

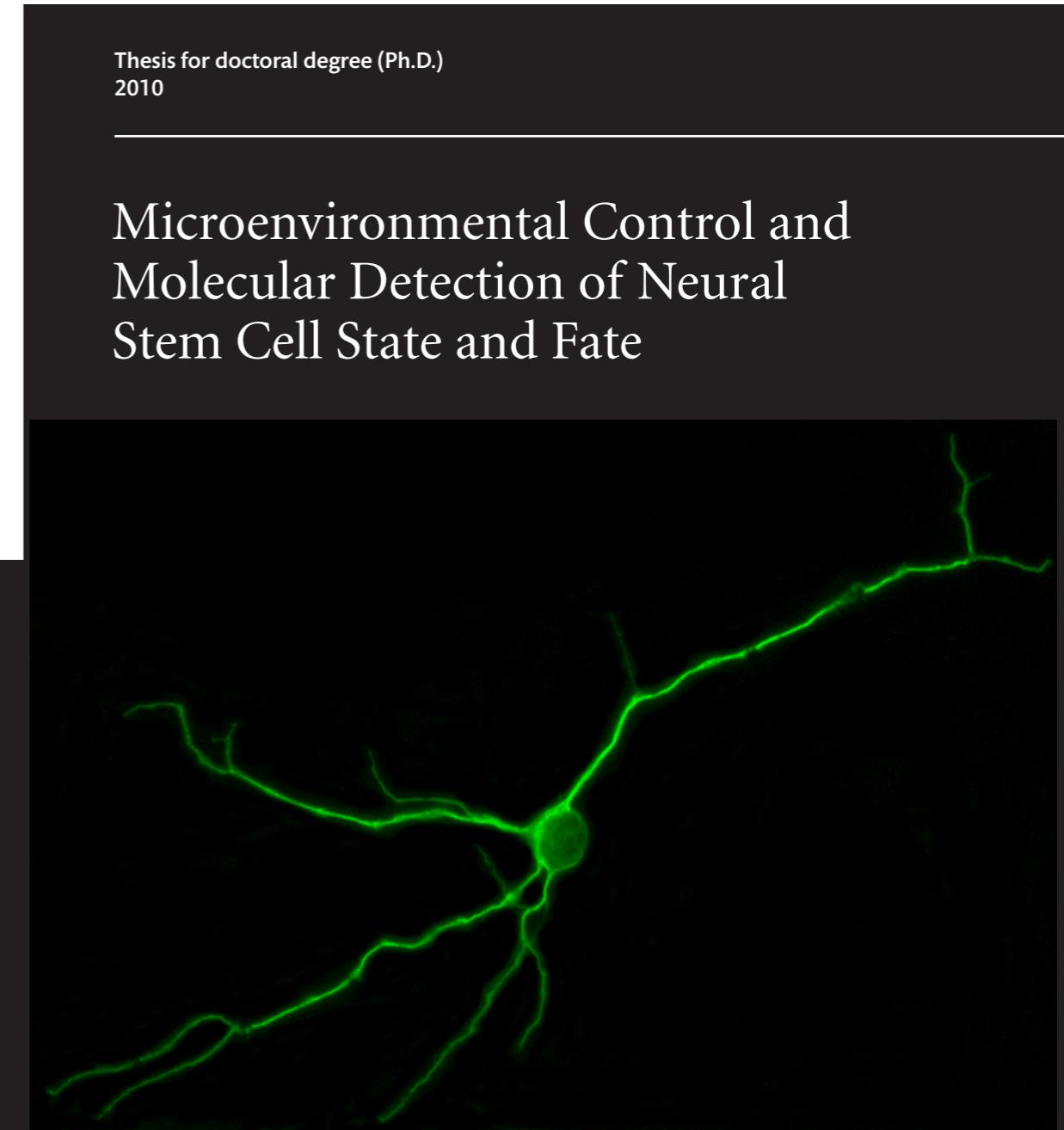
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Microenvironmental Control and Molecular Detection of Neural Stem Cell State and Fate

Shirin Ilkhanizadeh



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From Department of Neuroscience  
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## MICROENVIRONMENTAL CONTROL AND MOLECULAR DETECTION OF NEURAL STEM CELL STATE AND FATE

Shirin Ilkhanizadeh



Stockholm 2010

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## **ABSTRACT**

Neural stem cell growth and specialization is dependent on microenvironmental cues, such as growth factors, oxygen levels and substrate stiffness. Together these cues define the genetic and epigenetic program of neural stem cells. In our studies we have manipulated these cues in order to achieve better control of neural stem cell state and fate. In addition, we introduce a novel way of detecting neural stem cells and cancer stem cells in culture. Luminescent conjugated oligothiophenes (LCO) are short polymers, which cross the cell membrane and generate luminescence in the range of wavelengths of ordinary fluorescent probes. In paper I we present a novel LCO, p-HTMI, for detection of live neural stem cells but not differentiated cells. p-HTMI represents a new generation of molecular probes for immediate and non-invasive live detection of specific cell types. In paper II we show that the NADH-sensitive transcriptional co-repressor, CtBP, controls a switch between neuronal and non-neuronal cell fates in oxygen and BMP rich environments. CtBP molecularly integrates microenvironmental levels of BMP and oxygen through Notch mediated regulation of anti- and pro-neural genes. Furthermore, we manipulated growth factor presentation in the stem cell microenvironment through inkjet printing technology. In paper III, we show that inkjet printed macromolecules remained biologically active when printed on hydrogels and control the differentiation of neural stem cells. Finally, in paper IV we demonstrate that soft substrates with brain-like stiffness promote the maturation of neurons. This defines substrate stiffness as a crucial parameter in neuronal differentiation of neural stem cells.



## LIST OF PUBLICATIONS

- I. **Ilkhanizadeh S**, Simon R, Åslund A, Teixeira AI, Holmberg J, Uhlén P, Joseph B, Konradsson P, Hermanson O\*, Nilsson KP\*  
Live detection of neural stem and cancer stem cells by an oligothiophene derivative.  
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- II. Teixeira AI\*, Dias JM\*, **Ilkhanizadeh S\***, Duckworth JK, Rosenfeld MG, Ericson J, Hermanson O  
CtBP mediates Notch dependent control of neural stem cell fate by oxygen levels and BMP.  
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- III. **Ilkhanizadeh S**, Teixeira AI, Hermanson O  
Inkjet printing of macromolecules on hydrogels to steer neural stem cell differentiation.  
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- IV. Teixeira AI\*, **Ilkhanizadeh S\***, Wigenius JA, Duckworth JK, Inganäs O, Hermanson O  
The promotion of neuronal maturation on soft substrates.  
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## LIST OF ABBREVIATIONS

2DG	2-Deoxy-D-glucose
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSL	CBF1/Su(H)/LAG1
CtBP	C-terminal binding protein
Dll	deltalike
E	embryonic day
ECM	extra cellular matrix
EGF	epidermal growth factor
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
GFP	green fluorescence protein
HDAC	histone deacetylase
iPSC	induced pluripotent stem cell
LCO	luminescent conjugated oligothiophene
LCP	luminescent conjugated polythiophene
MAPK	mitogen activated protein kinase
MSC	mesenchymal stem cell
NAD	nicotinamide adenine dinucleotide
NSC	neural stem cell
NGN	neurogenin
NICD	notch intracellular domain
NRSF	neuron restrictive silencing factor
PDGF	platelet derived growth factor
PDMS	polydimethylsiloxane
PI3K	phosphatidylinositol-3-OH kinase
PNS	peripheral nervous system
REST	repressor element 1 silencing transcription factor
RhoA	ras homolog gene family, member A
ROCK	RhoA kinase
RTK	tyrosine kinase
SHH	sonic hedgehog
Sir2	silencing information regulator 2
SIRT	sirtuin; silent mating type information regulator 2 homolog 1
SMA	smooth muscle actin
T3	triiodothyronine hormone
TCPS	tissue culture polystyrene
TGF $\beta$	transforming growth factor $\beta$
TSA	trichostatin A
VPA	valproic acid

# 1 INTRODUCTION

## 1.1 STEM CELLS

### 1.1.1 History

The concept of stem cells originates from the field of hematolgy and was postulated in the beginning of last century by the Russian histologist Alexander Maximow (Konstantinov 2000). It was not until the 1960s when Altman and Das reported adult neurogenesis in the brain and McCollough and Till described self renewing cells in the bone marrow that there was any scientific evidence for mammalian stem cells (Till and McCulloch 1963; Altman and Das 1965; Altman and Das 1966; Altman and Das 1967). Friedenstein and coworkers further established bone marrow stem cells in a series of studies in the 1960s and 1970s (Friedenstein, Lalykina et al. 1967; Friedenstein, Chailakhjan et al. 1970; Friedenstein and Lalykina 1970; Friedenstein, Deriglasova et al. 1974; Friedenstein 1976; Friedenstein, Gorskaja et al. 1976). A great breakthrough in stem cell research was made when mouse and human embryonic stem cells derived from the inner cell mass was reported (Evans and Kaufman 1981; Martin 1981; Thomson, Itskovitz-Eldor et al. 1998). Many exciting discoveries in the field of stem cell research have been made in the 21<sup>st</sup> century involving greater control of stem cell growth and specification, offering new possibilities for treatment of diseases and understanding development of organisms. However, the expanding field of stem cell research raises as many questions as it generates discoveries, leaving many fundamental questions yet to be answered.

