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Studies on adult stem cells

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Abstract

While our bodies are aging, in quite a few tissues, new cells are born every day, till the day we die. As you are reading this sentence, there is perhaps a new neuron born in your brain (and some are dying). Where does it come from, why does it come now and will it do any good?
The field of adult stem cell biology deals with the basic functions of stem cells as found in most species and with their therapeutic potential. It is the stem cells found in each tissue that possess that almost magical regenerative ability.
Having endogenous cell replacement is not without risks. Most of us will at some point develop tumors; is there a connection between aging, stem cells and cancer? It is likely, but we don't know.
Accumulating evidence these days, including research presented in this thesis, points to the common molecular pathways that govern behavior of stem cells and cancer development. There are several ways one can study stem cells of the adult mammalian brain; our efforts have focused on employing unbiased searches for stem cell signatures, testing candidate genes for their importance in regulating adult stem cell characteristics and creating transgenic mice for the study of the stem cell progeny during health and injury.
The first two studies presented in this thesis aim at developing methods for the analysis of gene expression profiles of stem and progenitor cells. Based on a collaborative effort, we constructed cDNA libraries representing transcripts present in adult neural stem cells in vivo and in vitro as well as genes expressed by cells in the neurogenic microenvironment of the lateral ventricle. The cDNA libraries have further been used for the assembly of cDNA arrays for the quantitative analysis of gene expression in stem cells.
The collected sequencing data and gene expression profiles have been used in a bioinformatic search for genes that are preferentially expressed in neural stem cells. We have found several interesting targets that could serve as new stem and progenitor cell markers.
Studies III and IV are based on the same investigative principle: we chose two candidate pathways for the analysis of their influence on adult neural stem cell biology. A clinically important issue is the cellular origin of tumors. There is an emerging view in tumor biology that only a subset of the cells in a tumor supports the expansive cancer growth. Consequently, study III describes the functions of a tumor suppressor, p53, in adult neural stem/progenitor cells as a way of finding common mechanisms between stem cells and tumor formation. p53 is established as an important inhibitor of tumorigenesis and p53 mutations are found in most human cancers. We have found that p53 is an important negative regulator of self-renewal and proliferation of neural stem cells.
Study IV continues with a signaling pathway involved in stem cell identity and self-maintenance. We focused on elucidating the importance of RBP-J/Notch signaling in adult neural stem cells. Using viral approaches in vivo by ventricular injections of adenoviruses and lentiviruses expressing Cre recombinase in a conditional floxed RBP-J mouse line we have been able to pinpoint the effects of deleting RBP-J in a specific cell population, ependymal cells. The deletion of RBP-J in ependymal cells has uncovered their differentiation potential along the neurogenic pathway and the importance of Notch signaling in maintenance of ependymal cell quiescence.
Studies V and VI are based on transgenic mice for genetic tracing of candidate stem cell populations. We have constructed two mouse lines: a Nestin-CreER line and a FoxJ1-CreER line. Nestin-CreER expresses $CreER^{T2}$ in stem and progenitor cells of the central nervous system providing a lineage tool by temporal induction of recombination and thereby visualization of neurogenesis as well as temporal gene deletion. FoxJ1-CreER expresses $CreER^{T2}$ in ependymal cells of the brain and spinal cord and is used in study six for delineating the functions of ependyma-derived progeny in response to spinal cord injury.
In conclusion, by defining the molecular events that are instrumental in neural stem cell biology and following cell fates of candidate stem cell populations, I have contributed to our understanding of stem cell behavior.

List of Thesis Publications

I. Sievertzon M, Wirta V, Mercer A, **Meletis K**, Erlandsson R, Wikstrom L, Frisén J, Lundeberg J.
Transcriptome analysis in primary neural stem cells using a tag cDNA amplification method.
BMC Neurosci. 2005 Apr 15;6(1):28.

II. Williams C*, Wirta V*, **Meletis K**, Wikström L, Carlsson L, Frisén J, Lundeberg J.
Catalog of gene expression in adult neural stem cells and their in vivo microenvironment.
Experimental Cell Research. 2006 Mar 16; [Epub ahead of print]

III. **Meletis K**, Wirta V, Hede SM, Nister M, Lundeberg J, Frisén J.
p53 suppresses the self-renewal of adult neural stem cells.
Development. 2006 Jan;133(2):363-9.

IV. Carlén M, **Meletis K**, Cassidy R, Evergren E, Tanigaki K, Almendola M, Naldini L, Honjo T, Shupliakov O, Frisén J.
RBP-J maintains ependymal cell quiescence and inhibits neurogenesis in the adult brain.
Manuscript

V. Carlén M*, **Meletis K***, Barnabé-Heider F, Frisén J.
Genetic visualization of neurogenesis.
Submitted Manuscript

VI. **Meletis K**, Carlén M, Barnabé-Heider F, Evergren E, Shupliakov O, Frisén J.
Fate mapping of candidate stem cells in the adult spinal cord.
Manuscript

*equal contribution

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Abbreviations

APC	Adenomatous Polyposis Coli protein
Ara-c	cytosine arabinoside
bFGF	basic fibroblast growth factor
ß-gal	ß-galactosidase
bHLH	basic helix-loop-helix
BrdU	5-bromo-2-deoxyuridine
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CVADR	Coxsackie B and adenovirus receptor
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ES cell	embryonic stem cell
EST	expressed sequence tag
FACS	fluorescent activated cell sorter
FoxJ1	forkhead box J1
GFAP	glial fibrillary acidic protein
Hes	hairy and enhancer of split
Hey	hairy and enhancer of split-related with YRPW motif
HIV	human immunodeficiency virus
LVW	lateral ventricle wall
loxP	locus of crossing-over of P1 phage
Mash1	mammalian achaete-schute complex homolog-like 1
Mdm2	mouse double minute 2
mRNA	messenger ribonucleic acid
NICD	Notch intracellular domain
NS	neurosphere
NSC	neural stem cell
Rb	Retinoblastoma
RBP-J	recombination signal binding protein
SGZ	subgranular zone
SVZ	subventricular zone
TK	thymidine kinase
Wnt	wingless-related Murine Mammary Tumor Virus

Introduction

Complex multicellular and multiorgan life forms as found in the family of vertebrates generally go through a biological transition early in life and enter what is called adulthood. An adult exhibits biological features that are not found in the newborn or young animal. Adulthood represents status quo regarding body size, nevertheless cells in our bodies die on a daily basis. Cell death is found in all organs to varying degrees, and in order to counteract a rapid body degradation there is a need for a balancing act, and that regenerative entity is found in the progenitors and stem cells that reside in tissues of the adult body. The term “adult stem cells” thus describes a cellular origin that accounts for the continuous birth of cells in an already full-grown adult animal.

The brain as found in birds, mice or us humans is the organ responsible for executing vital functions such as breathing and movement control, but more interestingly, the brain links the outside physical world with our internal biological world. The cell that we think of as responsible for the fantastic mental capabilities we possess is the neuron. A neuron comes in different three dimensional forms and dimensions. What is common for all neurons is that they generate electric potentials, a “firing” property that in mysterious ways transduces and stores information of the external world. Firing of neurons makes a brain into a mind. We humans tend to think of ourselves as “I”, and that “I” is probably generated somewhere in each person’s brain. The “I” we use in language and thought is something we view as static and hence laymen and scientists have always naturally considered the brain to be rigid and conservative, a brain that is evolutionarily determined to stay the same and represent a continual identity during an animal’s lifetime. Today there is accumulating biological evidence that the brain is not static, it is plastic. New neurons are added to the adult brain in all species investigated. The neurons that are born during embryogenesis and adulthood, both derive from a population of cells that is called neural stem cells.

The identity of adult neural stem cells, their importance for adult life and some of the unanswered questions in stem cell biology is what I will focus my discussion on.

Adult Stem Cells

The term *cell* was first coined by Robert Hooke 400 years ago when looking at thin cork slices. It would be 200 years later that the cell was identified as the unit of life by Rene Dutrochet (1824). The concept of adult stem cells arose from work on irradiated mouse bone marrow in the early 1960's by McCulloch and Till. They could establish that bone marrow cells that gave rise to colonies of multiple differentiated cells were derived from a single common cellular ancestor as identified by a unique genetic mark (Becker et al., 1963).

Stem cells in adult tissues divide in order to generate several different mature cell types and still maintain the stem cell population. These two key properties of any stem cell are termed multipotential differentiation and self-renewal. The differentiated cells coming from adult stem cells replace dying cells and acquire similar functions to their predecessors. The possibility of endogenous cell replacement makes room for tissue plasticity, an important feature in a changing world. An extreme version of the dynamics produced by a continuously regenerating cellular system is the blood. Blood is composed of many specialized cell types with functions ranging from immune response to oxygen delivery; still all these cells have a common origin, the adult hematopoietic stem cell. A single hematopoietic stem cell produces millions of differentiated cells through several steps in a cellular lineage, and the balance of birth and death of cells is strictly controlled in order to meet demands. A fundamental difference between the brain and the blood system is that brain only regenerates to a small degree; there are rather a few evolutionarily conserved anatomical locations that contain endogenous stem cells in the adult. Stem cells in the adult brain are primarily found in the lining of the anterior ventricular system and in the hippocampus. The anterior lateral ventricle harbors cells that contribute with new neurons to the olfactory bulb, neurons that integrate into existing neuronal circuits and help process odor information for further signaling to the cortex. Cells in the subgranular layer of the hippocampal dentate gyrus divide and produce mature neurons that integrate and have equal functions to the pre-existing granule neurons, most likely involving functions related to memory formation.

