H727 cells that revealed an undetectable expression of TH-positive cells upon GDNF/TGF-β1 treatment, also showed a downregulation in PITX3. In contrast, in H1770 cells PITX3 expression remained unchanged and a significant induction of Nurr1 expression was detected after GDNF/TGF-β1 treatment (Table 3.29). These cells also revealed an increase in the number of TH-positive cells. Given that Nurr1 and Ptx3 (rodent homolog of PITX3) response elements have been identified in the TH promoter, it suggests that Ptx3 and Nurr1 may be implicated in the transcriptional control of TH gene expression during development [Sakurada et al., 1999; Cazorla et al., 2000; Iwawaki et al., 2000]. Therefore, it is possible that changes in TH levels are directly associated with PITX3 and Nurr1 activity in H727 and H1770 cells. In addition, TH has been reported being regulated by phosphorylation and feedback inhibition of catecholamines [Fitzpatrick, 2000]. Therefore, we could speculate that a loss of TH expression in H720, H835 and MC-11 cells could be due to the feedback regulation where catecholamines regulate the Shh pathway. However, these data clearly indicated that GDNF/TGF-β1 signaling interfered with catecholamine metabolism in NETs.

Taken together, NETs are a group of neuroendocrine neoplasms that secrete various neuroactive peptides (more than 30), including catecholamines. Here, we observed a remarkable correlation between utilization of Shh/Nurr1/Ptx3 pathways and
the regulation of catecholamine synthesis in NETs. Studies of the dopaminergic identity in the CNS have found that it is determined by a combination of pan-dopaminergic (e.g., Shh/FGF-8) and region-specific (Nurr1) mechanisms, since Nurr1 has no influence on the expression of other than TH dopaminergic markers [Sakurada et al., 1999]. Furthermore, FGF and Shh signals activate regulatory mechanisms involving Gli family of regulatory factors that control also serotonergic cell fate in developing CNS [Ye et al., 1998], as cells that are generated in the region flanking the floor plate, including dopaminergic and serotonergic neurons, were greatly reduced in number or absent in Gli-2 homozygous embryos [Matise et al., 1998]. In addition, ectopic expression of Gli-1 in the dorsal midbrain and hindbrain of transgenic mice mimics the effects of ectopically expressed Shh-N to the formation of ectopic dorsal clusters of dopaminergic and serotonergic neurons [Hynes et al., 1997]. Recent study evidenced that Shh promotes proliferation of neural crest derivatives of the sympathoadrenal lineage and supports TH induction of mature sympathetic neurons in vitro [Williams et al., 2000]. Consistent with these studies, our data suggested that pan-catecholaminergic (Shh) and region-specific (Nurr1/Ptx3) mechanisms affect at least L-tyrosine metabolism in NSCLCs. However, it is currently not clear whether this is an indirect consequence (reduced levels of TH resulting from feedback inhibition of reduced levels of catecholamines) [Fitzpatrick, 2000] or more direct effect of Shh and Nurr1 signaling pathways on TH expression. The consequences of altered Shh/Nurr1/Ptx3 signaling for L-tryptophan metabolism and also for other monoamines (bioactive peptides, hormones, biogenic amines, neuroactive compounds) upon growth factor in NETs has not been the focus of the study. Whatever their relationship to Shh/Nurr1/Ptx3 signaling, we suggested that NETs change their neurotransmitter profile under growth-factor treatment that it might contribute to different aspects of malignant growth (proliferation, differentiation, apoptosis) of these cells. Several opposite effects of serotonin (5HT) and dopamine on tumor growth have been reported. Depending on the small cell lung carcinoma type, 5HT receptor agonists have been found to inhibit the tumor growth or to contribute to cell proliferation [Vicaut et al., 2000]. Inhibition of growth of human small cell lung cancer by a dopaminergic agonist has been observed [Ishibashi et al., 1994]. In pituitary adenomas, thyroliberin (TRH) release is stimulated in the presence of dopamine while somatostatin is inhibited in the
presence of TRH [Peillon et al., 1989]. Generalizing, changes in the levels of synthesis of neurotransmitters directly affect metabolic activity of other bioactive compounds (hormones), which altogether may have significant impact in tumor phenotype and resistance/response to treatments.

### 3.7.4 Changes in TF expression profile of pulmonary neuroendocrine tumors upon GDNF/TGF-β1 treatment

Although the cooperative effects of GDNF and TGF-β1 were not responsible for inducing dramatic changes in the proliferation of NETs (Table 3.26) or expression of certain neural markers (Table 3.27), our study proceeded to test our second hypotheses as to whether extrinsic factors, GDNF/TGF-β1 will alter the expression of regulatory genes in NETs (as described in section 3.6).

To test our hypotheses, we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different NETs upon GDNF/TGF-β1 treatment. Since one of the aims of our study was to propose a way to improve the diagnostics of NETs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our purposes. Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

According to our results, two major adaptations were observed in the TF networks of GDNF/TGF-β1 treated NETs: The first adaptation constituted a group of genes that exhibited cell-autonomous regulation and showed no change in their expression upon GDNF/TGF-β1 treatment (Table 3.30). This group of genes comprised mostly of, 1) *early negative regulators of neural differentiation*, such as Chordin, Msx-1 and Msx-2; 2) *transcriptional regulators linking neural inducers to activation of proneural genes*, such as Irx-3; 3) *transcriptional regulators linking neural determination to the specification of neuronal identity, e.g. proneural and neurogenic genes*, such as hASH1,
NeuroD1, NeuroD3, NSCL-1, NSCL-2, Hes-1, Hey-2, HeyL, MyT-2, MyT-3 and Olf-1; 4) transcription factors characterizing the fate switches to specific neural lineages, such as LMO4, Dlx-2 and Otx-2.

