Isolation of multipotent astroglia form the adult stem cell niche and the injured brain

PhD Thesis

Submitted to

The faculty of Biology

Ludwig-Maximillian University, Munich.

Prepared in the group of Prof. Dr. Magdalena Götz

at the Helmholtz Zentrum Munchen/ Institute for Stem Cell Research

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Munich, February 2009

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die Dissertation von mir selbständing, ohne unerlaubte Beihilfe angefertigt ist.

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Dissertation eingereicht am 28. Februar 2009-07-29

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Tag der mündlichen Prüfung: 01. July 2009

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List of abbreviations

BMP	Bone morphogenetic protien
bFGF	Basic Fibroblast growth factor
CNS	Central Nervous System
Ctx	Cortex
Dapi	4'-6-Diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotides
DIENC	Diencephlalon
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
ES	Embryonic stem cells
ERT2	Estrogen receptor type2
E.*coli*	*Escherichia coli*
FACS	Fluorescence activated cell sorting
Flp-e	Flippase-enhanced
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate astrocyte-specific transporter
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HCL	Hydrocloric acid
HSCs	Haematopoeitic stem cells
NGS	Normal goat serum
NSCs	Neural stem cells
LB-medium	Luria Bertani medium
LIF	Leukemia inhibitory factor
LoxP	locus of X over Pi
MEF	murine embryonic fibroblasts
Neo	neomycin resistance
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDL	Poly-D-lysine
PDGFRα	Platelet derived growth factor receptor alpha
PFA	Paraformaldehyde
PSANCAM	Polysialylated neural cell adhesion molecule
RMS	Rostral migratory stream
RNAse	Ribonuclease
RT	Room temperature
RT-PCR	Realtime-polymerase chain reaction
SEZ	Subependymal Zone
SGZ	Subgranular Zone
SV40	simian virus 40
SW	Stab Wound
Wt	Wild type
TG	Transgene
X-GAL	5-bromo-4-chloro-3-indolyl β-D-galactoside

Abstract

Adult neural stem cells, as the source of life-long neurogenesis, reside in the subependymal zone (SEZ) in the lateral wall of the lateral ventricles and in the dentate gyrus of the hippocampus. In both neurogenic regions, subsets of glial fibrillary acidic protein (GFAP) expressing astrocytes are found, that have been shown to act as neural stem cells. So far, it is not known how to distinguish these stem cell astrocytes from other astrocyte populations within the SEZ. Towards this end we decided to isolate a subpopulation of adult SEZ astrocytes that expresses the CD133 by FACS. GFP-positive cells in the SEZ from hGFAP/eGFP mice that were also $CD133^{+ve}$ comprised all neurosphere-initiating cells that were self-renewing and multipotent from the SEZ. Moreover, single cell neurosphere analysis showed 70% efficiency in neurosphere formation. Further more Cre-mediated fate mapping of this double-positive population showed their contribution to adult neurogenesis. Transcriptional profiling of the GFP/CD133-double-positive cells allowed us to a) determine their similarity at the transcriptome level to both ependymal cells AND astrocytes and b) to identify their unique molecular neural stem cell signature.

We also discovered that astrocytes outside this neurogenic niche could go some way towards dedifferentiation into neural stem cells. We have previously described (Buffo et al., PNAS 2008) a population of astrocytes in the adult cerebral cortex after stab wound injury that dedifferentiates as far to form multipotent and self-renewing neurospheres. Now we succeeded to establish the factor responsible for this dedifferentiation and sufficient to elicit the dedifferentiation response even in cells that were not exposed to injury. These data will be presented. Taken together, our work allows for the first time, the identification and characterization of the astrocyte sub-types acting as neural stem cells.

1. Introduction

1.1 Adult Stem Cells

The discovery of stem cells was one of the landmarks in the history of medicine that opened up a whole new era of regenerative medicine. The pioneering work of McCulloch and Till in the 1960s on irradiated mouse bone marrow had shown that a common cellular ancestor could give rise to multi-lineage differentiated cells (Becker et al., 1963) and hence established the concept of adult stem cells.

It has long been known that there is a constant turnover of worn out cells in the body. Lower organisms can regenerate their lost body parts (for example tail/limbs) by regeneration. Regenerative capacity is very limited in higher vertebrates except for some organs like the liver, haematopoietic system and skin (Till and McCulloch, 1980; Cotsarelis et al., 1990). The mechanistic explanation for these processes came from the discovery of stem cells. Stem cells are defined by their ability to **self-renew** and their **ability to differentiate** into terminally-differentiated specialized cells. During embryonic development, the cells of the inner cell mass are called embryonic stem cells (ES cells). They are pluripotent and have the ability to generate and differentiate into all kinds of cells in the adult organism. Some stem cells are also present within developing and adult tissues. They are somatic stem cells. Unlike ES cells, they have a restricted differentiation potential; either they are pluripotent (like haematopoietic stem cells) or multipotent (like neural stem cells), as they can only give rise to cells of a single organ or tissue.

Adult stem cells have recently gained attention for a number of reasons. First, the plasticity of these tissue-specific stem cells was underestimated. Although debated, it is clear that adult stem cells can differentiate into other cell lineages different from their tissue of origin: a process called trans-differentiation. For example, cells from bone marrow were demonstrated to give rise to cells with neuronal or hepatocyte-like characteristics (Mezey et al., 2000; Schwartz et al., 2002). Second, adult stem cells were found in tissues other than blood or skin, which were not known before. The knowledge that adult stem cells exist in various organs has increased the scope and potential of stem cell research for various clinical purposes. Third, stem cells can be potentially used to replace diseased or lost tissue. By targeted gene therapy it is now possible to correct the

genetic defects in vitro and replace these genetically corrected cells into the patient. Once repopulated, they may reverse the diseased phenotype towards normalcy. For this approach adult stem cells are suitable because of their ability to differentiate into specialized cells.

Unlike the haematopoietic system, the regenerating capacity of the brain is very limited as neurons are terminally differentiated and non-dividing. Despite this, there are a few evolutionary conserved anatomical locations that contain endogenous stem cells in the adult brain. They are primarily found in the lining of the anterior ventricular system and in the hippocampus.

1.2 The nervous system

The nervous system is the most complex mammalian organ. It is responsible for perceiving information about the organism and its environment, processing this information, storing some of it in memory for future reference, and coordinating appropriate behavioral responses such as hormonal and metabolic activity, muscle movement, and speech. All of this is essentially accomplished by nerve cells and the connections between them. There are about 100 billion nerve cells (10^{11}) in an adult human brain and although they all share the same basic architecture, at least 1,000 different types of neurons can be distinguished (Kandel, 2000). All these nerve cells, or neurons, can establish hundreds of connections to other neurons by cellular protrusions called axons and dendrites. Electrical charges are transmitted between individual neurons via these axons and dendrites to mediate communication in the nervous system. The specific connections between individual neurons as well as the flexibility in forming and breaking these connections are thought to represent the basis for the outstanding performance of the nervous system.

The nervous system is divided into the central nervous system (CNS), comprising the brain and the spinal cord, and the peripheral nervous system (PNS). The nervous system originates from the ectodermal cells located along the dorsal midline of the early embryo, called the neural plate. Folding of the neural plate generates the neural tube. The caudal region of the neural tube develops into the spinal cord, whereas the rostral region becomes the brain. The cells on the inner wall of the neural tube are the neuroepithelial

stem cells. Massive, but highly regulated proliferation of these neuroepithelial stem cells, as well as folding and bending of the expanding neural tube, ultimately forms the adult brain. Deep within the brain remains a remnant of the folded neural tube, a system of connected cavities called ventricles, which are filled with cerebrospinal fluid. Epithelial cells called ependymal cells line the ventricles. During development the cell layer adjacent to the ventricles, which contains the neuroepithelial stem cells, is called the ventricular zone (Figure 1.1A). The region beneath the ventricular zone is called the subventricular zone (SVZ) and in adult as subependymal zone (SEZ). Both zones are reduced during postnatal development, leaving only an ependymal cell layer with a narrow SEZ in the adult brain (Figure 1.1B).

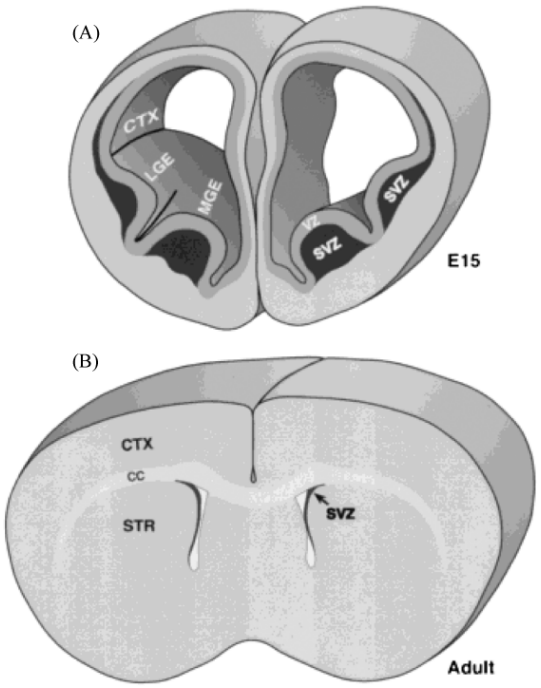


Figure 1.1. (A) Coronal section through the embryonic mouse forebrain at day 15 postconception. The proliferative ventricular zone (VZ) lines the lateral ventricles. A secondary proliferative region, the subventricular zone (SVZ), is prominent in lateral and medial ganglionic eminences (LGE and MGE) and transiently appears under the VZ of developing neocortex (CTX). (B) Coronal section through the adult mouse telencephalon. The embryonic VZ is transformed into a terminally differentiated ependymal layer lining the brain ventricles. The proliferative SVZ remains adjacent to the ependymal layer of the lateral walls of the lateral ventricles. This region is thought to be derived from the SVZ of the lateral ganglionic eminence (A). CTX: neocortex; CC: corpus callosum; STR: striatum. Adopted from Garcia-Verdugo et al., 1998.

Glial cells are non-neuronal cells in the brain, which are more abundant than neurons (approximately 10^{12} to 10^{13} in the adult human brain). Glial cells have traditionally been considered ancillary, satellite cells of the nervous system (Greek gliok = glue, slime), but their importance for proper brain function has also been increasingly appreciated (Nedergaard et al., 2003). Three glial cell types are recognized in the CNS: astrocytes, oligodendrocytes, and microglial cells, and each has a distinct morphology according to its respective function (Kandel, 2000). Astrocytes are the most numerous cells in the CNS and provide metabolic and trophic support for the neurons. In addition, they release and inactivate neurotransmitters and form the blood-brain barrier. A special type of astrocyte is the radial glial cell, which sends long cellular processes through the brain to guide migrating neurons during development. Oligodendrocytes are the myelinating glial cells of the CNS responsible for insulating axons for efficient propagation of action potentials during neuronal communication. In addition, there are microglial cells, which are derived from immune cells and become activated in response to injury or disease.

1.3 Stem cells of the nervous system

1.3.1 Neurogenesis in the adult rodent brain

It is widely recognized that damage to the mammalian PNS results in regeneration to some extent, whereas the CNS seems to be incapable of recovering from insults, resulting from trauma, ischemia or neurodegenerative disorders. In other words, as quoted by the famous Spanish neuroanatomist Ramón y Cajal (1852-1934): "in the adult centers the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated' (1913-1914)."

The first evidence for ongoing neurogenesis in the adult mammalian brain, specifically in the hippocampus and the olfactory bulb of the adult rat brain (*Rattus norvegicus*) was provided by Joseph Altman (Altman and Das, 1965; Altman, 1969). To classify the newborn cells as neurons Altman relied on morphological analysis, but at the time was not able to demonstrate their neuronal identity by immunohistochemical analysis due to a lack of appropriate marker proteins. However, in the early Nineties, Altman's findings were confirmed by two reports that demonstrated the presence of cells in the straitum of adult murine brain (*Mus musculus*) that were able to proliferate *in vitro* in the presence of

epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF), giving rise to cells positive for neuronal marker proteins, as shown by immunofluorescence analysis (Reynolds and Weiss, 1992; Richards et al., 1992). These cells proliferated in culture without adhering to the dish, and formed free-floating clusters of cells called neurospheres. By means of [^{3}H] thymidine birthdating, Lois and Alvarez-Buylla (1993) identified the subependymal zone (SEZ) adjacent to the walls of the lateral ventricles as a site where 'neural stem cells' reside and divide *in vivo* in mice.

Since these stem cells were obviously dividing in the adult brain ([^{3}H] thymidine labeling), the question arose as to where the newborn cells would integrate. By retroviral labeling of proliferating rat SEZ cells with an X-gal reporter gene, Marla Luskin demonstrated that their descendants migrate anteriorly into the olfactory bulb and acquire the morphology of granule and periglomerular neurons (Luskin, 1993). This phenomenon of extensive migration of newborn cells from the SEZ to the olfactory bulb was demonstrated in the adult mouse brain as well (Lois and Alvarez-Buylla, 1994) (Figure 1.2A). Periglomerular and granule cells are the two major types of interneurons in the *bulbus olfactorius* and are responsible for the modulation of incoming sensory signals before they are passed on to the olfactory cortex via the *tractus olfactorius*.

Newborn neurons from the adult murine SEZ migrate as chains in the so-called rostral migratory stream (RMS) towards the olfactory bulb. These migrating cells are immunoreactive for a polysialylated form of neural cell adhesion molecule (PSA-NCAM) (Rousselot et al., 1995) and early neuron-specific marker tubulin-β-III (Thomas et al., 1996). The chains of migrating neuroblasts are ensheathed by glial cells (Lois et al., 1996) (Figure 1.2B). These cells also synthesize the glial fibrillary acidic protein (GFAP), a widely used marker of astrocytes. The SEZ itself is also organized as an extensive network of chains of migrating neuronal precursors, which are also positive for PSA-NCAM and tubulin-β-III (Doetsch and Alvarez-Buylla, 1996). After reaching the olfactory bulb, neuroblasts exit the cell cycle and start to migrate radially towards their final destination.

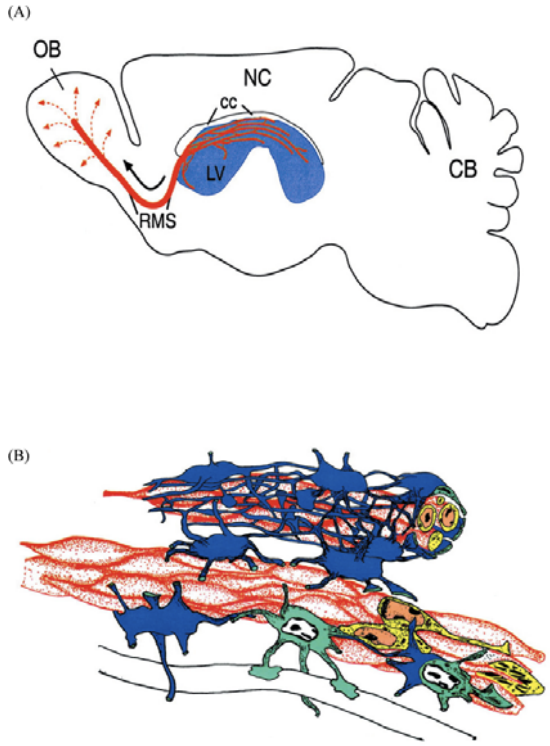


Figure 1.2. (A) Schematic sagittal view of the adult rodent brain depicting the lateral ventricle (LV) where new neurons are generated throughout the SVZ and aligned into long chains (red lines). The cells migrate as chains within the rostral migratory stream (RMS) towards the olfactory bulb (OB). In the olfactory bulb, cells disperse radially (dotted lines) as individual cells and differentiate into granule and periglomerular interneurons. CB, cerebellum; NC, neocortex; cc, corpus callosum. (B) Chain migration of young neurons (red) within the SVZ and the RMS. The chains of migrating neuroblasts are ensheathed by glial cells (blue) with astrocytic characteristics (connections to blood vessels, immunopositive for GFAP). In the bottom chain only few astrocytes are shown to illustrate the tight organization of neuroblasts into chains. Intracellular characteristics are illustrated in some astrocytes (light blue) and neuroblasts (yellow). Adopted from Alvarez-Buylla and Garcia-Verdugo, 2002.

1.3.2 Identification of the proliferating cells in the adult rodent brain

The discovery of proliferating cells and ongoing neurogenesis in the SEZ of adult rodents prompted questioning of which of these cells may act as stem cells. The cellular composition and three-dimensional organization of the SEZ in adult mice was described in great detail by means of electron microscopy, [^{3}H] thymidine autoradiography, and immunocytochemistry (Doetsch et al., 1997). Doetsch et al. found four major cell types in this region: migrating neuroblasts (type A cells), astrocytes (type B cells), undifferentiated putative precursor cells (type C cells), and ependymal cells. The type C

cells were most actively dividing and found in close proximity to clusters of type A neuroblasts, which were organized in chains. This suggested that type C cells are the highly proliferative, precursor cells for the neuroblasts. However, it remained unclear whether type C cells are the actual 'stem' cells, or only descendants of a slowly dividing 'stem' cell population. Morshead et al. had demonstrated earlier, that selective killing of the constitutively proliferating cells in the SEZ did not abolish the capacity of SEZ explants to form neurospheres *in vitro* (1994). They concluded that a relatively quiescent SEZ cell could repopulate the constitutively proliferating population *in situ* and give rise to neurospheres *in vitro*.

In 1999, the lab of Jonas Frisen claimed that the ependymal cells, which separate the ventricular lumen from the SEZ, are the neural 'stem' cells, and could generate new neurons *in situ* and neurospheres *in vitro* (Johansson et al., 1999a). They demonstrated that ependymal cells that were labeled with DiI generated multipotent neurospheres that could be passaged. Furthermore, individual ependymal cells that were isolated on the basis of the criteria that the cells expressed cilia and DiI were also found to generate neurospheres. More convincingly, Johannson et al. followed the fate of cells labeled by a contralateral, intraventricular injection of DiI. Using this protocol, cells lining the ventricular surface were specifically labeled. Over time, an increasing number of DiI-labeled cells were found within the SVZ and rostral migratory stream. After 10 days DiI-labeled cells that expressed βIII-tubulin or MAP-2, both markers for neurons, were observed in the olfactory bulb. Similar results were obtained with intraventricular injections of adenovirus expressing lacZ, whose receptor, CXADR, is specifically expressed by ependymal cells. Furthermore, labeled cells were also found in the olfactory bulb after the ependymal layer was infected with a BAG retrovirus carrying a lacZ reporter. In addition, when BrdU was administered to adult mice in the drinking water for 2 weeks, many ependymal and SVZ cells were labeled, but only ependymal cells were BrdU label-retaining, a proposed property of quiescent adult NSCs. Together, these data strongly suggested that some ependymal cells functioned as NSCs in vivo.

However, since then, their results have been questioned. Alvarez-Buylla and colleagues have shown that the SVZ astrocytes (type B cells) are the slowly dividing 'stem' cells *in situ* (Doetsch et al., 1999). Doetsch and colleagues used a number of techniques to refine their initial EM-based model of adult periventricular neurogenesis[80] wherein six cell

types were identified: 1. Type A cells (migrating neuroblasts), 2. type B1 astrocytes, 3. type B2 astrocytes, 4. type C cells (putative precursors), 5. type D cells(tanycytes), and 6. type E cells (ependymal cells). In this initial model, the actively proliferating type A, B2, and C cells were ruled out as NSC candidates based on the evidence that adult NSCs were relatively quiescent. Doetsch and colleagues built on this initial model by infecting SVZ astrocytes with adenovirus constructed to express GFP under the GFAP promoter. The infected SVZ, when dissociated and cultured, gave rise to multipotent GFPexpressing neurospheres, indicating that GFAP-expressing neural cells behaved like NSCs in vitro. To further demonstrate that GFAP-expressing SVZ cells functioned as NSCs in vivo, Doetsch and colleagues injected replication-competent avian leukosis virus into the SVZ of mice that expressed the receptor for the virus (not normally expressed by mammalian cells) under control of the GFAP promoter. This revealed that GFAP-expressing cells in the SVZ generate cells that migrate to the olfactory bulb and differentiate into neurons. Furthermore, EM analysis on mice brains 15 or 30 days after mice were injected with tritiated-thymidine revealed that label-retaining cells were not ependymal, but rather subventricular. Together, these experiments provided strong evidence that SVZ astrocytes function as adult periventricular NSCs. Other studies also confirmed their findings (Chiasson et al., 1999; Laywell et al., 2000; Capella and Temple, 2002). Therefore, the current model is that GFAP-positive SEZ astrocytes are the 'stem' cells in the adult rodent brain. They generate transit-amplifying cells (type C cells), which are the highly proliferative precursor cells of migranting neuroblasts (type A cells) (Figure 1.3). These neuroblasts align in chains and migrate anteriorly along the lateral ventricles through the SEZ by forming rostral migrating streams, towards the olfactory bulb, where they stop dividing, differentiate, and functionally integrate as interneurons (Figure 1.2).

The second region in the mammalian brain with substantial adult neurogenesis is the subgranular zone in the dentate gyrus of the hippocampus. In the subgranular zone of adult mice hundreds of cells divide daily and give rise to neurons which migrate into the adjacent granule cell layer, and become morphologically indistinguishable from the other surrounding pre-existing granule cells (Kempermann et al., 1997a). Interestingly, paralleling the results for proliferating SEZ cells, it was shown that new neurons in the adult hippocampus were derived from GFAP-positive astrocytes (Seri et al., 2001). These

cells can be propagated *in vitro* after attachment to an adhesive surface in the presence of bFGF (Gage et al., 1998).

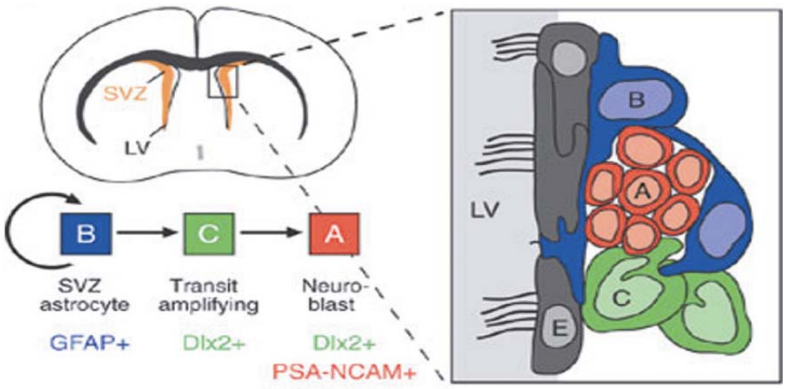


Figure 1.3. Organization of the adult rodent SVZ and cell types therein. Frontal scheme of the mouse brain showing the SVZ (orange), adjacent to the lateral ventricle (LV), and the SVZ cell types. Multiciliated ependymal cells (E, gray) line the lateral ventricle. The chains of neuroblasts (A, red) migrate through glial tunnels formed by SVZ astrocytes (B, blue). Rapidly dividing transit-amplifying cells (C, green) are scattered in small foci along the network of chains. Some SVZ astrocytes extend a process between ependymal cells that contacts the lateral ventricle. SVZ astrocytes (GFAP+) are 'stem' cells in this region and generate neuroblasts (GFAP-, Dlx2+, PSA-NCAM+) via the C cells (GFAP-, Dlx2+, PSA-NCAM-). Adopted from Doetsch, 2003

1.3.3 Cryoarchitecture of cells in adult neurogenic regions (SEZ and SGZ)

Heterogeneity among astrocytes is also observed in both the neurogenic niches, the SGZ and SEZ. In the SGZ, at least 2 populations of astrocytes have been identified (a) Nestin-positive mitotically active radial astrocytes, spanning their cytoplasmic processes into the granule cell layer (b) Nestin-negative, non-dividing, S100β positive horizontal astrocytes that extend their basal processes under the granule cell layer (Kronenberg et al., 2003; Seri et al., 2004). Astroglial populations in the SEZ are morphologically distinct. At the ultra-structural level, two types of astrocytes (type B1 and B2) are present that differ in their location, cytoplasmic and nuclear structure and proliferative ability (Doetsch et al. 1997). Additionally, stellate and bipolar astrocytes present in the SEZ have been shown to have different proliferation profiles (Garcia et al., 2004). Doetsch et al. in 1999

observed an interesting characteristic of a subpopulation of SEZ astrocytes; they occasionally protrude a cytoplasmic process, which intercalates between ependymal cells and comes into contact with the lateral ventricle, and thus is exposed directly to the CSF (Doetsch et al., 1999b). The number of astrocytes in contact with the lateral ventricle increases during regeneration and following an infusion of growth factors (Doetsch et al. 1999b, 2002; Conover et al. 2000). SEZ astrocytes in contact with the ventricle express a single 9+0 primary cilium, which is also found on neuroepithelial stem cells during development in mammals (Sotelo & Trujillo-Ceno´z 1958; Stensaas & Stensaas 1968), on radial glia (Tramontin et al. 2003; Spassky et al. 2005) and on primary precursors in adult songbirds (Alvarez-Buyalla et al. 1998). This primary cilium may be important for transduction of signals in the CSF and for stem cell function. The microanatomical detail of the adult neural stem cell niche has been illustrated recently (Mirzadeh et al., 2008) using special whole mount and three-dimensional imaging techniques. They could observe that neural stem cells in the SEZ (B1 astroglia) have fundamental properties of epeithelial cells as they show basal and apical compartmentalization. At the apical surface the B1 astroglia have a 'pinwheel' kind of organization with a core of pinwheel containing the small apical end of B1 cells directly in contact with the ventricle surrounded by ependymal cells at the periphery. In addition, at the basal end, these B1 astrocytes have a long process in close proximity to the blood vessels. Despite these molecular, biophysical and morphological differences, it is still unclear whether the different subpopulations of astrocytes in both adult neurogenic regions represent functionally distinct astrocytes or are at different stages in the lineage.

1.3.4 Adult neurogenesis in primates

In the late Nineties it also became clear that in the adult primate brain neurogenesis takes place as well. The first evidence for the production of new neurons in the hippocampus of adult macaque monkeys was found by Kornack and Rakic (1999). The generation of neuroblasts in the SEZ and chain migration of these cells towards the olfactory bulb was demonstrated in adult rhesus macaques (*Macaca mulatta*) and long-tailed macaques (*Macaca fascicularis*) (Kornack and Rakic, 2001; Pencea et al., 2001). Kirschenbaum and co-workers showed the first indication of neurogenesis in adult brain of Homo sapiens in 1994 with low capablility of producing neurons *in vitro* (Kirschenbaum et al.). They kept

human SEZ explants *in vitro* in the presence of [^{3}H] thymidine and found rare newborn cells, double positive for [^{3}H] thymidine and neuronal marker proteins. Treatment with bFGF and brain-derived neurotrophic factor (BDNF) strongly promoted neuronal outgrowth and survival of these cultured cells (Pincus et al., 1998). Gage and colleagues published evidence for ongoing neurogenesis in the hippocampus and the lateral ventricle wall of adult humans (Eriksson et al., 1998). They had analyzed postmortem tissue samples of cancer patients aged 57 to 72 years who had received BrdU treatment to label dividing cells for diagnostic purposes. The newborn cells were frequently positive for the neuronal marker NeuN and negative for the astrocyte marker GFAP. Johansson et al. (1999b) were able to generate neurosphere cultures from the lateral ventricle and the hippocampus of two female patients aged 16 and 19 years. These neurosphere cells were passaged as single cells and their progeny differentiated into neurons, astrocytes, and oligodendrocytes. This demonstrated the existence of self-renewing cells in the adult human brain that are multipotent *in vitro*. Such cells from the ventricular wall of adult humans were also shown to develop the distinct electrophysiological properties of neurons and glial cells *in vitro* (Westerlund et al., 2003). Taken together, these studies showed that, like rodents, the SEZ and the hippocampus of adult human brain contain progenitors that can give rise to neurons and glial cells *in vitro*.

Importantly, a detailed study of the human brain revealed no chains of migrating neuroblasts originating from the SEZ towards the olfactory bulb (Sanai et al., 2004). Instead, the human SEZ cytoarchitecture was found to be strikingly different from that of primates, rodents, dogs, cows, and sheep. A gap that contains no cell bodies separated the ependymal cells from a band of astrocytes referred to as an SEZ astrocyte ribbon (Figure 1.4). No chains of migrating neuroblasts and only few single tubulin-β-III-positive cells were observed in the SEZ of adult human brains. However, astrocytes in the ribbon co-expressed GFAP and the cell-division marker Ki-67, and they gave rise to neurospheres *in vitro*. Secondary neurospheres generated from these neurospheres were capable of differentiating into astrocytes, neurons, and oligodendrocytes analogous to rodent neurosphere cells. Therefore, the proliferating cells in the human SEZ, like in rodents and monkeys, seem to be astrocytes. The study by Alvarez-Buylla and colleagues also highlighted the importance of verifying the findings from rodents in the humans system, although rodents serve as valuable model organisms.

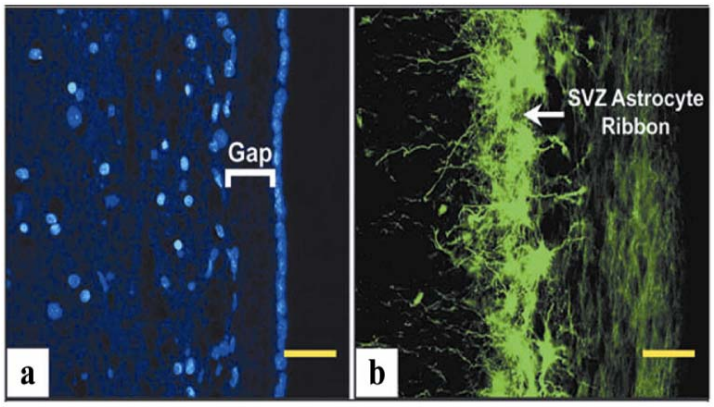


Figure 4. Dense ribbon of SVZ astrocytes in the adult human brain. (a) Coronal sections (6 μm) stained with the nuclear marker DAPI reveals a region of high cellularity that is separated from the ependyma by a gap. (b) Vibratome section showing GFAP expression in the SVZ astrocyte ribbon and GFAP-positive fibre bundles filling the subependymal gap. Adopted from Sanai et al., 2004.

Another study reported that some PSA-NCAM-positive cells were found in the adult human SEZ, although not aligned in chains. In addition, a few PSA-NCAM and nestin double-positive cells were detected in the olfactory bulb, indicating that these cells had migrated there in analogy to the rodent system (Höglinger et al., 2004). This is contradictory to the study by Arturo Alvarez-Buylla and colleagues and only further experiments can settle this conflict. However, one could speculate that migration of newborn cells from the SEZ towards the olfactory bulb does happen in humans, but is strongly reduced compared to other primates or rodents. It is well known that humans rely a lot less on their olfactory sense, and correspondingly both the size of the olfactory bulb and the number of functional olfactory receptor genes are significantly decreased in humans compared to other mammals (Kornack and Rakic, 2001; Gilad et al., 2003). Recently Curtis et al. (Curtis et al., 2007) also claimed extensive proliferation of neuronal progenitors along the lateral wall of the human lateral ventricles, massive neuroblast migration to the olfactory bulb (OB) through a putative rostral migratory stream (RMS), and a novel anatomical structure connecting the human subventricular zone (SVZ) to the OB via a continuous open ventricle. Irrespective of whether or not there is migration to the olfactory bulb, it is established that a substantial number of mitotically active cells exist in the adult human brain. However, the fate of these newborn cells from the human SEZ remains unclear.

1.4 Adult neural stem cell propagation

Extensive work over the past 10 years had shown convincingly that the embryonic ectodermal germ layer derived cell populations are present within the adult CNS that maintain proliferative capacity and generate new neurons and glial cells in adulthood (Gage, 2000; McKay, 1997). This population can be referred to as "adult neural stem cells" or aNSCs. The potential of such aNSCs to give rise to new neurons is the driving force behind the stem cell research for the amelioration or treatment of neurological disorders, stroke and brain injury-associated conditions.

Reynolds and Weiss first isolated and propagated cells from the adult mouse striatum with proliferative and differentiative abilities and introduced the concept of neurospheres. These cells, when grown *in vitro* under the influence of mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), form free-floating aggregates of cells called neurosphers (Renolds and Weiss, 1992). Neurospheres can be formed from single cells from the lateral wall of the lateral ventricle and passaged several times *in vitro*. Clonal neurospheres, when differentiated under appropriate conditions, can generate the three main cell types of the CNS: neurons, astrocytes and oligodendrocytes.

The neurosphere-forming assay has been used to detect the presence of stem cells in embryonic and adult neural tissues. Neurospheres can be generated from virtually all areas of the embryonic brain and along the ventricular neuraxis of the adult nervous system of rat brain (Wiess S 1996; Gritti A 2002). The identity of neurosphere forming cells in the embryo is poorly understood. However, their identity in the adult brain has been partially elucidated (Doetsch F 1999, 2002). A minority of cells within each neurosphere maintains their stemness character and is able to form a clonally-derived neurosphere upon single cell dissociation, representing an *in vitro* form of self-reneval. The cellular source of neurosphere formation has thus served as proof of stem cell identity. Growth factors such as EGF are essential for the induction of neurosphere formation. However, EGF signaling may also alter the cellular identity of SEZ cells in vitro. EGF is able to turn transit-amplifying progenitor cells into multipotent neurosphere-forming cells (Doetsch et al., 2002a) and ependymal cells acquire a radial glial morphology upon EGF administration *in vivo* (Gregg and Weiss, 2003).

Despite the fact that neurogenesis does occur in restricted areas of the adult brain and that cells from the SEZ and hippocampus can give rise to neurospheres capable of differentiating into neurons, astroglia and oligodendrocytes under the influence of growth factors *in vitro*, identification of a homogeneous cell population in the adult brain with long-term self-renewal properties remains elusive. One could argue that a progenitor cell population carries out adult neurogenesis with limited self-renewal capacity that exhausts itself over time, resulting in decreased neurogenesis with age. Perhaps that could explain the correlation between the observed decrease in hippocampal neurogenesis with age and the finding that the 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, the neurosphere assay provides an important tool in confirming the existence of stem cells in the mammalian CNS by exhibiting the properties of a self-renewing neural stem cell population *in vitro*. Due to the unique stem cell like behaviour of neurosphere cells, many research groups have focused their efforts on identifying the cellular source of the neurosphere-initiating population. So far neurospheres are the only functional read out for adult neural stem cells.

1.5 Prospective adult neural stem cell isolation

Flow cytometery and fluorescence activated cell sorting (FACS) are important tools for analyzing cells at the single cell level and isolating single populations based on their cell surface markers. Single cells can be analysed, classified and sorted based on a combination of cell surface markers, which has played a pivotal role in defining the stem cell population of the hematopoeitic system. Similar approaches have been used to isolate neural stem cells. In principle, different cell populations can be defined and isolated based on the combination of their characteristic cell surface markers. Fluorescent-conjugated antibodies against those epitopes are used to isolate a specific sub-population from a pool of cells. Unfortunately, it is not yet possible to reliably and easily isolate a homogeneous population of cells from freshly dissociated brain tissue that accounts for all the neurosphere-forming ability. While several attempts have been made to characterize the cell populations found in the SEZ based on extracellular markers, it is still difficult to purify a neurosphere-initiating population (Capella and Temple., 2002; Rietze et al., 2001; Uchida et al., 2000). Therefore, the search is still on to find new

surface/extracellular markers to distinguish stem cells from the progenitor cells which will be of great value to purify a homogeneous population of neurosphere-forming cells.

1.6 Potential Markers of the NSC

Identification of adult neural stem cells remains enigmatic as they are rare, and little is known about their unique characteristics. Although the SEZ stem cells have characteristics of astrocytes and express GFAP, additional markers that enable further characterization of the stem cells are lacking.

Genes expressed by adult CNS stem cells include Nestin, Musashi, Notch1, and GFAP (Sakakibara et al., 1996; Johansson et al., 1999; Doetsch et al., 1999a), but other CNS cell-types also express these markers. This severely limits the usefulness of such genes for characterization of adult neural stem cell. Moreover, many of these markers are intracellular and cannot be used for stem cell enrichment through FACS sorting. But, it has been shown that this problem can be overcome by creating transgenic mice with fluorescent reporter gene expression (Kawaguchi et al., 2001). A more generally useful marker would be a cell surface molecule allowing stem cell localization and purification from a wild-type mouse.

Several markers have been reported for isolating neural stem cells from the adult brain (Table1.1). The first example of immunoselection using a surface antigen was the use of antibodies against Notch1 (which was shown to label specifically ependymal cells of the SEZ) to enrich NSCs from the adult mouse brain (Johansson et al. 1999). In the same year Morrison et al. used p75 to isolate mouse neural crest stem cells. Uchida et al. (2000) also succeeded in isolating a population enriched for human fetal NSCs by FACS sorting the cells, which were CD133+, and mCD24^{-}. In another approach, Rietze et al. (2001) reported the isolation of one type of adult rat NSC from the periventricular area by selecting for cell size >12mM and collecting only those cells which were negative for peanut agglutinin (PNA)-binding activity, a marker for mouse HSCs (Salner et al., 1982), and also expressed low levels of CD24, a marker for neuronal progenitors and ependymal cells (Calaora et al., 1996; Shewen et al., 1996; Doetsch et al., 1999).

Table 1.1. Neural stem cells isolated using different surface antigens.

Surface markers	NSCs properties (*in vitro*)	NSCs properties (*in vivo*)	Expression in brain tissue	Reference
Notch 1	Yes	No	SEZ, Ependyma	Johansson et al. 1999
$CD133^{+VE}$ $mCD24^{-VE}$	Yes	Yes	Human foetal brain tissue	Uchida et al. 2000
P75	Yes	Yes	Neural Crest cells	Morrison et al. 1999
Lewis-x/ GFAP	Yes	No	SEZ, Astrocyte	Capella et al. 2002
PNAlo HSAlo Nestin +ve >12mM	Yes	Yes	SEZ, Astrocyte	Rietze et all 2001
PDGFRα/ GFAP	No	Yes	SEZ, Astrocyte	Jackson et al. 2006
CD133	Yes	Yes	SEZ, Ependyma	Coskun et al. 2008

Subsequently, Capella et al., (2002) came up with Lewis-X as a marker, a carbohydrate moiety expressed on the surface of embryonic stem cells, cells in the germinal zones of developing mouse brain, and on the surface of some adult neurosphere-forming astrocytes in the stem cell niche of adult brain. In 2006, Jackson et al. showed that a subset of astrocytes in the SEZ expresses PDGFRα and behave as neural stem cells. For the first time they could demostrate that PDGFRα signaling in SEZ is one of the key regulators in the stem cell lineage which determines the balance between oligodendrocytes and neuron generation *in vivo*. Recently Coskun et al. 2008 came up with cell surface molecule CD133, showing its expression exclusively in ependymal cells in SEZ, which has neural stem cell chaterteristics both *in vitro* and *in vivo*.

