Helsinki University Biomedical Dissertations No. 119

FROM NEURAL STEM CELLS TO PRECURSORS -
MOLECULAR REGULATION OF SELF-RENEWAL AND DIFFERENTIATION

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Faculty of Biosciences,
University of Helsinki, in Small Lecture Hall, Haartman Institute,
Haartmanin katu 3, Helsinki on 24th of April 2009 at 12.00 p.m.

Helsinki 2009
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ISBN 978-952-10-5455-6 (PDF)
ISSN 1457-8433
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2009
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original publications, which are referred to in text by their Roman numbers, and on some unpublished data.

I  Katja Piltti, Laura Kerosuo, Janne Hakanen, Minna Eriksson, Alexandre Angers-Loustau, Sirpa Leppä, Marjo Salminen, Hannu Sariola and Kirmo Wartiovaara

E6/E7 ONCOGENES INCREASE AND TUMOR SUPPRESSORS DECREASE THE PROPORTION OF SELF-RENEWING NEURAL PROGENITOR CELLS  

II  Laura Kerosuo, Katja Piltti, Heli Fox, Alexandre Angers-Loustau, Valtteri Häyry, Martin Eilers, Hannu Sariola and Kirmo Wartiovaara

C-MYC INCREASES SELF-RENEWAL IN NEURAL PROGENITOR CELLS THROUGH MIZ-1.  

III  Mikaela Grönholm, Tambet Teesalu, Jaana Tyynelä, Katja Piltti, Tom Böhling, Kirmo Wartiovaara, Antti Vaheri and Olli Carpen

CHARACTERIZATION OF THE NF2 PROTEIN MERLIN AND THE ERM PROTEIN EZRIN IN HUMAN, RAT, AND MOUSE CENTRAL NERVOUS SYSTEM.  
Molecular and Cellular Neuroscience 28: 683-693 (2005)

IV  Katja Piltti, Anu Plaken, Paula Haiko, Fares Zeidán-Chuliá, Nina Perälä, Anna Popsueva, Madis Jakobson, Petri Auvinen, Marika J Karkkainen, Maria Herranen, Hannu Sariola, Kari Alitalo, Mart Saarma, Kirmo Wartiovaara and Kirsi Sainio

VASCULAR ENDOTHELIAL GROWTH FACTOR C PROMOTES PROLIFERATION AND SURVIVAL OF EARLY SYMPATHETIC PROGENITORS.  
Submitted to Development
ABSTRACT

Stem cells are responsible for tissue turnover throughout lifespan. Only highly controlled specific environment, “the stem cell niche”, can sustain undifferentiated stem cell-pool. The balance between maintenance and differentiation is crucial for individual’s health: uncontrolled stem cell self-renewal or proliferation can lead to hyperplasia and mutations that further provoke malignant transformation of the cells. On the other hand, uninhibited differentiation may result in diminished stem cell population, which is unable to maintain tissue turnover. The mechanisms that control the switch from maintenance to differentiation in stem cells are not well known. The same mechanisms that direct the self-renewal and proliferation in normal stem cells are likely to be also involved in maintenance of “cancer stem cell”. Cancer stem cells exhibit stem cell like properties such as self-renewal- and differentiation capacity and they can also regenerate the tumor tissue.

In this thesis, I have investigated the effect of classical oncogenes E6/E7 and c-Myc, tumor suppressors p53 and retinoblastoma (pRb) family, and vascular endothelial growth factor (VEGF) subfamily and glial cell line-derived neurotrophic factor (GDNF) family ligands on behavior of neural stem cells (NSCs) and progenitors. The study includes also the characterization of cytoskeletal tumor suppressor neurofibromatosis 2 (NF2) protein merlin and ezrin-radixin-moesin (ERM) protein ezrin expression in neural progenitors cells and their progeny.

This study reveals some potential mechanisms regarding to NSCs maintenance. In summary, the studied molecules are able to shift the balance either towards stem cell maintenance or differentiation; tumor suppressor p53 represses whereas E6/E7 oncogenes and c-Myc increase the proportion of self-renewing and proliferating NSCs or progenitors. The data suggests that active MEK-ERK signaling is critical for self-renewal of normal and oncogene expressing NSCs. In addition, the results indicate that expression of cytoskeletal tumor suppressor merlin and ERM protein ezrin in central nervous system (CNS) tissue and progenitors indicates their role in cell differentiation. Furthermore, the data suggests that VEGF-C a factor involved in lymphatic system development, angiogenesis, neovascularization and metastasis but also in maintenance of some neural populations in brain is a novel trophic factor for progenitors in early sympathetic nervous system (SNS). It seems that VEGF-C dose dependently through ERK-pathway supports the proliferation and survival of early sympathetic progenitor cells, and the effect is comparable to that of GDNF family ligands.
ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ARN</td>
<td>anterior neural ridge</td>
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<tr>
<td>ARTN</td>
<td>artemin</td>
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<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<td>BLBP</td>
<td>brain-lipid-binding protein</td>
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<td>BMP</td>
<td>bone morphogenic proteins</td>
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<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
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<td>CD133</td>
<td>prominin 1</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
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<tr>
<td>CKI</td>
<td>cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CT-1</td>
<td>cardiotrophin 1</td>
</tr>
<tr>
<td>DβH</td>
<td>dopamine beta-hydroxylase</td>
</tr>
<tr>
<td>ES-cell</td>
<td>embryonic stem cell</td>
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<td>ERK</td>
<td>extracellular signal regulated kinase</td>
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<tr>
<td>E6</td>
<td>E6 oncogene</td>
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<tr>
<td>E7</td>
<td>E7 oncogene</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGFR2</td>
<td>fibroblast growth factor 2</td>
</tr>
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<td>GDNF</td>
<td>glial cell line derived neurotrophic factor</td>
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<td>GFL</td>
<td>GDNF family ligand</td>
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<td>GFR</td>
<td>GDNF family receptor</td>
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<tr>
<td>GLAST</td>
<td>astrocyte specific glutamate transporter</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>HPV 16</td>
<td>human papilloma virus type 16</td>
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<tr>
<td>Hox</td>
<td>homeobox gene</td>
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<tr>
<td>IL-6</td>
<td>interleukine 6</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal protein kinase</td>
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<tr>
<td>LIF</td>
<td>leukemia inhibitor factor</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Miz-1</td>
<td>Myc-interacting zinc finger protein-1</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated extracellular kinase</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
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<tr>
<td>NGF</td>
<td>neuronal growth factor</td>
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<tr>
<td>NF</td>
<td>neurofilament</td>
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<td>NCC</td>
<td>neural crest cell</td>
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<td>NCS</td>
<td>neural stem cell</td>
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<td>NSCM</td>
<td>neural stem cell medium</td>
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<td>Ngn</td>
<td>neurogenin</td>
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<td>NPC</td>
<td>neural progenitor cells</td>
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<td>NRTN</td>
<td>neurturin</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Nrp</td>
<td>neuropilin</td>
</tr>
<tr>
<td>NT</td>
<td>neurotrophin</td>
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<tr>
<td>OLP</td>
<td>oligodendrocyte precursor cell</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PDGFRα</td>
<td>platelet derived growth factor receptor α</td>
</tr>
<tr>
<td>PlGF</td>
<td>placental growth factor</td>
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<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol 3 kinase</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>Sema</td>
<td>semaphorin</td>
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<tr>
<td>SGC</td>
<td>sympathetic ganglia chain</td>
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<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
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<td>SPC</td>
<td>sympathetic progenitor cell</td>
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<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PSPN</td>
<td>persephin</td>
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<tr>
<td>RA</td>
<td>retinoid acid</td>
</tr>
<tr>
<td>TA cell</td>
<td>transient amplifying cell</td>
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<tr>
<td>TGF β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>Trk</td>
<td>tyrosine kinase receptor</td>
</tr>
<tr>
<td>Tuj-1</td>
<td>neuronal class β tubulin</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless/int</td>
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1 REVIEW OF THE LITERATURE

1.1 INTRODUCTION

NSCs and neural progenitor cells (NPCs) differentiate into new neural cells both during embryonic development and adulthood. The differentiation process involves the cell proliferation, but also capacity to respond to microenvironmental signals, exit the cell cycle and remain quiescent. The path from self-renewing NSC to committed progeny is thought to be initiated when cell divisions are switched from symmetric to asymmetric. The decision between self-renewal and differentiation is controlled by several cell intrinsic and extrinsic factors but the mechanisms are still poorly understood. Intrinsic modulators such as length of the cell cycle, orientation of the mitotic spindle, asymmetrically distributed molecules and tumor suppressors together with the epigenetic chromatin modifiers regulate the gene expression in NSCs. These signals either compete or collaborate with the cell extrinsic factors such as different growth factors and signals derived from surrounding microenvironment, extracellular matrix and neighboring tissues.
1.2 **NERVOUS SYSTEM DEVELOPMENT**

1.2.1 Development of central nervous system (CNS)

*Early events of the neural tube development*

CNS that includes the brain and spinal cord is one of the first organ systems formed during vertebrate embryo development (reviewed in Gilbert, 2008). CNS development and its intricate connections occur in several steps. First step is neural induction that results in formation of neural plate. In mammalian species, induction of the neural tissue is likely linked to patterning of the entire early embryo (reviewed in Levine and Brivanlou, 2007). This is followed by neurulation wherein increased cell proliferation makes the neural plate fold, elevate and bend to form a tube like structure (Figure 1).

![Figure 1. Left panel: Folding of the neural plate and the neural tube formation (Modified from Kandel et al. 2000). Right panel: Early events in brain development: formation of the primary vesicles (Modified from Gilbert 2008).](image)

Neural tube formation and maturation in anterior-posterior axis is not concurrent. The most anterior part of the neural tube is already undergoing changes that will eventually lead to the development of the brain while the posterior end is still closing. At this stage, the early neural tube consists of rapidly dividing neuroepithelial cells (Schoenwolf, 1991). Brain development is initiated by formation of three primary vesicles: prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain) (Figure 1B). Next the forebrain subdivides into two hemispheres and forms telencephalon (cerebrum) and diencephalon (interbrain). The cerebrum forms olfactory lobes, hippocampus, cerebral hemispheres and basal ganglia while the interbrain differentiates into thalamic and hypothalamic brain regions. The midbrain does not divide but the hindbrain subdivides into metencephalon (cerebellum) and myelencephalon (medulla...
oblongata). The hindbrain forms also segmental pattern called rhombomeres that later specify the locations where certain cranial nerves originate (reviewed in Kandel et al., 2000).

**Molecular control of CNS patterning**

CNS patterning and cell differentiation is guided in both anterior-posterior-, and ventral-dorsal–axis. The major signaling pathways that are involved in the anterior-posterior patterning are transforming growth factor β (TGF-β) family members, especially bone morphogenic proteins (BMPs) (Liem et al., 1997), fibroblast growth factor (FGF) family, Wingless (Wnt) and retinoid acid (RA) (Pierani et al., 1999) (Figure 2).

Forebrain characteristics are guided by follistatin, noggin and chordin that antagonize BMP signaling. Antagonization of BMP and Wnt signaling seems to be the most critical steps for the development of early anterior CNS (Gilbert, 2008). FGFs, Wnts and RA are involved in the induction of the posterior neural tissues (Gilbert, 2008) and they activate homeodomain protein family, the group of transcription factors that are encoded by homeobox genes (Hox-genes) (Houle et al., 2003). The highly conserved Hox-genes are also expressed in overlapping domains along the hindbrain and spinal cord axis (Gilbert, 2008). In the hindbrain, the Hox-genes control identity of cell boundaries in rhombomeres (Akin and Nazarali, 2008). One of the most studied signaling centers in the brain, the isthmus organizer, localizes between the midbrain and hindbrain area and secretes molecules such as FGFs and Wnts that guide cell patterning in the midbrain and hindbrain area (Partanen, 2007).

Although, the signals that define forebrain development are not as well known one important signaling center in forebrain patterning is anterior neural ridge (ARN) that localizes at junction between anterior neural plate and ectoderm and secretes signaling molecules such as FGF (Martinez-Barbera et al., 2001; Shimamura and Rubenstein, 1997). Gene expression studies indicate that the embryonic forebrain is subdivided along its anterior-posterior axis into transversal prosomeres. These boundaries coincide with the expression patterns of certain transcription factors and inductive signals. For example, Shh is expressed in the cell boundary between prosomeres 2 and 3, and the signals from this area are thought to control cell differentiation in the forebrain similarly as the isthmus organizer controls patterning in the mid- and hindbrain (reviewed in Kandel et al., 2000; Akin and Nazarali, 2008).
From neural stem cells to precursors  

Review of the Literature

Figure 2. Patterning of early neural tube in anterior-posterior-axis. In anterior neural tube, BMP antagonists chordin, noggin and follistatin to establish the forebrain characteristics. Hindbrain and the posterior neural tube are patterned by RA, Wnts and FGFs, which activate Hox-genes. (Modified from Kandel et al., 2000)

In the spinal cord and brain, the cell differentiation is also guided in ventral-dorsal-axis (Figure 3). Dorsal ectoderm secretes signals such as BMPs, which commits the roof plate cells in dorsal neural tube to secrete BMP-4, and this induces expression cascade of TGF-β family proteins such as dorsalin and activin in the adjacent cells (Liem et al., 1997). Whereas, the ventral neural tube is exposed to signals such as Sonic hedgehog (Shh) and RA derived from notocord and somites, respectively (Pierani et al., 1999). Shh induction commits the floor plate cells in ventral neural tube to initiate additional Shh expression (Briscoe et al., 1999; Roelink et al., 1995). The gradient of the roof- and floor plate secreted proteins together with their down-stream targets commit interneurons in the spinal cord (Lumsden and Krumlauf, 1996). The same dorsal-ventral signals are also involved in patterning of the hindbrain, midbrain and much of the forebrain (Kandel et al., 2000).
1.2.2 Development of peripheral nervous system (PNS)

The PNS consists of the somatic and autonomic nervous systems, and the latter can be divided to sympathetic-, parasympathetic- and enteric nervous systems. Unlike CNS, PNS is derived from the neural crest (Le Douarin et al., 2004).

Neural crest

In mammals, neural crest is a transient cell lineage that holds an extensive plasticity and high migration capacity. The neural crest is formed from the dorsal neuroectoderm in the area where ectoderm and neural tube connect. During neural tube formation, BMPs, Wnts, and FGFs induce expression of transcription factors, the neural plate border specifiers, such as Distalless-5 and Pax-3, which prevent the neural crest becoming either epidermis or neural plate (Huber, 2006). Consequently, this induces expression of another set of transcription factors, the neural crest specifiers, such as Snail, FoxD3 and RhoB. FoxD3 is critical for specification of ectodermal cells into neural crest cells (NCCs), and Snail and RhoB are required for neural crest migration (reviewed in Sauka-Spengler and Bronner-Fraser, 2006). Prior to the migration, the NCCs undergo epithelial-to-mesenchymal transformation and loose their attachment. RhoB is thought to be involved in cytoskeletal modifications needed for the migration, whereas Snail affects the cell-to-cell attachment (reviewed in Cheung et al., 2005).