The second adaptation constituted a group of genes that exhibited signal-dependent regulation and revealed expression that was influenced by GDNF/TGF-β1 treatment (Table 3.30). Specifically, 1) in early negative regulators of neural differentiation, the most interesting observation was the loss of Smad-6 and Smad-7 expression in H727 and H720 upon growth factor treatment. BMP-2 and BMP-6 expression was lost in MC-11 and H720, H1770, respectively. Growth factor treatment induced an up-regulation in Noggin and down-regulation in Dlx-7 expression, also in MC-11 as well as a down-regulation in expression of Nkx-2.5 in H727 cells; 2) transcriptional regulators linking neural inducers to activation of proneural genes, as GDNF/TGF-β1 affected the expressions of Gli-1 mRNA in H727, H720 and MC-11, Gli-2 mRNA in H720 and H1770, Gli-3 mRNA in H727, Zic-1 and Zic-2 mRNA in H727 and MC-11, Zic-3 mRNA in H727, Irx-2a mRNA in H727 and MC-11 and Sox-2 mRNA in H1770. According to these results, TFs linking neural inducers to activation of proneural genes, seemed to be altered mostly in H727 and MC-11 cell lines upon growth factor treatment; 3) transcriptional regulators linking neural determination to the specification of neuronal identity, e.g. proneural and neurogenic genes, as NN-1 mRNA was up-regulated in H727, MC-11 and H1770, whereas NN-3 mRNA was down-regulated in MC-11 and H1770 cells upon GDNF/TGF-β1 treatment. NeuroD2 mRNA expression was lost in H727, MC-11 and H1770 cells whereas Hes-5 expression was upregulated in MC-11 and down-regulated in H727. Growth factor treatment induced a down-regulation in the expression of Hey-1 mRNA in MC-11 and H727, while a down-regulation of MyT-1 mRNA was observed in MC-11 cells and that of Olf-1H in H720 and MC-11 cells; 4) transcription factors characterizing the fate switches to specific neural lineages, with GATA-2 and GATA-3 mRNA expression being down-regulated in MC-11 and up-regulated in H1770, respectively. Four of the NETs, but H1770, revealed a decrease in Pbx-1 mRNA expression upon growth factor treatment. An up-regulation was observed in H727 cells and a down-regulation in H1770 cells with respect to their expression of Sox-11 and Otx-1, respectively. Pax-6 mRNA was only altered in H835
cells, which revealed a down-regulated expression. Both Brn-4 and Brn-5 mRNA expression were downregulated upon GDNF/TGF-β1 treatment in H835 and MC-11 cells, respectively.
Table 3.30. mRNA expression profile of regulatory genes in untreated and GDNF/TGF-β1 treated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Control (untreated)</th>
<th>GDNF/TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H727</td>
<td>H720</td>
</tr>
<tr>
<td>BMP-2</td>
<td></td>
<td></td>
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<tr>
<td>BMP-6</td>
<td></td>
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</tr>
<tr>
<td>Noggin</td>
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<td></td>
</tr>
<tr>
<td>Chordin</td>
<td></td>
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<tr>
<td>Smad-6</td>
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<tr>
<td>Smad-7</td>
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<tr>
<td>Mx1</td>
<td></td>
<td></td>
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<tr>
<td>Mx2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dlx-7</td>
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<td></td>
</tr>
<tr>
<td>Nkx-2.5</td>
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<tr>
<td>Gli-1</td>
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<tr>
<td>Gli-2</td>
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<tr>
<td>Gli-3</td>
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</tr>
<tr>
<td>Zic-3</td>
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<td></td>
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<tr>
<td>Irx-2a</td>
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</tr>
<tr>
<td>Irx-3</td>
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<tr>
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<tr>
<td>NeuroD1</td>
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<td>Hey-2</td>
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<tr>
<td>HeyL</td>
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<tr>
<td>MyT-1</td>
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<tr>
<td>Olf-1</td>
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<tr>
<td>Olf-14H</td>
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<tr>
<td>GATA-2</td>
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<td>GATA-3</td>
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<tr>
<td>Pbx-1</td>
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<tr>
<td>LMO4</td>
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<td>Dlx-2</td>
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<tr>
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<tr>
<td>Pax-6</td>
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<tr>
<td>Brach-4</td>
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<tr>
<td>Brach-5</td>
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</tbody>
</table>

Table legend: mRNA expression data are show in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression
patterns of all genes were analyzed in untreated and GDNF/TGF-β1 treated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same NET, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

Therefore, according to our results signal transduction pathways that are inducibly engaged in neural differentiation during normal development, contribute to the initiation of neural differentiation programs also in pathologies, hereby NETs. The sequential expression of bHLH transcription factors is a characteristic feature of many differentiating lineages. Such sequential expression of bHLH genes is thought to underlie the linked processes of neural determination and differentiation. During development of the peripheral nervous system, precursors of the peripheral sensory neurons first express neurogenins, followed by one or more members of the NeuroD family [Anderson, 1999]. According to our data, GDNF/TGF-β1 signaling activated NN-1 in most of the NETs, suggesting the induction of sensory neurogenic pathways in these cells. Since all NETs with the exception of H835 expressed at least one member of the NeuroD family, however, most of them did not execute growth arrest upon cytokine-treatment, we concluded that because of the defects in regulatory networks these cells enabled uncoupled regulation of proliferation from neural terminal differentiation and thereof the sensory neurogenic potential remains unrealized.

In conclusion, the results of the effects of GDNF/ TGF-β1 on five human NETs, revealed the potential of applying the understanding of transcriptional regulatory networks/ mechanisms operational in tumors to predict the behavior of these malignant cells in response to an external stimuli. Because it is now possible to screen thousands of tumor samples for the full-genome expression using DNA microarray technology, monitoring of TF networks in tumors following the logic of cell development would simplify the task and present a more straightforward approach to understand and treat cancer.
3.8 The effect of retinoic acid treatment on human pulmonary neuroendocrine tumors

Clinical and research data of the efficiency of differentiating human non-small cell lung cancers (NSCLCs) with RA and its derivatives is quite contradictory [Treat et al., 1996; Lokshin et al., 1999]. However, one of the aims of our study was to employ a set of transcriptional regulators which are all related to the regulation of neurogenesis and to examine how the growth potential of five human NETs, of different origin, respond to an instructive signal (RA), that normally conveys neural differentiation.

3.8.1 Effects of RA on proliferation of pulmonary neuroendocrine tumors

First we examined the expression of RARβ, RXRβ and RXRα in NETs. The reason for this approach was due to the fact that the presence/absence of RA receptors in the NETs could possibly explain their cytostatic/non-cytostatic response to RA treatment. Thus, for this assay NET cell lines were cultured for six days on laminin-coated plates in the presence of 1μM RA (serum-free medium). Following culturing, cells were fixed and processed for RARβ, RXRβ and RXRα using immunocytochemical techniques. According to our data, all untreated NETs except MC-11 expressed RARβ (Table 3.31). RXRβ was not detected in H720 cells, however this cell line exhibited aberrant expression for other retinoid receptors upon RA treatment, which might render these cells unresponsive to RA-mediated differentiation. On the contrary, the restored expression of RXRα may enhance differentiating responses of H1770 cells upon RA treatment. Upon RA treatment the expression of RXRβ remained unchanged in all NETs (Table 3.31). Expression of RXRα, RXRβ and RARβ remained the same in RA-treated as in untreated H835 and H727 cells (Figure 3.8I, J, K). RA-treatment induced RARβ expression in MC-11 cells, but ceased the expression in H720 cells.