### 1.1.2 Definition and classifications

Stem cells are the foundation for every organ, tissue and cell in the body and are characterized by unique defining properties. These cells have the capability of dividing and renewing their population for long periods of time while remaining unspecialized cells that can give rise to specialized cells (Morrison, Shah et al. 1997). When stem cells divide, the two daughter cells have the potential to either remain stem cells (symmetric cell division), or generate one identical daughter cell and one differentiated cell with more specialized functions (asymmetric cell division) (Götz and Huttner 2005). Differentiation is a complex process during which, certain genes become

activated and other genes become inactivated. As a result the differentiated cell develops defining structures and performs specialized functions.

There are different types of stem cells, which can be categorized into embryonic stem cells, fetal stem cells and adult stem cells. The embryonic stem cells are derived from the early stage embryo (5 days old in humans) and are capable of generating any cell in the body under the right conditions. Fetal stem cells are derived from the fetus and are more tissue specific. An adult stem cell is an undifferentiated cell that is found in a differentiated tissue of an adult organism. *In vivo*, these cells can only give rise to the cells types of the tissue they are found in.

Stem cells can also be classified based on their functionality into the subgroups totipotent, pluripotent, multipotent and unipotent. Totipotent stem cells have the ability to divide and differentiate into all the cell types in the embryo as well as extra-embryonic tissues. Pluripotent stem cells can differentiate into cells of all three germ layers (mesoderm, endoderm and ectoderm), but cannot give rise to extra-embryonic tissues. Multipotent stem cells can generate cells of the germ layer or tissue where they originate. Unipotent stem cells can only maintain their own population and are classified as stem cells for their self-renewing properties.

### 1.1.3 Neural stem cells in the developing brain

Neural stem cells (NSCs) are cells in the nervous system that are capable of self-renewal and can give rise to neurons, astrocytes and oligodendrocytes. Isolation of stem-like cells in the embryonic mammalian central nervous system (CNS) was performed in early studies (Temple 1989; Cattaneo and McKay 1990; Reynolds, Tetzlaff et al. 1992; Kilpatrick and Bartlett 1993). In the embryonic nervous system neural stem cells have been isolated from the basal forebrain, cerebral cortex, hippocampus, cerebellum and the spinal cord (McKay 1997; Rao 1999; Gage 2000; Laywell, Rakic et al. 2000). In the peripheral nervous system (PNS), the neural crest holds a population of neural stem cells (Stemple and Anderson 1992). Neural crest stem cells can generate Schwann cells (glial cells of the PNS) and sympathetic and sensory neurons (neurons of the PNS). They can also give rise to craniofacial mesenchyme (smooth muscle cartilage and bone) (Etchevers, Vincent et al. 2001; Aybar and Mayor 2002).

#### 1.1.4 Neural stem cells in culture

NSCs can be isolated and cultured *in vitro* as adherent monolayer cultures or as free-floating neurospheres (Reynolds, Tetzlaff et al. 1992; Von Visger, Yeon et al. 1994; Johe, Hazel et al. 1996; Hermanson, Jepsen et al. 2002). Immunoreactivity to the intermediate filament nestin identifies NSCs (Lendahl, Zimmerman et al. 1990). Intrinsic and extrinsic signaling orchestrates NSCs state and fate. In order to understand neural development, manipulating key regulators by administration of factors or blocking of pathways have been performed. Proliferation of NSCs can be maintained by fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) (Reynolds, Tetzlaff et al. 1992; Gritti, Parati et al. 1996; Johe, Hazel et al. 1996). Notch signaling is an additional key regulator of maintaining stem cell state (Androutsellis-Theotokis, Leker et al. 2006).

In adherent monolayer cultures isolated from the telencephalon of the developing brain, the cells differentiate mainly into astrocytes and neurons but also into oligodendrocytes upon mitogen withdrawal (Johe, Hazel et al. 1996; Hermanson, Jepsen et al. 2002). Exposure to single signaling factors induces differentiation into various cell fates. Ciliary neurotrophic factor (CNTF) induces astrocytic differentiation, valproic acid (VPA) induces neuronal differentiation, bone morphogenetic protein (BMP) induces astrocytic differentiation as well as differentiation into smooth muscle cells and triiodothyronine hormone (T3) promotes oligodendrocytic differentiation (Gross, Mehler et al. 1996; Johe, Hazel et al. 1996; Hsieh, Nakashima et al. 2004). Smooth muscle cell differentiation can also be obtained by exposing neural stem cells to serum (e.g. fetal bovine serum (FBS)) (Tsai and McKay 2000; Ilkhanizadeh, Teixeira et al. 2007). PDGF (platelet derived growth factor) signaling promotes neuronal proliferation (Williams, Park et al. 1997; Erlandsson, Enarsson et al. 2001; Erlandsson, Brannvall et al. 2006). Additionally, activation of the Wnt signaling pathway leads to neuronal differentiation and maturation (Kleber and Sommer 2004; Lee, Kleber et al. 2004; Kleber, Lee et al. 2005).

#### 1.1.5 Notch signaling in neural stem cells

The Notch signaling pathway is highly conserved and is present in all metazoans, regulating signaling cascades by intercellular communication in adjacent cells. Notch

signaling is involved in fundamental processes such as differentiation, proliferation and apoptotic programs and is essential during development. The core components of Notch signaling in mammals includes Notch receptors (Notch1-4), Notch ligands (e.g. Deltalike1 (Dll1)) and Notch responsive transcription factors (e.g. CBF1/Su(H)/LAG1 (CSL)). During development of the mammalian nervous system the pro-neural bHLH genes murine *Achaete-Scute* homologue (*Mash1*) and Neurogenin 2 (*Ngn2*) produce products that initiate Notch ligand expression, which sequentially activate Notch signaling in adjacent cells (Castro, Skowronska-Krawczyk et al. 2006). Once activated, the transmembrane protein Notch releases the Notch intracellular domain (NICD), which translocates to the nucleus and complexes with CSL. The NICD-CSL complex induces the expression of bHLH factors Hes1 and Hes5 leading to the repression of pro-neural gene expression (Artavanis-Tsakonas, Rand et al. 1999). This cell-cell regulation is called lateral inhibition and maintains neighboring cells as progenitors.

At the outset of mammalian nervous system development neural progenitors merely proliferate. When neuronal differentiation starts in a subset of cells, the expression of pro-neural genes and Notch ligand genes are expressed in a salt and pepper pattern in the ventricular zone (Guillemot and Joyner 1993; Hatakeyama and Kageyama 2006; Hammerle and Tejedor 2007; Kageyama, Ohtsuka et al. 2008). Little is known regarding the exact mechanisms underlying this pattern formation of gene expression. The established view is that initially all cells express pro-neural gene products and Notch ligands at comparable levels whereupon some cells stochastically start expressing Notch ligands at higher levels. These cells would then activate Notch signaling in adjacent cells leading to elevated Hes levels, which consequently down-regulates expression of Notch ligand genes and pro-neural genes. The former cells would therefore further up-regulate pro-neural genes and Notch ligands causing them to exit cell cycle and differentiate into postmitotic neurons while maintaining the progenitor state in neighboring cells. However, this view has been challenged by data showing oscillatory levels of Notch effectors (Hes1, Ngn2 and Dll1) in neural progenitor cells of the developing mouse brain (Shimojo, Ohtsuka et al. 2008). Thus, the salt and pepper expression pattern of Notch effectors changes over time leading to the conclusion that a stochastic change in gene expression is unlikely to be the cause for progenitors to exit cell cycle. Lateral inhibition is nevertheless involved in maintenance of the progenitor pool but not in neural selection. The latter is more likely

to be determined by asymmetric cell division regulated by Numb (Cayouette and Raff 2002; Shen, Zhong et al. 2002; Johnson 2003).