Adult Neurogenesis

The field of adult neurogenesis has followed a typical evolution of scientific thought and paradigm shift as described by Thomas Kuhn. The giant of neuroscience and the proponent of the neuron doctrine at the turn of the 19th century, Santiago Ramón y Cajal, is often cited as being the person who introduced the notion that neurons are only born during prenatal stages. Ramón y Cajal used the Golgi technique for the study of the fine structure of the nervous system and could observe and draw exquisite images of neurons with elaborate and far-reaching axons and dendrites, and perhaps this static image of beautiful neurons provided ground for the aversion towards adult neurogenesis for almost a century. In the past decade, adult neurogenesis has become an established biological phenomenon and the accumulating evidence for its importance in adult physiology would probably surprise Ramón y Cajal.

As many times before in science, it was the introduction of a technical innovation that opened up the possibilities for a more detailed study of cell division in the adult brain. The introduction of radioactively labeled thymidine (H^3-thymidine) enabled the identification of cells that had undergone DNA synthesis during S-phase of the cell cycle based on autoradiographic exposure of tissue sections (Sidman et al., 1959). By administering H^3-thymidine at different time points in early post-natal life and adulthood in rodents, Joseph Altman in the 1960's could for the first time question the long-lived idea of "no adult-born neurons". By following and identifying cells that had incorporated H^3-thymidine and therefore had undergone DNA synthesis and cell division at the time of administration and then matured to specific cell types, it was now possible for the first time to uncover adult neurogenesis (Altman, 1962; Altman and Das, 1965). A crucial element was to convincingly demonstrate a nucleus with radioactively labeled genome that also exhibited neuronal characteristics. Resistance against the idea of life-long neurogenesis was coming from studies on non-human primates performed by Rakic in the 1980's (Rakic, 1985), although careful and detailed studies more than a decade later could overturn the negative results (Kornack and Rakic, 1999). Elegant work in canaries by Nottebohm further established that adult neurogenesis was a reality and that it probably had functional importance for the development of seasonal song learning since neurons generated in adulthood connected to pre-existing circuits, the first proof of functionality of adult-born neurons (Paton and Nottebohm, 1984). A number

of other technical innovations such as advanced microscopy (electron microscopy and confocal fluorescent microscopy), electrophysiological recordings, antibodies for the discrimination of cell types on sections and the introduction of another nucleotide analog, bromodeoxyuridine (BrdU), which is compatible with antibody staining procedures, helped pave the way to today's understanding of neurogenesis from rodents to humans. BrdU, for example, allowed for the simultaneous detection of cells that had undergone cell division with a detailed phenotypic characterization of the fate produced and in combination with confocal detection it was possible to unambiguously establish the case for adult neurogenesis.

There are two discrete anatomical locations where adult neurogenesis in many species occurs and is easily detectable: the olfactory bulb and the dentate gyrus of the hippocampus.

Lateral Ventricle Wall Architecture

In the most anterior part of the forebrain, the lateral wall of the lateral ventricle is a neurogenic niche. The four main cell types identified by electron microscopy in the rodent lateral ventricle wall are ependymal cells with the characteristic motile cilia facing the lumen of the ventricle, the type B cells expressing glial firbillary acidic protein (GFAP), the type C cells that make up the transit-amplifying progenitor population and the type A cells that are the neuroblasts (Doetsch et al., 1997). Type C cells proliferate rapidly and provide a continuous supply into the type A neuroblast lineage that form migratory chains from the subventricular zone (SVZ) to the olfactory bulb. Neuroblasts need 2 days up to a week to reach the core of the olfactory bulb, where they then migrate radially and further mature into granule neurons or to a lesser extent into periglomerular neurons.

The migration of neuroblast along the rostral migratory stream (RMS) is influenced by factors in the SVZ and the cerebrospinal fluid (CSF). The direction of neuroblast migration mimics the flow of CSF produced by the movement of ependymal cilia. Chemorepulsive factors produced by the choroid plexus such as members of the Slit protein family could account for the observed directionality of neuroblast migration (Nguyen-Ba-Charvet et al., 2004; Sawamoto et al., 2006). Surprisingly, one study reports that Slit1 is also expressed by type A and type C cells in the SVZ, complicating the interpretation of Slit function in neuroblast migration (Nguyen-Ba-Charvet et al., 2004).

GFAP-expressing B cells represent a neural stem cell candidate population as proposed by Alvarez-Buylla (Doetsch et al., 1999a) and this population may further be subdivided into a B1 and B2 cell population. B1 cells have intimate ependymal and ventricular contact and characterized by the presence of a single immotile cilium (Doetsch et al., 1997). The function of ependymal cells in rodents regarding adult neurogenesis has been conflicting with evidence pointing to a slowly-proliferating cell with adult neural stem cell capacity (Johansson et al., 1999) or a post-mitotic glial cell with no contribution to adult neurogenesis (Spassky et al., 2005). As a general principle, stem cells themselves divide rather rarely in most studied organs, and it is the progenitor population that actively divides and generates the amplification effect that leads to the production of new differentiated cells. Using this stem cell characteristic it is possible to deplete a stem cell niche of its rapidly proliferating cell population in order to test the identity and differentiation potential of the remaining slowly dividing cells. Administration of a toxic anti-mitotic agent, cytosine arabinoside (Ara-C), for six days to the adult

brain only spares ependymal cells and type B cells in the lateral ventricle wall (Doetsch et al., 1999b). The regenerative capacity of the SVZ is remarkably rapid and within ten days the stem cell niche is fully restored. It is possible based on such a progenitor depletion regime to identify the initiating cell in a lineage. The published study based its lineage conclusions on cell morphology as assessed by electron microscopy. Directly after Ara-C removal many type B cells appear to be ventricle contacting and as described are the only cells that enter the cell cycle, demonstrated by H^3-thymidine incorporation. The conclusion of Doetsch et. al. is that type B cells have the potential to give rise to type C transit-amplifying cells and in consequence type A neuroblasts, establishing the stem cell potential of B cells after injury. It is not possible, based on this study, to account for the function of ependymal cells since the ependymal definition is morphologically centered. If ependymal cells for example would adopt type B cell morphology before cell division, then it would be impossible to detect ependymal contribution the SVZ lineage. To resolve the issue of cell lineage initiation we need strict lineage marking using genetic methods to confidently study the contribution of each cell population to the adult neurogenic lineage.

Olfactory Bulb Neurogenesis

The sense of smell is first mediated through neurons in the olfactory epithelium expressing odorant receptors that send axons into the olfactory bulb where further processing of the incoming odor information takes place. The olfactory bulb of a mouse is proportionally larger to the corresponding structure in humans. The newborn neurons that integrate in the circuits of the olfactory bulb are arriving after a long journey through the rostral migratory stream which has its source in the lateral wall of the anterior lateral ventricle. Continuous proliferation of progenitors and rapidly amplifying precursor cells in the SVZ creates a stream of neuroblasts that are guided in chains by the functions of various molecular repellents and attractants (Lois et al., 1996). The olfactory bulb itself does not seem to be required for the correct proliferation and migration of neuroblasts towards their target (Kirschenbaum et al., 1999). There are generally low numbers of mature astrocytes and oligodendrocytes produced in this region, the adult SVZ is mainly a neurogenic niche. Regarding primates, adult neurogenesis originating in the SVZ and destined for the olfactory bulb is similar to rodents for non-human primates (Pencea et al., 2001) but is not yet fully established for humans, although there are reports supporting that also adult humans have olfactory bulb neurogenesis (Bedard and Parent, 2004). The

architecture of the human SVZ is different from rodents (Quinones-Hinojosa et al., 2006) including a "GFAP-ribbon" between ependymal cells and deeper cell layers with limited proliferation (Sanai et al., 2004).

The interneurons that are produced in the adult olfactory bulb have been studied using replication-incompetent retroviruses that label the progeny of progenitors in the lateral ventricle wall SVZ. Both morphological and electrophysiological maturation stages have been described along the maturation path (Carlen et al., 2002; Carleton et al., 2003; Petreanu and Alvarez-Buylla, 2002). Already the immature neurons with growing dendrites receive synaptic input from other neuronal assemblies, a process that might be important for the correct maturation and integration of the newly arrived neurons. In conclusion, adult-born neurons go through similar maturation steps as their embryonic counterparts in terms of gene expression and membrane properties.

Hippocampal Neurogenesis

The hippocampal formation is involved in processing of incoming information and when damaged will negatively affect the formation of new memories.