Supported by others, the abovementioned data revealed the critical role of retinoid receptors (RARβ, RXRβ) in mediating growth inhibitory effects of RA in lung cancers [Li et al., 1998].
Table 3.31. Effect of retinoic acid on RAR and RXR expression in untreated and RA-treated pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th></th>
<th>Control (untreated)</th>
<th>RA treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H720</td>
<td>H727</td>
</tr>
<tr>
<td><strong>RARβ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RXRa</strong></td>
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</tr>
<tr>
<td><strong>RXRβ</strong></td>
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</tr>
</tbody>
</table>

Table legend: NET cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 medium supplemented with B27 in presence of 1μM RA. Following six days of culturing, cells were fixed and processed for RARβ, RXRa and RXRβ using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The percentage of NETs that exhibited expression of RARβ, RXRa and RXRβ, over a six-day period, are presented as a number (0) or “filled box”. ABBREVIATIONS: 0, no expression; “filled box”, 100% expression.

Figure 3.8. Expression of retinoic acid receptors in five different human pulmonary neuroendocrine tumors (see opposite page for figure).

Figure Legend: Retinoic acid receptor expression identified by immunofluorescence in untreated and RA-treated NETs. NETs were plated on laminin-coated 24-well plates in DMEM/F12 medium (with or without 1μM RA) at a density of 2-3 × 10^4 cells/well - incubated for 24 hours. Following this incubation, cells were fixed and analyzed for retinoic acid receptors, using immunocytochemical techniques. ABBREVIATIONS: +RA, RA-treated.

Following the determination of retinoid receptor expression in NETs, these five cell lines were cultured again for six days on laminin-coated plates, in the presence of serum-free medium and 1μM RA. After six days, cells were dissociated and counted using a hemocytometer. The effect of RA on NET cell proliferation was measured using [3H]thymidine incorporation assay (see materials and methods).

According to our results, only one of the five NETs, H1770, exhibited severely inhibited (40-fold decrease) mitotic activity (Table 3.32).
Table 3.32. Effect of RA on proliferation of pulmonary neuroendocrine tumors.

<table>
<thead>
<tr>
<th></th>
<th>H727</th>
<th>H720</th>
<th>H835</th>
<th>MC-11</th>
<th>H1770</th>
</tr>
</thead>
<tbody>
<tr>
<td>+RA treatment</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>39.8±0.2↓</td>
</tr>
</tbody>
</table>

Table legend: Cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 media supplemented with B27 in the presence of 1μM RA. After six days cells were dissociated and an aliquot of cells counted using a hemocytometer. The effect of RA on cell proliferation was measured using a [³H]thymidine incorporation assay. Cells were plated on 12-well plates at a density of 2-3 x 10⁴ cells/well and treated with 4μCi of [³H]thymidine, and lysed in 0.2N NaOH. [³H]thymidine incorporation into the DNA was quantified using a β-counter. Each data point was performed in quadruplicates. Values represent the relative change (in folds) in cell number normalized to the cell number of untreated cultures. Three independent experiments were performed to assess the changes in cell number. ABBREVIATIONS: ↑ - enhanced proliferation; ↓ - reduced proliferation; NE, -no effect compared with control.

As observed for decreased proliferation of H727 cells upon GDNF/TGF-β1, the suppressed proliferation of H1770 upon RA treatment might reflect both cytostatic as well as cytotoxic effects due to the administered drug/growth factor and therefore a cell viability assay, like tryphan blue, would have been a highly accurate approach to distinguish between these two outcomes (cytostatic and cytotoxic). When considering that NET control cultures were grown for six days in serum-free, RA-free medium, there was no significant cell death and the majority of cells, about 80%, of cells survived. Therefore, we considered it unnecessary to examine the cytotoxic or cytostatic nature of RA, any further. Although we do agree that estimates of total cell viability would be necessary to exclude cell death as a cause of the reduction of overall cell number, it should be mentioned that thorough analyses of the mechanisms of the effects of the drugs (RA) were planned as a continuation of the studies presented in the present thesis. Therefore, the identification of possible regulatory routes and respective involvement of TFs following application of RA to NETs was only discussed and not subjected to experimental analyses.

To conclude, we might speculate that there is no correlation between retinoid receptor expression and RA-induced proliferation in the five human NETs analyzed, since the
poor RA-induced proliferation is not representative of the retinoid receptor expression in these NETs.

3.8.2 Effects of RA on differentiation of pulmonary neuroendocrine tumors along neural lineages

To identify whether the pathways that connect transcriptional regulators to neural differentiation, are active-functional in these NETs, we determined the protein level for signaling pathways using immunocytochemical techniques. Protein determination in this case, seemed like the most favorable approach due to the availability of very good antibodies against receptors and members of these signaling pathways. Therefore, the effect of RA on differentiation of NETs was determined through culturing of cells for six days on laminin-coated plates in the presence of serum-free medium supplemented with 1μM RA. Following six days of exposure to RA, NET cells were fixed and subjected to immunocytochemical analyses using antibodies that are known astrocytic and neuronal markers. According to our data, these markers revealed that RA increased the expression of non-phosphorylated forms (NF-L 311) of neurofilaments in all NETs, including H720 cells which despite their decreased expression of retinoid receptors and unchanged mitotic activity, were stimulated by RA treatment. Non-phosphorylated forms of neurofilaments showed a weak immunoreactivity in about 50% of the untreated population of H720, H727, H835 and MC-11 cells, however 100% immunoreactivity in H1770 cells. High levels of expression of phosphorylated neurofilaments (NF-L Phos) was observed throughout the population of RA treated and untreated NETs (Figure 3.8G). It is possible that the expression of our phophorylated and non-phosphorylated NF results be explained by a study of Christen et al., in which it was revealed that the prevalence of phosphorylated neurofilament epitopes (100% expression in our untreated NETs), compared with non-phosphorylated forms (50% expression in our untreated NETs), in the cell bodies of tumor cells implicate abnormal phosphorylation states in the formation of these intermediate filament aggregates [Christen et al., 1987]. Another interesting finding involved EAT2 downregulation in about 40% of H1770 cells (Table 3.33; Figure 3.8M and N). Studies using mouse astrocytic cultures, have revealed that the transcripts for GLT-1 (EAT2) messages are detectable only in
embryonic astrocytes in comparison with GLAST (EAT1) messages that are highly expressed in E18 and P1-P4 astrocytic cultures, declined in P10-P21, and was undetectable in P50 astrocytes [Stanimirovic et al., 1999]. Accordingly, the lack of EAT1 immunoreactivity in RA treated H1770 cells, revealed the activation of signaling pathways involved in astrocytic maturation (Table 3.36). Upon treatment with RA, H727 and MC-11 cells began to express smooth muscle actin (approximately 60% and 5%, respectively) (Figure 3.8O and P). Figures 3.8O and P showed smooth muscle actin expression before and after RA-treatment in H727 cells. Since all RA treated NETs expressed desmin (Figure 3.8H) and showed no immunoreactivity for GFAP, it is suggestive that all tumors, particularly H727 and MC-11 cells, have acquired strong phenotypic traits characteristic of smooth muscle cells.

