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Neural Stem and Progenitor Cells

- Cellular Responses to Known and Novel Factors

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2010

ISSN 1651-6206
ISBN 978-91-554-7671-7
urn:nbn:se:uu:diva-110722

Dissertation presented at Uppsala University to be publicly examined in B21, (BMC) Biomedical Center, Husargatan 3, Uppsala, Friday, January 15, 2010 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract
Larsson, J. 2010. Neural Stem and Progenitor Cells. - Cellular Responses to Known and Novel Factors. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 504. 63 pp. Uppsala. ISBN 978-91-554-7671-7.

Neural stem cell self-renewal and differentiation are tightly regulated events during CNS development, leading to cell division into new neural stem cells or the formation of neurons and glial cells. This thesis focuses on the cellular responses induced by known and novel factors in neural stem and progenitor cells (NSPCs).

Platelet-derived growth factor (PDGF) signaling has previously been implicated in NSPC regulation as well as in tumor formation. In order to evaluate the differentiation process and find new regulators of NSPCs a micro-array screen was performed, evaluating transcription during normal differentiation and the effect of PDGF-AA in this process. The transcriptional profile of PDGF-AA treated NSPCs was shown to be an intermediate between the profiles of neural stem cells and their progeny. The NSPC transcriptome was also found to have similarities with that of experimental glioma. A previously non-characterized transcript, the nuclear receptor binding protein 2 (NRBP2), was identified and found to be expressed in the developing and adult mouse brain and in medulloblastoma. NRBP2 down-regulation rendered neural progenitors sensitive to induced cell death.

Different PDGF ligands interact with different combinations of PDGF receptors. Therefore NSPCs were stimulated with either PDGF-AA or -BB to further evaluate cellular responses with regard to the two specific isoforms. A divergent effect between the two isoforms in long-term proliferation and cell survival was found, with PDGF-BB being the most efficient stimulator.

Stem cell factor (SCF) has previously been identified as a regulator in the hematopoietic system and we showed that SCF induces a migratory response in NSPCs. In addition, SCF positively affected cell survival but had no effect on NSPC differentiation. Insights into the regulatory mechanisms involved in neural stem cell signaling are needed to develop diagnostic tools and novel treatments.

Keywords: neural stem cell, differentiation, proliferation, migration, PDGF, SCF, NRBP2, medulloblastoma

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ISSN 1651-6206
ISBN 978-91-554-7671-7
urn:nbn:se:uu:diva-110722 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-110722)

To Kattis and Valle

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Demoulin, J-B., Enarsson, M., **Larsson**, **J**., Essaghir, A., Heldin, C-H., Forsberg-Nilsson, K. (2006) The gene expression profile of PDGF-treated neural stem cells corresponds to partially differentiated neurons and glia. *Growth Factors*, 24(3):184-196.

II **Larsson**, **J**., Forsberg, M., Brännvall, K., Zhang, X-Q., Enarsson, M., Hedborg, F., Forsberg-Nilsson, K. (2008) Nuclear receptor binding protein 2 is induced during neural progenitor differentiation and affects cell survival. *Molecular and Cellular Neuroscience*, 39(1):32-39.

III **Larsson**, **J**., Bergström, T., Forsberg-Nilsson, K. A divergent effect of PDGF-AA and -BB on differentiating neural stem and progenitor cells. *Manuscript.*

IV Erlandsson, A., **Larsson**, **J**., Forsberg-Nilsson, K. (2004) Stem cell factor is a chemoattractant and survival factor for CNS stem cells. *Experimental Cell Research*, 301:201-210.

Additional publications by the author

Gustavsson, M., Barmark, G., **Larsson**, **J**., Murén, E., Ronne, H. (2008) Functional genomics of monensin sensitivity in yeast: implications for post-Golgi traffic and vacuolar H^+-ATPase function. *Molecular genetics and genomics*. 280 (3):233-48.

Contents

Abbreviations

BMP	bone morphogenetic protein
caspase	cysteine-aspartic acid protease
CNTF	ciliary neurotrophic factor
DAG	diacylglycerol
DG	dentate gyrus
E	embryonic day
EGF	epidermal growth factor
Erk	extracellular regulated kinase
ES	embryonic stem
FGF-2	fibroblast growth factor 2
GFAP	glial fibrillary acidic protein
Gsk-3	glycogen synthase kinase 3
HGF	hepatocyte growth factor
ICM	inner cell mass
IP3	inositol 1,4,5-trisphosphate
IPS	induced pluripotent stem
LIF	leukemia inhibitory factor
Lrp	lipoprotein receptor-related protein
MAP-2	microtubule-associated protein 2
NICD	notch intracellular domain
NRBP2	nuclear receptor binding protein 2
NSPC	neural stem and progenitor cell
PCD	programmed cell death
PDGF	platelet-derived growth factor
PDK-1	phosphoinositide-dependent protein kinase
PI3K	phosphatidylinositol 3-kinase kinase
PINK1	PTEN-induced kinase 1
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PLCγ	phosphlipase Cγ
PTB	phosphotyrosine binding
PTCH	patched
RMS	rostral migratory stream
RTK	receptor tyrosine kinase
SCF	stem cell factor
SGZ	subgranular zone

SH2 and SH3	src-homology 2 and 3
Shh	sonic hedgehog
Sl	steel
SMO	smoothened
SVZ	sub ventricular zone
VEGF	vascular endothelial growth factor
W	white spotting

Introduction

The cells of an organism, independent of cellular function, initially have the same information encoded in their DNA. The genetic code determines the components of the cellular machinery needed to form the whole spectra of cells present in an organism. Although cells have the same genetic content, only some of the cells are capable of functioning as founding cells in the developing and adult organism. These cells are referred to as stem cells.

The term stem cell is collectively used for cells present in many niches and with different developmental capacities. However, the primary task for stem cells is being responsible for the formation and maintenance of a functional organism. If the regulatory control is lost, the proliferation capacity of these cells in combination with additional mutations can be turned against the organism in the form of a tumor. In order to learn more about neural stem cells, this thesis focuses on the regulation of neural stem cells during development.

Stem cell properties

Stem cells have the capability to self-renew and are thus able to divide symmetrically or asymmetrically. In symmetrical cell division, a cell divides into two identical daughter cells that are exact copies of the mother cell. During asymmetrical cell division, one cell is maintained in the same state as the mother cell whereas the other daughter cell becomes more mature (Gotz and Huttner, 2005). This makes it possible to provide the new cells needed for building the organism and to replace old or damaged cells, without depleting the stem cell pool (McKay, 1997; Doetsch et al., 1999).

Stem cells are often categorized into groups depending on the potency of a stem cell to contribute to cells of different lineages. The fertilized egg is the totipotent stem cell because it can form a new individual. After fertilization of the egg, the zygote divides and at day 3.5 a mouse embryo is at the developmental stage of a blastocyst. The blastocyst contains the inner cell mass (ICM), a pluripotent cell population which has the potential to form all cell types of a new organism (Smith, 2001). Through various types of signals different genetic programs are initiated in the ICM cells, resulting in intermediate maturation to stem cells with a more limited repertoire, as well as postmitotic cells of different lineages. In comparison to totipotent and pluri-

potent stem cells, multipotent stem cells are formed after the blastocyst stage and constitute a diverse class of cells with a more restricted differentiation potential. The multipotent stem cells are found in large numbers in the developing embryo, producing the cells needed for organogenesis and growth. These stem cells are characterized by their ability to form all or several cell lineages of a specific organ. Multipotent stem cells are also present in many organs in the adult, such as the liver, skin, intestines, and brain. However, in the adult organism there are significantly fewer multipotent stem cells than in the developing embryo (Sadiq and Gerber, 2004).

Embryonic stem cells

The blastocyst contains two major cell populations, the inner cell mass (ICM) and the trophectoderm. The ICM consists of pluripotent stem cells and gives rise to the embryo. The trophectoderm develops into the extra embryonic tissue. The possibility to isolate and culture cells from mouse and human ICM was the start of a new era in stem cell research (Figure 1.1) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). These ICM derived cells maintain their pluripotency in culture and are referred to as embryonic stem (ES) cells. The definition of a mouse ES cell is a cell that can be expanded in culture with sustained pluripotency, and if transplanted they should form teratomas, a tumor containing cells from the three germ layers as well as undifferentiated stem cells. In addition, if injected into a blastocyst, ES cells should contribute to all germ layers of the embryo and form functional gametes (Figure 1.2) (Smith, 2001).

Mouse blastocyst derived ES cells are usually cultured in the presence of leukemia inhibitory factor (LIF), a cytokine that keeps ES cells proliferating and inhibits differentiation (Smith et al., 1988). ES cells can be cultured in the presence or absence of feeder cells that provide factors and structural support. The human ES cell counterpart has different growth criteria, being dependent on factors such as FGF-2, Activin and Nodal for propagation (Vallier et al., 2005). Mouse ES cells represent a cell population isolated from an earlier developmental stage, which could explain this difference (Tesar et al., 2007).

ES cells are widely used in research for the production of knock-out mice (Doetschman et al., 1987). These genetically modified mice have had great impact on medical science, enabling detailed molecular studies during both normal development and different disease states. In addition, *in vitro* differentiated ES cells can be used as a source for experimental transplantation and replacement therapies (Odorico et al., 2001). In recent years several protocols have emerged that enable selective expansion and differentiation of ES cells into neural cells (Figure 1.3) (Okabe et al., 1996; Conti et al., 2005; Glaser et al., 2007). It is also possible to direct ES cells towards dif-

ferent lineages of neurons. For example Lmx1 was identified as an inducer of ES cell differentiation to dopaminergic neurons (Andersson et al., 2006).

Recently, important progress has been achieved by reprogramming somatic cells into ES cell like cells called induced pluripotent stem (IPS) cells (Figure 1.7). Reprogramming is induced in cell culture by over-expression of the four factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This new field of research is expanding rapidly, and protocols are constantly being published modifying the reprogramming parameters. This is necessary due to initial problems with factor misexpression leading to tumor formation in animals produced from IPS cells (Figure 1.8) (Yamanaka, 2008). The hope is that the reprogramming technique will make each individual a possible allogenic source of IPS cells. These cells could be genetically modified and transplanted back to the host as the desired cell type (Wernig et al., 2008; Soldner et al., 2009).