### 1.1.6 Neural induction

Spatiotemporal control of NSCs is essential for specification, expansion and differentiation of the nervous system. Neural induction was first described by Spemann and Mangold in the early 1920s (Spemann and Mangold 1924). In their study, a small bit of tissue from the dorsal side of one amphibian embryo at gastrula stage was transplanted to the ventral region of a second amphibian embryo. At the transplantation site they could observe a secondary body axis including a neural tube, notochord and somites. They named the transplanted piece the “organizer” as it could organize its environment. The great challenge was subsequently to identify the underlying molecules inducing neural fate in vertebrate embryos. In order to identify neural fate inducing genes, screening techniques for expression were used. Novel genes that induce neural fates were identified. The organizer has been shown to secrete proteins including noggin, chordin and follistatin (Piccolo, Sasai et al. 1996; Sasai, Lu et al. 1996; Zimmerman, De Jesus-Escobar et al. 1996; Fainsod, Deissler et al. 1997). The ventral side of the embryo expresses BMPs where they help to form the ectoderm whereas noggin and chordin are expressed on the dorsal side inducing neural fates (Lamb, Knecht et al. 1993; Sasai, Lu et al. 1995; Wilson and Hemmati-Brivanlou 1995). These results indicate that dorsal suppression of BMP signaling is essential for formation of neural tissue. Additionally, FGF signaling has been proven to be important for neural induction. Induction of neural fate involves both activation of FGF signaling and repression of BMP signaling (Streit, Berliner et al. 2000; Wilson, Graziano et al. 2000; Delaune, Lemaire et al. 2005; Londin, Niemiec et al. 2005). Even though inhibition of BMP signaling is essential for initial neural fate determination, at later stages it regulates CNS development. In the developing spinal cord, BMP proteins are secreted from the roof plate (dorsal) and Shh from the floor plate (ventral). These proteins are expressed as morphogens and form opposing gradient, creating dorsoventral patterning of the neural tube (Ericson, Muhr et al. 1995; Ericson, Briscoe et al. 1997; Briscoe, Pierani et al. 2000). The most dorsal cell type of the developing spinal cord is the Math1 positive population, which is lost upon loss of BMP signaling (Chesnutt, Burrus et al. 2004).

## **1.2 GLIOMA STEM CELLS**

### **1.2.1 Gliomas**

Gliomas are the most common type of brain tumors in humans and can be divided into the subgroups ependymomas, astrocytomas and oligodendrocytomas based on their cell type. Mixed gliomas (e.g. oligoastrocytomas) consist of a mixed cell population and are less common. Astrocytomas are the most common form of gliomas in humans and can be classified based on malignancy grade I-IV, according to World Health Organization (WHO) guidelines (Louis, Ohgaki et al. 2007). There is a direct relationship between tumor grade and tumor malignancy. Glioblastomas (glioblastoma multiforme) are grade IV gliomas and the most aggressive type of brain tumors. Molecular and genetic studies have led to better understanding of glioma biology and glioma formation, helping the discovery of several genetic abnormalities involving mainly cell cycle arrest control and signal transduction. Glioma development and progression involves alterations in core signaling pathways, including activation of receptor tyrosine kinases (RTK), phosphatidylinositol-3-OH kinase (PI3K) and AKT signaling (Maher, Furnari et al. 2001; Grzmil and Hemmings 2010). Mutations in the tumor suppressor gene TP53 in astrocytomas are frequent, interfering with cell cycle progression, DNA repair and apoptosis (Ohgaki and Kleihues 2009). In addition, deletions in chromosome 1p, 19q and 10q are common genetic mutations in gliomas (Cairncross, Ueki et al. 1998; Jeon, Park et al. 2007).

### **1.2.2 The cancer stem cell hypothesis**

Many cancers eventually reoccur even after efficient treatments when the tumor has been ablated. The cancer stem cell hypothesis supports the idea that there is a rare, slow cycling, population of cells within the tumor that possess limitless self-renewing abilities (Bonnet and Dick 1997; Reya, Morrison et al. 2001). It is hypothesized that cancer therapy directed towards these stem-like cells could decrease relapse of cancers. There are several ideas regarding the origin of glioma stem cells. In theory, these cells could originate from mature glia, which dedifferentiate into a more native state, unipotent neural progenitors or multipotent neural progenitors.

Multipotent neural stem cells have been isolated from the subventricular zone (Sanai, Tramontin et al. 2004), the lining of the lateral ventricles, the dentate gyrus (Eriksson,

Perfilieva et al. 1998), within the hippocampus and the subcortical white matter (Nunes, Roy et al. 2003) of the adult brain. The machinery for self-renewal is activated in neural stem cells, facilitating reprogramming (Reya, Morrison et al. 2001). Multipotent neural stem cells may possibly be the cell of origin for brain tumors supported by the fact that neural stem cells and brain tumor cells are regulated by the same signaling pathways and therefore display the same characteristics.

The case of mature glia as tumor progenitors is obstructed by the notion that glial cells would have to dedifferentiate into a more immature state. A recent study presents evidence that mature peripheral astrocytes are not able to give rise to brain tumors (Jacques, Swales et al. 2010). Additionally, there are technical difficulties studying the mechanisms of dedifferentiation of adult astrocytes both *in vitro* and *in vivo*. The most common marker for mature astrocytes, GFAP, is also expressed in some neural stem cells, astrocyte progenitors and cortical astrocytes (Doetsch, Caille et al. 1999). This in addition to the fact that it is exceptionally intricate to culture adult cortical astrocytes and that retroviral expression vectors infect only proliferating cells leads to uncertainties in studying terminally differentiated astrocytes and biasing towards immature mitotic astrocytic progenitors. However, induced pluripotent stem cell (iPSC) technology, where genetic cocktails of a few transcription factors can transfer fibroblasts into pluripotent cells bordering on ES cell plasticity (Okita and Yamanaka 2006; Okita, Ichisaka et al. 2007; Takahashi, Okita et al. 2007; Wernig, Meissner et al. 2007; Nakagawa, Koyanagi et al. 2008) has given credibility to the theory of an *in vivo* dedifferentiation of mature astrocytes.

The theory that “restricted” unipotent neural progenitors in the adult brain could be the source of glioma stem cells is supported by the fact that committed oligodendroglial progenitors can be reprogrammed *in vitro* (Kondo and Raff 2000). This reprogramming results in chromatin remodeling and reactivation of the stem cell marker Sox2 (Kondo and Raff 2004). Similar mechanisms might be implicated in reprogramming of neural progenitors in view of the fact that Sox2 is frequent in human gliomas (Schmitz, Temme et al. 2007). Within the cycling neural progenitor pool resides the multipotent NG2 progenitor population, which are cells expressing the chondroitin sulfate proteoglycan, NG2 (Levine and Stallcup 1987; Diers-Fenger, Kirchhoff et al. 2001); (Belachew, Chittajallu et al. 2003; Liu, Han et al. 2007). It has been reported that NG2 cells can differentiate into astrocytes and neurons *in vitro* (Liu, Han et al. 2007) and a

vast majority of the cells express Olig2. Theoretically, it is possible that NG2 cells could be the source of glioma stem cells.

### **1.3 LUMINESCENT POLY- AND OLIGOTHIOPHENES**

#### **1.3.1 Conjugated polymers and oligomers**

Polymers and oligomers consist of repeating structural units, monomers. The difference between the two lies in the number of monomers that the molecule is made up of. Oligomers are composed of only a few monomers while polymers in theory can consist of an unlimited amount of monomers. The physical properties of oligomers are readily altered by small modifications in monomer number. In contrast, adding or removing monomers from the backbone of polymers leads to minor effects their physical properties. The Nobel Prize in chemistry was awarded to Alan J. Heeger, Alan G. Macdiarmid and Hideki Shirakwa in 2000 ‘*for the discovery and development of electrically conductive polymers*’. The remarkable discovery that polymers, commonly used as insulators, can be made conductive has lead to the development of flat screen technology, solar cells and electromagnetic shielding of computer. Conjugated polymers have a polymer backbone with alternating single and double bonds and can be used in many applications such as solar cells and display screens. Apart from giving the molecule its rigidity, the single and double bonds also contribute to the ability of the molecule to absorb and emit light as well as transporting electrical charges. These events occur as electrons or holes, moving along the backbone and between molecules.

#### **1.3.2 Luminescent poly- and oligothiophenes for recording biological events**

Luminescent conjugated polymers are semiconductive and were first reported in 1990 (Burroughes, Bradley et al. 1990). Absorption of a photon happens when an electron is excited from its ground state to an excited state and relaxation of the electron back to the ground state can lead to light emission. The difference in energy between the absorption peak and the emission peak is called the Stokes shift. Luminescent conjugated polymers and oligomers commonly have a large Stokes shift, which is beneficial as it reduces noise from the excitation light source in the emission spectra (Aslund, Nilsson et al. 2009). Luminescent conjugated oligothiophenes (LCOs) and luminescent conjugated polythiophenes (LCPs) have the ability to adopt different

geometrical shapes as a result of their flexible backbone. A twist in the backbone gives a blue shift in color whereas a planar configuration leads to a red shift (Leclerc 1999; Ho, Dore et al. 2005). Utilizing these features, LCPs have been used for detection of hybridized, double-stranded DNA (dsDNA) (Ho, Boissinot et al. 2002; Ho, Najari et al. 2008). Due to the flexibility of its backbone, the LCP can adopt twisted or planar configurations, yielding color shifts visible for the eye. Initially, when free in solution the LCP had a twisted configuration, mixing it with a single-stranded DNA (ssDNA) strand the configuration changed to planar and there was a color shift from yellow to red. Adding an additional complementary ssDNA strand to the solution led to the formation of a double-stranded oligonucleotide giving the LCP a twisted configuration again and the color changed back to yellow. Further, LCPs and LCOs can be used as optical tools to visualize peptide conformation (Nilsson, Rydberg et al. 2003; Nilsson, Rydberg et al. 2004) and specific proteins (Ho and Leclerc 2004). Further, it has been shown that LCPs can be used to distinguish between native and fibrillated proteins *in vitro* (Herland, Nilsson et al. 2005; Nilsson, Herland et al. 2005). Formation of protein aggregates is associated with many neurodegenerative conditions such as Alzheimer's, Parkinson's and prion diseases. These and other protein aggregation diseases can be studied utilizing LCP/ LCO technology. In 2006, lucid patterns of amyloid deposits were stained in *ex vivo* pancreas islet amyloids on tissue slides (Nilsson, Hammarstrom et al. 2006). The same group has shown plaque formation staining by LCPs in brain sections from a transgenic mouse model of Alzheimer's disease (Nilsson, Aslund et al. 2007).