**Table 3.33.** Effect of RA on expression of neural markers in pulmonary neuroendocrine tumors.

<table>
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**Table legend:** NET cells were grown for six days on laminin-coated 24-well plates in DMEM/F12 medium supplemented with B27 in presence of 1 μM RA. Following six days of culturing, cells were fixed and processed for neuronal and glial markers using immunocytochemical techniques. All cells were counterstained with DAPI to reveal the nuclei. Four fields of 200 cells were counted for evaluation. The percentage of NETs that exhibited expression of neuronal and glial markers, over a six-day period, are presented as numbers or a "filled box". ABBREVIATIONS: 0, no expression; "filled box", 100% expression; 60, 60% expression; 5, 5% expression; 50, 50% expression; internex, internexin-α; bIIItub, 119
βIIIItubulin. The reason for adding "red" blocks is to distinguish the 100% (all) or 0% (nothing) expression from the percentage of expression between 0 and 100%.

**Figure 3.8.** Expression of neural marker expression in five different human pulmonary neuroendocrine tumors (see opposite page for figure).

**Figure Legend:** Neural marker expression identified by immunofluorescence in untreated and RA-treated NETs. NETs were plated on laminin-coated 24-well plates in DMEM/F12 medium (with or without 1µM RA) at a density of 2-3 x 10^4 cells/well - incubated for 24 hours. Following this incubation, cells were fixed and analyzed for several neural markers, using immunocytochemical techniques. ABBREVIATIONS: +RA, RA-treated; NF-P, phosphorylated neurofilament; SM-a, smooth muscle actin.

The co-expression of neuronal filaments (NFs, βIIIItubulin, MAP-2) (Figure 3.8F and G) with desmin (Figure 3.8H), A2B5 (Figure 3.8E), in some cases internexin-α (Figure 3.8D) and smooth muscle actin (Figure 3.8O and P), revealed that these NETs exhibited aberrant regulation of structural proteins. However, the aberrant expression of intermediate filaments is not a feature unique to a group of highly unusual tumors or due to a tissue-culture phenomenon, but is found throughout the spectrum of lung cancers [Gatter et al., 1987]. Additionally, NSE, which is a characteristic molecular marker for NETs, was induced in three of the five NET cell lines upon RA treatment (Table 3.33).

### 3.8.3 Changes in the TF expression profile of pulmonary neuroendocrine tumors upon RA treatment.

Although RA was not responsible for inducing dramatic overall changes in the proliferation of NETs (except for H1770) (Table 3.32) or in the expression of neural markers (Table 3.33), our study proceeded in testing our second hypothesis which stated that the expression of regulatory genes in five human NETs was altered upon RA treatment. To test our hypotheses, we analyzed the mRNA expression levels (using qualitative RT-PCR) of about 50 regulatory genes in five different NETs upon RA treatment. Since one of the aims of our study was to propose a way to improve the diagnostics of NETs, we determined only at mRNA level whether a specific gene was expressed or not and how it changed upon treatment. Determination of the level of protein expression is necessary for therapeutics, however, not critical for our purposes.
Even though our study was only concerned with mRNA expression, we are aware that mRNA expression is not a strict predictor of protein concentration [Anderson and Seilhamer, 1997; Gygi SP et al., 1999] and that quantitative protein determination is important to measure the final expression product rather than an intermediate [Lockhart DJ and Winzeler EA, 2000].

As revealed in the results of GDNF/TGF-β1 treated NETs (section 3.7.3), the two major adaptations were also observed in the transcriptional profile of RA treated NETs (Table 3.34): The first which constituted a group of genes exhibiting cell-autonomous regulation, showed no change in their expression upon RA treatment. Regulatory genes related to this group constituted, 1) **early negative regulators of neural differentiation**, such as BMP-2, Noggin, Chordin, Smad-7, Msx-1, Msx-2, Nkx-2.5; 2) **transcriptional regulators linking neural inducers to activation of proneural genes**, such as Zic-3 and Irx-3; 3) **transcriptional regulators linking neural determination to the specification of neuronal identity** – e.g. proneural and neurogenic genes, such as NN-1, hASH-1, NeuroD1, NeuroD3, NSCL-1, NSCL-2, Hes-1, Hes-5, Hey-1, MyT-2, MyT-3, Olf-1; 4) **transcription factors characterizing the fate switches to specific neural lineages**, such as GATA-2, Pbx-1, LMO4, Dlx-2, Sox-11, Otx-1, Otx-2, Pax-6. The second adaptation consisted of a group of regulatory genes that exhibited signal-dependent regulation and revealed expression that was affected by RA treatment. Changes in expression were observed in, 1) **early negative regulators of neural differentiation**, where RA induced a down-regulation in the expression of BMP-6 mRNA and an up-regulation in Smad-6 and Dlx-7 mRNA of H720 and H835, respectively; 2) **transcriptional regulators linking neural inducers to activation of proneural genes**, where Gli-1 mRNA expression was induced in H835 and H1770 but disappeared in MC-11 upon RA treatment. No expression was observed for Gli-2 mRNA expression in any of the five NETs exposed to RA treatment. Changes were also observed in the mRNA expression of Gli-3, Zic-1, Zic-2, Irx-2a and Sox-2; 3) **transcriptional regulators linking neural determination to the specification of neuronal identity** – e.g. proneural and neurogenic genes, where the only changes in regulatory gene expression occurred in NeuroD2, Hey-2, HeyL, MyT-1, Olf-1H. NeuroD2 mRNA expression was upregulated in H835 cells upon RA treatment while Hey-2 and HeyL expression were both downregulated in H720 and H727, respectively.
RA treatment also induced downregulation in MyT-2 and Olf-1H mRNA expression of H720 and MC-11 cells, respectively; 4) *transcription factors characterizing the fate switches to specific neural lineages*, where only three regulatory genes, GATA-3, Brn-4, Brn-5, responded to RA treatment. Upregulation in mRNA expression of GATA-3 and Brn-4 occurred in H1770 and MC-11, respectively, whereas downregulation of expression was observed for Brn-4 and Brn-5 in H720 and MC-11, respectively.
Table 3.34. mRNA expression profile of regulatory genes in untreated and RA-treated pulmonary neuroendocrine tumors.