However, among all these markers used, none could enrich the NSCs pool to 100%, revealing that not all the cells selected by above mentioned potential neural stem cell markers are stem cells.

1.6.1 CD133: molecule of the moment – a potential stem cell marker

CD133 (prominin-1) was the first identified member of the prominin family of pentaspan membrane proteins. Although the ligands and specific functions of the prominins are still unclear, they show restricted expression within plasma membrane protrusions, such as epithelial microvilli and epididymal ductal epithelial sterocilia, hence deriving their name from latin *prominere* meaning 'to be prominent'. Due to its expression by haematopoietic progenitors (Yin et al., 1997), interest has been directed towards the potential of CD133 as a cell surface marker of adult stem cells. CD133 is expressed by a number of stem cells, including myogenic, hematopoietic and intestinal stem cells (Miraglia et al., 1997;

Liqin et al., 2009). Additionally, CD133 is also expressed on endothelial progenitor cells (EPCs), which play a role in angiogenesis and neovasculogenesis during both tumour growth and wound healing (Ribatti D et al., 2004). CD133 is also a promising stem cell marker in several other tissues (Table 1.2). Uchida et al. used an antibody directed against the unglycosylated form of CD133 to isolate stem cells from human neural tissue, which were capable of successful engraftment, migration, proliferation, and neural differentiation for a prolonged period following transplantation into the brains of NOD/SCID mice (Uchida et al., 2000). Lee et al. isolated CD133+lineage− cells, which constituted approximately 0.1–0.3% of cells in the developing cerebellum of mouse, and showed that these cells were capable of clonal expansion to form neurospheres *in vitro* and expressed other neural stem cell markers including Musashi, Sox-2, and GFAP (Lee et al., 2005). These cells underwent clonal multi-lineage differentiation both *in vitro* and *in vivo* (Lee A et al., 2005). Recent data of Corti *et al* also confirm the potential of murine $CD133^{+VE}$ cells to undergo multi-lineage differentiation to form both neurons and glia *in vivo* (Corti S., 2007). Barraud *et al* showed that the $CD133^{+VE}$, stage-specific embryonic antigen 4+ (SSEA4+) fraction isolated from the human embryonic forebrain could be used to enrich neural progenitors (Barraud P et al., 2007). More recently, CD133 has gained increased attention as a surface marker to isolate a subpopulation of cells called ‘cancer stem cells’ from glioblastoma, medulloblastoma, ependymoma, (Singh SK et al., 2004; Taylor MD et al., 2005; Bao S et al., 2006; Piccirillo SG et al., 2006), prostate and colon cancer (Collins AT et al., 2005; O’Brien CA et al., 2007; Ricci-Vitiani L et al., 2007), which are believed to be responsible for initiating and sustaining tumor growth.

Although CD133 has been well characterized in terms of haematopoetic and cancer stem cells, its role has yet to be fully understood in the adult neurogenic niches. Recently, it has been shown that CD133 is expressed in the adult SEZ and contributes to adult neurogensis. Therefore, it would be very relevant to further characterize this molecule in detail in adult neurogenic niches.

Table 1.2. Cells expressing prominin family members, often in combination with a repertoire of other markers, are widely expressed by multiple tissues and function as stem cells

Antigenic phenotype	Origin	Stem cell action	Reference
AC133 or CD133	Human adult blood, bone marrow, cord blood, and fetal liver	Human haematopoietic reconstitution	Yin AH, 1997; Gallacher L, 2000; Lang P 2004; Bitan M 2005
AC133	Human peripheral blood	Myogenesis in mouse model of Duchenne's muscular dystrophy	Torrente Y, 2004
CD133	Human peripheral blood	Endothelial and cardiomyocytic differentiation *in vitro*	Bonanno G, 2007
CD133	Adult human kidney	Endothelial and epithelial differentiation, human renal regeneration	Bussolati B, 2005
CD133	Human bone marrow	Human liver regeneration, mechanism unknown	Am Esch JS 2nd, 2005
CD133	Human and mouse brain	Neural differentiation in mice	Uchida N., 2000; Lee A., 2005; Corti S., 2007
CD133, SSEA4	Mouse embryonic forebrain	Neural differentiation *in vitro*	Barraud P, 2007
AC133-2, β1-integrin	Human neonatal foreskin	Keratinocyte differentiation *in vitro*	Yu Y, 2002
CD133, β1-integrin	Human prostate basal cells	Prostatic acinar differentiation in mice	RichardsonGD, 2004
$CD133^{+}CD34^{-}CD45^{-}$ $Ter119^{-}$	Ductal epithelium neonatal mouse pancreas	Multiple lineage differentiation in mouse pancreas	Oshima Y, 2007
CXCR4, NGN-3, nestin, CD133, Oct-4, Nanog, ABCG2, CD117	Human pancreas	Islet differentiation *in vitro*	Koblas T, 2007

1.7 Concept of the neurogenic niche

Several lines of evidence suggest that neurogenesis in the mature CNS is dependent upon a permissive microenvironment. The first evidence came from the observation that,

neural stem and progenitor cells, when transplanted into non-neurogenic regions, did not generate neurons. Rather, they either died or differentiated into glial cells (Suhonen et al. 1996). Transplantation studies have shown that progenitors from non-neurogenic regions give rise to neurons when transplanted into neurogenic regions. Moreover, the generated neurons differentiate in a region-specific manner (Gage et al. 1995a; Suhonen et al. 1996; Takahashi et al. 1998; Shihabuddin et al. 2000). The SEZ precursor cells generate hippocampal neurons when transplanted into the hippocampus and the SGZ precursor cells generate olfactory interneurons after transplantation into the RMS (Suhonen et al. 1996). Strong influence of the local microenvironment on the outcome of differentiation suggests that neurogenesis requires neurogenic niches, but an elaborate understanding of the cellular and molecular mechanisms at play are still lacking.

The composition of the neurogenic niche is being extensively studied, and it appears that all of its constituents, including endothelial cells lining the blood vessels, ependymal cells, extracellular matrix, astrocytes, mature neurons, and even neural stem and progenitor cells, contribute to neurogenic permissiveness. In culture, endothelial cells release soluble factors that promote neuronal proliferation and generation from neural stem cells (Shen et al., 2004), which seems to take place *in vivo* as well (Ramirez-Castillejo et al., 2006). In addition, endothelial cells upregulate neurotrophic factors and increase angiogenesis in response to injury (Gotts and Chesselet, 2005; Ohab et al., 2006), features that are thought to strongly influence the neurogenic niche. Furthermore, neurogenic niches of dividing progenitors are in close proximity to blood vessels (Shen et al., 2008; Palmer et al., 2000), suggesting a role for the vasculature in promoting neurogenesis. In addition, circulating VEGF has a survival-promoting effect on neuronal cells (Schanzer et al., 2004). Radial glias, such as astrocytes in the subgranular zone, contact blood vessels with their vascular endfeet and are in direct communication with endothelial cells (Filippov et al., 2003). Multi-ciliated ependymal cells of the SEZ constitute the barrier between the CSF-filled ventricles and the SEZ and are potent regulators of the neurogenic niche. In accordance with this, ependymal cells have been shown to produce the BMP antagonist noggin, thereby promoting neurogenesis (Lim et al., 2000). Recently, ependymal cells have also been demonstrated to harbor the capacity to dedifferentiate and act as neural stem cells, contributing to the overall production of neurons (Zhang et al., 2007; Coskun et al., 2008). Further studies confirming these findings are needed to establish the function of ependymal cells as neural stem cells in the

SEZ neurogenic zone. The extracellular matrix of the neurogenic niche is enriched with molecules that influence a neurogenic environment, such as Tenascin C and Reelin (Garcion et al., 2004; Zhao et al., 2007). Laminins not only provide anchorage, but also influence proliferation, differentiation, and migration of stem and progenitor cells (Campos, 2005). Local astroglia in this region secrete cytokines and chemokines, thereby modulating the neurogenic environment (Barkho et al., 2006). Astrocytes also produce basic morphogenic signals in the neurogenic niches, such as sonic hedgehog (Jiao and Chen, 2008) and the Wingless type (Wnt) glycoprotein. Sonic hedgehog can even increase neurogenic permissiveness in the otherwise non- neurogenic neocortex (Jiao and Chen, 2008). In the hippocampus, Wnt 3a is expressed by astrocytes and supports neuronal production (Lie et al., 2005). In both the subventricular zone and hippocampus, astrocytes express the cell surface receptor Notch, which maintains them in an undifferentiated state and capacity for self-renewal (Tanaka et al., 1999; Alexson et al., 2006; Givogri et al., 2006; Breunig et al., 2007). Interaction between stem cells and their progeny also constitutes a part of the neurogenic niche. Neuroblasts in the niche function within a negative feedback loop, where inhibitory GABA signals from neuroblasts on radial glia-like astrocytes reduce the production of additional neuroblasts (Liu et al., 2005). Synaptic activity from mature neurons strongly affects the production of and integration of new cells within the neurogenic niche, especially in the hippocampus (reviewed Ming and Song, 2005). It has also been shown recently that the neurogenic zone in the adult SEZ is not restricted to the cells adhering to the lateral wall of the ventricle. Rather, it consists of a large extended area, starting from the SEZ to the core of the adult olfactory bulb (OB). Both Merkle et al. (2007) and Alonso et al. (2008) confirmed the presence of astrocyte- like progenitors in the RMS giving rise to OB interneurons, creating a microenvironment for neural precursor cells capable of cell division and neuronal commitment. However, permanent suppression of SEZ neurogeneis following whole brain radiation (Panagiotakos et al., 2007) indicates that the self-renewal of neural precursors in the RMS lacks long-term capacity to repopulate the OB interneurons, at least when the neurogenic potential of the SEZ is inactivated. Perhaps most interesting, in the context of regenerative medicine, is the ability of the brain, under certain circumstances, such as injury, to produce local neurogenic niches in non-neurogenic regions. This has been demonstrated in the case of stroke (Ohab et al., 2006) and also by modulating factors, which act as negative regulators for neurogenesis outside neurogenic niches, like ephrins (Jiao et al., 2008).

1.8 Diverse functions of astrocytes in neurogenic and non-neurogenic niches

Astrocytes were previously considered part of the connective tissue of the brain. They have multiple functions like maintaining the local ion concentrations, interacting with endothelial cells to form the blood–brain barrier and taking up neurotransmitters at the synaptic cleft. Recently, it has been shown that these so called 'support cells' can act as dynamic regulators of many processes, including synaptogenesis and synaptic efficacy (Ullian et al. 2001; Christopherson et al. 2005). Additionally, a subpopulation of astroglia cells expressing GFAP in the adult brain (SEZ and SGZ) have characteristics of neural stem cells (Doetsch et al. 1997; Doetsch et al. 1999a; Seri et al., 2001; Seri et al., 2004; Steiner et al., 2004; Gotz et al., 2002; Laywell et al. 2000; Imura et al. 2003; Morshead et al. 2003; Garcia et al. 2004; Sanai et al. 2004; Ahn & Joyner 2005). Thus, astrocytes are emerging as key mediators of brain development, function and plasticity, highlighting the critical need to better characterize the heterogeneity and developmental specification of different subpopulations of astrocytes both within adult neurogenic regions and throughout the brain (Bachoo et al. 2004; Bonaguidi et al. 2005; Muroyama et al. 2005; Imura et al. 2006; Lim et al. 2006; Sakaguchi et al. 2006).

In addition to their role as stem cells in the neurogenic niches, astrocytes also help in sensing and regulating the environment of the niche. Long appendages of astrocytes allow them to contact all cell types and structures in the niche (Doetsch et al. 1997; Seri et al. 2004), including blood vessels and the basal lamina (Mercier et al. 2002). Furthermore, astrocytes are often coupled via gap junctions and have the ability to form a syncytium (reviewed in Giaume & McCarthy 1996; Giaume & Venance 1998). Thus, they integrate the diverse signals originating from different cell types and provide direct cell-to-cell communication within the niches, thereby regulating activation and differentiation of stem cells.

In contrast to astroglia from neurogenic niches, cortical astroglia are postmitotic non-dividing cells under normal conditions. It is not clear whether these cells can be induced to function as stem cells in adult animals. Interestingly however, young astrocytes isolated from multiple brain regions before postnatal day 10 can give rise to

neurospheres— neural stem cells *in vitro* (Laywell et al., 2000). The relationship and defining characteristics of germinal astrocytes versus parenchymal ("differentiated") astrocytes remains to be determined. Nevertheless, cortical astrocytes upon CNS damage have been shown to be more plastic than neurons and oligodendrocytes; they possess the potential to proliferate and migrate (Fawcett and Asher, 1999; Norton et al., 1992). Cortical astrocytes are the major cell-type that responds to any kind of injury; by reactive astrogliosis. This process is characterised by hypertrophy, cell migration, cell proliferation and increased expression of glial fibrillary acidic protein (GFAP) leading to the formation of a glial scar (Eddleston and Mucke, 1993; Fawcett and Asher, 1999; Norton et al., 1992; Wu and Schwartz, 1998; Wu et al., 1998). Glial scars, predominantly comprised of astrocytes, impede repair processes, preventing axonal growth and remyelination of demyelinated axons (Fawcett and Asher, 1999; Fok-Seang et al., 1995). A better understanding of the functional biology of astrogliosis will be very important for therapeutic interventions that can be used in treating patients.

1.9 Induction of neurogenesis in the non-neurogenic regions of the brain

Neurogenesis in non-neurogenic regions could be induced in one of at least three possible ways: (i) activation of local parenchymal precursor cells to generate new neurons upon a stimulus of either a pathological event or a set of directed molecular and/or genetic interventions; (ii) migration of precursors from the neurogenic niches to the parenchyma upon stimulation and (iii) De-differentiation and reprogramming of reactive astroglia (major cell type proliferating upon any insult) to multipotent neural stem cells either *in vivo* or *in vitro*. The intriguing possibility that multipotent neural stem cells may be present in injured tissue therefore it needs to be further investigated, and is one of the aims of this thesis. A number of studies provide evidence for each of the above outlined possibilities.

A few studies have shed light on the possibility of neural stem cells in the adult rodent neocortex. In 2001, Arsenijevic et al. isolated adult neural stem cell-like cells from the cortex and amygdala of adult human epileptic patients. Subsequently, Nunes et al. in 2003 isolated multipotential oligodendrocyte precursor cells using the A2B5 surface antigen from human subcortical white matter. These adult human white-matter progenitor

cells (WMPCs, A2B5+) could be passaged as neurospheres *in vitro* and could always generate functionally competent neurons and glia both *in vitro* and after xenograft to the foetal rat brain. Gross and colleagues, in the late 90's, reported that the progenitor cells arising from the SEZ, could migrate through the white matter in to neocortical regions where they differentiate into mature neurons (Gould et al., 1999). However, two studies performed in macaques were not in agreement with these data. Rather, they demonstrated that mitotically active cells detected in the neocortex are in fact satellite glial cells closely apposed to resident neurons (Kornack and Rakic, 2001; Koketsu et al., 2003). These discrepancies underscore the necessity to perform detailed confocal analysis and three-dimensional reconstructions to address unambiguously the origin of newly generated neurons and glia in the adult CNS.

Magavi and colleagues (2000) reported for the first time that neurogenesis can be induced from endogenous precursors in adult rodent cortex by manipulating the microenvironment in the non-neurogenic regions of the adult CNS. They selectively killed the neurons by apoptosis, thereby inducing endogenous precursors to divide and differentiate into neurons in mammalian neocortical layer VI. However, this lesion destroyed only the targeted neurons without affecting the surrounding tissue. Nevertheless, this work served as a 'proof of concept' that neurogenesis can be induced in non-neurogenic area of adult CNS. Based on this work, other groups extended this general concept and reported similar and complementary results in the hippocampus (Nakatomi et al. 2002) and striatum (Arvidsson et al. 2002; Parent et al. 2002b). Nakatomi and colleagues used an *in vivo* ischaemia model in which they observed that repopulation of neurons in the CA1 region of the hippocampus is possible following their elimination (Nakatomi et al. 2002). Recently, Chen et al. (2004) demonstrated the induction of low-level neurogenesis of corticospinal motor neurons from endogenous precursors in adults, with progressive extension of axons into the cervical spinal cord over three to four months. Taken together, these results demonstrate that endogenous neural precursors can be induced to differentiate into CNS neurons in a region-specific manner.

Reactive astroglial cells are the major cell-type that responds to any kind of injury. The most prominent hallmarks of reactive astrocytes are the upregulation of GFAP and vimentin, which are also expressed by astrocytes in the stem cell niche. Reactive glia also

begins to re-express Nestin and synemin (Clarke et al., 1994; Eliasson et al., 1999, Jing et al., 2007). However, a subpopulation of reactive astrocytes also shows proliferation indicated by pairs of BrdU-positive cells (Magavi et al., 2000). It has been suggested that these dividing astrocytes may arise from endogenous progenitors (Miyake et al., 1998; Alsono et al., 2005; Buffo et al., 2005; Sofroniew et al., 2005). Leavitt et al. in 1999 observed the re-expression of RC2-positive radial glia cells following the transplantation of embryonic neurons into neuron deficient regions of the adult neocortex (Leavitt et al., 1999). Dedifferentiation of astrocytes into radial glial cells has also been observed after chronic hypoxia (Ganat et al., 2002). These observations suggest the possibility that radial glial cells de-differentiating from astrocytes in the injured cortex may act as the endogenous neuronal progenitors, which are present in the embryonic brain during development. It is not yet known in which aspects the activated radial glia in the adult brain and radial glia in the embryonic brain differ, but these studies raise the exciting possibility that manipulation of extrinsic cues in the adult brain may be sufficient to convert astrocytes to an early stage of neurogenic radial glia after injury and thereby allow repair from endogenous precursors. Therefore, the quest for a better understanding of the radial glia-astroglia diversity at the molecular and cellular level should be a key element of future research that focuses on neuronal regeneration in the adult brain following injury.

1.10 Aims of the present study

As discussed above, astrocytes are the main player responsible for neurogenesis in most regions in the adult brain. Moreover, astrocytes are the responsive cells following injury. They are not only involved in containment of damage upon injury or inflammation to a restricted area but they also give rise to precursor cells that generate new neurons and thereby help in restoring the damaged part of the brain. The present study was carried out with an astrocentric viewpoint and aimed at getting a better understanding of astroglia function in neurogenic, non-neurogenic and in the injured environment by examining their behavior and transcriptome.

In order to exploit the potential of astrocyte-like endogenous neural stem cells for therapeutic intervention a better understanding of the basic biology of adult neural stem cells and neurogenesis in the adult brain is necessary. A substantial part of the present study was devoted to understanding the role of astrocytes in neurogenesis in the adult

brain at cellualar and molecular level.

The second part of the present study was aimed at addressing whether de-differentiation of reactive glia occurs in vitro to such a degree that they may reacquire stem cell potential. If so, the next aim was to determine the factors responsible for this dedifferenciation.

The present study was conducted with the following objectives:

1) Prospective isolation of adult neural stem cells
2) To characterize the neural stem cells and astrocyte-like cells in the SEZ by gene expression profiling.
3) To identify additional markers that can distinguish the stem cells from the progenitor cells in the SEZ.
4) To ascertain whether de-differentiation of reactive glia occurs *in vitro* and what endogenous factors are required for this process.
5) Generation of new mouse line for in vivo labeling of astrocytes in different regions of brain.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers and solutions

(A) Immunohistochemistry

Name	Protocol
BrdU-Solution in *drinking water*	1mg/ml 5mg/ml (w/v) BrdU in H20
BrdU-Solution for *i.p.*-Injection	5mg/ml (w/v) BrdU in 1x PBS
HCl (2.4 N)	2.4 N HCl (37 % (w/v)); 1:5 dilution in ddH20
NaN_3-PBS (0.05%)	0.05% (w/v) NaN_3 in 1x PBS
PBS (Phosphate buffered salt solution) 1x pH 7.4	137 mM NaCl, 2.7 mM KCl, 80.9 mM Na2HPO4, 1.5 mM KH2PO4 in ddH2O
PFA (2%)	2% (w/v) paraformaldehyde in 1x PBS
PFA (4%)	4% (w/v) paraformaldehyde in 1x PBS
Saline (isotonic)	0.9% (w/v) NaCl in ddH20
Sucrose-PBS-solution (30%)	30% (w/v) sucrose in 1x PBS
Triton X-100 (0.1% / 0.5%)	0.1% / 0.5% (v/v) Triton X-100 in 1x PBS

(B) Isolation of genomic DNA

Proteinase K lysis buffer:	100mM Tris, pH8.0-8.5 5mM EDTA, pH8.0 2% SDS 200mM Sodium chloride

(C) DNA agarose gels

TAE buffer (50 x stocks):	2M Tris 50mM Glacial acetic acid 50mM EDTA
Loading buffer DNA:	100mM EDTA 2% SDS 60% Glycerol 0.2% Bromine phenol blue

(D) Southern blot

Depurination

(Fragments >=10kb): 1.1% HCl in H_2O

Denaturation (all gels):	87.66g Sodium chloride 20.00g NaOH 1000ml H_2O (final volume)
Neutralization (all gels):	87.66g Sodium chloride 60.50g Tris 1000ml H_2O (final volume) pH 7.5 with HCl conc. (approx. 11ml)
Transfer, 20x SSC (all gels):	88.23g Tri-sodium-citrate 175.32g Sodium chloride 1000ml H_2O (final volume) pH 7-8
Hybridisation buffer:	1M Sodium chloride 50mM Tris, pH7.5 (at 37°C) 10% Dextransulfate 1% SDS 250µg/ml Salmon Sperm DNA sonificated Store 30ml aliquots at -20°C
Washing buffers	2x SSC / 0.5% SDS 1x SSC / 0.5% SDS 0.1% SSC / 0.5% SDS
Stock solutions:	20x SSC 175.3g Sodium chloride 88.2g sodium citrate, pH7.0 20% SDS 200g SDS

(E) Bacteria and culture media

Bacteria

E. coli K12 EL350 (Lee *et al*., 2001)

Culture media

LB medium (lysogeny broth; Bertani, 1951)

LB agar (lysogeny broth; Bertani, 1951)

Supplemented with 100µg/ml ampicillin

25µg/ml kanamycin

1000ml H_2O (final volume)

2.1.2 Enzymes and kits

1) Superscript II (Invitrogen)

2) Restriction enzymes (NEB GmbH, Frankfurt a. M.; Fermentas GmbH, St. Leon-Rot)

3) DNA-Polymerases (DNA Polymerase I, Large (Klenow) Fragment, NEB GmbH, *Taq* DNA Polymerase; Qiagen,; *Pfu* DNA Polymerase, invitrogen)
4) RNase-free Dnase I (Promega GmbH, Mannheim)
5) Ligase (T4 DNA ligase; NEB GmbH, Frankfurt a. M.; M0202)
6) Phosphatase (T4 Polynucleotide Kinase, NEB GmbH)
7) QIAquick PCR Purification Kit (Qiagen)
8) QIAquick Gel Extraction Kit (Qiagen)
9) QIAgen Maxi Kit (Qiagen)
10) QIAgen Mini Kit (Qiagen)
11) RNeasy Micro Kit (Qiagen)
12) Labelling Kit (Roche Holding GmbH, Applied Science, Mannheim)
13) QuantiTect™ SYBR Green PCR Kit (Qiagen)

2.1.3 Culture media

(A) ES cells

MEF: DMEM (Gibco, Invitrogen™ Cooperation, Carlsbad, CA), supplemented with 2mM L-glutamine (200mM Gibco, Invitrogen™ Cooperation, Carlsbad, CA), 15% FCS (PAN Biotech GmbH, Aidenbach), 0.1mM β-mercaptoethanol(50mM, Gibco, Invitrogen™ Cooperation, Carlsbad, CA), 1x MEM (non-essential amino acids, 100x; Gibco, Invitrogen™ Cooperation, Carlsbad, CA).

TBV2: DMEM (Gibco, Invitrogen™ Cooperation, Carlsbad, CA), supplemented with 2mM L-glutamine (200mM Gibco, Invitrogen™ Cooperation, Carlsbad, CA), 15% FCS (PAN, Biotech GmbH, Aidenbach), 0.1mM β-mercaptoethanol(50mM, Gibco, Invitrogen™ Cooperation, Carlsbad, CA), ESGRO® (LIF) (107U/ml; Chemicon, Millipore, Schwalbach), 1x MEM(non-essentiell amino acids, 100x; Gibco, Invitrogen™ Cooperation, Carlsbad, CA).

ES CELL SELECTION: TBV2 supplemented with 300µg/ml G418 (50mg/ml Geneticin; Gibco, Invitrogen™ Cooperation, Carlsbad, CA).

(B) Primary Cell Culture

(i) Factor Dilutions

EGF (epidermal growth factor, Roche 855573 grade I, 100 μg)
100 μg/10ml DMEM: F12 medium for 1hr to dissolve at room temp
100 μl aliquot =10ng/μL (- 20^{o}C, store)

bFGF (bovine basic fibroblast growth factor, Roche 10555400, μg)
10 μg /1ml DMEM: F12 medium for 1hr to dissolve at room temp
100 μl aliquaot =10ng/μL (-20^{o}C, store)

Shh-N (R&D systems, 461-SH/CF)
25 μg/125μl in 0.1% BSA (-20^{o}C, store)

(ii) Tissue Preparation Buffer: HBSS (Gibco, 1X; 500ml), HEPES (Gibco, 1M; 5ml)

(iii) Dissociation media: 5ml Solution 1 supplemented with 1ml of 0.05% Typsin+EDTA (Gibco, Cat.no. 25300-054).
(iv) Neurosphere Isolation Solutions

Solution 1: D/Glucose(Sigma, stock: 300 mg/ml; 9ml), HEPES (Gibco, 1M; 7.5 ml), HBSS (Gibco, 1X; fill up to 500ml), *Adjust pH to 7.5*

Solution 2: Sucrose (Sigma; 154g), HBSS (Gibco, 1X; fill up to 500ml), *Adjust pH to 7.5*

Solution 3: HEPES (Gibco, 1M; 10 ml), BSA (Sigma, A4503; 20g), EBSS (Gibco, 1X; fill up to 500ml), *Adjust pH to 7.5*

(v) Neurosphere Medium: DMEM/ F12 (Gibco Invitrogen™ Cooperation; 47ml), B27 supplement (Gibco Invitrogen™ Cooperation; 1ml), Pen/Strep (Gibco, Cat.No 5140-122; 0.5ml), Hepes buffer, 1M (Gibco Invitrogen™ Cooperation; 0.4 ml), EGF (10μg/ ml; 50μl), bFGF (10 μg/ ml; 50μl)

(vi) Staining Solution FACS: FCS (Foetal Calf Serum, sigma; 50ml), Sodium Azide (stock 10%NaN_3 in PBS; 100 μl), DMEM/F12 (Gibco, LT 31331-028, fill upto 50ml)

(vii) PDL coated cover-slips: 1mg/ml PDL stock dilute 1: 100 in PBS add 0.5ml/well (24 well plates with cleaned autoclaved coverslips, diameter of coverslips 13mm), incubate 1-24 hrs at 37 degrees, wash with sterile water 4 times 1ml in each well thoroughly.

2.1.4 Primers

Primers for Genotyping	Name	Sequence
hGFAPeGFP	GFAP-LZ	actccttcataaagccctcg
	GFP-2	aagtcgatgcccttcagctc
Glast:: CreERT2	GLAST	gaggcacttggctaggctctgagga
	F8	gaggagatcctgaccgatcagttgg
	GLAST	ggtgtacggtcagtaaattggacat

Primers for qRT-PCR	Type	Sequence
WD repeat domain 78	fw	cagaccaggtcagggtgttt
	rv	ggcttctttcctccactcaa
centrin 2	fw	tagtgcctggcattttgtga
	rv	gtgctcttgttctgccctgt
Fox J1	fw	gagtgagggcaagagactgg
	rv	caggctggaaggtttgtagc
calcium/calmodulin-dependent protein kinase	fw	aagaccaaccgtgagtggac
	rv	ctcagggattctgtggcatt
calpain	fw	gagatgcgaaatgcagtcaa
	rv	agctctgaacatcccttcca
parvalbumin	fw	gggcctgaagaaaaagaacc
	rv	ttcttcaaccccaatcttgc
melanoma inhibitory activity 1	fw	gggccaagtggtgtatgtct
	rv	tcccattgatcggtcttcat
Forkhead box O1	fw	tgccctgtgtcacgctgtcatagt
	rv	gtagtaggaggcgaaccccagtca
BMPER	fw	gcatgtcctgcactgaagtc
	rv	aaacgtagatggcccctagc
Bmpr1b	fw	aacccttgccaaaatgtcag
	rv	cccaaacagtttctcctcctc
Bmpr1a	fw	ctcctggctgtaaggagtgg
	rv	aaggaaagaacagagcacaacc
IFT57	fw	aatggcggagtaactgaacg
	rv	tgaagtagcgtgtgctccac
desmulin	fw	gcgtggggacagtttatacg
	rv	cgcacctattttcctttcca
ciliary rootlet coiled-coil, rootletin	fw	ctcgaggagcaggtatccac
	rv	ggcaggctatgctacaaagg

Primers for qRT-PCR	Type	Sequence
cyclin G2	fw	ggaggctaccccggagaatgataa
	rv	cagacgccaatgcaggacaggt
cAMP responsive element binding protein 1	fw	agcccctgccatcaccact
	rv	accccatccgtaccattgttagc
Fgfr2	fw	gcccggccctccttcagtttag
	rv	acctcattgtgggcgttggattc
cyclin-dependent kinase 4	fw	gctcccgggcccagataaag
	rv	tcccataggcaccgacaccaa
Fgfr3	fw	cgtggccgtgaagatgctgaaa
	rv	gccgcgcccgaaggaac
Fgfr4	fw	ccttcccggccctagtctcctgt
	rv	gcggccgttgctggttttcttat
Fgfr1	fw	agtgccgccgggagtcgtg
	rv	tggcgcggcgggtgtct
cyclin D2	fw	cgctcgcccaccttccactc
	rv	ccagccggccaccactcg
Bmp6	fw	ttgactctcggtggtgtgag
	rv	ggagccagtgttcctgatgt
RIKEN cDNA 1700027A23 gene	fw	ccagactggtctcaggttcc
	rv	acgaaatgggcagacaagag

2.1.5 Antibodies

(i) Primary antibodies

Name	Host-animal	Marker	Supplier
β-tubulin-III	Mouse, monoclonal IgG2b	Postmitotic neurons	Sigma
BrdU	Rat	S-Phase marker	Abcam
β-galactosidase	Rabbit	Reporter gene	Cappel
DCX	Guineapig	Immature neurons	Chemicon
GFAP	Rabbit	Astrocytes	DAKO
GFAP	Mouse, monoclonal IgG1	Astrocytes	Sigma
GFP	Chicken	Reporter gene	Aves LABS
O4	Mouse, monoclonal IgM	Mature oligodendrocytes	Gift from Jack Price
PDGFRα	rabbit	Immature oligodendrocytes	Spring Biosciences
S100β	Mouse, monoclonal IgG1	Astrocytes	Sigma

(ii) Secondary antibodies

Name	Supplier
Anti-rabbit Ig FITC / TRITC / biotinylated	Boehringer Ingelheim
Anti-rabbit Ig Cy2 / Cy3/Cy5	(Vector Laboratories)
Streptavidin AMCA	(Vector Laboratories)
Anti-mouse IgG+M Cy3	EuroPath Ltd.
Anti-mouse IgG1 FITC / TRITC / biotinylated	(Southern Biotechnology Associates)
Anti-mouse IgG2b FITC / TRITC / biotinylated	(Southern Biotechnology Associates)
Anti-mouse IgM FITC / TRITC / biotinylated	(Southern Biotechnology Associates)
Anti-guinea pig Ig Cy2 / Cy3	Dianova Immundiagnostics
Anti-rat FITC / TRITC	(Jackson ImmunoResearch)

(iii) Antibodies for FACS

Type	Name	Supplier
Isotype control	PE™ Rat IgG1, κ Isotype Ctrl antibody	BD Bioscience
Anti-CD133	Rat, monoclonal IgG1	BD Bioscience
Isotype control	Pacific Blue™ Rat IgG2b, κ Isotype Ctrl antibody	Biolegend
Anti-CD24	Pacific Blue™ anti-mouse CD24	Biolegend

2.1.6 Mouse lines

C57Bl/6 (inbred strain) hGFAPeGFP (background FVB/N; Malatesta et al., 2003; Nolte et al., 2001; Buffo et al., 2008)

Glast::Cre/ERT2/ZeG (background C57Bl/6; Mori et al., 2006; Ninkovic et al., 2007)

2.2 Methods

2.2.1 Methods in Molecular Biology

(A) Preparation of nucleic acids: DNA preparations

(a) Plasmid preparations according to the QIAGEN Plasmid Kits

Plasmid preparations were carried out using the QIAGEN Mini Kit .The DNA pellet was resuspended in up to 50µl dist. H_2O, TE buffer or EB buffer. For larger amounts of DNA the QIAGEN Maxi Kit was used and the DNA was resuspended in 150-300µl TE buffer.

(b) Preparation of genomic DNA from cells or tissue

The preparation of genomic DNA from cells or tissue, in principle, consists of three steps: the complete lysis of the cells, the precipitation of the DNA and its subsequent

purification. The lysis is carried out using SDS-containing buffers, which is freshly supplemented with proteinase K to remove proteins, especially DNA associated proteins. The incubation is done at 55°C, the optimal temperature for the proteinase activity. By adding salt and ethanol to the solution after complete lysis the DNA is precipitated. Afterwards, the DNA is washed with ethanol, dissolved in water or buffer and, if necessary, purified by an additional phenol-chloroform extraction. This last cleaning step is only necessary when subsequent restriction digests are performed with especially sensitive restriction enzymes.

(i) Isolation of genomic DNA from cells in 96-well plate format

The purification of DNA with phenol-chloroform can be disregarded in 96-well format because of the effort. Therefore, the following restriction digests should be tested with DNA prepared according to the same protocol. To obtain enough material, the extraction of DNA from cells in 96-well format should be started when the cells are confluent and the medium (100µl/well) turns yellow or orange within one day. Directly before starting, the preparation the lysis buffer was supplemented with proteinase K in a concentration of 100µg/ml, because the proteinase will digest itself over time. The cells were washed twice with PBS–$Mg^{2+/}Ca^{2+}$ to completely get rid of the medium. The PBS was removed and 50µl of lysis buffer were added to each well. The plate was sealed with parafilm and incubated at 55°C in a humid chamber overnight. The next day, 150µl 5M sodium chloride was mixed with 10ml 100% ice-cold ethanol and 100µl of this mixture was added to each well for precipitation. The plate was incubated at room temperature for 30min without moving. To decant the liquid, the plate was inverted carefully and slowly (in about one minute). The rest of the liquid was removed by inverting the plate on a paper towel upside-down. Subsequently, the DNA was washed three times using 150µl 70% ice-cold ethanol per well. For each wash, the plate was inverted as previously described. At this point the DNA can be stored in 70% ethanol at -20°C. After the last washing step, the DNA was dried at RT for 10-15min. Then 25µl TE buffer or water was added to the pellet and the DNA was dissolved at 4°C overnight or at 37°C, shaking, for 1h in a humid chamber.

(ii) Isolation of genomic DNA from mouse-tail biopsy

The preparation of genomic DNA from mouse-tail tips provides the basis for genotyping. For efficiency reasons the genotyping is carried out by PCR and therefore it is important

that preparations are done as clean and carefully as possible to prevent contamination. Each individual mouse tail tip with a length of approximately 4mm was put into a single Eppendorf reaction tube and was stored at -20°C if it could not be processed the same day. The lysis buffer for mouse tail tip DNA was freshly supplemented with proteinase K (final concentration: 100µg/ ml) and each mouse tail tip was lysed in 500µl of this buffer at 55°C overnight. The next day, the complete lysis was visually checked and the suspension mixed properly by vortexing. By centrifugation at 14.000rpm (centrifuge: 5417 C; Eppendorf) for 10min the mouse hairs and the other remaining insoluble constituents were pelleted. The supernatant containing the DNA was transferred into a new Eppendorf reaction tube containing 500µl isopropanol. To precipitate the DNA the solution was mixed well by stringently shaking. Subsequently, the DNA was pelleted at 14.000 rpm (centrifuge: 5417 C; Eppendorf) for 20min, the supernatant was decanted and discarded, the DNA was washed once with 70% ethanol (centrifugation as before for 5min) and the pellet was air-dried at room temperature for 10-15min. Finally, the pellet was dissolved in up to 500µl H_2O or TE buffer and incubated at 4°C overnight or at 37°C, shaking, for 1h.

(B) Preparations of nucleic acids: RNA preparations

RNA preparations are carried out analogously to DNA preparations and can be divided into three basic steps: complete lysis of the cells, precipitation and purification of the RNA. During the RNA extraction it is important to work as fast as possible and especially RNase-free. The use of RNase inhibitors and RNA stabilizers is highly recommended for low amount of cells. For instance, cells can be lysed using buffers containing β-mercaptoethanol; a chemical that solubilizes the cells and inhibits the activity of Rnases. RNA should be dissolved in Rnase free water or diethylpyrocarbonate (DEPC) H_2O. DEPC inactivates RNases irreversibly by destroying their histidine residues. DEPC, however, is non- compatible with tris or HEPES buffers and should not be used in those cases. Extracted RNA is most stable when stored at -80°C.

(i) Additives for isolation of very small RNA samples

N-carriers (that protect against RNases and surface adsorption in spin column purifications) and P-carriers (for alcohol precipitation) are used in combination for better recovery of small amount of RNA. Lysis buffer is supplemented with N and P-carriers (1µl each).

(ii) Preparation of total RNA according to the QIAGEN RNA Micro Kit
Total RNA preparations were carried out using the QIAGEN RNA Micro Kit. RNA was stored at -80°C.

(C) Determination of the concentration and quality of DNA and RNA solutions
(i) The concentrations of nucleic acid in solution were determined measuring the extinction at 260nm with a photometer (NanoDrop). This specialized photometer allows the measurement of concentrations with extremely small amounts of solution (1µl).
To control the purity of the nucleic acid solution the extinction at 280nm (band of absorption by proteins) was determined. The quotient E_{260nm}/E_{280nm} should be in between 1.9-2.0 for clean RNA solutions and between 1.8-1.9 for clean DNA solutions.

(ii) For microarray analysis further evaluation of the RNA quality was done by using the Agilent Bioanalyzer and Lab-on-a-Chip. Electrophorograms were created that detect degradation and measure the ribosomal 5S, 18S, and 28S bands. Agilent has developed software that assigns a specific quality number to the RNA sample based on its electrophoretic profile. The RNA Integrity Number (RIN) ranges from 1 (totally degraded RNA) to 10 (completely intact RNA). Only high quality RNA, with RIN greater than 8 and $A_{260/280}$ greater than 1.8 were used for microarray analysis.