## 1.4 THE TRANSCRIPTIONAL CO-REPRESSOR CtBP

### 1.4.1 CtBP family proteins

C-terminal binding proteins (CtBPs) are highly conserved proteins that were discovered in 1993 when CtBP1 was found as a 48 kD cellular phosphoprotein that bind to the C-terminus of the adenovirus E1A oncoprotein (Boyd, Subramanian et al. 1993; Schaeper, Boyd et al. 1995). Transcriptional activation and oncogenesis of E1A is repressed by CtBP (Sollerbrant, Chinnadurai et al. 1996). Identification and cloning of the *Drosophila* homolog of CtBP (dCtBP) further strengthened the role of CtBP as a transcriptional repressor (Nibu, Zhang et al. 1998; Poortinga, Watanabe et al. 1998). The genome of the vertebrate contains two distinct gene loci (CtBP1 and CtBP2) while the genome of plants and invertebrates contain only one. CtBPs play essential roles in

animal development. Mammalian CtBP1 and CtBP2 can be functionally redundant and perform unique activities during development (Katsanis and Fisher 1998; Hildebrand and Soriano 2002). In mice, CtBP1 and CtBP2 encompass diverse regulatory roles and differ in expression and cellular localization.

The *CtBP1* gene is expressed throughout embryogenesis and into adulthood whereas *CtBP2* is expressed in the forming placenta and is limited to embryogenesis (Furusawa, Moribe et al. 1999). During development of the mouse embryo, CtBP1 is significantly expressed in the spinal cord, while CtBP2 is expressed in cephalic and dorsal root ganglia, the limb buds as well as the spinal cord. Another difference is that while CtBP1 is both cytoplasmic and nuclear, CtBP2 is almost solely found in the nucleus (Zhao, Subramanian et al. 2006). Both CtBP1 and CtBP2 are widely expressed in most human tissues (Sewalt, Gunster et al. 1999). Located on chromosome 4 in humans (chromosome 5 in mice) the *CtBP1* gene expresses two major transcripts in consequence of alternate RNA splicing, encoding two isoforms of CtBP1 (CtBP1-S and CtBP1-L). Apart from an amino acid region in the N-terminus the isoforms are identical. The *CtBP2* gene codes for three protein isoforms and is located on chromosome 10 in humans (chromosome 7 in mice). The three isoforms are CtBP2-S (Katsanis and Fisher 1998), CtBP2-L (Verger, Quinlan et al. 2006) and RIBEYE. The third isoform, RIBEYE has a large N-terminal domain (unrelated to CtBP) and is mostly expressed in sensory neurons (Schmitz, Konigstorfer et al. 2000; Zenisek, Horst et al. 2004).

#### 1.4.2 CtBP in transcriptional repression

In an early study it was shown that deletion of the CtBP-binding domain in E1A increased its transcriptional activity and was the first evidence for CtBP as a repressor of transcription (Sollerbrant, Chinnadurai et al. 1996). The identification and cloning of *Drosophila* CtBP (dCtBP) initiated several studies giving CtBP its definitive role as a mediator of transcriptional repression. During embryonic development of *Drosophila*, dCtBP has been shown to interact with repressors as Knirps, Snail, Krüppel and Hairy and function as a transcriptional co-repressor (Nibu, Zhang et al. 1998; Nibu, Zhang et al. 1998; Poortinga, Watanabe et al. 1998). Moreover, CtBP has been found in complex with a number of DNA-binding transcription factors involved in processes and pathways such as Wnt and BMP/TGF $\beta$  signaling (Melhuish and Wotton 2000; Izutsu,

Kurokawa et al. 2001). Other proteins shown to interact with CtBP include CoREST, histone methyltransferases, and a polyamine oxidase (LSD1) (Shi, Sawada et al. 2003). In addition to interacting with DNA-binding proteins, CtBPs have been shown to interact with polycomb group proteins suggesting a role in chromatin remodeling and transcriptional repression (Sewalt, Gunster et al. 1999). Transcriptional repression mediated by CtBP seems to act both in HDAC (histone deacetylase) dependent and independent manner. Several *in vitro* and *in vivo* studies have revealed interaction between CtBP and numerous HDACs (Sundqvist, Sollerbrant et al. 1998; Koipally and Georgopoulos 2000; Dressel, Bailey et al. 2001). The HDAC inhibitor Trichostatin A can block the CtBP dependent repression (Criqui-Filipe, Ducret et al. 1999; Izutsu, Kurokawa et al. 2001). Conversely, CtBP dependent repression can take place even in the presence of trichostatin A (Koipally and Georgopoulos 2000; Sundqvist, Bajak et al. 2001). Taken together, these data indicate that CtBP can employ various mechanisms in its role as a transcriptional regulator.

Mutations in CtBP1 and CtBP2 in mouse models have indicated both unique and redundant roles for the two genes during mouse embryogenesis (Hildebrand and Soriano 2002). CtBP1 null mice are viable and fertile although 30% smaller than wild type and heterozygous littermates. Additionally, 23% of the homozygous mice die by postnatal day P20. In contrast, CtBP2 null mice are not viable and die by embryonic day E10.5. These embryos are smaller in size, have axial truncations, delayed neural development and defects in heart morphogenesis. Some of these malformations might be due to reduced levels of Brachyury expression; a direct target of Wnt3A, suggesting CtBP2 to be a regulator of Wnt mediated signaling *in vivo*.

#### 1.4.3 Regulation of CtBP

Nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) is a dinucleotide found in all living cells and is involved in metabolic redox reactions. When  $\text{NAD}^+$  accepts electrons from other molecules, it becomes reduced and the reaction forms NADH, which in turn can donate electrons and acts as a reducing agent. This process is also involved in Adenosine-5'-triphosphate (ATP) synthesis. Additionally,  $\text{NAD}^+/\text{NADH}$  levels can regulate genes through energy homeostasis, demonstrated for instance by the  $\text{NAD}^+$ -dependence of the histone deacetylase, Sir2 (silencing information regulator 2) (Imai, Armstrong et al.

2000; Landry, Sutton et al. 2000). Relatively little is known about how CtBP is regulated. The ability of CtBP to bind NADH is possibly a way for CtBP to be modulated by cellular energy homeostasis. It has been proposed that by detecting changes in nuclear NAD<sup>+</sup>/NADH ratio, CtBP can act as a transcriptional redox sensor (Zhang, Piston et al. 2002; Fjeld, Birdsong et al. 2003). Mimicking hypoxic environment through chemical treatment increases the levels of nuclear NADH. This increase was shown to enhance the binding of CtBP with target transcription factors and resulted in elevated repression of transcription (Zhang, Piston et al. 2002). In a different study it was shown that 2-Deoxy-D-glucose (2DG), a glycolytic inhibitor, blocked expression of a number of neuronal genes in a CtBP dependent manner (Garriga-Canut, Schoenike et al. 2006). The 2DG associated decrease in NADH was shown to lead to an increase in recruitment of CtBP2 by the neuronal repressor neuron restrictive silencing factor (NRSF, also known as REST (repressor element 1 silencing transcription factor)), a key regulator of pluripotency and neuronal differentiation. In a different study, CtBP was shown to have an opposite effect on repression after 2DG treatment (Zhang, Wang et al. 2007). This study demonstrated that 2DG treatment leads to decreased interaction between CtBP and the tumor suppressor HIC1 as a result of decreased NADH levels resulting in SIRT1 (mammalian homolog of Sir2) expression. Opposing effects of hypoxia on SIRT1 expression was observed due to increased free nuclear NADH concentration. Together these studies suggest that NADH might regulate CtBP differently depending on the transcriptional regulator that recruits CtBP.

## 1.5 INKJET PRINTING

### 1.5.1 Inkjet technologies

The mechanism by which a liquid stream breaks into droplets was first described in the end of the 1870s (Rayleigh 1878). Siemens Elema in Sweden patented the first inkjet device based on this principle 70 years later. The technique was further refined in the 1960s by technology from Stanford University and was known as continuous printing (Sweet 1965). The next decade IBM implemented continuous printing for their computer printers. Concurrently, researchers at Lund Institute of Technology in Sweden independently developed continuous inkjet printers. Continuous inkjet technology is very fast but expensive and difficult to operate. This technology is therefore mostly utilized in high-speed industrial printers. In late 1970s, the drop-on-demand printing technology was invented and further developed. As the name suggests,

droplets are dispersed only when needed, increasing the consistency of inkjet printing technology. The drop-on demand technology can be categorized based on how the drop formation is processed and the major methods used are: thermal, piezoelectric, electrostatic and acoustic inkjet. Canon developed the bubble jet technology in 1979; these were a new generation of printers that ejected ink from the nozzle with the help of a water vapor bubble. Hewlett-Packard launched their version a few years later and named it Thinkjet (thermal inkjet). The Thinkjet printheads were inexpensive and could therefore be used as disposable units. The following decades inkjet printing techniques were refined in order to achieve higher resolution, lower cost and increased printing speed.