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<th>A. Control (untreated)</th>
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<td><strong>Early negative regulators of neural differentiation</strong></td>
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**Table legend:** mRNA expression data are show in matrix format with each row representing the RT-PCR of a single cDNA and each column representing the measured expression levels for all genes in a single sample (tumor). Gene names are shown in the left (see Appendix for Genbank ID numbers). Expression
patterns of all genes were analyzed in untreated and RA-treated pulmonary neuroendocrine tumors. To visualize the results, the expression level of each gene is represented by a color, with black ("filled box") representing expression and white (0), no detectable expression. Three cell samples, from the same NET, were analyzed three months apart and these samples were highly correlated in gene expression with each other, suggesting that the observed pattern of gene expression reflect intrinsic differences between the tumors, rather than variation in handling or experimental artifacts.

To date, the role of hASH-1, a basic helix-loop-helix transcription factor homologous to the Drosophila achaete-scute complex, is suggested to contribute to the expression of NE markers in small-cell lung cancers (SCLCs) [Borges et al., 1997]. Another study showed that hairy-enhancer-of-split-1 (Hes-1), also a helix-loop-helix transcription factor, is abundant in most human non-SCLCs (NSCLCs), whereas it is virtually absent in hASH-1 expressing SCLCs, suggesting that deficiency of Hes-1 activity leads to the constitutive expression of hASH-1 in SCLCs [Chen et al., 1997]. During normal development, hASH-1 and NN-1 are expressed in complementary patterns [Fode et al., 2000; Ma et al., 1997 whereas in some SCLCs, NN-1 (NeuroD3) have been detected to be co-expressed with hASH-1 [Rostomily et al., 1997]. All these factors, the proneural achaete-scute genes and the neurogenic genes of the Notch pathway (constituting the Hes, Hey and MyT families) are widely understood as being essential for early neurogenesis.

For most of the five NETs, we examined that RA application was sufficient to overwrite the expression patterns of many transcriptional regulators and consequently the developmental programs inherent for the tumors that were active before the treatment. Unsuccessful treatment is highly likely to result in driving the tumor phenotype into more malignant derivatives. In most cases upon RA treatment, the induction of certain neural features was observed in all tumors. However, the completion of neural differentiation by inhibition of abnormal growth upon RA treatment is achieved only in H1770, which expressed the largest number of postmitotic neural regulators, including all three NeuroD family members. It is therefore possible that monitoring of the presence of NeuroDs and some other postmitotic neural regulators (NSCLs etc.) is sufficient criteria to assess the potential of a certain NET to neural differentiation-based treatment, by means of different drugs/pharmacons, including RA.
Taken together our study revealed that RA inhibited the proliferation and induced neural differentiation of certain human NETs but not all. Neural differentiation upon RA-treatment occurred in NETs, which exhibited the expression of many transcriptional regulators and neuronal markers that are associated with the differentiated histiotype.

Finally, our study determined whether the different gene expression profiles of these tumors as well as gene expression variability observed in untreated and treated (GDNF/TGF-β1 and RA) NETs, was statistically significant. To accomplish this, we firstly performed the analyses in all five treated (GDNF/TGF-β1 and RA) and untreated NETs in the four different gene groups by counting the number of genes expressed in each gene group for each tumor, also upon treatment. Secondly, the expression data of all four groups of genes were combined in order to finally determine the statistical significance of total gene expression differences between the five human NETs as well as between treatments applied. The analyses were performed using histograms to reveal number of genes expressed, and two-way analysis of variance (ANOVA)/F-test (95% level of confidence) to reveal the statistical significant difference between tumors and treatments in a specific gene group. Upon identification of a significant difference in a specific gene group, a Bonferroni post hoc test was performed to identify between which tumors or treatments the significant differences was observed. Given that every gene, belonging to a gene group, has a specific individual function apart from the main function of the group, therefore, in order for us to perform statistical analyses of the data, we have to assume that all genes in a given gene group perform the same function which is the main function of that specific gene group.

**Gene Group 1: Early negative regulators of neural differentiation.**

As revealed previously, the group of early negative regulators of neural differentiation consisted of 10 genes that included signals that can suppress neural differentiation and promote epidermogenesis. It included the genes BMP-2, BMP-6, Noggin, Chordin, Smad-6, Smad-7, Msx-1, Msx-2, Dlx-7 and Nkx-2.5. The histogram (Figure 3.9), revealed the total of group 1 genes expressed in the five untreated, GDNF/TGF-β1 and RA-treated NETs. According to the results of this histogram, untreated H727 and H720 revealed the highest expression out of 10 genes for group 1. H727 and H835 showed
the highest gene expression upon GDNF/TGF-β1 treatment, whereas the highest expression upon RA-treatment was observed in H727 and H835. Upon performing a two-way analysis of variance, it was clear that there was a significant difference ($P = 0.004$) between the five NETs in regard to the number of group 1 genes expressed. However, a significant difference ($P = 0.013$) was also observed between the gene expression patterns for each treatment. Since a significant difference was found in both, gene expression among tumors as well as treatments, we determined between which tumors and treatments were the significant differences observed, using a Bonferroni post hoc test. According to our results, the number of genes expressed in H727 was significantly different from H720 ($P = 0.0351$), H835 ($P = 0.0351$) and H1770 ($P = 0.020$). Also was there a significant variation between the number of genes expressed in H720 and H1770 ($P = 0.038$), which also revealed a significant difference between treatments in these two tumors ($P = 0.031$).

**Figure 3.9.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing expression of early negative regulators of neural differentiation (Gene Group 1).

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents early negative regulators of neural differentiation (Gene Group 1). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated NETs. To visualize results, the total number of genes expressed in Gene Group 1, by a single NET sample, is represented by a bar. The number of genes was expressed as mean ± SD ($n = 10$),
calculated for three independent experiments. SD values were calculated between tumors of the same treatment. H720, H835 and H1770 were significantly different from H727, and H720 significantly different from H1770 when analysis of variance showed an F value with $P < 0.04$.

Therefore, according to our analyses, there is a significant difference between these tumors in regard to their expression of early negative regulators of neural differentiation. The higher number of genes expressed in H727 and H720 tumors, compared to the rest of the NETs, was a possible indication of suppressed neural differentiation and promotion of epidermogenesis. Therefore, we can conclude, as in NBs that other than neural developmental programs was also active in H727 and H720 NETs.