(D) Microarray
The quality of purified RNA was examined using the Agilent Bioanalyser and revealed high quality of all RNA preparations. Total RNA (2 ng) was amplified for the first round with the MessageAmp II aRNA Amplification Kit (Ambion). For the second round, the MessageAmp II-Biotin Enhanced kit (Ambion) was employed. 10 µg of amplified aRNA were hybridized on Affymetrix MOE430 2.0 arrays containing about 45,000 probe sets. Staining and scanning was done according to the Affymetrix expression protocol. In total, 8 samples distributed over three cell types were analyzed. All housekeeping genes were present and the number of present calls was determined as 40% or higher. To process the data, we calculated probe set summaries (according to RMA (Bolstad et al., 2003) and normalized the data (lmp, nonlinear transformation employing the LOESS smoother (Cleveland, 1981)). To test the quality and reproducibility of the samples, hierarchical clustering was used to find (dis) similarities between the samples, showing that the

replicates of each group of cells analyzed were clustering together. Hierarchical clustering was performed on normalized data (RMA) using packages available from http://cran.r-project.org. Statistical analysis of the microarrays was performed using the statistical program R (R Development Core Team), using packages available from http://cran.r-project.org and mips.gsf.de/mips/staff/mader/software. Briefly, probe set summaries were calculated according to RMA. A log-scale transformation (log, basis 2) using normalization against the mean of all samples employing the LOESS smoother operating on M-A scale was carried out. Genewise testing for differential expression was done with the Bioconductor software package implemented in Carma web with the Limma *t*-test and Benjamini-Hochberg multiple testing corrections (FDR < 0.1). The analysis of pathways, transcription factor binding sites and GO term analysis was done with Bibliosphere (Genomatix)/DAVID.

(E) Reverse transcription

(i) Reverse transcription with oligo dT-primers

With reverse transcription, RNA is transcribed into DNA (cDNA). Using oligo-dT-primer polyadenylated mRNA is transcribed. For each RNA sample one preparation with and one without reverse transcriptase should be made, especially when the primers used in the following PCR do not distinguish genomic DNA and cDNA (without intron), meaning that the primers are not intron spanning. The following mixture was used for one cDNA preparation:

2.0µl RNA (from DNAse digest =1ng)
1.0µl Oligo-dT primer (500ng/µl)
11.0µl filled up with DEPC-treated H_2O

The preparations were incubated at 70°C for 10min to denature the RNA and allowed for the annealing of the primers. Afterwards, the mixture was put on ice and the following components were added:
4.0µl 5x transcriptase buffer
2.0µl DTT (0,1M > 10mM)
1.0µl dNTPs (je 10mM > 0,8mM)
1.0µl RNA inhibitor (40U/µl > 40U)

The mixture was incubated at 42°C for 2min. Then 200U SuperScript (200U/µl > 1µl) was added, followed by an incubation at 42°C for 50min. To stop the reaction the enzyme was inactivated at 70°C for 15min. The cDNA was stored at -20°C.

(ii) Quantitative RT-PCR

For validating results obtained from array analyses we used a quantitative RT-PCR method using SYBR Green (Quiagen). SYBR Green provides the simplest and most economical format for detecting and quantifying PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The only disadvantage of SYBR Green is that it binds to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. The following mixture was made for Quantitative RT-PCR:

12.5µl 2x QuantiTect SYBR Green PCR Master Mix

1 µl (0.3 µM) Primer A

1 µl (0.3 µM) Primer B

Variable RNase-free water; filled with the final volume of 25µl

Variable (≤10µg) Template DNA

Real-time cycler conditions for OPTICON machine

The protocol consists of two programs: amplification of cDNA and melting curve analysis for product identification.

Denaturation for 15 s at 94°C

Annealing for 30 s at 50–60°C (Approximately 5 to 8°C below *T*m of primers)

Extension for 30 s at 72°C (Perform fluorescence data collection, unless an additional data acquisition step has been integrated)

Cycle number 35–40 cycles (Cycle number depends on the amount of template DNA)

The temperature ramp was 20°C/seconds. At the end of the extension step fluorescence of each sample was measured to allow quantification of the RNA. After amplification a melting curve was obtained by heating at 20°C/seconds to 95°C, cooling at 20°C/seconds to 60°C and slowly heating at 0.1°C/seconds to 98°C with fluorescence data collection at 0.1°C intervals.

Quantitation of light cycler data:

The quantitation approach used is termed the comparative C_t method. This involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. ((The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where $[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$. Here, $[delta]C_{t,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene. For the [delta][delta] Ct calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how [delta] Ct varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiency of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

Melting curve Analysis for Primer specificity:

To carry out melting curve analysis, the temperature is increased very slowly from a low temperature (e.g., 65°C) to a high temperature (e.g., 95°C). At low temperatures, all PCR products are double stranded, so SYBR Green binds to them and fluorescence is high, whereas at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence. The fluorescence is measured continuously as the temperature is increased and plotted against temperature. A curve is produced, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures, as the melting temperatures of nonspecific and specific PCR products are reached. The detection systems calculate the first derivatives of the curves, resulting in curves with peaks at the respective T_ms. Curves with peaks at a T_m lower than that of the specific PCR product indicate the formation of primer-dimers, while diverse peaks with different T_ms or plateaus indicate production of nonspecific products or a smear.

(F) Restriction analysis of DNA

Restriction analyses for characterization of DNA are based on enzymes, which are able to cut DNA. Originally they were a defensive mechanism of bacteria against foreign DNA (e.g. DNA of phages). They recognize palindromic sequences on the DNA strand and they cut the DNA either approximately 100 nucleotides after the recognition site (restriction endonucleases class 1), directly at the recognition sequence (restriction endonucleases class 2) or at a defined distance from the recognition site (restriction endonucleases class 3). After cutting, restriction enzymes can leave DNA fragments with 3´ or 5´ overhang (sticky ends) or plain ends without any overhang (blunt ends). If the concentration of a restriction enzyme in one reaction exceeds a certain level (>5-10% of the reaction volume) the risk of STAR activity, unspecific cutting by the enzyme, may occur. With the decrease of the salt concentration of the solution the specificity of the enzyme decreases also; concentrations, which are too high, can eventually lead to a failure of the reaction.

(i) Restriction digestion of plasmid
A typical reaction for a restriction digest for plasmid DNA is as follows:
DNA from mini preparation (ca. 500-100ng) 5.0µl
10 x buffers 2.0µl
10x BSA (optional) 2.0µl
Enzyme (5.000U) 0.5µl
dist. H2O 10.5µl
Σ 20.0µl
The digest was incubated in 1.5ml Eppendorf tubes at 37°C or 25°C for 1.5h.

(ii) Restriction digests of genomic DNA
In principle, the same rules are valid for restriction digests of genomic DNA used for Southern blot analysis as for restriction analysis of plasmid DNA. The suitable enzyme buffer and BSA, if necessary, are added to the reaction. RNaseH can also be used to get rid of RNA background and thereby raise the efficiency of the enzyme activity. Additionally spermidine (in a final concentration up to 2mM) makes sure that the digest will be complete. The completeness of the digest is controlled visually on the gel before blotting. The DNA should appear on a lower molecular level and repetitive sequences should emerge in a distinct band.

A typical mix for a restriction digest of genomic DNA was carried out as follows:
25µl genomic DNA
1x restriction buffer
1x BSA
1x spermidine (1mM)
50-100µg/ml RNaseH
H_2O to fill up approx.
50U restriction enzyme
The reaction was incubated at the enzyme-dependent optimal temperature ON. Prior to loading, samples were partially evaporated to obtain a smaller volume.

(G) Gel electrophoresis

Gel electrophoresis is a tool for the analysis of the size of molecules, e. g. DNA, RNA and proteins. The gel forms a fine-pored net depending on the percentage of the agarose (for DNA and RNA) or polyacrylamide (PAA; for proteins). The sample is transported through this net using electricity. The velocity of the sample is correlated with the size of the molecule and its three-dimensional shape (ideal case: linear), its net charge, the power of the electric field, the pore size of the gel and the temperature.

(i) Agarose gel electrophoresis for analysis
A typical analytical agarose gel was prepared according to expected fragment size with 0.8-2.0% agarose in TAE buffer. Heating in the microwave dissolved the agarose. After the solution was cooled down, 5µl ethidiumbromide (EtBr)-solution (EtBr=1mg/µl, final concentration: 0.005%) were added, mixed by swirling and poured into a prepared gel chamber without bubbles. Separation of samples from PCR or restriction analysis was carried out at U=100V for ca. 30-45min. The band patterns of the samples were documented under UV light.

(ii) Agarose gel electrophoresis for DNA extraction
Preparative gel electrophoresis was carried out analogously to the analytical gel electrophoresis, but the bands with the right size were cut out and documented under UV with low intensity to avoid UV- light induced mutations.

(H) Generation of blunt ends

For cloning purposes, it might be necessary to use two enzymes which create overhangs that are not compatible. Because non-compatible sticky DNA ends cannot be ligated, these ends have to be converted to blunt ends. There are in principle two ways to achieve blunt ends from sticky ends: either the strand is filled up (for 5‘-overhangs) or it is blunted by cutting of the overhanging bases (for 3‘-overhangs) using Klenow fragment. Typical mixes for blunting using Klenow fragment are shown.

5.0µg DNA
1.0µl Klenow fragment (5U/µl)
1.0µl 10x buffer suppl. with 33µM dNTPs
1.0µl ATP (10mM)
2.0µl H_2O
Σ 10.0µl

(I) Dephosphorylation of linearised DNA

For cloning it might be necessary to dephosphorylate vector DNA if the overhangs left by the restriction enzymes after digestion are compatible. Otherwise, the efficiency of the ligation of the insert into the vector decreases because religation of the vector will be energetically favoured. A typical mix for the dephosphorylation of linearized plasmid DNA is as follows:

1.0µg DNA
1.0µl 10x enzyme buffer cend = 1x enzyme buffer
1.0µl alkaline phosphatase (1U/µl) cend = 1U enzyme/reaction (1µg DNA)
7.0µl H_2O
Σ 10.0µl

(J) Ligation

Ligations of DNA fragments can be done with compatible ends left over by restriction enzymes. It does not matter if these overhangs are 3’ or 5’ or if there is a blunt end. T4 DNA ligase is an enzyme extracted from the phage T4 that can ATP-dependently ligate DNA fragments with at least one phoshorylated 5’ end. The buffer supplied with enzyme contains 10mM ATP and should therefore be kept frozen. Vector and Insert should be

used in a ratio 1:3 for best results in a ligation with sticky DNA ends. In a blunt end ligation a ratio 1:1 vector: insert is recommended.
A typical mix for the ligation of vector with insert is the following:
1.0µl vector DNA (e.g.; see below)
0.5µl insert DNA (e.g.; see below)
1.0µl 10x T4 ligation buffer cend = 1x enzyme buffer
0.5µl T4 ligase (10U/µl; NEB) cend = 5U enzyme/reaction
7.0µl dist. H2O (add at 10µl with dist. H2O)
10.0µl
The used volume of insert can be calculated with the following equation:
Ratio: vector/insert = 1/3
Vector concentration: c(v) [ng/µl] = x ng/µl size (v) [bp] = y bp
Insert concentration: c(i) [ng/µl] = a ng/µl size (i) [bp] = b bp
Used amount of vector-DNA: 100-400ng > used for ligation: v ng / w µl
Used amount of insert-DNA: intron [ng] = 3 * (b bp / y bp) * w µl > intron DNA [µl]

(K) Transformation of bacteria

The term transformation marks the transfer of foreign DNA e. g. a plasmid or BAC (Bacterial Artificial Chromosome) into organisms, e. g. bacteria. For this kind of manipulation of bacteria there are in principle two common methods: chemical transformation and electroporation. The chemical transformation is based on the assumption that the membrane gets porous and instable through Ca^{2+} ions and heat, so that the pick up of DNA sticking to the membrane is possible. Using electroporation, another method of transformation of bacteria and eukaryotic cells, the cells are made permeable for plasmids or other vectors in a salt-free solution by shortly applying a high electric tension. This method allows an efficient transfer of big DNA fragments into diverse organisms but by which the vitality certainly is relatively low. Independent of the kind of transformation the bacteria or cells should have a regeneration time of a few cell cycles (generally 1-3 cycles) in normal medium and under normal incubation conditions. Only then they should be transferred to selection media.

(i) Transformation of bacteria using heat shock
For the transformation of bacteria with heat shock, one aliquot of bacteria per construct to be transferred was thawed on ice. A suitable amount (e.g. 4µl of a ligation) of Vector was

mixed with the bacteria and incubated on ice for 30min. For heat shock, the mixture was put on 42°C in a water bath for 90s. Immediately after the heat shock, the bacteria were put back on ice, diluted with 1ml LB medium and incubated for regeneration (850rp, Eppendorf shaker; 37°C; 30-60min).

(L) Southern Blot

The Southern blot, a method in molecular biology developed by Edwin Southern in 1975, is a widely used technique to identify specific genomic sequences within a complex mixture of DNA (e.g. the whole genome of an individual). The DNA is digested into smaller pieces using restriction enzymes and separated by gel electrophoresis. The pieces of DNA are blotted on a membrane and the fragment of interest is detected by hybridisation to a radioactive- or fluorescent labelled complementary DNA probe. In the case of a radioactively labeled probe, it can be visualized later by exposing the hybridised membrane to a film. Additionally the Southern blot can be used to show correct integration of a piece of DNA by homologous recombination. In this case it is important that the knock-in allele shows a different restriction pattern in comparison to the wild type allele. That means that the band, which can be detected with the probe, has to have a change in the fragment size.

(i) Gel electrophoresis

First, a photo of the gel was taken under UV light. A ruler was used for reference length, put next to the DNA ladder. If the digest is complete, a distinct band under UV light can easily detect repetitive sequences.

(ii) Blot

For the detection of larger fragments (≥10.000kb), the gel was depurinized by incubating it in 989ml H_2O + 11ml HCl (conc.) for 15-20min while shaking. This was followed by a denaturation step in 0.4M sodium hydroxide and 0.6M sodium chloride again while shaking. For neutralization, the gel was incubated in a solution of 0.5M tris and 1M sodium chloride (pH 7.2) for 15-20min once again while shaking. The blot was built up in 20x SSC as follows (see figure 47) and the transfer was carried out overnight. Note that the gel lies upside down. The next day the blot was taken apart and the slots of the gel were marked on the membrane with a ballpoint pen. Afterwards, the membrane was dried and cross-linked in between two layers of Whatman 3 MM paper for 30min at 80°C

or treated in the cross-linker (UV Stratalinker 1800; Stratagene) for 1min, respectively.

(iii) Hybridisation

I. Prehybridisation

30ml hybridisation buffer per hybridisation tube (Hybridizer HB 100; ThermoHybaid, Thermo Electron Cooperation) were preheated to 65°C in a water bath. The membrane was rolled and put into the hybridisation tube and the preheated buffer was carefully poured into the tube without generating bubbles between membrane and tube. The membrane was prehybridised turning in an oven at 65°C for at least 1.5 – 2h.

II. Preparation of samples – radioactive labelling of the probe

While the membrane was prehybridizing, the radioactive labeling of the probe was started. First, 100ng of the linearised probe DNA was diluted to a final volume of 24µl with dist. H_2O in a 1,5ml Eppendorf reaction tube. Second, 10µl of random oligonucleotides were added and the mixture was boiled for denaturation in a water bath for 5min. Subsequently, the tube was put on ice to let the solution cool down, centrifuged to collect the liquid on the bottom of the tube and then put on ice again. 10µl 5x buffer (d*CTP-buffer for 32P labelled CTP) that already incorporates the correct mixture of nucleotides and salts were added. In the radioactive lab labelled dCTP (50Cu) and 5U Klenow-enzyme (Exo(-) Klenow, 1µl, 5U/µl) were added, the mixture carefully vortexed or well-mixed, shortly centrifuged and labelled at 37-40°C for 2-10min. For completion of the labeling, 2µl STOP mix were added. This buffer contains the chelating agent EDTA for binding of Mg^{2+} and Ca^{2+} ions and thereby inhibits the activity of the polymerase. For the purification (removal of proteins and residual nucleotides), the reaction mixture was loaded on a prepared column (centrifuged without sample for 2min at 400xg) and centrifuged at 400xg for 2min in a tabletop centrifuge. Afterwards, the sample was put on ice, shortly mixed and centrifuged before measuring and 1µl was used to measure the activity. For denaturation of the probe, 500µl salmon sperm DNA (10mg/ml) were denatured at 100°C in a water bath for 10min, put in a 50ml Falcon tube and stored on ice. The hot probe was added in a final concentration of 1x106 counts per 1ml hybridisation buffer, which means 2x107 counts per 20ml buffer. By adding 50µl 10N sodium hydoxyl, the sample was denatured while the tube was shaken carefully to mix. Further on swinging, 300µl 2M tris, pH8.0, and afterwards 475µl 1M HCl were pipetted drop wise into the tube for neutralisation.

III. Hybridisation

At the end of the prehybridization 10ml hybridization buffer were decanted from the tube and discarded. The radioactive-labelled and chemically denatured DNA probe was pipetted into the glass hybridization tube and the tube was put back into the oven at 65°C. The incubation was carried out while rotating at 65°C ON (12-24h). The following day, the probe was decanted from the glass tube and the membranes were washed with 2x SSC/0.5% SDS (approx. 250ml, RT) in a plastic bowl. For the first washing step, the membranes were incubated 5min at RT while shaking. After 5min the membranes were taken from the SSC solution and the buffer was discarded. 500ml fresh 2x SSC / 0.5% SDS preheated to 65°C were poured into the bowl and the membranes were washed up to 30min while shaking in a 65°C warm water bath. The washing buffer was replaced as necessary. If a new probe was used control measurements were carried out every 5min. When obtaining a signal that was still high but relatively weak (approx. 100-200x102 counts) the washing step was simply repeated. The stringency of the washing steps could be raised by lowering the concentration of SSC (1x SSC / 0.5% SDS or 0.1x SSC / 0.5% SDS, respectively) if higher signals were measured (> 200 x102 counts). Finally, at approx. 35x102 counts (depending on, among other things, the size of the membrane and the probe and its CTP content), the membrane was wrapped tightly in saran wrap and fixed in a film cassette. A film (Kodak, BioMax) was applied, stored at –80°C ON and developed the following day (AGFA Curix 60).

IV. Preparation of samples (radioactive labelling of the probe according to Roche) Working with reagents of the Roche labeling kit, the following changes were applied: 100ng linearised DNA were filled up to 9µl final volume with dist. H_2O and denatured in a boiling water bath for 5min. Afterwards, it was shortly put on ice to cool down, centrifuged and put on ice again to prevent renaturation. 1µl dATP, dGTP and dTTP each and 2µl hexanucleotide-mix were added. The addition of radioactive labeled dCTP (50uCi) and Klenow-enzyme was carried out as described above; however; the incubation was done at 37°C for 1h. Terminating the reaction was achieved adding 2µl 0.2M EDTA, pH8.0, and the probe was diluted to a final volume 50µl using 28µl TE buffer for loading on the column. The remaining steps are as described above.

2.2.2 Methods in Cell Biology

(A) ES cell culture

Murine ES cells self-renew under certain conditions in culture. They need a murine embryonic feeder layer as support. ES cell tested FCS and supplementation of the medium with LIF to prevent them from differentiation.

(i) Culture of primary murine embryonic fibroblasts

Murine embryonic fibroblasts (MEF) were split every three to five days 1:4 – 1:6 depending on their growth speed and density. Therefore, 15cm dishes of MEFs were washed with at least 10ml PBS – $MgCl_2$ and treated with 7ml trypsin-EDTA at 37°C for 5min. The reaction was stopped by adding 7ml MEF medium. A single cell suspension was achieved by pipetting up- and down 10 times. Afterwards, the cell suspension was transferred to a 15 or 50ml Falcon tube and centrifuged at 250xg for 5min. The supernatant was discarded and the cells were resuspended in a suitable volume of MEF medium and plated on 5-6 new 15cm dishes.

(ii) Treatment of murine embryonic fibroblasts (MEF) with mitomycin C (MMC)

Murine embryonic fibroblasts are incubated with mitomycin C (MMC) to inhibit their growth in coculture with ES cells. MMC is an inhibitor of mitosis. To treat MEF with MMC, the cells were trypsinized as described before. Then the cells of five 15cm dishes were transferred to a 50ml Falcon tube in 20ml MEF medium and treated with 200µl MMC (1mg/ml) at 37°C for 45min. Every 15min the tube was inverted to prevent cells from attaching to the plastic of the tube. After 45min of incubation, the cells were pelleted by centrifugation at 250xg for 5min. The supernatant was discarded and the cells were washed twice with MEF medium to remove MMC. Afterwards, the cells were plated on cell culture plates for direct use as a feeder layer for ES cells or they were frozen for later use

(iii) Seeding of murine embryonic fibroblasts (MEF) for ES coculture

After treatment with mitomycin C the MEFs were seeded for ES cell culture on plates and dishes in the following density:

10cm $1.5x10^6$ cells

6cm $0.5x10^6$ cells

(iv) Thawing of ES cells

To thaw ES cells, a Falcon tube containing 10ml pre-warmed ES medium was prepared. A cryovial of ES cells was thawed in a 37°C waterbath while carefully shaking. The cells were then transferred into the prepared Falcon tube and pelleted at 250xg for 4min. Cells were then resuspended in a suitable volume of ES cell medium (e.g. 5ml for one 6cm dish) and cultured on feeder cells at 37°C and 5-7% CO_2 in a humid incubator.

(v) Passaging of ES cells

After two days (approximately 48 hours) in culture, ES cells need to be split to prevent differentiation and to expand them. They can be split from 1:2 to 1:30 depending on their division rate. The medium was removed and the cells were washed with a suitable volume of PBS (-$Mg^{2+/}Ca^{2+),}$ approximately 5ml. After removing the PBS, the cells were incubated with trypsin-EDTA (1ml per 6cm dish or 3ml per 10cm dish) for 5min at 37°C, 5-7%CO_2 in a humid incubator. The dissociation of the cells was briefly checked under the microscope and the reaction was stopped using at least the same amount of TBV2 cell medium compared to the trypsin solution (4ml for one 6cm dish). To get a single cell suspension, cells were well suspended by pipetting up and down at least 10 times and transferred to a Falcon tube. The cells were centrifuged for 4min at 250xg. The medium was discarded and the cells were resuspended in fresh ES cell medium. Dilutions of cells were plated on new feeder cells layers according to the splitting ratio determined previously. Cells were incubated for approximately 48h at 37°C and 5-7% CO2 in a humid incubator.

(vi) Cryoconservation of ES cells

For cryoconservation in liquid nitrogen, ES cells are removed from the plate as described above and centrifuged. Instead of resuspending them in TBV2 medium they were resuspended in pre-cooled freezing medium and transferred into cryovials. These cryovials were put into freezing boxes and stored at –80°C for at least 4 hours to cool the cells down to –80°C carefully (1°C/min). After 4 hours or incubation over night the vials were transferred into liquid nitrogen (N2).

(B) Homologous recombination in ES cells

Homologous recombination is the basis for targeted mutagenesis and the generation of

knock-in mice. Therefore, ES cells are transformed by electroporation with the linearized targeting vector, selected for insertion and transformed colonies are picked and expanded.

(i) Transformation of ES cells by electroporation

For one electroporation one 10cm dish ES cells (70-80% confluent) was used. The cells were trypsinized as described before. The single cell suspension was then transferred to a Falcon tube and centrifuged at 250xg for 5min. The cells were then washed with 10ml PBS (-$Mg^{2+/}Ca^{2+}$) at room temperature and centrifuged under the same conditions. After centrifugation, the cell pellet was resuspended in 1.5ml ice-cold PBS (Mg^{2+}/Ca^{2+}) and 0.7ml of this cell suspension were mixed with 0.1ml vector (25µg) in PBS (-Mg^{2+} /Ca^{2+}) to a final volume of 0.8ml. The mixture was then transferred into a pre-cooled cuvette and the electroporation was carried out under the following conditions. (2 pulses, stored on ice in between for 1min; Programm: 220 V; 500 µF; Resistance: ∞)

After electroporation, the cuvette was kept on ice for 5min. Then the cells were transferred into a pre-warmed dish with a feeder layer and ES cell medium (0.4ml of the cell suspension was put into a 10cm dish, the volume of one electroporation is therefore sufficient for two 10cm dishes). The medium was exchanged daily. After 24h, the selection was started with neomycin (G418) in a final concentration of 300µg/ml or puromycin in a concentration up to 2µg/ml. Clones could be picked after 6-8 days.

(ii) Picking of ES cell clones

Picking of ES cell clones was done in two conical 96-well plates with 60µl PBS –$Mg^{2+/}Ca^{2+}$ per well and four normal 96-well plates, two of which were coated with gelatine (0.1%) and filled with 100µl ES selection medium and two with a feeder layer and 100µl ES selection medium. The medium of one 10cm dish with ES cell clones was removed and 10ml PBS –Mg^{2+}/Ca^{2+} were added. Under the stereo microscope, clones that looked compact and round were picked with a 100µl pipette set on 20µl. The clones were detached by tapping with the tip of the pipette and then sucked and transferred into one well of the prepared 96-well dishes filled with PBS –Mg^{2+} /Ca^{2+}. Care was taken not to mix clones by detached cells. After one 96-well plate was filled, 30µl trypsin-EDTA was added per well (total volume 110µl) and the cells incubated at 37°C for 15 minutes. After incubation, the cells were pipetted 10 times up and down with a multi-channel pipette to achieve a single cell suspension. 50µl were then transferred to each of the two prepared 96-well plates (one with feeder layer, which is the master plate for freezing, one with

gelatine-coating which is the template for DNA preparation).

(iii) Expansion of ES cell clones

The cells on 96-well plates were incubated at 37°C and 6% CO2 in a humid incubator. After 2-4 days when the cells are dense enough that the master plate was frozen. When the medium of the DNA plate turned yellow in one day, DNA was prepared.

(iv) Cryoconservation of ES cell clones in 96-well-plates

For the cryoconservation of ES cell clones in 96-well plates 2x freezing medium (10ml per 96-well plate) was prepared and cooled down to 4°C.

2x ES freezing medium:

4ml ES-cell medium

4ml FCS

2ml DMSO

10ml

The medium was removed and the cells were washed once with 200µl PBS –Mg2+/Ca2+. Then 40µl trypsin-EDTA per well were added and cells were incubated at 37°C for 5min. With 60µl cold medium the reaction was stopped and the cells were resuspended by pipetting up and down 10 times (as fast and cold as possible). Per well 100µl2x freezing medium were added as fast as possible. The plates were closed with parafilm, put in napkins and then in a box that was stuffed with paper towels. The box was stored at –80°C for 6-8 weeks.

(C) Neurosphere Culture

The neurosphere assay is an in vitro assay used for determining the existence of neural stem cells in a particular region of brain.

Neurosphere cultures were generated from adult WT, hGFAPeGFP, GLAST::CRE-ERT2/ZE/G mouse lines. Briefly, animals were anesthetized with ether, and then killed by cervical dislocation. The brain was exposed, surgically removed, and then placed on an ice-cold sterile tissue preparation buffer (see above in materials, HBSS+HEPES). SEZ or cortical regions of adult brain were dissected and minced into small pieces with a sterile scalpel, and placed in an ice-cold 5ml Solution 1 (see above in materials,

HBSS+HEPES+D-glucose). Minced tissue in Solution 1 was then supplemented with 0.05% Trypsin /EDTA (Gibco, 25200-056), then incubated at 37ºC for 30 minutes. In between the incubation time, tissue was mechanically triturated with a fire polished Pasteur pipette. The enzyme activity was stopped by addition of equal volume of Solution 3 (see above in materials, 4% BSA in EBSS) and filtered with 70µm cell strainer. The cells were centrifuged at 1200 rpm for 5 min, resuspended in Solution 2 (0.9 M sucrose in 0.5 HBSS), and centrifuged for 15 min at 2000rpm. The cell pellet was resuspended in 2 ml of Solution 3, placed on top of 10 ml Solution 3 (4% BSA in EBSS), and centrifuged at 1500rpm for 9 minutes. The cells were washed in DMEM/F-12 at 1200 rpm for 5 minutes at 4ºC, and re-suspended in neurosphere medium (see above in materials). The cells were plated out in non-adhesive 24-well plates/Terasaki plates/ T75 flask at varing density depending upon experiment. Cultures were supplemented with EGF and bFGF (20 ng/mL and 10 ng/mL, respectively), every second day.

(i) Single cell culture in Tarasaky plate

For single cell culture of sorted cells, firstly cells after sorting were pelleted down by a brief centrifugation of 5 mins and then resuspended in 100µl of neurosphere media. Serial dilution of sorted cells was performed in such a way that each Tarasaky well should have single cell. Counting of cells (10µl of the resuspended cells) was done on Tarasaky plate's follwed by serial dilution. After serial dilution cells were plated from each population in ensuring the presence of single cell per tarasaky well in 25 µl of neurosphere media.

(D) Passaging or Differentiation of Neurospheres

For passaging, neurospheres were spun down and dissociated either enzymatically or mechanically. For enzymatic dissociation, the spheres were incubated in trypsin solution at 37ºC for 3 minutes, dissociated with 200µl pipette 10-20 times for single cell suspention and then washed with solution 3 (see materials). For mechanical dissociation,

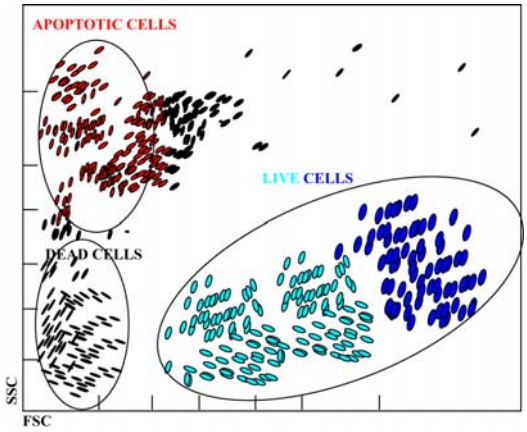
a 200µl pipet tip was used to triturate the cells (30 times up 30 times down). Dissociated cells were again resuspended in neurosphere media. For differentiation assay, single neurospheres were directly plated on PDL-coated glass coverslips without dissociation with DMEM: F12 media for 7-10 days. In this case, cells migrated from the core of sphere and differentiated.

(E) Immunostaining of cells for FACS

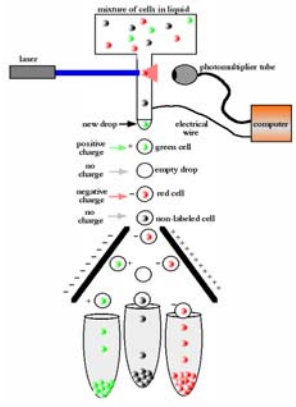
For immunostaining of adult SEZ cells, the dissociation was done as described above (section 1.3). Single dissociated cells were incubated in the dark with staining solution (see materials) containing primary antibody directly conjugated with the flurophore for 20 minutes. Cells were washed with staining solution without any antibody and centrifuged for 5 mins at 1200rpm. The pellet of stained cells was resuspended in neurosphere media and processed for FACS analysis/sorting.

(F) Fluorescence Activated Cell Sorting (FACS)

FACS is a technique that allows detection, discrimination and the sorting of cells in a cell mixture according to their size, their complexity or marker expression (detected with fluorescence/ fluorescent antibodies). In flow cytometry a beam of light (usually laser light) of a single frequency (colour) is directed onto a hydrodynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescence detectors. Each suspended particle, passing through the beam, scatters the light in some way, and fluorescence chemicals in the particle may be excited into emitting light at a lower frequency than the light source. The detectors in flow cytometry pick up this combination of scattered and fluorescence light. By analyzing fluctuations in brightness at each detector it is possible to deduce various facts about the physical and chemical structure of each individual particle. *FSC* correlates with the cell volume. *SSC* depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).



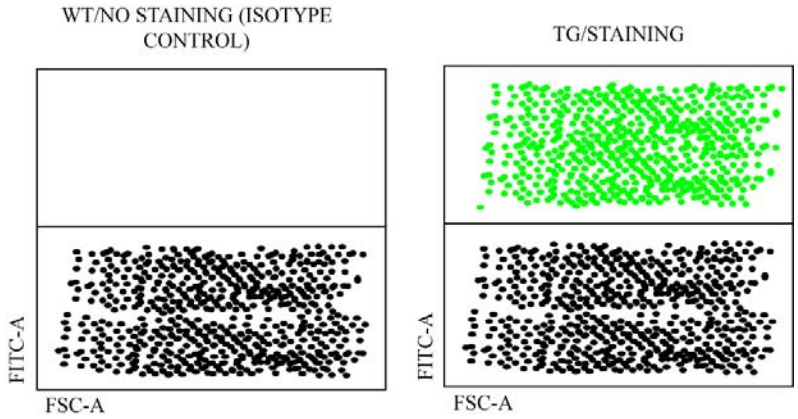
To separate a subpopulation of cells, cells can be tagged with an antibody linked to a fluorescent dye. The antibody is bound to a protein that is uniquely located on the surface of the cells one wants to separate. The laser light excites the dye, which emits a color of light that is detected by a photomultiplier tube, the light detector. By collecting the information from the light (scatter and fluorescence) a computer can determine which cells are to be separated and collected. Sorting of cells is accomplished by electrical charge. The computer determines how the cells will be sorted before the drop forms at the end of the fluid stream passing through the flow cell. As the drop forms, an electrical charge is applied to the stream and the newly formed drop will form with a charge. This charged drop is then deflected left or right by charged electrodes and into waiting sample tubes. Drops that contain no cells are sent into the waste tube. The end result is three tubes with pure subpopulations of cells. The number of cells is each tube is known and the level of fluorescence is also recorded for each cell.



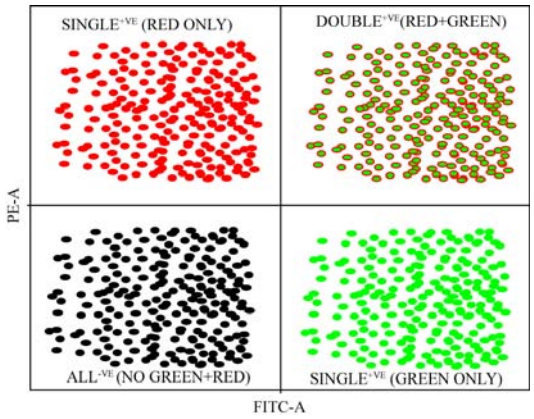
Quantifying FACS data:

FACS data collected by the computer can be displayed in two different ways.

For single stained cells, the florescence intensity of the green or red fluorescence is plotted on the Y-axis and the different sizes of cells can be plotted on the X-axis. The gating for green or red positive cells in a sample was done by using a sample without any primary antibody but with isotype control with same flurophore conjugation (red or green).



In the case of staining the sample with two colors (red and green), one can plot the intensity of green fluorescence in X-axis and red fluorescence in Y-axis. Like this, one can see cells labeled with single color (only green /only red) or marked with both the colors (red and green). Additionally, unstained cells can be visualised by this plot. This method of graphing the data is especially useful if cells are present that have been labeled both red and green.



When multiple fluorophores, whose emission signals overlap, are used, compensation may be required. Compensation is usually done for pairs of adjacent channels. For example, assuming that 10% of FITC signal (FL1 channel, green) spills into the FL2

channel (orange) being used for PE-signal, the true PE-signal is judged by subtracting 10% equivalent of the FL1 signal from the FL2 signal. Proper compensation requires 'compensation control' samples. For this, cells are stained for only one fluorophore. For example, in an experiment in which both PE and FITC fluorophores are used, the compensation controls to set up would be a tube with only the PE fluorophore and another one with only the FITC fluorophore. Proper compensation would be achieved when the median PE (and FITC) fluorescence of the sample using both FITC and PE fluorophores is same as that of the control sample using just the PE (or FITC) fluorophore.

(G) Immunocytochemistry

Immunostainings allow proteins to be detected by the use of antibodies. The first antibody detects and binds to the protein; the second antibody detects the bound primary antibody. The second antibody is coupled to a fluorescent dye so that it is possible to detect it with a fluorescence microscope. It is also possible to directly label the first antibody. First, the cells plated after FACS/neurosphere were washed with PBS and then fixed in 2% paraformaldehyde for 15min at room temperature. Afterwards, they were washed three times with PBS for at least 5min for each washing step. At this point the cells were either stored at 4°C (covered with PBS) or directly used for a staining. For staining, the cells were incubated with the primary antibody always containing 0.5% Triton X-100 and 10% normal goat serum (NGS blocks the unspecific binding of the antibody) overnight at 4°C. The following day, cells were thoroughly washed with PBS + 0.5% Triton X-100 three times for 10min at RT. Non-specific binding of the primary antibody was removed by washing the cells in PBS. Cells were then incubated with the secondary antibody containing 0.5% Triton X-100 and 10% normal goat serum (NGS) for 2h at room temperature, then they were washed again as described. Finally, they were sprinkled with a few drops mounting medium and covered with a cover slip. Pictures were taken at the inverse fluorescence microscope (Zeiss, AxioVert M200).

2.2.3 Methods associated with in vivo studies

A) Genotyping of hGFAPeGFP mice

The PCR protocol for genotyping of the hGFAP-GFP and the hGFAP-eGFP mouse line was adapted from previous publications (Nolte et al., 2001; Zhuo et al., 1997). PCR was carried out using about 40ng of genomic DNA (~1µl) and 0.4µM of the primers in a 30µl reaction volume containing 0.2mM dNTPs, 1.5 U of Taq-DNA-polymerase, 3µl 10xPCR-buffer and 3µl 5xQ-solution. Cycling conditions were: 4 minutes at 94°C, followed by 30 cycles at 94°C for 30 seconds, at 61.5°C for 30 seconds and at 72°C for 1 minute. Finally, amplicons were extended at 72°C for 5 minutes. 15µl of each PCR-product was analyzed on a 1 % agarose-TBE-gel. The amplicon obtained from hGFAPeGFP transgenic animals is 498bp long.

(B) Genotyping of Glast::CreERT2 mice

PCR was carried out using approximately 40ng of genomic DNA (~1µl) and 1µM of the primers in a 30µl reaction volume containing 0.2mM dNTPs, 1.5 U of Taq-DNA-polymerase, 3µl 10x PCR-buffer and 6µl 5x Q-solution. Cycling conditions were: 2 minutes at 94°C, followed by 35 cycles at 94°C for 20 seconds, at 55°C for 30 seconds and at 72°C for 30 seconds. Finally, amplicons were extended at 72°C for 5 minutes. 15µl of each PCR-product was analyzed on a 1 % agarose-TBE-gel. The amplicon obtained from WT-DNA is 700bp and that from Glast::CreERT2-DNA is 400bp.