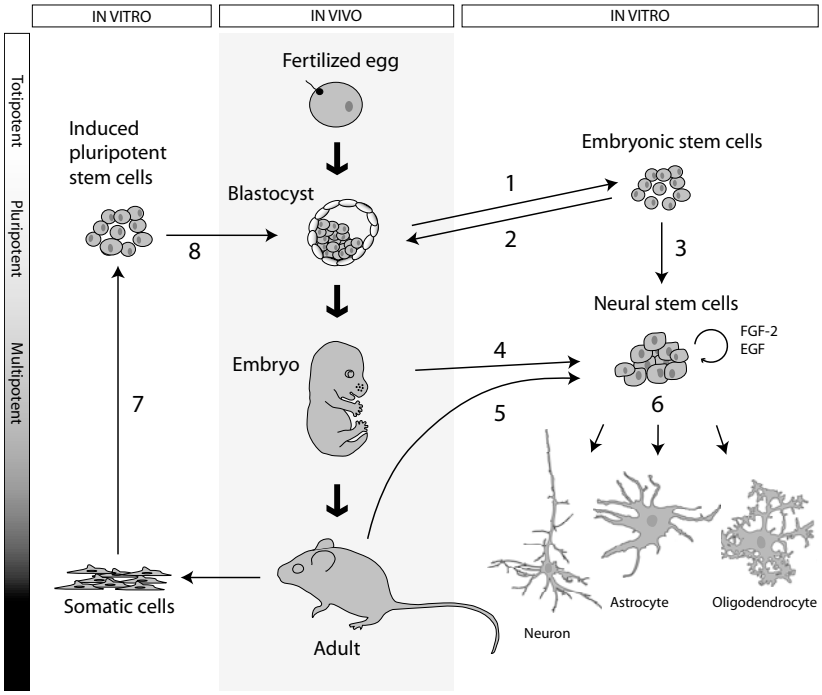


Figure 1. Schematic overview of how important events during development can be studied using experimental system *in vitro*. Thick arrows describe the normal developmental process. Thin arrows indicate different experimental procedures that can be undertaken *in vitro* and which are referred to in the text as Figure 1.1-8.

Neural stem cells

The development of an adult organism from the fertilized egg is an extremely complicated process. This process is guided by a pattern of signaling molecules released by nearby structures or cells, and is needed to determine positional and cellular identity. Neurulation is the initial step of CNS formation and occurs when the neural plate thickens and folds inwards to form the neural tube. This is the structure that contains neural stem cells (NSCs) and subsequently develops into the brain and spinal cord (Panchision and McKay, 2002; Greene and Copp, 2009).

A NSC has both the ability to self renew and the potential to differentiate into neurons, astrocytes and oligodendrocytes, which constitute the mature cell types of the CNS. When the NSC start to differentiate in response to, or in the absence of, various signals it is referred to as a progenitor cell. The progenitor cell has a more restricted differentiation potential than its stem cell origin (McKay, 1997).

In the mammalian brain, radial glial cells have been identified as NSCs during cortical development. Radial glia also give rise to the adult NSCs in the sub ventricular zone (SVZ). Besides being a stem cell in the developing CNS, radial glia direct migration of newborn neurons in cortical development (Noctor et al., 2002; Merkle et al., 2004). NSCs are most abundant during the embryonic development of the brain, where they form new neurons (neurogenesis) and glial cells (gliogenesis). In the cerebral cortex of rodents, neurogenesis mainly occurs during embryonic day (E) 11 to E16. Neurogenesis is followed by gliogenesis, which starts at E16 and continues until several weeks after birth (Sauvageot and Stiles, 2002). Isolation of NSCs at various embryonic time-points, starting at E10, shows that fewer neurons are produced in favor of glial cells over time. This also applies to NSCs in culture, where cells isolated at E10-E11 almost exclusively give rise to neurons if differentiated. However, during culturing these NSC gradually change their qualities, enabling the differentiation into both neurons and glial cells (Qian et al., 2000).

NSCs can be isolated and cultured from various embryonic brain regions either as free-floating neurospheres or as monolayer cultures (Figure 1.4) (Reynolds et al., 1992; Von Visger et al., 1994; Johe et al., 1996). NSCs are also present in the adult mammalian brain, located to the SVZ of the lateral ventricles and the dentate gyrus (DG) of the hippocampus (Figure 1.5 and Figure 2) (Reynolds et al., 1992; Lois and Alvarez-Buylla, 1993; Weiss et al., 1996; Palmer et al., 1997). The cells within the subependymal region lining the lateral ventricle in the adult mouse brain can form neurospheres. If the proliferating cells in this region are selectively depleted, the ability of neurosphere formation diminishes (Morshead et al., 1994). The NSC found within the SVZ is positive for the astrocytic marker GFAP and is defined as a B-cell. It gives rise to a transit amplifying progenitor cell (C-cell) that in

turn can develop into a neuroblast (A-cell) (Doetsch et al., 1999). The A-cell is able to form new functional neurons in the olfactory bulb (Carlen et al., 2002). Furthermore, the B-cells can also give rise to oligodendrocytes (Menn et al., 2006). In addition, after injury ependymal cells lining the SVZ can act as a reservoir of cells with NSC differentiation capacity (Carlen et al., 2009).

Through fate mapping the stem cell population has been visualized in the subgranular zone (SGZ) of hippocampus. Besides the normal NSC attributes, such as self-renewing capacity and the ability to form both neuron and glia precursors these cells express the markers Sox2, Nestin and GFAP (Fukuda et al., 2003; Suh et al., 2007). Neurogenesis in the SGZ leads to functional integrated neurons in the hippocampus (van Praag et al., 2002). Environmental factors as well as genetic alterations affect the NSCs and thereby influence neurogenesis (Kempermann and Gage, 2002). For example enriched environments as well as exercise has been shown to increase neurogenesis (van Praag et al., 1999; Suh et al., 2007). Though neurogenesis occurs in the adult rodent brain it decreases during aging (Kuhn et al., 1996). The decrease in neurogenesis in the SVZ has been linked to the expression of p16, a tumor suppressor affecting senescence in the SVZ stem cells (Molofsky et al., 2006).

NSCs are also found in the adult human SVZ and the SGZ of the hippocampal DG (Eriksson et al., 1998; Johansson et al., 1999). Under normal conditions neurogenesis is restricted to these regions in the adult human brain (Spalding et al., 2005; Bhardwaj et al., 2006).

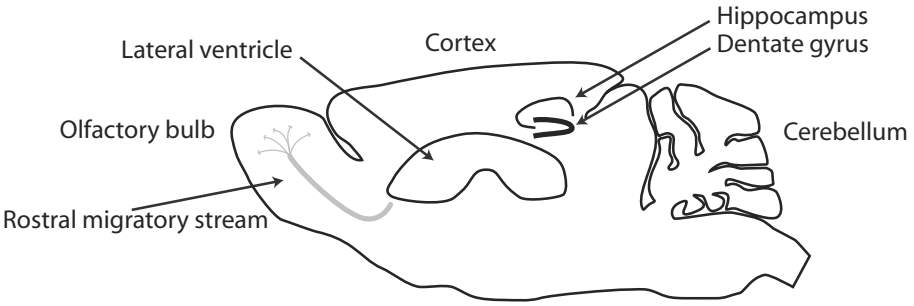


Figure 2. Brain regions in the adult mouse. Neural stem cells are found in the dentate gyrus of the hippocampus and in the walls of the lateral ventricles.

Neural stem cell proliferation and differentiation

NSCs are controlled by both extrinsic and intrinsic signals that are needed for spatial and temporal regulation. These signals enable the NSCs to stay in an undifferentiated proliferative state or direct them to their final differenti-

ated cell fate (Panchision and McKay, 2002). Attempts to elucidate the function of different signaling pathways have, to a large extent, been done using both genetically modified animals and primary NSC cultures propagated from various brain regions and time-points. Administration of factors or inhibitors of key players in signaling pathways has provided important information about regulators of growth and differentiation.

Several factors regulate NSC proliferation after binding to specific receptors. Fibroblast growth factor 2 (FGF-2) (Gritti et al., 1996; Johe et al., 1996), epidermal growth factor (EGF) (Reynolds et al., 1992) and Notch (Androutsellis-Theotokis et al., 2006) all promote proliferation and prevent differentiation of NSCs. FGF-2 treatment of cells is thought to increase the responsiveness to EGF by up-regulating the amount of EGF receptors (Lillien and Raphael, 2000). In addition, platelet-derived growth factor (PDGF) promotes progenitor cell proliferation and delays differentiation. However, PDGF cannot replace FGF-2 or EGF as mitogen in NSC cultures (Johe et al., 1996; Erlandsson et al., 2001).

The NSCs are important during development and they differentiate to a wide range of specialized cells needed during the formation of the CNS. The differentiated progeny of NSCs are neurons, astrocytes and oligodendrocytes (Figure 1.6). Astrocytes and oligodendrocytes are collectively referred to as glial cells. Neurons are the signaling cells and are equipped with dendrites and axons for receiving and transmitting signals. Signaling is mediated between cells by the release of neurotransmitter substances. Astrocytes have many diverse functions in the brain, including formation of the blood brain barrier, supplying nutrients to neurons and providing structural support. They also regulate synaptic activities by for example modulation of the potassium ion concentration in the vicinity of the neurons. Oligodendrocytes are the producers of myelin in the CNS. Myelin bundles provide the neurons with insulation needed for efficient transmission of electrical action potentials (Fields and Stevens-Graham, 2002).

The proportion of the different mature cell types formed during NSC differentiation can be influenced by soluble factors provided by co-cultured cells (Morrow et al., 2001; Song et al., 2002), or by the addition of exogenous factors. Examples of such extracellular factors are ciliary neurotrophic factor (CNTF) and bone morphogenetic protein (BMP), which both promote the astrocytic lineage, and triiodothyronine hormone, which promotes the formation of oligodendrocytes (Gross et al., 1996; Johe et al., 1996). The Wnt pathway is one of few extracellular signaling pathways whose activation leads to an increase in neurogenesis (Hirabayashi et al., 2004; Kleber and Sommer, 2004; Lie et al., 2005). PDGF signaling has been suggested to increase the amount of neurons, however this induction is more likely an effect of proliferation on all the neural cell lineages (Johe et al., 1996; Williams et al., 1997; Erlandsson et al., 2001; Erlandsson et al., 2006). Several reports show that PDGF-AA and -BB stimulation of neural stem and pro-

genitor cells *in vitro* leads to an increased amount of oligodendrocytes in the culture (Johe et al., 1996; Frost et al., 2003; Woodruff et al., 2004; Hu et al., 2008; Appolloni et al., 2009; Rao et al., 2009). Cell culturing densities are important for the NSC differentiation process. For example in low-density cultures containing serum, cortical NSCs can differentiate to smooth muscle cells. These smooth muscle cells were derived from the same clones as GFAP positive astrocytes (Tsai and McKay, 2000). In addition, inhibiting chromatin-remodeling proteins such as histone deacetylases will interfere with the differentiation process, leading to neuronal in favor over glial differentiation (Hsieh et al., 2004).

Migration

Migration is the phenomenon of cellular movement. This is a common event during development, where cells respond to different cues in order to reach their final destination. Migration is guided by a complex pattern of extracellular factors, cell-to-cell adhesion and interactions with extracellular matrix molecules. The effects of extracellular factors are often pleiotropic and a factor promoting migration in a cell at one time can later on stimulate a different response (Sobeih and Corfas, 2002). Migration has been extensively studied using different types of labeling methods to track cells. This has historically been done using traceable components, and more recently by genetic approaches enabling selective labeling of different cell populations (Nagy, 2000). In addition, several methods for analyzing migration *in vitro* have been used. One example is the Boyden chamber where cells can actively move through a membrane with defined pore size (Behar et al., 1994).