The only location so far where human adult neurogenesis has been clearly demonstrated is the dentate gyrus of the hippocampus (Eriksson et al., 1998). Cells located in the subgranular zone (SGZ) proliferate and give rise to increasingly differentiated progenitor cell types. Neuronal progenitors consecutively develop into immature neurons that migrate deeper into the granular layer where further maturation takes place. In rodents, several thousand of new granule neurons are added every day (Cameron and McKay, 2001; Christie and Cameron, 2006), making place for functional plasticity of the dentate gyrus. Several groups have in different ways investigated the integration and electrophysiological function of newborn neurons, reaching the conclusion that the neurons born in adulthood resemble and in many cases are identical to the neurons born during embryogenesis (van Praag et al., 2002; Zhao et al., 2006). Several environmental factors influencing animal behavior have been associated with effects on dentate gyrus neurogenesis. The number of adult-born neurons in the dentate gyrus correlates positively with an enriched environment and more specifically physical activity (Kempermann et al., 1997; van Praag et al., 1999). In contrast, a stressful environment, aging and elevated corticosteroids during aging decrease neurogenesis in the adult hippocampus (Cameron and McKay, 1999; Kuhn et al., 1996; Mirescu and Gould, 2006).

Adult Neural Stem Cells

The central nervous system (CNS) undoubtedly contains a cell population or several different cell populations derived from the embryonic ectodermal germ layer that in adulthood maintain proliferative capacity and generate new neurons and glial cells (Gage, 2000; McKay, 1997). I will refer to this somewhat theoretical population as "adult neural stem cell" or aNSC. The promises of such aNSC for the amelioration or treatment of neurological disorders is the driving force behind society's willingness to invest in stem cell research.

The isolation and propagation of cells from adult mouse striatum with proliferative and differentiation ability introduced a new concept: the neurosphere (Reynolds and Weiss, 1992). Neurospheres are free floating aggregates of cells that under the influence of mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) continue to grow in size. Neurospheres can be formed from single cells from the lateral wall of the lateral ventricle, passaged in vitro several times and clonal neurospheres when differentiated under appropriate conditions generate the three main cell types of the CNS: neurons, astrocytes and oligodendrocytes. Neurospheres can be established from other sites all along the rostrocaudal neuraxis, for example the spinal cord (Weiss et al., 1996). Studies aiming at the identification of the endogenous adult neural stem cell have heavily relied on the in vitro assay for neural stem cell properties, the neurosphere assay. A minority of cells within each neurosphere maintains their stemness character and is able upon single cell dissociation to form a clonally derived neurosphere, representing an in vitro form of self-renewal. The cellular source of neurosphere formation has thus served as a proof of adult neural stem cell identity. Due to the application of growth factors such as EGF for the induction of neurosphere formation it has been postulated that EGF signaling might alter the cellular identity of SVZ cells. EGF is able to turn transit-amplifying progenitor cells into multipotent neurosphere-forming cells (Doetsch et al., 2002a) and ependymal cells acquire radial glial morphology upon EGF administration in vivo (Gregg and Weiss, 2003).

To my knowledge, there is no evidence thus far of an identifiable homogeneous cell population in the adult brain with long-term self-renewing properties, so the search continues. One can argue that adult neurogenesis could be a developmental remnant of a progenitor cell population with limited self-renewal that exhausts itself during adulthood, resulting in decreasing neurogenesis with age. Perhaps that could explain the correlation

between the observed decrease in hippocampal neurogenesis with age and the finding that hippocampus at least in vitro mainly gives rise to progenitors and not self-renewing stem cells (Bull and Bartlett, 2005; Seaberg and van der Kooy, 2002).

In summary, neurospheres exhibit in vitro the properties of a self-renewing neural stem cell population. Due to the unique stem cell-like behavior of neurosphere cells, many research groups have focused their efforts on identifying the cellular source of the neurosphere-initiating population. Through these efforts it has become increasingly clear that we need to focus our attention on the behavior of each cell population in their in vivo context.

Stem Cell Biology Instruments

Isolation

Single cells can be analyzed and sorted based on different fluorescent spectra using a flowcytometer, fluorescent activated cell sorter (FACS). Understanding of the blood stem cell system is based on the delineation of the blood cell lineage by detecting extracellular epitopes with fluorescently conjugated antibodies and the same approach has been employed for neural stem cell isolation. Unfortunately, we do not yet have a reliable and commonly accepted method of isolating a homogeneous population of cells from freshly dissociated brain tissue that accounts for all the neurosphere-initiating activity of the tissue and where most cells isolated form neurospheres. Several attempts have been made to characterize the cell populations found in the SVZ based on extracellular markers and find ways of purifying a neurosphere-initiating population (Capela and Temple, 2002; Rietze et al., 2001; Uchida et al., 2000). Therefore, it is still of great value to find new extracellular markers for stem and progenitor cells.

Detection of Proliferation

Before a cell undergoes cell division it faithfully duplicates its chromosomes during S-phase of the cell cycle in order for the two cells produced to inherit a complete chromosome setup. The DNA synthesis occurring during S-phase utilizes free nucleotides and thereby builds a copy of a chromosome strand. In the presence of nucleotide analogs such as H^3-thymidine or BrdU, the newly synthesized chromosome will be labeled and this marks the nuclei originating from proliferating cells. By varying the length and amount of BrdU availability and the interval between BrdU administration and detection, one is able to study the dynamics of the cell cycle itself, the differentiation of progeny arising from cell divisions and the survival of newborn cells (Magavi and Macklis, 2002). Since BrdU only provides information of DNA synthesis and not cell division per se, it is good practice to exclude BrdU incorporation due to DNA repair mechanisms. BrdU incorporation and detection has revolutionized adult stem cell biology with the price of introducing controversial findings (Rakic, 2002). On the other hand, controversy is not without merit since it brings interest and competition in a field and forces people to be creative.

Viral Marking

Tracing of cells originating from cell divisions can be achieved by infection of a proliferative pool with retroviruses that integrate in the genome only during mitosis. Such a retrovirus is the Muloney murine leukemia virus (MMLV) that lacks nuclear import signals and therefore does not integrate in non-dividing cells. Retroviral vectors based on MMLV can be constructed to express fluorescent proteins which facilitates the three-dimensional visualization of newborn cells such as neurons and further makes it possible to detect action potentials and firing patterns using electrophysiological recordings (Morshead et al., 1998; Zhao et al., 2006). The possibility to study the electrophysiological properties of adult-born neurons of different maturational stages provides a basis for the understanding of interplay between neuronal communication and successful integration into existing circuits.

Lentiviruses are specialized retroviruses and newly engineered variants are based on the HIV genome. Lentiviruses have the advantage of high viral titer production and integration in quiescent cells as well as dividing cells (Blomer et al., 1997; Consiglio et al., 2004; Naldini et al., 1996). The latest generation of such viral vectors has been designed to carry a viral coat derived from viruses with wide tropism (vesicle stomatitis virus VSV-G pseudotyped) based on phospholipid binding and therefore enhancing transduction of all cell types in any species. Currently used lentiviruses contain minimal genetic information from the wild-type virus backbone, reducing safety concerns regarding viral amplification/replication or immune responses against viral proteins (Zufferey et al., 1998). Viruses can further be genetically engineered to express fluorescent proteins, Cre recombinase or other genetic tools under the control of a specific cellular promoter, further increasing specificity of use (Lois et al., 2002).

Adenoviruses are double-stranded DNA viruses and therefore do not integrate in the genome of the infected cell, in contrast to retroviruses (Davidson and Bohn, 1997). Replication-deficient adenoviruses carrying different reporters or Cre have successfully been used by several groups for the specific infection of ependymal cells lining the lateral ventricle since these cells express high levels of the receptor CVADR needed for internalization of adenovirus (Bajocchi et al., 1993; Hauwel et al., 2005; Tomko et al., 2000; Yoon et al., 1996). In general, there are several different types of viruses suited for specific experiments providing a powerful tool for the study of cell lineage and maturation of newborn cells in the adult brain, with one drawback: the injection of virus into the tissue of interest will always

be a concern due to the injury reaction complicating the interpretation of any finding.

Genetically Engineered Mice

The common mouse has served biology as an important animal model for decades. Mice have the advantage over other vertebrate models in that there are several genetic tools available for sophisticated analysis of gene function and cell lineage. Mouse embryonic stem cells (ES cells) were the first stem cells to be established and propagated in vitro something that led to an explosion in mouse biology (Evans and Kaufman, 1981). In principle, by manipulating ES cells or the fertilized egg one is able to construct mouse lines carrying any favorite genetic element. Mouse ES cells are cultured in vitro and by homologous recombination using a selectable drug resistance marker it is possible to uniquely target any specific site in the mouse genome (Ramirez-Solis et al., 1993). The use of homologous recombination in ES cells has made it possible to delete genes and study the loss-of-function phenotype during embryonic development or in adult mice and to use the endogenous genetic enhancers for directed expression of markers (such as fluorescent proteins) or genetic tools such as Cre recombinase (see Tracing chapter).