Figure 3. Dorsal-ventral patterning of the neural tube is guided by gradient of BMPs and Shh. (Modified from Gilbert, 2008)
The neural crest can be divided into four regions that have their own derivatives: cranial, trunk, vagal/sacral, and cardiac neural crests (Figure 4). In mammals, the cranial neural crest starts to migrate while the neural tube is still folding whereas in the trunk area the migration is initiated when the neural tube is closing (reviewed in Gilbert, 2008). The cranial NCCs migrate dorsolaterally and form the facial and neck structures including a proportion of cranial neurons and glial cells. Also NCCs that differentiate into melanocytes migrate dorsolaterally (Figure 4) into embryonic ectoderm and towards ventral midline (Gilbert, 2008). In addition to pigment cell formation, this NCC population can also differentiate into neurons, cartilage and connective tissue. Contrary to cranial NCCs, the cells in trunk, vagal, sacral and cardiac regions migrate along the ventral migration route (Figure 4) (Gilbert, 2008). The cardiac neural crest, which is located between the cranial and trunk area, contributes to formation of the septum and the walls of large arteries in heart. Whereas, sensory (dorsal root ganglia) and sympathetic ganglia are derived from the trunk neural crest, and parasympathetic and enteric ganglia from vagal and sacral regions (Gilbert, 2008). The epinephrine secreting chromaffin cells in adrenal medulla are also derived from trunk neural crest.
Sympathetic nervous system development

The sympathetic nervous system (SNS) is derived from trunk NCCs that migrate through the ventrolateral migratory pathway (Fraser and Bronner-Fraser, 1991). During the migration, the NCCs are surrounded by extracellular matrix proteins, such as laminin, fibronectin, tenasin, different types of collagen and proteoglycans that promote the migration (Testaz and Duband, 2001). The extracellular matrix protein trombospondin is expressed in the anterior but not in the posterior part of the somites and is important in guiding the migration as well (Tucker et al., 1999). Permissive signals as well as repulsive signals are essential to NCC migration. EphrinB1 is one of the inhibitory transmembrane proteins that localizes in the cells in posterior part of the somite (Wang and Anderson, 1997). Blocking of either EphrinB1 or its receptor EphB2 leads to disrupted formation of the sympathetic ganglia (Kasemeier-Kulesa et al., 2006). Class 3 secreted semaphorin Sema3F is also expressed in the posterior somite while the migrating NCCs express its receptor Neuropilin 2 (Nrp2) (Chen et al., 2000; Giger et al., 2000). Nrp-2-deficiency results in loss of segmental NCC migration pattern while the cells migrate through both anterior and posterior part of the somite (Gammill et al., 2006). In addition, Sema3F is also involved in the repulsive guidance of the axons (Chen et al., 2000; Giger et al., 2000). Also Sema3A and its receptor Nrp1 play role in sympathetic ganglia chain migration since both Sema3A and Nrp1-deficient mice show dislocation of in sympathetic neurons and their progenitors (Kawasaki et al., 2002).

After the migration, these pre-sympathetic progenitors aggregate laterally to dorsal aorta, form immature sympathetic ganglia chain and undergo neuronal and catecholaminergic differentiation. The early sympathetic progenitors initiate expression of neuronal class β tubulin, neurofilament (NF), SCG10 and other neuronal markers. Also the expression of enzymes needed for the synthesis of noradrenaline such as tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DbβH) is initiated (reviewed in Huber, 2006; LaBonne and Bronner-Fraser, 1999). Cell adhesion molecule N-cadherin is transmembrane protein needed for the patterning of the sympathetic ganglia (Kasemeier-Kulesa et al., 2006). Sympathetic progenitors start express N-cadherin prior to aggregation of the ganglia (Pla et al., 2001), and a dominant negative form of N-cadherin has been shown to disturb the normal ganglia compaction (Kasemeier-Kulesa et al., 2006).

Also several growth factors such as GDNF family members GDNF and artemin (ARTN), neurothropins nerve growth factor (NGF) and Neurotropin-3 (NT-3) and their receptors and are needed for normal SNS development (Figure 5). BMPs are known to promote differentiation of sympathetic neurons and bHLH transcription factors such as Mash-1, Phox2a and Phox2b, and zinc finger transcription factors GATA3 are required for initiation and maintenance of noradrenergic phenotype in sympathetic cells (Figure 5). These factors and their role in SNS development will be discussed in differentiation of sympathetic lineage cells and growth and survival factors of GDNF-family chapters.
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Figure 5. Factors that regulate development of sympathetic nervous system. GDNF family growth factors GDNF and ARTN and their receptors are important for migration, proliferation and survival of early sympathetic progenitors. Migration and aggregation of the cells is guided by Sema3A and Sema3B, EphrinB1 and their receptors Nrp1, Nrp2 and EphB2, respectively. N-cadherin is needed for aggregation of early SGC. Dorsal aorta (DA) derived BMPs and neuregulins promote SPC differentiation and transcription factors such as Mash-1, Phox2a, Phox2b and GATA3 are required for initiation and maintenance of noradrenergic phenotype in sympathetic cells. Later, while SGC matures, the GFLs switch to promote survival and target innervation of mature sympathetic neurons and NGF and NT-3 function as target-derived survival or differentiation factors. NCCs = neural crest cells, SGC = sympathetic ganglia chain, NT = neural tube.

1.3 NEURAL STEM CELLS

Neural stem cell (NSC) can be defined as a cell 1) that is nervous system derived or can generate neural tissue, 2) it has proliferation- and self-renewal capacity, 3) and multipotency to differentiate into three different neural cell lineages: neurons, astrocytes and oligodendrocytes (Gage, 2000; McKay, 1997). NSCs can be derived not only from nervous system but also from pluripotent embryonic stem cells (ES cells) or reprogrammed fibroblasts (Brustle et al., 1999; Thomson et al., 1998; Wernig et al., 2008).

NSCs and neural progenitors (NPCs) are commonly cultured as neurospheres that are a heterogenous population of undifferentiated NSCs, NPCs and cells at the various stages of differentiation (reviewed in Jensen and Parmar, 2006, Kornblum, 2007). The neurosphere culture system was the first in vitro system to demonstrate that the adult brain contains cells that have characteristics of true NSCs (Reynolds et al., 1992, Reynolds and Weiss, 1996). The system is still a useful tool to analyze proliferation, self-
renewal capacity and multipotency of NSCs and NPCs (Jensen and Parmar, 2006). However, the major problems of neurosphere assay are that it is sensitive to culture methods used. Different concentration of factors in the medium, method and frequency of passasing, sphere size, number of passages after isolation lead to different cell properties within the spheres (Jensen and Parmar, 2006). Moreover, variations in cell density alter the microenvironment and this may affect cell proliferation for instance (reviewed in Jensen and Parmar, 2006). This sensitivity may lead to difficulties to compare the data between different research groups or even to interpret the results within the same study (reviewed in Jensen and Parmar, 2006).

Regardless of these weaknesses the neurosphere culture system is valuable tool to study neurogenesis and neural development in vitro. With this system, it is possible to assess intrinsic cell specification at various developmental timepoints without interference of the extrinsic cues provided by an in vivo environment. Moreover, the intrinsic cell properties can be easily modulated, for example by using viral vectors, and the extrinsic cues can be changed by modifying the culture condition. In addition, NSCs and NPCs cultured in neurospheres have shown to behave as their temporal and spatial in vivo correlates in fetal brain and maintain expression of many developmental genes by region specific manner after several passages depending on the area of collection. However, as is the case for any other cell, culturing NSCs changes their properties so it cannot be assumed that the studies necessarily imply similar mechanisms in vivo (Kornblum, 2007).

1.3.1 NSC niche

In the nervous system, NSCs and progenitors occupy a tissue specific niche. In the adult CNS, the majority of NSCs are located in the subventricular zone (SVZ) of the telencephalic lateral ventricles next to ependymal cells that separate SVZ from lateral ventricles and cerebrospinal fluid secreted by endothelial cells in choroid plexus. In SVZ, NSCs are closely associated with microvessels (Palmer et al., 2000; Ward and Lamanna, 2004) and the endothelial cells have shown to promote maintenance of NSCs (Mompeo et al., 2003; Shen et al., 2004). Neurons and endothelial cells can communicate with each other via VEGF-A, neurotrophins and their cognate receptors (Chow et al., 2001; Kim et al., 2004a). Therefore, the neurovascular niche may play important role in adult stem cell maintenance.

1.3.2 CNS stem cells and progenitors

*Stem cells during CNS development*

In embryos, NSCs are found in several regions such as cerebellum, hippocampus, cerebral cortex, basal forebrain and spinal cord (reviewed in Temple, 2001). Primary sphere-formation assays indicate that the stem cells are prevalent at early developmental stages but the number of CNS NSCs declines rapidly during embryonic development (Mayer-Proschel et al., 1997; Qian et al., 2000). In rats, 50% of spinal cord cells are able
to form neurospheres at embryonic day (E) 8 (Kalyani et al., 1998; Mayer-Proschel et al., 1997). However, by E12 the number sphere-forming cells had decreased to 10%, and at first postnatal day (P1) only 1% of the cells showed sphere formation (Kalyani et al., 1998; Mayer-Proschel et al., 1997).

During early development, the neuroepithelial cells express markers such as transmembrane protein prominin 1 (CD133) (Weigmann et al., 1997) and intermediate filament protein nestin (Hartfuss et al., 2001). In mouse, the neuroepithelial cells give rise to radial glia that replace most of the neuroepithelial cells by E12 (Hartfuss et al., 2001; Kriegstein and Gotz, 2003; Noctor et al., 2002). Radial glia express markers such as CD133, astrocyte specific glutamate transporter (GLAST), Ca^{2+}-binding protein S100β, glial fibrillary acidic protein (GFAP), vimentin, and brain-lipid-binding protein (BLBP) (Kriegstein and Gotz, 2003). Most of the radial glial cells form neurons during neurogenesis and later either astrocytes or oligodendrocytes (Malatesta et al., 2000; Noctor et al., 2002; Noctor et al., 2004). Therefore, the majority of neurons are derived either directly or indirectly from radial glial cells (Anthony et al., 2004; Malatesta et al., 2003). In developing CNS, a third cell type appears at the onset of neurogenesis and it is called as basal progenitor or neuroblast. Basal progenitors originate from the neuroepithelial and radial glial, and they form SVZ and contribute to neurogenesis (Haubensak et al., 2004; Noctor et al., 2004). Basal progenitors differ from neuroepithelial and radial glial cells by the expression of specific transcription factors such as TRB2 (Englund et al., 2005), Cux1 and Cux2 (Nieto et al., 2004).

**Stem cells in adult CNS**

In the adult CNS, NSCs are found only in certain locations such as SVZ of telencephalic lateral ventricles and subgranular zone (SGZ) of hippocampal dentate gyrus (Cassidy and Frisen, 2001; Doetsch et al., 1999). Some other regions contain also NSC-like cells that show lower differentiation potential than NSCs (Kornblum, 2007; Jensen and Parmar, 2006; Temple, 2001; Gage, 2000; McKay, 1997). Four cell types are found in adult SVZ: ependymal cells that are located in ventricle wall, slowly proliferating astrocytes that show radial glia-like features (type B cells), fast-proliferating intermediate cell type C, and neuroblasts or neural progenitors (type A cells) (Sohur et al. 2006; Alvarez-Buylla et al., 2001; Doetsch et al., 1999) (Figure 6).

SVZ astrocytes express markers such as GFAP, nestin and vimentin (Jankovski and Sotelo, 1996; Peretto et al., 1999). Both astrocytes and neural progenitors (B and A cells) form neurospheres in vitro (Sohur et al. 2006; Alvarez-Buylla et al., 2001; Doetsch et al., 1999). Since radial glia function as NSCs in the developing CNS, it has been hypothesized that perhaps also SVZ astrocytes could function as stem cells or progenitors in adult CNS (for review see Yadrigi and Marino, 2009; Sohur et al. 2006; Temple, 2001).
1.3.3 PNS stem cells and progenitors

Prior the migration NCCs can be considered pluripotent cells. However, only certain areas of the neural crest can contribute to formation of the PNS (reviewed in Le Douarin et al., 2004). In chicken, the vagal neural crest located between somites 1 and 7 gives rise to the enteric ganglia, and the thoracic neural crest located between somites 18 and 24 gives rise to the sympathetic ganglia chain and adrenal medulla (Le Douarin et al., 2004). Several chicken-quail grafting experiments have shown that NCC fate is determined by the environmental signals during the migration and at the final destination (Le Douarin et al., 2004).

During embryogenesis, both sensory and autonomic ganglia contain undifferentiated non-neural cells that can be considered as putative neural crest stem cells since they proliferate, remigrate and differentiate when transplanted into environment of the younger host (Duff et al., 1991; Hagedorn et al., 1999; Hagedorn et al., 2000; Le Douarin et al., 2004). Peripheral nerves and enteric plexuses (Bixby et al., 2002; Kruger et al., 2002; Morrison et al., 1999; Nataf and Le Douarin, 2000) also contain these undifferentiated pluripotent cells. The progenitors can be found in these areas until late in development, and even during postnatal and adult life (Kruger et al., 2002). At least, the cells isolated from enteric-, and sciatic nerves and DRG boundary caps show capacity for self-renewal (Bixby et al., 2002; Kruger et al., 2002; Morrison et al., 1999). Also epidermal melanocytes and peripheral Schwann cells show reciprocal transdifferentiation (Dupin et al., 2000; Dupin et al., 2003). Endothelin-3 treatment of melanocytes or Schwann cells induces cell proliferation and gives rise of clonal progeny that contains both melanocytes and Schwann cells indicating that these cells can reverse their fate to bipotent glia and melanocyte producing progenitors (Dupin et al., 2000; Trentin et al., 2004). Adult mouse Schwann cells can also generate melanocytes after severe peripheral nerve injury (Rizvi et al., 2002).
1.4 DIFFERENTIATION

Differentiation of nervous system cells is a multi-step process that includes proliferation, migration and terminal cell cycle exit. The formation of new neurons (neurogenesis) involves also neurite outgrowth, synapse formation and apoptosis. It has been estimated that the human nervous system contains hundreds of different types of neurons, but glial cells such as astrocytes and myelinating oligodendrocytes or Schwann cells in PNS are still most predominant cells in adult nervous system.

1.4.1 NSC progeny in CNS

Neurogenesis

During embryo development, neuroepithelial cells that are connected to the lumen of the neural tube (ventricular germinal zone) start to differentiate and migrate “inside-out” in CNS (Chenn and McConnell, 1995). In mouse CNS, most of the neurons are formed between embryonic stage E10 and E13. After the onset of neurogenesis, the neuroepithelial cells give rise to radial glia that show residual neuroepithelial and astroglial properties and replace the neuroepithelial cells (Kriegstein and Gotz, 2003). Generic neural fate is promoted by certain basic helix-loop-helix (bHLH) transcription factors that activate the cascade of neuronal genes, which support the cell cycle exit and inhibit gliogenesis.

One essential transcription factor in neural differentiation in both CNS and PNS is Mash1 (Porteus et al., 1994, Guillemot et al., 1993). In CNS, Mash1-deficient mice show severe brain defects in basal ganglia, cerebral cortex and olfactory bulb due to loss of neuronal progenitor cells (Casarosa et al., 1999; Cau et al., 2002). Another group of neuronal bHLH transcription factors are neurogenins (Ngns), Ngn1 and Ngn2, which define distinct progenitor populations and are highly expressed in dorsal ventricular zone in the developing nervous system (Sommer et al., 1996). Transient expression of Mash1 and Ngns is sufficient to initiate neuronal differentiation also in mouse embryonic carcinoma cells (Farah et al., 2000). Mash1 and Ngns not only activate the expression of neuronal bHLH genes such as NeuroD and Math3 (Cau et al., 2002; Ma et al., 1998), but they also promote cell cycle exit. All of these factors can inhibit the cell cycle by inducing the expression of cyclin dependent kinase inhibitors, including p27 (Farah et al., 2000). Mash1 and Ngns are also involved in inhibition of gliogenesis since Mash1/Ngn2-deficient mice show compensatory premature generation of astrocytic progenitors both in vivo and in vitro (Nieto et al., 2001). Ngn1 has been shown to suppress leukemia inhibitory factor (LIF)-induced gliogenesis through Smad1-p300 complex in cultured progenitors (Sun et al., 2001).