During the formation of the forebrain, extensive migration occurs. These migration events can be divided into radial and tangential migration. Migration from the progenitor zone towards the surface of the brain is an example of radial migration, whereas in tangential migration cells migrate orthogonal compared to radial migration (Marin and Rubenstein, 2003). Radial glial cells facilitate radial migration by acting as migrational guides for newborn neurons migrating between the ventricular zone and the pial surface (Rakic, 1972; Misson et al., 1988). Besides being important for guiding neurons, radial glial cells are also considered to be a source of neurons by asymmetrical cell division (Noctor et al., 2002; Anthony et al., 2004) and a source of NSCs in the adult brain (Merkle et al., 2004).

Tangential migration occurs in parallel to the lateral ventricle (Fishell et al., 1993). An example of this is the rostral migration of neuroblasts derived from the NSCs of the SVZ to the olfactory bulb in rodents (Lois and Alvarez-Buylla, 1993). There is also evidence that this rostral migratory stream (RMS) is present in the adult human brain (Curtis et al., 2007).

As described above, neuronal progenitors migrate extensively during the development of the brain. Data also show that the NSCs respond to migra-

tion stimuli (Forsberg-Nilsson et al., 1998). One interesting property of neural stem and progenitor cells is their ability to migrate towards an injury or different types of tumors. The reason for this migration toward tumors is based, at least in part, on release of exogenous factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), EGF and stem cell factor (SCF) from the tumors. This induced migration of NSCs has therefore been suggested as a possible way to deliver different types of inhibitory compounds to tumors (Aboody et al., 2000; Serfozo et al., 2006; Kendall et al., 2008).

Survival

Programmed cell death (PCD) helps the organism to maintain cellular homeostasis by removing unhealthy or excess number of cells during development. Although the terms PCD and apoptosis cannot be used as synonyms, PCD often manifests several of the morphological features of apoptosis (Kroemer et al., 2009). If the regulation of apoptosis is disturbed a number of pathological conditions may occur, such as cancer or neurodegenerative disease (Fadeel et al., 1999). The apoptotic machinery can be induced either extrinsically by death receptors or intrinsically by the mitochondrial pathway. In both pathways key proteins are the cysteine-aspartic acid proteases (caspases). The pathways lead to the cleavage and activation of various caspases in a cascade-like manner, resulting in the activation of the effector caspases 3, 6 and 7. Activation of these caspases leads to further recruitment and activation of enzymes that in a series of events disassemble the cells (Khosravi-Far and Esposti, 2004).

During development of the nervous system, apoptosis is one of the key pathways that regulate NSC numbers. Neurons and glia are produced in excess and cells that do not make the correct interactions will be removed (Jacobson et al., 1997). For example, in the developing mouse cortex a high percentage of dying cells can be detected during neurogenesis. This widespread cell death is initially located to proliferative zones and later also detected in the postmitotic regions (Blaschke et al., 1996). The importance of PCD is demonstrated in mice lacking caspase 3 or 9 as this results in hyperplasia in the CNS due to a decreased apoptosis (Kuida et al., 1996; Pompeiano et al., 2000). However, although the intrinsic apoptotic pathway is fully functional, the extrinsic pathway involving death receptors does not seem to be operating in NSCs (Tamm et al., 2004). Interestingly, in NSCs, the activation of the death receptor Fas during growth factor starvation instead promotes NSC survival (Knight et al., 2009).

Cell signaling via receptor tyrosine kinases

Cell signaling is mediated through a variety of different types of receptors located in different cellular compartments *e.g.* the cell surface. Some of the most well studied cell surface receptors are receptor tyrosine kinases (RTKs). Examples of such are the FGF, EGF, SCF and PDGF receptors. These receptors bind their corresponding soluble ligand or ligands in different combination. The affinity of a ligand to its receptor is sometimes affected by co-factors and matrix components that act as key regulators for the interaction. Binding of a ligand to a cell surface receptor can mediate signaling in different ways.

Cell signaling via tyrosine kinase receptors starts by receptor-ligand interactions, leading to receptor dimerization or clustering. Receptor dimerization is often followed by phosphorylation of the receptor itself, so called autophosphorylation, enabling the binding and phosphorylation of other signaling molecules (Heldin et al., 1995). Examples of epitopes mediating binding to phosphorylated RTKs are the Src-homology 2 and 3 (SH2 and SH3) domains and phosphotyrosine binding (PTB) domains. Proteins containing SH2 can be divided into proteins with enzymatic activity and proteins acting as adaptors of signal transduction containing only binding domains. Examples of proteins with SH2 binding sites and enzymatic activities are Src kinases with protein tyrosine kinase activity, Shp2 with phosphatase activity and PLCγ with phospholipase 3 activities. Adaptor proteins containing SH2 and SH3 domains that interact with RTK are Grb, Nck, Crk and Shc (Schlessinger, 2000). Independently of how the signaling cascade starts, the endpoint is often a down-stream effector that activates different transcriptional programs. Many transcription factors can be regulated by several different signaling pathways, and many pathways also converge *e.g.* in the cytoplasm and affect each other.

Two RTK-mediated signaling pathways of great importance for NSCs are the Ras/Map kinase pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (Figure 3).

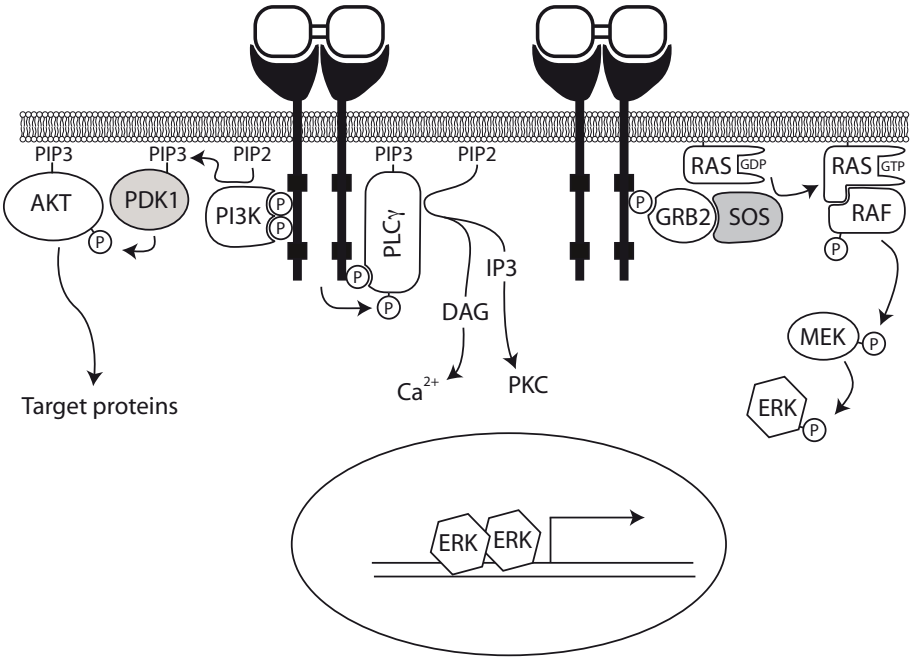


Figure 3. Signaling pathways induced by receptor tyrosine kinases. The ligand bound receptor dimer gets activated by autophosphorylations, which subsequently leads to the formation of binding sites and phosphorylations of target proteins. The left receptor shows signaling by the PI3K and PLCγ pathways. The right receptor shows signaling mediated by the Ras/Map kinase pathway.

The Ras/Map kinase pathway

One of many ways to activate the Ras/Map kinase pathway is through receptor tyrosin kinases such as FGF receptors, PDGF receptors and the SCF receptor c-Kit. Activation of this signaling pathway in NSCs can influence a variety of cellular events, such as proliferation, differentiation, migration and survival.

In the case of PDGF signaling the Ras/Map kinase pathway is activated by ligand binding, followed by auto-phosphorylation of the dimerized receptor. This leads to the activation of Ras via the complex of the adaptor protein Grb2 and the guanine nucleotide exchange factor Sos. The binding of the Grb2-Sos complex directly or in combination with other adaptor proteins to the RTK mediates the activation of membrane bound Ras (Chardin et al., 1993). This starts a phosphorylation cascade where activated Ras binds Raf and this binding mediates the phosphorylation of Raf. Activated Raf in turn activates Mek by phosphorylation. All these previously mentioned signaling events occur in the cytoplasm, however the phosphorylation of extracellular regulated kinase (Erk) by Mek leads to dimerization and nuclear translocation of Erk. In the nucleus Erk activates multiple targets, including transcrip-

tion factors by phosphorylation (Karin and Hunter, 1995; Murphy and Blenis, 2006).

The PI3 kinase and PLCγ pathways

The phosphatidylinositol 3-kinase kinase (PI3K) pathway utilizes membrane lipids as signaling molecules and is one of the major pathways in cell signaling. As for the Ras/Map kinase pathway, many different types of receptors are responsible for mediating signals through the PI3K pathway. In addition there is extensive crosstalk between signaling pathways. For example the PI3K pathway is linked to Raf, the main mediator for Map kinase signaling (Wittinghofer and Nassar, 1996).

RTK can activate the PI3K signaling pathway by recruitment of PI3K by its SH2 domains. This will lead to the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 enables binding of phosphoinositide-dependent protein kinase (PDK-1) and Akt by their PH domains. PDK-1 phosphorylation activates Akt, enabling further activation of a variety of downstream signaling molecules dependent on Akt kinase activity.

Phospholipase Cγ (PLCγ) can also be recruited to the phosphorylated PDGF receptor where it converts PIP2 to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). For this to occur the PI3K product PIP3 needs to interact with the PH domain of PLCγ (Falasca et al., 1998). The secondary messengers DAG and IP3 influence Ca^{2+} levels and PKC activity (Schlessinger, 2000).

Fibroblast growth factor 2

FGF-2 is one of 22 members of the FGF family (Yamashita et al., 2000; Dono, 2003). FGFs are structurally similar proteins with a variety of functions during normal development, wound healing and tumor development. FGF-2 signals through FGF receptors 1-4, which are tyrosine kinase receptors. These receptors can be further modulated through alternative splicing (Powers et al., 2000). The binding of FGF-2 to the receptor as a monomer is mediated through an interaction with heparan sulphate acting as a co-factor (Nurcombe et al., 1993; Brickman et al., 1995).