### 1.5.2 Inkjet printing for biological applications

Inkjet printing has, apart from its conventional application as a personal or office tool, also been used to print electronic materials and biological molecules (Collier, Janssen et al. 1996; Lemmo, Rose et al. 1998; Hughes, Mao et al. 2001; Wang, Zheng et al. 2004). More than 20 years ago inkjet printing was for the first time used to print cell adhesion proteins and monoclonal antibodies onto substrates in order to control cell adherence (Klebe 1988). In this initial study, the ink of a Hewlett-Packard printer was replaced with selected cell adhesion proteins in order to form positive or negative patterns of cells. Immobilized patterns of growth factors in two dimensions have been proven to be a viable approach to study cellular response to spatially defined growth factor concentrations using techniques such as photolithography (Ito, Kondo et al. 1997; Ito, Chen et al. 1998) and microcontact printing (Bernard, Delamarche et al. 1998; Kane, Takayama et al. 1999) as well as inkjet printing (Turcu, Tratsk-Nitz et al. 2003; Watanabe, Miyazaki et al. 2003; Sanjana and Fuller 2004; Campbell, Miller et al. 2005). Interestingly, inkjet technology can also be used to print viable cells, which opens the possibility of three-dimensional layering to create structures (Sanjana and Fuller 2004; Nakamura, Kobayashi et al. 2005; Xu, Jin et al. 2005; Eagles, Qureshi et al. 2006; Ringeisen, Othon et al. 2006). Moreover, DNA molecules have been printed on glass substrates for the purpose of high density DNA microarrays (Okamoto, Suzuki et al. 2000; Hughes, Mao et al. 2001). The conventional technology for making protein or DNA chips includes photolithography (Southern 1996; Gao, LeProust et al. 2001) and pin arrays (Shalon, Smith et al. 1996; Haab, Dunham et al. 2001; Wilson and Nock 2002). With the use of inkjet technology, computer programs can easily generate

patterns as well as creating gradients to manipulate the concentration of the printed bioink. Other advantages include the ability to work with hydrated samples, the high-throughput capacity and the possibility to print multiple samples simultaneously. However, the resolution is lower in inkjet printing (100  $\mu\text{m}$ ) compared to micro-contact printing (less than 100 nm) (Pardo, Wilson et al. 2003; Renault, Bernard et al. 2003).

## 1.6 SUBSTRATE STIFFNESS

### 1.6.1 Mechanosensing

The roles of the extra cellular matrix (ECM) was historically believed to merely be to provide structural support to tissues and act as a scaffold for cells to bind to. It is now recognized that cells receive signals from the ECM, dictating their behavior. The extracellular matrices of tissues are composed of many components, which together cooperate to convert internal and external mechanical cues into alterations in tissue configuration and function. This process is called mechanochemical transduction and is necessary during development and important throughout adulthood for bodily movements. Many cellular and extracellular components are responsive to mechanical stimuli such as fluid shear stress, pressure and elongation. Cells can sense and respond to external mechanical stimuli in multiple ways. All eukaryotic cells are subject to direct mechanical stretching on integrin binding sites at the cell surface. Conformational changes in the ECM lead to changes in integrin structure and subsequent activation of secondary messenger pathways within the cells. The effects of activating such pathways may cause changes in cell division and alterations in genes regulating ECM proteins. Similar pathways are involved when gap junctions with calcium sensitive stretch receptors are deformed, leading to transduction of mechanical signals through activation of secondary messengers. Additionally, mechanochemical transduction occurs upon activation of cell membrane ion channels.

Tension within the cell membrane walls is created thanks to cellular focal adhesion points that anchor to their surrounding substrate (Beningo and Wang 2002). The cell generates tension differently depending on the properties of the surrounding matrix. A stiff matrix opposes the pulling force of the cell, causing rigidity and extension in the periphery of the cell (Schwarz, Balaban et al. 2002). These forces generated by cells are by means of mechanosensing converted into signals leading to cell migration, proliferation and differentiation.

### 1.6.2 Cellular response to substrate stiffness

Early studies showed that mesenchymal cells changed shape depending on the viscosity of their surrounding medium (Weiss and Garber 1952). The possibility to visualize cytoskeletal filaments by fluorescence lead to the observation that the structure and assembly of the cytoskeleton was different when cells were grown on glass as compared to collagen gels (Tomasek, Hay et al. 1982). In a different study, single cells were grown on islands of adhesive squares and formed bundles of actin filaments diagonally along the cells (Marek, Kelley et al. 1982). Fibroblasts have been reported to display improved spreading with increased substrate stiffness (Keese and Giaever 1991). Cell proliferation has been reported to depend on substrate stiffness in several studies, showing that soft materials suppress proliferation (Wang, Dembo et al. 2000; Paszek, Zahir et al. 2005; Hadjipanayi, Mudera et al. 2009; Winer, Janmey et al. 2009). The mechanisms behind this observation still need to be elucidated, thus clarifying *in vivo* diseases and cellular dysfunctions. The fact that cells are able to apply traction forces to their substrates has been shown to have implications in cellular motility, cytoskeleton organization, proliferation, differentiation and protein secretion. Fibroblast cell migration has been proven to be slower on stiff substrates compared to soft gels. However, when presented with a boundary between stiff and soft materials, cells seem to prefer to migrate towards the stiffer areas (Lo, Wang et al. 2000). This process, termed durotaxis, has also been observed in three dimensional collagen matrices as cells migrate to the stiff side of the matrix (Hadjipanayi, Mudera et al. 2009). Nevertheless, the impact of stiffness on cell behavior seems to be cell type specific. As fibroblast (Gray, Tien et al. 2003) and epithelial cells (Saez, Ghibaudo et al. 2007) prefer to migrate toward stiffer regions, prostate carcinoma cells migrate in the direction of decreasing substrate stiffness (Zaman, Trapani et al. 2006).

Neurons extend their neurites further and faster with decreased substrate stiffness (Flanagan, Ju et al. 2002; Kostic, Sap et al. 2007; Jiang, Yurke et al. 2008). Bone marrow derived mesenchymal stem cell (MSC) differentiation is dependent on substrate stiffness demonstrated by growing MSCs on gels with a wide range of elasticities. The stiffest substrates supported MSCs expressing osteogenic markers, cells on the intermediate gels expressed myogenic markers and cells on the softest substrates expressed neuronal markers. This demonstrates the importance of

appropriate stiffness matching. However, there are studies showing that stiffness *per se* is not sufficient to fully differentiate cells, but augments the rate of differentiation in concert with chemical induction factors (Hsiong, Carampin et al. 2008; Saha, Keung et al. 2008; Winer, Janmey et al. 2009).

Matching substrate stiffness with *in vivo* tissue elasticity has proven to be successful for maturation of neurons. For example, neurite branching is increased with decreasing substrate stiffness in primary neurons (Flanagan, Ju et al. 2002) and PC12 cells (Gunn, Turner et al. 2005). A negative correlation between increasing stiffness and axonal length as well as expression of focal adhesion kinase (FAK) was observed (Jiang, Yurke et al. 2008). A difference between neurons and glia was reported with less glial growth on soft substrates in contrast to neurons growing well even on the softest substrates (Flanagan, Ju et al. 2002; Georges, Miller et al. 2006). Co-cultures of astrocytes and neurons grown on soft gels supported approximately 80%  $\beta$ 3-tubulin expressing cells as compared to less than 45% when grown on stiff substrates (Flanagan, Ju et al. 2002; Georges, Miller et al. 2006). Neurons were observed to grow only on top of glia on stiff substrates but could grow independently on the soft gels, partly explaining the difference in number of neurons. Finally, substrate stiffness has great impact on both adult (Saha, Keung et al. 2008) and embryonic (Teixeira, Ilkhanizadeh et al. 2009) NSC differentiation.

Intracellular signaling may be regulated through substrate stiffness by means of ECM-integrin-actin connections through mitogen activated protein kinase (MAPK) pathways. The activity of MAPK has been positively correlated with increased substrate stiffness and increased osteogenic gene expression in osteoblasts (Khatiwala, Peyton et al. 2007). Regulation of calcium levels by RhoA (Ras homolog gene family, member A) and its effector RhoA kinase (ROCK) may also be affected by substrate stiffness. This is shown by reduced RhoA activity on soft gels as well as inhibition of calcium oscillation frequency by ROCK, demonstrating that substrate stiffness regulated calcium oscillations may be mediated through RhoA and ROCK (Kim, Seong et al. 2009).

## **2 AIMS OF THE THESIS**

The overall aim of the work presented in this thesis was to molecularly detect neural stem cells and to engineer microenvironmental cues in order to control their genetic and epigenetic programs.