In several cell types including the brain cells, differentiation is also regulated by CCAAT/enhancer-binding protein (C/EBP) transcription factor family (Cao et al., 1991; Yeh et al., 1995, Sterneck and Johnson, 1998). Activation of C/EBPs results in enhanced neural differentiation in cortical progenitors (Davis, 1995). Moreover, overexpression of
active C/EBP has been shown to enhance neurogenesis by directly activating neuronal genes such as neuronal class β tubulin and by preventing ciliary neurotrophic factor (CNTF) induced gliogenesis (Menard et al., 2002).

**Astrocyte differentiation**

Most of the astrocytes are produced in a second wave of neural differentiation that takes place at E12-P0 in mouse. Both astrocytes and oligodendrocytes are originated from the same progenitors, which become progressively more restricted. Tripotential glial-restricted progenitors (GRP) arise directly from NSCs and can generate two different types of astrocytes and oligodendrocytes (Herrera et al., 2001). GRPs differentiate further into more restricted progenitors, which differentiate into one type of astrocytes and oligodendrocytes (Skoff and Knapp, 1991).

In CNS, factors such as Notch, CNTF, LIF and BMP-2 influence glial fate decisions (Morrison et al., 2000; Morrow et al., 2001). Notch promotes both NSC maintenance and gliogenesis by activating transcription of Hes genes (Corbin et al., 2008; Morrison et al., 2000), and BMP-2 and LIF induce astrocyte differentiation in synenergy by activating respectively Smad1/4 and STAT1/3 transcription factors in telencephalic NPCs (Nakashima et al., 2001). Also CREB-binding protein (CBP)/p300 transcriptional co-activator, the adaptor molecule of STAT-Smad interaction, can directly activate glial factors such as GFAP (Nakashima et al., 1999). BMP-Smad pathway induces expression of bHLH transcription factors Hes5 and inhibitor of differentiation (Id)-family that can interfere the action of proneural transcription factors (Nakashima et al., 2001). Ids have been shown to prevent premature differentiation of neurons and oligodendrocytes but not astrocyte differentiation (Ross et al., 2003).

**Generation of oligodendrocytes**

Formation of oligodendrocytes occurs later in development starting at E16 in mouse and ending postnatally, when the synaptic connections have been established and neurons are surrounded by supportive glia. However, oligodendrocyte progenitors (OPs) can be found as early in development as at E11 in mouse (Thomas et al., 2000). In rodents, OPs are first found in the ventral part of the developing neural tube and brainstem and have been found to express platelet derived growth factor receptor α (PDGFRα) (reviewed in Nishiyama et al., 2009). The PDGFRα positive OPs are derived from a subset of ventral neuroepithelial cells (Tekki-Kessaris et al., 2001) under influence of notochord derived Shh that is required for oligodendrocyte differentiation (Lu et al., 2000). Soon after OPs start show NG2 expression followed by Olig-2 and immature oligodendrocyte antigen O4 expression (reviewed in Nishiyama et al., 2009). These immature oligodendrocytes downregulate NG2 and O4, and initiate galactocerebroside expression as the cells differentiate further into premyelinating oligodendrocytes. Mature myelinating oligodendrocytes express markers such as galactocerebroside and myelin basic protein (MBP) but Olig-2 expression is downregulated (Nishiyama et al., 2009). Transcription
factor Sox10 have been implicated in oligodendrocyte differentiation and its expressed in OPs and throughout the oligodendrocyte development (Nishiyama et al., 2009; Stolt et al., 2002)

1.4.2 Differentiation of sympathetic lineage cells in PNS

Differentiation of sympathetic neurons is promoted by BMPs derived from dorsal aorta whereas neuregulin directs the cells into glial fates (Reissmann et al., 1996; Shah et al., 1996). In sympathetic cells, BMPs activate bHLH transcription factors including Mash1 (Lo et al., 1991), eHand (Cserjesi et al., 1995), dHand, paired homeodomain transcription factors Phox2a and Phox2b (Pattyn et al., 1997), and zinc finger transcription factors GATA2 and GATA3 (Groves et al., 1995). According to previous studies, Mash1 is not required for neuronal fate decision in sympathetic cells but it may promote the development of already committed cells. In Mash1-deficient mouse embryos, the early sympathetic cells migrate and aggregate to dorsal aorta normally and undergo at least part of their normal differentiation program before becoming apoptotic (Guillemot et al., 1993). Mash1-deficiency leads to a loss of PNS neuronal markers such as SCG10 and peripherin (Sommer et al., 1995). Moreover, Mash1 has been shown to activate Phox2a that positively regulates noradrenergic characteristics and is required for TH and DβH expression (Hirsch et al., 1998). However, Mash1-deficiency does not affect the expression of early sympathetic markers like Phox2b, receptor tyrosine kinase c-Ret, NF and Tuj-1 (Howard et al., 2000; Huber et al., 2002; Huber, 2006). Phox2b is expressed in all peripheral and CNS noradrenergic neurons and in the majority of neurons that are integrated in autonomic reflex pathways (Pattyn et al., 1997). Phox2b-deficient mouse embryos die at midgestation, and they have abolished neurogenesis (Huber, 2006). Phox2b has shown to regulate Hand2 expression, which is needed for the maintenance of noradrenergic phenotype in sympathetic cells (Howard et al., 2000; Huber et al., 2002). Mouse sympathetic cells express also both GATA2 and GATA3, and in GATA3-deficient mouse embryos, the primary sympathetic chain seems to be largely intact but in later stages the neurons loose TH expression and cells show increased apoptosis (Takahashi et al., 2000; Tsarovina et al., 2004).

Development and maintenance of the sympathetic cells are also dependent on certain growth factors. NGF is important for differentiation of sympathetic cells and NGF-mutant mice suffer dramatic loss of both sensory- and sympathetic cells (Crowley et al., 1994). Lack of NGF high affinity receptor TrkA (Meakin and Shooter, 1992) leads to similar phenotype in sensory and sympathetic neurons (Smeyne et al., 1994). NT-3 is also one of survival or differentiation factors essential for maintenance of mature sympathetic neurons during later development (Kuruvilla et al. 2004). GDNF family members such as ARTN and GDNF are involved in sympathetic ganglia chain development and maintenance (reviewed in Francis and Landis, 1999).
1.5 SELF-RENEWAL

Self-renewal is clonal proliferation that generates one or two daughter cells that acquire the same cell fate as the dividing cell. Self-renewing cells are important for replenishing specialized cells in tissue repair but also maintain the normal turnover of regenerative organs. Sphere-forming capacity is commonly used to analyze NSC or NPC self-renewal (reviewed in Jensen and Parmar, 2006). Due to lack of specific markers it is difficult to precisely determine that NSC has produced a true copy of itself and therefore self-renewal is often operationally defined as the degree of to which NSC can raise to colonies e.g. neurospheres (reviewed in Kornblum, 2007). The neurosphere forming assay is the best available functional assay for NSC self-renewal as long as it is carefully performed, tested over serial clonal passaging and followed by in vitro differentiation to show multipotency of the individual spheres (reviewed in Jensen and Parmar, 2006).

It is known that only small percentage of cells within the neurosphere has the capacity for self-renewal (Reynolds and Weiss, 1992) and even a smaller percentage of the cells meet the definitions of true NSC. Ensuring the clonality is sometimes difficult because the culture conditions to generate individual cells are quite harsh and floating cells tend to aggregate especially if plated in too high density (reviewed in Kornblum, 2007). Several groups have shown sorting of NSCs based on such as cell size or on LeX antigen in murine samples (Rietze et al. 2001, Capela and Temple, 2002), or prominin (CD133) expression in fetal human samples (Uchida et al. 2000). These methods are frequently used to enrich self-renewing NSCs (Kornblum, 2007). Unfortunately, these techniques still can lead to cell loss and therefore may diminish the yield of NSCs (Kornblum, 2007). To date there is no set of markers that precisely identifies a NSC from more limited progenitors (Kornblum, 2007).

1.5.1 Symmetric and asymmetric cell division

In vertebrate cortical differentiation, symmetric and asymmetric cell divisions are distinguished by orientation of mitotic spindle towards the cell cleavage plane (Chenn and McConnell, 1995) (Figure 7). In developing neural tube, the number of neuroepithelial cells is first increased by the proliferative symmetric cell divisions. In symmetric cell division, the mitotic spindle and cleavage plane are vertical to respect of apical (ventricle side) plasma membrane (Gotz and Huttner, 2005). Later during differentiation, the cells begin to show also asymmetric cell divisions that occur in horizontal cleavage plane and result in one mother-cell-like cell and a second more differentiated cell, such as a progenitor or neuron. In spinal cord, symmetric cell divisions have shown more regularly to be related to production of two identical daughter cells, either progenitors or neurons (Wilcock et al., 2007) (Figure 7).

Stem cell self-renewal capacity is unlikely limited to only one certain cleavage plane orientation even though the symmetrically dividing cells show higher tendency for self-renewal. The different orientation of mitotic spindle allows different partition of the cell fate determinants into daughter cells (Haydar et al., 2003). The asymmetrically
distributed molecules play important role in cell fate determination and some of these factors will be discussed in the next chapter. It has been hypothesized that also the cell cycle length is involved in cell fate determination (Gotz and Huttner, 2005). If the cell cycle length is short, the cell fate determinants may not have enough time to induce differentiation, and both daughter cells adopt the stem cell or progenitor fate and continue proliferation. Consequently, in longer cell cycle, the cell fate determinants have more time to affect, which leads to differentiation and asymmetric or symmetric cell division depending on the cell cycle length (Gotz and Huttner, 2005). The mechanisms that determine spindle orientation, and transition from symmetric to asymmetric divisions are still poorly understood.

Figure 7. A) Symmetric and asymmetric cell divisions. B) When NSCs differentiation starts the asymmetric cell divisions take place producing progenitors and neurons. C) Symmetric cell divisions more regularly occur in production of two identical cells: for example two self-renewing progenitors or two neurons (Modified from Huttner and Kosodo, 2005, and Gotz and Huttner, 2005).
1.5.2 Control of NSC self-renewal

1.5.2.1 Transcription related factors

Notch

Notch signaling pathway and its downstream targets play important role in NSC fate decision. In Drosophila, lateral inhibition mediated by Notch receptor controls the production of all neuronal cells (Artavanis-Tsakonas et al., 1999). In mammals, during CNS development Notch1 is expressed in SVZ and ventricular zone (VZ) cells, and cultured neurospheres and its ligands delta and jagged are expressed in differentiating cells (Corbin et al. 2008; Irvin et al., 2001). Inhibition of Notch pathway causes premature neurogenesis, whereas expression of active form of Notch suppresses differentiation (Hitoshi et al., 2002). Moreover, Notch signaling also induces glial differentiation (Gaiano et al., 2000).

The mechanism how Notch inhibits neural differentiation and maintains undifferentiated cell fate is understood in some detail. Binding of Notch ligands and activation of Notch receptor leads to transcriptional activation of its target genes such as Hes genes (Iso et al., 2003). Both Hes1 and Hes5 are expressed in SVZ and in cultured NPCs, and they have been shown to be required for Notch signaling and the maintenance of NSC pool (Ohtsuka et al., 2001). Hes-transcription factors can repress the activity of genes such as proneural Mash1 by binging to N and E box regulatory region of the target genes (Iso et al., 2003). Notch pathway is also important in other tissues: in mouse intestine Notch amplifies progenitor pool and inhibits differentiation (Fre et al., 2005), and in muscle Notch is a key determinant of muscle regenerative potential that declines with age (Conboy et al., 2003). Notch signaling is also important for angiogenesis and vascular development (see VEGF subfamily chapter) during normal development and in tumors (Rehman and Wang, 2006). Moreover, Notch signaling is implicated at least in T-cell leukemia, breast and colon adenocarcinomas, and cervical cancer and it also has a potential role in brain tumorigenesis (Rehman and Wang, 2006). Recently it was shown that Notch gradient in apical-basal interkinetic nuclear migration regulates neurogenesis in retina (Del Bene et al., 2008).

Numb

Numb and Numb-like (Numbl) proteins are asymmetrically distributed in the daughter cells and are thought to play a key role in NSC fate decisions (Cayouette and Raff, 2002). In mammals, Numb localizes at the ventricular surface especially near adherens junctions and after asymmetric division it is segregated to the cell that remains as progenitor (Kim and Walsh, 2007; Petersen et al., 2002). Numb-deficient mouse embryos die at E11 showing premature neurogenesis in forebrain (Zhong et al., 2000). In mouse, conditional knockout of Numb in Numb-like-deficient background results in transient over-production of neurons and severe loss of progenitors at E10, which indicates that Numb is required for self-renewal (Petersen et al., 2002). It has also been suggested that Numb has
different functions during nervous system development. In early development, it has a role in maintaining progenitors (Petersen et al., 2002) but later it also promotes neuronal differentiation (Zilian et al., 2001). In Drosophila, Numb is shown to antagonize Notch signaling in formation of interneurons (Spana and Doe, 1996).

**Bmi-1**

Bmi-1 is one of the polycomb group proteins (PcGs) that are known as epigenetic chromatin modifiers. PcGs silence genes by means of deacetylation, methylation and possibly also by inhibiting the transcription initiation machinery itself (Valk-Lingbeek et al., 2004, Dellino et al., 2004). PcGs play role in both self-renewal and cancer development. Bmi-1 is needed for self-renewal and proliferation of several stem cell types including cerebellar NPCs. Bmi-1 effects on self-renewal are mediated through the inhibition of certain tumor suppressor proteins in adults (Molofsky et al., 2003). Bmi-1 is also expressed in several cancers and cancer stem cells (Bea et al., 2001; Hemmati et al., 2003; Kim et al., 2004b). In cerebellum, Bmi-1 is extracellularly regulated by Shh (Leung et al., 2004). Moreover, Bmi-1 regulates three Hox genes in NSCs: Hoxd8, Hoxd9 and Hoxc9 (Molofsky et al., 2003).

1.5.2.2 Cell cycle

Cell cycle is the period from cell division to another and it has four phases: G1, S, G2 and M. During G1 the cell grows and prepares for DNA replication. This is followed by S-phase when DNA is synthesized and centrosomes are duplicated. In G2-phase, the cell prepares for mitosis and during M-phase the cell divides. The cell cycle of post mitotic, differentiated cells is arrested from G1 to G0, the quiescent stage (Figure 8).

In eukaryotic cells, the cell cycle is regulated by a small number of heterodimeric protein kinases that contain regulatory subunits cyclins and catalytic subunits cyclin-dependent kinases (CDKs). Cyclin concentrations vary depending on cell cycle phase and their association with CDKs leads to activation of several cell cycle related proteins and transcription of multiple genes (Galderisi et al., 2003) (Figure 10). One group of activated genes is E2F transcription factors that are required for G1-S transition and expression of several important genes, such as CDK2, cyclins A and E (Stiegler et al., 1998).