Common Regulators with Cancer Cells

The self-renewal and differentiation ratio of adult stem cells help maintain tissue homeostasis. Therefore, a natural conclusion is that deregulation of stem/progenitor cell dynamics and behavior may lead to tumorigenesis. In recent years, the association between stem cell biology and cancer biology has been strengthened by several findings of common molecular regulators of cancer formation and stem cell maintenance (Reya et al., 2001). Analysis of the cellular constituents of brain tumors has revealed great heterogeneity and some tumors contain cells that display in vitro or in vivo stem cell characteristics (Hemmati et al., 2003; Ignatova et al., 2002; Tunici et al., 2004; Uchida et al., 2004). There are two aspects of stem cells and their involvement in cancer formation: is cancer in some instances a stem cell disease and do tumors contain cancer stem cells? Since stem and progenitor cells proliferate in vivo which also a subset of a tumor does, hence they are expected to share some common mechanisms of cell cycle control. Most of the anti-cancer agents used today clinically including radiotherapy target the proliferative properties of the tumor, resulting in severe side-effects on the normal proliferation of stem cells responsible for the maintenance of vital functions of blood, intestine and skin. Is there reason to believe that it is possible to exclusively target cancer stem cells leaving normal tissue stem cells unaffected? Regarding the seemingly blunt attacks performed today, there must be room for progress, and cancer patients facing a soon death as the alternative are probably interested in small improvement. Valuable information on the effects of anti-cancer treatments on endogenous stem cells of the brain can be derived by studying post-mortem tissues of patients treated for tumor in other organs.

Glioblastoma is the most common tumor in adult brains. Glioblastomas are highly malignant with poor survival rates; still we know little of their cellular origin. It has been assumed that astrocytes residing in brain parenchyma are the tumor precursors. Several gene mutations or gene overexpressions have been identified and the affected pathways usually involve EGF receptor signaling (Libermann et al., 1985) and cell cycle regulators (p53, Mdm2, Rb, PTEN) (Rasheed et al., 1999). Autocrine or paracrine enhanced EGF activity occurs in a majority of glioblastomas and a mouse model based on astrocytic expression of a truncated and constitutively active EGF receptor has been developed (Holland et al., 1998). The majority of glioblastomas have defects in the p53 network, either by direct mutations in p53 or in deregulation in

components of the p53 pathway such as Mdm2 (Ichimura et al., 2000). Another important signaling factor that is deregulated in glioblastoma cells is PTEN (Duerr et al., 1998). PTEN catalyzes the conversion of phosphatidylinositol (3,4,5)-trisphosphate to phosphatidylinositol (4,5)-bisphosphate, linking growth factor signaling with intracellular activation of Akt (Cantley and Neel, 1999; Maehama and Dixon, 1998; Stambolic et al., 1998). PTEN has been found to be an important regulator of adult stem cells. Neural progenitors deficient for PTEN have a shorter cell cycle and create an enlarged brain (Groszer et al., 2001). PTEN deficiency further enhances proliferation of neural stem cells in vitro. In glioma cells PTEN regulates p27 and phosphorylation of Rb for the induction of cell cycle arrest (Gottschalk et al., 2001). Interestingly, the importance of PTEN as a tumor suppressor and regulator of self-renewal is connected to p53 as PTEN is a direct target of p53 transcriptional activation (Stambolic et al., 2001; Tang and Eng, 2006).

Bringing current knowledge of the association between stem cells and tumors, a recent classification of gliomas based on their gene expression profiles has revealed common pathways with neural progenitors as being important for the establishment of clinical prognosis (Phillips et al., 2006).

Another conserved pathway that is important for neural stem cell maintenance and tumor formation is the Hedgehog pathway (Rowitch et al., 1999). Medulloblastomas are malignant cerebellar tumors frequently found in children and inactivation of the Hedgehog receptor Patched which results in activation of the downstream Hedgehog signaling is often found in medulloblastomas (Pietsch et al., 1997; Raffel et al., 1997).

The Wnt pathway was initially discovered as oncogenic in a mouse breast cancer model (Nusse and Varmus, 1982). The classical downstream events of Wnt signaling target ß-catenin and APC, two molecules with functions in tumors and stem cells (Bienz and Clevers, 2000; Chenn and Walsh, 2003; van de Wetering et al., 2002).

In summary, tumor cells exhibit alterations in signaling pathways that are shared with stem cells.

Molecular Regulators of Proliferation

Most cells in our body remain in a non-dividing state and then there are cells like stem cells, progenitor cells and in a few cases differentiated cell types that undergo cell divisions. The cell cycle is a multistep process resulting in one cell dividing and giving rise to two cells. The mechanisms controlling cell cycle progress are heavily conserved between species since it is a fundamental principle of multicellular organism to grow and create organs by cell division. An essential function of the cell cycle is to faithfully replicate the cell's genome and pass on a copy of it to the next generation. In principle there are three types of protein complexes: complexes that promote cell cycle, complexes that inhibit cell cycle progression and proteins with control functions (Grana and Reddy, 1995).

In summary, the retinoblastoma family of proteins (Rb, p107, p130) acts as inhibitors of cell cycle entry and when phosphorylated by a cyclin-dependent kinase (Cdk) activity release a positive factor, E2F. E2F then activates the transcription of genes necessary for transition to the next stage of the cell cycle: DNA synthesis. Cdk activity is at the core of cell cycle progression and is in constant antagonism with the family of Cdk inhibitors (Elledge, 1996). Cdk inhibitors consists of two families of proteins: four members in the INK4A family (p15, p16, p18, p19) (Lowe and Sherr, 2003) and three members in the WAF1/KIP family (p21, p27, p57) (Harper et al., 1993; Vidal and Koff, 2000). The choice between quiescence and cell cycle entry is an essential component for the correct dynamics of a continuously dividing population in order to meet the demands of a regenerating tissue without creating a tumor. Small imbalances between quiescence and cell cycle entry on a population level will rapidly lead to amplified deregulation of tissue homeostasis. This is clearly manifested in human cancers where mutations and alterations in cell-cycle regulators is a common tumor-initiating event.

Growth factors and mitogens mediate their proliferative and survival effects through receptor signaling, and in many cases the intracellular pathways used for transmitting the signals from the extracellular milieu are very similar and share common transactivators. Growth factor induced PI3-kinase/Akt signaling redistributes Mdm2 protein to the nucleus resulting in p53 degradation and suppression of p53 function. Furthermore, Akt affects cell cycle progression by phosphorylation of p27 and p21, which segregates these cell cycle inhibitors from the positive cell cycle-promoting Cdk activity (Liang et al., 2002; Rossig et al., 2001; Shin et al., 2002).

p53 is at the core of several cell cycle associated processes: cell cycle progression control, DNA-damage surveillance and apoptosis. p53 activity is deregulated in most human cancers providing evidence for the importance of p53 for maintaining tissue balance and counteracting tumor formation (Sung et al., 2000; Vogelstein and Kinzler, 2004; von Deimling et al., 1992).

p53, when activated, binds a specific DNA sequence, termed a p53-responsive element, and regulates the expression of a multitude of genes (el-Deiry, 1998; el-Deiry et al., 1992; Hoh et al., 2002; Kern et al., 1991). An important part of the p53 network is the p21 protein (el-Deiry et al., 1993; Harper et al., 1993) which is a direct target of p53 transcriptional activation. p21 is a Cdk inhibitor and targets one of the first Cdk activity present in cells entering cell cycle, the cyclin E/Cdk2 complex. Inhibition of the cyclin E/Cdk2 complex results in Rb hypophosphorylation, which in turn causes cell cycle arrest. p21 further has p53-independent functions and is expressed in differentiated cell types (Parker et al., 1995).

There are seven established negative regulators of p53 activity (Mdm2, Cop1, Pirh2, cyclin G, Wip1, p73 delta N and Siah1) and three positive regulators (PTEN/Akt, p14/19 and Rb). All of these proteins form autoregulatory loops with p53 and their activitites are finely balanced in order to ensure proper cell cycle progression. The ubiquitin ligase Mdm2 and p53 create an autoregulatory loop where p53 stimulates the expression of Mdm2 and Mdm2 in turn translocates p53 to the cytoplasm for degradation through the ubiquitin-proteasome pathway and loss of p53 reverses the phenotypes associated with Mdm2 deficiency (Jones et al., 1995; Kubbutat et al., 1997).

Previous studies have pointed to other oncogenes and tumor-suppressors as being important for stem cell self-renewal and maintenance. One example is Bmi1 which is highly expressed both by aNSC and other stem cells. Bmi1 is a transcriptional repressor of the Polycomb group initially identified because of its oncogenic effects in cooperation with c-myc (van Lohuizen et al., 1991). Lack of Bmi1 induces expression of p16 and p19, and Bmi1-deficient neurospheres show elevated p21 levels (Jacobs et al., 1999; Molofsky et al., 2003).