FGF-2 is present early during brain development and is important for regulation of cell survival and NSC proliferation and differentiation both *in vitro* and *in vivo* (Cavanagh et al., 1997; Raballo et al., 2000). Intraventricular administration of FGF-2 during late neurogenesis increases the cortical volume. In addition, removal of FGF-2 during mouse development leads to a decrease in cell density in the motor cortex (Ortega et al., 1998; Vacarino et al., 1999). This is a somewhat milder phenotype than what was expected and can partly be explained by redundant function between FGF family members due to structural similarities. Mouse knock-out studies show that FGF recep-

tor 1 and 2 are both essential for survival, whereas FGF receptor 3 and 4 are not (Coumoul and Deng, 2003). This has been further evaluated in developing mouse cortex, where the loss of the FGF receptors one by one increases the truncation of telencephalon (Paek et al., 2009).

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) exists in four monomeric variants that dimerize to form five different isoforms: AA, BB, AB, CC and DD. The PDGF receptor tyrosine kinase receptors exist in three isoforms composed of the homodimers αα and ββ, or the heterodimer αβ. PDGF-AA binds only to the αα receptor, PDGF-AB and PDGF-CC bind both to the αα and the αβ receptor, PDGF-BB binds to all three receptors, and PDGF-DD binds to the ββ and αβ-receptor (Figure 4) (LaRochelle et al., 2001; Li and Eriksson, 2003). Receptor dimerization causes receptor auto-phosphorylation, leading to an increase in the receptor kinase activity and binding affinity for a variety of signaling molecules such as proteins containing Src-homology 2 and phosphotyrosin binding domains, which are common mediators of signaling (Heldin et al., 1998).

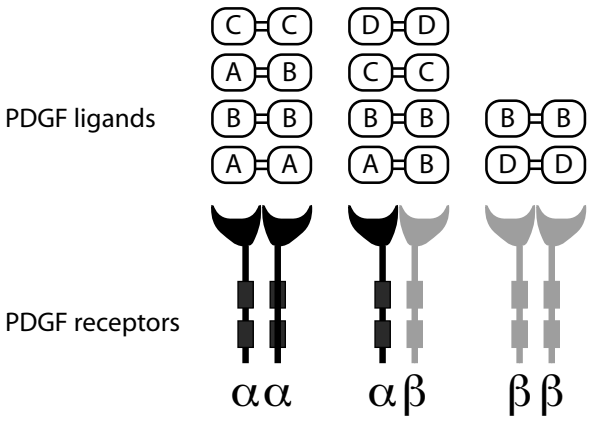


Figure 4. PDGF ligands and their receptors. Different combinations of ligands (top) bind to and activate different PDGF receptors (bottom).

The PDGF ligands and receptors are widely expressed during the formation and organization of the embryo. PDGF signaling provides both migratory and proliferative signals (Betsholtz, 1995), and as a result, PDGF signaling is important for proper development of several organs, such as kidneys, testis, placenta, lungs and brain. In the developing brain, the mRNA for PDGF-A and -B, as well as the α- and β-receptors are present (Reddy and Pleasure, 1992). Expression of PDGF-A has more specifically been localized to neu-

rons of the embryonic and adult mouse brain (Yeh et al., 1991). Immunohistochemical studies show that the PDGF β-receptor is expressed in a wide range of neurons throughout the CNS (Smits et al., 1991), and that the PDGF α-receptor is detected mainly on the surface of oligodendrocyte precursors (Pringle et al., 1992). In addition, a population of NSCs in the adult mouse brain is positive for the α-receptor and responds to PDGF signals by an increase in cell numbers (Jackson et al., 2006).

The importance of PDGF signaling in embryonic development can be seen in PDGF ligand or receptor knock-out mice, where deletion of either receptor is embryonic lethal (Soriano, 1997; Kaminski et al., 2001). However, conditional β-receptor deletions in neurons have no effect on mouse viability. Nevertheless, these mice are more sensitive to brain trauma, suggesting that PDGF signaling is important in protecting neurons against stress induced apoptosis (Ishii et al., 2006). PDGF-B null mice die during early development (Levéen et al., 1994; Kaminski et al., 2001), which is also true for some of the PDGF-A null mice. The PDGF-A knock-out mice that survive show a decreased number of oligodendrocytes and dysmyelination (Boström et al., 1996; Fruttiger et al., 1999). Adult mice injected with PDGF as well as transgenic mice over-expressing the PDGF ligand in the brain display hyperplasia (Forsberg-Nilsson et al., 2003; Jackson et al., 2006).

Rodent NSCs cultured *in vitro* express the PDGF α-receptor. If differentiation is induced in the NSC culture, the α-receptor expression remains constant while the β-receptor expression level increases (Erlandsson et al., 2001). Furthermore PDGF stimulates progenitor cell proliferation and cell survival, leading to an increase in immature neurons (Johe et al., 1996; Erlandsson et al., 2001) and oligodendrocytes (Noble et al., 1988; Frost et al., 2003; Woodruff et al., 2004). Neural stem and progenitor cells in culture have been shown to synthesize their own PDGF-B. This production of PDGF-B stimulates neural progenitor proliferation, and inhibition of the endogenous PDGF leads to a faster maturation of progenitors to neurons and oligodendrocytes in particular (Erlandsson et al., 2006).

Stem cell factor

Stem cell factor (SCF) can be either membrane bound or proteolytically cleaved to a soluble form (Anderson et al., 1990). Signaling is initiated when the SCF in its dimeric form binds to the receptor c-kit. Receptor signaling via the RTK c-kit is conducted in a fashion similar to that of other receptors in the RTK family (Yarden et al., 1986; Sattler and Salgia, 2004).

Developmental studies of SCF signaling have been performed on mouse mutants lacking either c-kit or SCF. These mutations are located in the white spotting (W) locus coding for c-kit, and in the steel (Sl) locus encoding the SCF (Russell, 1949; Chui et al., 1976; Chabot et al., 1988; Geissler et al., 1988; Zsebo et al., 1990). Both the W and the Sl mutants show similar ab-

normalities in melanocyte, germ cell and mast cell development. The lack of c-kit completely removes the mast cell population, whereas SCF removal leads to mast cells unable to expand. In addition SCF signaling induces migration in mast cells (Meininger et al., 1992; Nilsson et al., 1994).

The c-kit receptor and the SCF ligand are widely expressed in various regions during development and also in adult organisms. For example, both the receptor and the ligand are expressed during neural tube formation and in the developing cerebral cortex (Keshet et al., 1991; Soumiya et al., 2009). SCF ligand and receptor expression by neurons and glial cells affect neuroprotection, angiogenesis and migration (Dhandapani et al., 2005; Sun et al., 2006). In addition neural derived cultures of astrocytes and neurons as well as glioma cell lines express the SCF ligand and receptor (Stanulla et al., 1995; Zhang and Fedoroff, 1997). Endogenous expression of SCF by neurons after brain injury or administration of SCF to the mouse brain induces migration of neural progenitor cells toward the SCF (Zhang and Fedoroff, 1999; Sun et al., 2004). There is also evidence for an endogenous production of SCF from gliomas. This secretion of SCF is higher in aggressive tumors where it is involved in inducing angiogenesis (Sun et al., 2006).

Additional signaling pathways important for neural stem cells and CNS development

Besides signaling mediated by receptor tyrosine kinases, several other pathways are crucial for NSC regulation. In this section a brief description of additional important ways to mediate signaling and cellular responses in NSCs will be discussed. The crosstalk between these pathways is extensive and necessary for a proper cellular response.

Notch signaling

Notch signaling is crucial for many processes during adult and embryonic development. Signaling is mediated by a cell-cell interaction, where four membrane bound Notch receptors interact with the membrane bound Jagged or Delta ligands presented on a neighboring cell. This interaction subsequently leads to cleavage of the receptor by γ-secretase, releasing the Notch intracellular domain (NICD) that acts directly in the nucleus as a transcription factor. The transcriptional program induced by NICD is mediated by interaction with transcriptional co-activators and through inhibition of transcriptional repression (Borggrefe and Oswald, 2009). One of the transcriptional repressors inhibited by NICD is RBP-J (Oswald et al., 2001). The interaction between NICD and RBP-J stops repression and promotes transcription of the Hes gene family (Jarriault et al., 1995). Hes1 and 5 promote NSC maintenance by repressing neuronal specific genes, such as neurogenin 1 and 2, Mash1 and Math (Kageyama et al., 2005). This represents a simpli-

fied description of the Notch pathway. The complexity of Notch signaling is, as for many signaling pathways, increased by a variety of ligands and receptors, co-activators and repressors. Many of these Notch pathway interactions have been evaluated in the CNS using mouse mutants (Yoon and Gaiano, 2005). For example, deletion of Notch1 or 2, which have a similar embryonic expression pattern are in contrast to Notch3 or 4 deletions embryonic lethal (Swiatek et al., 1994; Hamada et al., 1999). Furthermore, conditional deletion studies of the activators in Notch signaling such as Notch1, RBP-J, HES1 and/or HES5 show that lack of Notch signal leads to precocious neuronal differentiation, depleting the stem cell pool (de la Pompa et al., 1997; Hitoshi et al., 2002; Hatakeyama et al., 2004; Carlen et al., 2009). In addition, expression of the intracellular domain of Notch1 or 3 in E9.5 telencephalic progenitors induces a radial glial like phenotype (Gaiano et al., 2000; Dang et al., 2006). This further strengthens the hypothesis that radial glial cells are the source of adult NSCs in the SVZ.

Wnt signaling

As most of the signaling pathways discussed in this thesis, the Wnt pathway also induces a variety of cell responses, including an effect on embryonic patterning. Furthermore, Wnt signaling is divided into canonical and non-canonical pathways. Canonical Wnt signaling includes cellular responses leading to fate determination, whereas noncanonical signaling leads to cell movement and polarization of tissues (Mikels and Nusse, 2006; Katoh and Katoh, 2007). In this thesis only the canonical pathway will be mentioned further.

In mammals the Wnt family consists of 19 members that bind to the Frizzled family of receptors. Simplified, the canonical Wnt pathway starts by binding of the Wnt ligand to the Frizzled receptor. This binding is further enhanced by low-density lipoprotein receptor-related protein (Lrp) co-receptors. The Frizzled activation subsequently leads to the stabilization and translocation of the transcripton factor β-Catenin into the nucleus. The stabilization of β-Catenin depends on the inhibition of glycogen synthase kinase 3 (Gsk-3). The Dishevelled protein that is activated by the Wnt receptor Frizzled mediates this inhibition of Gsk-3. Translocation of β-Catenin to the nucleus induces transciption in combination with the DNA binding Tcf/Lef family of proteins (Peifer and Polakis, 2000). A non-stimulated Wnt pathway allows binding of β-Catenin to the Axin/Apc complex in the cytoplasm, leading to β-Catenin phosphorylation and ubiquitination followed by proteasome degradation.