Cell division and DNA replication are monitored at four key checkpoints. Cyclin kinase inhibitors (CKIs) and tumor suppressors p53 and pRb molecules can silence the cell cycle in G1/G0 for example due to DNA damage. Unlike other cells, NSCs and progenitors undergo interkinetic nuclear migration between apical (ventricle) and basal cell surface so that M-phase always occurs at the apical surface and S-phase in more basal locations (Murciano et al., 2002). The relevance of the interkinetic nuclear migration is currently unknown.
From neural stem cells to precursors  

Review of the Literature

Figure 8. Cell cycle and its regulation. Cyclin concentrations vary depending on phase of the cell cycle and their association with CDKs leads to activation of several cell cycle related proteins and transcription of multiple genes. p16\(^{INK4a}\) inhibits the activity of cyclin D-CDK4/6 and keeps pRb hypophosphorylated, which prevents E2F activation and blocks the cells from exiting the G1 phase. p19\(^{Arf}\) blocks the cell cycle by inhibiting Mdm2 (Sherr and Weber, 2000), the inhibitor of tumor suppressor p53. p53 promotes cell cycle arrest predominantly by transcriptionally activating p21\(^{Cip1}\), which subsequently inhibits CDK activity and blocks the cell cycle at G1 or G2.

\[\text{Cyclin kinase inhibitors (CKIs)}\]

INK4/Arf tumor suppressor proteins p16\(^{INK4a}\), p15\(^{INK4b}\), p1\(^{INK4c}\) and p19\(^{Arf}\) can arrest the cells at G1 phase. Tumor suppressor p16\(^{INK4a}\) inhibits the activity of cyclin D-CDK4/6 and keeps pRb hypophosphorylated, which prevents E2F activation and blocks the cells from exiting the G1 phase. p19\(^{Arf}\) blocks the cell cycle by inhibiting Mdm2 (Sherr and Weber, 2000), the inhibitor of tumor suppressor p53 (Bates et al., 1998; Xiao et al., 1995). Loss-of-function mutations in Ink4a/Arf locus are common in cancers, especially in glioblastoma (Ekstrand et al., 1991; Hayashi et al., 1997). Increased expression of p16\(^{INK4a}\) and p19\(^{Arf}\) is associated with Bmi-1-deficiency, and loss of p16\(^{INK4a}\) partially rescues the self-renewal capacity in Bmi-1-deficient NSCs, which suggests that p16\(^{INK4a}\) controls also NSC self-renewal (Bruggeman et al., 2005; Molofsky et al., 2003).

In mammals, the cell cycle arrest can also be initiated by CDK inhibitor proteins (CIPs) p21\(^{Cip1}\), p27\(^{Kip1}\) and p57\(^{Kip2}\) that inhibit CDK1-, CDK2, CDK4 and CDK6-cyclin complexes. p21\(^{Cip1}\) is one of the best studied CIPs which responses to DNA damage by
inhibiting CDK2 and arresting the cell cycle at G1 or G2 (Galderisi et al., 2003). p21\textsuperscript{Cip1} plays an important role in cell cycle arrest, differentiation, DNA repair, cell senescence and apoptosis (reviewed in Sherr and Roberts, 1999), and its expression is regulated by several transcription factors, such as p53, C/EBP, STATs and Smads (reviewed in Gartel and Tyner, 1999).

**Tumor suppressor p53**

The p53 transcription factor and tumorsuppressor responds to multiple cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Also the other p53 family proteins, p63 and p73, can transactivate p53-responsive genes due to similar DNA binding domain and cause cell cycle arrest or apoptosis (reviewed in Murray-Zmijewski et al., 2006). However, p53, p63 and p73 not entirely functionally redundant, and genetic knockout models suggest that these proteins have their own roles in development and growth (Murray-Zmijewski et al., 2006). p53 activity is modulated by several posttranslational modifications such as phosphorylation and acetylation. In unstressed conditions, p53 is kept inactive essentially by ubiquitin ligase Mdm2 that inhibits transcription of p53 and promotes the protein degradation (Bourdon et al., 2007). All p53 family proteins have several isoforms due to multiple splicing and alternative promoters. p53 promotes cell cycle arrest predominantly by transcriptionally activating p21\textsuperscript{Cip1}, which subsequently inhibits CDK activity (el-Deiry et al., 1993).

Besides controlling cell proliferation p53 also affects the cell differentiation. Several in vivo and in vitro studies have shown that addition of exogenous p53 promotes differentiation of different cell types (Almog and Rotter, 1997). In mouse, p53-deficiency frequently leads to spontaneous tumors, decreased fertility and neural tube closure defect in fore- and midbrain area that is called an excencephaly (Armstrong et al., 1995). Terminal differentiation of several cells including neurons involves changes such as cell cycle arrest, which is considered an irreversible step in differentiation (Miller et al., 2003). Arguably the most important function of p53 in neurons is to prevent cell cycle re-entry (Miller et al., 2003). Moreover, nuclear p53 can activate transcription of proapoptotic genes whereas cytoplasmic p53 directly activates proapoptotic proteins and induces apoptosis (Chipuk et al., 2005). p53 is mutated in approximately 50% of all the cancers and loss of p53 activity is considered to be universal to all cancers (Murray-Zmijewski et al., 2006). The function of p53 is more complicated than has been thought since human p53 gene has been found to encode at least nine different isoforms (Bourdon et al., 2005). p53 variants are expressed in tissue dependent manner in several normal tissues and they seem to have distinct biochemical activities. Preliminary studies indicate that the balance between p53 and its different isoforms regulates cell fate in response to p53 activation (Bourdon et al., 2005). p53 also seems to accelerate the ageing of several tissues (Tyner et al., 2002).
Rb family proteins

Rb family tumor suppressor proteins, pRb, p107 and p130, repress the cell cycle and their function is mainly accomplished by binding to E2F family members (Stiegler et al., 1998). Rb family members are posttranslational regulated proteins and the phosphorylation leads to their functional inactivation. Phosphorylation of Rb family proteins is cell cycle related phenomenon and dependent on CDK4, CDK6 and CDK2 (Sherr et al., 1996). pRb amino acid sequence is approximately 35% identical to p107 and p130, whereas p107 and p130 share 50% amino acid identity to each other (Claudio et al., 2002). Rb family proteins can compensate each other at some level but they have distinct binding preferences to E2Fs and have tissue specific expression patterns (reviewed in Giacinti and Giordano, 2006). pRb preferentially binds to E2F1, E2F2, and E2F3, whereas p107 and p130 bind to E2F4 and E2F5 (Galderisi et al., 2003). Moreover, pRb represses gene transcription required for G1 to S phase transition (Wang et al., 2001). pRb also affects transcription by remodeling chromatin structure through interactions with factors such as histone deacetylase (Luo et al., 1998), methyltransferase (Robertson et al., 2003) and PcG proteins (Dahiya et al., 2001).

Several human tumors show mutations or functional inactivation of pRb gene (Giacinti and Giordano, 2006), and pRb has a central role in various differentiation processes including CNS and PNS neurons, epidermal-, hair-, and muscle cells, melanocytes and in hepatocytes (Giacinti and Giordano, 2006). In developing brain, pRb is expressed in dividing progenitors and post mitotic cells (Lipinski and Jacks, 1999). pRb-deficient mouse embryos show severe defects in the CNS and hematopoietic system, and the phenotype is embryonic lethal at E14 (Lee et al., 1992; Macleod et al., 1996; Slack et al., 1998). In neurons, pRb play a role at least in processes related to cell cycle (cell-autonomous) and differentiation (non-cell-autonomous) (Liu et al., 2004).

Both p107 and p130 have also role in neural differentiation. p107 expression is restricted to brain ventricular zone and subependyma, and it becomes downregulated at the onset of neurogenesis (Vanderluit et al., 2004). p107-deficient adult mice show increased self-renewal and proliferation of neural progenitors but the tissue homeostasis is maintained by increased apoptosis (Vanderluit et al., 2004). In neural cells, highest p130 expression is found during and after terminal mitosis (Jiang et al., 1997; Yoshikawa, 2000). In some mouse strains p130-deficiency leads to no obvious defects (Cobrinik et al., 1996) but in others the phenotype is embryonic lethal at E11-E13 (LeCouter et al., 1998). The p130-deficient mouse embryos display arrested growth, disorganization of the neural tissues, increased proliferation and apoptosis in CNS, reduced numbers of the neurons in the neural tube and in dorsal root ganglia (LeCouter et al., 1998).
Cytoskeleton, cytoskeletal tumor suppressor protein merlin and ERM protein ezrin

The cytoplasm of eukaryotic cells contains a complex, dynamic network of filamentous proteins that form a structure called cytoskeleton. Actin is one of the three types of protein filaments found in the cytoskeleton, and it is essential for several cellular functions including in cell migration, morphogenesis, cytokinesis, establishment of cellular polarity, vesicle trafficking and intracellular signal transduction (reviewed in Ramaekers and Bosman, 2004). Such diverse roles are possible due to spatial and temporal control of actin filament assembly by several actin binding proteins (reviewed in Ayscough, 1998). In a variety of diseases, interactions between the actin cytoskeleton and cell membrane components are deregulated (reviewed in Ramaekers and Bosman, 2004). In tumor cells, changes in actin expression, in actin associated proteins and in cell membrane-actin linker proteins are also involved in abnormal growth, metastasis and tissue adhesion (reviewed in Jordan and Wilson, 1998).

Merlin and ezrin serve as linker proteins between the actin cytoskeleton and cell membrane components. They are involved in cell adhesion, cell movement, cell growth, cell membrane trafficking and signaling. Merlin, a cytoskeletal tumor suppressor protein (reviewed in Sun et al., 2002) is encoded by NF2 gene (Rouleau et al., 1993; Trofatter et al., 1993) and it belongs to BAND 4.1 protein superfamily. Lack of functional merlin is involved in tumorigenesis in dominantly inherited neurofibromatosis (NF2) disease (Louis et al., 1995). Merlin is a cytoskeleton-associated membrane organizing protein and therefore a unique type of tumor suppressor. In primary schwannoma cells, merlin overexpression reduces cell proliferation and promotes G0/G1 arrest (Schulze et al., 2002). Respectively, suppression of merlin induces proliferation in schwannoma cells (Huynh and Pulst, 1996). In vitro, merlin is localized underneath the plasma membrane and it is mainly seen in membrane ruffles and filopodia (Gonzalez-Agosti et al., 1996; Sainio et al., 1997). In tissues, merlin is widely expressed but the expression levels are low especially in adults. The cell type distribution and subcellular localization are not well known.

During mouse embryogenesis, merlin is highly expressed in heart, nervous system, extraembryonic tissue and skeleton (Huynh et al., 1996). In adult mouse and human merlin is expressed in lung, muscle, intestine, CNS, lens, spleen and kidney (Claudio et al., 1995; den Bakker et al., 1999). Nf2-deficient mouse embryos die at E7 due to collapsed extraembryonic region and absence of organized extraembryonic ectoderm (McClatchey et al., 1997). Heterozygous Nf2-mutants spontaneously develop several tumors such as osteosarcomas and hepatocellular carcinomas that show a loss of the wild type allele (McClatchey et al., 1997). In mice, conditional deletion of Nf2 in Schwann- and arachnoidal cells leads to schwannomas and meningeal neoplasias, respectively (Giovannini et al., 2000; Kalamardides et al., 2002). The mechanism by which merlin functions as tumor suppressor is not fully understood but it has been shown that merlin undergoes cycle-dependent nucleo-cytoplasmic shuttling (Muranen et al., 2005) and binds to cyclin B binding protein and cell cycle regulator HEI10 (Gronholm et al., 2006).
Ezrin (Gould et al., 1989; Turunen et al., 1989) is another member of BAND 4.1 protein superfamily and structurally related to merlin. Similarly to merlin also ezrin, the ERM (ezrin, radixin, moesin) family protein, is a linker protein between the actin cytoskeleton and cell membrane components (Bretscher et al., 2002). Ezrin has shown to have partial functional homology and able to form interactions with merlin (Gronholm et al., 1999). However, ezrin has the opposite effect on cell proliferation than merlin. Ezrin has been related to enhanced cell growth and metastasis and its expression is altered in several tumors (Bohling et al., 1996; Geiger et al., 2000; Yu et al., 2004). Overexpression of ezrin in fibroblasts increases the cell proliferation through loss of contact inhibition (Kaul et al., 1996). Ezrin has been shown also to participate in cell survival signaling through PI3K/Akt pathway (Gautreau et al., 1999).

1.6 CLASSICAL ONCOGENES

1.6.1 Human Papilloma Virus 16 E6/E7 oncogenes

Traces of viral DNA are regularly found in human tumors, and some viruses and their oncogenes are directly implicated in cancer development (Lowe et al., 2007; Nevels et al., 2001; Tognon et al., 2003). Human papilloma viruses (HPVs) are double stranded nonlytic DNA viruses and their life cycle is tightly linked to differentiation program of the infected epithelial cells. HPVs are designated in high or low risk types according to malignant progression of the epithelial lesions derived after the viral infection. HPV 16 is the most prevalent high risk HPV type (reviewed in Munger et al., 2004).

HPVs infect the basal epithelial cells that form the actively proliferating cell layer in the epithelium. When the cells differentiate and migrate towards surface of the epithelium, the viruses replicate to high copy numbers and new viruses are released into surrounding environment. One of the key events in HPV induced transformation is the integration of viral DNA into host genome. This leads to constantly maintained expression of viral E6 and E7 genes (Fehrmann and Laimins, 2003) whereas the other viral DNA is often deleted or its expression repressed (reviewed in Munger et al., 2004). DNA from different HPVs has been found approximately in 99% of the cervical and in 20% of the oropharyngeal cancers (Longworth and Laimins, 2004).

E6 and E7 oncogenes degrade tumor suppressor p53 and pRb/p107/p130, respectively (Boyer et al., 1996; Davies et al., 1993; Scheffner et al., 1990). E7 expression and its interaction with pRb are critical in formation of the microenvironment that promotes viral genome replication. The epithelial cells that have integrated E6/E7 in their genome show selective growth advantage over normal cells or cells that express episomal HPV genes (Jeon et al., 1995). E6/E7 oncogenes immortalize and transform at least keratinocytes, epithelial and endothelial cells (Coursen et al., 1997; Hawley-Nelson et al., 1989). The constant expression of E6/E7 oncogenes is necessary for maintenance of the transformed phenotype in cervical cancers (Goodwin and DiMaio, 2000).
Besides the degradation of the tumor suppressor, E6 and E7 oncogenes have also other cellular functions: E6 activates human telomerase transcription (Veldman et al., 2001) and increases mitogen-activated protein kinase (MAPK) signaling in epithelial cells (Chakrabarti et al., 2004). Whereas, E7 overrides growth inhibitory activity of CDK inhibitors p21cip1 and p27kip1 (Funk et al., 1997; Jones et al., 1997) and also associates with chromatin modifying enzymes, particularly with histone deacetylases and histone acetyl transferases (Brehm et al., 1999). In addition, E7 is can integrate with transcription factors, cell cycle regulators and metabolic enzymes (reviewed in Munger et al., 2004), but the biological relevance of these interactions is still unclear. E7 has been also reported to interact with transcriptional co-activators p300, CBP and pCAF (Bernat et al., 2003). Both E6 and E7 oncogenes can independently induce genomic instability in normal human cells (reviewed in Munger et al., 2004). E6 and E7 have also been shown to directly activate Notch signaling by upregulating expression of both Jagged-1 and its receptor Notch-1 in cervical carcinoma cells (Veeraraghavalu et al., 2004; Weijzen et al., 2003).