The function of E2F1 as an important factor for the regulation of proliferation and apoptosis of progenitors in both the SVZ and SGZ has been described in mice deficient for E2F1 which leads to a decrease in neurogenesis in the adult olfactory bulb and dentate gyrus (Cooper-Kuhn et al., 2002). The Cdk inhibitor p27 is important for the controlled proliferation of the transit-amplifying type C progenitors since lack of p27 leads to an increase in progenitor proliferation and a concomitant decrease of proper differentiation

(Doetsch et al., 2002b). Interestingly, many of the identified self-renewal influencing factors partly converge on the p53 and p21 network which could help explain some of the regulatory properties observed on the different stem/progenitor populations (Pomerantz et al., 1998).

Based on the well-established functions of p53 as a tumor-suppressor we chose to investigate in study III how loss-of-function of p53 influences the behavior of stem and progenitor cells in the adult mouse brain.

The initial observation of the importance of p53 for adult neural stem/progenitor cells was the dramatic increase in neurosphere size in the absence of p53. In order to understand p53 function in vivo we analyzed p53 expression and the proliferation properties of endogenous neural progenitors in the SVZ. We could establish that p53 is a negative regulator of the adult neurogenic program. Similar functions are evident for p53 in vitro where p53-deficient neurospheres display increased proliferation rate and increased self-renewal without deleterious effects on cell differentiation. Using our own developed cDNA array we could identify p21 as the most deregulated transcript, with p21 levels being 20-fold down-regulated in neurospheres without p53. In line with our findings, p21 has been reported to positively regulate quiescence of hematopoietic stem cells (Cheng et al., 2000b), and that lack of p21 increases the stem cell population size and their cycling rate whereas lack of p27 rather affects the size of the progenitor pool (Cheng et al., 2000a). p21 has also been found to be important for the regulation of in vivo proliferation of adult neural progenitors (Kippin et al., 2005). p27 functions as a sensor of mitogen signaling and an intrinsic timer, regulating the number of divisions and time of differentiation (Durand et al., 1998) which is illustrated by the increasing body size of mice with decreasing amounts of p27 (Kiyokawa et al., 1996; Nakayama et al., 1996). The observed effects of p53 deficiency on neural stem/progenitor proliferation and self-renewal are most likely not only attributable to lower levels of p21 protein. Several other pathways are affected and an interesting candidate for mediating non-p21 effects is Nucleostemin, a p53-interacting protein, part of a GTP binding protein family with functions in the nucleolus (Tsai and McKay, 2002). The nucleolus is known for ribosomal RNA biogenesis, but it may also have functions in control of proliferation as Nucleostemin and Mdm2 both are found in this compartment (Bernardi et al., 2004; Carmo-Fonseca et al., 2000). Similar results regarding loss of p53 activity in neural progenitors have been published by other groups (Gil-Perotin et al., 2006; Piltti et al., 2006)

An interesting concept is that the aging process could, at least in part, be the result of tumor suppressor functions. Perhaps proteins like p53 and p21 act as endogenous timer or clock factors, limiting the number of cell divisions a cell will go through as a defense mechanism against tumor development. Limiting the number of cell divisions of stem and progenitor cells could result in malfunction of tissue regeneration, ultimately leading to what we observe as aging. One would then assume that in the absence of p53 and p21, a deregulation of cell cycle numbers would eventually lead to an accumulation of cell cycle associated errors that in turn are tumorigenic. The observed lengthening of the cell cycle in aging neural progenitors may arise from such a repressor function of p53 and p21 (Luo et al., 2006). The impact of stem cell function and dysfunction on aging is an important and fascinating issue that deserves further attention.

In Vivo Tracing

Using genetic means of investigating cell lineage has the advantage of identifying physiological events in an undisturbed system. Tracing of cells and their progeny has been most successfully applied to organisms such as the worm and the fruitfly where powerful genetic marking and identification of cells has paved the way for our view of mammalian cell lineages. Inserting genes into the genome of the mouse has been advanced with the application of transgenic and knock-in technologies. Transgenic approaches rely on the fusion of a transcription promoter and a gene of interest. Such a construct is then injected into pronuclei of fertilized mouse eggs, where integration events occur thereby leading to the birth of mice bearing the designed genetic elements. Knock-in technology is a more sophisticated and time-consuming approach where one is able to insert any genetic element into any desired site of the genome utilizing endogenous promoters or enhancers present in the genome. In order to do so, one needs to first target the genome of ES cells with the desired construct and select cells with correct integrants, which are then injected into a developing mouse embryo at the blastocyst stage. A chimeric mouse consists of cells derived from injected ES cells and of the cells present in the blastocyst and is used for transmission of the targeted allele by further mating. Germ cells derived from the injected ES cells will fertilize an oocyte and lead to the development of a mouse heterozygous for the targeted allele. A useful genetic tool is the protein Cre recombinase that recognizes specific 34 base pair long DNA sequences, called loxP, and catalyzes excision of DNA between loxP sites (Nagy, 2000; Sauer, 1993). Cre can therefore be used as a genetic tool for the removal of any DNA sequence between two loxP sites. In order to further control Cre activity, expression of Cre can be targeted to specific tissues and cell populations using specific gene promoters or enhancer elements (Gu et al., 1994; Lakso et al., 1992). For the temporal control of recombination initiation it is possible to use a fusion between Cre and the estrogen receptor (ER) binding domain (Feil et al., 1996; Metzger and Chambon, 2001). Such a CreER protein fusion will have a cytoplasmic localization and therefore genomic DNA containing loxP sites will not be accessible for recombination. The administration in vivo or in vitro of a ligand for ER, such as 4-hydroxytamoxifen, leads to a rapid translocation of the CreER protein to the nucleus and hence induction of loxP recombination. Recently, mutagenesis of CreER has led to the development of protein variants with higher ligand

affinity and less background recombination of which CreER^{T2} is most commonly used (Indra et al., 1999). For tracing of Cre recombination events at a cellular level it is usual to mate a mouse expressing Cre or CreER^{T2} with a "reporter" mouse. The progeny of such a mating will have expression of Cre and the reporter construct for detecting recombination. A reporter construct includes a promoter driving the expression of two different proteins before and after recombination, allowing for retrospective identification of Cre activity. Since all cells share the genomic DNA carrying a reporter locus but only the cells having Cre or CreER^{T2} expression have the possibility of recombination we can use this system to track the progeny or migration of cell populations. The development of fluorescent proteins such as EGFP has revolutionized cell biology since it has become possible to visualize and isolate cells based on fluorescence (Tsien, 1998). A limiting factor for genetic tracing experiments is our knowledge of unique genetic markers for each subpopulation of interest, since the system relies on targeted expression of Cre.

Many laboratories have relied on GFAP as a marker of an adult stem/progenitor population both in the SVZ and SGZ (Doetsch et al., 1999a; Seri et al., 2001). Therefore, transgenic mice have been developed that utilize part of the human or mouse GFAP promoter for the expression of either Cre recombinase or thymidine kinase (TK) derived from Herpes simplex. TK is an enzyme that metabolizes gancyclovir and other pyrimidine analogs to toxic nucleotide analogs resulting in the death of TK-expressing cells undergoing DNA synthesis. The laboratory of Sofroniew has investigated whether GFAP expressing cells in the adult brain are the main source of both in vivo and in vitro neurogenesis (Garcia et al., 2004; Imura et al., 2003). To this end they have developed two transgenic mouse lines expressing either TK or Cre under the mouse GFAP promoter. As is the case for all experiments using transgenic mice it is important to analyze in depth the expression of the transgene and conclude if it faithfully recapitulates the endogenous GFAP expression. Interestingly, studies using the GFAP-TK transgenic mice reveal that administration of ganciclovir in vitro during the neurosphere establishment period or in vivo for 4 days before the isolation of the tissue greatly diminishes the number of neurospheres produced. That would argue for GFAP-TK expression both in the neurosphere initiating population in vivo and in the proliferating cells in vitro. An alternative explanation would be that the in vitro isolation of neurospheres and the injury reaction produced during the 4 days of elimination of proliferating GFAP-TK cells could result in the upregulation of GFAP in other cell types of the SVZ. It is therefore necessary

to validate these results using other approaches. To elucidate the importance of the GFAP-positive population found in the SVZ and SGZ, I would propose to make use of the temporally controlled and tamoxifen-dependent CreER^{T2} recombinase under the control of GFAP promoter elements, preferably using a knock-in approach in order to minimize misexpression. A mouse with controllable induction of Cre recombination would allow for the study of the lineage generated in adulthood from GFAP-expressing cells and allow for the elucidation of whether such a population is a long-term self-renewing stem cell population or if it is a transient progenitor-like cell population. An interesting and important tool would also be a mouse line with a fluorescently labeled GFAP-positive population that would allow for FACS based isolation of cells for the study of neurosphere forming abilities and the transcriptional profile of SVZ GFAP-cells. Fluorescent labels such as EGFP have further the advantage of discriminating with the aid of FACS between cells with differential fluorescent intensity, cell size and cell granularity.