(C) Genotyping of ZE/G reporter mice

PCR was carried out using approximately 40ng of genomic DNA (~1µl) and 1µM of the primers in a 30µl reaction volume containing 0.2mM dNTPs, 1.5 U of Taq-DNA-polymerase, 3µl 10x PCR-buffer and 6µl 5x Q-solution. Cycling conditions were: 2 minutes at 94°C, followed by 35 cycles at 94°C for 20 seconds, at 55°C for 30 seconds and at 72°C for 30 seconds. Finally, amplicons were extended at 72°C for 5 minutes. 15µl of each PCR-product was analyzed on a 1 % agarose-TBE-gel. The amplicon obtained from WT-DNA is 700bp and that from Glast::CreERT2-DNA is 400bp.

(D) Free floating sections

Animals were anesthesized with 500ml of 5% cloralhydrate and perfused with 4% PFA-PBS. The brains were removed from the perfused animals and cryprotected in 30% sucrose in PBS overnight. For cutting free-floating sections, the cryostat chamber was set to – 4°C and object support to –35/40°C. The brain was mounted on the metal chuck with Tissue Tek and inserted into the object holder. When the brain was frozen, the support temperature was altered to –12°C. Sections were cut frontally at 30µm thickness and collected in the wells of a 24-well plate filled with fresh PBS. These free-floating sections were stored in storing solution (30% glycerol, 30%ethylen glycol, 30% distilled water, 10% 10x PO_4 buffer) at -20°C.

(E) Whole-Mount preparation and immunostaining

After cervical dislocation, the brain was extracted fresh in Preparation Buffer. The lateral wall of SEZ was dissected very carefully to avoid the striatal tissue. The dissected lateral wall was fixed in 2% PFA for 15 min at room temperature washed 3 times for 5 min each with PBS to remove the PFA completely. After that, tissue was directly processed for immunostaining. For staining, the tissue was incubated with the primary antibody always containing 0.1% Triton X-100 and 10% normal goat serum (NGS) for 48 hrs at 4°C. After 2 days, tissue was thoroughly washed with PBS three times for 10min at RT. To remove non-specific binding of the primary antibody, tissue was washed in PBS. Subsequently, the tissue was incubated with the secondary antibody containing PBS and 10% normal goat serum (NGS) for 7h at room temperature, and then washed in PBS. Finally a few drops of mounting medium were used to mount the tissue between two coverlips with spacers used to prevent the tissue from being damaged.

(F) Tamoxifen Administration

Tamoxifen (SIGMA, T-5648) was dissolved in corn oil (SIGMA, C-8267) at 20mg/ml and 1mg was injected intraperitoneally (i.p) twice a day for 5 consecutive days (Mori et al., 2006). Animals were sacrificed 10 days after the last tamoxifen application.

(G) Stab-Wound Injury

Animals were anesthetized (100 mg/kg, Ketavet; Amersham Pharmacia, Erlangen, Germany, and xylazine, 5mg/kg, Rompun; Bayer, Leverkusen, Germany) and underwent a stab wound in the right cerebral neocortex (Bregma from -0.9 mm to -2.7 mm, latero-lateral 1.5-2.5 mm) using pointed blade.

(H) BrdU Labeling

For detection of proliferating cells, the DNA base analogue 5-bromodeoxyuridine (BrdU, Sigma) was injected i.p. (100 mg/kg body weight, dissolved in 0.9% NaCl with 0.4 N NaOH) 1h before perfusion to label fast proliferating cells (short pulse). To label slow dividing stem cells in the SEZ, BrdU was given in the drinking water (1mg/ml) for 2 weeks followed by another 2 weeks with BrdU-free drinking water.
For BrdU immunostaining the sections were treated with 2N HCl for 15 minutes at 37ºC for DNA denaturation followed by one washing step of 10 minutes in PBS and were treated with 0.1M Sodium-tetra-borat (pH: 8.5) for neutralization.

(I) DNA-labelling

For the detection of condensed chromosomes for the analysis of mitotic cells and the orientation of their spindle-apparatus at the ventricular surface, cryostate-sections were counterstained with DAPI (4,6-diamino-2-phenyl-indol; (Naimski *et al.* 1980), propidium-iodide (PI; (Crissman *et al.* 1976) . DAPI and PI are dyes that intercalate into nucleic acid molecules of DNA and RNA. DAPI-staining results in blue and PI in red fluorescence. DNA staining (DAPI 1µg/ml in PBS; PI 5µg/ml in PBS containing 50U/ml RNAseA) was performed after immunohistochemistry/FACS in sections or cells for 5-10 minutes at RT followed by three consecutive washing steps in PBS at RT.

3 Results

3.1 Isolation and characterization of stem cells form SEZ.

3.1.1 A sub-population of astrocytes from the SEZ expresses CD133

In order isolate astrocytes from both neurogenic and non-neurogenic niches I used the hGFAPeGFP (human GFAP promoter driven enhanced GFP) *(astrocyte specific)* transgenic line. This line shows a strong eGFP signal in astrocytes of the adult SEZ consistent with the abundance of GFAP-expressing cells in this neurogenic niche and diencephalic astrocytes, one of the non-neurogenic areas in the brain *in vivo*. Therefore I focused on these 2 regions to make a comparison analysis between the astrocyte populations. To see the expression pattern of the potential stem cell marker CD133 amongst these 2 regions I performed CD133 immunolabeling on the adult hGFAPeGFP sections. Immunostaining for CD133 revealed expression localized to the lining of the walls of the lateral ventricles and some striatal cells. However, when sections were co-immunostained with anti-GFP antibodies, only a subset of GFP^{+VE} SEZ astrocytes were found to be expressing CD133. The CD133 signal was enriched in the astrocytes having close contact to the lateral ventricle consistent with the suggestion that astrocytes having access to ventricle might be the stem cells. The cells showed CD133 immunoreactivity only on their surface facing the ventricle (Figure 3.1A). In contrast, CD133 expression was not present in the postmitotic astrocytes of diencephalon. Thus, two populations of SEZ astrocytes and parenchymal diencephalic astrocytes were found in hGFAP-eGFP mice, which exhibit differences in their CD133 expression (Figure 3.1A). Also, I immunostianed the hGFAPeGFP sections with GFAP and S100b (marker for astrocytes) to examine weather the eGFP transcript in this trangenic line is specifically targeted to astrocytes. Both in the SEZ and diencephalon most of eGFP expressing cells revealed astrocytic features (Figure 3.1B, 3.1C). Due to differential expression of astrocytic markers in different regions of the adult brain, two different markers were used for examining the astrocytic features of eGFP-positive cells. In the adult SEZ, usually a monoclonal GFAP antibody was used as the polyclonal GFAP and S100β antibody marks cell types in this region of the adult brain (ependyma) other than astrocytes. In the case of diencephalon, to assess the astrocytic feature of eGFP-positive cells, S100β immunoreactiviy was done, as GFAP immunoreativity is very little in this region of the

brain.

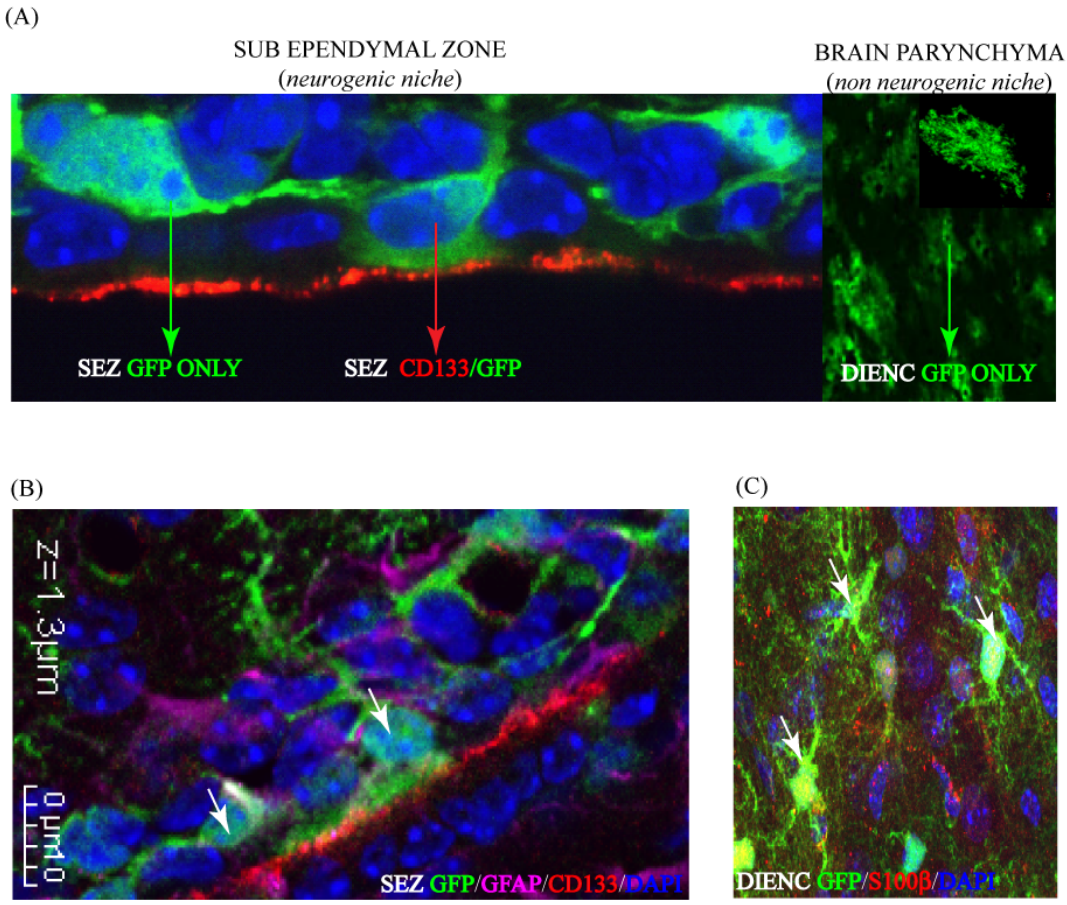


Figure 3.1 Astrocytes sorted from hGFAPeGFP transgenic line (astrocyte specific) from both neurogenic and non neurogenic niches. (A) Astrocytes were sorted from the sub ependymal zone, one positive for CD133/GFP and another positive only for GFP from the neurogenic area, and as a control astocytes from completely non-neurogenic area were taken that are postmitotic and have no stem cell properties like dienchephalic astrocytes. (B, C) Most of the GFP positive cells from hGFAPeGFP transgenic line express astrocytic markers like GFAP and S100β.

3.1.2 FACS analysis of cells in the adult mouse SEZ

To determine which cells in adult mouse SEZ possess characteristics of neural stem cells, I isolated subpopulations and monitored them *in vitro*. I utilized Fluorescent activated Cell Sorting (FACS) to isolate cells from SEZ. Firstly, adult sorting was estabilished by optimising the critical step of tissue dissociation into single cells. I used several methods of dissociation, starting with nonenzymatic to increasing incubation times of three

enzyme preparations: papain, 0.05% trypsin, and DnaseI. Initial comparisons were done with 2 weeks old brain tissue and extended to 8 weeks old (adult) mouse subependumal zone (SEZ), cortex and diencheplalon. The enzymetic reaction was stoped after a certain interval of time and the tissue passed through a cell strainer and then centrifuged through a sucrose gradient (for removal of debris). Cells were harvested, washed and resuspended in neurosphere media. To assess viability, we incubated the dissociated cells with PI (propodium iodide) to identify apoptotic and dead cells in FACS. In FACS, the first graph has Low-angle forward scatter (often called simply "forward scatter") is roughly proportional to the diameter of the cell. Orthogonal, 90^{o} or "side scatter" is proportional to the granularity: neutrophil granulocytes have higher side scatter than do lymphocytes, which are agranular. Thus, in the FACS, each cell shows up: size, granularity, plus green, red, and far-red fluorescence intensities. Usually we want to see data only from single, viable cells. Typically one wishes to eliminate data (fluorescent intensities) from cell debris (particles smaller than cells), dead cells, and clumps of 2 or more cells. Subcellular debris and clumps can be distinguished from single cells by size (estimated by the intensity of low angle forward scatter). Dead cells have lower forward-scatter and higher side-scatter than living cells and are PI stained. Our flow cytometric analysis showed the poorest results in dissociating the cells with growth medium or HBSS alone (nonenzymetic; Figure 3.2A). In contrast, each of the three enzyme preparations greatly improved dissociation and viability, both in terms of percentage of the main population (intact cells) and the total population (all detected events, including debris). Cell aggregates measured by high forward scatter, were common in nonenzyme- dissociated cells but minimal in the enzyme-treated preparations. The best results in terms of single cell suspention and viability came from 0.05% Trypsin (Figure 3.2B, 3.2C).

After setting up the FACS dissociation protocol for adult mouse tissue, I microdissected the SEZs and diencephalon of adult hGFAPeGFP mice and dissociated the tissue by using 0.05% trypsin for half an hour, as it gave us the best results in terms of viability and single cell suspension of adult tissue.

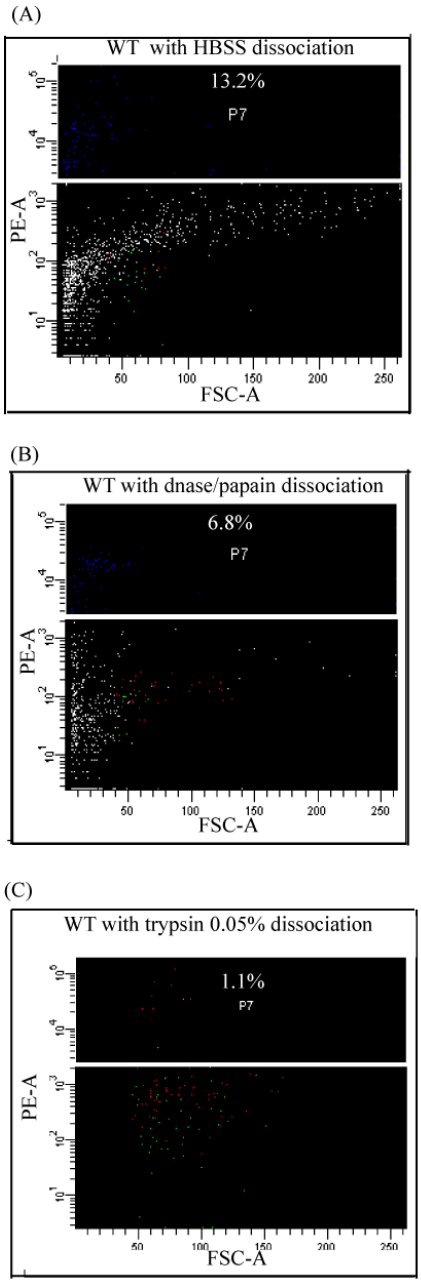


Figure 3.2: Flow cytometric analysis of adult mouse tissue for cell viability using different dissociation protocols. (A) Adult mouse SEZ/CTX/DIENC was dissociated with HBSS alone , (B) 12 units/ml preactivated papain/Dnase1 in HBSS and (C) 0.05% trypsin in HBSS. Cells from all the three dissociation protocols were than stained with PI to access viability of live cells. Dissociation with 0.05% of trypsin yielded the lowest amount of dead cells.

To see the CD133 expression in the different populations I incubated the dissociated cells with the PE-CD133 conjugated fluorescent antibody, which is detectable by the FACS machine. Background fluorescence was measured using unlabeled cells and cells labeled

with isotype control or secondary antibody alone, which set gating parameters between positive and negative cell populations. Cell aggregates and small debris were excluded from analysis or isolation on the basis of side scatter (measuring cell granularity) and forward scatter (measuring cell size). Dead cells were excluded from analysis on the basis of viability dye fluorescence like PI. Following immunostaining for CD133, four different populations of cells were observed in FACS profile: ALL^{-VE} (refers to population negative for all markers) (70.1%), $CD133^{+VE}$ (CD133-positive; 12.4%), GFP^{+VE} (GFP-positive; 13.4%), $CD133^{+VE}/GFP^{+VE}$ (positive for both GFP and CD133; 4.1%; Figure 3.3A). In the previous reports, CD133 was suggested to label mainly ependymal cells (Cosima V. Pfenninger et al., 2007;Coskun et al., 2008), but here I could observe a subpopulation of CD133 immunoreactive cells having astrocytic characteristics in terms of eGFP expression. To revisit this finding, the dissociated SEZ cells from hGFAPeGFP transgenic line were coimmunostained for PE-CD133 and Pacific-Blue-CD24 (an antigen present exclusively in ependymal cells and some neuroblasts in this niche) and analyzed by FACS. Interestingly, the CD133-positive population (Figure 3.3B) and the CD24-expressing population did not overlap, and hence the population expressing both CD133 and GFP (mainly astrocytic population) never colocalise with CD24 (i.e., 0% cells). Apart from FACS analysis, I wanted to plate the cells to look at neurosphere forming efficiency of differnent sorted population, and further to do a microarray analysis of neurosphere enriched pool of cells. For this, we needed to check the purity of cells after sorting. The resorting of all the intersting populations was analysed namely SEZ $CD133^{+VE}/GFP^{+VE}$, SEZ GFP^{+VE} ONLY, SEZ $CD24^{+VE}$, $CD133^{+VE}$ ONLY and DIENC GFP^{+VE} ONLY.

The resorted cells, which included eGFP expression, yielded more than 85 % of purity apart from SEZ $CD24^{+VE}$ ONLY and $CD133^{+VE}$ ONLY sorted cells, which had more than 95 % purity. This might be due to cell stress resulting from adult sorting, which may have led to a loss of eGFP expression.

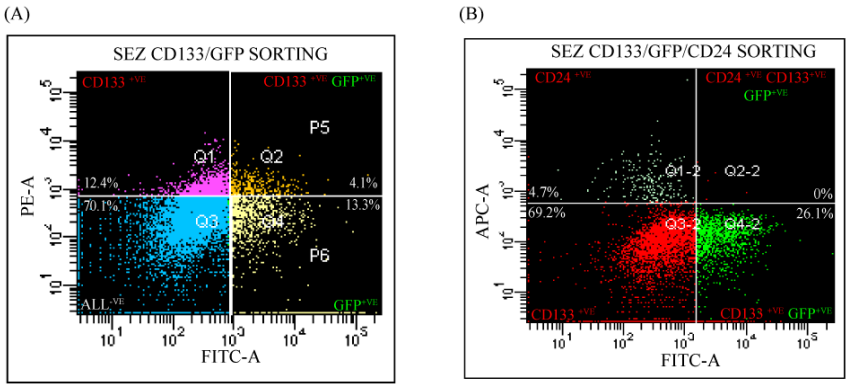


Figure 3.3: FACS profile of different cell popultions in SEZ.(A, B) Representative FACS profiles of SEZ cells from hGFAPeGFP (astrocyte specific) transgenic line stained for CD133 and CD24.

3.1.3 Neural Stem Cell characterisation of cells in the adult mouse SEZ sorted cells

To determine which population of FACS sorted cells actually contained the neural stem cells (NSCs), I performed neurosphere formation assays. This assay involves a culture system in which NSCs isolated from the adult SEZ can proliferate and keep their stem cell identity in the presence of growth factors (epidermal growth factor = EGF and basic fibroblast growth factor = bFGF) *in vitro* without differentiating. Proliferating stem cells make sphere like structure that are called neurospheres (see materials and methods). Within the neurosphere, the stem cells mostly stay undifferentiated and increase there number by self-renewing. Neurospheres can be then dissociated into single cells and maintained in the same conditions to get secondary neurospheres, which may be used as an outcome of self-renewing capacity (Reynolds and Weiss, 1992).

For this assay, the sorted cells were counted and plated at a density of 10 cells/ml, number of primary neurospheres whose diameter was >5cm was counted 7–10 days after plating. Interestingly, the proportion of neurosphere-initiating cells was highest from the $CD133^{+VE}$ /GFP^{+VE} fraction (8 % ± 2) followed by the GFP^{+VE} only (1.5% ± 1), $CD133^{+VE}$ only (0.3% ±0.2) and $CD24^{+VE}$ only (0.1% ± 0.1). In contrast, the pool of sorted cells that were negative for all three markers had very few neurosphere forming cells with high standard deviation (Figure 3.4A). However, this relatively low efficiency of neurosphere formation from these sorted cells could be most likely due to death of the

cells after sorting.

To confirm the further stem cell characteristics of the neurosphere forming cells, I dissociated the primary neurospheres and either plated the dissociated cells for 5–7 days in the absence of mitogens or incubated the dissociated cells again with the mitogens for analysis of multipotency and self-renewing capacity, respectively. I performed immunostaining on the differentiated cells with antibodies against neuronal (βIII-tubulin), astrocytic (GFAP), and oligodendrocytic (O4) antigens. All of the colonies belonging to $CD133^{+VE}$ /GFP^{+VE} and GFP^{+VE} only pool of sorted cells (n = 5–10 in each fraction) contained these three cell types, indicating that the sorted neurosphere-initiating cells were multipotent. The cells of $CD133^{+VE}$ only and $CD24^{+VE}$ only pools were poor in their differentiation per se. In some occasions they gave rise to GFAP-positive cells (Figure 3.4B, 3.4C).

To examine the self-renewal capacity of the sorted cells, primary neurospheres were mechanicaly dissociated into single cells, then cultured (1cell/µl) for 7 days in mitogen containing medium and assessed for neurosphere formation at different passages. Only $CD133^{+VE}$/GFP^{+VE} cells could form secondary (13% ± 2), tertiary (15% ± 2), quaternary (12% ± 3) and even quinary neurosphere formations (11% ± 3), indicating that only these cells could self-renew for prolonged time points *in vitro*. Conversely, GFP^{+VE} only and $CD133^{+VE}$ only cells failed even to form secondary neurospheres (Figure 3.4D).

To further ensure that each neurosphere is formed from a single cell, sorted cells were firstly pelleted down by a brief centrifugation of 5 mins and then resuspended in 100µl of neurosphere media. Serial dilution of sorted cells was performed in such a way that each Tarasaky well should have 1-2 cells in 25µl of neurosphere media. Counting of cells (10 µl of the resuspended cells) was done on Tarasaky plate's follwed by serial dilution. After serial dilution, cells were plated from each population, followed by a visual inspection of each Tarasaky well to ensure the presence of a single cell per well (see materials and methods). Single live cells were counted in each Tarasaky well and were monitored for neurosphere formation. The neurosphere forming efficiency was dramatically increased by singly plating the cells: among the $CD133^{+VE}$/GFP^{+VE} cells

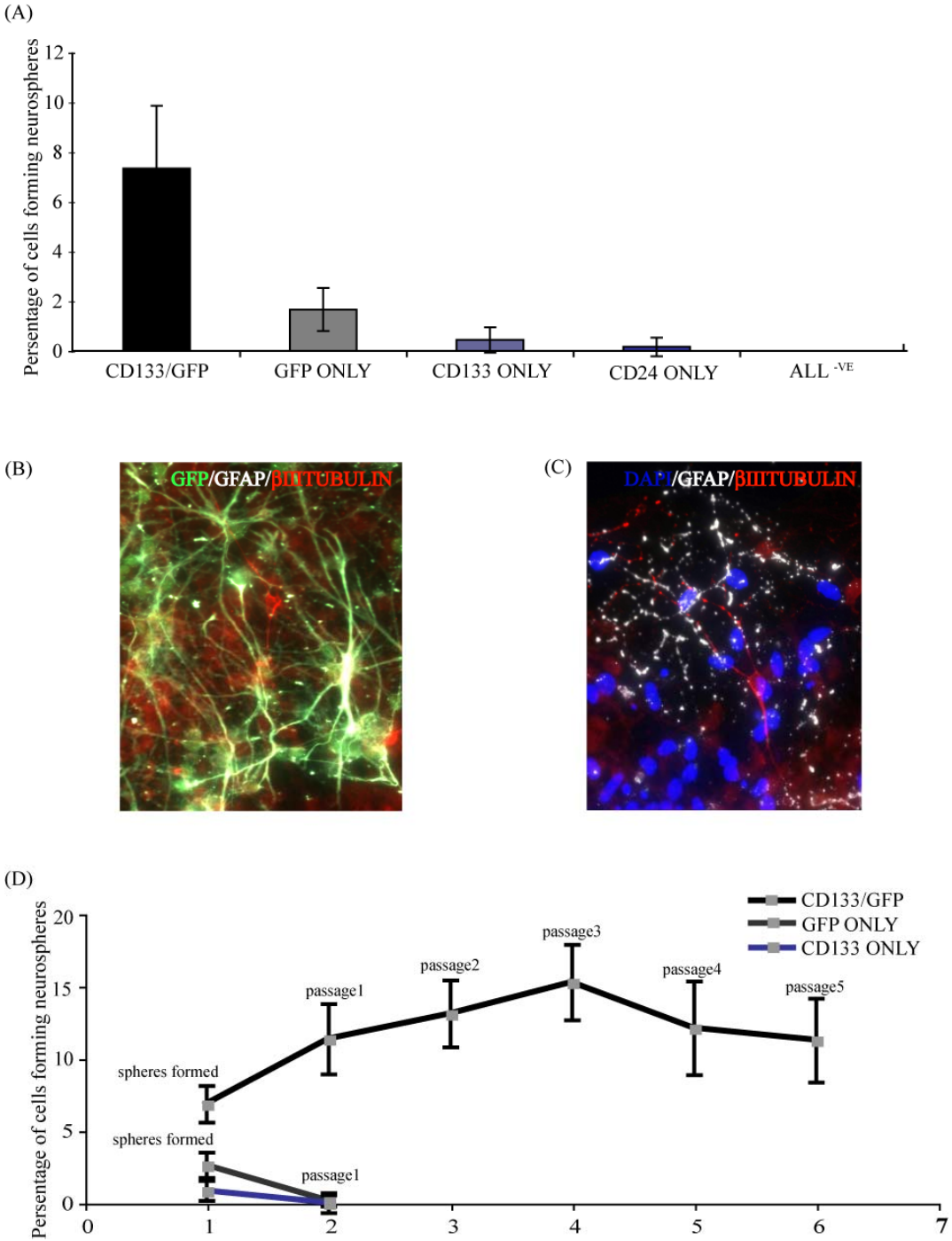


Figure 3.4: Neurosphere forming capacitiy, multipotency and self-renewal of different sorted cell popultions in SEZ. (A) Percentage of primary neurospheres originated clonally from the cells obtained from each fraction of sorted cells. (B, C) CD133/GFP-positive neurospheres showed multipotency upon differenciation giving rise to neuron, astrocytes and oligodendrocytes. (D) Relative percentages of neurospheres that can be self renewed originated from CD133/GFP, GFP ONLY and CD133 ONLY populations.

72% ± 2 formed neurospheres which could also be passaged for secondary neurosphere formation (59%± 2). Notably, the GFP^{+VE} only (15% ± 2) and $CD133^{+VE}$ only (9% ± 2) cells also formed neurospheres in higher efficiency. However, the neurospheres derived from these sorted populations did not cantain self-renewing stem cells, as they never formed secondary neurospheres (Figure 3.5A).

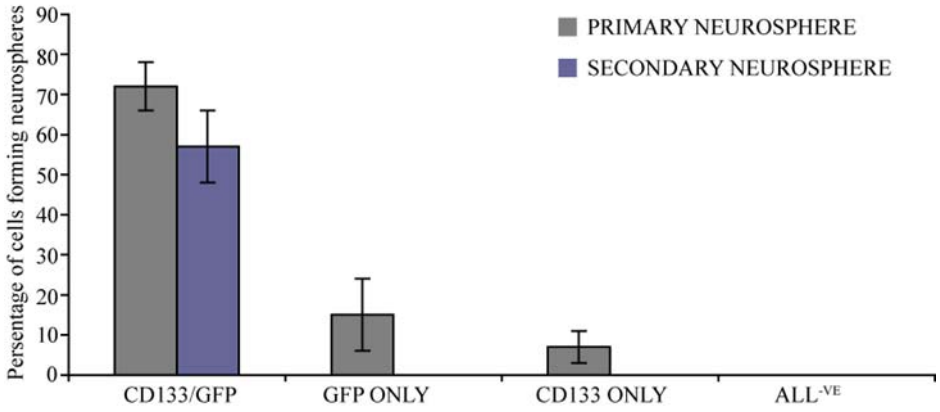


Figure 3.5 Self renewal of singly plated cells. Sorted CD133/GFP, GFP ONLY and CD133 ONLY cells plated singly on tarasaky wells showed neurosphere formation with high efficiency.

Taken together, CD133/GFP-positive cells comprise all SEZ cells forming self-renewing multipotent neurospheres i.e., the exclusive population of cells exhibiting neural stem cell identity in vitro.

In order to further examine whether CD133 would colocalise with other astrocyte specific protiens, I repeated the experiment with the GLAST::CreERT2 mouse line. This mouse line has previously been shown to label astrocytes in the SEZ that give rise to the majority of new born neuroblasts for over 9 months, indicating that it labels a large fraction of in vivo neurogenic stem cells (Ninkovic et al., 2007). In the GLAST::CreERT2 mouse line the inducible form of Cre (it's a 38 kDa recombinase protein from bacteriophage P1 which mediates intramolecular (excisive or inversional) and intermolecular (integrative) site specific recombination between loxP sites) is expressed in the locus of the astrocyte-specific glutamate transporter (GLAST). The fusion of Cre to the ligand binding domain of the modified estrogen receptor (ERT2) is restricted to the cytoplasm and translocates only upon ligand binding (ligand=tamoxifen)

into the nucleus where it can then mediate recombination. Thus, inducible Cre (CreERT2) in the locus of GLAST allows mediating recombination at specific time points, specifically, in astroglial cells. To follow the recombined cells, the GLAST::CreERT2 mouse line was crossed to a reporter line (Z/EG reporter, where the GFP gene is under a ubiquitous promoter. To report Cre recombination, a stop cassette flanked by loxP sites is placed in front of the GFP gene. Only in the presence of Cre, the stop cassette is deleted and the GFP gene is transcribed into the enzyme β-galactosidase (Figure 3.6A, 3.6B, 3.6C). After TM application (see methods) in 2 month old GLAST::CreERT2/Z/EG mice, GFP immunoreactivity was observed in a considerable proportion of GFAP-positive astrocytes as early as 5 days in the adult SEZ. The expression of CD133 was checked in these GFP labelled astrocytes by FACS. The total number of induced cells expressing GFP was low (3.4%), probably reflecting the low recombination efficiency of the reporter mouse line. However, among these cells 65% were double positive for CD133 (Figure 3.7A). After sorting these double positive cells, single cells were plated and only CD133/GFP positive cells formed most of the neurospheres.

Among those singly plated cells 39% formed neurospheres while only 3% of GFP^{+VE} ONLY cells gave rise neuropheres and the $CD133^{+VE}$ ONLY cells formed neurospheres with 10% frequency. Additionally, some neurospheres were generated by the CD133 negative, GFP negative pool of sorted cells (ALL^{-VE} pool 2%). Thus, the above experiments confirmed the astrocytic nature of CD133/GFP-positive neurosphere initiating cells (Figure 3.7B).

3.1.4 Cellular composition of FACS sorted cells

To identify the composition of the FACS sorted $CD133^{+VE}/GFP^{+VE}$ cells from the SEZ, two populations were collected, one positive for both CD133 and GFP and a second positive for GFP only. Additionally, as a control, cells from the diencephalon (DIENC) were sorted to examine the normal parenchymal astrocytes from a non-neurogenic region. GFP expression was high in these cells, reflecting the presence of astrocytes but no CD133 immunostaining was observed in this region. The sorted cells were plated on cover slips coated with poly-D-lysine at a very low density (2000 cells per cover-slip).

The cells were allowed to attach to the cover-slips for 2 hours and then fixed with 2% paraformaldehyde for immunohistochemical analyses. For all the three-sorted populations, SEZ $CD133^{+VE}/GFP^{+VE}$, SEZ GFP^{+VE} ONLY and DIENC GFP^{+VE} ONLY, the purity of sorted cells was first verified by resorting of the cells after sorting (see above).

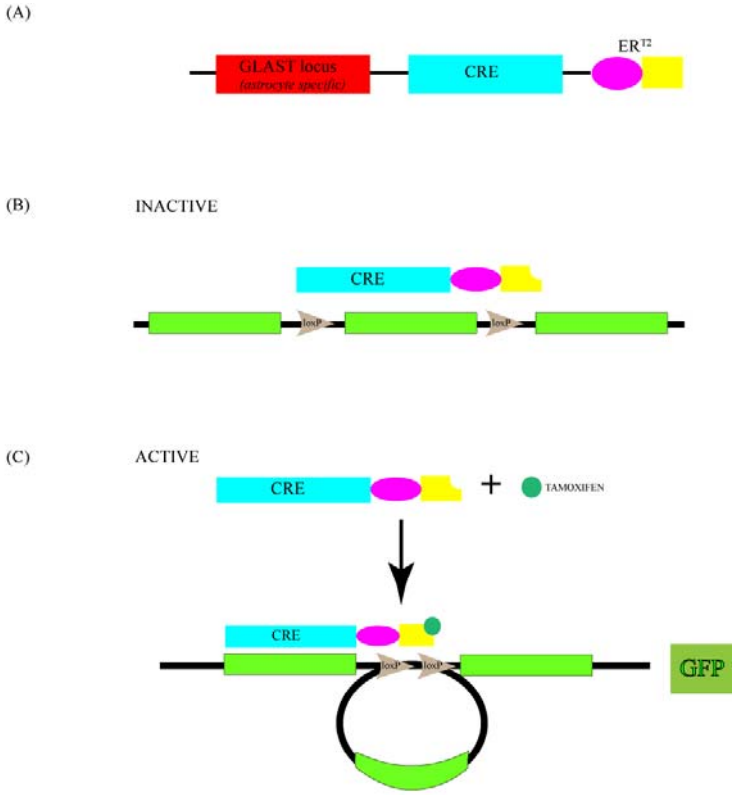
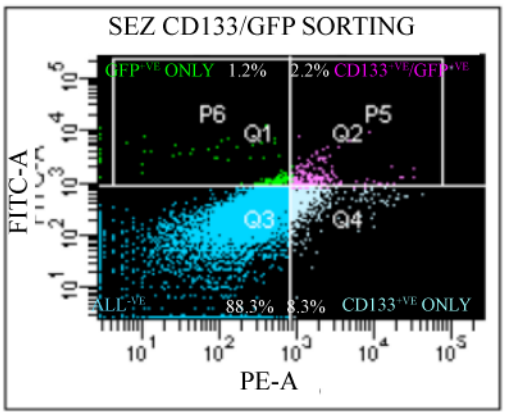


Figure 3.6: Schematic diagram of tamoxifen based Cre/LoxP system for fate mapping. (A) Illustrates Cre protien fused with receptor binding domain of estrogen (ERT2). This domain carries T2 mutation such that it binds specifically the estrogen analog tamoxifen but not the endogeneous estrogen. The CreERT2 is knocked in astrocyte specific GLAST locus. In absence of tamoxifen CreERT2 stays in cytoplasmana cannot achieve recombination (B) whereas in the presence of tamoxifen CreERT2 goes to the nucleus and recombine at loxp sites(C).

(A)



(B)

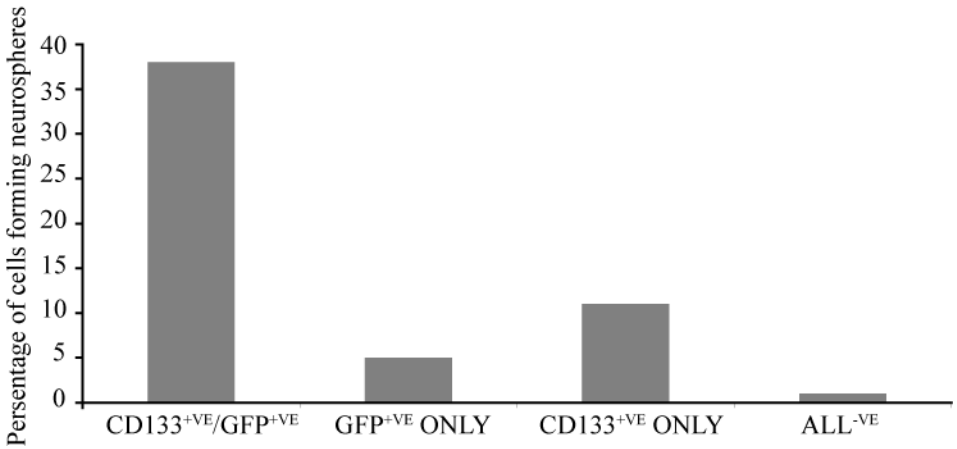


Figure 3.7: Genetically labeled stem cell like astrocytes contributing to adult neurogenesis have CD133 expression and are neurosphere formng cells in vitro.

(A) FACS profile dipicting amoung 3.4% of total GFP labeled astrocytes nearly half expresses CD133 (1.2%, CD133+VE/GFP+VE). Out of all four-sorted fraction of cells CD133+VE/GFP+VE cells made most of the neurospheres (37%)(B).

To examine whether the hGFAPeGFP fluorescence faithfully reflected the expression of GFAP, FACS sorted cells were immunolabeled with antibody against GFAP (an astrocyte specific marker). When SEZ $CD133^{+VE}/GFP^{+VE}$ sorted cells were immunostained with GFAP, the majority of cells were positive for GFAP (84%) confirming that they are astrocytes, leaving 16% marker negative cells (Figure 3.8A). Also apart from GFAP, most of these sorted cells had expression of other astrocytic

markers like GLT1 (85%) and GLAST (81%). With this immunolabeling I could confirm the astrocytic nature of the SEZ CD133^{+VE}/GFP^{+VE} sorted cells. Expression of GFAP in the SEZ GFP^{+VE} ONLY cells was 82%, which was lower in comparison with 92% of the DIENC GFP^{+VE} ONLY sorted cells (Figure 3.9A, Figure 3.10A). This can be due to neuronal contamination in SEZ GFP^{+VE} ONLY sorted cells (below result), confirming the fact that the SEZ GFP^{+VE} ONLY sorted population are more heterogeneous in having neuroblasts and transient amplifying cells apart from astrocytes.

To further reveal the composition of the three FACS sorted populations, DCX (a marker of new born neurons) immunolabeling was done, confirming any neuronal contamination among the sorted cells from hGFAPeGFP trangenic line. Interestingly, DCX immuno-staining never overlapped with CD133/GFP cells, confirming the fact that they might be a pure astrocytic pool of cells (Figure 3.8B). In the SEZ GFP^{+VE} ONLY sorted population there was a small contamination of DCX^{+VE} cells (19%; Figure 3.9B). This is most likely due to the strong stability of GFP that remains in newborn neurons derived from stem cell like astrocytes (GFP^{+VE}) in the SEZ. GFP sorted cells from diencephalon (DIENC) also showed no overlap with DCX (0%) showing no neuronal contamination (Figure 3.10B). Amoung the control sorted cells (SEZ GFP^{+VE} ONLY and DIENC GFP^{+VE} ONLY) DIENC GFP^{+VE} ONLY cells were more pure and stable than SEZ GFP^{+VE} ONLY. As eGFP should label mostly astrocytes (hGFAP promoter; astrocyte specific) both, SEZ CD133^{+VE}/GFP^{+VE} and DIENC GFP^{+VE} ONLY sorted cells ensured their astrocytic feature compared to the SEZ GFP^{+VE} ONLY sorted population.