1.6.2 Myc-family

Myc family members, c-Myc, n-Myc and l-Myc, are proto-oncogenes that belong to bHLH leucine zipper transcription factors. The Myc proteins are active during the embryo development and they show high functional redundancy for each other (Hirvonen et al., 1990, Murphy et al., 2005). c-Myc-deficient mouse embryos show hematopoietic, vascular and placental defects and the phenotype is embryonic lethal before E10 (Trumpp et al., 2001). Lack of n-Myc leads to dramatic abnormalities in heart, liver, kidney, limb bud, lungs, CNS and PNS, and the phenotype is embryonic lethal (Sawai et al., 1991; Sawai et al., 1993). l-myc-deficient mice are viable and show no obvious defects (Hatton et al., 1996).

C-Myc together with its partner Max regulates transcription through several mechanisms, including recruitment of chromatin modifiers and remodeling factors, and interaction with transcriptional factors and functions that are involved in at least cell cycle control, growth, cell adhesion, differentiation and apoptosis (Coller et al., 2000; Dang et al., 2006; Grandori et al., 2000). c-Myc interacts also with Myc-interacting zinc finger protein-1 (Miz-1) (Bouchard et al., 1999; Coller et al., 2000), which represses transcription of the cell cycle inhibitors p21cip1, p15INK4b and p57kip2 (Adhikary et al., 2003; Seoane et al., 2002; Staller et al., 2001). Moreover, Myc represses proteins also involved in cytoskeleton and cell adhesion (Coller et al., 2000, Gebhardt et al., 2006).

All Myc family members are expressed during fetal brain development (Hirvonen et al., 1990). Targeted loss of n-Myc in nestin positive NPCs severely disrupts their ability to expand, differentiate and populate the brain even though the mice survive to adulthood (Knoepfler et al., 2002). Whereas, in mouse CNS c-Myc overexpression has been shown to increase NPC proliferation (Fults et al., 2002). Furthermore, c-Myc is also expressed at the neural plate border and have been shown to be important in neural crest development (Bellmeyer et al., 2003). In Xenopus, c-Myc downregulation by using morpholinos has
revealed that it is required for induction of the neural crest markers and formation of the neural crest derivatives (Bellmeyer et al., 2003). Overexpression of c-Myc causes genomic instability in several cell types (Wade and Wahl, 2006), and amplification of Myc can be detected in several human and animal cancers (Grandori et al., 2000). In nervous system derived tumors, both c-Myc and n-Myc overexpression are implicated in pathogenesis of medulloblastoma, glioma and neuroblastoma (Eberhart et al., 2004; Schwab, 2004; Su et al., 2006).

Biological functions of c-Myc are pleiotrophic and highly cell-type specific (Murphy et al., 2005). In fibroblasts, exogenous Myc promotes short G1-S transition (Leone et al., 2001, Karn et al., 1989). Activation of Myc has also shown to permit prolonged expression of cyclin E/CDK2 by upregulation of cyclin D and CDK4, by increasing pRb phosphorylation and by repressing CDK inhibitors (Bouchard et al., 1999; Coller et al., 2000). In skin, c-Myc is expressed in the basal layer that contains proliferating epithelial stem cells or progenitors and transient amplifying (TA) cells and it is needed for self-renewal (Bull et al., 2001, Zanet et al., 2005).

Also in ES-cells, c-Myc maintains self-renewal through LIF/STAT3 pathway (Cartwright et al., 2005). Ectopic c-Myc expression together with three other factors Oct4, Sox-2 and Klf4 can dedifferentiate adult fibroblasts into pluripotent ES-cell-like cells (Takahashi et al., 2006), and therefore it is considered one of the factors driving “stem cell properties”. c-myc-deficient ES-cells show severely inhibited formation of both embryoid bodies and secondary erythroid colony-forming units, which outlines its importance for hematopoiesis and vasculogenesis (Baudino et al., 2002; Knies-Bamforth et al., 2004; von Rahden et al., 2006). In addition, c-myc-deficient ES-cells show downregulated VEGF-A levels and c-Myc is able to upregulate VEGF-A in several tissues, which indicates that VEGF-A is one of its transcriptional targets (Baudino et al., 2002; Knies-Bamforth et al., 2004; von Rahden et al., 2006).

In skin and bone marrow, c-Myc has been proposed to indirectly affect the differentiation by altering the interactions between the stem cells and the niche (Murphy et al., 2005). In adults, Cre–loxP induced lack of c-Myc forced the expanding hematopoietic progenitors into quiescent G0-phase, whereas the long-term HSCs accumulated in the bone marrow (Wilson et al., 2004). c-myc-deficient HSCs overexpress N-cadherin and several integrin αL, α5 and β2 adhesion receptors that have been previously implicated in HSC homing and mobilization (Murphy et al., 2005). It has been suggested that increased adhesion or altered cell migration preserves the c-myc-deficient HSCs in the stem cell niche, which leads to differentiation defect (Wilson et al., 2004). c-Myc may also control the first differentiation step of HSCs since c-Myc overexpression results in loss of these cells likely due to premature differentiation (Wilson et al., 2004).
1.7 GROWTH- AND SURVIVAL FACTORS OF GDNF AND VEGF-C-FAMILY

GDNF family

GDNF family ligands (GFLs): GDNF (Pichel et al., 1996), ARTN (Balah et al., 1998), neurturin (NRTN) (Kotzbauer et al., 1996) and persephin (PSPN) (Milbrandt et al., 1998) belong to the TGF β superfamily. They are secreted proteins, which are first produced as preproteins and then cleaved to their active forms that show approximately 40% similarity to each other (reviewed in Airaksinen and Saarma, 2002). In general, GLFs have important role in neurogenesis of the several neural subtypes.

In CNS, GDNF promotes growth of the dopaminergic neurons, spinal motoneurons and noradrenergic neurons (Airaksinen and Saarma, 2002). Whereas, in PNS GDNF regulates survival and growth of other neural subpopulations including sympathetic-, parasympathetic and enteric neurons (Airaksinen et al., 1999; Airaksinen and Saarma, 2002). Gdnf-deficient phenotype in mouse is embryonic lethal and the embryos lack enteric neurons and kidneys, and show defects in sensory neurons (reviewed in Airaksinen and Saarma, 2002). GDNF has role also in maintaining the spermatogonia stem cells in testis (Meng et al., 2000).

During embryo development, ARTN is expressed in smooth muscle cells of the central and peripheral blood vessels. Artn-deficient mouse embryos have defects in axonal outgrowth of sympathetic neurons and approximately 30% decrease in the size of sympathetic ganglia chain (Balah et al., 1998; Nishino et al., 1999). Recently, ARTN was shown to support growth of Peyer’s Patches, the major components of gut associated lymphoid tissue (Veiga-Fernandes et al., 2007).

The third family member, NRTN has been shown to be important in development and maintenance of the enteric-, sensory- and parasympathetic neurons (Heuckeroth et al., 1999), and Nrtn-deficiency leads to defects in enteric, parasympathetic nervous systems and innervation defects of the cholinergic sympathetic neurons (Hiltunen and Airaksinen, 2004; Rossi et al., 1999). PSPN promotes survival of several types of neurons including midbrain dopamine neurons and motoneurons (Sariola and Saarma, 2003).

GDNF family receptors

GFLs bind to the non-kinase glycosylphosphatidylinositol-linked (GPI) GDNF family receptors (GFRs) GFRα1, GFRα2, GFRα3 and GFRα4 and signal through c-Ret (Ret) (reviewed in Sariola and Saarma, 2003) (Figure 9). GLFs have their preference in GFR binding: GDNF binds to GFRα1, and ARTN, NRTN and PSPN bind to GFRα3, GFRα2 and GFRα4, respectively. However, in vitro GFRα1, GFRα2 and GFRα3 have shown weak redundancy according their ligands (reviewed in Sariola and Saarma, 2003). GFRs are normally bound to plasma membrane but at least GFRα1 is biologically active in soluble form (Worley et al., 2000). GFL-GFR interaction activates Ret and its downstream signaling pathway (reviewed in Airaksinen et al., 1999).
Ret is a proto-oncogene and transmembrane receptor, which has three domains: an extracellular domain with four cadherin-like repeats and a cystein rich domain and a cytoplasmic tyrosine kinase domain (Anders et al., 2001). Ret has three alternatively spliced isoforms, 51, 43 and 9, (Myers et al., 1995; Tahira et al., 1990). Ret isoforms 51 and 9 are the most predominant and they have distinct functions (de Graaff et al., 2001; Tsui-Pierchala et al., 2002). Ret-deficient mouse embryos and newborns show mislocated superior cervical ganglia and defects in sympathetic ganglia chain, and the phenotype is lethal at P1 (Enomoto et al., 2001). Ret-deficient sympathetic neurons show retarded differentiation, defects in target innervation and increased neuronal death during embryonic development (Pichel et al., 1996; Enomoto et al., 2001). Ret-deficient mice have also defects in the migration of enteric neurons (Pichel et al., 1996). Similarly to GFLs, Ret also has role outside of the nervous system such as in kidney development (Pichel et al., 1996; Sariola and Saarma, 2003) and in testis (Meng et al., 2000).

GDNF can signal Ret-independently through GFRα1 and Src-family kinase or neural cell adhesion molecule (NCAM) (Paratcha et al., 2003; Popsueva et al., 2003). Gfrα1-deficient mouse embryos have defects in sensory neurons and they lack both the enteric neurons and kidneys, and the phenotype is embryonic lethal (reviewed in Airaksinen and Saarma, 2002). While, a lack of GFRα2 results in defects in both the enteric and parasympathetic nervous systems, the mutant mice show also innervation defects of the cholinergic sympathetic neurons (Hiltunen and Airaksinen, 2004; Rossi et al., 1999). Gfrα3-deficiency leads to approximately to a 30% decrease in size of the sympathetic ganglia chain (Nishino et al., 1999), and the mutant mice also show decreased numbers of cells in gut associated lymphoid tissue (Veiga-Fernandes et al., 2007). Gfrα4-deficient mice have no other defects than reduced production of thyroid calcitonin (Lindfors et al., 2006).

![Diagram of GDNF and Ret interaction](image-url)

Figure 9. GFLs and interaction with their receptors. (Modified from Sariola and Saarma, 2003)
VEGF subfamily

Platelet derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) family members are predominantly secreted glycoproteins, which form either disulfide-linked or non-covalently bound homo- and heterodimers with anti-parallel arranged subunits (Muller et al., 1997; Stacker and Achen, 1999). VEGF subfamily consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PLGF, and they all share VEGF homology domain (VHD). In general, all VEGF subfamily members are essential for vascular development (vasculogenesis) and blood vessel sprouting (angiogenesis) in both embryo and adult, but they have also been implicated in vascular permeability and metastasis (reviewed in Tammela et al., 2005).

VEGF-A (Jin et al., 2000; Maurer et al., 2003; Schratzberger et al., 2000; Sondell et al., 1999) promotes proliferation and survival of endothelial cells, and increases microvascular permeability. Moreover, VEGF-A is involved in several biological processes such as mobilization of intracellular calcium, plasminogen synthesis, monocyte migration, expression of cell adhesion molecules, and induction of nitric oxide mediated vasodilation and hypotension (Ferrara, 1999; Neufeld et al., 1999; Zachary, 1998). In humans, VEGF-A has five isoforms encoded by distinct mRNA splice variants and named by their amino acid length; 121, 145, 165, 189 and 206. All isoforms promote mitogenic activation in endothelial cells, however the biological activity, receptor specificity and affinity for the low affinity receptor cell surface- and extracellular matrix-associated heparin sulfate proteoglycans between the isoforms is different. VEGF121 and VEGF165 are the predominant forms of VEGF-A even though most of the tissues express several VEGF-A isoforms simultaneously (Ferrara, 1999). Both VEGF121 and VEGF165 are soluble but VEGF165 is mostly confined to the cell surface and extracellular matrix proteoglycans.

In embryonic tissues, VEGF-A expression is associated with the vascular system, but it is also found in several tumors and tumor cell lines (Eggert et al., 2000; Fakhari et al., 2002; Komuro et al., 2001; Meister et al., 1999). Lack of a single allele of Vegf-a in mice leads to embryonic death at E11-E12, and the embryos showed several anomalies and defects in cardiovascular development (Ferrara et al., 1996). The phenotype of VEGF-A targeted gene disruption knockout mice also indicates that the protein is critical for the survival and development of the cardiovascular system and angiogenesis (Raab et al., 2004, Haigh et al, 2000). Recently, VEGF-A was shown to be one of the factors required for expansion of the ES-cell derived blood cell precursors (Pick et al., 2007). VEGF-A and BMP increased frequency of ES-cell derived hematopoietic progenitors and the numbers of immature and mature hematopoietic cells.

Postnatally, VEGF-A is required for the growth, organ development, growth plate morphogenesis and endochondral bone formation. In adults, VEGF-A is involved in wound healing and the development of corpus luteum (Ferrara, 1999; Neufeld et al., 1999). VEGF-A expression is regulated transcriptionally and post-transcriptionally. and Hypoxia, several hormones, interleukins and cytokines such as EGF and TGF-β can enhance VEGF-A expression (Ferrara, 1999). Besides of the role in endothelial cells,
VEGF-A also attracts and guides developing neurons (Carmeliet et al., 2005). In CNS, VEGF-A signal is mediated by VEGFR-2 and it leads to expansion of the neural progenitors in the hippocampus (Jin et al., 2002). In retinal progenitors, VEGF-A increases proliferation and decreases neurogenesis by stimulating bHLH factor Hes1 (Hashimoto et al., 2006). In ES-derived neural progenitors, VEGF-A promotes survival and proliferation of the FGF and EGF-dependent definitive progenitors instead of the primitive LIF-dependent progenitors (Wada et al., 2006).

VEGF-B (Silvestre et al., 2003) has two alternatively spliced isoforms, 167 and 186, which share approximately 44% amino acid sequence homology with VEGF-A (Olofsson et al., 1996a; Olofsson et al., 1996b). Both isoforms can form heterodimers or homodimers with VEGF-A. VEGF-B is primarily expressed in cardiac and skeletal tissues in embryos and adults (Joukov et al., 1997; Stacker and Achen, 1999), and Vegf-b-deficient mice show dysfunction of the coronary vasculature, reduced size of the heart and impaired recovery after cardiac ischemia (Bellomo et al., 2000). VEGF-B is also expressed in the brain especially after brain injury (Nag et al., 2002), and it can reduce hypoxic death of cultured cerebral cortical neurons in vitro (Sun et al., 2004) and increase proliferation of neural progenitors in adult CNS (Sun et al., 2006).

VEGF-C (Joukov et al. 1996) is produced as pre-protein that undergoes extensive proteolytic modifications, which result in a mature VEGF-C that shows approximately 30% identical amino acid sequence with VEGF-A. The mature VEGF-C can bind to both VEGFR-3 and VEGFR-2 and promote either lymphangiogenesis or angiogenesis respectively depending on the receptor (Joukov et al., 1997; Makinen et al., 2001; Mandriota et al., 2001; Marconcini et al., 1999). Partial proteolytic processing produces an intermediate form of VEGF-C, which shows increased affinity for VEGFR-3 receptor (Joukov et al., 1997).

During development, VEGF-C is expressed in allantois, in the wall of dorsal aorta, jugular area and metanephric kidneys in temporal and spatial pattern (Joukov et al., 1997; Karkkainen et al., 2004). VEGF-C is involved in regulation of lymphangiogenesis by promoting the migration and survival of lymphendotelial progenitors, and Vegf-c-deficient mice lack lymphatic vessels, develop lymphoedema and die at E15-E16 (Karkkainen et al., 2004). Vegf-c-heterozygous mutants are viable, however, they also show lymphoedema during embryogenesis. VEGF-C overexpression induces growth of the hyperplastic lymphatic vessel network in skin (Jetlsch et al., 1997).