*Specific aims listed according to each paper:*

- I. Live detection and staining of neural stem cells and cancer derived stem cells by a novel molecule.
- II. Elucidate the roles of CtBP co-repressor activities in neural stem cell fate decisions by means of oxygen and BMP levels.
- III. Using inkjet printing technology to obtain functional immobilized patterns of printed proteins to steer neural stem cell differentiation.
- IV. Manipulating neural stem cell differentiation by substrate stiffness.

### **3 RESULTS AND DISCUSSION**

#### **3.1 PAPER I**

Neural stem cells (NSCs) dissociated from the telencephalon of E15.5 rat embryos form uniform cultures of nestin positive cells, indicating neural stem cell identity (Lendahl, Zimmerman et al. 1990), when grown on plates coated with poly-L-ornithine and fibronectin in culture medium supplemented with fibroblast growth factor (FGF2) (Teixeira, Duckworth et al. 2007). Upon mitogen withdrawal, NSCs differentiate into a mixed population of neurons, astrocytes and oligodendrocytes (Johe, Hazel et al. 1996). Soluble factors in the culture medium can induce NSC differentiation into the neuronal subtypes as well as smooth muscle cells (Johe, Hazel et al. 1996; Teixeira, Duckworth et al. 2007).

Oligothiophenes are molecules in which two or more thiophene rings are linked together. These compounds possess interesting optical properties as they can fluoresce and emit light upon excitation. We have used two oligothiophene probes, p-HTMI and p-HTES, similar in structure but targeting cells differently. In order to characterize p-HTMI and p-HTES in a NSC context, we utilized several differentiation protocols to obtain a variety of cell types derived from NSCs. Astrocytic differentiation was induced by CNTF treatment, cells differentiated into smooth muscle cell by FBS treatment and a combination of BMP4 and Wnt3a induced neuronal differentiation. In undifferentiated NSCs, p-HTMI generated fluorescence at a wavelength common to green fluorescent proteins, accumulated in the cytoplasm of the cells, within 10 minutes after application of the molecule. The differentiated cells displayed a significantly lower or no signal. Conversely, p-HTES did not target undifferentiated NSCs in any way; a weak signal was however obtained in fully differentiated smooth muscle cells and mature astrocytes. Fluorescence activated cell sorting (FACS) is commonly used to quantitatively record fluorescent signals from individual cells and to separate cells based on fluorescence. We stained FGF2 treated undifferentiated NSCs with either p-HTMI or p-HTES and used FACS to sort unstained cells, p-HTMI stained cells and p-HTES stained cells. p-HTES stained cells displayed only background fluorescence, comparable to unstained cells. Interestingly, p-HTMI stained cells generated a clear fluorescence peak that did not overlap with background fluorescence levels. In order to control for cell-to-cell leakage of the probes we stained cells separately with p-HTMI

and p-HTES. Next, the cells were mixed and sorted with FACS. Two distinct fluorescence peaks were generated, proving that there was no cross contamination of the probes. These results show that p-HTMI selectively stains undifferentiated NSCs in contrast to p-HTES that displays weak staining in mature astrocytes and smooth muscle cells.

We have used rat C6 glioma cells as an experimental model system. It has previously been shown that glioma stem cell lines maintained as adherent cultures display stem cell properties and harbor tumor-specific characteristics (Pollard, Yoshikawa et al. 2009). In order to put the stem cell properties of C6 glioma cells to test, we added FGF, CNTF, valproic acid (VPA) or FBS to the culture medium of cells grown in DMEM supplemented with 10% FBS. Immunocytochemistry showed that C6 glioma cells are not plastic and do not differentiate into any alternate fates. We hypothesized that C6 glioma cells grown as NSCs would dedifferentiate and obtain stem cell characteristics. We tested this hypothesis by growing the C6 glioma cells with the same protocol as NSCs. C6 glioma cells were cultured on poly-L-ornithine and fibronectin coated plates and in N2 medium with supplements. The C6 derived NSCs (C6NSCs) grown in the presence of FGF2 formed uniform layers of nestin positive cells and CNTF treatment induced astrocytic differentiation (immunoreactive to GFAP). VPA induced TuJ1 expressing cells, indicating neuronal identity. Consistently, mRNA levels of GFAP were elevated upon CNTF treatment and Tubb3 mRNA levels were elevated upon VPA treatment. Therefore, C6 glioma cells grown as NSCs acquire stem cell characteristics.

The percentage of cancer stem cells in the C6 glioma cell line is controversial, some studies show that 1-4% of the C6 glioma cells are cancer stem cells (Kondo, Setoguchi et al. 2004; Setoguchi, Taga et al. 2004) whereas other studies claim that all cells in the C6 glioma cell line are cancer stem cells (Zheng, Shen et al. 2007). When staining C6 glioma cells with p-HTMI, we observed staining in 1-2% of the cells. The stained cells were smaller and more round in their shape. Conversely, p-HTES stained the majority of the C6 glioma cells. We further stained C6NSCs grown in the presence of FGF2 by either p-HTMI or p-HTES. All cells stained by p-HTMI displayed a strong green signal; wheras p-HTES did not stain any cells. These results indicate that p-HTMI can stain a subpopulation of C6 glioma cells and that these cells might be cancer stem cells.

Moreover, p-HTMI stains all FGF2 treated C6NSCs. In contrast, p-HTES stains the majority of C6 glioma cells but does not stain any C6NSCs.

It has previously been shown that conjugated polymers can be used as optical probes for studying biological processes and structures (Ho, Boissinot et al. 2002; Nilsson and Inganäs 2003; Nilsson, Rydberg et al. 2003; Dore, Dubus et al. 2004; Nilsson, Rydberg et al. 2004; Herland, Nilsson et al. 2005; Nilsson, Herland et al. 2005; Aslund, Herland et al. 2007; Nilsson, Aslund et al. 2007). Most of these studies were generally done in clear-cut systems with the molecule of interest dissolved in a buffer. The first study using polythiophene probes in cultured cells targeted cellular DNA in chromatin as well as nuclear and cytoplasmic vesicles in fibroblasts, macrophages and leukocytes (Bjork, Peter et al. 2007). The probes used in the study could not target cytoplasmic vesicles in transformed cells such as melanoma or neuroblastoma cell lines. Additionally, the study presented an oligothiophene probe for live staining of cells, visualizing the actin skeleton of fibroblasts. In our study, we show for the first time specific live molecular detection of neural stem cells and cancer stem cells by a novel oligothiophene probe.

### 3.2 PAPER II

C-terminal binding proteins (CtBPs) are transcriptional co-regulators with repressive activity. Members of the CtBP family have been implicated both in repression of neuronal gene expression, by their association to CoREST (Wang, Scully et al. 2007), as well as promotion of neuronal differentiation as silencers of Notch target genes (Oswald, Winkler et al. 2005). We first explored CtBP1 and CtBP2 expression pattern in the developing chick spinal cord, observing a gradient of expression along the dorsal ventral axis of the spinal cord (Hamburger Hamilton stage 17, HH17). Additionally, an elevation in expression was observed in the dorsal region extending to anterior regions of the neural tube, including the chick hindbrain and the developing choroid plexus in mouse. Interestingly, CtBP1 and CtBP2 were expressed both in Sox3 positive cells and in TuJ1 positive cells, suggesting a role in both progenitor cells and postmitotic neurons. A fusion protein of CtBP1 and the Herpes Simplex transactivation domain VP16 (VP16-CtBP1) activates CtBP target genes (van Grunsven, Michiels et al. 2003). We used either full length CtBP1 or VP16-CtBP1 to electroporate stage 10 chick neural tubes and observed immense alterations, 24h after electroporation (24hae), in the morphology of the neural tube. The electroporated half of the neural tube displayed

severe thinning compared to the non-electroporated side. The VP16 labeled cells were negative for the neuronal progenitor marker Sox3 but expressed the neuronal markers NeuN and TuJ1. Conversely, on the control side, few cells expressed NeuN and most cells were Sox3 positive. Over-expression of CtBP1 did not change the size of the neural tube but induced a consistent decrease in post-mitotic neurons. It has been reported that CtBPs have a role in pro-apoptotic gene programs (Grooteclaes, Deveraux et al. 2003). However, we could not observe a significant change in the number of cleaved caspase-3, a marker for cells undergoing apoptosis, positive cells by electroporation of either VP16-CtBP1 or CtBP1. Hence, CtBP does not seem to be involved in the regulation of pro-apoptotic gene programs in the early chick neural tube.

Misexpression of VP16-CtBP1 in the non-neurogenic dorsal-most region of the chick neural tube induced expression of NeuN and TuJ1 24hae. In addition, the premigratory neural crest marker, Slug, was lost. Over-expression of CtBP1 did not induce misexpression of neuronal markers in the roof plate. Thus, activating genes regulated by CtBP caused a premature differentiation of neural progenitors throughout the dorsoventral axis of the spinal cord and induced neuronal fates in the non-neurogenic domains. The fact that the roof plate holds high levels of BMPs lead us to study the CtBP regulation of NSCs exposed to BMP signaling. NSCs cultured at low densities differentiate mainly into astrocytes and smooth muscle cells, in the presence of BMP4. We show that nuclear CtBP1 and CtBP2 are expressed endogenously by NSCs. An increase in TuJ1 positive cells was observed upon nucleofection of VP16-CtBP1 but not by over-expression of CtBP1. Similarly, mRNA levels of Tubb3 were up-regulated upon VP16-CtBP1 over-expression. However, over-expression of CtBP1 did not induce alterations of Tubb3 but instead lead to a clear induction of smooth muscle actin (SMA). Moreover, we knocked down CtBP1 and CtBP2 by siRNA and observed an increase in the expression of Tubb3, mimicking the effects of VP16-CtBP1 over-expression. Together, these results indicate that CtBP activity is required for NSC ability to undergo non-neuronal differentiation in the context of high BMP levels.