The studies I present in this thesis involving cell lineage tracing, are based on two transgenic mouse lines, both expressing CreER^{T2}. In one mouse line, CreER^{T2} is under the control of a neural-specific enhancer found in the 2nd rat Nestin intron (Zimmerman et al., 1994) and in a second mouse line CreER^{T2} is regulated by a human FoxJ1 ciliogenic transcription factor promoter (Ostrowski et al., 2003). Nestin was the first marker to be identified as specific for neural stem and progenitor cells during nervous system development and in adult brain (Lendahl et al., 1990). The choice of a promoter specific for ciliated cells arose from a long search for promoters that would exhibit specific expression in ependymal cells. Foxj1 has been reported to be expressed in several cell types with motile cilia and is important for the development of the cilium itself (Blatt et al., 1999; You et al., 2004). Tracing of stem cell proliferation and of the stem cell descendants will provide answers regarding the function of adult neurogenesis and the differentiation potential of stem cells in their normal environment. Plasticity of neural stem cells with the potential to generate cell types of other germ layer origin than ectoderm has been proposed to be possible although all published reports are based on an in vitro expansion of the studied cells (Bjornson et al., 1999; Clarke et al., 2000; Galli et al., 2000; Wurmser et al., 2004). It is therefore important as a next step in understanding neural stem cell differentiation potential to investigate the differentiation and lineage choices of adult stem cells in vivo which is now possible using the genetic tools described in this thesis.

Molecular Regulators of Identity

When studying a process it is of importance to identify the individual components under study. In the case of adult neural stem cells it is essential to define and identify such a population in the brain. The first efforts in defining different cell types in the developing CNS based on epitopes finally opened up for the identification and isolation of neural stem and progenitor cells (Frederiksen et al., 1988; Frederiksen and McKay, 1988; Hockfield and McKay, 1985). We have good understanding of the cell lineage leading from the transit-amplifying progenitor population to the neuroblast and finally the fully integrated neuron. What we are lacking, is a common definition of adult neural stem cells in vivo and the elucidation of the cellular hierarchy present under physiological conditions. Molecular markers have been established for discriminating between mature cell types of the brain and the progenitor population as found in the SVZ and SGZ.

Three ultra-conserved signaling pathways that regulate cellular behavior from flies to humans are known as the Notch, Hedgehog and Wnt pathways (Artavanis-Tsakonas et al., 1999; Ingham and McMahon, 2001; Polakis, 2000). Generally, when a scientist sets out to identify important biological events and regulators it is always a good start to focus on evolutionarily conserved mechanisms, since evolution is a good guide for pointing out fundamental principles. Shortly, evolution does not change horses in midstream. The Notch signaling pathway is classically viewed as a differentiation inhibitor and consists of three main factors: a membrane-bound Notch receptor, a membrane-bound ligand, and the nuclear mediator of Notch signaling RBP-J (also known as CSL) (Hansson et al., 2004). In vertebrates there are four different Notch receptors (Notch1-Notch4) and five ligands (Delta-like1- Delta-like3, Jagged1, Jagged2). When a cell expressing a Notch receptor is found next to a cell expressing a Notch ligand, the binding of receptor and ligand will lead to proteolytic events and transportation of the intracellular domain of the Notch receptor (NICD) to the nucleus (Mumm and Kopan, 2000; Schroeter et al., 1998). The nuclearly localized NICD interacts with the transcriptional repressor RBP-J which turns RBP-J to an activator of transcription (Hsieh et al., 1996; Tamura et al., 1995). Genes that are known to be directly regulated by RBP-J form a family of transcription factors known as Hes/Hey (Iso et al., 2003; Jarriault et al., 1995). Hes/Hey transcription factors contain a protein domain with DNA binding affinity called basic helix-loop-helix (bHLH) and a cellular identity

based on Notch signaling is established through their interaction with downstream targets and transcriptional regulation. In conclusion, Notch signaling through RBP-J activates transcription of Hes/Hey genes that in turn act as transcriptional repressors of genes involved in cell differentiation. One can thus argue that the stem/progenitor fate is a very fragile state in need of constant maintenance and support, otherwise differentiation occurs.

As expected, both the Hedgehog (Lai et al., 2003; Palma et al., 2005) and Wnt (Lie et al., 2005) pathways have been shown to be important in maintaining stem cell identity in several tissues, during development and in adulthood (Reya and Clevers, 2005; Taipale and Beachy, 2001).

In study IV we have initiated an effort of understanding Notch signaling in the adult ventricle wall. As an initial step, we undertook to establish the expression patterns of the Notch signaling components, and in combination with published data from other groups (Givogri et al., 2006; Stump et al., 2002) we concluded that ependymal cells express Notch1 and bind the ligand Delta-like1. Since active Notch signaling is viewed as a differentiation brake, and ependymal cells have been described as having neural stem cell potential in spite of their differentiated morphology (Johansson et al., 1999) we became interested in eliminating Notch activity specifically in ependymal cells. Previous studies, where forced Notch signaling in embryonic neuroepithelium resulted in ependymal character (Gaiano and Fishell, 2002; Ishibashi et al., 1994) further spurred our interest. In order to specifically target ependymal cells lining the lateral ventricle in the adult mouse brain, we chose to use intraventricular injections of two different types of viruses: a replication-incompetent adenovirus expressing Cre recombinase under a general CMV-promoter that specifically transduces ependymal cells (Davidson and Bohn, 1997; Doetsch et al., 1999a) and a replication-incompetent lentivirus with a specific FoxJ1 promoter driving the expression of an EGFP/Cre fusion protein. Expression of Cre from both viruses after intraventricular injections induces recombination, and when injected in the ventricle of mice carrying a conditional allele of RBP-J ($RBP^{f/f}$)(Han et al., 2002) and a recombination reporter (R26R) (Soriano, 1999), RBP-J will be deleted and cells that have undergone recombination will be marked by constitutive expression of ß-galactosidase (ß-gal). Loss of RBP-J will result in an inability at the cellular level to receive any Notch signaling. As reported before, we found adenovirus infection to be specific for ependymal cells and we could also conclude that the lentivirus with the Foxj1 promoter specifically induced recombination in ependymal cells. Injection of adenovirus expressing Cre (Adeno-Cre) in mice with one conditional allele $RBP^{f/w}$ and the

R26R reporter resulted in ependymal cells with ß-gal expression and 2 weeks after injection the only cells with detectable ß-gal were ependymal cells. The same result was observed after injection of the lentiviral FoxJ1-Cre vector. In contrast, when either Adeno-Cre or FoxJ1-EGFP/Cre lentivirus was injected in $RBP^{f/f}$/R26R mice, we could detect many recombined ß-gal positive cells in the SVZ, lacking ependymal cell markers. Loss of ependymal cell character upon RBP-J deficiency prompted our further phenotypic analysis of recombined cells. A part of the ß-gal positive cells were proliferating as assessed by the presence of DNA in metaphase and staining for a mitosis marker, phosphorylated H3 (Crosio et al., 2002). Further, ß-gal cells stained with intermediate neuronal precursor markers such as Mash1 (Casarosa et al., 1999) and entered the neurogenic pathway in the SVZ and the rostral migratory stream as identified by co-staining with the neuroblast markers, doublecortin and ß-III-tubulin (des Portes et al., 1998; Gleeson et al., 1998; Menezes and Luskin, 1994). Whether ependymal cells were induced to recombine and delete RBP-J by adenoviral or lentiviral expression of Cre, the end result was the same: ependymal cells that had undergone recombination as assessed by ß-gal would lose ependymal character and enter the SVZ, thereafter contributing to the neuroblast lineage and finally arriving in the olfactory bulb and becoming morphologically mature neurons. Investigating the effects of RBP-J depletion from ependymal cells at later timepoints (8 weeks), we could observe that the lateral ventricles were depleted of recombined ependymal cells and most ependyma-derived cells had migrated and differentiated into neurons. Our experimental approach non-reversibly deletes RBP-J and inhibits downstream Notch signaling in all lineages; it is therefore expected that the targeted ependymal cells do not contribute to a self-renewing cell population since Notch signaling is required for stem cell maintenance.

The result of this study points to Notch signaling as a potential rheostat controlling the number of transit-amplifying cells and neuroblasts present in the SVZ, thereby functioning as a regulator of the neurogenic output. Ependymal cells that are not normally participating in the daily production of new neurons could be recruited either long-term to renew a short-term stem cell population or after injury, as a backup cell population. This mechanism is similar to the reported function of a skin stem cell population, the bulge stem cells, that apparently contribute to the regeneration of epidermis only after injury (Ito et al., 2005). Molecular interventions affecting the proliferation rate of endogenous progenitors, the lineage choices made and the direction of

neuroblast migration could serve as therapeutic factors for neurodegenerative disease.

In conclusion, we have used genetic tools in adult mice for following the effects of RBP-J deletion in ependymal cells. Our results uncover a cell lineage in vivo in a defined adult neural cell population, establishing that Notch signaling is important for the maintenance of the quiescent character of ependymal cells and when removed, ependymal cells readily contribute to the neuronal lineage.