In later development and adulthood, VEGF-C may maintain differentiated lymphatic endothelium (Ferrara, 1999). VEGF-C shows also angiogenic properties, and promotes the growth and migration of endothelial cells by signaling through VEGFR-2 in vitro (Eichmann et al., 1998). In CNS, VEGF-C promotes proliferation of OLPS in optic nerve and olfactory bulb progenitors by signaling through VEGFR-3 (Le Bras et al., 2006). Furthermore, VEGF-family members and their receptors are found in several cancers including sympathoadrenal-lineage-derived human neuroblastomas (NBs) (Eggert et al., 2000; Fakhari et al., 2002; Komuro et al., 2001; Meister et al., 1999). In aggressive NBs,
VEGF-C expression is also found to be associated with $n$-Myc amplification (Eggert et al., 2000).

VEGF-D (Achen et al., 1998; Orlandini et al., 1996) is structurally and functionally most closely related to VEGF-C. Similarly to VEGF-C, also VEGF-D undergoes proteolytic processing (Achen et al., 1998; Chen et al., 2000; Joukov et al., 1997). During embryo development, VEGF-D is expressed in heart and lungs in temporal and spatial pattern whereas in adults its expression is mostly restricted to skeletal muscles. VEGF-D also induces mitogenic responses in endothelial cells in vitro.

VEGF subfamily member VEGF-E is orf-virus-encoded and shows approximately 20% identity with VEGF-A at the amino acid level. Viral infection leads to pustular dermatitis that may involve endothelial cell proliferation and vascular permeability (Ferrara, 1999; Stacker and Achen, 1999). VEGF-F was recently isolated from snake venom (Suto et al., 2005) and it consists of two VEGF-related proteins that show 50% structural homology with VEGF$_{165}$ and binds selectively to VEGFR-2 (Suto et al., 2005).

Placental growth factor (PlGF) has three isoforms generated after alternative splicing and those can form heterodimers or homodimers with VEGF-A (Hauser and Weich, 1993; Maglione et al., 1993). PlGF has poor capacity to induce angiogenesis or proliferation of endothelial cells when compared to VEGF-A. During embryogenesis PlGF is primarily expressed in the placenta and in trophoblastic giant cells of the pariental yolk sac together with VEGF-A (Stacker and Achen, 1999).

**VEGF receptors**

VEGFs signal predominantly through transmembrane receptor tyrosine kinases VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) (Figure 10). VEGF-A binds to both VEGFR-1 and VEGFR-2 (Ferrara and Davis-Smyth, 1997; Shibuya et al., 1990; Terman et al., 1991) whereas VEGF-B and PlDGF have selective affinity for VEGFR-1 (Olofsson et al., 1998). Both VEGF-C and VEGF-D signal through VEGFR-2 and VEGFR-3 (Achen et al., 1998; Joukov et al., 1996; Kukk et al., 1996; Ristimaki et al., 1998; Saaristo et al., 2002).

VEGFR-1 contains an extracellular region with seven immunoglobulin (Ig)-like domains, a transmembrane domain and a tyrosine kinase domain (Shibuya, 2006). Organization of VEGFR-2 (Terman et al., 1991) and VEGFR-3 (Pajusola et al., 1992) is similar to VEGFR-1 and the receptors show 80% identity to VEGFR-1 tyrosine kinase domain. Unlike other VEGFRs, one of the Ig-like loops in VEGFR-3 is replaced by a disulfide bridge (Pajusola et al., 1994). All VEGFRs undergo alternative splicing that results in several isoforms of the receptors (Ebos et al., 2004; Hughes, 2001; Kendall and Thomas, 1993). During embryo development, VEGFR-1 negatively regulates angiogenesis whereas in adults it promotes angiogenesis (reviewed in Shibuya and Claesson-Welsh, 2006). Vegfr-1-deficient mouse embryos show overgrowth in endothelial cells, blood vessels disorganization, and the embryos die at E8-E9 (Fong et al., 1995). VEGFR-1 is expressed in endothelial cells and in the monocyte-macrophage cell lineage where it
regulates the cell migration (Clauss et al., 1996; Sawano et al., 2001). VEGFR-1 is also expressed in dendritic cells, osteoclasts and trophoblasts (Dikov et al., 2005; Kaipainen et al., 1993) but its function in these cell types is not clear. VEGFR-2 stimulates endothelial growth and angiogenesis, and Vegfr-2-deficient mice die at E8 to 9 due to lack of vasculogenesis and poor hematopoietic development (Shalaby et al., 1995). VEGFR-3 is involved in both lymphatic and blood vessel function, and Vegfr-3-deficiency causes abnormal remodeling of the primary vascular plexus and leads to death at E9 (Dumont et al., 1998). Early lymphatic development is VEGFR-3 independent but the sprouting of the lymphatic endothelial cells from the cardinal vein is mediated by VEGF-C and VEGFR-3 around E10-11 (Karkkainen et al., 2004).

GPI-linked non-kinase neuropilin (Nrp) receptors Nrp1 and Nrp2 (Karkkainen et al., 2001) are co-receptors for VEGF subfamily members. At least, VEGF-A, VEGF-B and VEGF-C can bind Nrp1 and Nrp2 (reviewed by Matsumoto and Claesson-Welsh, 2001; Neufeld et al., 2002). In endothelial cells, Nrp2 can enhance VEGF-C-induced VEGFR signaling (Soker et al., 1998). Whereas, in nervous system Nrp1 bind class 3 semaphorins (Sema) and guide developing axons by chemo-repulsion and -attraction (Chen et al., 1998; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Accordingly, Nrp1-deficient mouse embryos show defects in neuronal projection in CNS and PNS, dislocation of sympathetic neurons and their progenitors, and also cardiovascular defects (Bagri and Tessier-Lavigne, 2002; Kawasaki et al., 1999; Klagsbrun et al., 2002). In mouse, lack of Nrp2 leads to mild defects in cranial and spinal nerve organization and also to a reduction in the numbers of small lymphatic vessels and capillaries (Chen et al., 2000; Giger et al., 2000; Yuan et al., 2002). Both Nrp1 and Nrp2 are expressed in mouse and human neuroblastoma cells (Beierle et al., 2003; Fakhari et al., 2002; Jogi et al., 2004; Marcus et al., 2005), and at least Nrp1 mediates Sema3A signaling during sympathetic nervous system patterning (Kawasaki et al., 2002).

![Figure 10](image.png)

Figure 10. VEGF-family ligands and interaction with their receptors. (Modified from Hicklin and Ellis, 2005)
2 AIMS OF THE STUDY

The aim of this study is to investigate how certain intrinsic and extrinsic factors affect self-renewal and differentiation of NSCs and NPCs. The studied cell intrinsic factors are the tumor suppressor p53 and pRb–family members, which are involved in cell proliferation and differentiation of several tissues, the proto-oncogene c-Myc and the cytoskeletal tumor suppressor NF2 protein merlin and the ERM protein ezrin. The investigated cell extrinsic factors are the classical viral oncogenes HPV E6/E7 that degrade both p53 and pRb–family members, and the lymphangiogenic growth factor VEGF-C, which has several roles during development and in tumors. The specific questions addressed by this study are:

I) How do tumor suppressors p53 and pRb-family or human papilloma virus oncogenes E6/E7 affect neural progenitor behavior?

II) Does proto-oncogene c-Myc have a role in the maintenance of neural progenitors?

III) What is the localization and expression of NF2 protein merlin and ERM protein ezrin in the developing and adult brain, and in the brain derived neural progenitors?

IV) Does lymphangiogenesis related vascular endothelial growth factor C (VEGF-C) affect the sympathetic nervous system development?
3 MATERIALS AND METHODS

The methods used in this study are described in the respective publications or their references. The experimental procedures that required the use of laboratory animals were performed according to ethical guidelines approved by the local authorities.

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Neurosphere culture system, self-renewal capacity assay and in vitro differentiation

Neural stem cells and progenitors were collected from mouse embryos at E11 (I, III & IV) or at E12 (II) and cultured as free-floating neurospheres in neural stem cell medium (NSCM): Dulbecco’s MEM (DMEM) supplemented with nutrient mixtures F12 (Sigma-Aldrich) and B27 (Invitrogen), Glutamax (Invitrogen), growth factors FGF2 (20 ng/ml; Sigma-Aldrich) and EGF (40 ng/ml; Sigma-Aldrich). The neurospheres were passaged mechanically twice a week (I, II and III) and the experiments were performed with cells passaged for <15 (I and III) or <20 times (II). However, neuropheres were maintained for >30 passages without signs of senescence.
Self-renewal capacity of NSCs and NPCs was measured by clonal divisions: the neurospheres were dissociated mechanically (I, II and III) or enzymatically with Trypsin-EDTA 0.25% (IV) into single cells that were plated into 96-well plates in a density of 15 cells/well. The number of new neurospheres was counted after 7–10 days. The data is shown as percentage of self-renewing cells, which was calculated by dividing the number of new neurospheres with the number of originally plated cells (I and II). SPCs did not form spheres but grew in loose cell clusters in NSCM and therefore the number of the clusters and the number of the cells in the clusters were counted after 7-10 days (IV).

For in vitro differentiation, small neurospheres or single cells were plated on poly-D-lysine or poly-L-lysine (Sigma-Aldrich) coated glass coverslips and cultured in FGF2 and EGF-free NSCM and supplemented with 2% or 5% fetal calf serum (FCS) or fetal bovine serum (FBS) (PromoCell) (I, II and III) or 1% FBS and 100 ng/ml NGF (Sigma-Aldrich) (IV).

MTT cell proliferation analysis and ApopTag apoptosis assay

Cell proliferation kit I (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Roche) was also used for measuring cell proliferation in NPCs. For MTT analysis the non-differentiated cells were washed three times for 30 min with FGF2-EGF free medium before plating in a density of 20 000-30 000 cells/well into 96-well plate. Cells were cultured 3 to 4 days before adding MTT labeling- and solubilization reagents. The MTT absorbance was measured with multi-well spectrophotometer by using wavelengths of 585 nm and 750 nm for reference. ApopTag fluorescein in situ apoptosis kit (Chemicon) was used to detect apoptosis in NPCs and the immunocytochemistry was performed according the manufacturer’s protocol.
4 RESULTS AND DISCUSSION

4.1 Oncogenes and proto-oncogenes increase stem cell characteristics in CNS progenitors (I, II)

HPV 16 E6/E7 oncogenes

Several viral oncogenes such as HPV E6/E7 (Boyer et al., 1996; Davies et al., 1993; Scheffner et al., 1990), SV40 large-T antigen (DeCaprio, 1999), BKV TAg (Harris et al., 1998), and adenovirus E1B/E1A oncogenes (Dyson et al., 1993; Whyte et al., 1988) are able to degrade the tumor suppressor p53 and pRb-family members. In this study, we used E6/E7 as classical viral oncogene model in order to study the effect of oncogenic exposure on mouse fetal CNS progenitors that were cultured as free floating neurospheres. In NPCs, expression of E6/E7 was found to increase the capacity for self-renewal and proliferation (Figure 1 in I). In culture conditions that promote differentiation, E6/E7 oncogenes delayed the cell cycle exit and gave NPCs the ability to remain multipotent, but did not block the differentiation process (Figure 2, 3 and 4 in I).

These functions seem to be at least partly linked to degradation of p53 and pRb-family proteins in NPCs (I). Furthermore, E6/E7 oncogenes induced slight upregulation of MEK-ERK signaling, which seems to be important for NPC self-renewal (Figure 7 in I). In NPCs, E6 oncogene expression led to a clear reduction in the level of p53 whereas E7 oncogene degraded all pRb-family members, pRb, p130 and p107 (Figure 1 in I). Both E7 and E6/E7 expressing NPCs showed increased proliferation and shorter population doubling time when examined by BrdU incorporation (Figure 1 in I) and MTT assay (Roche) (Figure 10). Moreover, E6/E7 expression caused 5 fold increase in NPC self-renewal capacity when analyzed by secondary sphere formation assays (Figure 1 in I), and the secondary E6/E7 spheres were found to be bigger than the controls (Figure 11), which is likely due to an enhanced cell proliferation rate.

The self-renewal capacity was also increased in E7 expressing NPCs and slightly but not significantly in E6 expressing NPCs (Figure 1 in I). These clonally formed secondary spheres were all able to produce neurons and astrocytes, which indicates that the spheres were derived from multipotent progenitors and not from more committed precursor cells.
In the environment that promotes differentiation, E6-, E7-, E6/E7 expressing NPCs and the controls attached and developed morphology of differentiated neuronal cells. When the culture conditions were switched back to promote growth and proliferation after two weeks, E6/E7 expressing cells showed frequent re-formation of the spheres (>2000 spheres/200 000 plated cells) (Figure 2 in I). These re-formed spheres could be expanded in culture and after in vitro differentiation the cells showed the ability to form both neurons and astrocytes. Only low numbers of re-formed spheres were detected in all E6, E7 and the control cells (<10 spheres/200 000 plated cells) (Figure 2 in I), which indicates that cells showed non-reversible and complete differentiation.

To study the cause of the sphere re-formation after differentiation, the cells were BrdU-pulse labeled for 24 hrs during each day of the differentiation. The analysis revealed that the controls and E6 expressing progenitors silenced their cell cycle within first 48–72 hrs of the differentiation (Figure 3 in I). However, a high fraction (~50%) of NPCs expressing both E6/E7 oncogenes entered S-phase still as late as on eighth day of the differentiation (Figure 3 in I). In E7 expressing cells, the terminal mitosis was also markedly delayed but complete after four days differentiation (Figure 3 in I).

Even if E6/E7 expression delayed or partly inhibited the terminal mitosis in NPCs, it did not block the differentiation in vitro or in vivo (Figure 4 and 5 in I). Undifferentiated control and E6/E7 expressing progenitors had similar expression pattern of the neural bHLH transcription factors Mash1, Hes1, Hes5 and Id2 (Farah et al., 2000; Ross et al., 2003; Toma et al., 2000) when studied by RT-PCR (Figure 4 in I). Both control and E6/E7 NPCs were also able to form Tuj-1 positive neurons and GFAP positive astrocytes during 96 hrs in vitro differentiation (Figure 4 in I). Both control and E6/E7 expressing cell showed high proportion of nestin positive cells after 96 hrs differentiation in vitro indicating that cell fate determination was still on going, even though in the control group
the cells had exited the cell cycle. Beside of the delayed or partly inhibited terminal mitosis, E6/E7 expressing NPCs showed increased apoptosis during in vitro differentiation when analyzed with ApopTag apoptosis kit (Chemicon) (Figure 12). A similar type of behavior has been seen in pRb-deficient cortical progenitors that showed enhanced apoptosis during both in vitro and in vivo differentiation, which was most likely due to problems to undergo terminal mitosis (Callaghan et al., 1999; Slack et al., 1998).

Figure 12. The number of apoptotic cells after 4 days in vitro differentiation of the NPCs. The chart represents an average of 3 experiments, *P<0.05 (Student’s T test) and error bars indicate SD between the experiments.