Hes1 is a repressor of neurogenic genes and is highly expressed in the dorsal neural tube during development (Baek, Hatakeyama et al. 2006). Hes genes have been implicated in the repression of pro-neural bHLH transcription factors in the choroid plexus, maintaining a non-neural region (Imayoshi, Shimogori et al. 2008). The neural

progenitor domain ventrally abutting the roof plate gives rise to the dorsal-most population of spinal cord interneurons (DI1) and express the pro-neural bHLH transcription factor Math1 (Atoh1). Math1 over-expression resembles VP16-CtBP1 misexpression in the chick spinal cord, promoting neuronal differentiation and preventing neural crest formation (Ebert, Timmer et al. 2003). We could confirm the presence of CtBP1 in the Hes1 complex by performing GST-CtBP1 pull-down. Additionally, CtBP1 and Hes1 were both detected in the promoter of Math1, in a region that presents Hes binding sites. siRNA knockdown of CtBP1 and CtBP2 in BMP4 treated NSCs induced Math1 expression. We used the  $\gamma$ -secretase inhibitor DAPT in order to decrease Hes1, leading to an increase in Math1 expression. Moreover, Hes1 over-expression could not rescue the induction of Math1 by CtBP knockdown, suggesting that Hes1 repression of Math1 is dependent on CtBP availability. These results indicate that CtBP is required for Hes mediated CtBP repression of Math1 in the context of high BMP signaling.

To further investigate the roles for CtBP proteins in the chick spinal cord, we knocked down multiple CtBP proteins simultaneously. An increase in Math1 was observed upon knockdown of CtBP1, CtBP2 and cCtBP3 (a novel protein showing high homology to CtBP1) in the chick spinal cord. This shows that CtBP acts as a repressor of Math1 expression both in NSCs and in the chick dorsal spinal cord. Surprisingly, in the more ventral parts of the neural tube, CtBP knockdown caused downregulation of the pro-neural bHLH transcription factors Mash1 and Ngn2. These observations dispute the model of CtBP as a general repressor of neuronal differentiation.

Our study establishes CtBP as both a repressor and promoter of pro-neural bHLH transcription factor expression. The promoter occupancy of CtBPs determines their role in cell fate decisions and is set by the cellular context. It has previously been shown that the repressive activity of CtBP can be regulated by NADH levels (Zhang, Piston et al. 2002). Cells exposed to hypoxic conditions translate into an elevated cellular content of NADH. We have used EF5, a compound that acts as a sensor of hypoxia, in the chick spinal cord in order to establish the oxygen tension profile. A dorsal to ventral gradient of oxygen in the dorsal neural tube was observed, following the BMP gradient. Interestingly, the Math1 domain displayed higher levels of oxygen than the more ventral parts. Hence, oxygen levels may regulate CtBP activity and induce opposing roles for CtBP in the regulation of pro-neural genes. BMP4 treated NSCs exposed to

hypoxic conditions promote the recruitment of CtBP to the Hes1 promoter, suggesting that CtBPs promote neuronal differentiation in hypoxia. This observation was further confirmed by up-regulation of Tubb3 levels in BMP4 treated NSCs exposed to hypoxia. Additionally, NSCs exposed to hypoxia hampered BMP4 mediated mesenchymal differentiation of NSCs.

The roof plate is a signaling center that expresses both BMPs and Wnts and plays a significant role in patterning subpopulations of neuronal progenitors. Our study proves that CtBP co-repressor activity is crucial for the exclusion of neurogenesis from this domain. We replicated these results in NSCs exposed to BMP signaling, suggesting a general role for CtBP to inhibit neuronal differentiation in BMP rich environments. Furthermore, we have shown that these repressive effects were mediated by repression of pro-neural bHLH transcription factors. The Notch target Hes1 is a repressor of bHLH proteins and is strongly expressed in the dorsal spinal cord and in BMP4 treated NSCs. We show that both Hes1 and CtBP1 are present in promoter regions of Math1. Moreover, we have shown that microenvironmental variables orchestrate the promoter occupancy of CtBPs, determining its definitive role as pro-neural or anti-neuronal. Hes levels are elevated upon BMP treatment, leading to recruitment of CtBP to the Math1 promoter. Our study demonstrates that CtBP molecularly integrates microenvironmental levels of BMP and oxygen and that these signals are interpreted through Notch mediated regulation of anti-neuronal and pro-neuronal genes.

### 3.3 PAPER III

In order to obtain spatial control of NSC differentiation inkjet printing was used to immobilize extrinsic factors onto a hydrogel matrix. We modified a BJC-2100 Canon printer to print biologically active proteins onto poly-acrylamide-based hydrogel coated glass slides (Hydrogel<sup>TM</sup>-coated slides from PerkinElmer). After extensive cleaning of the ink cartridges, the ink was substituted with extrinsic factors in solution. The printer was computer controlled and PowerPoint was used to print patterns. Cy5-conjugated protein was used to print the word “JET” (Arial, 8 pt) and subsequently imaged by fluorescence microscopy. Visualization of the printed pattern demonstrated the feasibility of protein printing using this set up and revealed the potential for inkjet printing of extrinsic factors onto hydrogels for guiding stem cell differentiation. Due to clear cell fate readouts when added to NSC cultures (Johe, Hazel et al. 1996;

Hermanson, Jepsen et al. 2002), FGF2 and CNTF were chosen as model extrinsic factors in this application. As stated earlier in this thesis, FGF2 maintains NSC identity and CNTF induces astrocytic differentiation, identified by immunoreactivity to nestin or GFAP respectively. HydroGel<sup>TM</sup>-coated glass slides were printed with FGF2 and CNTF on bordering areas subsequently seeded with NSCs and incubated for 22h. Interestingly, GFAP positive cells could only be found on the side printed with CNTF as assessed by immunocytochemistry. The FGF2 printed side displayed only nestin positive cells and a clear spatial threshold of GFAP expressing cells was obtained. The CNTF printed side harbored 12-16% GFAP positive cells indicating that the inkjet printed proteins were immobilized and biologically active yielding in an appropriate cellular response.

Further exploring the possibilities of inkjet printing of bioinks was put to the test by printing a grayscale pattern of Cy5-conjugated transferrin. The gradient could be visualized in the microscope and was shown to consist of dots with large dots at high concentration end of the gradient, getting progressively smaller and more parse towards the more diluted part of the gradient. In order to evaluate the degree of protein immobilization, the printed slide was immersed in culture medium and incubated for 22h at 37°C. The gradient pattern was clearly visible 22h after incubation indicating partial immobilization of the protein. Next, a gradient of CNTF was printed using the same grayscale image in order to obtain a gradient of NSC differentiation. The underlying printed CNTF induced a gradient of GFAP positive cells, decreasing linearly from 16% to 4%. Additionally, FBS was printed in a uniform layer on the hydrogel in order to induce smooth muscle cell differentiation of NSCs. After 22h of incubation, cells were expressing smooth muscle actin (SMA). Adjacent areas of FGF2 and FBS were printed as well, inducing SMA expressing cells only on the side printed with FBS. The results so far show that inkjet printed proteins are biologically active, that NSCs respond to these and that multiple cell fates can be obtained simultaneously by printing different extrinsic factors. Next, we wanted to test if genetically modified NSCs could grow on hydrogels and respond to printed extrinsic factors. A construct containing the SV-40 promoter driving the expression of the smooth muscle specific gene SM22 fused to green fluorescence protein (GFP), peGFP-hSM22, was used to nucleofect NSCs. The nucleofected NSCs were then seeded on a FBS printed hydrogel slide and incubated for 22h. An expression of the SM22 gene, reported by GFP fluorescence and co-localization with SMA expression was observed. Hence, applying

extra stress on NSCs by gene delivery did not obstruct their ability to respond to underlying extrinsic factors.