In NSCs Wnt signaling has been suggested to promote NSCs towards the neuronal lineages as well as to support progenitor proliferation (Hirsch et al., 2007). In line with this, enlarged brains and increased neurogenesis has been detected in mice over-expressing a stabilized form of β-Catenin in neural progenitors (Chenn and Walsh, 2002, 2003). Furthermore, Wnt7a or β-

Catenin over-expression induces neuronal differentiation of cultured NSCs derived from embryos at day E11.5. The transcription factor Neurogenin1 has been suggested to promote this effect (Hirabayashi et al., 2004). In contrast, another study has presented data that Wnt signaling mediates self-renewal of neural stem and progenitor cells (Kalani et al., 2008). This dual role of Wnt signaling is further shown in adult hippocampal stem and progenitor cells. The Wnt3 over-expression has been shown to induce neurogenesis (Lie et al., 2005), whereas another study showed that an endogenous autocrine loop in these cells maintain cells undifferentiated and that over-expression of Wnt1 leads to a decrease in neurons (Wexler et al., 2009).

Sonic hedgehog signaling

The high degree of conservation in the Sonic hedgehog (Shh) gene between species indicates its importance during development. Shh is expressed by the notochord and the floorplate and acts as a morphogen during development, where it induces different cell fates in a dose dependent manner (Ericson et al., 1996). Deletion of Shh in mouse embryos is lethal and due to the loss of regional specific Shh signaling a variety of phenotypes are induced (Chiang et al., 1996).

The Shh pathway signals via the Patched (PTCH) receptor, where binding of the Shh ligand to PTCH disturbs the inhibition of Smoothened (SMO). A non-inhibited SMO enables signaling via the transcription factors Gli1-3. Multiple targets have been identified for Gli mediated transcription (Vokes et al., 2007). Although Gli1-3 can act as transcriptional activators, Gli-3 in a cleaved form also acts as a transcriptional repressor.

Shh signaling has been linked to the expression of the transcription factors Olig1 and Olig2, important for the development of the oligodendrocytic lineage (Lu et al., 2001; Zhou et al., 2001). Impaired Shh signaling in nestin positive progenitor cells leads to a reduction in brain size, increased cell death in the SVZ and a reduced number of NSCs in the adult stem cell regions. Furthermore, both the B- and the C-cells of the SVZ have an increase in differentiation during the initial period after Shh signaling removal. This differentiation does not as expected lead to an initial increase in neurogenesis, but rather to a decrease in neurogenesis (Machold et al., 2003; Balordi and Fishell, 2007).

Shh signaling is crucial for the cerebellar development as it stimulates proliferation in granule cell precursors (Wechsler-Reya and Scott, 1999). Activating mutations in the Shh signaling pathway is one of the main causes of medulloblastoma formation (Goodrich et al., 1997). Shh signaling in adult NSCs in the DG is dependent on primary cilia, a distinct structure in the cell membrane (Han et al., 2008). Disturbance of the cilia structure is important also for signaling in medulloblastoma (Han et al., 2009).

Bone morphogenetic protein signaling

Bone morphogenetic proteins (BMPs) are a group of signaling proteins belonging to the TGFβ-superfamily. BMPs are crucial for embryonic patterning and are expressed in non-neural ectoderm and the roof plate of the neural tube during early development.

Activation of this pathway is mediated through the BMP receptor types I and II. The type II receptors mediate ligand binding, and the signaling is facilitated mainly through the type I receptors. Activation of the pathway leads to phosphorylation of Smad1, 5 and 8. Each of these phosphorylated Smads can form heterodimers with Smad4. Such complexes translocate to the nucleus where they promote transcription. Besides this canonical Smad pathway the MAP kinase pathway can be activated by the ligand bound receptors (Chen and Panchision, 2007). In the BMP pathway several exogenous inhibitors of signaling have been identified, such as Chordin and Noggin which both inhibit the effect of BMP2 and 4 (Jones and Smith, 1998).

The BMP pathway induces a variety of cellular responses that in many cases have opposing effects. This difference in response often correlates with the developmental state of the cells when they are exposed to BMPs. For instance *in vitro* data show that BMP2, 4, 5, 6 and 7 induce astrocytic differentiation in NSCs cultured from the SVZ at E17 (Gross et al., 1996). The same is also true for NSCs dissected and cultured from the ganglionic eminences at E18.5 (Bonaguidi et al., 2005). In contrast cells retrieved from earlier embryonic time-points differentiate to neurons in the presence of the same BMPs (Li et al., 1998; Li and Grumet, 2007). In line with this, the BMP inhibitor Noggin partly inhibited neuronal differentiation in E12-13 cultures (Li and LoTurco, 2000).

In the adult there are contradictory reports regarding the role for BMP signaling in the neurogenic regions. BMP signaling in a neurosphere assay decreases the amount of neurons after differentiation. The decrease in neurogenesis was also shown *in vivo* using retroviral expression of BMPs in ependymal cells (Lim et al., 2000). Furthermore, the BMP inhibitor Noggin is critical for the expansion of NSC from the SGZ and differentiation of these cells in the presence of BMP leads to a decrease in the amount of neurons. Inhibition of BMP signaling positively affected the formation of secondary spheres isolated from SGZ but had no effect on primary sphere formation. In addition, transgenic Noggin expression *in vivo* increased the amount of stem cells in the SGZ (Bonaguidi et al., 2008). The TGFβ signaling molecule Smad4 is important for neurogenesis. Smad4 is activated by BMPs and conditional deletions of Smad4 as well as Noggin infusion leads to a reduction in neurogenesis and an increase in oligodendrogliogenesis in the SVZ (Colak et al., 2008).

CNS tumors and neural stem cells

Brain tumors constitute a heterogeneous group of cancers where the gliomas are the most common type of tumor. There are different subgroups of gliomas, *e.g.* astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. This classification is based on the morphologic appearance of the tumor. However, the cell of origin of these tumor types has not been identified (Louis, 2006).

CNS tumors are further histologically separated into grade I-IV according to the World Health Organization guideline. In general, patients with glioma tumors of lower grade have a better chance of survival compared to patients with higher-grade gliomas. Multiple genetic alterations are involved in glioma formation. Among these are loss of heterozygosity in chromosome 1p and 19q. In addition, misexpression of several growth factors and receptors of the PDGF and EGF families are frequently detected in gliomas. Tumor suppressors such as PTEN, RB, P53, P16 and P14 are also commonly down-regulated either by deletion, loss of heterozygosity or promoter methylation (Gladson et al., 2009).

Another type of tumor in the CNS is medulloblastoma, which is the most common childhood brain tumor. Medulloblastomas have a cerebellar origin and are further characterized into subgroups depending on histological appearance. Several genetic alterations in signaling pathways, such as the Wnt and Shh, have been shown to cause medulloblastomas (Gilbertson and Ellison, 2008). Both gliomas and medulloblastomas have been suggested to contain cancer stem cells. This is a much-debated phenomenon, which will be further discussed below.

Linking PDGF to tumor formation

In many of the gliomas, aberrant expression of the PDGF ligands and receptors has been detected. This is of particular interest since tumor PDGF signaling is thought to act in an autocrine fashion (Hermanson et al., 1992). In addition, misexpression or amplification of the EGF and PDGF receptors has frequently been found in gliomas (Fleming et al., 1992), primitive neuroectodermal tumors and medulloblastomas (Smits et al., 1996). In transgenic mice that over-express PDGF at various stages of CNS development and adulthood, no tumors have been found (Calver et al., 1998; Forsberg-Nilsson et al., 2003). However, if the same ligand is expressed in neural progenitors in combination with an additional genetic modification, gliomas are formed (Uhrbom et al., 1998; Dai et al., 2001; Appolloni et al., 2009; Hede et al., 2009; Lindberg et al., 2009). These genetic disturbances can be mediated either by transgenic techniques or retroviral integration. Furthermore, a study investigating the genomic localization of the inserted PDGF expressing retrovirus, has provided valuable information of genes involved in glioma for-

mation (Johansson et al., 2004). This suggests that PDGF by itself may not be sufficient to induce tumors, but the combinatorial effect of insertional mutagenesis and PDGF over-expression may induce the formation of glioma. There are reports, however, suggesting that solely over-expression of PDGF can induce tumors *in vivo* (Assanah et al., 2009). Furthermore, glioma cell lines treated with PDGF receptor inhibitors exhibit an increase in cell death, decrease in proliferation, and reduced tumor formation after transplantation into nude mice (Kelly et al., 2002; Lokker et al., 2002). This altogether implicates the importance of PDGF signaling in tumor formation.

Cancer stem cells

It is of great importance for the understanding and efficient treatment of tumors to identify the cell type in the brain from which the malignant cells arose. It has been suggested that a subpopulation of cells within the tumors possess NSC characteristics and that these cells may be the founders of the tumor. Gliomas often express markers such as nestin and GFAP that are associated with NSCs and their progeny (Dahlstrand et al., 1992; Tohyama et al., 1992). These "cancer stem cell-like cells" have characteristics different from the bulk tumor cells and may therefore require other approaches for tumor ablation.

Further studies have shown that there are cells within gliomas that in some cases form neurospheres in culture, which is a common characteristic of NSCs. These neurospheres can be dissociated mechanically a number of times, upon which new spheres form that have maintained expression of nestin and a staining pattern similar to that of the original tumor. If differentiation of these cells is induced by removal of mitogens, the proliferation of the tumor cells, just like for normal NSCs, decreases and the cells start to express neuronal and glial markers. However, some tumor cells, unlike normal NSCs, co-express markers typical for other neural cell types. Transplantation of tumor-derived spheres into rat brains leads to the formation of neuron and glial-like cells that also continue to proliferate (Hemmati et al., 2003; Galli et al., 2004).

The hematopoietic and NSC marker CD133 (Uchida et al., 2000) has been used to identify brain tumor initiating cells in various CNS tumor types. These CD133 positive cells found in brain tumors have been isolated and transplanted in small numbers into immune deficient mice, where they form new tumors, wheras tumor derived CD133 negative control cells did not (Singh et al., 2003). However, in contrast to this study CD133 positive as well as negative cells derived from gliomas, form tumors in nude rats. In addition, the CD133 negative cells become CD133 positive after transplantation (Wang et al., 2008). A subset of the glioma-derived neurospheres expresses CD133, and this population of cells shows a higher proliferation index compared CD133 negative spheres (Beier et al., 2007). Tumors that

contain cells from which spheres can be retrieved are more tumerogenic compared to tumors that contain cells that grow adherently (Gunther et al., 2008). The cancer stem cell-like cell population has been found in the vicinity of the endothelial cells of the vasculature. The interaction between cancer stem cell-like cells and endothelial cells promotes proliferation of undifferentiated cancer cells (Calabrese et al., 2007). Interestingly, genetic and viral approaches selectively targeting neural progenitors at different stages also implicate progenitors as the founder cells in experimental gliomas (Rao et al., 2004; Uhrbom et al., 2005).

Isolation and propagation of primary neural stem and progenitor cells

The key discoveries enabling *in vitro* culturing of neural stem and progenitor cells were that the factors FGF-2 and/or EGF stimulated proliferation and self-renewal of multipotent CNS precursors from embryonic striatum, hippocampus and cortex (Reynolds et al., 1992; Reynolds and Weiss, 1992; Vescovi et al., 1993; Davis and Temple, 1994; Johe et al., 1996; Tropepe et al., 1999). The isolated NSCs can be grown under serum free conditions with the addition of the mitogens described above either as monolayer cultures (Johe et al., 1996), or as free-floating aggregates referred to as neurospheres (Reynolds et al., 1992; Reynolds and Weiss, 1992). Differentiation of NSC cultures is induced by the removal of mitogens or addition of serum, and leads to the generation of mixed cultures composed of neurons, astrocytes and oligodendrocytes.