In vivo transplantation of EGFP positive E6/E7 and control NPCs into brain ventricles of mouse embryos at E14 in utero revealed that the cells engrafted throughout the brain tissue. Immunohistochemistry after serial cryostat sections of the brains revealed that E6/E7 expressing and control NPCs were able to form all three types of neural cells: neurons, astrocytes and oligodendrocytes in 96 hrs after the transplantation (Figure 5 in I). Both E6/E7 and control cells were negative for the neural progenitor marker nestin (data not shown) indicating that the cells were committed to differentiate. Contrary to the in vitro data, E6/E7 expressing NPCs did not show inhibited terminal mitosis after in vivo differentiation since no EGFP and Ki-67 double positive cells were detected (data not shown).

It is possible that the specific microenvironment of the developing brain is able to overcome the oncogenic signals of E6/E7 genes. Another feasible option is that the transplanted NPCs that had delayed or inhibited the cell cycle exit were killed by apoptosis. It has been shown that rapid turnover of G1/S and G2/M-phases due to degradation of p53 often leads to chromosomal duplications and centrosomal abnormalities in E6/E7 expressing cells (Coursen et al., 1997). However, both control and E6/E7 expressing NPCs showed euploidy and normal cell cycle distribution when analyzed with propidium iodide staining (PI) as detected by fluorescence-activated cell sorting (FACS) flow cytometer (Figure 4 in I). The higher proliferation rate of E6/E7 expressing NPCs was also seen in here, since the cell numbers in S and G2/M-phases were increased when compared to controls (Figure 4 in I).

In epithelial cells, E6/E7 oncogenes up-regulate MAPK signaling (Antinore et al., 1996; Chakrabarti et al., 2004). Some of the MAPK pathway molecules were also changed in
E6/E7 expressing NPCs. In NPCs, E6/E7 expression caused upregulation of c-Jun and slight increase in extracellular signal-regulated kinase (ERK) activation, but no changes in JNK activation were detected (Figure 7 in I). c-Jun is involved in cell proliferation, differentiation, tumor transformation and apoptosis in various cell types (Herdegen et al., 1997). During differentiation, E6/E7 expression decreased ERK phosphorylation but increased the expression of JunD transcription factor (Figure 7 in I).

We focused on studying the MEK-ERK pathway that in neural cells functions at least in regulation of the proliferation, differentiation, neurite outgrowth and synaptic plasticity (reviewed in Grewal et al., 1999). Also in several tumors, Ras-Raf-MEK-ERK intracellular signaling cascade is often found to be constitutively active. In NPCs, MEK–ERK inhibition by U0126 (Favata et al., 1998) led to approximately 50% decreased in cell proliferation but almost completely blocked the self-renewal (Figure 7 in I). This suggests that MEK/ERK pathway is essential for maintaining the stem cell characteristics in NPCs. Previously MEK/ERK has been shown to be important for the self-renewal of immature avian erythroid progenitor cells (Dazy et al., 2003). MEK-ERK inhibition during in vitro differentiation was able to decrease the excess apoptosis of E6/E7 expressing NPCs perhaps by facilitating the cell cycle exit of the proliferating progenitors (Figure 13) (Piltti et al. unpublished data).

Self-renewal is cell proliferation dependent and CDKs are essential for all types of cell division (Galderisi et al., 2003). NPCs express at least CDK-2, -4, -5 and -6, and all of these except CDK-5 are involved in cell cycle regulation (Ferguson et al., 2000). In NPCs, inhibition of CDKs by R-Roscovitine (Meijer et al., 1997), which targets CDK-1, -2, -5, -7 and -9 almost equally (IC50 values >1 mM) (Bach et al., 2005) decreased both proliferation and self-renewal the same magnitude of 50% (Figure 7 in I) suggesting that self-renewal and proliferation pathways can be separated.
c-Myc

Overexpression of Myc family members can be detected in several cancers (Grandori et al., 2000). In nervous system derived tumors, both c-Myc and n-Myc overexpression have been implicated in pathogenesis of medulloblastoma, glioma and neuroblastoma (Eberhart et al., 2004; Schwab, 2004; Su et al., 2006). In CNS, c-Myc is expressed in the walls of lateral ventricles, the area where self-renewing NSCs and NPCs are located (Figure 1 in II). Embryonic CNS NPCs transduced with modified viruses expressing tamoxifen inducible c-Myc (MycER) showed increased cell proliferation and self-renewal when c-Myc expression was activated (Figure 2 in II). After 24 hrs of BrdU incorporation 29% of the control NPCs and 77% of tamoxifen induced MycER expressing NPCs were found to be in S-phase (Figure 2 in II). In self-renewal assays, tamoxifen increased the proportion of self-renewing cells five fold from 4.2% in control to 22.5% in MycER expressing neurospheres (Figure 2 in II). Immunocytochemistry with antibody against annexin revealed that Myc does not enhance apoptosis in NPCs (Figure 7 in II) unlike in some other cell types (Meyer et al., 2006). However, PI-stainings revealed that one of the transgenic Myc NPC populations had become tetraploid (Figure 7 in II) reflecting the ability of Myc to induce genomic instability (Wade and Wahl, 2006).

Beside of the enhanced self-renewal capacity c-Myc overexpression significantly increased the ratio of the NPCs that expressed Bmi-1 and nestin, the markers related to undifferentiated neural progenitors. 84% of the control NPCs showed Bmi-1 expression and Myc overexpression increased this to 93% (Figure 2 in II). Bmi-1 has been shown to be important for self-renewal and proliferation of several stem cell types, including cerebellar NPCs and to be also one of the c-Myc transcription targets (Guccione et al., 2006). The effects of Bmi-1 on self-renewal is at least partly mediated by its capacity to inhibit p16^INK4a/p19^Arf, the tumor suppressors upstream from p53 and pRb (Bruggeman et al., 2005; Molofsky et al., 2003), and Bmi-1 induced inhibition of p21^Cip1 is essential for NSC self-renewal during development (Fasano et al., 2007). In both control and Myc overexpressing NPCs, Bmi-1 was downregulated during in vitro differentiation (Figure 3 in II). Myc overexpression also increased percentage of nestin expressing NPCs from 44% to 74% (Figure 2 in II). Similarly to Bmi-1 expression nestin expression also was downregulated almost totally during differentiation in both control and Myc-expressing cells (Figure 3 in II). Increased self-renewal capacity with increased percentage of cells expressing immature neural progenitor markers suggests that c-Myc is able to increase the pool of self-renewing NPCs and shift the differentiation balance towards a more primitive cellular identity. In astrocytes, Myc has been previously shown to drive the cell fate of GFAP-expressing cells towards a less differentiated, nestin-expressing progenitor-like fate (Lassman et al., 2004).

Even though undifferentiated Myc overexpressing neurospheres proliferated twice as much as the control NPCs, during in vitro differentiation the majority of the cells were able to respond to the surrounding environmental cues and exit the cell cycle. BrdU incorporation revealed that Myc overexpressing cells contained a minor cell population (4%) that continued proliferating on third day of differentiation when all the control cells.
had exit the cell cycle (Figure 3 in II). Most of the attached control cells showed either neuronal or astrocytic morphology after 4 days in vitro differentiation, and immunocytochemistry revealed that the cells also expressed Tuj-1 and GFAP (Figure 3 in II). The Myc overexpressing cells also attached and showed Tuj-1 or GFAP expression but their morphology varied when compared to the control cells (Figure 3 in II). When NPCs were plated into 96-well plates in different densities and cultured in differentiation promoting conditions approximately 1-2% of the Myc overexpressing cells were able to form new neurospheres in two weeks (Figure 4 and 5 in II). Control NPCs did not show the neurosphere formation but differentiated permanently (Figure 4 in II). Sphere reformation in Myc expressing cells seemed to be density-dependent phenomenon since it took place only when the cells were plated in the density of more than 200 cells/well (Figure 5 in II). After reaching this seemingly critical number, the higher cell densities did not lead to an increase in the new neurospheres. When re-formed Myc-expressing spheres were replaced into culture medium supplemented with EGF and FGF the cells showed similar proliferation, self-renewal and differentiation capacity as the primary Myc overexpressing NPCs (Figure 5 in II). Western blott analysis of c-Myc and its downstream target nucleolin revealed that the re-formation of the spheres was not due to selection of clones that express more Myc (Figure 5 in II).

In CNS, similarly to Myc also its binding partner Miz-1 is found to be expressed in the walls of lateral ventricles, the area where self-renewing NSCs and NPCs are located (Figure 8 in II). Myc interaction with Miz-1 has been shown to represses transcription of the cell cycle inhibitors such as p21\textsuperscript{CIP1} (Adhikary et al., 2003; Seoane et al., 2002; Staller et al., 2001). In keratinocytes, Myc regulates also adhesion and differentiation in the epidermal stem cell niche via binding to Miz-1 (Gebhardt et al., 2006). Similarly to wild type Myc NPCs, also NPCs transduced with modified retroviruses carrying a mutant form of Myc (MycV394D) that is unable to bind to Miz-1 (Herold et al., 2002) showed increased cell proliferation (Figure 6 in II). However, the self-renewal capacity of MycV394D overexpressing NPCs were similar to control NPCs (Figure 6 in II). Unlike wild type Myc NPCs MycV394D overexpressing NPCs also failed to re-form spheres during the differentiation (Figure 6 in II). This suggests that in NPCs c-Myc effect on self-renewal is at least partly mediated through Miz-1. However, the downstream targets of Myc-Miz interaction that are responsible of these effects remain to be revealed. Both E6/E7 and c-Myc data indicates that the stem cells or progenitors that are exposed to oncogenes or proto-oncogenes can shift their differentiation towards more primitive, highly proliferating cell population that is potential target for additional mutations needed for tumorigenesis.

### 4.2 Tumor suppressors decrease both proliferation and self-renewal (I)

p53 and pRb are the major tumor suppressor pathways that prevent cancer formation. Beside of controlling the cell proliferation, p53 has role in cell differentiation (Almog and Rotter, 1997). During embryogenesis and postnatally, p53, p27\textsuperscript{kip1}, and p16\textsuperscript{INK4a} mRNAs are highly expressed SVZ zone in rats (van Lookeren Campagne and Gill, 1998). Arguably the most important function of p53 in neurons is to prevent cell cycle re-entry
(Miller et al., 2003). Besides of differentiation, increase of p53 seems to accelerate the ageing of several tissues but the mechanisms are still unclear (Tyner et al., 2002). CNS NPCs collected from p53-deficient mouse embryos at E11 showed both increased proliferation and self-renewal (Figure 1 in I). During differentiation, terminal mitosis of p53-deficient NPCs was also delayed but completed after four days in culture (Figure 3 in I). p53-deficient NPCs showed ability to re-enter the cell cycle and re-form large numbers of spheres (>400 spheres/200 000 plated cells) after two weeks of differentiation if the growth factors, which promote cell proliferation were re-introduced (Figure 2 in I). This suggests that p53 not only has a role in tumor suppression but also a role in controlling NSC self-renewal.

Also pRb has central role in various differentiation processes, including the neural differentiation in both CNS and PNS (reviewed in Giacinti and Giordano, 2006). In developing brain, pRb is expressed in dividing progenitors and post mitotic cells (Lipinski and Jacks, 1999). Mutations or functional inactivation of pRb gene are also found in several human tumors (reviewed in Giacinti and Giordano, 2006). In E6/E7 or in E7 expressing NPCs, restored pRb expression decreased excess cell proliferation but did not affect self-renewal (Figure 6 in I), which suggests that pRb controls cell proliferation instead of self-renewal.

Previously, p16<sup>INK4a</sup>, the tumor suppressor upstream from p53-pRb proteins was shown to control NPC self-renewal since inactivation of p16<sup>INK4a</sup> partially rescued the incompetent self-renewal of Bmi-1-deficient NPCs (Molofsky et al., 2005). p16<sup>INK4a</sup> inhibits the activity of cyclin D-CDK4/6 and keeps pRb-family members hypophosphorylated, which prevents E2F activation and blocks the cells from exiting G1-phase (Ashizawa et al., 2001; Sherr and Weber, 2000). In E7 or in E6/E7 expressing NPCs, restored pRb did not upregulate the expression of the other degraded Rb-family members p107 and p130 (Figure 6 in I).

In the brain of adult mice, unlike other Rb-family proteins, p107 expression is restricted to NPCs in the ventricular zone (Jiang et al., 1997; Vanderluit et al., 2004). Furthermore, p107 has been shown to be one of the factors that regulate NSC self-renewal (Vanderluit et al., 2004). In mouse CNS, p107-deficiency increases the number of slowly proliferating stem cells and enhances NPC self-renewal capacity in vitro (Vanderluit et al., 2004). Moreover, p107 modulates Notch-pathway that suppresses the differentiation of the neural cells (Hitoshi et al., 2002). p107 has been shown to bind to E2F consensus sites of Notch1 regulatory sequences and p107 overexpression downregulates Notch1 activation (Vanderluit et al., 2004). In addition, p107-deficient mice show upregulated expression of Notch1 and its ligands Delta-like1 and Hes1 in SVZ (Vanderluit et al., 2004). Therefore it is likely that the increase in self-renewal in E7 expressing NPCs is at least partly due to degradation of p107. In NSCs, pRb-family members are temporally regulated during the differentiation: p107 is expressed in uncommitted NSCs or NPCs and becomes rapidly downregulated when the cells undergo differentiation (Callaghan et al., 1999). Whereas, the pRb that is required for terminal mitosis is upregulated and activated when the differentiation is initiated (Callaghan et al., 1999; Ferguson et al., 2000; Jiang et al., 1997). p130 shows upregulation once the differentiation is complete.
and the expression is believed to maintain the cells differentiated (Callaghan et al., 1999; Ferguson et al., 2000; Jiang et al., 1997).

p53 and pRb-family are not the only tumor suppressors, which affect the self-renewal and proliferation of NSCs. Loss of function of phosphatase and tensin homolog deleted on chromosome 10 (Pten) has been found in several human cancers including brain tumors (Vivanco and Sawyers, 2002; Xu et al., 2004). A lipid phosphatase and tumor suppressor Pten negatively regulates phosphatidylinositol 3-kinase (PI3K)/Akt pathway and modulates G1 progression (reviewed in Endersby and Baker, 2008). Pten has been shown to regulate proliferation of NSC (Groszer et al., 2001, Li et al., 2003). Pten-deficient mice have enlarged and abnormal brains, and mutant brain derived neurospheres showed increased cell numbers suggesting an increased in the number of NSCs (Groszer et al., 2001, Li et al., 2003).

Also loss of tumor suppressor lethal giant larvae (Lgl) leads to massive tissue disorganization, tumor-like growth and lethal phenotypes in both Drosophila and mouse (Vasioukhin, 2006; Klezovitch et al., 2004). Lgl-deficient cells displayed disrupted cell polarity, failure of asymmetric cell division and defects in cell fate determination (Klezovitch et al., 2004; Ohshiro et al., 2000). Newborn Lgl-deficient mice suffer from severe hydrocephalus and die neonatally. A large proportion of Lgl-deficient NPCs fail to exit the cell cycle and differentiate which leads to continued proliferation and apoptosis (Klezovitch et al., 2004). Moreover, Lgl-deficient NPCs fail to localize Notch inhibitor Numb asymmetrically during the cell divisions (Klezovitch et al., 2004).

4.3 Cytoskeletal tumor suppressor NF2 protein merlin and ERM protein ezrin are restricted into different cell lineages during CNS development (III)

The expression pattern of merlin and ezrin in the CNS suggests that these proteins may also be involved in normal brain development. Western blotting revealed widespread expression of merlin and ezrin in various regions in adult human, rat and mouse brain tissues (Figure 1,2,3 and 4 in III). Analysis of mouse embryonic, postnatal, and adult brain protein lysates revealed that merlin expression started at E11 stage and it was highly expressed until P1 (Figure 1 in III). After that merlin expression was decreased but stilldetectable until one year of age (Figure 1 in III). Interestingly, merlin expression starts at the timepoint when neurogenesis is initiated in mouse. Various levels of ezrin was detected in mouse embryonic brain starting at E5 up to one year of age and the expression seemed to peak at E8, E9, E11, E12, at E18 and P1, and at P14, P21 and P28. Ezrin expression was highly decreased after P28 (Figure 1 in III).