Thus all the sorted cells consisted largely of GFAP+VE astrocytes with only the SEZ GFP^{+VE} ONLY cells contianing small proportion of neuroblast contamination.

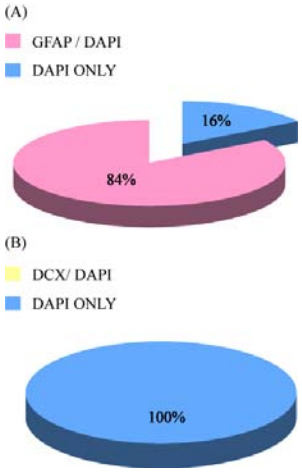


Figure 3.8: Cellular composition of subset of Sub Ependymal Zone astrocytes isolated from hGFAPeGFP transgenic line. (A) The pie chart represents the most of CD133/GFP sorted cells expresses GFAP (marker for astrocytes) with no neuronal contamination as immunostaining for DCX (neuronal marker) showed no expression in these sorted population (B).

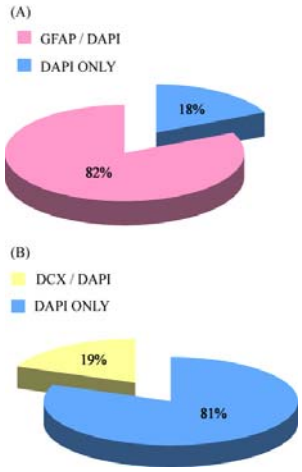


Figure 3.9: Cellular composition of subset of Sub Ependymal Zone astrocytes isolated from hGFAPeGFP transgenic line. (A) The pie chart represents the purity of GFP ONLY sorted cells, majority of sorted cells express GFAP marker for astrocytes (A) but few of the GFP ONLY positive cells also express DCX (neuronal marker) showing some contamination of neuroblasts in this pool of sorted cells (B).

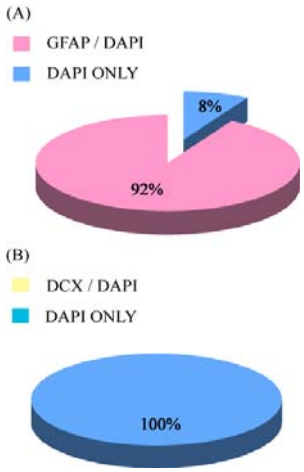


Figure 3.10: Cellular composition of subsets Diencephalic astrocytes isolated from hGFAPeGFP transgenic line. (A) The pie chart represents the purity of GFP ONLY sorted cells with none of the GFP ONLY positive cells are expresing DCX (neuronal marker) (B) , but do expressing (A) GFAP marker for astrocytes.

3.1.5 Transcriptome analysis of astrocytes from the Diencephalon and SEZ CD133^{+VE}/GFP^{+VE} stem cell like Astrocytes

As demonstrated before, we observed three subpopulations of astrocytes using the hGFAP-eGFP transgenic mouse line. Two types of astroglia subpopulations emerged from the SEZ of hGFAP-eGFP transgenic mice. One subpopulation was double positive for CD133 and GFP, and formed multipotent and self-renewing neurospheres *in vitro*. Thus, this first population was comprised of cells with noval stem cell characteristics. The second subpopulation, which was positive for only GFP gave rise to non self-renewing neurospheres *in vitro*. This could be an intermediate precursor and not a true stem cell. A third subpopulation, consisting of astrocytes taken from a completely non-neurogenic area, the diencephalon, where GFP expression was high, showed no proliferative tendency and was thus postmitotic. To characterize the three distinct astroglial cell populations, we investigated the gene expression differences between them.

To address this question, total RNA was prepared from the three-astroglia populations isolated by FACS. Several rounds of FACS sorting were done to obtain a considerable amount of cells for RNA isolation. Usually one sorting from fifteen transgenic animals yielded 20, 000 events and in order to have good quality RNA we needed 100,000 events

in total. Hence 5 different sortings were pooled to get one RNA sample. Because of this I made 5 different replicates of RNA samples from the three sorting populations: namely SEZ CD133/GFP, SEZ GFP ONLY, and Dienceplaon GFP ONLY. The RNA quality (Bioanalyser, see methods) of these samples was checked and the 3 best samples with higher quality were used to generate labeled cRNA using a two-step linear amplification protocol with poly (A) primers that amplify the 3' end of the mRNA. This labeled cRNA was hybridized to Affymetrix Mouse 430 2.0 Arrays containing oligonucleotide probe sets complementary to the 3'-ends of the RNA transcripts (3'-arrays). Each array contained 45,037 oligonucleotide probe sets representing 20,832 unique genes. The MAS 5.0 algorithm to generate expression values and absent/present (A/P) calls and RMA software were used for statistical analysis and clustering. This experiment was carried out using four best independent biological samples.

Firstly the hierarchical divisive clustering of SEZ CD133/GFP, SEZ GFP ONLY and DIENC GFP ONLY (Diencephalon) purified cell-type samples were done. The clustering revealed heterogenity among the sorted astrocytes derived from the same niche and from different niches. Both for Diencephalon and SEZ GFP ONLY sorted cells we could include only two biological replicates, as other two biological replicates from these samples did not culster together within the same sample set. Whereas for SEZ CD133/GFP where could use all four biological replicates.

Finaly, in total for Diencephalon GFP ONLY and SEZ GFP ONLY, two biological replicates were used and for SEZ CD133/GFP four replicates. All of these samples in clustering showed high reproducibility between biological replicates and strong similarity within a cell-type (Figure 3.11).

When we compared the first two sorted subsets of astroglia, the SEZ $CD133^{+VE}/GFP^{+VE}$ and the SEZ GFP^{+VE} ONLY cells at the transcriptome level, we obtained 319 differentially expressed genes (very stringent significance test, p value < 0.0001). We also compared the astroglia that were $CD133^{+VE}/GFP^{+VE}$ derived from the SEZ with those derived from the diencephalon that was exclusively GFP^{+VE}. This comparison yielded 349 differentially regulated genes following the same stringency tests. Amoung all the significant genes in the above comparisons, 80 genes were common in the pool of SEZ

CD133^{+VE}/GFP^{+VE} cells when compared to both SEZ GFP^{+VE} ONLY and DIENC GFP^{+VE} ONLY cells. This pool of stem cell enriched genes showed cilia and calcium specific genes dominating.

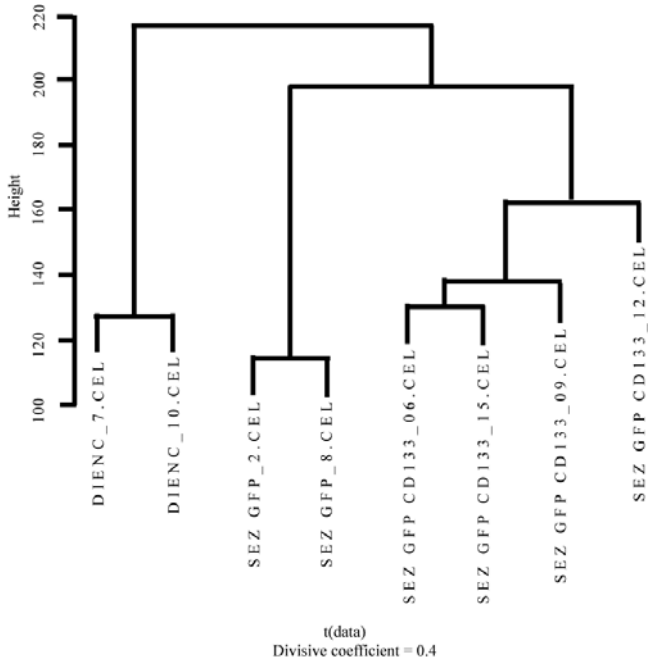


Figure 3.11: Divisive clustering of all the probe sets on normalized rma data. The above clustering reveals the similarity and reproducibility between the biological replicates as they are clustering together. The above clustering also reflects the distiction between different cell type sorted in terms of thier trancriptome.

The heat map generated from unsupervised hierarchical gene clustering of all significant expressed genes FDR < 10% shows cell type gene expression patterns at the gene level. Heat maps were generated for both of the comparisons: SEZ CD133^{+VE}/GFP^{+VE} Vs SEZ GFP^{+VE} ONLY (Figure 3.12) and SEZ CD133^{+VE}/GFP^{+VE} Vs DIENC GFP^{+VE} ONLY (Figure 3.13).

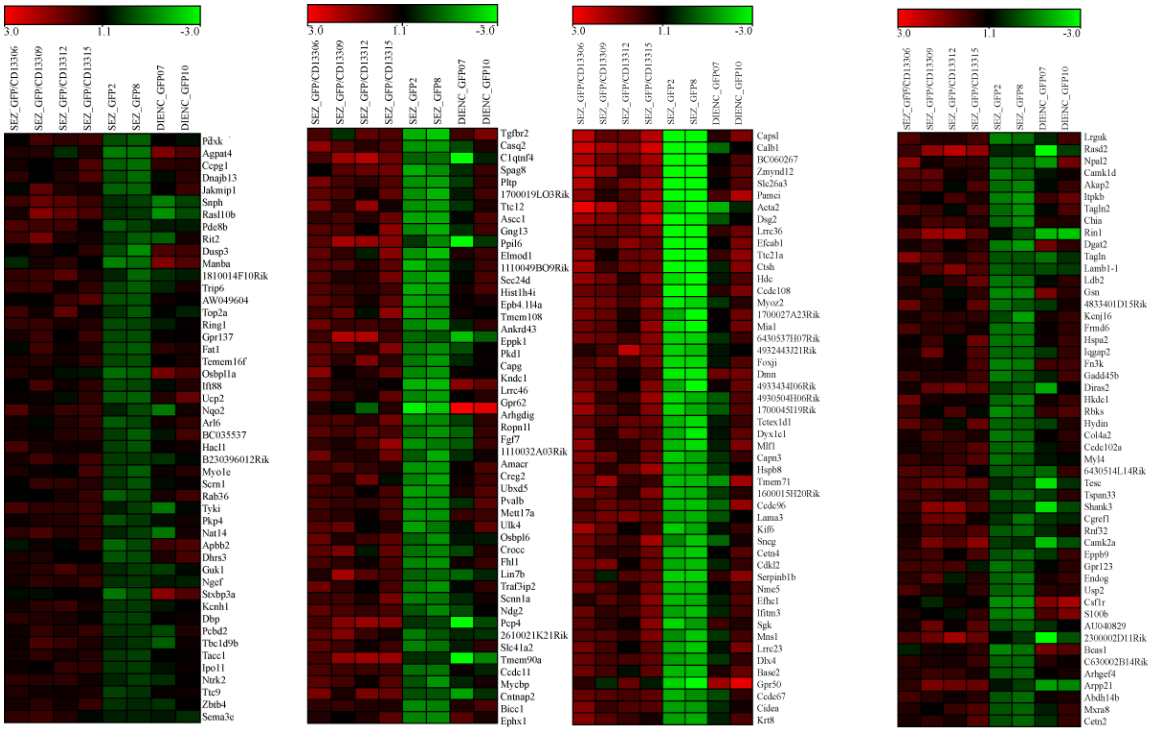


Figure 3.12: Expression of genes enriched in stem cell pool (SEZ CD133^{+VE}/GFP^{+VE} Vs SEZ GFP^{+VE} ONLY). Heat map of significant probe sets, FDR<10% found between SEZ CD133/GFP Vs SEZ GFP and DIENC GFP sorted astrocytes. Red indicates increased and green decreased expression levels.

3.1.6 Gene Ontology Analysis Identifies Cilia and Calcium Related Genes as Prominent Biological Events in the SEZ CD133^{+VE}/GFP^{+VE} Astrocytes versus SEZ GFP^{+VE} and DIENC GFP^{+VE} Astrocytes

To translate the gene expression data into functional profiles, we used Gene Ontology (GO) analysis. GO provides an organized vocabulary of terms that can be used to describe a gene product's attributes. GO terms are organized into three categories: biological process, cellular component, and molecular function. We used the DAVID gene annotation program to analyze the GO terms.

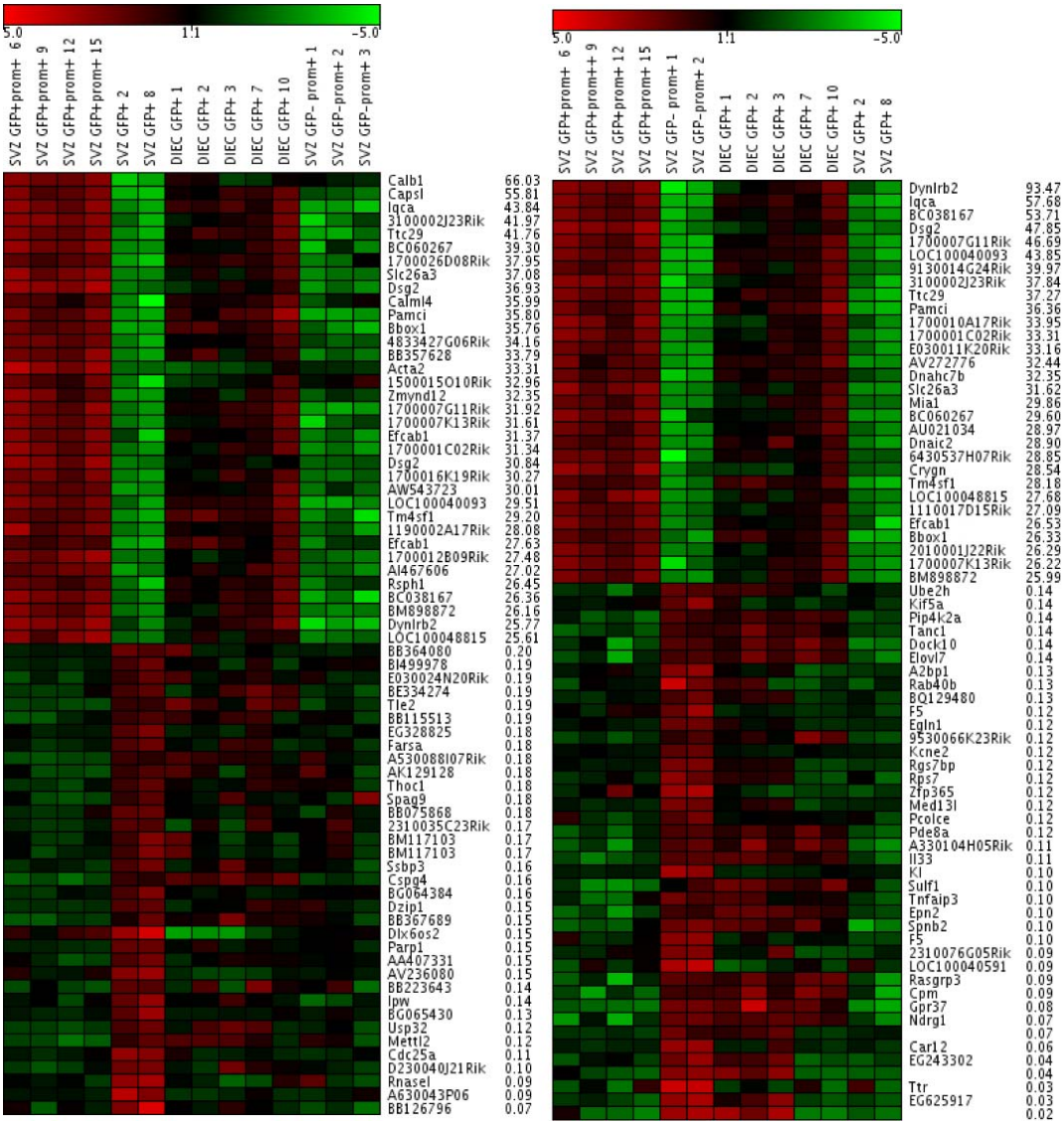


Figure 3.13: Expression of genes enriched in stem cell pool (SEZ CD133^{+VE}/GFP^{+VE} Vs DIENC GFP^{+VE} ONLY). Heat map of significant probe sets, FDR<10% found between SEZ CD133/GFP Vs SEZ GFP and DIENC GFP sorted astrocytes. Red indicates increased and green decreased expression levels.

3.1.6.1 Genes upregulated in Stem Cell Population (SEZ CD133^{+VE}/GFP^{+VE})

When the SEZ CD133^{+VE}/GFP^{+VE} pool of cells was subjected to GO analysis and compared with the GO analysis from SEZ GFP^{+VE} ONLY and DIENC GFP^{+VE} ONLY astrocyte profiles, many cilia (7%) and calcium (10%) related genes were particularly the most prominent categories in the stem cell population (SEZ CD133^{+VE}/GFP^{+VE}) (Figure

3.14) besides unknown and other genes. This finding pointed to the fact that neural stem cells bear cilia and cilia mediated signaling which is a hallmark for their transcriptome.

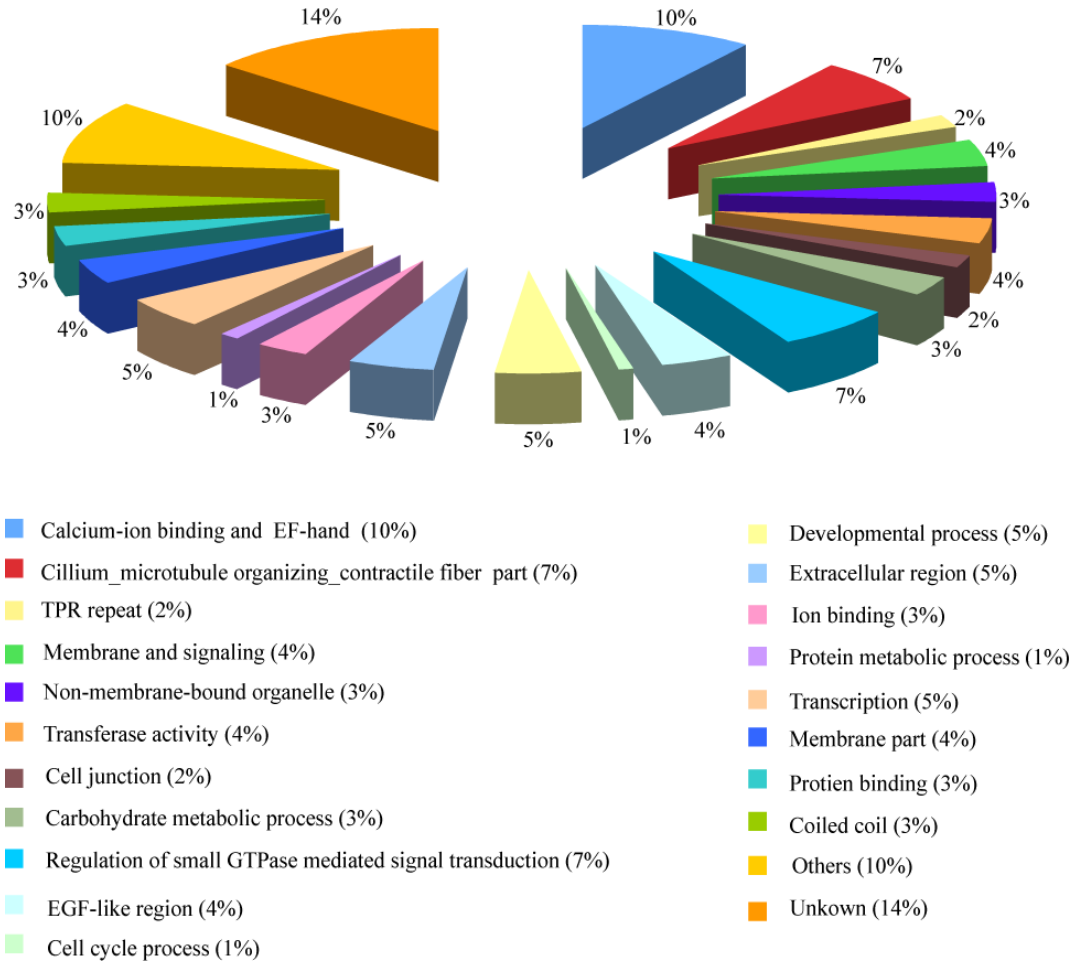


Figure 3.14: Temporal analysis of Gene Ontology of upregulated genes in SEZ CD133^{+VE}/GFP^{+VE} pool of sorted cells compared to SEZ GFP^{+VE} ONLY cells. Total number of genes differentially expressed at least 2-fold within each ontological category, the SEZ CD133/GFP sorted cells have an enrichment of cilia and calcium specific genes.

The genes showing high significance in the MAS t test program and with a high fold difference in the microarray (at least more than 2 fold) were validated by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). All Intrafalgellar transport mRNA (IFTs) were checked, as they are the major cilia components important for specialized intracellular trafficking between the cell body and the tip of the cilia. IFT is a highly conserved process, as mutations in the IFT motors or particles result in the loss of cilia

from single-celled organisms to mammals. Most of the IFTs showed higher expression in the SEZ CD133^{+VE}/GFP^{+VE} cells (stem cell pool) compared to SEZ GFP^{+VE} only cells (non-stem cell pool). However, among all the IFTs only IFT88 showed the strongest differential expression and statistically significant difference by qRT-PCR. IFT88 also showed high significance in the MAS t test program in the microarray (Figure 3.15). All other cilia-related genes like foxj1, centrin2, WD repeat domain 78, desmulin, ciliary rootlet coiled-coil, Riken cDNA 1700027A23, that showed significantly higher expression in the stem cell pool, were confirmed by qRT-PCR (Figure 3.16).

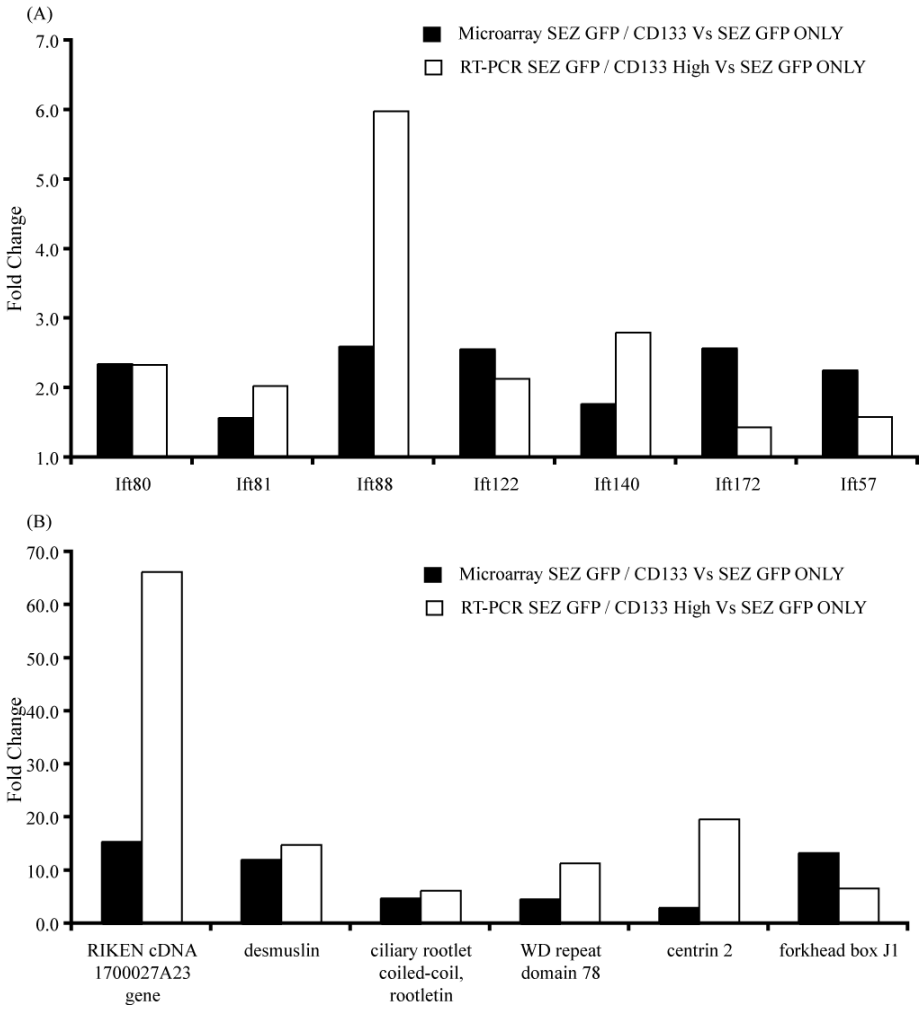


Figure 3.15: Differential Expression of Cilia mRNA between SEZ CD133/GFP vs SEZ GFP population sorted. (A) mRNAs of IFTs showing higher expression in SEZ CD133/GFP sorted cells in microarray analysis, also validated by quantitative RT-PCR.(B) Also other genes related to cilia upregulated in microarray in SEZ GFP/CD133 sorted pool was validated by RT-PCR.

Also, number of calcium related genes were found to be significantly and differentially expressed by SEZ CD133^{+VE}/GFP^{+VE} cells (stem cell pool) in comparison with SEZ GFP^{+VE} only cells (non-stem cell pool). This was of particular interest as calcium is one of the mechanosensors used by cilia in sensing the extracellular environment (Satir P et al., 2007). Genes like calcium/ calmodulin-dependent protein kinase ID, calpain3 and parvalbumin involved in calcium homeotasis were confirmed by qRT-PCR showing higher levels of expression in the SEZ CD133^{+VE}/GFP^{+VE} cell pool (Figure 3.17).

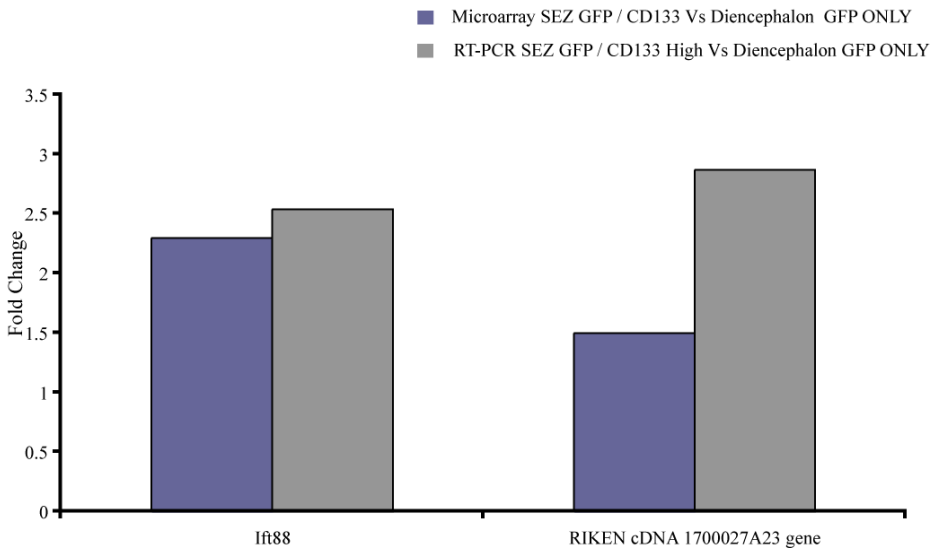


Figure 3.16: Expression and confirmation of Cilia genes upregulated in SEZ CD133^{+VE}/GFP^{+VE} Vs Diencephalon GFP^{+VE} ONLY sorted population. (A) Microarray data showed very few of cilia specific mRNAs higher in SEZ CD133/GFP sorted pool of astrocytes when compared to Diencephalic astrocytes having lower fold change but some of them was confirmed by quantitative RT-PCR.

As Bmp signalling is also known to regulate neural stem cells in the SEZ (Colak D et al., 2008) we examined whether these genes are also upregulated in our stem cell population. Melanoma inhibitory activity1 that regulates BMP activity and BMP6 were confirmed by qRT-PCR in the SEZ CD133^{+VE}/GFP^{+VE} stem cells compared to the SEZ GFP^{+VE} only cells. When stem cells were compared with diencephalic astrocytes the BMP-binding endothelial regulator protein precursor (BMPER) which was upregulated in stem cells in microarray was also validated by qRT-PCR in the SEZ CD133^{+VE}/GFP^{+VE} population.

BMPER is a secreted protein that directly interacts with BMP2, BMP4, and BMP6 and antagonizes BMP4-dependent Smad5 activation (Moser M et al., 2003) (Figure 3.18A, 3.18B).

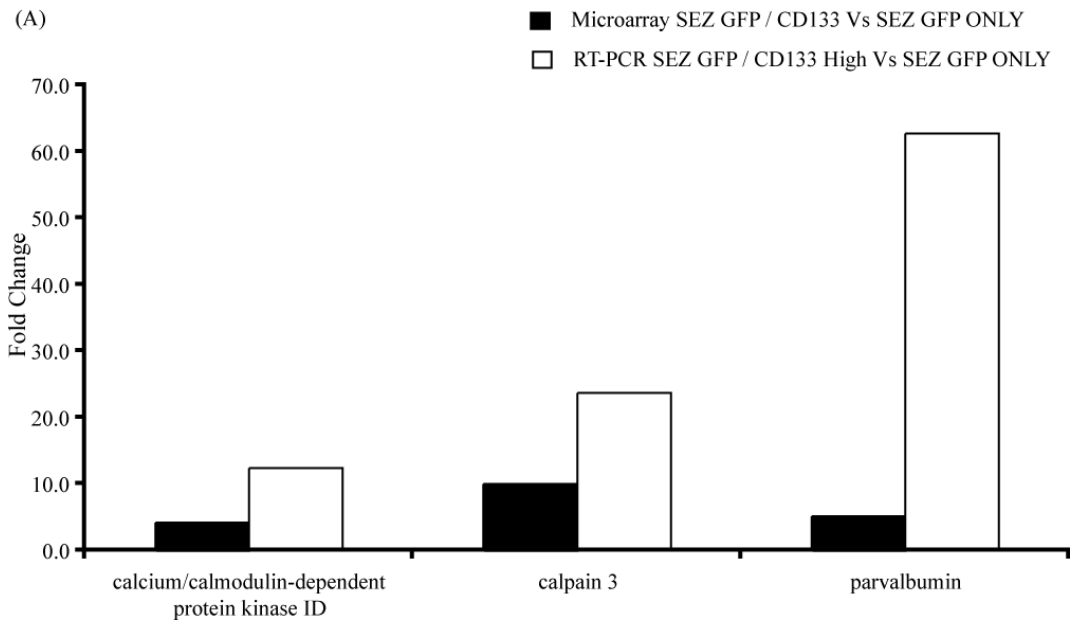


Figure 3.17: Expression of Calcium genes enriched in SEZ CD133/GFP sorted population compared with SEZ GFP population. mRNAs calcium related genes highly expressed by SEZ CD133/GFP cells in microarray analysis also,validated by quantitative RT-PCR.

Other genes like LIM domain-containing proteins, which have been shown to direct neuronal lineage from embryonic stem cells (Hwang M et al., 2008), were upregulated in SEZ $CD133^{+VE}/GFP^{+VE}$ cells compared to SEZ GFP^{+VE} only cells in the microarray and were confirmed by qRT-PCR. Also, I checked in qRT-PCR the Fibroblast Growth Factor Receptors (FGFR1-4) expression in the stem cell pool as its one of the main factors, together with EGF (epidermal growth factor), which induce the neural stem cell charecteristics *in vitro*, however, the FGF-responsive cells *in vivo* have not been identified. Even though FGF-receptors did not pass the stringent statestical test in microarray, I performed qRT-PCR but, none of them could be confirmed at RT level (Figure 3.19).

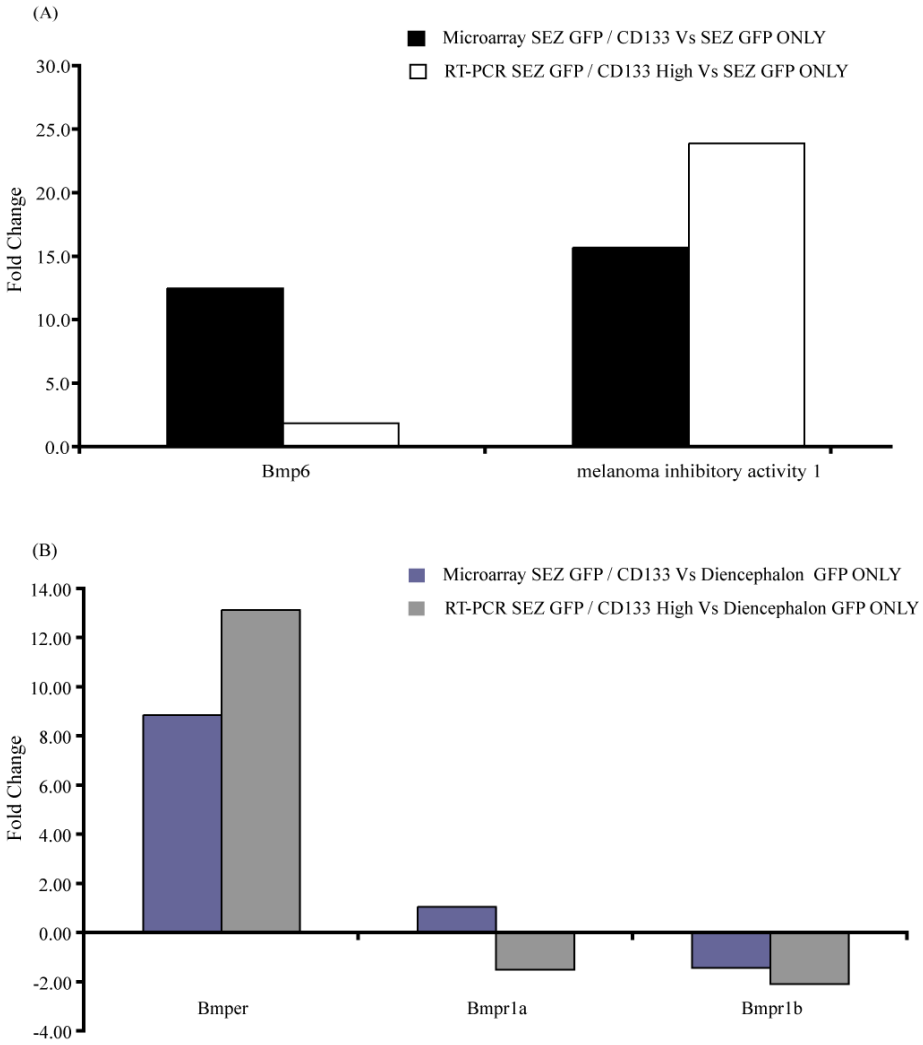


Figure 3.18: Differential Expression of BMP signaling genes between SEZ CD133/GFP vs SEZ GFP and SEZ CD133/GFP Vs Dienc GFP population sorted. (A, B) mRNA fold change in microarray is dipicted from the cells sorted from SEZ CD133/GFP astrocytic pool compared with SEZ GFP and Dienc GFP astrocytes and some of them was confirmed by RT-PCR.

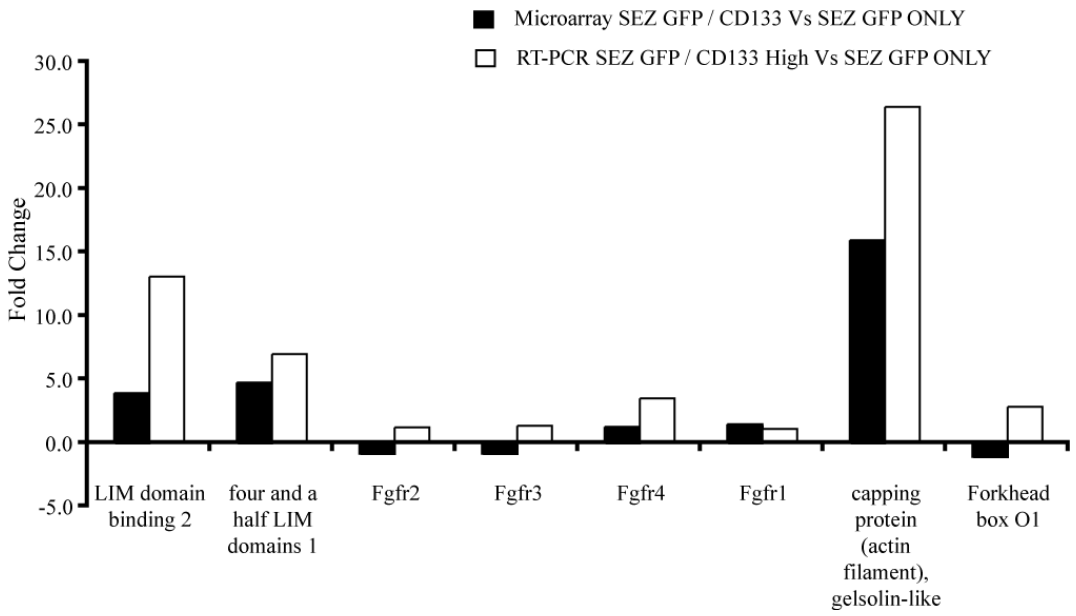


Figure 3.19: Expression and confirmation of genes in SEZ CD133/GFP vs SEZ GFP population. mRNAs predicted to be differentially expressed by microarray analysis and only genes with higher fold change in microarray was validated by quantitative RT-PCR.

3.1.6.2 Genes downregulated in Stem Cell Population (SEZ CD133^{+VE}/GFP^{+VE})

Apart from the genes upregulated in stem cells, I also examined genes highly expressed in the SEZ GFP^{+VE} population. SEZ cells that exclusively express GFP most likely include transient amplifying precursors and some neuroblasts due to the stability of GFP in the hGFAPeGFP transgenic line. GO analysis showed that cell cycle (8%), post-translational protein modification (9%), neurogenesis and developmental process (7%) genes are prominent in the GFP^{+VE} only pool (Figure 3.20). Quantitative RT-PCR confirmed that cell-cycle genes are differentially regulated in SEZ GFP^{+VE} only cells compared to the SEZ CD133^{+VE}/GFP^{+VE} pool, which is in line with the microarray data (Figure 3.21). These data are consistent with various works showing that transient amplifying precursors (TAPS) and neuroblasts divide much faster than the stem cells themselves.