One of the attributes of a NSC is its immaturity, and often NSC identity is confirmed by the cell fate of its progeny. Various markers are used for identifying NSCs, however as true specific stem cell markers are still lacking, NSC pools isolated in different studies are probably both heterogeneous and variable. Therefore the term NSC has gradually changed to neural stem and progenitor cells (NSPCs). The actual proportion of NSCs present in an *in vitro* expanded neurosphere is suggested to be as low as 2-3% (Reynolds and Rietze, 2005). Previously, the amount of “true” NSCs in a neurosphere was over-estimated due to technicalities in the neurosphere clonality study. A true NSC should by definition be able to produce a new sphere during clonal expansion, where most cells in the sphere should continue to be multipotent. However, the assays used for evaluating clonality have previously used as much as five cells or more per µl cell medium. A recent report shows an extensive occurrence of stem and progenitor cell motility and sphere merging, illustrating the importance of culturing single cells in single wells when studying clonality (Singec et al., 2006). One problem with growing cells under clonal conditions is that cell-cell interactions and the paracrine signal-

ing are lost, which may to some extent explain the low success rate in sphere formation from single cell cultures (Louis et al., 2008).

Markers used for studying neural stem cells and their progeny

To analyze NSC proliferation and differentiation, markers are needed that can be used to determine the cell type and developmental state of a cell. Some of the commonly used markers for identifying NSCs are nestin, musashi1, Sox2 and CD133. However, as discussed previously, none of these markers can be considered exclusive for NSCs.

Nestin is an intermediate filament initially identified by its presence in neuroepithelial stem cells *in vivo* (Lendahl et al., 1990). Nestin expression is also detected in cultured NSCs, and during differentiation the expression declines (McKay, 1997). There are several transgenic mouse models expressing reporter genes such as lacZ and GFP under the nestin promoter, visualizing the expression of nestin in NSCs (Zimmerman et al., 1994; Mignone et al., 2004). Although nestin is the most commonly used marker to stain NSCs, it is not exclusively expressed in NSCs but also found in neural progenitor cells, in cells in the myotome and in vascular endothelial cells (Sejersen and Lendahl, 1993). There is therefore a need for additional NSC markers. Glial fibrillary acidic protein (GFAP), which also is an intermediate filament protein, has served as a marker for the detection of astrocytes (Eng et al., 2000). Although GFAP is an excellent astrocytic marker, it can also stain a specific pool of NSCs that has been identified in the adult mammalian SVZ (Doetsch et al., 1999). Another NSC marker is CD133, which is a plasma membrane bound protein containing five transmembrane regions. CD133 has previously been identified as a marker for hematopoietic stem cells (Miraglia et al., 1997; Yin et al., 1997) and has also successfully been used for sorting NSCs (Uchida et al., 2000). In addition, the transcription factor Sox2 can bee used as a marker for NSPCs both *in vivo* and *in vitro*. Transgenic mice that express GFP under the Sox2 promoter have shown that the expression of Sox2 is directed to NSC regions both in young and adult animals (Brazel et al., 2005). Musashi1 is an RNA binding protein that can be used for staining of NSCs, however as for all the markers for NSCs there is cross reactivity with other more mature cell types (Kaneko et al., 2000).

For the identification of the differentiated progeny of NSCs there are markers that are commonly accepted as exclusive for the respective cell lineage. Early neurons can be identified using microtubule-associated protein 2 (MAP-2) and β-III-tubulin (Geisert and Frankfurter, 1989), which both are structural proteins expressed in neurons (Izant and McIntosh, 1980; Burgoyne, 1986). Oligodendrocytes are identified by expression of the cell sur-

face antigen O4 (Sommer and Schachner, 1981). NG2 is a proteoglycan that is also expressed on the cell surface of oligodendrocytic progenitors, while for more mature oligodendrocytes the proteolipid protein (PLP), which is a major component in myelin, can be used as a marker (Griffiths et al., 1998).

Present investigation

Neural stem cell proliferation, differentiation and migration are tightly regulated during CNS development. The understanding of these fundamental cellular processes, and the factors that guide them, is of great importance to understand normal brain development as well as pathological conditions such as cancer and neurodegenerative disease. This thesis discusses the cellular response of neural stem and progenitor cells to known and novel factors.

Aims

Paper I
To investigate the transcriptional changes in primary neural stem and progenitor cells upon the shift from proliferation to differentiation, with or without the addition of PDGF-AA.

Paper II
To characterize the expression and investigate the function of NRBP2, a gene identified as up-regulated in Paper I, in mouse brain, neural progenitor cells and a pediatric brain tumor.

Paper III
To identify differences between cellular responses to the PDGF isoforms A and B in neural progenitor cultures.

Paper IV
To evaluate cellular responses mediated by stem cell factor in neural stem cells.

Results and discussion

Paper I) The gene expression profile of PDGF-treated neural stem cells corresponds to partially differentiated neurons and glia

PDGF has previously been identified as a NSC regulator and some studies claimed that PDGF could act as a neurogenic factor while other reports suggested that PDGF stimulation is involved in keeping neurons immature. To study the PDGF-response process further we analyzed the transcriptional changes occurring during NSPC differentiation. We performed a micro-array analysis comparing the RNA expression between proliferating NSPCs and cells differentiated in the presence or absence of PDGF-AA. The NSPCs used in this study were derived from E14.5 rat cortices. These cells were grown in serum free medium containing FGF-2, and differentiation of NSPCs was induced by the removal of FGF-2.

In general a micro-array approach provides a lot of information, making the evaluation complex. In our case this was further complicated by the use of a mouse array for the analysis of rat cDNA. However at the time when the experiments were performed, rat arrays were not common, especially not with the same characteristics as the mouse array used in Paper I. The gene profile of the mouse array and the similarities between the coding regions of the two species still made this an attractive choice. The accuracy of our array results was further confirmed by examining key genes using real-time PCR.

The results showed that the transcriptional program for cells differentiated in the presence of PDGF was delayed compared to control cells. Furthermore, addition of PDGF *in vitro* to differentiating NSPCs does not alter the proportions of mature cell lineages. The NSPCs differentiated in PDGF-AA however, stayed in the cell cycle for an increased time, and as a consequence had a slower differentiation compared to control cells. No genes were found to be differently regulated between the two conditions +/- PDGF during NSPC differentiation, meaning that no gene was up-regulated in the PDGF condition and down-regulated in the control or *vice versa*. This was somewhat surprising since PDGF exposure has been shown to have a drastic effect on the morphology of neurons (Erlandsson et al., 2001). However, PDGF-BB also binds the PDGF α-receptor and the recent findings that PDGF-BB is produced by NSPCs themselves in an autocrine/paracrine fashion could therefore partly explain the lack of differences in the expression profile of the two conditions (Erlandsson et al., 2006). Consideration should also be taken to the heterogeneous cell population that is formed during NSPC differentiation. A small down-regulation in one cell type could easily be compensated by the over-expression in other cell populations. However, the results from the array show a slower up- or down-regulation of genes associated with all three neural cell lineages, indicating that PDGF is not, as previously reported, a neurogenic factor. PDGF can in this system be de-

scribed more as a general-acting mitogen for the progenitor pool rather than an instructive factor towards the neuronal cell lineage.

Although only a general difference on transcription was detected between control and PDGF treated cell populations, we could use the experimental setup to evaluate genes involved in the shift from proliferation to differentiation. We were able to identify genes that have been associated with the cellular events of proliferation and differentiation previously, but some genes were found that are less well studied. For example, we identified PTEN-induced kinase 1 (PINK1) and nuclear receptor binding protein 2 (NRBP2) as genes being up-regulated during NSC differentiation. This up-regulation was confirmed using real-time PCR. PINK1 is a gene that during the last years has been of interest due to its suggested role in neurodegenerative disease. NRBP2 is a non-characterized gene with no known function, which will be further described in Paper II in this thesis.

Recently, cells from CNS tumors have been shown to have neurosphere forming capacity, as well as a number of NSC properties. We therefore compared the array data from the NSPCs to previously published arrays of experimental glioma. Although comparisons of data from different groups with different experimental conditions should be used with caution, we noted that genes up-regulated during NSPC differentiation were less expressed in tumors. The glioma transcriptional profiles therefore more resemble NSPCs than their differentiated progeny. This can be seen as a reflection of the immature cells present in gliomas.

Paper II) Nuclear receptor binding protein 2 is induced during neural progenitor differentiation and affects cell survival

In this study we investigated one of the genes that was found up-regulated during NSPC differentiation in the micro-array screen previously described in Paper I. The gene of interest was recently assigned the name nuclear receptor binding protein 2 (NRBP2), and was a previously non-characterized gene. We characterized the expression of NRBP2 in the developing mouse brain, in NSPC cultures and on sections of pediatric medullablastoma. The expression analysis was done using RNA *in situ* hybridization and immunostaining on cells and tissues.

To be able to analyze NRBP2 protein expression we produced an antibody directed against a peptide from the C-terminal end of the predicted protein. Using this antibody we identified the NRBP2 protein as a larger protein than what was predicted in public databases. Further analysis of the mRNA revealed the presence of two different transcripts. Both these transcripts will in theory give rise to a protein containing the same C-terminal, but the two proteins differ significantly in size. In our study we could only detect the larger protein of approximately 55 kDa. Further use of the NRBP2

antibody revealed that, in line with previous RNA data from Paper I, the NRBP2 protein was up-regulated during both primary cultured and ES cell derived NSPC differentiation.

Expression analysis revealed the presence of NRBP2 in the developing CNS from E12.5 and onward. During early postnatal development NRBP2 was most abundant in the walls of the third and forth ventricles, the CA3 region of the hippocampus and the Purkinje cells in the cerebellum. However, the expression of NRBP2 is not exclusively found in the CNS. RNA expression can also be seen in organs such as the lung, tongue and eye. Immunostainings on cultured NSPCs and paraffin sections of mouse brains revealed a cytoplasmatic localization for the NRBP2 protein. To further investigate the function of NRBP2 we used siRNA to down-regulate the gene in ES cell derived NSPCs. Transient down-regulation of NRBP2 during neural progenitor differentiation rendered the cells more sensitive to staurosporin-induced apoptosis. However no changes in cell fate choice were found. We also found clusters of NRBP2 positive cells, which were surrounded by GFAP positive cells, within sections of a pediatric medullablastoma. These NRBP2 positive cells within the tumor also stained positive for the neuronal marker Neurofilament. This is of particular interest since medulloblastomas are believed to originate from immature cells of the cerebellum, which also is a region where we find strong NRBP2 expression.