Human brain tissue arrays showed that merlin was predominantly detected in neurons and ezrin in astrocytes in most parts in the human brain (Figure 3 in III). Mouse fetal CNS derived NPCs that were cultured as spheres showed partial co-localization of merlin and ezrin (Figure 6 in III). In the beginning of in vitro differentiation when the spheres adhere to culture slide and the cells start to migrate out from sphere, ezrin was predominantly found in the outermost first migrating cell layer in short filopodia whereas merlin was
detected in the cell bodies (Figure 6 in III). Tuj-1 positive neurons that were spontaneously differentiated in spheres did not express ezrin but expressed merlin. Similarly as in human brain tissue arrays, merlin staining was strongest in Tuj-1 positive immature neurons that were ezrin negative after 5 days in vitro differentiation (Figure 7 in III). Immunocytochemistry of in vitro differentiated progenitors also revealed that ezrin was expressed in all the GFAP positive cells that showed only weak merlin expression (Figure 7 in III). In rat primary hippocampal cell cultures, ezrin expression was concentrated in cell bodies and fine filopodia of the astrocytes (Figure 8 in III). The cells showed also weak merlin expression and it was mainly detected in cell bodies. Similarly to mouse NPC derived neurons, rat hippocampal neurons did not show ezrin expression but merlin was detected in the cell somas and extensions (Figure 8 in VI).

In accordance of the previous data (Gutmann et al., 1995; Huynh et al., 1996), this study suggests that the cytoskeletal tumor suppressor NF2 protein merlin and ERM protein ezrin may be developmentally regulated. Huynh et al. 1996 detected merlin expression in wide range of mouse embryonic tissues starting at E10, and the expression appeared to be increased in fully differentiated tissues suggesting that merlin is involved in morphogenesis and organogenesis. We also detected changes in expression levels of merlin and ezrin during neural differentiation; primary cultures of rat hippocampal cells showed both merlin and ezrin expression in GFAP- and Tuj-1 positive cells in one to three days cultures (data not shown). However the neuronal expression of ezrin was not detected after three days cultures when the cells start to mature. Closer look at merlin’s subcellular localization in neurons revealed that the expression did not colocalized with presynaptic marker synapsin I positive structures but with some of the structures positive for postsynaptic density marker PSD-95 (Figure 8 in III). Moreover, merlin expression in postsynaptic junctions colocalized also with its binding partner RIβ (Gronholm et al., 2003) (Figure 8 and 9 in III). Targeted disruption of gene encoding RIβ causes defections in hippocampal synaptic plasticity (Brandon et al., 1995; Huang et al., 1995). Therefore is a possibility that merlin has a role in signaling related to learning and memory.

4.4 VEGF-C promotes cell proliferation and survival in developing sympathetic nervous system (IV)

VEGF-C is essential for the early events of lymphangiogenesis since it promotes migration of the lymphatic endothelial precursors out from the cardinal veins to periphery prior to formation of the primary lymph sacs (Karkkainen et al., 2004). VEGF-C functions are not only restricted to endothelial cells because it can also promote proliferation in both OLPs in optic nerve and in NPCs in olfactory bulb (Le Bras et al., 2006). Similarly to blood vessels and neurons the lymphatic- and peripheral nervous systems also share ligands that concurrently support development of the each other. In mouse embryo at E10-E13, VEGF-C is expressed adjacently to the areas where early sympathetic progenitor cells (SPCs) are located (Figure 1 in IV).
Immunohistochemistry of matched coronal sections of thoracic SGC revealed that Vegf-c-deficient embryos had reduced SGC size due to a 48% decrease in number of early Tuj-1 positive sympathetic progenitors when compared to wild type controls at E13-E14 (p<0.01, Student’s t-test) (Figure 1 in IV). Respectively, Vegf-c--heterozygous embryos showed a 22% decrease in SGC size (p<0.05, Student’s t-test) (Figure 1 in IV). In cultured mouse SPCs that were isolated from premature sympathetic chain, VEGF-C increased proliferation and survival dose-dependently and ligand specifically (Figure 2 and 3 in IV) suggesting that VEGF-C is acting as a paracrine factor in the developing SGC. In addition, VEGF-C and GFLs seem to function together in SNS development since in vitro VEGF-C and Ret ligands ARTN and GDNF induced similar growth levels in sympathetic ganglion-like structures and proliferation in SPCs (Figure 2 in IV). VEGF-C unlikely affects the cell migration, self-renewal, neurite outgrowth, or lineage decision in embryonic SPCs (Figure 3 in IV). However, similarly to several other growth factors VEGF-C also seems to change its functions while SNS matures since at P1 exogenously added VEGF-C did not increase cell survival but enhanced neurite outgrowth in sympathetic cervical ganglia (Supplemental figure 1 in IV).

Our data suggests that unlike in endothelial cells or CNS progenitors (Joukov et al., 1996; Le Bras et al., 2006), the VEGF-C signal in embryonic SNS is not mediated through known VEGF-C receptors (Figure 4 in IV). In SPCs, Vegfr-2 and Vegfr-3 expression was found to be virtually absent (≤0.01) and Tuj-1 positive immature sympathetic chain in Vegfr3/lacZ+/- mouse embryos did not show Vegfr-3-promoter driven β-galactosidase activity (Figure 4 in IV). As earlier reported by (Chen et al., 1997 and Kolodkin et al., 1997), embryonic SPCs expressed high levels of Nrp1 and Nrp2 (Figure 4 in IV). Nrps have not been shown to signal on their own (He and Tessier-Lavigne, 1997) but they are potential co-receptors for VEGF-C in embryonic SNS.

Despite of lack of VEGFR-2 and VEGFR-3, VEGF-C stimulus enhanced ERK phosphorylation in SPCs (Figure 4 in IV). In neural cells, MEK/ERK intracellular signaling cascade can be activated by several receptors and it is implicated in multiple cellular responses such as proliferation, self-renewal, survival and cell death, and differentiation (Colucci-D'Amato et al., 2003; Thomas and Huganir, 2004). In SPCs, VEGF-C induced MEK/ERK pathway activation is related to cell proliferation since specific MEK1/2 inhibitor decreased VEGF-C induced proliferation to the same level with the uninduced controls (Figure 4 in IV). In SPCs, VEGF-C provided cell survival is unlikely mediated through PI3K/Akt that has been related to sympathetic cell survival (Crowder and Freeman, 1998) since VEGF-C stimulus did not change the pathway activation (Figure 4 in IV). VEGF-C stimulus neither resulted in changes in JNK/c-Jun pathway activation (Figure 4 in IV), which has been implicated in neural cell death, stress and degeneration (Harper and LoGrasso, 2001).

GFLs and the general signaling receptor Ret are critically needed for the migration, proliferation and differentiation of several neural crest-derived PNS cell populations including the sympathetic progenitors (Enomoto et al., 2001; Pichel et al., 1996). Ret-deficient mouse embryos (Schuchardt et al., 1994) show decreased sympathetic chain size when compared to wild type embryos at E11 and during later development (Enomoto et al., 2001).
et al., 2001). Since Ret-deficient and Ret-heterozygous SPCs were not able to respond to VEGF-C we hypothesize that VEGF-C acts downstream of GFL/Ret in the signaling cascade of growth factors needed for SNS development. The lack of VEGF-C response in Ret-deficient SPCs was not due to changes in the expression levels of the conventional VEGF-C receptors since the amount of Nrps and VEGFRs did not differ from that in wild type SPCs. Previously, Ret phosphorylation has shown to take place in presence of exogenous VEGF-A in ureteric bud cell line (Tufro et al., 2007), but we gained no evidence of upregulated Ret phosphorylation in VEGF-C stimulated SPCs. Nevertheless, this suggests that Ret is required for the specification of sympathetic cells prior the VEGF-C.
5 CONCLUDING REMARKS AND PERSPECTIVES

This study suggests that tumor suppressors regulate not only NPC proliferation but also their self-renewal. The mechanisms by which tumor suppressors or their downstream targets inhibit the self-renewal capacity remain still unclear. Moreover, signaling related to NPC proliferation and self-renewal are connected but can be also separated since active MEK-ERK pathway seems to be more necessary for the self-renewal than for the proliferation. Interestingly, E6/E7 expressing NPCs showed slightly higher levels of ERK phosphorylation levels than the controls, and the inhibition of MEK-ERK pathway decreased the excess proliferation and self-renewal capacity. Respectively, several tumors have constitutively active MEK-ERK pathway and hypothetically this could also contribute to the cancer stem cell maintenance. Furthermore, oncogenic exposure or loss of p53 function in NPCs seemed to diminish their sensitivity for microenvironmental cues. A small proportion cells maintained the stem cell characteristics in conditions, which normally promote differentiation and terminal cell cycle exit.

c-Myc interaction with Miz-1 seems to be critical for its capacity to promote self-renewal in NPCs. However, it is still uncertain whether the effect is mediated by transcriptional inhibition of CDK inhibitor proteins such as p21\textsuperscript{Cip1} or by some other mechanisms. Developing brain and NPCs express cytoskeletal tumor suppressor NF2 protein merlin and ERM protein ezrin but the expression is mostly restricted into different neural cell lineages during the differentiation. Merlin expression in mouse brain also peaks at the time point when neurogenesis is initiated suggesting that merlin may have a role in neural differentiation and brain development.

Beside tumor suppressors and oncogene studies, this thesis revealed that lymphangiogenic growth factor VEGF-C is novel paracrine growth factor for developing sympathetic nervous system. VEGF-C and GDNF family ligands seem to function together in sympathetic ganglia chain formation. Moreover, c-Ret, the signaling receptor for GDNF family ligands, is required for the specification of sympathetic lineage cells prior to function of VEGF-C. Unlike in CNS progenitors or endothelial cells, the VEGF-C signal in sympathetic progenitors is mediated through VEGFR-2 and VEGFR-3 independent alternative receptor or receptor complex that remains to be discovered.
6 ACKNOWLEDGEMENTS

This research was carried out at the Developmental Biology laboratory, Institute of Biomedicine, University of Helsinki during 2002-2008. I want to express my gratitude to the Head of the Institute, professor Esa Korpi, for providing excellent facilities and administrative support: without quality infrastructure great scientific research would be impossible.

I am grateful to my supervisors for all the mentoring that I have received these past years. I thank Dr. Kirmo Wartiovaara for his valuable contribution to my thesis especially during my first four years of my studies, and professor Hannu Sariola for his encouragement and excellent scientific advice, based on his long-term experience in developmental biology and medical research. I am very grateful to Dr. Kirsi Sainio for her advice and guidance during my final years of my study; her constant and strong support helped me to push this process to its end. I warmly thank my thesis committee members, professor Tomi Mäkelä and Dr. Marjo Salminen for their recommendations and helpful advice. I would also like to kindly acknowledge Dr. Juha Partanen and Dr. Ari Ristimäki for their constructive comments upon reviewing my thesis. Professor Päivi Ojala, Tomi Mäkelä, Ms. Aija Kaitera and Dr. Elina Värtö at the Helsinki Biomedical Graduate School administration are warmly thanked for all the opportunities provided to me during the past years. We were lucky enough to share the Centre of Excellence position of the Academy of Finland during the first years of my studies: professor Irma Thesleff as the head of the Centre is acknowledged for the excellent scientific atmosphere the program has provided to all those involved. Professor Juha Voipio, my custos, and Dr. Jonna Katajisto at the Faculty of Biosciences are acknowledged for their kind assistance with the thesis related details and paper work. Without your constant guidance throughout this process I would have been lost. My most valuable collaborators have been professors Mart Saarma, Kari Alitalo and Olli Carpen. They provided me with the tools that were critical in carrying out this study successfully to the end – with luck I hope that this fruitful collaboration continues in the future. I warmly thank my postdoctoral advisors Dr. Aileen Anderson and Dr. Brian Cummings and their sweet daughter Camryn for the patience and the support while I have been at University of California, Irvine, and my current colleague Manuel Galvan for the language review of my thesis.

I am grateful to all my co-authors and collaborators for their individual contributions and great teamwork spirit, especially to Laura Kerosuo, Mikaela Grönhoml, Janne Hakanen, Anu Plaken, Paula Haiko, Minna Eriksson, Marika Kärkkäinen, Mart Saarma, Kari Alitalo, Olli Carpen and Sirpa Leppä, Marjo Salminen and Meilahti Experimental Animal Facility staff – your help has been valuable. I also want to acknowledge Drs. Anu Wartiovaara, Nina Napponen and Juha Klefström Marika Linja, Tanja Lepikhova for their advice and assistance. I want to thank all my friends and co-students in HBGS and the other graduate schools, especially Anna Kiialainen, Kilian Guse, Kari Vahtomeri, Tea Blom, Taru Muranen, Thomas Westerling, Pekka Katajisto, João Dias, Iulia Diaconu, Milla Mikkola, Can Hekim, Johanna Partanen, Tuomas Tammela, Katja Helenius, Nora Pöntynen, Marko Uutela, Topi Tervonen, Ana-Marie and Niklas Pakkasjärvi for all the events and parties that we shared together, and the Finnish Developmental Biologists in Oulu, Turku and Helsinki for the unforgettable moments in Tvärminne and Hyytiälä. I also want to thank Heli Lindeberg and the other people
affiliated with Kuopio University for all the good old times and for giving me the spark that has led me here.

I want to thank all the present and past members of the Circus Sariola for all the shared moments and cooperation – I will miss the crayfish parties, the fondue and wine tasting get-togethers, picnics, afternoon skiing and all the other great activities that we did together. I thank Laura for all the shared conference trips - we indeed chose the best ones. Valtteri and Samer are truly thanked for being my friends and labmates, even the busiest moments at the work were joyful with you guys. I thank Fares and Roxana for bringing lots of colourful attitude and laughter into the lab. Madis, Alexandre, Heli, Anna, Marjo, Nina, Marianna, Tiina, Satu, Anastasia, Maria, Pauli, Antti, Anita, Eric, Maxim, Jonna and Jouni - it was a pleasure to work and share the same labspace with you. My sincere thanks go to the excellent technical support, especially to Agnès but not forgetting Lea, Virpi, Jetta, Valtteri Harri, and Jukka Suokas for all their technical assistance.

I grace all my good friends, especially Anna P, Anna K, Mikko, Kilian, Genevieve, Heli, Heikki, Marja and Gustavo - you were always there for me! Your friendship and support is very precious to me. I also want to thank Markus, Laura and Lotta for the gourmet dinners, delightful parties and joyful activities including the famous summer Olympic games. Thank you for the great times we have shared together – those shall continue!

Last but surely not least, my dearest acknowledgements belong to my Mother and my Father, grandmother and all the rest of my kin, thank you for your love and support. I hope that some day this thesis will encourage my godchildren Iiris, Erno and Elina to reach far in their own studies.

I have been financially supported by grants from the Helsinki Biomedical Graduate School, Biomedicum Helsinki Foundation, Lilly Foundation (Lilly Säätiö), Orion Pharma Research Foundation, Finnish Concordia Fund, Vetenskapsstiftelsen för Kvinnor and University of Helsinki Funds.

Irvine, March 2009

Kaja Pietti
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