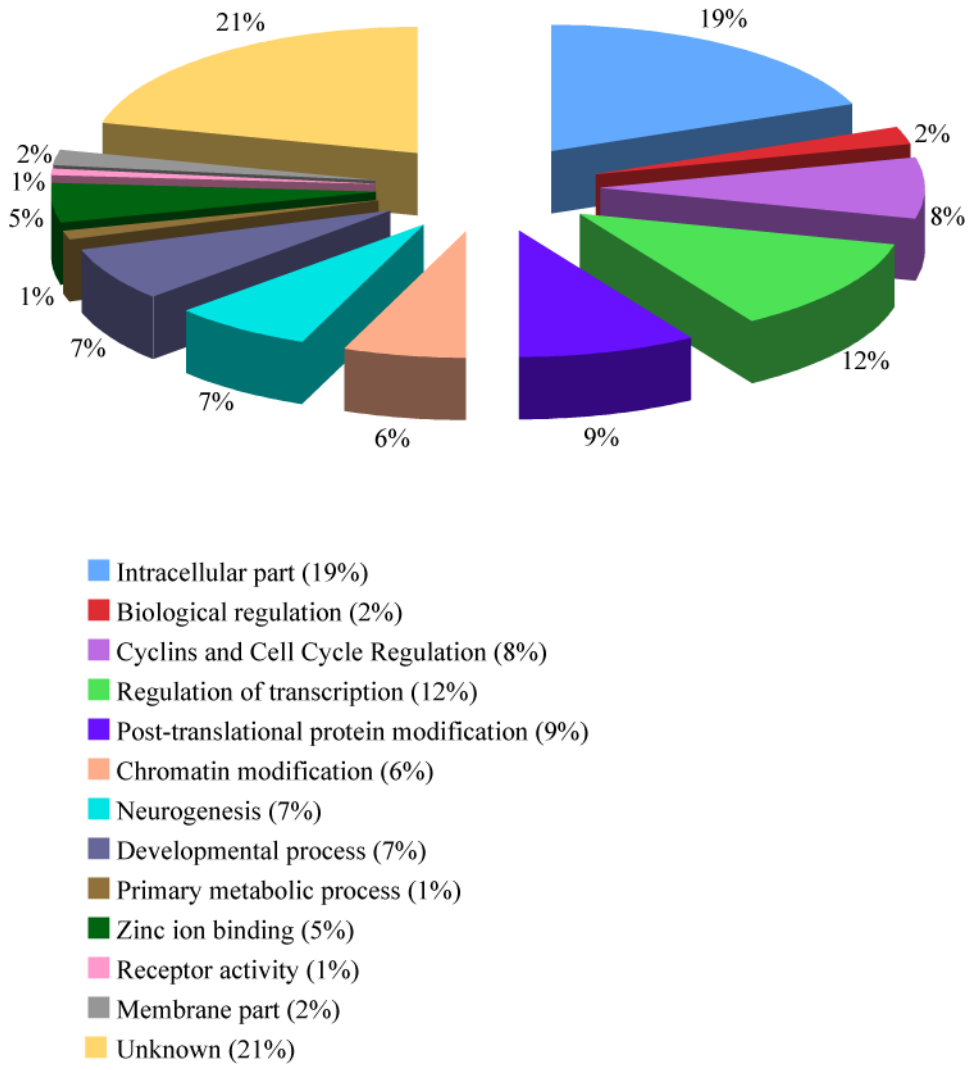


Figure 3.20: Temporal analysis of Gene Ontology of upregulated genes in SEZ GFP ONLY sorted cells. Total number of genes differentially expressed at least 2-fold within each ontological category, the SEZ GFP sorted cells have a contamination of neuronal genes of about 7%.

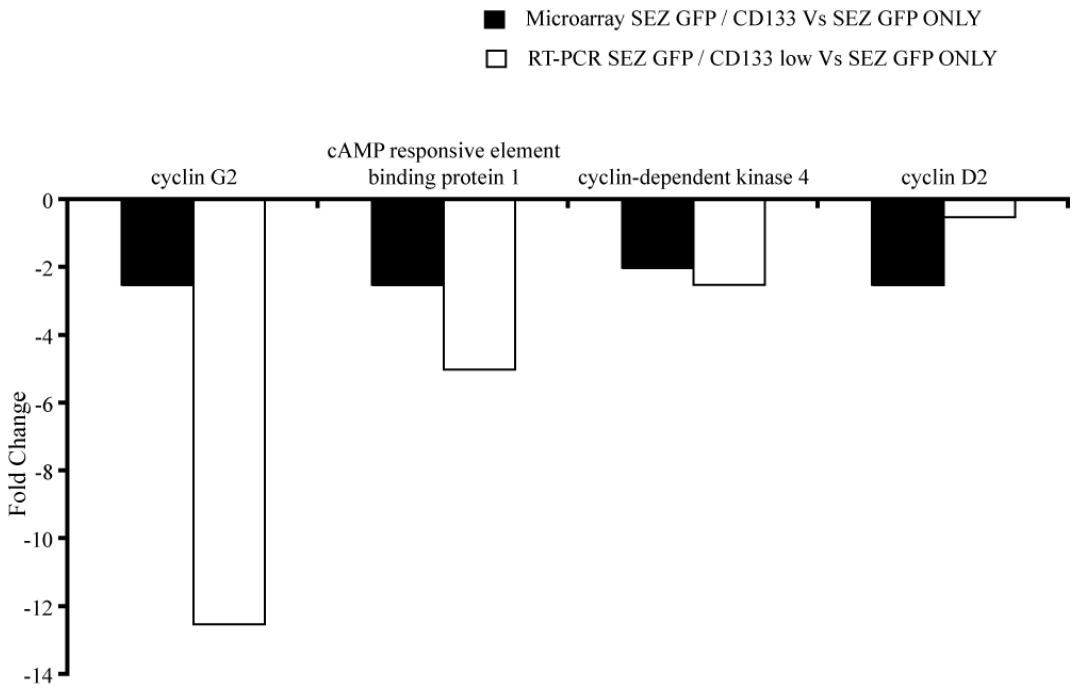


Figure 3.21: Expression and confirmation of Cell Cycle genes in SEZ CD133/GFP vs SEZ GFP population. mRNAs predicted to be differentially expressed by microarray (negative value in microarray means genes are downregulated in the camparison, for instance here cell cycle genes are very low expressed in SEZ CD133/GFP pool of sorted cells compared to SEZ GFP ONLY sorted cells) analysis was validated by quantitative RT-PCR.

3.1.7 SEZ CD133^{+VE}/GFP^{+VE} Cells Are More Similar to DIENC GFP^{+VE} Only at Transcriptome Level

To investigate whether the transcript level differences between the three sorted cell populations are consistent or random events, a Venn diagram was created. The SEZ CD133^{+VE}/GFP^{+VE} and DIENC GFP^{+VE} sorted populations share 1026 genes, while the SEZ CD133^{+VE}/GFP^{+VE} and SEZ GFP^{+VE} ONLY sorted cells have 909 genes in common. The stem cell pool (SEZ CD133^{+VE}/GFP^{+VE}) had a greater degree of similarity to the DIENC GFP^{+VE} pool at the transcriptome level than the SEZ GFP^{+VE} ONLY pool. One possible explanation for this is that DIENC GFP^{+VE} cells make up a purer astrocytic population than SEZ GFP^{+VE} ONLY, which likely consist of a mixture of astrocytes, transit amplifying cells and some neuroblasts. Thus the stem cell pool population (SEZ CD133^{+VE}/GFP^{+VE}) has more similarity to the DIENC GFP^{+VE} astrocytes (Figure 3.22 A).

Additionally, to examine the reliability of our astrocyte analysis we compared our gene expression data with the astrocyte transcriptome database created by Ben Barres and colleagues. SEZ CD133^{+VE}/GFP^{+VE} and DIENC GFP^{+VE} sorted populations showed a high degree of similarity with the genes of the astrocyte database. When 250 top astrocyte enriched genes from Cahoy et al database (Cahoy et al 2008) were compared to our SEZ CD133^{+VE}/GFP^{+VE} and DIENC GFP^{+VE} sorted populations (where RMA data for these sorted cells was filtered for average expression ≥ 50) SEZ CD133^{+VE}/GFP^{+VE} stem cells showed 82% similarity sharing 204 genes with 250 astrocyte specific genes. Additionally, our control sorted cells (DIENC GFP^{+VE}), which had pure astrocytic characteristics shared 215 genes similar with 86% congruence (Figure 3.22B, 3.22C).

Also the GO analysis of SEZ CD133^{+VE}/GFP^{+VE} astrocytes (neurogenic) verses dienceplalic astrocytes (DIENC GFP+VE ONLY; non-neurogenic) revealed many cell cycle (23%) and intracellular part (34%) genes being upregulated in stem cells. These data again point to the fact that SEZ CD133^{+VE}/GFP^{+VE} astrocytes are dividing more slowly compared to parenchymal astrocytes (DIENC GFP^{+VE} ONLY) as shown in the above analysis when SEZ CD133^{+VE}/GFP^{+VE} cells were compared to SEZ GFP^{+VE} ONLY cells and their cell cycle genes were higher in SEZ GFP^{+VE} ONLY (Figure 3.23).

3.1.8 A novel gene PitchFork expressed in SEZ CD133^{+VE}/GFP^{+VE}, adult neural stem cell pool

To charecterize novel genes in the stem cell population (SEZ CD133^{+VE}/GFP^{+VE}), we examined our gene expression database to identify specific genes with the highest levels of mRNA expression in the neugenic astrocyes (SEZ CD133^{+VE}/GFP^{+VE}). One such gene which I followed, was Riken cDNA 1700027A23 (PitchFork), which has been shown recently to be expressed in tissues rich in highly ciliated cells, such as olfactory sensory neurons, and is predicted to be important to cilia (Timothy S. McClintock et al., 2008). Also this RIKEN has enriched expression in the node of the early developing mouse embryo. As it is expressed in cilia, we decided to explore its expression and role in adult neural stem cells (SEZ CD133^{+VE}/GFP^{+VE}).

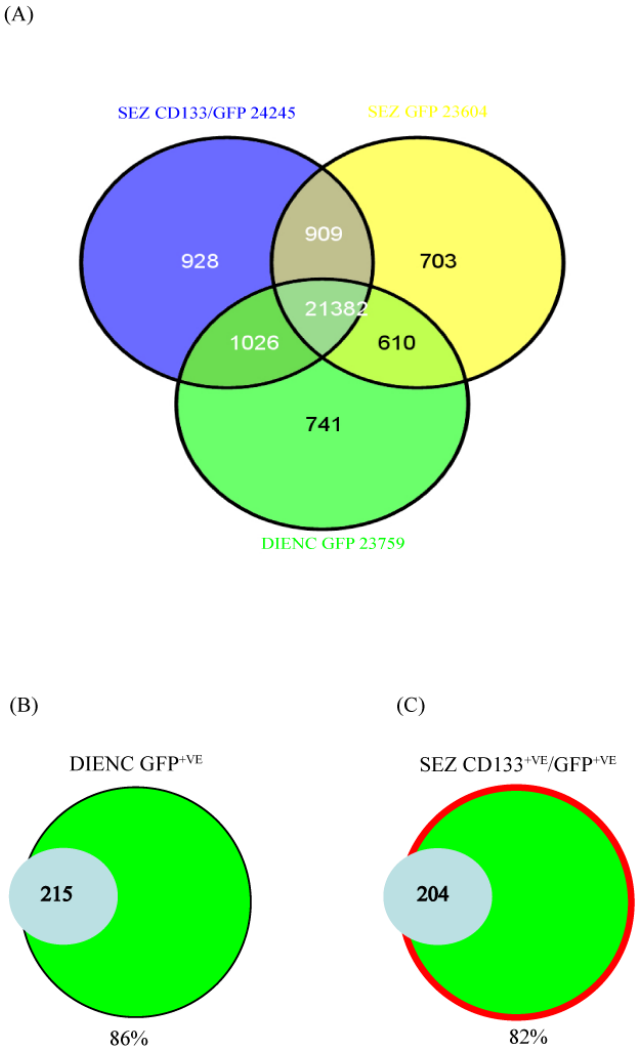


Figure 3.22: Venn diagrams representing shared and specific genes expressed in sorted populations. (A) Displaying the number of shared and unique astrocytic genes in the SEZ CD133/GFP -SEZ GFP ONLY, SEZ CD133/GFP-DIENC GFP ONLY and SEZ GFP ONLY-DIENC GFP ONLY comparisons. (B, C) Venn diagram showing the number of genes enriched in SEZ CD133/GFP-DIENC GFP ONLY overlap strongly with genes identified in common astrocytes from Cahoy et al.

To analyse the Pifo (pitchfork) in more detail, firstly at mRNA level with qRT-PCR, I confirmed pitchfork (shown above) was upregulated in the SEZ CD133^{+VE}/GFP^{+VE} sorted stem cell pool as compared to SEZ GFP^{+VE} ONLY and DIENC GFP^{+VE} sorted populations (control cells). To look at pitchfork at the protien level in the adult neurogenic area, namely SEZ *in vivo*, where in FACS sorted cells it was enriched in CD133/GFP-positive population at the mRNA level. The immunostaining with Pifo

(pitchfork) antibody revealed its strong expression both the neurogenic niches in adult brain namely SEZ (subependymal zone) and SGZ (sub granular zone) like a doty structure (Figure 3.24A, 3.24B). As this was an affinity-purified antibody, I used the preimmune serum as control to validate the specificity of the staining. I could never observe in the sections any doty staining using the primmune serum, which confirmed the specificity of our antibody (Figure 3.24C).

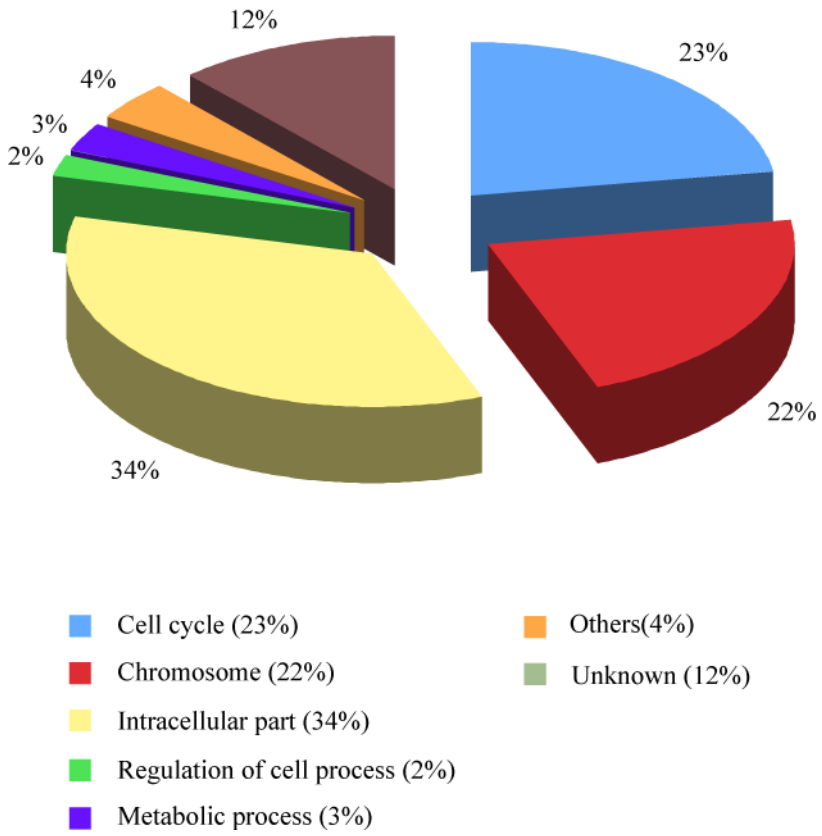


Figure 3.23: Temporal analysis of Gene Ontology of upregulated genes in SEZ CD133/GFP astrocytic pool compared to DIENC GFP ONLY sorted cells. Total number of genes differentially expressed with highest significance within each ontological category. In this analysis genes related to cell cycle, choromosome and Intracellular part were having high expression in SEZ astrocytes (SEZ $CD133^{+VE}/GFP^{+VE}$).

Apart from this I also prepared the whole mounts of adult SEZ, as whole mounts provide an en face veiw to the ventricular surface, which will facilitate the better veiw of ventricle-contacting cells. To visualize the cilia at immuno level I stained the whole mounts of SEZ with acytylated-tubulin, which labels the microtubules structure and cilia.

To localize the protien expression of pitchfork double immunolabeling of pitchfork with acytylated-tubulin was done. Pitchfork showed its expression at the tip of the cilia like a doty structure (Figure 3.24D).

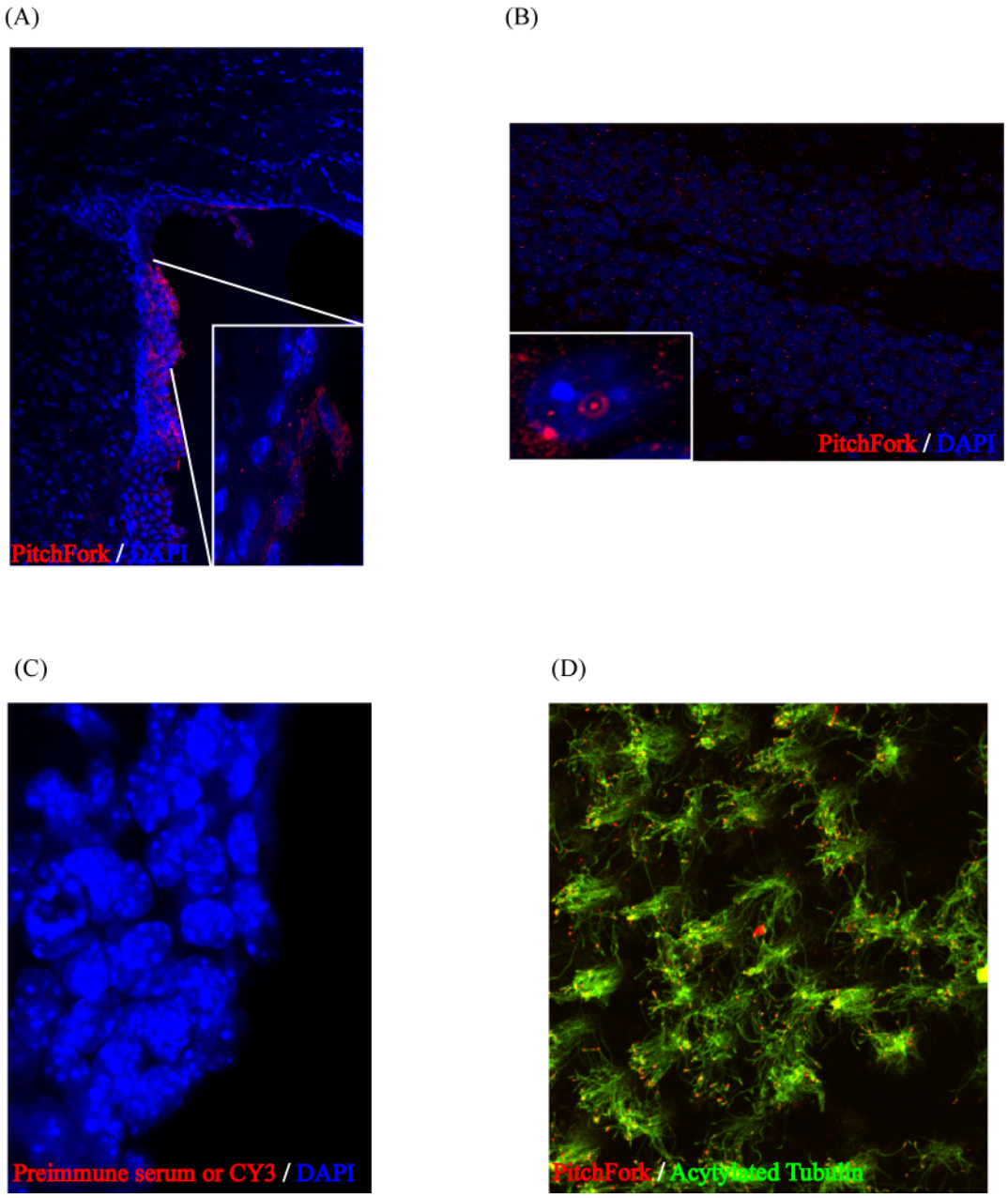


Figure 3.24: Pitchfork expression in adult neurogenic niches. (A, B) Both neurogenic areas (SEZ and SGZ) have PitchFork immunostaining. (C) As this was self-made antibody, the negative control with preimmuserum/secondary antibody alone revealed the specificity of antibody as no immunostaining was observed. (D) Whole mount preperation of SEZ revealed the localisation of pitchfork at the tip of the acytylated tubulin positive cilia.

3.2 Origin and progeny of reactive multipotent astroglia

3.2.1 Cells from the injured adult cortex acquire multipotency *in vitro*

Given the astrocyte identity of neural stem cells and the well known upregulation of factors present in the adult neurogenic niche after injury like Shh and FGFs , I examined weather cortical astrocytes reacting to cortical stab wound injury can reveal stem cell potential *in vitro* using the neurosphere assay. It was already known that the white matter of adult cortex harbors a pool of NG2-positive progenitor cells, which in culture can give rise to neurospheres (Nunes MC et al., 2003). Therefore it was important to do the injury only in the cortical grey matter (above the corpus callosum) to avoid contamination by this known source of neurosphere forming cells. Three days post injury, I dissected, the cortex from both the injured and the contralateral side, dissociated it into single cells and cultured it for 10-12 days in uncoated flasks in serum-free medium supplemented with EGF and FGF-2 every alternate day (Figure 3.25). After 7-10 days *in vitro*, the cells isolated from the injured cortex, formed small spheres with all the typical morphological properties of neurospheres (1 in 1100 cells; Figure 3.26A, 3.26B). To determine whether cells within the spheres could continue to proliferate in secondary cultures, these spheres were mechanically dissociated and replated at density of 10,000 cells in 1ml of neurosphere media in the presence of mitogens (EGF/FGF2). The cortical neurospheres proliferated and formed new spheres without changing their morphology and phenotype for 3-10 weeks (5-10 passages; Figure 3.27A). Further differentiation of this self-renewing neurospheres was initiated by removal of the mitogens and plating the cells onto poly-L-lysine.

After 10 days *in vitro*, the majority of cells (30%) acquired morphologic and phenotypic characteristics of astrocytes ($GFAP^+$), 20% acquired oligodendrocytic characteristics ($O4^+$), and 9% acquired neuronal characteristics (βIIItubulin$^+$; Figure 3.27B-D). Among all the neurospheres formed, 46% were multipotent, giving rise to cells with morphological and antigenic properties of all the principal cell types of the CNS and the remaining 54% were bipotent, giving rise to only glia, no neurons (Figure 3.27E).

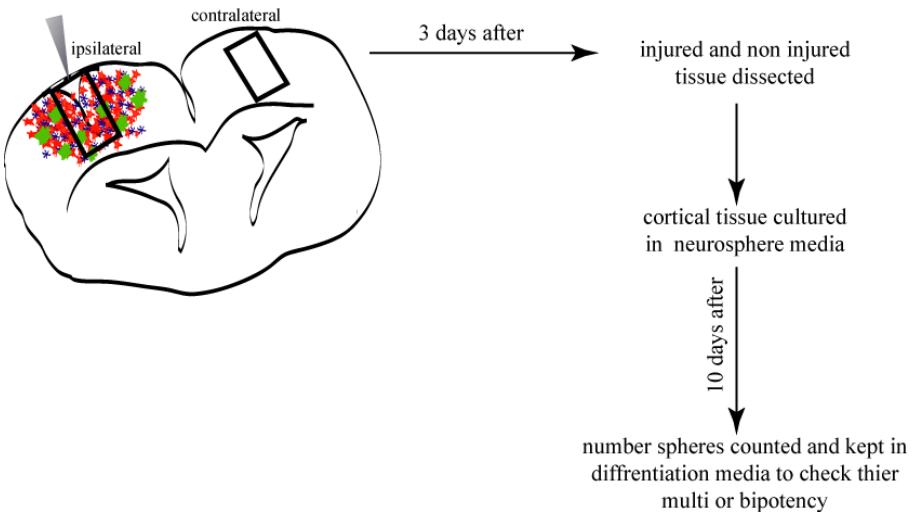


Figure 3.25: Schematic drawing of cells cultured in vitro upon injury. The picture depicts that upon stab wound injury cell types like astrocytes, oligodendrocytes and microglia proliferate to make the glial scar and inhibit neurogenesis in this area, this tissue upon injury is cultured to see their potential in vitro.

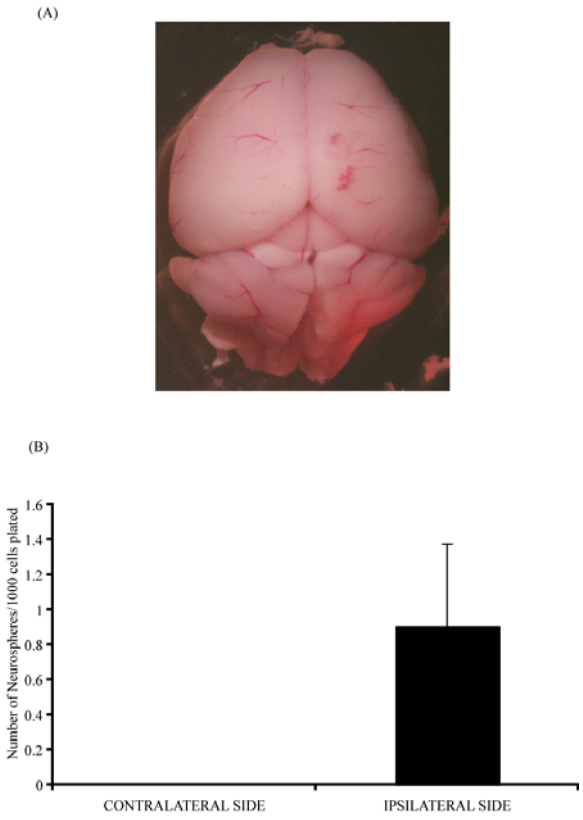


Figure 3.26: Cells from injury side gives rise to neurospheres. (A) Whole mount of a brain 3 days post lesion dipicting both conralateral side (left) and ipsilateral side (right) of injury. (B) Micrograph

showing only cells from ipsilateral side of injury giving rise to neorospheres.

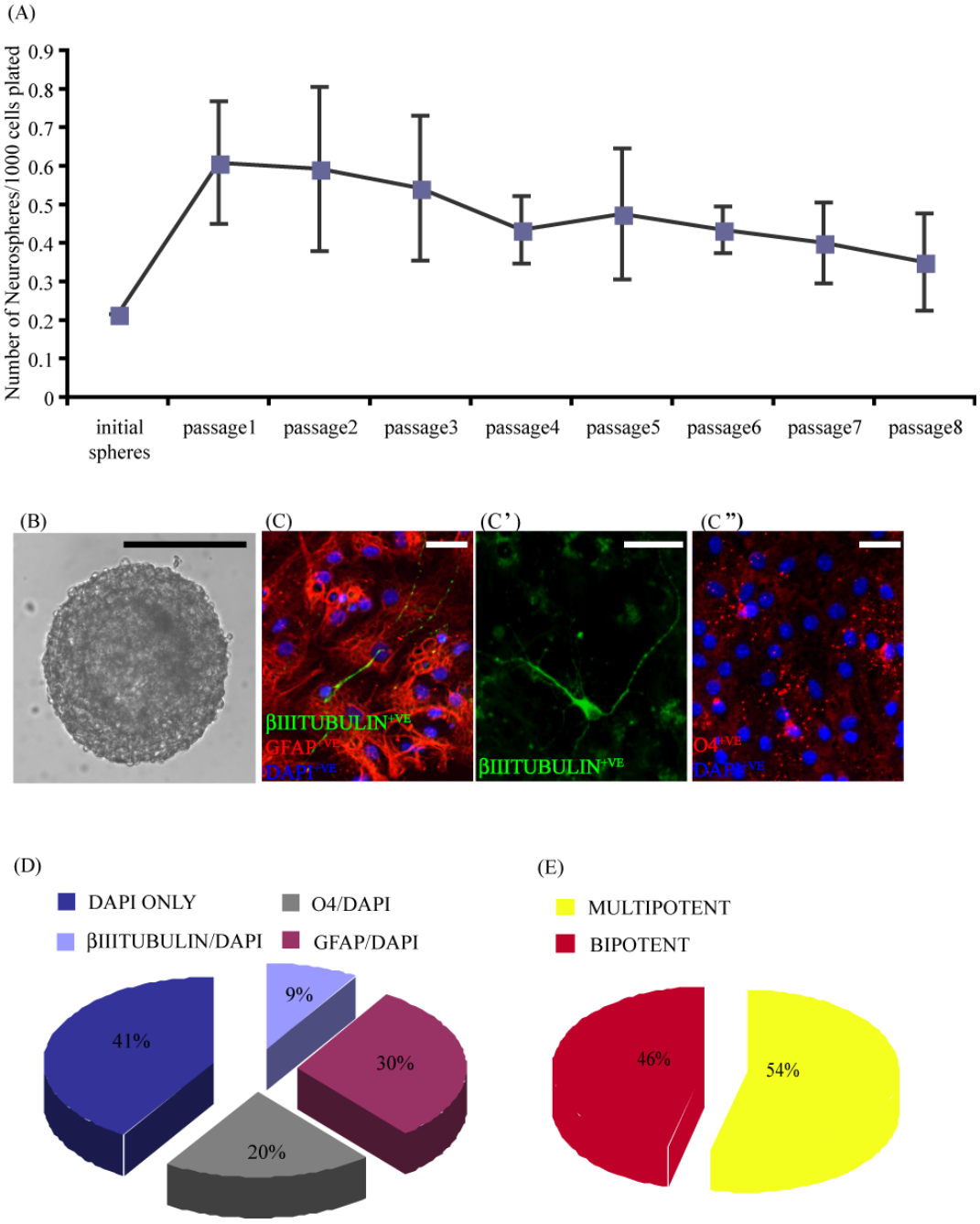


Figure 3.27: Neurosphere forming cells upon injury are self renewing and multipotent. (A) Neurospheres formed from the injury site can be passaged for several rounds in vitro. (B) Micrograph depicting example of neurosphere formed by cells isolated from the lesioned cortex (10 days in vitro) and their progeny upon differenciation (C, C', C''). Only half of neurospheres are multipotent (E) giving rise to astrocytes, neurons and oligodendrocytes in vitro (D). Scale bar (A, D) 50mM, (B, C) 20mM.

3.2.2 Quiescent astrocytes in the injured adult cortex resume proliferation *in vivo* and acquire multipotency *in vitro*

As astrocytes are the major cell type in any kind of brain insult, the next question I asked weather the neurosphere forming cells are reactive astrocytes or $NG2^{+VE}$ cells. In order to examine the cellular origin of the multipotent astroglia, we used a knock-in line in which the tamoxifen-inducible form of the Cre recombinase (CreERT2) was targeted to the locus of the astrocyte-specific glutamate transporter GLAST (Slc1a3) that allows inducing recombination in adult astrocytes *in vivo* (Mori T *et al et al., 2006*). Recombination can be monitored in the reporter lines R26R (Soriano P et al., 1999) or Z/EG (Novak A et al., 2000). Using these mouse lines, it was possible to monitor the fate of astrocytes following injury. When R26R was used as an indicator of recombination, β-galactosidase (β-gal) was expressed mostly in astrocytes positive for GFAP (Figure 3.28A). Upon 6-8 days of tamoxifen- induction in the adult mouse stab wound injury was done. 3 days post stab wound injury the number of β-gal+ cells/mm^2 was increased by 30-50% within 150 μm from the lesion track, consistent with the increase in astroglial cells after injury compared to uninjured site. To monitor whether cell division by the reporter+ cells contributed to this increase in number, BrdU was provided in the drinking water starting at the time of lesion to label all cells dividing upon injury. The vast majority of the BrdU/β-gal double-labeled cells expressed GFAP (Figure 3.28B), indicating that about half of the formerly non-proliferating astrocytes start to divide following injury. To look at the neurosphere forming capacity of these reactive astrocytes I isolated these tamoxifen-induced reactive astrocytes expressing GFP (Z/EG reporter line) by FACS sorting (Figure 3.28C) and cultured them in expansion media with mitogens for the neurosphere assay. Both GFP^{+VE} and GFP^{-VE} cells were sorted from the injury site formed multipotent neurospheres (1 in 675 GLAST::CreERT2;Z/EG-positive cells and 1 in 2500 GLAST::CreERT2;Z/EG-negative cells) and gave rise to neurons (7%), astrocytes (45%) and oligodendrocytes (16%; Figure 3.28D-G). The presence of GFP^{+VE} neurospheres thus clearly demonstrates that astrocytes labeled prior to injury acquired the capacity to form neurospheres and that the GFP^{+VE} neurospheres were not derived from the small proportion of GFP^{-VE} cells included in the sort as the purity of sortings was 95%. Also the GFP^{-VE} cells forming the spheres could be explained by the

low efficiancy of recombination using the Z/EG repoter mice in the GLAST locus.

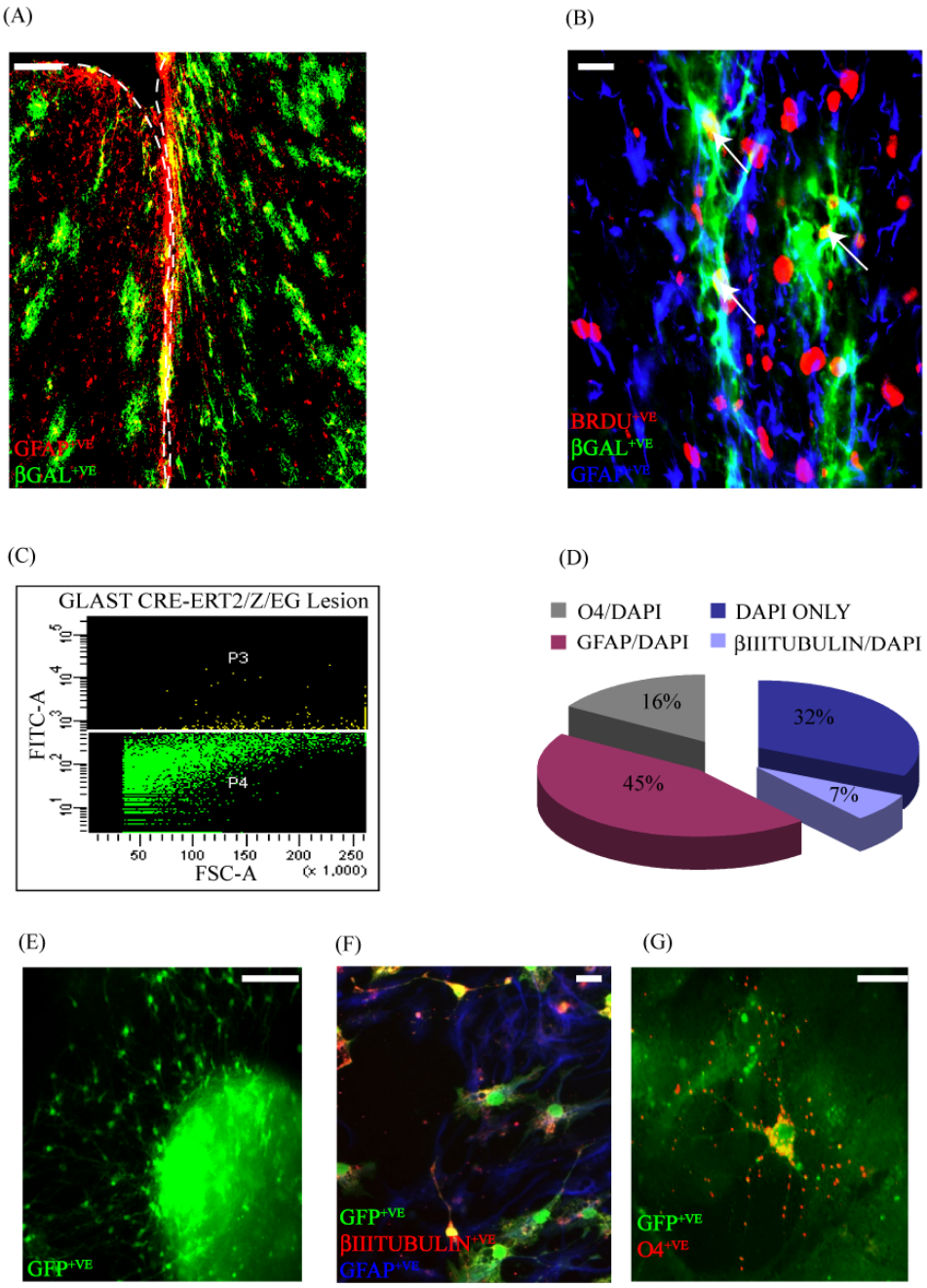


Figure 3.28: Proliferation and fate of genetically labeled postmitotic astrocytes in the injured mouse neocortex. (A, B) A large number of genetically labeled β-gal+ve astrocytes are GFAP immunoreactive at the lession site also they incorporate BrdU (arrows) 3dpl. (C) Representative FACS profile of cortical cells isolated 3dpl from injury site of GLAST: : Cre-ERT2;Z/EG (astrocyte specific) inducible line with GFP^{+VE} signal in yellow. GFP^{+VE} astrocytes give rise to multipotent neurospheres in vitro (D-G). Scale bars (A) 100µm (B) 40µm (E-G) 20µm

In addition to GLAST::CreERT2;Z/EG mouse line I also used hGFAP-eGFP transgenic

mouse line (Nolte C *et al., 2001)* in which astroytes shows strong eGFP expression in neurogenic niche region (SEZ and SGZ) and in non-neurogenic region (dienchephalic and cerebellar) of the adult CNS. Using hGFAP-eGFP transgenic line, astrocytic response to CNS insults can be directly monitored by a fluoresence increase. Within the first few days after cortical stab wound injury Nolte et al found an increased number of eGFP expressing astrocytes lining the stab wound canal, relating to an increased expression of GFAP within the astrocytic process. In addition to strong eGFP expression around the lesion tract I could observe that some astrocytes began to proliferate *in vivo*, as evidenced by their incorporation of the DNA-base analogue 5-Bromo- 2'deoxyuridine (BrdU) at the injury site (Figure 3.29A), thus confirming the view that there is a pool of astrocytes capable of entering the mitotic cycle. Based on green fluorescence, reactive astrocytes from the injured cortices of the hGFAP-eGFP transgenic mouse line can be sorted by FACS (fluorescence-activated cell sorting; Figure 3.29B). Injured cortices from the hGFAP-eGFP transgenic line 3 days after lesion were dissociated into single cells and passed through the FACS sorter. Both the GFP^{+VE} and GFP^{-VE} cells from injured area were sorted and cultured in serum free media supplemented with EGF/FGF2 for the neurosphere assay. After 7-10 days *in vitro* spheres were obtained from both GFP^{+VE} and GFP^{-VE} sorted cells from the injury site. However, both the GFP^{+VE} and GFP^{-VE} cells formed neurospheres but the efficiency of neurosphere formation from GFP^{+VE} cells was 10-20x stronger (1 in 376 hGFAP-eGFP cells) compared to GFP^{-VE} cells (1 in 1148 cells). In general, the efficiency of neurosphere forming cells from injured cortices was low compared to neurospheres from SEZ $CD133^{+VE}/GFP^{+VE}$ sorted cells where almost every cell plated formed neurospheres (1 in 1,4 cells; 72%).