At present we do not know how the NRBP2 expression can be correlated to the function of NRBP2 during development and adulthood. Further clues on NRBP2 function could be gained from the studies published on the related protein NRBP1 or its mouse ortholog MADM. NRBP1 was first identified as an adaptor protein (Hooper et al., 2000), which was confirmed in reports showing interactions between NRBP1 and RAC3, Jab1 (De Langhe et al., 2002; Wang et al., 2006) and a factor mediating the phosphorylation of myeloid leukemia factor 1 (Lim et al., 2002). Interestingly, in Xenopus a MADM-like protein has been identified as an important component in retina and lens development (Elkins and Henry, 2006). This is of special interest since we detected NRBP2 in the retina of the mouse embryo. The NRBP2 protein is well conserved between mouse, rat and human, but nothing has previously been known about its function. Whether NRBP2 indeed is a nuclear receptor binding protein remains elusive since we could not detect its expression in the nucleus, but it contains one of two motifs important for nuclear receptor binding. The NRBP2 protein is also suggested to contain a putative kinase domain, in which a mutation was recently identified in a patient with malignant glioma (Hunter et al., 2006). However, since this kinase domain found in NRBP2 is probably not functional, the role of NRBP2 in tumor formation still has to be elucidated.

Paper III) A divergent effect of PDGF-AA and -BB on differentiating neural stem and progenitor cells

In this part of the thesis we evaluated the PDGF-dependent cellular response during extended differentiation of rat primary NSPCs. In addition, we were interested to see if a divergent effect could be detected in cells stimulated with either PDGF-AA or PDGF-BB. This was tested at different time-points during normal differentiation, and during differentiation that was disrupted by a mechanical passage after 6 days.

We could show that the PDGF β-receptor was expressed by the cells during the whole differentiation process. During the first days of differentiation, PDGF-AA seemed to exert a larger proliferative effect than PDGF-BB. However, after 6 days of differentiation, cells were proliferating more extensively in the PDGF-BB treated cultures. This is an indication that the effect on cultured NSPCs differs between the two PDGF ligands. PDGF-AA and -BB partly bind different receptors, which could explain the differential effect. PDGF-AA binds only to the PDGF receptor homodimer αα, whereas PDGF-BB binds all the available PDGF receptor combinations (αα, αβ and ββ).

Evaluation of PDGF signaling components shows that a sustained presence of growth factor mediates signaling in NSPCs. NSPCs differentiated for 12 days in PDGF-AA or -BB were morphologically different from control cells. Both PDGF isoforms delayed differentiation, which was shown by neurons having a rounded morphology and fewer processes compared to differentiation in the absence of growth factor. A similar morphology was previously detected at 6 and 8 days of differentiation in the presence of PDGF-AA or PDGF-BB (Erlandsson et al., 2001; Erlandsson et al ., 2006).

By introducing a mechanical passage after 6 days of differentiation, we evaluated the extent of remaining progenitor cells that could divide if continually treated with PDGF-AA or -BB. This mechanical passage selectively removes cells not able to repopulate a new plate. This selection to a large extent removed neuronal cells, whereas astrocytic and oligodendrocytic precursors were able to recover after passaging. The ability of cells to recuperate was greatly enhanced by PDGF-BB in comparison to PDGF-AA and untreated conditions. Cells that had been differentiated in PDGF-BB and later on, after the passage, subjected to PDGF-AA had a higher degree of proliferation than cells initially primed in PDGF-AA and shifted to PDGF-BB. Furthermore, it was evident that PDGF-BB treatment increased the survival rate of passaged cells, compared to PDGF-AA.

This population of cells arising after the late passage was to a large extent positive for the oligodendrocytic progenitor marker NG2. PDGF-BB was mainly responsible for the increase in NG2 expressing cells. That PDGF treatment enhances oligodendrocyte differentiation is previously known, but that PDGF-BB was able to promote a glial population of cells with a better

ability to survive and proliferate than PDGF-AA is intriguing. This leads to the conclusion that the PDGF β-receptor in combination with the α-receptor signaling generates specific cellular responses in neural progenitors compared to only α-receptor signaling.

Paper IV) Stem cell factor is a chemoattractant and survival factor for CNS stem cells

In this study we investigated the cellular responses of NSCs to stem cell factor (SCF). SCF signaling via its receptor c-kit is a potent inducer of proliferation and migration in the hematopoietic system. NSCs and other multipotent stem cells have the ability to migrate, and NSCs in particular are known to migrate towards injury as well as tumors in the CNS. SCF has been suggested to be one among many of the signaling molecules that are involved in these processes.

To examine the effect of SCF on NSCs we started by showing that the c-kit receptor was present in both proliferating and differentiating NSCs derived from E14.5 rat cortices. In a migration assay SCF was shown to induce a strong migratory response in cultured NSCs. This migratory response was comparable to known inducers of migration, such as PDGF and FGF-2. In addition migration could be efficiently inhibited by the addition of a SCF antagonistic antibody. Furthermore, we could show that SCF to a certain degree positively affects cell survival. Although a striking effect on proliferation has been detected in other cell types, no such effect was detected in NSCs. When NSCs were subjected to SCF, no effect could be detected on their differentiation repertoire into neurons, astrocytes and oligodendrocytes.

Identifying SCF as one of the factors that induces migration of NSCs is an important step for understanding developmental processes and the signaling cues activated by disease or injury. This is necessary for further development of vector-based systems for delivering drugs or genetically modified cells to tumors.

Future perspectives

The approach to study cellular responses *in vitro* has been extremely helpful, providing many clues to how and why NSCs respond as they do in a given situation. The thought is basically to be able to simplify the *in vivo* situation, enabling the understanding of a NSC in a stepwise manner. In this thesis, mainly cells derived from embryonic rat cortices were used. This is a cell system that in the NSC field is considered as a classical adherent system (Johe et al., 1996). The introduction of new ways to derive NSCs *in vitro*, besides demanding less animal sacrifices, may provide systems of more homogeneous character. This would provide more detailed information in screenings aiming to find key events that are restricted to one cell lineage. One example of a more homogeneous system is the ES cell derived NSCs (Conti et al., 2005). However, before addressing the cellular response to different exogenous stimuli the presence of the necessary receptors first needs to be evaluated.

To be able to dissect the transcriptional and signaling events mediated by PDGF in detail, there is a need to further separate the signaling pathways for the different ligands. This is especially important since NSCs have been shown to produce their own PDGF-BB (Erlandsson et al., 2006). Separation of the signaling events can be accomplished either by using cells that lack either the receptors for PDGF signaling, or by using PDGF receptor type specific inhibitors.

Interestingly there are reports showing that PDGF αα in contrast to ββ receptor signaling occurs only in the vicinity of primary cilia in fibroblasts (Schneider et al., 2005). Primary cilia are structures present in only one copy on almost all cells, this is also the case for NSCs (Alvarez-Buylla et al., 2001). Furthermore, this structure has been shown to be essential for Shh signaling in adult NSCs (Han et al., 2008). Evaluation of this regional difference in PDGF receptor distribution in NSCs may give important clues to the divergence in PDGF signaling between the ligands. This enables the possibility of selective inhibition of structures important in α-receptor signaling by other means than targeting the receptor directly. One of the proteins important in PDGF α-mediated signaling is the NHE1 protein, a Na^+/H^+ exchanger that can be selectively inhibited, causing blocking of PDGF-AA mediated migration in fibroblasts (Schneider et al., 2009).

There is a solid link between brain tumor formation and PDGF. This has been evaluated in animal models, and PDGF signaling has been detected in

human cancers. Therefore it is important to investigate the normal cells present in the brain and their response to PDGF. In paper II we showed that a previously uncharacterized gene expressed in neural progenitors also could be detected in human brain tumors. Although we cannot provide detailed information of the NRBP2 function, we could detect a higher sensitivity to induced cell death in neural progenitors after NRBP2 down-regulation. To explore the cellular function of NRBP2, conditional knock out studies and cell lines over-expressing NRBP2 would be of interest to resolve the developmental role for this gene. NRBP2 contains a potential kinase domain, but as this is truncated, it may not be functional. Instead there is a possibility that this protein may act as an adaptor protein rather than a kinase. Identifying interactions between NRBP2 and other proteins would be key findings in elucidating the role of NRBP2.

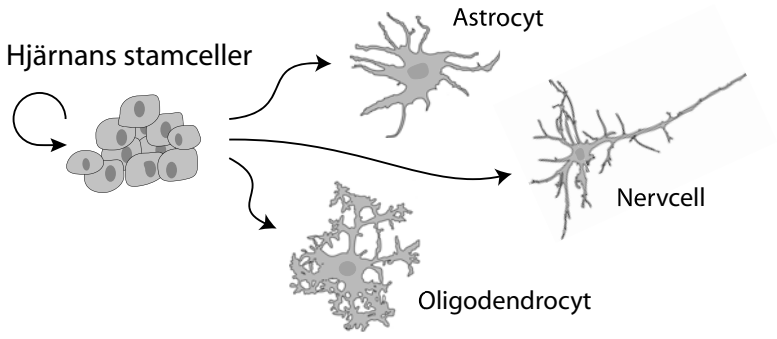
In conclusion, research is a long-time investment and the global research on NSCs will continuously deliver important information about the normal development as well as hopefully discover potential cures for different diseases. My hope is that this thesis will, in the end, provide information that contributes to this process.

Populärvetenskaplig sammanfattning

Hjärnan - en plats för stamceller

Trots att celler i kroppen kan ha helt olika uppgifter så innehåller varje enskild cell i en individ identiskt DNA. DNA-molekylen är dubbelsträngad och sammansatt av baserna A, T, G och C i oändligt många kombinationer. DNA-koden bildar gener som läses av cellens maskineri och omvandlas till budbärarmolekylen RNA. Den här processen kallas transkription och kommer att diskuteras mer senare i denna sammanfattning. Även RNA molekylen används som mall för att bilda en kedja bestående av aminosyror som formas till ett protein. Proteiner är de molekyler, som med vissa undantag, utför arbetet i cellerna och hjälper till att bygga upp cellstrukturerna.

Stamceller är celler som har möjlighet att vid celldelning återskapa en cell som har samma egenskaper som modercellen. Stamcellen måste också kunna mogna ut till de celltyper som representerar det organ de finns i. En stor del av den embryonala hjärnan består av stamceller som sedermera mognar ut till de celler som återfinns i den utvecklade hjärnan, d.v.s. nerv- och gliaceller. Nervcellerna finns i ett stort antal i hjärnan och står för den ytterst komplicerade nervsystemssignaleringen. Gliaceller är ett samlingsnamn för oligodendrocyter och astrocyter. Oligodendrocyterna bildar de fettskidor som finns på nervcellernas utskott och är viktiga för att underlätta signalering mellan nervceller. Astrocyter har många olika funktioner i hjärnan där de bland annat verkar som stödjevävnad.



Stamcellerna i hjärnan delar sig och kan mogna ut till nervceller, astrocyter och oligodendrocyter.