**Gene Group 2: Transcriptional regulators linking neural differentiation to activation of proneural genes.**
The nine genes constituting transcriptional regulators linking neural differentiation to activation of proneural genes are responsible for promoting primary neurogenesis. These genes are activated upon suppression of BMP signaling by neural inducers [Sasai, 1998]. Gene group 2 consists of Gli-1, Gli-2, Gli-3, Zic-1, Zic-2, Zic-3, IIX-2a, IIX-3 and Sox-2. As observed in our histogram (Figure 3.10), H727 revealed the highest untreated number of genes expressed, with H1770 revealing the lowest. Gene expression upon treatment differed somewhat in each tumor, when compared to untreated expression profile in that tumor sample. However, according to our statistical analyses, it was clear that there was no significant difference ($P = 0.342$) among the five NETs in regard to the amount of group 2 genes expressed. Also did we observe that the gene expression patterns for each treatment was not significantly different ($P = 0.123$) from each other.
**Figure 3.10.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing expression of transcriptional regulators linking neural differentiation to activation of proneural genes (Gene Group 2).

![Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing expression of transcriptional regulators linking neural differentiation to activation of proneural genes (Gene Group 2).](image)

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents transcriptional regulators linking neural differentiation to activation of proneural genes (Gene Group 2). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated NETs. To visualize results, the total number of genes expressed in Gene Group 2, by a single NET sample, is represented by a bar. The number of genes was expressed as mean ± SD (n = 9), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. No significant P values could be derived from the analysis of variance for the five tumors, as well as the different treatments.

**Gene Group 3: Transcriptional regulators linking neural determination to specification of neurogenic genes – e.g. proneural and neurogenic genes.**

Genes, constituting transcriptional regulators linking neural determination to specification of neurogenic genes, that is operational during normal development, is well applicable for monitoring the activated developmental programs in NETs and involved proneural and neurogenic genes of the bHLH family. Gene group 3 consisted of 18 regulatory genes namely, NN-1, NN-3, hASH-1, NeuroD1, NeuroD2, NeuroD3, NSCL-1, NSCL-2, Hes-1, Hes-5, Hey-1, Hey-2, HeyL, MyT-1, MyT-2, MyT-3, Olf-1 and Olf-1H. According to the histogram for gene group 3 (Figure 3.11), the expression patterns for the 18 genes of this group varied in each tumor with the highest number of genes expressed observed in H1770 cells, also upon treatment. The two-way analysis of variance data revealed that there was a significant difference (P = 0.0009) between the
five NETs in regard to the amount of group 3 genes expressed. However, no significant difference ($P = 0.573$) was observed between the gene expression patterns for each treatment. The differences among the tumors expressing group 3 genes, as observed with the two-way ANOVA, allowed us to perform a Bonferroni post hoc test to determine between which tumors were the significant differences observed. Data from the post-test revealed that H727 showed significant variation in the number of group 3 genes expressed when compared to H720 ($P = 0.009$) and MC-11 ($P = 0.032$). Also did our results reveal that H1770 was significantly different from H720 ($P = 0.020$) and MC-11 ($P = 0.006$).

**Figure 3.11.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing expression of transcriptional regulators linking neural determination to specification of neurogenic genes (Gene Group 3).

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents transcriptional regulators linking neural determination to specification of neurogenic genes (Gene Group 3). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated NETs. To visualize results, the total number of genes expressed in Gene Group 3, by a single NET sample, is represented by a bar. The number of genes was expressed as mean ± SD ($n = 18$), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. H720 and MC-11 were significantly different from H727, and H1770 significantly different from H720 and MC-11 when analysis of variance showed an $F$ value with $P < 0.04$. 

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Based on the prominence of neural features, our results, which revealed a significant difference in gene expression between these five NETs, indicated that the H1770 tumor would classify this as developmentally more advanced/mature than the rest of the tumors because of its high expression of Group 3 genes. Its characteristic set of expressed transcriptional regulators included many which are typical of postmitotic neural cells, including all NeuroDs, NSCL-1 and NSCL-2, and the structural proteins - NFs.

**Gene Group 4: Transcription factors characterizing fate switches to specific neural lineages.**

The 11 genes constituting transcription factors that characterize fate switches to specific neural lineages are Brn-4, Brn-5, Dlx-2, Gata-2, Gata-3, LMO4, Otx-1, Otx-2, Pax-6, Pbx-1, and Sox-11. During normal development, cells that are selected to become neurons begin to express NeuroD1 [Korzh et al., 1998; Lee et al., 1995]. However, numerous other regulators are needed to provide fine positional and specification information to maturing neurons. Therefore, we can assume that gene group 4 consisted of transcriptional regulators that have previously been identified to specify various neuronal phenotypes. Our histogram (Figure 3.12) revealed only slight changes in the number of genes expressed between each tumor. In agreement with our histogram presentation, the statistical analyses revealed no significant difference ($P = 0.265$) between the five NBs in regard to the amount of group 4 genes expressed. Also was there no significant difference ($P = 0.116$) observed between the gene expression patterns of each treatment.
**Figure 3.12.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing expression of transcription factors characterizing fate switches to specific neural lineages (Gene Group 4).

![Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs](image)

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the number of genes expressed in the group of genes that represents transcription factors characterizing fate switches to specific neural lineages (Gene Group 4). Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated NETs. To visualize results, the total number of genes expressed in Gene Group 4, by a single NET sample, is represented by a bar. The number of genes was expressed as mean ± SD (n = 11), calculated for three independent experiments. SD values were calculated between tumors of the same treatment. No significant P values could be derived from the analysis of variance for the five tumors, as well as the different treatments.

Upon combining the number of genes expressed in all four groups of genes, for each tumor, we observed that (Figure 3.13) all five NETs showed different untreated and treated gene expression patterns. According to our statistical analyses, the five NETs are significantly different (P = 0.022) from each other in regard to their expression of the 48 regulatory genes, analyzed in our study. Our data revealed that the gene expression patterns for each treatment were also significantly different (P = 0.011) from each other. These results allowed us to determine between which tumors and treatments were the significant differences observed, in regard to their expression of the 48 regulatory genes. According to the Bonferroni post hoc test, the only significant differences were observed between H727 and MC-11 (P = 0.012). The post hoc test on the treatment results revealed a significant difference in untreated NET gene expression versus
GDNF/TGF-β1 ($P = 0.018$) treated NETs as well as RA-treated gene expression profile versus that of GDNF/TGF-β1 ($P = 0.022$) treated. No significance was found between the gene expression profiles of untreated versus RA-treated NETs.

**Figure 3.13.** Histogram of untreated, GDNF/TGF-β1 and RA-treated NETs revealing number of genes expressed for all four groups of genes, combined.