The combination of conditional gene deletion and cell lineage analysis provides us with a powerful tool for an in depth analysis of adult neurogenesis and cell identity.

Forebrain Neural Stem Cells

Nestin was identified as the first marker of embryonic and adult neural stem and progenitor cells (Lendahl et al., 1990; Reynolds and Weiss, 1992; Tohyama et al., 1992). In the adult brain Nestin expression is present in several stem cell candidate populations and progenitor cells, something that complicates the definite identification of an endogenous adult neural stem cell population. The genetic marking of different candidate populations makes it possible to follow the progeny and long-term behavior of each population under undisturbed physiological conditions.

As a first step in characterizing at a cellular level the adult lateral ventricle neurogenic program, we constructed a transgenic mouse line that expresses the tamoxifen-dependent CreER^{T2} recombinase under a Nestin enhancer (Nestin-CreER). The enhancer found in the second intron of the Nestin gene directs transgenic expression to neural stem and progenitor cells (Lendahl, 1997; Roy et al., 2000; Zimmerman et al., 1994). Our analysis of the Nestin-CreER mouse line established the correct expression of CreER^{T2} in the stem/progenitor population both in the embryonic and adult brain, mimicking Nestin expression. Using the Nestin-CreER mouse line one can achieve two things: one is to delete genes at specific time points in neural stem/progenitors and their progeny and second to follow the fate of Nestin-positive cells at different time points thereby testing in vivo the multipotentiality of the Nestin population. We can provide evidence for the applicability of the Nestin-CreER mouse by studying the birth of cortical neurons during development and the birth of olfactory bulb neurons during adulthood. By administering tamoxifen to Nestin-CreER embryos carrying a neuron-specific reporter expressing nuclear ß-gal and membrane-associated GFP after Cre recombination (Hippenmeyer et al., 2005), we could specifically label cortical neurons born at late embryonic time points. A systematic analysis using Nestin-CreER driven recombination at different embryonic stages can provide us with a detailed neuroanatomical map of the time of birth of different neuronal populations and their axonal targets. It will be of great value to use the Nestin-CreER mouse together with a fluorescent reporter mouse such as we performed with Z/EG (Novak et al., 2000) and then study the electrophysiological properties of maturing neurons in the olfactory bulb and other neurogenic areas or in neurons born for example after ischemia (Arvidsson et al., 2002; Nakatomi et al., 2002). It is a matter of controversy whether neurogenesis occurs in other parts of the CNS than

hippocampus and SVZ under normal conditions. Using our Nestin-CreER approach for visualization of adult neurogenesis, we could potentially resolve whether there is adult neurogenesis in areas such as the substantia nigra (Frielingsdorf et al., 2004; Lie et al., 2002; Zhao et al., 2003) or in cortical areas (Magavi et al., 2000).

It is possible using a Nestin-CreER/ Z/EG mouse to study the self-renewal properties of the Nestin positive forebrain stem cells by analyzing the long-term contribution of the marked cells to the neurogenic pathway and further performing FACS isolation of the GFP-positive cells remaining in the SVZ after different recombination intervals. Isolation of Nestin-CreER derived cells remaining in the SVZ is an initial enrichment for a possible stem/progenitor cell population and can serve as cellular source for gene expression analysis and the study of in vitro properties.

It is our hope that the Nestin-CreER mouse line will become a valuable alternative for deleting genes in the CNS with the advantages of temporal control and the possibility to level the amount of recombination. Further studies using similar genetic marking of other subpopulations in the adult brain will shed light on the lineage and potential of different cells under normal and diseased conditions.

Spinal Cord Neural Stem Cells

The spinal cord assembles all ascending and descending nerves conducting muscle and sensory information from skin to brain and back for processing, integration and feedback of sensorimotor information.

Spinal cord injury induces degeneration of transected axons and loss of sensorimotor function, along with effects on the supporting glial cells. The gliosis associated with a spinal cord injury is identified by the presence of reactive astrocytes that upregulate expression of many proteins, such as cytokines and growth factors as well as GFAP and Nestin (Frisen et al., 1995; Ridet et al., 1997). The mechanisms and reasons behind spinal cord degeneration and lack of regeneration are unknown, although inflammation and the resulting glial scar are thought of as detrimental to axonal regeneration or survival. The presence of inhibitory molecules associated with myelin in the mature spinal cord that hinder the growth of axons further complicates recovery after injuries (Schwab, 2002). The mobilization of endogenous spinal cord neural stem cells for the functional recovery after injury would be an attractive approach. We know that it is possible to derive neurospheres from the adult spinal cord; therefore some cells in the spinal cord retain stem cell ability (Represa et al., 2001; Weiss et al., 1996).

Transplantation of spinal cord neurospheres into hippocampus induces neurogenesis, whereas transplantation of the same cells into spinal cord induces gliogenesis (Shihabuddin et al., 2000). Spinal cord environment is inhibitory to neuronal differentiation of neural stem cells (Cao et al., 2001). Not only is the spinal cord inhibitory to neurogenic differentiation, it also inhibits the regeneration and growth of axons. Spinal cord axons do not grow in the injured tissue, but if axons are allowed to encounter nervous tissue from the peripheral nervous system such as the sciatic nerve, then there is axonal growth arguing for an intrinsic inhibitory mechanism present in spinal cord tissue (Aguayo et al., 1981; David and Aguayo, 1981). In summary, spinal cord environment seems to be quite a hostile place for axons to regenerate or for stem and progenitor cells to appropriately differentiate (Aguayo et al., 1981; David and Aguayo, 1981; Schwab et al., 1993).

Proliferation of adult ependymal cells has been more often reported in caudal parts of the neuraxis and especially in the spinal cord rather than in the forebrain. Interestingly, the occurrence of ependymomas is more prevalent in spinal cord (Bruni, 1998; Bruni and Anderson, 1987; Bruni and Reddy, 1987).

Since ependymal cells of the forebrain have the capacity to enter the neurogenic lineage after deletion of RBP-J as shown by study IV, we wanted to investigate the differentiation potential of ependymal cells upon spinal cord injury. The behavior of ependymal cells in terms of migration, amplification and differentiation after spinal cord injury has not been well characterized. Ependymal cells line the central canal of the spinal cord and are induced to proliferate in a few hours after a spinal cord insult (Frisen et al., 1995; Matthews et al., 1979) and there is some migration towards the injury site (Guth et al., 1985). In contrast, in the intact spinal cord there is a limited immature NG2-positive cell population that proliferates and produces mature astrocytes and oligodendrocytes (Adrian and Walker, 1962; Horner et al., 2000).

In study V, we have traced the fate of ependymal cells after injury using a mouse line with ependyma-specific CreER^{T2} expression based on a FoxJ1 promoter (FoxJ1-CreER). The tamoxifen-dependent recombination of a reporter locus, R26R, induces the expression of ß-galactosidase, providing a positive identification mark of all cells descending from ependymal cells. The injury model we have chosen is a longitudinal transection of the dorsal funiculus at thoracic levels. By analyzing tissue sections from injured spinal cords after different time intervals, we could appreciate the dynamics of cell migration and differentiation. Four days after injury, we could detect proliferation of ependymal cells lining the central canal and many ependyma-derived ß-gal positive cells were forming migratory clusters. Migration was also evident at later time points (2-6 weeks), but the ependyma-derived cells did not fill the glial scar, they were rather sparsely scattered with a few cells adopting GFAP expression. Surprisingly, not all ependyma-derived cells that migrate towards the injury site adopted a GFAP-positive phenotype. We could identify clusters of ß-gal positive cells in the spinal cord tissue that had lost ependymal cell identity as assessed by the downregulation of the FoxJ1-CreER transgene expression but were negative for GFAP expression or the oligodendrocyte precursor marker NG2. We have performed the same type of analysis on our Nestin-CreER mouse line and have found similar results.

Current ideas on how to deal with spinal cord injuries on a regenerative stem/progenitor plane deal with two issues: remyelination of axons with new oligodendrocytes and replacement of all dead cells using stem cells (Brustle et al., 1999; Horner and Gage, 2000; Okano et al., 2003). We present data that ependymal cells or other endogenous stem and progenitor cells could be used for in situ migration and differentiation as an alternative to cell transplantation.

Systems Stem Cell Biology

There is increasing awareness in modern biology that we need to dig deeper and see wider. The "one-protein - one-professor" approach of science has been successful in the founding of modern biology, nevertheless today we are reaching a transition period where a more comprehensive view of biological phenomena needs to be established. The first large non-hypothesis driven efforts to collect vast amounts of data was the sequencing of genomes, from humans to worms (Lander et al., 2001; Sulston et al., 1992; Venter et al., 2001). A pre-genome era is almost unconceivable today, as the easy access to any genomic information through the public databases is part of everyday life in most laboratories. Another technical innovation that has in some ways revolutionized modern biology is array profiling of gene expression through oligonucleotide microarrays (Lockhart et al., 1996) or cDNA arrays (Schena et al., 1995). A majority of laboratories has at some timepoint conducted (or thought of) an array experiment. There have been several improvements and iterations on the array technology, from arrays of tissue staining (Kononen et al., 1998) to arrays for transcription factor binding sites (Boyer et al., 2005; Bulyk et al., 2001; Iyer et al., 2001).