Utöver närvaron under utvecklingen återfinns stamceller även i specifika regioner i den vuxna hjärnan där man antar att de bidrar till skapandet av nya nervceller vid inlärning och skada. Detta sker dock i begränsad utsträckning och endast i vissa regioner av hjärnan. Även om nya nervceller inte bildas i stor skala i den vuxna hjärnan så kan takten på förnyelse av nervceller ökas hos möss genom ökad mängd motion och stimulerande miljöer.

Under det senaste årtiondet har ett flertal protokoll tagits fram för att isolera, expandera och studera hjärnans stamceller utanför kroppen. Jag har använt dessa metoder för att ta fram stamceller från den embryonala råtthjärnan. Cellerna används sedan för att studera effekterna av externa tillsatta faktorer, såsom PDGF och SCF, samt den interna faktorn NRBP2. Kunskapen om hur stamceller regleras av externa och interna faktorer är av stor vikt för att förstå såväl normal utveckling som olika sjukdomstillstånd. Detta gäller framförallt hjärncancer där högt uttryck av PDGF i kombination med mutationer i stamceller tros vara en möjlig orsak till att dessa uppstår.

Studie I) Hur påverkas hjärnans stamceller av PDGF-behandling?

PDGF är ett protein som utsöndras av cellen och som kan påverka ursprungcellen eller angränsande celler genom att PDGF-proteinet binder till speciella receptorer på cellytan. På detta sätt påverkar PDGF bland annat det centrala nervsystemet under utvecklingen. I isolerade stamceller är effekten av PDGF tydligast på den ökade celldelningen, men man ser även ett mer omoget utseende hos cellerna. I detta delarbete undersöktes hur uttrycket (transkriptionen) av gener påverkades av behandling med PDGF under mognadsprocessen mot nerv- och gliaceller. En generellt lägre upp och nedreglering kunde påvisas i celler som behandlats med PDGF. Detta innebär förenklat att PDGF hjälper alla celler att fortsätta dela sig under mognadsprocessen. Den transkriptionella profilen hos den PDGF-behandlade cellpopulationen liknade även till viss del transkriptionen i hjärntumörer. Utöver dessa resultat hittades även många gener som regleras upp eller ned under skiftet från en delande stamcell till en mer terminalt utmognad nerv- eller gliacell. Vissa av dessa gener har ingen känd funktion och detta ledde vidare till delarbete II.

Studie II) Var finns och hur påverkar den nyligen funna faktorn NRBP2 hjärnans stamceller?

Nuclear receptor binding protein 2 (NRBP2) identifierades i delarbete I som en hittills okänd gen som fick ökad RNA-transcription under utmognaden av stamceller. Precis som för RNAt från NRBP2 genen fann vi även NRBP2-proteinet i större mängd i mer mogna celler jämfört med hjärnans stamceller. När vi studerade lokaliseringen av NRBP2-proteinet fann vi ett uttryck främst i nervceller i lillhjärnan och hippocampus, en region i storhjärnan viktigt för inlärning och minne. Uttrycket av NRBP2 gav oss anledning att titta närmare på medulloblastom, en tumör som uppstår i lillhjärnan. Vi kunde detektera NRBP2 i kluster av celler i dessa tumörer. Vidare funktionella

studier av NRBP2 visade att om vi tog bort NRBP2 uttryck i celler blev cellerna mer känsliga mot ämnen som inducerar celldöd.

Studie III) Finns det skillnader i reaktioner mellan celler behandlade med olika PDGF-isoformer?

PDGF finns i flera olika former och två av dessa är PDGF-AA och -BB som båda påverkar utvecklingen av hjärnans stamceller. Huruvida dessa faktorer påverkar stamcellerna på olika sätt är dock okänt. I detta delarbete studerade vi skillnader mellan PDGF-AA och PDGF-BB behandling under utmognaden från stamceller till nervceller och gliaceller. Vi visade att celler som kontinuerligt stimuleras av PDGF-BB hade en betydligt större möjlighet att återväxa i kultur efter mekanisk delning än celler som behandlats med PDGF-AA. Närvaron av PDGF-BB gav en ökning i överlevnad och även antalet gliaceller av oligodendrocytursprung blev fler. Sammanfattningsvis så visar vi att det finns en skillnad mellan hur neurala stamceller under utmognad svarar på stimuli från PDGF-AA och -BB.

Studie IV) Hur påverkas hjärnans stamceller av SCF?

Utvecklingen av blodstamceller regleras av SCF. I detta delarbete undersöktes i vilken utsträckning SCF påverkar hjärnans stamceller isolerade från råttembryon. Vi kunde visa att stamcellerna och de utmognande nerv- och gliacellerna bar receptorn som möjliggör SCF-aktivering. Behandlingen med SCF ledde till en markant ökning av cellernas förmåga att aktivt röra sig. Denna förmåga till riktad rörelse kunde hävas genom att blockera SCF och dess receptor. Dessutom visade sig SCF-behandlingen öka cellernas överlevnad. Att behandla mognande stamceller med SCF påverkar dock inte proportionerna av nerv- och gliaceller som bildas.

Acknowledgements

I would like to express my gratitude to all the persons that have supported me. In particular I would like to thank:

Karin Forsberg-Nilsson for accepting me as a PhD student. For educating me and introducing me to the life as a researcher. For always having a positive angle and believing in me. Without you nothing of this would have been possible.

Maud Forsberg for being my co-supervisor and friend during these years. For sharing your knowledge about ES cells and cloning. Du är en Klippa!

Kristofer Rubin for being my co-supervisor. For always finding time for me whenever I have questions.

Hans Ronne for first introducing me to science. For being my examiner and for letting me participate in the monensin project. Also, many thanks to past and present members of your research group who took care of me as a project student, especially Mattias Ö, Eva, Marie and Gunilla. Även ett stort tack till övriga i *cerevisiae* och *pombe* grupperna för alla trevliga diskussioner i samband med luncher och undervisning.

Karin Holmqvist (Brännvall) för din vänskap och för att du visat mig hur en ytterst kompetent och produktiv forskare ska vara. Tack även för att du höll mig i form under alla år som vi tränade löpning ihop.

Tobias Bergström för att du alltid varit behjälplig i så många avseenden och för att du gjort mina figurer presentabla. Den som ändå hade haft dina förkunskaper när man började doktorandutbildningen. För att inte göra detta stycke längre än nödvändigt och inte sväva i allt för yviga ”känslomässiga” resonemang väljer jag att summera dina egenskaper i ett ord. Kompetent!

Ulrika Wallenquist min doktorandkollega sedan urminnes tider. Även om våra projekt inte varit direkt kopplade till varandra så har jag alltid känt din vänskap och stöd. För att du är så optimistisk och har en helt annan tidsuppfattning än mig. För att du är en varm människa. Jag menar verkligen tack för att du utstrålat värme under kalla vinterdagar.

Lena Kjellen för att du är så trevlig och kunnig. För att du och dina kollegor undervisat mig i sockrets betydelse, jag börjar faktiskt tycka det börjar bli intressant...

Staffan Johansson för att du givit mig en inblick i integrinsignalering. För att du ibland delat dina löprundor med mig, och nej: det blir ingen gympa på torsdag.

Xiao-Qun Zhang för att du alltid är så glad och hjälper mig när jag behöver det.

Mia Niklasson (Enarsson) och Anna Erlandsson för att ni tog emot mig med öppna armar när jag kom till labbet. För att ni välkomnade mig till era projekt och för att ni varit så framgångsrika, ni är en inspirationskälla.

Magnus Essand, Björn, Wing, Helena och Berith för att ni gav mig möjligheten att utveckla mina kunskaper inom immunologi. För att ni bjöd på en härlig stämning under min tid som student hos er.

Cecilia Annerén för att du utökat mina kunskaper om ES-celler och signalering. Du och din grupp har verkligen tillfört mycket positivt sedan ni kom till korridoren.

Christoffer Tamm för att du är så driven och kompetent. För att du delar med dig av din vida kunskaper samt mycket annat praktiskt på labb. För att du gjorde de mogna cellerna till figurerna i kappan till denna avhandling.

Per-Henrik Edqvist för att du lär mig saker om ögon och håller diskussionerna igång.

Catharina Svensson och Birgitta Jönzén for att ni möjliggjorde forskarskoleutbildningen i Uppsala och gav oss deltagare chansen att visa vad vi kunde. Tack även alla andra UGSBRare.

Anders Dagälv, Inger Eriksson, Katarina Garpenstrand, Pernilla Carlsson och Birgitta Wärmegård för all hjälp, alla trevliga luncher, all vänlighet och goda råd som jag fått under åren. Ett speciellt tack till Anders för att du återgett alla sportresultat från Leksandstrakten och för ständiga diskussioner om rykande färska händelser. Du har möjliggjort min dominans i doktorandrummet när det gäller DNs nutidstest.

Sammarbetspartners, medförfattare, kollegor vid IMBIM och i korridoren D9:4, jag har uppskattat att jobbat tillsammans med er.

Anna Eriksson för att du är så ytterst smart och vass. Du har redan lämnat ett stort avtryck i familjen Larsson.

Sofia Gustafsson (Svahn) för att du blivit min, Kattis och Valles vän. För att du och Daniel alltid ställer upp. För att ni infört socialt umgänge och andra bekanta i våra liv.

Elina tack för de 10 år som vi har studerat i närheten av varandra och även för att du inte körde över mig med bilen trots att du hade chansen.

Agnieszka, Anders, Sofia, Tobias och Hannah för att jag haft möjligheten att handleda er under er studietid. Jag hoppas jag lyckades lära er något, det har varit en lärorik tid för mig i alla fall.

Per Jemth för att du gillar att hänga och delge dina tips om hur forskning och löpning ska genomföras. För att du är en så bra förebild i papparollen och för att du har en så härlig familj.

Familjerna Karlsson, Forsberg, Matsson/Entesarian och Eklund/Svensson för att ni alltid finns tillgängliga för umgänge, rekreation och avlastning. Det är alltid lika roligt att ses.

Grabbarna från studietiden
Danne, John, Erik, Johan, CJ, Anders och Staffan för att ni gjort min grundutbildning till en så trevlig erfarenhet.

Anders Hellström för att du sedan kursstart år 2000 eller snarare två veckor efter kursstart delat mina mödor stora som små. Har vi blivit äldre? -Ja! Har vi blivit bittrare? -Ja! Är vi samma gamla goa killar? -Ja det tror jag!

Jon Ramsell för att du möjligen en aningen motvilligt varit min polare under de senaste åren. Det är ingen ide att kämpa emot, det vet du…

Larssons för att ni tar så väl hand om oss när vi är på besök och vilar upp oss.

Lilla familjen Larsson: Kattis för att du är min stora kärlek en underbar mor en ytterst kompetent, smart och driven forskare. Tack även för att du tagit dig an och läst och korrigerat denna avhandling.

Valle, min grabb, du är fin du!

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