![Histogram of gene expression](image)

**Figure legend:** mRNA expression data are shown in histogram-format with each bar representing the total number of genes expressed for all four groups of genes, combined. Three bars grouped together represent a single tumor sample. Expression patterns of genes were analyzed in untreated (black bars), GDNF/TGF-β1 (red bars) and RA (blue bars) treated NETs. To visualize results, the number of genes expressed in all four groups of genes combined, in a single NET sample, is represented by a bar. The number of genes was expressed as mean ± SD ($n = 48$), calculated for three independent experiments. SD values calculated between tumors of the same treatment. H727 and MC-11 were considered significantly different from each other when analysis of variance showed an $F$ value with $P < 0.02$. Also did the analysis of variance reveal that untreated NETs were significantly different from GDNF/TGF-β1 treated NETs and RA treated significantly different from GDNF/TGF-β1 treated NETs with $P < 0.03$.

According to our results obtained of the five human NETs analyzed in this study, we are able to reject the null hypotheses and accept our first hypotheses in that each of the five analyzed NETs is molecularly different from the other based on their expression of the four groups of neurogenesis-involved regulatory genes. Based on our statistical analyses, we could again reject the null hypotheses and therefore accept our second
hypotheses in that the NETs exhibited significantly different responses to drug (GDNF/TGF-β1 and RA) treatment.

To conclude, our study revealed that the five NBs and NETs are molecularly different from each other based on their expression of the 48 regulatory genes and that drug treatments applied (GDNF/TGF-β1 and RA) induced changes in the expression profile of NETs. Although the only statistical significant data we could derive were based on the number of genes expressed in each tumor, also upon treatments, it was sufficient enough to identify changes between these tumors and to emphasize the importance of identifying genetic markers for effective treatment of each individual tumor. Since the main goal of our study was only to show that NBs and NETs are molecularly different based on their expression of regulatory genes and that these expression profiles will change upon drug treatments, our study was therefore not designed to allow the identification of significant differences between individual genes in each tumor. Therefore, follow-up studies are necessary and will be designed to obtain quantitative measures, such as mRNA (quantitative RT-PCR or microarrays) and protein expression, to determine the level of individual gene expression in each tumor and how these genes (specifically the expression levels) respond to various treatments. Given that the main goal of these studies will be to identify genetic markers for individual tumors, understanding the logic of cellular development will be essential, first for recognizing errors in the molecular machinery (level of gene expression) that leads to cancer, and second to assess the best way to overcome these errors (effect of various treatments on gene expression levels).
Chapter 4: Summary and Conclusion

Summary: Although the induction of tumor differentiation through manipulation of regulatory genes is a relatively unexplored area of cancer therapy, it might suggest an additional approach for tumor-specific treatment [Tallman et al., 1997]. Therefore, in view of this idea, we have investigated the following hypotheses in that 1) individual NBs and NETs are molecularly different based on their expression of regulatory genes and that 2) exposure of NBs and NETs to extrinsic factors, such as GDNF/TGF-β1 and RA, will alter their growth potential. To test our hypotheses, we analyzed about 50 neurogenesis-involved regulatory genes in untreated, GDNF/TGF-β1 and retinoic acid-treated NBs and NETs, using RT-PCR to reveal the gene expression patterns in these five human NB and five NET cell lines. However, in order to get an idea of the efficiency of the extrinsic factor treatment, an understanding of the cellular potential of these tumors was necessary. Therefore, our study also monitored which signaling pathways and whether multi-neurotransmitter synthesis is active and/or activated/blocked in these NBs and NETs.

According to our results, each individual NB and NET reflected a certain stage of neural development, characterized by the expression of discrete sets of regulatory genes. Two major adaptations were observed in the TF networks of both NBs and NETs upon GDNF/TGF-β1 as well as RA treatment. In NBs, the first adaptation included genes that exhibited cell autonomous regulation and showed no change in their expression upon GDNF/TGF-β1 and RA treatment. Upon GDNF/TGF-β1 treatment, this group comprised mostly of TFs linking neural differentiation to activation of proneural genes (no change in 56% of genes in this group) (Table 3.12), whereas upon RA-treatment, it consisted mostly of TFs linking neural determination to specification of neurogenic genes (no change in 61% of genes in this group) (Table 3.19). The second adaptation included genes that exhibited signal-dependent regulation and their expression was affected by GDNF/TGF-β1 and RA treatment. GDNF/TGF-β1 and RA treatment induced changes mostly in the group of TFs that characterized fate switches to specific neural lineages (55% change in genes of this group) (Tables 3.12 and 3.19). In NETs, upon GDNF/TGF-β1 treatment, the first adaptation comprised
mostly of TFs linking neural determination to specification of neuronal identity (no change in 61% of genes in this group) (Table 3.30), whereas RA treatment induced mostly no change in the group of genes constituting early negative regulators of neural differentiation (no change in 73% of genes in this group) (Table 3.34). For the second adaptation in NETs, GDNF/TGF-β1 and RA treatment mostly induced changes in the group of TFs linking neural differentiation to activation of proneural genes (89% change in genes of this group upon GDNF/TGF-β1 treatment and 78% change in genes of this group upon RA treatment) (Tables 3.30 and 3.34). Our study also determined statistically the significant difference in gene expression among these tumors, by counting the number of genes expressed in each tumor. According to these results, we observed a significant difference in the number of neurogenesis-involved regulatory genes expressed in NBs, although the changes in the number of genes expressed upon GDNF/TGF-β1 and RA treatment were not significantly different (Figure 3.6). As in NBs, we could also observe a significant difference in the number of genes expressed among the five NETs. Also did these tumors reveal a significant difference in the number of genes expressed upon GDNF/TGF-β1 and RA treatment (Figure 3.13).

Upon interpretation of our results, the most important finding to emerge from the present study is that each tumor is molecularly unique within the diagnostic category (NB or NET), and has a characteristic profile of gene expression implicated in neurogenesis (resembling neural stem/progenitor cells). The study suggested that individual NBs and NETs reflected a certain stage of neural development, characterized by the expression of discrete sets of regulatory genes. The present study is unique in the sense that we could demonstrate that these distinct sets of transcriptional regulators, implicated in neurogenesis, were affected upon growth factor and retinoic acid treatment, and that some changes lead to induced proliferation of NB and NET cells, whereas others resulted in the initiation of differentiation and cell death. Therefore our results, even though obtained from a very limited source of material, support the important role of this gene expression study in evaluating tumor molecular patterns, which could suggest the appropriate treatment.
**Conclusion:** Although embryologists have long used morphological characteristics, and more recently marker genes to identify neural tissue and to test neural-inducing activity of specific cell populations and signaling molecules, results from the present study are the first to demonstrate that the application of existing knowledge of TF networks/marker genes, to NBs and NETs, is informative enough to form the basis of tumor-specific diagnostics and therapy.