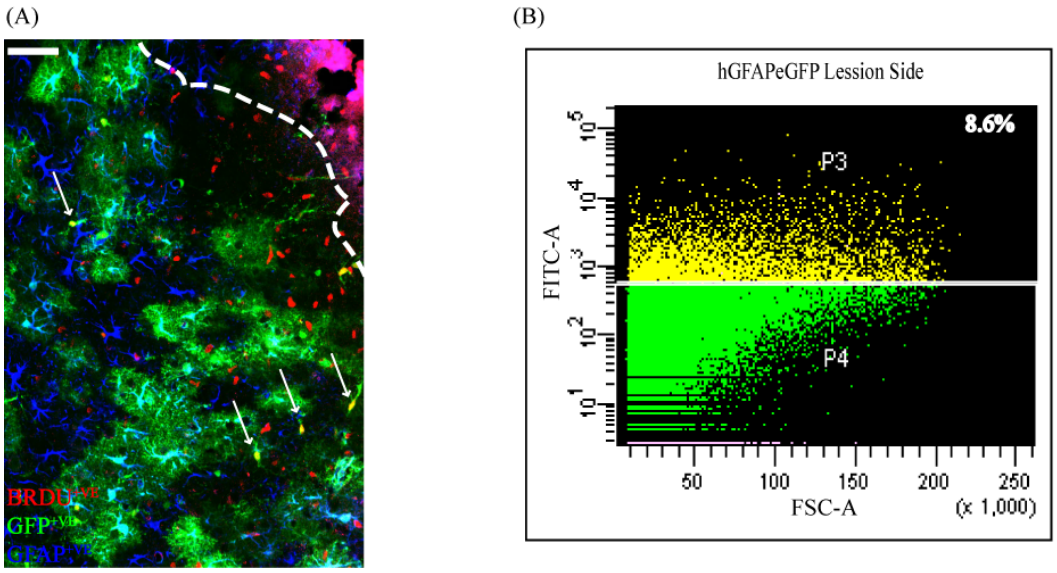


Figure 3.29: Isolation of neurosphere forming cells from hGFAPeGFP transgenic line.(A) Using hGFAPeGFP transgenic line majority of activated astrocytes are labeled 3-day post injury (dpl), these astrocytes proliferate in vivo (by arrow).(B) FACS sorting profile from hGFAPeGFP transgenic line shows at injury site 8.6% cells are labeled with GFP. Scale bar (A) 100µm

There is, however, always a risk in these experiments that some cell aggregates may form by cells attaching to each other rather than by cell proliferation. To test the potential of individual adult cortical GFP^{+VE} cells more stringently, dissociated cells were FACS sorted for GFP and cultured as single cells. I carefully plated out only one cell per micro-well of a Terasaki plate. Each well containing a single GFP $^{+VE}$ cell (n=329) was visually inspected daily for 7-10 days (Figure 3.30A-A'''). Strikingly, 1 in 18 GFP $^{+VE}$ cells gave rise to a multipotent neurosphere generating neurons, astrocytes and oligodendrocytes (Figure 3.30C). Thus, much of the relatively low efficiency in neurosphere formation, seen in the previous experiments, seems to be due to death of the cells after sorting. Amongst the healthy GFP^{+VE} astroglial cells more than 5% form neurospheres.

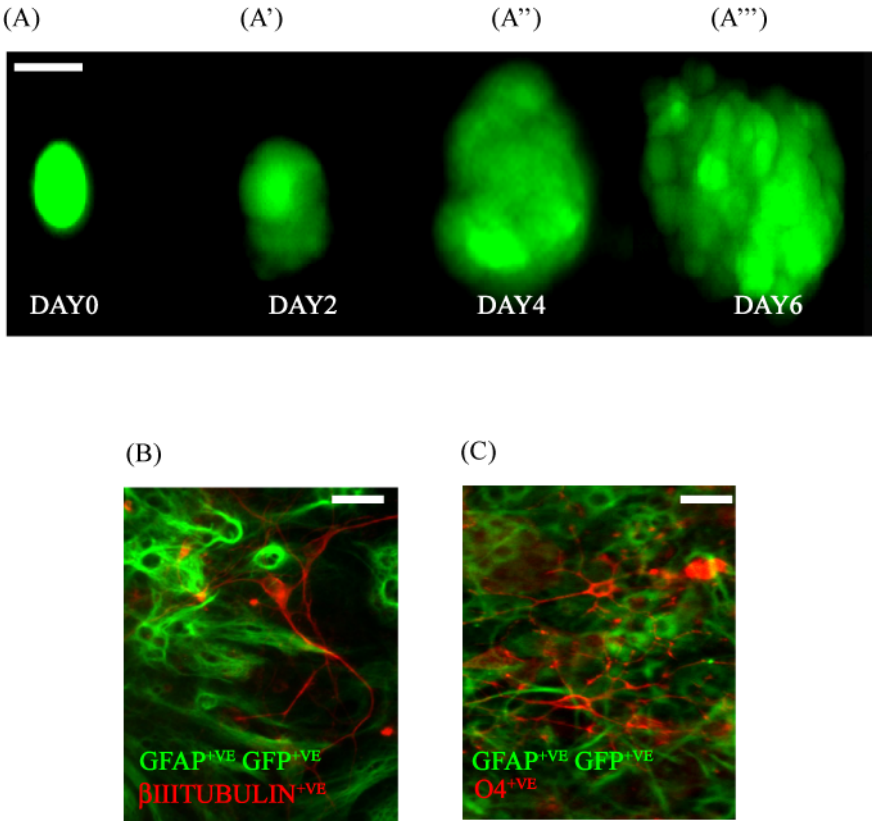


Figure 3.30: Single cell forms a multipotent neurosphere. (A-A''') Single cell plated, captured at different time points, dipicting a sphere generated from a single cell. (B, C) Neurosphere generated from single cell gives rise to astrocyte, neurons and oligodendrocytes.scale bar (A) 10µm (A'-A''', B, C) 20µm.

In the hGFAP-eGFP transgenic line the human GFAP promoter was used to target the expression of eGFP to astrocytes. As it is a human GFAP promoter and a transgenic line, it is possible that not all astrocytes activate this promoter and there might be an ectopic activation of eGFP in some non-astrocytic cells. To confirm the molecular identity of GFP^{+VE} FACS sorted cells, these cells were sorted and plated on PDL coated cover slips for 1 hour, fixed with 2% paraformaldehyde and stained for astrocytic markers, like GFAP and S100β, which are expressed *in vivo* by astrocytes upon injury. Examining the presence of these markers, I could observe diffenrences in their expression: S100β immunolabeled 75% of sorted cells and only 63% of the total sorted cells were stained for GFAP. Co-immunolabeling with GFP showed an overlap of only 59% with S100β and 45% with GFAP (Figure 3.31A, 3.31B). This low number of double positive cells might be due to the leaking out of GFP during plating and fixing. But still it goes in line to our finding that 100% of neurospheres from the GFP^{+VE} sorted cells were

immunopositive for astrocytic markers like GFAP or S100β and gave rise to all three principal CNS cell-types upon differentiation (Figure 3.31C-E).

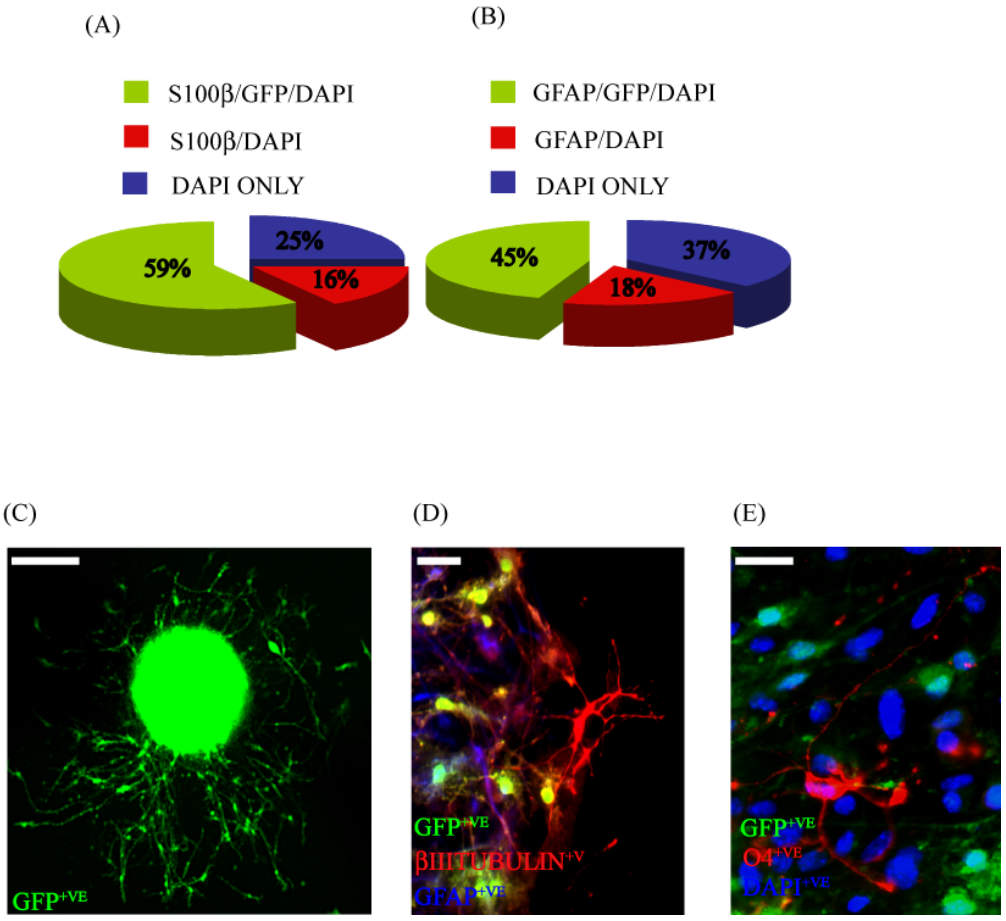


Figure 3.31: Astrocytes after injury are multipotent. (A, B) Sorted GFP^{+VE} cells upon plating mostly expresses S100β and GFAP marker for astrocytes. GFP^{+VE} neurosphere when plated for differentiation gave rise to both neurons and glia (C, D, E). Scale bar (C-E) 20μm.

GFP^{+VE} and GFP^{-VE} cells from the contralateral side of the injury, where astrocytes are neither reactive nor proliferative, were also sorted. No neurospheres were formed by GFP^{+VE} or GFP^{-VE} cells (0 in 60,000 hGFAP-eGFP cells; 0 in 90,000 cells). With these experiments we could show that there are cells that start to proliferate after stab wound injury and that these cells are astrocytes.

3.2.3 Proliferating oligodendroglial precursors do not acquire multipotency *in vitro*

To determine whether other glial populations also acquire the capacity to form multipotent neurospheres following injury, we isolated cells from the injury site by NG2 or PDGFRα surface staining. The NG2/ PDGFRα immunostaining was done on hGFAPeGFP transgenic line. As expected and explained above we could observe eGFP expression in some of the NG2/ PDGFRα immunostained cells. This is due to the human GFAP promoter and transgenic line, which causes ectopic expression of eGFP in some non-astrocytic cells (Figure 3.32A, 3.32B). But upon plating $NG2^{+VE}/GFP^{+VE}$ and $NG2^{+VE}$ ONLY or $PDGFR\alpha^{+VE}/GFP^{+VE}$ and $PDGFR\alpha^{+VE}$ ONLY no multipotent neurospheres were obtained (0 of 35,000 and 25,000 respectively), even upon addition of PDGF to these cultures (Chojnacki A et al., 2004). The NG2 or PDGFRα positive cells in culture did form some spheres, but these spheres were very small in size even after 7-10 days in neurosphere media supplemented with growth factors. Irrespective of their size these spheres were plated on PDL coated coverslips for differentiation for 10-12 days. Most of these spheres were ball-like aggregates of cells. They never gave rise to any neurons and only occasionally gave rise to some astrocytes (Figure 3.32C, 3.32D).

3.2.4 Viral tracing of stem cell-derived lineages originating in the subependymal zone lining the lateral ventricle

To test the possibility that the neurosphere-forming GFP^{+VE} cells from the cortical injury site actually originated from the subependymal zone (SEZ), a region of continuous adult neurogenesis, we virally traced the lineage of the SEZ stem cells. We injected GFP-containing VSVG-pseudotyped lentiviral vector into the SEZ ipsilateral to the site of stab wound injury (Hack MA *et al., 2005*) in C57BL/6 mice. Three days post injury, most GFP^{+VE} cells were detected within the SEZ and the rostral migratory stream (RMS) with few cells migrating into the white matter (WM) (Figure 3.33A), but none had entered the cortical grey matter (GM) surrounding the site of injury (Figure 3.33B). To ensure that smaller numbers of GFP^{+VE} cells did not escape detection, we used FACS analysis and found that 2.6% of SEZ cells were GFP^{+VE} (Figure 3.33C), while no GFP^{+VE} cells were detectable in the tissue isolated from the injury site (Figure 3.33D). This analysis demonstrates that the neurosphere-forming cells that occur with a frequency higher than 5% are not derived from stem cells in the SEZ.

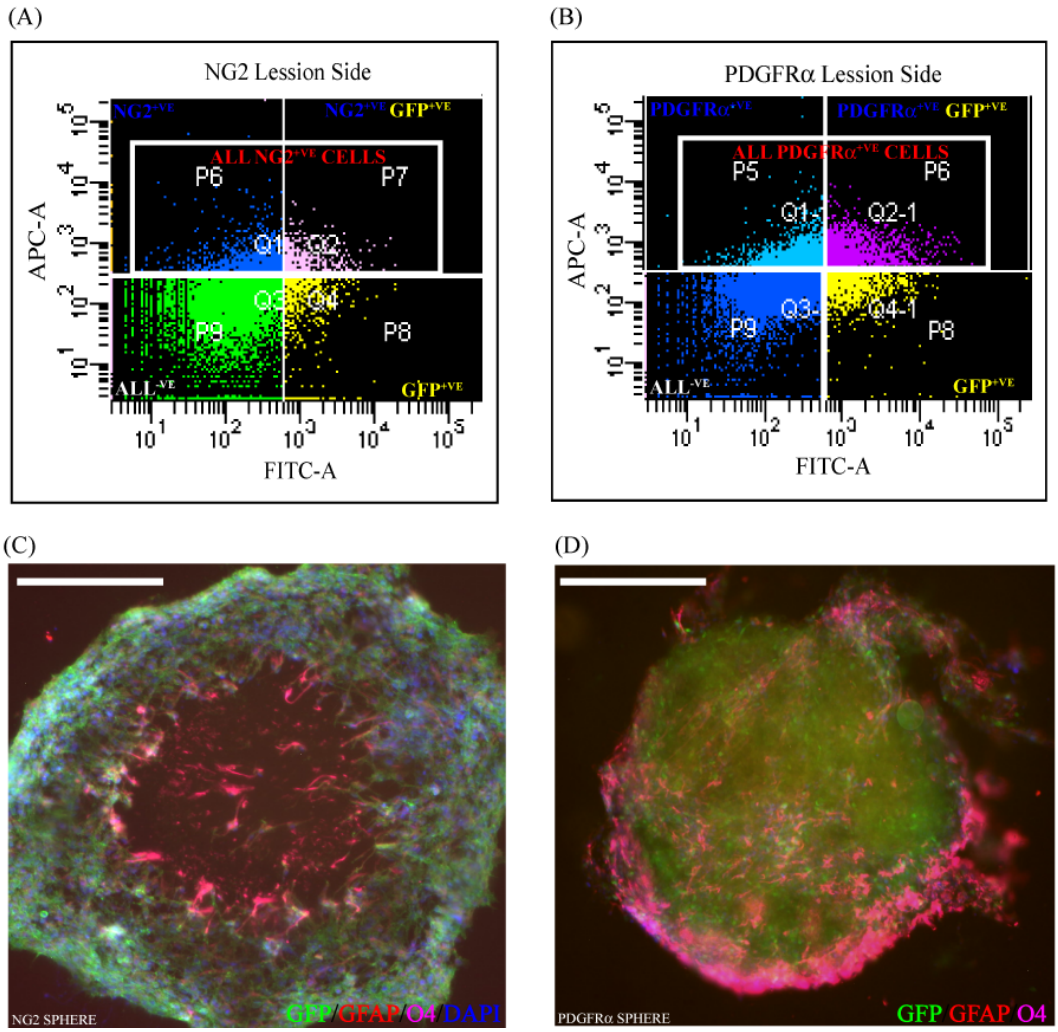
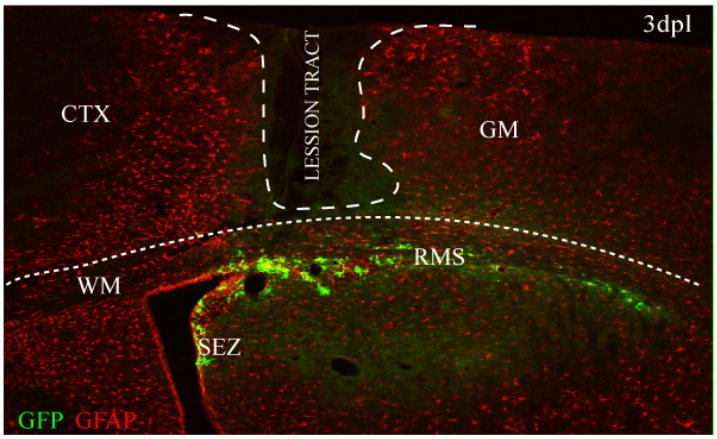
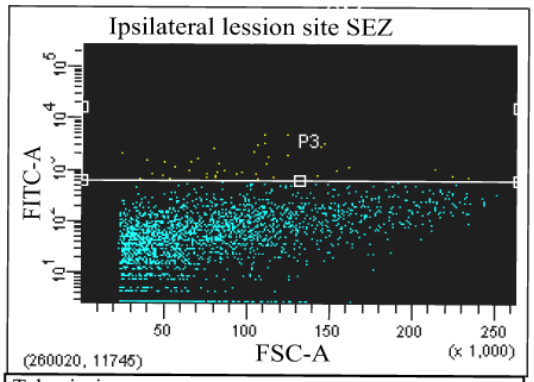


Figure 3.32: Capacity of other glial precursors upon injury. (A, B) Representative FACS profile of cortical cells isolated 3dpl from injury site of hGFAPeGFP transgenic line (astrocyte specific) stained with NG2 and PDGFRa.Few NG2+ve and PDGFRa +ve cells are also stianed for GFP (pink signal in 2ndquadrant of FACS profile). But the spheres formed from all NG2+ve and PDGFRa +ve cells when plated were always bipotent(C, D). Scale bar (C-D) 50µm

(A)

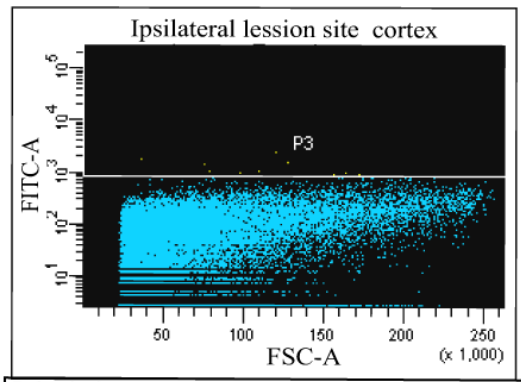


(B)



Tube: ipsi sez Population	Events	%Parent	%Total
All Events	10,000		100.0
P1	4,010	40.1	40.1
P2	2,599	64.8	26.0
P3	65	2.5	0.6

(C)



Tube: ipsi ctx Population	Events	%Parent	%Total
All Events	130,000		100.0
P1	56,0902	43.1	43.1
P2	26,877	47.9	20.7
P3	10	0.0	0.0

Figure 3.33: No infiltration of proginitors from stem cell niche. To clarify whether the neurosphere-forming GFP+ cells present in the cortex injury site may originate from the subependymal zone (SEZ), one of the regions of continuous adult neurogenesis, VSVG-pseudotyped lentiviral vector coontaining GFP was injected into the SEZ ipsilateral to the site of SW injury. 3dpl, most GFP+ cells were detected within the SEZ and the rostral migratory stream (RMS) with few cells migrating into the white matter, but none had entered the cortical GM surrounding the site of injury (A). To ensure that smaller, numbers of GFP+ cells did not escape detection, FACS analysis was done and only SEZ cells were GFP+ (B), while no GFP+ cells were detectable in the tissue isolated from the injury site (C).

3.2.5 Soluble factors from the injury site elicit neurosphere formation

To investigate whether soluble factors released from the lesioned area were responsible for the multipotency of reactive astroglia *in vitro*, I dissected the injured cortex 1 day post lesion (dpl) from C57BL/6 WT mice, chopped the cortex in small pieces and cultured them in basic media (DMEM) for 2 days. As a control, non-injured cortex was dissected and cultured using the same conditions. On the third day, cortical cells from normal adult mice from a transgenic line in which GFP is ubiquitously expressed by all cells, (GPI-anchored transgenic line) were dissociated into single cells, plated at a very low density (50,000 cells/5ml) and cultured in 5ml flasks supplemented with conditioned media collected from both injured and non injured cortices and growth factors (EGF/bFGF) for 7-10 days (Figure 3.34).

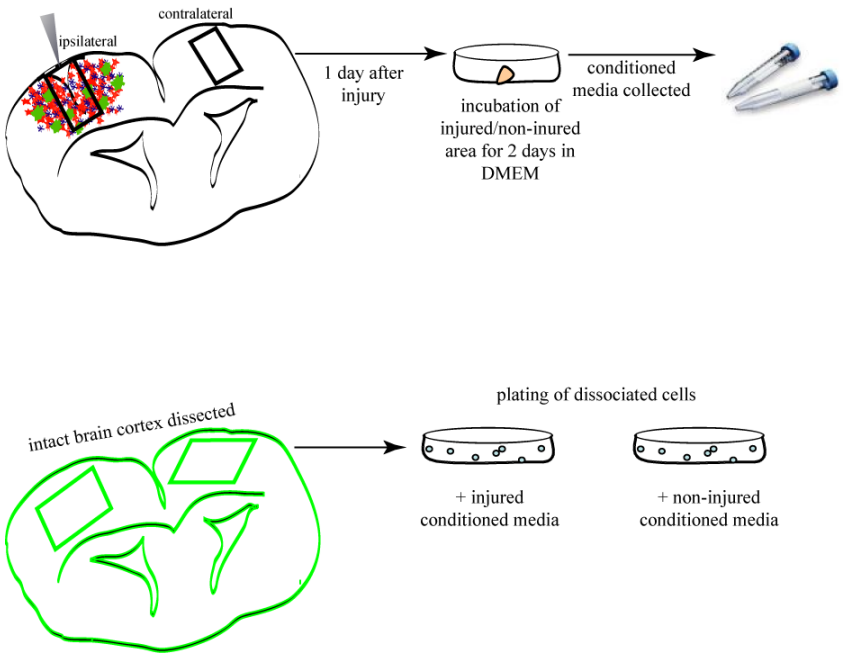


Figure 3.34: Schematic drawing representing the culturing of adult cortical cells for identifying the endogeneous signals screated by cells.

Remarkably, the cortical cells cultured with the lesion-conditioned media (LCM) gave rise to GFP^{+VE} multipotent neurospheres giving rise to neurons, astrocytes and oligodendrocytes upon differenciation (Figure 3.35A), suggesting that indeed some neurogenic signal produced from the LCM may lead to neurosphere formation from the adult cortex. None of the spheres were GFP^{-VE}, ruling out the possibility of contamination

by some live cells that might be present in the LCM. In contrast, the adult cortical cells treated with conditioned media from the un-injured cortex (contralateral side of injury, CCM) or with no conditioned media formed no or very few neurospheres (Figure 3.35B). Thus, diffusible factors released from the injured cortex are sufficient to elicit neurosphere formation from adult cortical cells.

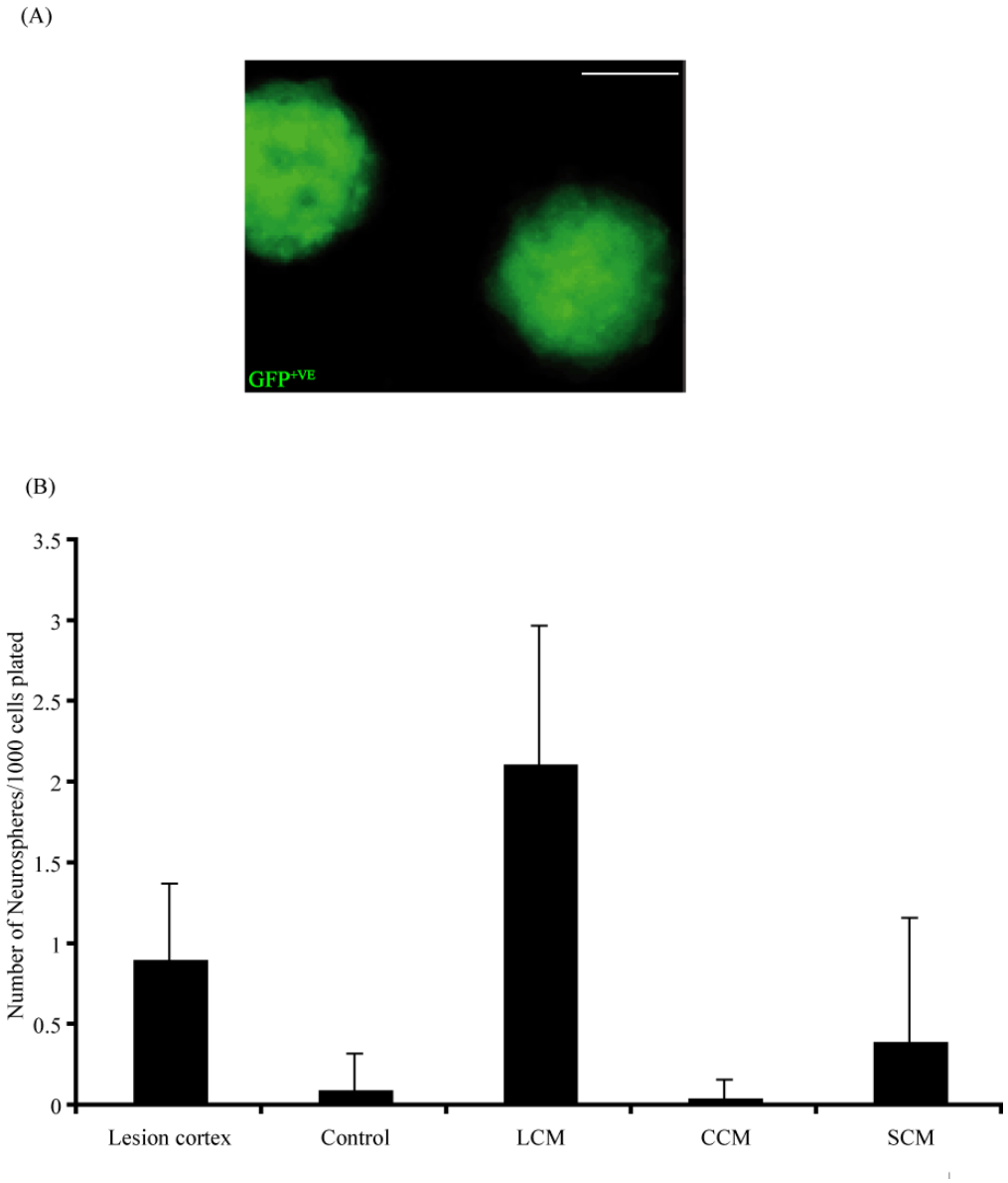


Figure 3.35: Neurospheres from Lesioned Conditioned Media. (A) The picture dipicts a GFP+VE neurosphere arising from adult cortical cells treated with lesioned conditioned media (LCM). (B) Adult cortical cells treated with LCM giving rise neurospheres with highest efficency compared to cortical cells supplimented with no conditioned media (control) or contralateral conditioned media (CCM) and even more than the injured cortical tissue.scale bar 50μm in (A).

3.2.6 Sonic Hedgehog Signaling (shh) triggers multipotency of cells around the lesioned area

To identify the signal produced by cells upon cortical stab wound lesion that might be a cause of neurosphere formation of these cortical cells *in vitro*, I screened for signaling molecules that were upregulated in the microarray analysis of neurogenic astrocytes (SEZ $CD133^{+VE}/GFP^{+VE}$) in the SEZ niche when compared to non neurogenic astrocytes from the same niche (SEZ GFP^{+VE} ONLY) and from another parenchymal niche (DIENC GFP^{+VE} ONLY) where astrocytes are completely post mitotic. Among many signaling molecules I focused on Shh, which is known to regulate proliferation of adult neural stem cells (Lai K et al., 2003; Machold R et al., 2003). Pharmacological inhibition or stimulation of Shh signaling in the adult brain also leads to decreased or elevated neural stem cell proliferation in the hippocampus and SEZ, respectively (Lai K et al., 2003;Machold R et al., 2003). I therefore examined weather Shh may be upregulated upon cortical stab wound injury. First, I analysed expression of Shh by qRT-PCR in the cells one-day post injury both in the injured (ipsilateral side) and non-injured sites (contralateral side). Shh expression was strongly upregulated in the ipsilateral side of the injury (Figure 3.36A) similar to cells in the neurogenic niche that showed stem cell characteristics (SEZ $CD133^{+VE}/GFP^{+VE}$) *in vitro* for neurosphere assay. Then I examined weather Shh is also required for triggering neurosphere formation in non-neurogenic CNS regions, as it was one of the potent proliferating factor in neurogenic adult CNS regions. I dissected the adult cortical grey matter (the region where usually stab wound lesion is done) without any injury, dissociated, plated at low density, and examined for their ability to proliferate in clusters of clonally related cells in presence of Shh in addition to EGF and bFGF which are already present in the culture media. I could observe that addition of Shh was sufficient to stimulate the proliferation of neural progenitors and sphere formation from adult cortical cell cultures (Figure 3.36B). Also, if Shh is required for cortical neurosphere formation, one could predict that by using Shh antagonists the sphere-forming effect of cortical cells could be attenuated. To directly test the requirement of endogenous Shh signaling on adult cortical cell proliferation and self-renewal using neurosphere assay, I added cyclopamine to the LCM. Cyclopamine is a plant alkaloid that selectively inhibits Shh signaling (Incardona et al., 1998; Cooper et al., 1998). Treatment of cortical cells with cyclopamine (5μM) led to an inhibition of cortical neurosphere formation. Indeed, by cyclopamine treatment I could block neurosphere

formation in the cortical cells supplemented with lesioned conditioned media (LCM).

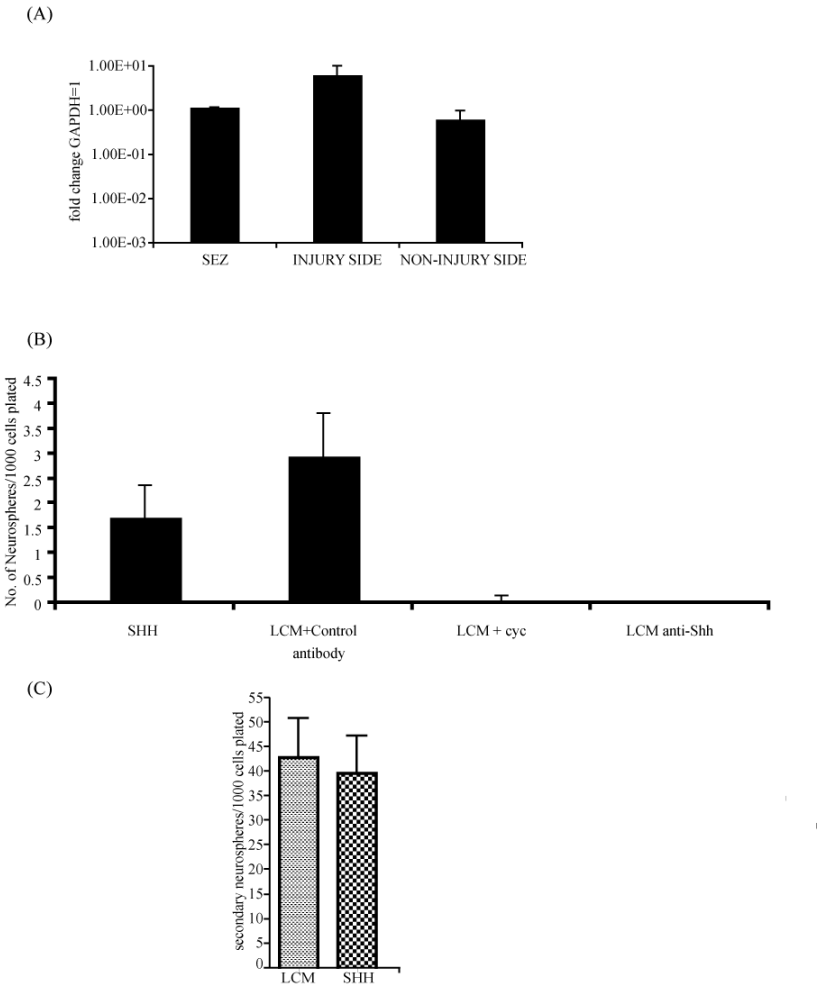


Figure 3.36: Shh as a component triggering neurosphere formation from adult cortical cells. (A) Shh expression by neurogenic astrocytes (SEZ) compared to non-neurogenic astrocytes upon injury. Representative qRT-PCR analyses Shh expression in SEZ and the cerebral cortex with and without injury. Shh is a positive regulator for neural progenitor cell proliferation in culture. Quantification of neurosphere formation in dissociated cortical cell cultures in the presence of Shh (10 ng/ml) and neurogenic capacity of LCM could be bocked by anti-Shh (1 mg/ml), while control antibody yielded similar number of neurospheres as LCM, additionaly its inhibitor Cyclopamine (5 μM) diminished neurosphere formation in cortical cultures (B) both LCM and Shh driven spheres gives rise to secondary neurospheres (C).

Furthermore, Shh requirement for cortical neurosphere formation was confirmed by using anti-Shh monoclonal antibody (4μg/ml). By adding anti-Shh antibody, the neurosphere forming capacity in cortical cells supplemented with LCM was attenuated .This confirmed Shh as a growth signal, which is required to stimulate neural progenitor

proliferation of cortical cells *in vitro*. However, these cells can be passaged in presence of FGF or EGF into secondary neurospheres. Strikingly, cells supplemented with both LCM and Shh formed secondary neurospheres (Figure 3.36C). Dissociating these clusters and allowing cells to attach and differentiate can demonstrate the multipotency of these Shh induced neurospheres. Cells dissociated both from primary and secondary neurospheres readily attached and began to differentiate. After 8 days, the sphere-derived cells displayed morphologies of neurons, astrocytes, and oligodendrocytes (Figure 3.37). Thus we could demonstrate that Shh is an essential component in the LCM to elicit neurosphere formation and hence stem cell properties of adult cortical cells in vitro.

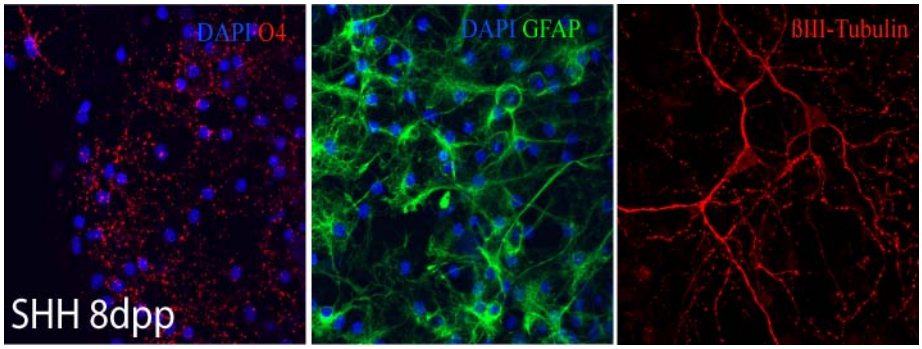


Figure 3.37: Shh triggered neurospheres from adult cortical cells are multipotent. Shh-derived spheres upon differentiation could give rise to neurons, astrocytes, and oligodendrocytes in culture.

3.3 Generation of GLAST::Venus/Tomato (astrocyte-specific) knock-in mice

3.3.1 Design and generation Venus/Tomato knock-in construct in *GLAST* locus

The targeting vector was designed (Figure 3.38A, 3.38B) at the exon2 of the GLAST locus, 66 base pairs upsteam of the translation initiation site with two constructs one containing VENUS another containing Tomato in order to allow the in situ localization of astroglia in normal adult mouse brain. Both targeting constructs contained after the polyadenylation signal (PA) the neomycin resistance gene (PGK-neo-poly-A) flanked by FRT sequences in opposite orientation for positive selection of targeted ES cells. A DTA

cassette without poly-A signal was also added at the 3' end of each targeting vectors for negative selection.