Precise and reproducible complex patterning of biologically active molecules can be achieved inexpensively by using computer-controlled inkjet printing techniques. When printing molecules for biological purposes several important criteria need to be fulfilled. The sensitive nature of biological molecules calls for systems that can operate rapidly. It is important that no cross-contamination between samples occur. In addition, the system must be able to work with hydrated samples. The above-mentioned touchstones are typical features of inkjet printing. In addition, the flexibility of the system allows printing of proteins, cells, and extracellular matrices as well as a mixture of those (Boland, Xu et al. 2006). There are however practical issues concerning inkjet printing including clogging of printer heads and need of extensive cleaning of the system leading to leakage. The biological materials passing the printer head nozzles are exposed to high shear stress and localized heating possibly leading to protein degradation. Even if partial protein denaturation might occur, our study proves that samples are biologically active after printing presented by a proper NSC response. The hydrogel in this study was chosen based on its compatibility with NSC cultures and ability to efficiently immobilize proteins. The mechanical properties (e.g. pore size) and chemical characteristics of the poly-acrylamide-based gel together result in efficient protein binding and immobilization. There is however a major drawback of the hydrogel used in this study since it loses its mechanical properties already after 24h in cell culture media making it a poor fit for longterm cell culture. The system allows the use of alternative substrates. In fact, tailoring of hydrogels to match *in vivo* conditions in combination with inkjet printing could improve stem cell culture techniques and lead us one step closer to cell therapy in the nervous system.

### **3.4 PAPER IV**

Substrate stiffness has proven to be a major determinant involved in cell migration, proliferation and differentiation (Engler, Sen et al. 2006; Kostic, Sap et al. 2007; Saez, Ghibaudo et al. 2007). In order to assess NSC differentiation as a result of substrate rigidity, cells were grown on substrates with a wide range of stiffnesses. Polydimethylsiloxane (PDMS) is a biocompatible and transparent elastomeric polymer widely used in micropatterning and microfluidics. NSCs grown on PDMS were viable

and maintained stem cell characteristics. The stiffness of PDMS can be tuned by altering the ratio of crosslinker to base. The stiffest PDMS substrate used in this study was prepared with a ratio of crosslinker to base of 1:10, which is the standard dilution recommended by the manufacturer. We prepared PDMS substrates with ratios of crosslinking agent to base of 1:10, 1:50, 1:70 and 1:100 with respective Young's Modulus of  $750\pm170$  kPa,  $18\pm15$  kPa,  $12\pm4$  kPa and close to zero. Mechanical properties vary across tissues and developmental stages. The adult brain parenchyma stiffness is in the order of hundreds of Pa (Lu, Franze et al. 2006), corresponding our softest substrates. NSCs grown on all PDMS substrates, in the presence of FGF2, proliferated and expressed nestin at levels comparable to cells grown on tissue culture polystyrene (TCPS).

To explore the effect of substrate stiffness as such on NSC differentiation, cells were grown for seven days in the absence of growth factors on TCPS or PDMS substrates. We found that total cell number was unaffected by substrate stiffness but was lower on all PDMS substrates compared to TCPS. Notably, we observed an increase in astrocytic differentiation with decreasing PDMS substrate stiffness. The softest PDMS substrates harbored three times more GFAP positive cells than the stiffest PDMS substrates after seven days in culture. Consistently, mRNA expression of GFAP increased linearly with decreasing substrate stiffness. It has previously been shown that astrocytic differentiation of NSCs requires functional Notch signaling (Ge, Martinowich et al. 2002; Hermanson, Jepsen et al. 2002). Inhibiting Notch signaling in NSCs by the  $\gamma$ -secretase inhibitor DAPT resulted in eradicated astrocytic differentiation both on TCPS and PDMS substrates. Hence, substrate stiffness could not override the requirement for Notch signaling in astrocytic differentiation.

The percentage of cells expressing TuJ1 (class III  $\beta$ -tubulin) was higher on all PDMS substrates than on TCPS, suggesting that the PDMS material promotes neuronal differentiation. Further, the percentage of TuJ1-positive cells was unaffected by substrate stiffness. Consistently, Tubb3 mRNA levels were constant on the different PDM substrates. The length of the longest neurite was measured in TuJ1-positive cells to assess the level of neuronal maturity. We observed a strong correlation between increasing neurite length and decreasing substrate stiffness. However, oligodendrocyte spreading was observed to decrease with substrate stiffness. In addition, neurite length was higher on all PDMS substrates comparing to TCPS. In addition to neurite length,

neuronal maturation was assessed by expression of the presynaptic marker synaptotagmin. Neurons differentiated on TCPS and stiff PDMS substrates did not express presynaptic synaptotagmin and displayed an immature morphology with short neurites. Conversely, neurons differentiated on the softest substrates expressed synaptotagmin and showed mature morphology. Taken together, these results suggest that neuronal maturation is promoted by soft substrates with brain-like stiffness.

Many studies indicate the importance of matching *in vitro* substrate stiffness to the target tissue *in vivo*. Using similar mechanical properties as the native tissues has improved adult neural (Saha, Keung et al. 2008), cardiac muscle (Krahenbuehl, Zammaretti et al. 2008) and mesenchymal (Engler, Sen et al. 2006) stem cell differentiation. This study elucidated the NSC dependence on mechanical stimuli and that brain-like stiffness enhances neuronal maturity. However, increased neurite outgrowth was not due to a general mechanism through which soft substrates promote spreading. Our study showed that oligodendrocyte spreading followed an opposite trend with more spreading on stiff substrates. In a previous study opposite responses to substrate stiffness has been reported for hippocampal neurons and fibroblasts (Kostic, Sap et al. 2007). In contrast to our study, adult NSCs has previously been shown to form more neurons and less astrocytes on soft substrates (Saha, Keung et al. 2008), suggesting that stem cells respond differently to substrate stiffness based on developmental stage. Additionally, substrate stiffness related differences in glial and neuronal growth have been reported (Flanagan, Ju et al. 2002; Georges, Miller et al. 2006). Both studies found reduced astrocyte adhesion on softer substrates whereas neurons could grow well on all substrates. The cells in these studies were however fully differentiated before they were seeded onto substrates with varying stiffness. It is possible that the differences in glial and neuronal growth can be attributed to decreased adhesion or increased detachment of astrocytes. The neurons in co-cultures grown on stiff substrates were observed to grow on top of astrocytes, whereas they could grow independently on soft substrates. This could partly explain the observed survival of neurons on soft substrates. Astrocytes have been reported to regulate synapse formation and maturation (Slezak and Pfrieger 2003) and might therefore be responsible for the maturation of neurons on soft substrates in our study. Taken together, these results show that stem cells integrate mechanical cues with intrinsic programs during differentiation and that substrate stiffness is a crucial parameter in neuronal differentiation.

## 4 CONCLUDING REMARKS

The work presented in this thesis has implicated microenvironmental cues such as growth factors, substrate stiffness and oxygen levels as major determinants in neural stem cell fate decisions. In addition, a novel way of detecting neural stem cells and cancer stem cells in culture has been presented.

The limitation for the potential of stem cell research is many times restricted by technical difficulties. For example, when studying stem cells it is crucial to know precisely what cell type you are studying in the dish. Identification and characterization of stem cells are commonly carried out by immunohistological techniques, such as antibody stainings. However, most markers have limitations and are not specific enough to detect stem cells in a complex system and are in need of secondary methods for visualization. Most often, several markers have to be utilized in order to identify stem cells. For example, NSCs must be positive for a combination of marker in order to be distinguished from other cell types, which can be a time consuming task. In addition, these techniques cannot be used in live cultures. In paper I we present a novel molecule for selective and specific identification of live NSCs and cancer stem cells. This opens possibilities for a new generation of smart molecules to be used as non-invasive probes for detection of live cells.

Signals from microenvironmental levels of proteins and oxygen can be integrated by cells and alter their genetic and epigenetic states, leading to stem cells fate decisions. In paper II we established CtBP as a co-regulator involved in such alterations of cellular gene programs. Further, we showed that there is a gradient of oxygen levels in the chick spinal cord following the BMP gradient, giving CtBP its dual role as a regulator of pro- and anti-neuronal genes. These results suggest oxygen levels to have a possible role in the patterning of the spinal cord. Thus, it is of great importance to study gradients to mimic *in vivo* situations.

In paper III we propose inkjet technology as a viable approach to create patterns of proteins to control NSC differentiation. By printing growth factor onto hydrogels we could achieve spatial control of NSC differentiation. This approach could be used for complex patterning of biomolecules, allowing cells in the same dish to be exposed to different levels of molecules. Inkjet technology can be combined in applications where

cells are grown on alternative substrates as well as in three-dimensional cell culture. Therefore, printing of molecules on to scaffolds is an attractive approach for future development of drug delivery systems and cell therapy applications.

Cells pull on their surrounding environments and can thereby respond to its stiffness. In paper IV we show that NSCs can sense and respond to mechanical properties. Neuronal maturation was promoted solely by culturing NSCs on soft substrates. Stem cells are commonly exposed to expensive factors to induce neuronal differentiation and maturation. For example, a combination of BMP4 and Wnt3a can induce neuronal differentiation. However, the factors need to be added daily to the cell cultures for up to 14 days to obtain mature neurons. In our study, NSCs grown on soft substrates for 7 days were differentiated into mature neurons without any added factors to the cell medium. It is not surprising that cells of neural origin need to see brain-like stiffness in order to develop into mature neurons. There is still little known about the exact mechanisms behind mechanosensing, suggesting a need for better understanding of mechanochemical pathways. Moreover, there is a need for novel biologically relevant elastic substrates, replacing the rigid polystyrene commonly used in cell culture. Finally, careful considerations to substrate stiffness need be taken when designing scaffolds for tissue repair or cell therapy.

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