We have chosen to approach neural stem cell biology from a wider point of view in studies I and II by analyzing genes expressed in the lateral ventricular wall and neurospheres from the same corresponding tissue. The method we employed was the assembly of two cDNA libraries deriving from RNA isolated from adult lateral ventricular wall and from small neurospheres. Our aim was to manufacture cDNA arrays representing an adult neural stem cell transcriptome and its in vivo niche. For this purpose we isolated the lateral wall of the anterior lateral ventricle and used it directly for RNA isolation. The same procedure was applied on small neurospheres of few passages in order to avoid gene expression alterations associated with multiple passaging or neurosphere size. The choice to construct a cDNA library on unsorted tissue was based on a decision to include the whole neurogenic niche and also on the lack of extracellular neural stem cell markers which could be used for FACS. A cDNA library will also provide information on the gene expression profile of the initial population from which RNA was isolated by performing a high-throughput cDNA sequencing although such information does not provide a quantitative aspect of expression levels (Adams et al., 1991). EST sequencing of the two cDNA libraries provides us with an unbiased detection of genes expressed with the possibility of uncovering new transcripts. As an

initial validation of our method we performed in study I a basic transcriptome profiling of neurospheres from different isolations, different passage numbers and differentiating neurospheres. A comparison of the expression profiles obtained from undifferentiated and differentiated neurospheres demonstrated the validity and applicability of our amplification method and of the array. Several transcripts that are expected to be upregulated upon neural differentiation were detected as differentially expressed (Mbp, Dlx1, Mag, Gabrb1). The stem cell oriented cDNA array that we have produced has been used for several studies and proven to be of great value for the identification of candidate genes in experimental set-ups (Meletis et al., 2006; Richter et al., 2006; Sievertzon et al., 2005).

Several other laboratories have tried to identify factors important for the function of stem cells, so called "stemness" factors (Ivanova et al., 2002; Phillips et al., 2000; Ramalho-Santos et al., 2002; Sato et al., 2003; Terskikh et al., 2001). The large amounts of information gathered from our sequencing served as a template for a bioinformatics comparison in study II with previously published stem cell-associated datasets. The aim of all "stemness" studies is to identify important molecular regulators of self-renewal, proliferation and survival that are in common for several stem cell populations. It is still a matter of debate whether stem cells from different tissues share common molecular identities. Sequencing of the cDNA library from lateral ventricular wall (LVW-cDNA) yielded 14884 ESTs and 5417 Unigene clusters. Sequencing of the neurosphere cDNA library (NS-cDNA) resulted in 25501 ESTs that assembled into 7848 Unigene clusters. A direct comparison with the number of published ESTs per transcript and the tissue of origin provides a good platform for the identification of low-abundance transcripts that are present in our neurosphere and lateral ventricle wall cDNA libraries. A statistical analysis of the number of ESTs present in our libraries that match the same mRNA transcript is informative as to how frequent a transcript is in each population and if such a transcript is rarely found in other tissues then that would be considered an interesting candidate (see Table 4 in study II). We further performed an analysis on chromosomal localization of the sequenced transcripts and could conclude that there was a statistically significant accumulation of transcripts on a part of chromosome 11, at the same location as a previously published major quantitative trait locus associated with hematopoietic stem cell turnover (Bystrykh et al., 2005). Using the information we have gathered on genes expressed in neurospheres and the SVZ it is possible to extract potential candidates that are important for the regulation of stem/progenitor behavior both on a cell-

autonomous level and through the neurogenic niche. In our study of gene expression changes induced by p53 deficiency in neurospheres we could compare our results with previously published array data.

The finding of Nestin ESTs in LVW-cDNA (2 ESTs) and in NS-cDNA (22 ESTs) served as a control for the successful application of our procedure and established that it is possible to detect stem and progenitor markers as significantly enriched in our sequencing data. Another example of the usefulness of the data we have collected is our enrichment for the Gpr56 transcript (13 ESTs in NS-cDNA and 1 EST in LVW-cDNA) that now is known to be expressed in neural progenitors and important for human brain cortical formation (Piao et al., 2004) and Gpr56 is also upregulated in gliomas (Shashidhar et al., 2005).

A comparison of EST frequency in our two cDNA libraries further confirmed the importance of tumor-associated transcripts in stem cells such as p53 (8 ESTs in NS-cDNA and 1 EST in LVW-cDNA), Akt1 (14 ESTs in NS-cDNA and 2 ESTs in LVW-cDNA) and PTEN (1 EST in NS-cDNA and 1 EST in LVW-cDNA). The importance of the stem cell niche was evident from the presence of several receptor signaling pathways most prominent being the FGF pathway with FgfR3 (8 ESTs in NS-cDNA and 2 ESTs in LVW-cDNA) and FgfR1 (6 ESTs in NS-cDNA and 2 ESTs in LVW-cDNA). A feature of the transcripts that are considered interesting and with potential stem cell regulatory functions is a limited expression in vivo in our LVW-cDNA library and other tissues combined with a more abundant expression in neurospheres that are enriched for stem and progenitor cells. Transcripts with many ESTs represented in the LVW-cDNA library most likely have general cellular functions such as metabolism or translation and are found in a majority of cells. As example of candidate genes with previously uncharacterized functions that would be of interest to examine closer, I can mention three Unigene sequences with limited number of ESTs in the Unigene database and still found to be overrepresented in our stem cell focused libraries. Mm.297496 has 100 ESTs that derive mostly from nervous system and we find 14 ESTs in NS-cDNA and 1 EST in LVW-cDNA, Mm.33042 has 48 Unigene ESTs and we find 14 ESTs in NS-cDNA and 1 EST in LVW-cDNA and Mm.41661 has 57 Unigene ESTs and we find 12 ESTs in NS-cDNA and 1 ESTs in LVW-cDNA.

A first step towards defining the role of new genes will be a careful assaying of their expression patterns on tissue level using in situ hybridization. Together with other studies on transcriptional profiles of the SVZ (Easterday et al., 2003; Lim et al., 2006) and publicly available in situ hybridization data

we will approach a wider understanding of neural stem cell biology and its microenvironment.

Our effort is part of a larger development of data acquisition that accumulates in public databases. There is a healthy amount of skepticism regarding such large-scale efforts, since the end result is not always obviously interpretable. We have shown that array analysis of neural stem cells is a valid approach for uncovering major factors responsible for an observed phenotype as is the case for p21 deregulation in p53 deficiency.

A major endeavor will be the assimilation and digestion of information from different experiments and research groups with an output that in comprehensible to the human mind. We have not yet reached the point where we easily can overview large datasets of gene expression profiles or protein interactions.

Final Thoughts

During the past decade the scientific community has accumulated information about the identity and possible function of adult stem cells. Still, several controversial issues remain and our understanding of stem cell biology is mainly limited by technological issues as often is the case in science. An issue that remains unanswered both regarding brain and blood stem cells is the definite characterization of the most primitive stem cell and its lineage under physiological conditions and after injury. It is further necessary to unveil the genetic program underlying stemness and couple that knowledge to the differentiation pathways. A comprehensive description of the proteins expressed by different adult stem cells and their functions and interactions is crucial for understanding cellular behavior in a deeper sense, providing good theoretical grounds for the development of targeted therapeutic applications using endogenous stem/progenitor cells. Since stem cells and tumor cells appear to share molecular and functional features, it is of great interest to elucidate whether tumors in some cases arise from stem or progenitor cells or if tumors gain stem cell characteristics as a prerequisite for neoplasia. A molecular distinction between stem cells and tumor cells is necessary for the development of accurate targeting of tumorigenesis and not of healthy stem cell regeneration. Cancer therapeutics based on unique properties of cancer stem cells could provide a more refined attack on tumors, avoiding injury of healthy tissue.

What is “function” in functional adult neurogenesis? It is possible using available genetic methods to strictly eliminate adult neurogenesis while maintaining proliferation in the adult brain and thereby assess the behavioral consequences of adult neurogenesis deficiency. Interestingly, we do not yet have an understanding of how neuronal action potentials and firing contribute to information processing since we do not know the basic information entity, how information is coded and stored in our brains. We are able to follow and record currents and spikes in neurons, but ultimately we do not understand the nature of these phenomena. In summary, we can record the electrophysiological properties of adult-born neurons and conclude that they mimic the behavior of embryonic-born neurons, without really knowing what the spikes and firing patterns mean. It is important to find out what the role of adult neurogenesis is and if disturbed adult neurogenesis is casual in the development of neurological disorders.

Visualization of neurogenesis using transgenic mice is now possible and the genetic tools under development will greatly expand our knowledge of cellular hierarchy in adult neurogenesis and the differentiation potential of adult stem cells of the nervous system. The uncovering of cell lineage relationships and of master regulators governing self-renewal and neuronal differentiation will provide us with a basis for the development of therapeutics for neurological disorders by recruitment of endogenous precursor cells.

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