Mapping of NBs systematically overexpressed genes corresponding to the earliest stage of neural development. This set of genes constituting gene group 4 (*transcription factors characterizing fate switches to specific neural lineages*) and is essential for cell survival and proliferation in early CNS development. NBs also underexpressed genes corresponding to later stages of neural development. This set was enriched in genes constituting gene group 2 (*transcriptional regulators linking neural inducers to activation of proneural genes*) that is responsible for promoting primary neurogenesis. These data therefore imply that NBs are arrested in certain stages of differentiation characterized by specific sets of transcriptional regulators. On the other hand, NETs overexpressed genes corresponding to gene group 3 (*transcriptional regulators linking neural determination to specification of neuronal identity – e.g. proneural and neurogenic genes*) that is predicted to act as neuronal commitment factors and underexpressed genes corresponding to gene group 2. These results could therefore entail a developmental stage of NETs that corresponded to a certain stage of neurogenesis.

In the present study, identification of transcriptional regulators allowed a qualitative view of the changes that accompany at the genomic level and enabled deeper insight into the molecular bases of the NBs and NETs. Also may some of the TF expression alterations identified in this study, prove useful in the development of novel diagnostic strategies. Specifically, the expression signature of Zic, Gli and Irx families of factors could be exploited as developmental stage designation markers for classification of NBs and NETs. The results from this study can also be employed

The ability to discriminate morphologically similar NBs and NETs on the basis of their gene expression profiles has the potential to guide the selection of therapeutic strategies, thereby maximizing therapeutic efficacy and minimizing toxicity.
On the basis of current knowledge, it is already quite evident that monitoring of large sets of genes (e.g. full genome), using DNA microarray technology [Ross et al., 2000; Perou et al., 1999; Iyer et al., 1999], similarly to that of individual transcription factors and/or isolated cascades of factors is not sufficient enough to understand the logic of cellular development, since it provides only a massive amount of data without any significant interpretation. Therefore, an understanding of the logic of cellular development is essential, first for recognizing errors in the molecular machinery (network of transcriptional regulators) that leads to cancer, and second to assess the best way to overcome these errors. Since the focus of the present study was to obtain such an understanding of the logic of cellular development, it is very possible that with follow-up studies the groundwork laid by our study might allow for customization of DNA microarrays as a diagnostic tool for the extensive molecular classification of NB and NET biopsies or resected tumor samples. Also may the innovative approach and results of the present study identify specific modifiable genes in other tumors of mixed neuro-glial origin such as astrocytomas, glioblastoma multiforme and oligodendrogliomas.
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Appendix

MATERIALS

1. Cell Culture Media

1.1 Neuroblastomas (SK-N-MC and SK-N-SH)
   - 500ml Minimal essential medium (MEM) (Life Technologies)
   - 50ml Fetal bovine serum (FBS) (Life Technologies)
   - Penicillin/Streptomycin (Pen/Strep) (Life Technologies)
   - 5ml 1mM Pyruvate (Sigma Aldrich)
   - 5ml 1mM Non-essential amino acids (Sigma Aldrich)
   - Store at 4°C

1.2 Neuroblastomas (SK-N-AS; SK-N-BE2; MC-1XC)
   - 500ml MEM (Life Technologies)
   - 50ml FBS (Life Technologies)
   - Pen/Strep (Life Technologies)
   - Store at 4°C

1.3 Pulmonary neuroendocrine tumors (H720 and H1770)
   - 500ml DMEM/F12 (Life Technologies)
   - 10ml B-27 supplement (Life Technologies)
   - Pen/Strep (Life Technologies)
   - 100ul (10ug/ml) Heparin (Sigma Aldrich)
   - 20ng/ml Leukemia inhibitory factor (LIF) (Peprotech)
   - 20ng/ml basic Fibroblast growth factor (bFGF) (Peprotech)
   - Prepare 50ml aliquots and store at -20°C

1.4 Pulmonary neuroendocrine tumors (H727; MC-11; H835)
   - 500ml DMEM/F12 (Life Technologies)
   - 50ml FBS (Life Technologies)
   - Pen/Strep (Life Technologies)
   - Store at 4°C
2. **Neurotrophins/Growth Factors/Cytokines**

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<td>2.3 NT-3</td>
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3. **Antibodies**

3.1 **Primary Antibodies**

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3.1.18 Mouse Internexin-α 1:100 Chemicon, CA
3.1.19 Mouse NeuN 1:100 Chemicon, CA
3.1.20 Mouse CNPase 1:500 Sigma Aldrich,
3.1.21 Mouse Actin 1:5,000 Chemicon, CA
3.1.22 Mouse GFAP 1:400 Chemicon, CA
3.1.23 Mouse MAP-2 1:200 Chemicon, CA
3.1.24 Mouse βIII-tubulin 1:200 Chemicon, CA
3.1.25 Mouse Desmin 1:25 Chemicon, CA
3.1.26 Mouse NSE 1:10 Chemicon, CA
3.1.27 Mouse DBH 1:300 Chemicon, CA
3.1.28 Mouse O4 1:100 Chemicon, CA
3.1.29 Mouse N-CAM 1:100 DAKO, CA
3.1.30 Rabbit GABA 1:300 Signature Immunol.
3.1.31 Rabbit L-Glutamate 1:300 Signature Immunol.
3.1.32 Rabbit L-Glutamine 1:300 Signature Immunol.
3.1.33 Rabbit Glutathione 1:300 Signature Immunol.
3.1.34 Rabbit Glycine 1:300 Signature Immunol.

3.2 Secondary Antibodies
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3.2.2 Mouse Cy3 1:300 Chemicon, CA
3.2.3 Goat Cy3 1:300 Chemicon, CA

4. RNA Isolation Reagents

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5. Reverse Transcription (cDNA preparation) Reagents

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- dGTP: 2'-deoxyguanosine 5'-triphosphate, sodium salt
- dATP: 2'-deoxyadenosine 5'-triphosphate, sodium salt
- dTTP: 2'-deoxythymidine 5'-triphosphate, sodium salt
- dCTP: 2'-deoxycytidine 5'-triphosphate, sodium salt

Add 20\mu l of each, a, b, c and d to 120\mu l of RNA-free water to make 200\mu l of a 10mM dNTP-mix.

5.3 Superscript Kit Life Technologies, CA

### 6. RT-PCR Reagents

#### Product Name

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