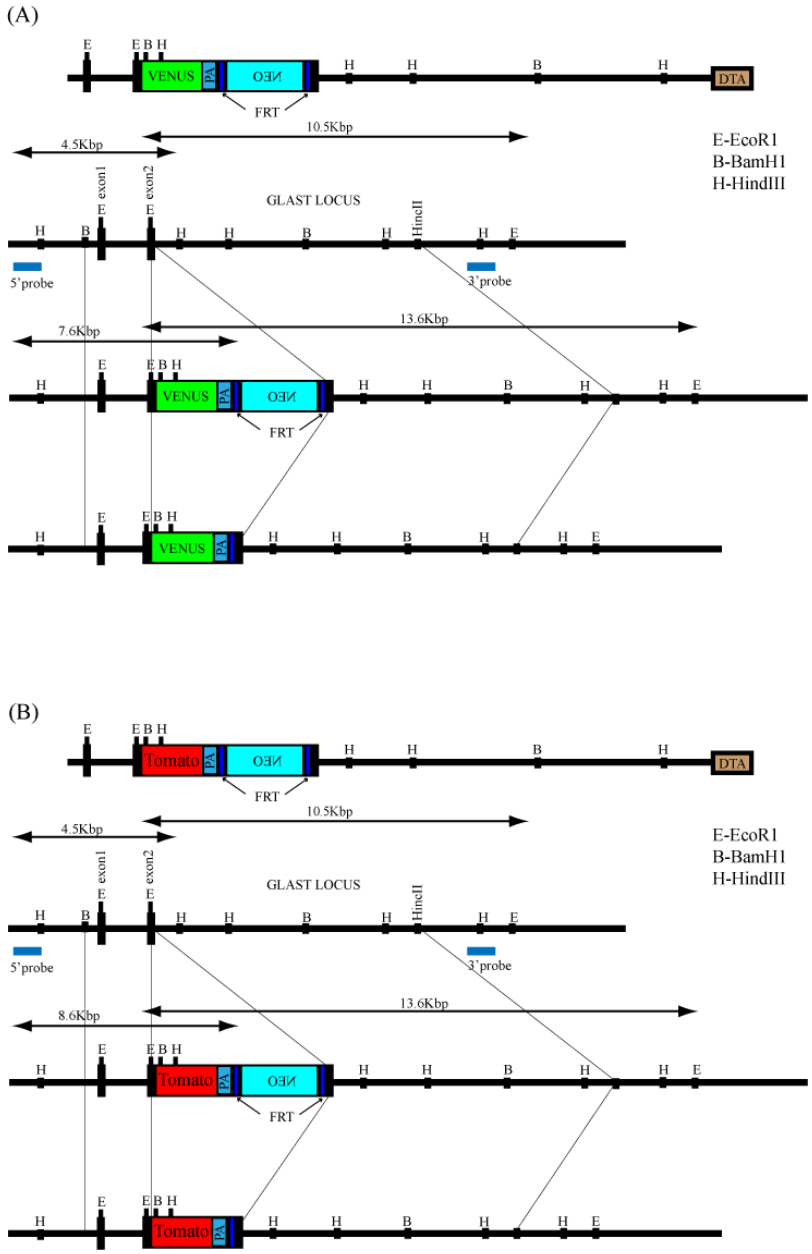


Figure 3.38: Targeting strategy of VENUS/Tomato in GLAST locus.

(A,B) A targeting vector was used to introduce VENUS/Tomato into exon 2 of the GLAST gene and the selection cassette was removed by Flp-mediated excision. GLAST exons (black boxes) are numbered. The external 5' probe and the internal Cre probe for Southern analysis are as indicated on the figure. Restriction enzyme sites for Not1 are shown. Homology regions to generate the targeting constructs are indicated as 5' and 3' retrieval (Abbreviations: FRT = sites of Flp-recombination; NEO = neomycin resistance gene; PA = Simian Virus 40 polyadenylation signal).

To accomplish homologous recombination in mouse ES cells the *NotI*-linearized p4VENUSfneolDTA/p4TomatofneolDTA-targeting vector was transferred into TBV2 ES cells (Wiles *et al*., 2000) via electroporation. Neomycin resistant clones were selected using 300µg/ml G418 (Invitrogen, 50mg/ml). Homologous recombination for the VENUS targeting vector specifically at the *GLAST* locus was confirmed by Southern blot analysis of *HindIII*-digested genomic DNA using the *GLAST* 5´-probe (1.8bp; generated by PCR; for sequence see Material and Methods) located outside of the targeting vector (Figure 3.39). By this way 2 out of 120 neomycin (neo) resistant clones were isolated as homologous recombinants and confirmed.

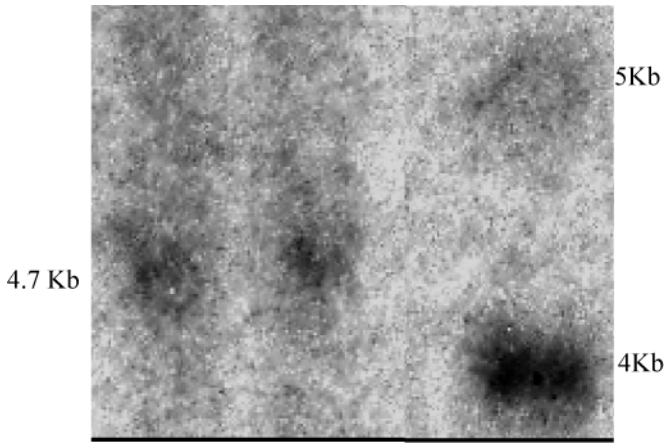


Figure 3.39: Southern Blot on ES cells and mice - Verification of homology recombined clones. Southern blot of ES cell (lane 1) and mousetail DNA (lane 3) digested with Not1 and hybridized with the external 3' probe, showing wild type allele (4.5kb).

4 Discussion

The aim of this study was to seek out astroglia function in neurogenic, non-neurogenic zones of the intact and injured brain.

The first part of my work was to look for additional markers for adult neural stem cells in the SEZ, apart from GFAP and to perform gene expression profiling these astrocyte-like cells. For that I focused on CD133, which is known to be one of the most broadly accepted markers for stem cells, including embryonic neural stem cells in humans (Ucida et al., 2000). The location and identity of CD133 expressing cells in the adult CNS has been elusive. I was able to show that a subpopulation of astrocytes in the adult SEZ expresses the CD133 antigen, and these CD133-GFAP positive cells ($CD133^{+VE}/GFP^{+VE}$; hGFAPeGFP transgenic line where eGFP colocalises mostly to GFAP-positive cells) upon sorting contained almost all neurosphere-forming cells in vitro. Furthermore, I performed transcriptional profiling of these neurogenic $CD133^{+VE}$ astrocytes and compared them to $CD133^{-VE}$ astrocytes in the SEZ niche and outside the niche, to examine the intrinsic differences between these neurogenic astrocytic populations to non-neurogenic.

The second part of my work deals with the question of whether de-differentiation of reactive glia is possible *in vitro* and which endogenous factors are reponsible for this phenomenon. For this study I examined astroglia following cortical stab wound injury. I could show in this study that astrogila upon injury acquire stem cell like properties *in vitro* by forming multipotent self-renewing neurospheres. Also, I could show that Shh as one of the potent enodogeneous neurogenic factors that elicits this response in astrocytes.

4.1 Prospective characterisation of exclusive neural stem cell fraction

The true identity of neural stem cells in the adult brain is elusive, and there are many studies on neural stem cells that are contradictory and arrive at seemingly mutually exclusive conclusions. Cells with a self-renewal capacity and the potential to generate

neurons, astrocytes and oligodendrocytes can be propagated *in vitro* from the adult nervous system (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Many studies have addressed the identity of the cells that display stem cell potential *in vitro* by performing neurosphere assays in the quest to find neural stem cells *in vivo*. The neurosphere comprises heterogeneous populations of cells, including cells characterized as NSCs, which can be demonstrated retrospectively by neurosphere reformation and differentiation into multiple lineages. Prospective methods for identifying NSCs and/or neural progenitors have been developed over the past several years (Uchida et al., 2000; Roy et al., 2000; Rietze et al., 2001; Kawaguchi et al., 2001). Analysis of NSC membrane properties, particularly compared with those of HSCs, has also been studied (Cai et al., 2003, 2004). Apart from this, others have used the absence of PNA lectin binding and CD24 expression to select for neural stem cells (Rietze et al. 2001). Uchida and colleagues have used AC133/CD133 immunocytochemistry for positive selection. Temple and colleagues have used SSEA1/Lewis-X antigen expression, while Hulspas and colleagues used Hoechst labeling to isolate stem cell populations (Hulspas and Quesenberry 2000; Uchida et al. 2000; Capela and Temple 2002). Here we have systematically examined the expression of some of these markers to enrich adult neural stem cells from the SEZ. First, during this work I sucessfully estabilished the prospective isolation of adult neural stem cells. After that I analysed the expression of CD133/ Lewis-X /CD24 and neurosphere forming capacity of SEZ cells expressing these antigens in the hGFAPeGFP transgenic line where eGFP corresponds mainly to astrocytes. In our experiments a sub-population of both CD133/Lewis-X coexpressed eGFP showing astrocytic identity. CD24, which is known to be an ependymal marker, never colocalised with eGFP. CD133/GFP-double positive cells collected by FACS constituted 4%–6% of the total SEZ population. When these CD133/GFP-double positive cells were seeded singly in Terasaki wells 72% of the purified CD133/GFP-double positive FACS sorted population made multipotent and self-renewing neurospheres. Conversely, CD133 only, GFP only, CD24 only, Lewis-X/GFP, Lewis-X only sorted cells sometimes formed few neurospheres but these spheres could not be passaged, i.e, contained non self renewing cells. Our data using CD133/GFP selection allowed for the isolation of all neurosphere-forming cells. This isolation enriches adult NSCs up to 72% in comparison to other studies done so far. For instance, Capella et al., (2002) used Lewis-X antigen for NSC enrichment. Their experiments showed that the neurosphere forming efficiency of Lex/ssea-1-positive cells was only 22.1%, whereas that for Lex/ssea-1-negative cells was

less than 1% in the adult SEZ. Interestingly other gorups like Kawaguchi et al. (2001) obtained much lower enrichment of SEZ neurosphere forming cells (0.31%) using eGFP expression under the control of the nestin enhancer. The group of Rietze et al., in 2001 used sequential steps based on cell size binding of the lectin PNA, and mCD24^{low} to separate a highly enriched (80%) population of SEZ stem cells. However, only 63% of the total stem cells were selected, leaving behind a significant subpopulation with a different phenotype. In our study, the CD133/GFP-positive selection encompasses all neurosphere forming stem cells allowing a more complete isolation of the stem cell population. Moreover, selecting for CD133/GFP-positive population is a simpler, one-step enrichment method that reduces manipulation of the stem cells.

4.2 Identity of adult neural stem cells – astrocytes or ependymal cells?

We could demonstrate that the cells expressing the stem cell marker CD133 belong to a subpopulation of astrocytes *in vivo*. Our data do not argue against the observation (Coskun et al., 2008) that CD133 marks a subset of ependymal cells as well. However, with respect to the neural stem cell criteria in the neurosphere assay only the CD133/GFP-positive (GFP expressed by astrocytes in the SEZ hGFAPeGFP trangenic line; GLAST::CRE-ERT2) pool of sorted cells gave rise to multipotent self-renewing neurospheres *in vitro*. Our data is in line with a recent study (Mirzadeh et al. 2008) where 30% of the adult SEZ astrocytes were shown to express CD133. In contrast, the Coskun et al. study proposed CD133+ve ependymal cells to be the neural stem cells apart from GFAP and/or LeX expressing cells in the SEZ both *in vitro* as neuropheres and *in vivo* by injecting/ electroporating a plasmid (mP2 plasmid) having the CD133 P2 promoter region that contained a GFP-Cre fusion protein expression cassette into the ventricles of ROSA26-LacZ mice. The transfected CD133-expresing cells generated β-galactosidase and PSA-NCAM positive neurons in the olfactory bulb. However they could not exclude the fact that at least some of the SEZ astrocytes could also express the CD133 antigen. In our study we show that the astroglia lining the ventricle and expressing CD133 are the cells that contribute to neurosphere formation. Additionally, when we seeded cells from the SEZ that only expressed CD133; we did not observe any neurosphere formation. Thus, our observations open up the possibility that the neural stem cells identified by Mirzadeh et al. and Coskun et al. might represent the same stem cell population.

4.2.1 Genes enriched in stem cells ($CD133^{+VE}/GFP^{+VE}$)

We used Affymetrix GeneChips to identify gene sets associated *in vivo* with our CD133/GFP-positive neurogenic astrocytes compared to other non-neurogenic astrocytes in the same niche area and outside the area. This gave us a database to explore the basic unique transcriptional characteristics of neural stem cells. Our transcriptome analysis of CD133/GFP-positive cells hinted towards the possibility that neural stem cells also have some ependymal features apart from having astrocytic charecteristics. First, when we compared our microarray data with Cahoy et al's astrocyte transcriptome we could confirm that the CD133/GFP pool which formed most of the neurospheres *in vitro* had astrocytic characteristics. Among 250 top astrocytic genes from Cahoy et al., CD133/GFP-positive sorted cells also expressed 219 of these genes. Also, the transcriptome analysis of the $CD133^{+VE}/GFP^{+VE}$ stem cells and their comparison to our diencephalic astrocytes revealed they have both astrocytic and cilia features. At the gene expression level we could observe genes like GLAST, GLT and connexins being present in stem cell ($CD133^{+VE}/GFP^{+VE}$) pool that are known to be expressed by astrocytes. Also, genes related to astrocytic functions were expressed in stem cell sorted pool. Astrocytes are known to show essential role in brain metabolism (Hertz et al., 2007). Among the metabolic roles of astrocytes, the preferential storage of glycogen and glycoylsis in astrocytes has long been known (Hertz, 2004). Astrocytes are thought to play a key role in degrading glutamate to glutamine and in coupling synaptic activity and glucose utilization (Magistretti, 2006). Genes related to synthesis of glycogen granules were expressed by our stem cell pool ($CD133^{+VE}/GFP^{+VE}$) of sorted cells. Additionally, astroglia also provide an array of supportive, nutritional, developmental, and homeostatic functions to surrounding neurons and can also modulate synaptic transmission (Allen and Barres, 2005; Fitzgerald, 2005; Araque, 2006; Haydon and Carmignoto, 2006). A key and specific process of astroglial function is the glutamate–glutamine **shuttle**, which includes the production of glutamate and the uptake of the excess extrasynaptic glutamate (via astroglial enzyme GS). The shuttle also allows for the release of glutamine from astroglia, which is then taken up by neuronal elements to replenish the supply of glutamate (Zwingmann and Leibfritz, 2003; Hertz and Zielke, 2004; Fonseca et al., 2005), which plays a major role in central sensitization (Dubner and Basbaum, 1994; Sessle, 2000; Woolf and Salter, 2000). The stem cell transcriptome also revealed genes related to glutamate–glutamine **shuttle** pointing to the fact indeed these cells have astrocytic

features (Figure 4.1A-C). However, the CD133/GFP-positive stem cell pool of sorted cells apart from having astrocytic features had a unique molecular signature. For example, many mRNAs of cilia genes were enriched in the CD133/GFP-positive stem cell population particularly all the IFTs (intraflagellar transport machinary, important for ciliary function).

Cilia are membrane-bounded, centriole-derived, microtubule-containing projections from the cell surface. The microtubule cytoskeleton of the cilium, the ciliary axoneme, grows from and continues the ninefold symmetry of the centriole, which is nearly identical to, and often becomes, a ciliary basal body. Mammalian ciliary axonemes are formed with two major patterns: 9+2, in which nine doublet microtubules surround a central pair of singlet microtubules, and 9+0, in which the central pair is missing (Satir P et al., 2005). Usually, 9+0 cilia are also missing the molecular motors, axonemal dyneins, which are responsible for ciliary movement. Such cilia are therefore non-motile and function primarily as chemo- or mechanosensors. In contrast, 9+2 cilia are motile. Cells residing in the SEZ have both motile (e.g., on ependymal cells in the brain that generate the force necessary for cerebrospinal fluid (CSF) flow) and non-motile primary/sensory cilia). Both cilia use specialized intraflagellar transport machinary (IFT) for intracellular trafficking between the cell body and tip of the cilia. The genes related to IFTs (IFT88, IFT172, IFT157, and IFT52) were all upregulated in the transcriptome of CD133/GFP-positive cells. This pointed to the fact that the cells, which we think are the stem cells, have cilia. Whether these cilia are motile or primary could not be addressed. Interestingly, the transcription factor FoxJ1, which is known to be exclusively in multi-ciliated ependymal cells, was highly enriched in our CD133/GFP-positive stem cell pool leading to the fact that this stem cell pool also has ependymal features at least at the transcriptome level. Also, we could observe (collaboration with W.Huttners lab) at the electron microscopic level the GFP-positive cell from hGFAPeGFP transgenic line showing ultrastructural charecteristics of astrocytes, with multiple processes that intercalate extensively between other cells. Morever, this GFP-positive astrocyte like cells had cilia with a 9+2 axoneme, in which the nine doublet microtubules surround a central pair of singlet microtubules (Figure 4.2). Cilia having 9+2 pattern are usually motile and they usually function in transducing messengers such as cAMP and Ca2+ into increased beat frequency or changes in beat form for efficient mucociliary transport. Taken together, a comparison of the transcriptome of the CD133/GFP-positive stem cell

pool to Cohoy et al's astrocytic genes and our astrocytes from SEZ GFP only /Diencephalon GFP only pools, suggests that stem cells in the SEZ are specialized cells having both astrocytic and ependymal characteristics.

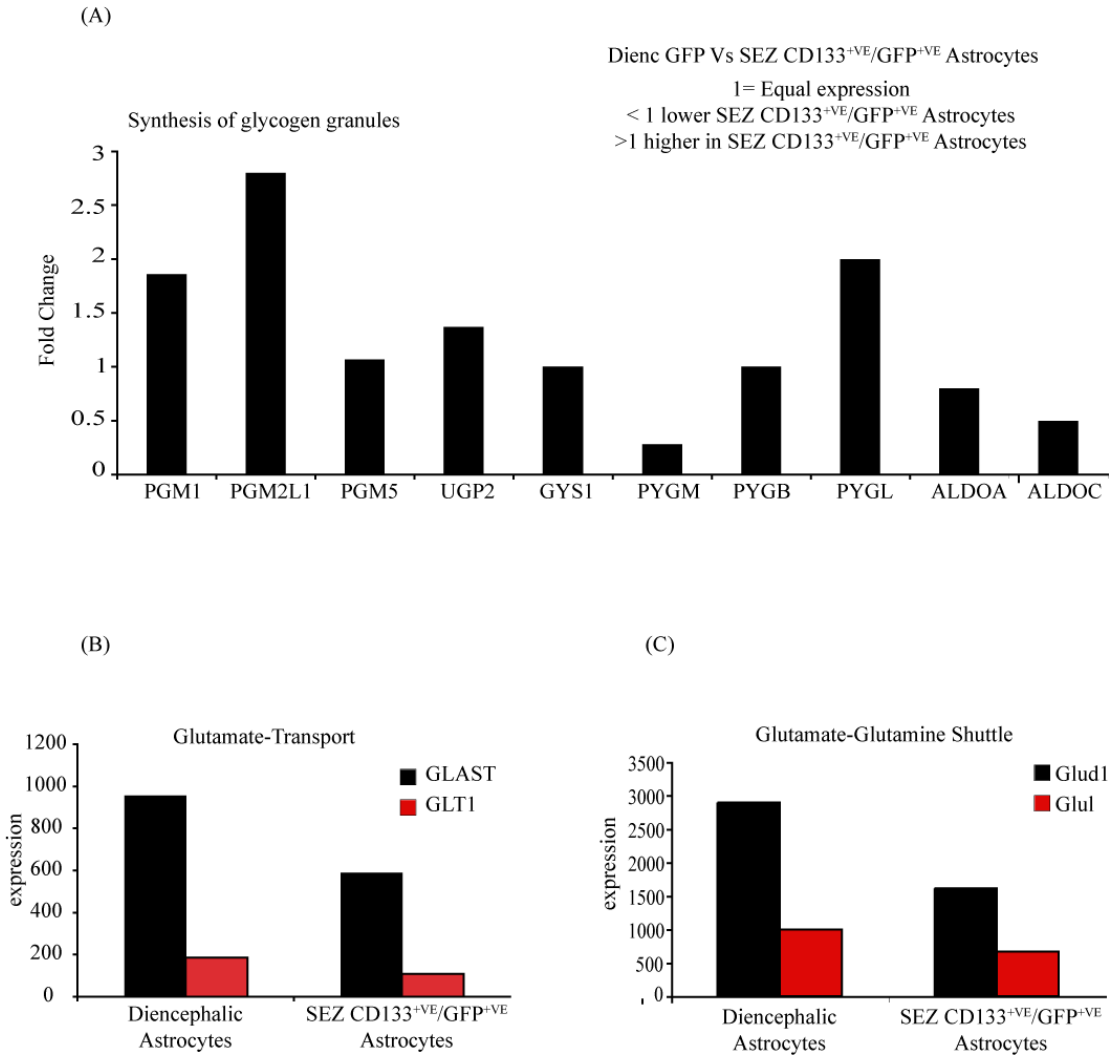


Figure 4.1 Genes related to astrocytic functions were expressed in stem cell pool. Astrocytes are known to be play role in metabolic functions and energy homeostatsis of brain, most of the genes related to glycogen synthesis (A), Glutamate-Transport (B) and Glutamate-Gutamine shuttle (C) were expressed by stem cells, giving astrocytic identity to stem cell pool at transcriptome level.

Apart from cilia genes, many calcium-related genes were also expressed at high levels in our stem cell (CD133/GFP) transcriptome. Ciliogenesis and ciliary movement have been suggested to be calcium-dependent (Ueno H et al., 2003; Praetorius & Spring, 2001), and it is noteworthy that another B9/C2 calcium/lipid-binding domain-containing protein, MKS1, has recently been localized to the ciliary basal body (Dawe HR et al., 2007).

Enriching calcium signalling could be very important for proper ciliary movement in receiving and coordinating extracellular signals for stem cell maintenance.

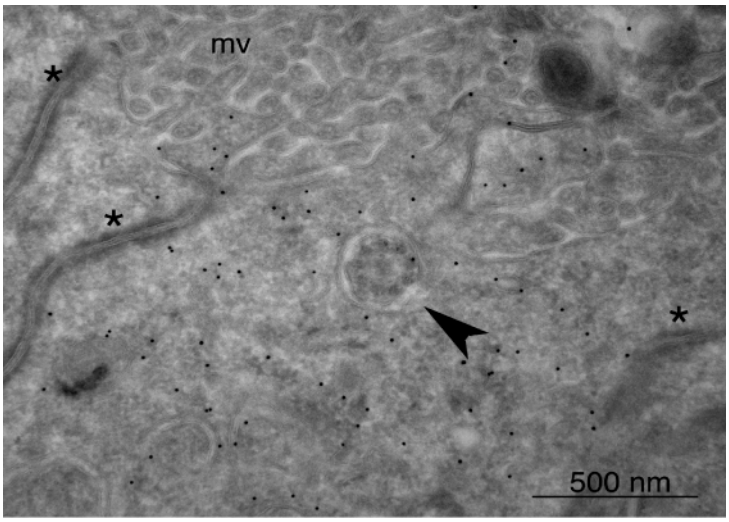


Figure 4.2 Stem cells in SEZ also have ependymal featutes. Electron micrograph shows GFP-positive cells (dot like precipitate is a staining for GFP) having 9+2 pattern of cilia.

Also, some studies have shown recently the role of Ca^{2+} signaling in stem cell development. In the case of haematopoeitic stem cells, extracellular Ca^{2+} present in the endosteal surface of the bone marrow are sensed by the seven transmembrane- spanning Ca^{2+}-sensing receptor (CaR) which has been shown to be important in the migration and homing of mammalian hematopoietic stem cells (HSCs; Adams et al., 2006). In this case, however, Ca^{2+} signaling does not appear to influence the ability of HSCs to proliferate or differentiate. As stem cells corresponding to other cell types are studied, it will be interesting to determine the involvement of Ca^{2+} signaling in stem cell specification and maintenance.

4.2.2 Pathways upregulated in stem cells (CD133/GFP)

The CD133/GFP-positive stem cell transcriptome that we have identified also provides new insights into pathways responsible for stem cell function and maintenance in this niche. For instance, genes related to Shh, TGFβ , and Wnt – signalling pathways were expressed at higher levels in the stem cell population (SEZ CD133/GFP-positive) compared with GFP only astrocytes from the SEZ and diencephalon. It is not surprising

that these signaling pathways were upregulated in SEZ CD133/GFP-positive astrocytic stem cell pool. It is already known that members of the Wnt family play critical roles in various physiological processes in the adult. Wnt signalling regulates stem cell self-renewal in several stem cell niches (reviewed in Kleber & Sommer 2004). In adult neurogenic niches, Wnt signalling has thus far been shown to regulate neurogenesis in the SGZ (Lie et al. 2005). Increasing or decreasing Wnt activity *in vivo* leads to an increase or decrease of SGZ neurogenesis, respectively. Another group of early neural morphogens, the bone morphogenic proteins (BMPs), belongs to the TGF-β superfamily, which includes TGF-β, activins, and the relatives named growth / differentiating factors. These glycoproteins play a crucial role in bone remodeling and in the regulation of dorso-ventral patterning of the neural tube and cell fate during embryonic development. It has been shown recently that BMP signaling is active in neural stem cells and is required for neurogenesis in the adult SEZ but not in the SGZ (Colak et al., 2008). Sonic hedgehog (Shh) is another important morphogen in development, it is a signalling glycoprotein that acts through the Patched 1-Smoothened (Ptc1- Smo) receptor complex to trigger various events during CNS development, including determination of ventral neural phenotypes, induction of oligodendrocyte precursors, proliferation of specific neuron progenitor populations, and modulation of growth cone movements (Marti E and Bovolenta P., 2002). In the adult SEZ, Shh regulates the proliferation of SEZ astrocytes (type B cells) and transit-amplifying type C cells (Machold et al., 2003; Ahn & Joyner 2005; Palma et al., 2005). Shh also affects proliferation in the SGZ (Lai et al., 2003; Machold et al., 2003).

The Shh and the canonical Wnt signaling pathways have a number of striking similarities (Nusse, 2003; Lum and Beachy, 2004). Both rely on serpentine receptors for pathway activation (Smo; Frizzleds). Both depend on regulation of proteosome-dependent events (processing of Gli3; regulated degradation of β-catenin). There is now a lot of evidence supporting the notion that both Shh and Wnt signaling pathways require cilia for their function. In the case of Shh, it has recently been demonstrated that key proteins in the Sonic hedgehog (Shh) signal transduction pathway are localized to primary cilia, including the transmembrane receptor Smoothened and Su (fu), a suppressor of Shh signaling (Scholey and Anderson, 2006). Cilia growth and the transport of protein cargoes occur through intraflagellar transport (IFT), using the same retro- and anterograde motors found in axonal transport (Rosenbaum and Witman, 2002).

Functional IFT machinery has been shown to be critical not only for phenotypically normal Shh signaling (Huangfu et al., 2003) but also for proteolytic processing of the transcription factor Gli3 (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Tran et al., 2008), in which the full-length activator form of Gli3 is cleaved to form a transcriptional repressor (Wang et al., 2000). Taken together this molecular signature provides an attempt to understand adult neurogenesis at the molecular level. Our data hint to the group of genes or pathways enriched in stem cells (CD133/GFP-positive) and share both ependymal and astrocytic features. For instance, as discussed above, for proper stem cell maintenance many signalling molecules like Shh/BMP might be required and in order to access these molecules probably from the CSF, the SEZ stem cells have cilia.

We propose that adult neural stem cells are a unique entity enwrapping both astrocytic and ependymal characteristics. Also examination of the transcriptome revealed for the first time the *in vivo* molecular description of SEZ neurogenic astrocytes and thus is a launching point for future studies into the regulation of adult neural stem cells.

4.3 De-differentiation of astrocytes after injury

In the adult cerebral cortex mature astrocytes lack expression of GFAP, nestin, vimentin and Tenascin-C and do not proliferate compared to astrocytes behaving as stem cells in the SEZ. However these proteins begin to be expressed in some reactive astroglial cells upon any kind CNS insult and some of these astroglia also start to proliferate (Buffo A et al., 2008). During development immature glia such as radial glia and postnatal glial progenitors proliferate *in vivo* and give rise to multilineage precursors or neural stem cells (Ganat YM et al., 2006; Pinto L et al., 2007; Götz M et al., 1997). We observed proliferation upon cortical stab wound injury in the hGFAPeGFP (labels mostly astrocytes) transgenic line in some of the GFP-GFAP cells. However, as this was a transgenic line where eGFP starts expressing in astrocytes during development (Nolte et al. 2001), mediated by human GFAP promoter elements, the cellular origin of these proliferative astrocytes in adult mice could not be addressed using this mouse line. It has been previously suggested that these proliferating astrogial cells might arise from endogenous progenitors present in the adult brain (Alonso G et al., 2005; Magnus T et al., 2007; Buffo A et al., 2005; Chen ZJ et al., 2002). If the dividing GFAP+ cells in reactive

gliosis originate from previously proliferating cells, such as the widespread NG2+ progenitors (Alonso G et al., 2005; Magnus T et al., 2007; Chen ZJ et al., 2002), it implies that mature astrocytes like other permanently postmitotic cells, such as oligodendrocytes and neurons do not resume proliferation. Conversely, if reactive proliferating GFAP+ cells originate from astroglial cells that resume proliferation after brain injury, it implies that astrocytes can undergo a certain degree of dedifferentiation towards an immature state. Indeed, proliferating astroglial cells in specific brain regions act as adult neural stem cells (Doetsch F et al., 1999; Seri B et al., 2001) and a dedifferentiation of astrocytes towards earlier developmental stages, such as in the postnatal (Ganat YM et al., 2006) or embryonic brain (Gotz M et al., 2005) may have obvious implications for attempts to reconstitute neurons after brain injury. Using the GLAST::CRE-ERT2 inducible (Mori T et al ., 2006) line we determined the origin of proliferating astroglia upon stab wound injury. Following induction with tamoxifen at adulthood, astrocytes were mostly labelled in the brains of GLAST::CreERT2 mice. Our fate mapping analysis revealed that reactive astroglia are derived from mature astrocytes, suggesting the fact that astrocytes exposed to injury may indeed resume properties of glia present at earlier developmental stages (Buffo A et al., 2008). Thus, in contrast to mature oligodendrocytes and neurons, mature astrocytes are not permanently postmitotic but rather retain the capacity to resume proliferation and up-regulate developmental features, a process that we refer to as 'dedifferentiation'.

Even more importantly, we could demonstrate that these changes occurring in astrocytes are accompanied by the acquisition of stem cell properties, as some of the astrocytes labeled prior to injury acquire the capacity to form multipotent and self-renewing neurospheres. Notably, astrocytes lose the potential to form neurospheres after the second postnatal week (Laywell ED et al., 2000), further supporting the concept that reactive astrocytes activate properties of earlier developmental stages. Indeed, we could also show that neither the astrocytes proliferating after injury nor the neurosphere-forming astrocytes are derived from the neurogenic SEZ by viraly labeling the stem cells residing in SEZ. We injected GFP-containing VSVG-pseudotyped lentiviral vector into the SEZ ipsilateral to the site of stab wound injury (Hack MA *et al., 2005*). Three days later, most GFP+ cells were detected within the SEZ and the rostral migratory stream (RMS) with few cells migrating into the white matter but none had entered the cortical GM surrounding the site of injury. To ensure that smaller numbers of GFP+ cells did not

escape detection, we even used FACS analysis and found that 2.6% of SEZ cells were GFP+, while no GFP+ cells were detectable in the tissue isolated from the injury site. This analysis therefore clarifies that the neurosphere-forming cells that occur with a frequency higher than 5% are not derived from stem cells in the SEZ.

These data therefore demonstrate, for the first time, a novel source of multipotent cells in the adult cerebral cortex after brain injury. However, the efficiency with which the cortical astrocytes made neurospheres was much lower (1 in 376 cells) than the SEZ $CD133^{+VE}$ /GFP^{+VE} astrocytes (1 in 1.4 cells; 72%), suggesting that only some mature astrocytes revert to a neural stem cell fate. Strikingly, this is not a general feature of other glial cells reacting to injury. Glial progenitors that are known to be actively proliferating in the adult cortex even prior to injury, such as NG2+ or PDGFRa+ cells (Alonso G et al., 2005; Magnus T et al., 2007; Buffo A et al., 2005; Horner PJ et al., 2000), were not capable of forming multipotent neurospheres *in vitro*. But this property was completely different from the potential of these progenitor cells at postnatal stages (Belachew S et al et al., 2003) and in adult white matter tracts (Nunes MC et al., 2003) where NG2 cells were multipotent and could even generate functional neurons *in vitro*. Thus, astrocytes showed to be the only cells that react to brain injury by partial dedifferentiation and even acquire multipotency.

Our fate mapping analysis further (Buffo A et al., 2008) demonstrates that these reactive astrocytes – despite being multipotent when isolated *in vitro* – cannot pursue their full potency *in vivo* and rather remain within their astroglial lineage. This is despite the activation of the same growth factor pathways (FGF2 and EGF) that are used to expand neurosphere cells after injury *in vivo* (Clarke WE et al., 2001; Smith GM et al., 2005). Although these signals can trigger neuronal repair at specific sites *in vivo* (Nakatomi H et al., 2002), in most other CNS regions the infusion of EGF and FGF2 are not sufficient to elicit a considerable degree of neurogenesis locally (Ohori Y et al., 2006). This situation can be improved after transduction with potent neurogenic transcription factors (Buffo A et al., 2005; Ohori Y et al., 2006), but the response is still rather limited. Obviously the anti-neurogenic environment present in the adult brain parenchyma (Seidenfaden R et al., 2006) contributes to the predominant glial fate *in vivo* with Notch (Yamamoto S-i et al., 2001) and BMP-signaling (Hampton DW et al., 2007) acting as potential candidates for this fate restriction. In addition, our results now suggest that the limited neurogenic

response even after overexpression of neurogenic factors may also be due to targeting glial cells with less plasticity than the multipotent subset of reactive astrocytes. Indeed, the viral vectors used in previous studies target predominantly NG2+ progenitors (Buffo A et al., 2005; Ohori Y et al., 2006). Our data now propose reactive astrocytes as a promising source of multipotent cells within the injury site that may be particularly suited to elicit neuronal repair in brain regions far away from zones of adult neurogenesis.

4.4 Endogenous neurogenic signals responsible for de-differentiation of cells in the adult cerebral cortex

As discussed earlier we could observe that upon injury astroglia resume proliferation *in vivo* and acquire multipotency *in vitro*. We could demonstrate that there are some factors released by the cells after injury that have neurogenic potential in terms of neurosphere formation. When we seeded un- injured cortical cells with conditioned media obtained from injury we could observe multipotent and self-renewing neurospheres *in vitro*. Interestingly, the efficiency with which the neurospheres were generated using LCM (2.1 in 1000 cells; lesion conditioned media) was double than the spheres obtained from injured cortical grey matter (0.9 in 1000 cells). This could be explained, by the enrichment of factors released upon injury being more concentrated in LCM compared to the injured tissue.

These results propose the new concept that when the lesioned tissue is cultured for 48hrs multipotent progenitors could also be instructed upon by factors released by injury in adult non-neurogenic CNS regions like cortex rather than being restricted to neurogenic regions. Moreover, different signaling molecules have been shown to play crucial roles in maintaining the neurogenic potential of proginitors residing in neurogenic areas compared to non-neurogenic areas. This data further suggests that the critical differences between neurogenic and non-neurogenic regions in the adult brain might not reflect the differences in the intrinsic properties of the progenitor cells but rather differences in their microenvironment. It has been known for more than a decade that in the adult central nervous system (CNS), constitutive neurogenesis is retained in only two regions—the subgranular zone (SGZ) and subependymal zone (SEZ), which give rise to neurons of the hippocampal dentate gyrus and olfactory bulb (Alvarez-Buylla A et al., 2002; Lie DC et al., 2004; Kornack DR et al., 2001). In contrast, most adult CNS areas outside the SGZ

and SEZ do not exhibit constitutive neurogenesis *in vitro* or *in vivo* under normal conditions. Exceptions to this are the optic nerve, striatum, hypothalamus, and subcortical white matter (Kokoeva MV et al., 2005; Lie DC et al., 2002; Nunes MC et al., 2003; Markakis EA et al., 2004) where some glial progenitors with neurogenic potential *in vitro* have been found. In fact limited neurogenesis observed by injury-induced signals (Magavi SS et al., 2000) or by specific neurogenic cues (Kokoeva MV et al., 2005; Markakis EA et al., 2004; Pencea V et al., 2001; Palmer TD et al., 1999) in non-neurogenic areas of brain *in vivo* suggested the fact that local environmental cues might be very important in allowing neurogenesis in the certain adult CNS regions. Compelling evidence points to the fact that the neurogenic behavior of SEZ and SGZ progenitors are determined by signals restricted to their niches. SEZ cells when transplanted to another SEZ (neurogenic area) generated large numbers of neurons that integrated into the recipient olfactory bulb, but when transplanted to non-neurogenic brain regions their neurogenic potential was severely reduced (Gage et al., 2000; Lim et al., 2000). However, the molecular signals in the neurogenic niches that regulate the ability of neural stem cells to self-renew and differentiate are largely unknown. In our microarray analysis some of the signaling pathways like Shh, Tgfb and Wnts were upregulated in the stem cell like astrocytes (CD133/GFP) from SEZ. Both Shh and BMP have been shown to be important regulators in adult neurogenesis (Ahn S et al., 2005; Colak D et al., 2008). When we looked at the Shh expression upon injury we could observe the upregulation of Shh at the injury site of the cortex compared to non-injured site. However, there was no differential regulation of BMP signaling observed at the injury site of the cortex (Colak et al., 2008), which goes in line with a recent study showing upregulation of Noggin after knife cut injury (very similar to stab wound injury) and inhibition of BMP activity (Hampton et al., 2007) in the adult cortex. As BMP signaling favored adult neurogenesis in the SEZ (Colak et al., 2008) and was also upregulated in neurogenic astrocytes in our microarray, this suggests the fact that the lack of BMP signaling after cortical injury might be a cause of no neurogensis after injury. When we used Shh in our adult cortical cultures we could observe mutipotent self-renewing neurospheres. We could also block the neurosphere formation of adult cortical cells by adding the Shh antagonist cyclopamine. Our data indicates that astrocytes might be a source of Shh at the injury site as they are in the neurogenic area. And our data further elucidates a role for Shh as one of the key components dictating the neurogenic potential of cells both in neurogenic or non-neurogenic areas. These findings suggest that it may be possible to manipulate

endogenous progenitors in diverse CNS areas to resume multipotency.

4.5 Conclusion and Future Prospects

The microarray data obtained from the neurogenic SEZ astrocytes (CD133/GFP-positive) and non-neurogenic astrocytes from the SEZ and diencephalon (SEZ GFP only; DIENC GFP only) identify genes, signaling pathways and biological processes differentially regulated between neurogenic vs. non-neurogenic astrocytes. This differential transcriptome data provides unique molecular signatures for neurogenic astrocytes (CD133/GFP) that can be explored for future studies at functional level. This data set can also be utilized in understanding the contribution of individual genes in the context of neural stem cell biology. Additionally, many unknown genes expressed exclusively by neurogenic astrocytes can be explored. This would be of great help as until now neural stem cells have no known unique molecular identity.

Upon cortical stab wound injury we found reactive astrocytes to be the source of multipotent stem cells apart from SEZ and SGZ. Our data also suggested that factors (Shh) released by injured cells are sufficient to instruct cells for stem cell potential. These data indicate that reactive astrocytes are the potential source of producing new neurons in non-neurogenic CNS regions in the adult brain. Therefore, these cells and factors could be used as a future tool for manipulation in inducing neurogenesis in non-neurogenic areas. By manipulating the reactive astrocytes following injury, novel therapeutic strategies may be employed.

The construct for GLAST-Venus/Tomato mice will be usefull to further examine the transcriptome of astrocytes in the regions of the brain lacking the activity of the human GFAP promoter. Therefore, these new knockins into GLAST locus provide a tool to examine gene expression differences between astrocytes of different brain regions. These mouse lines will also allow in dissecting the molecular pathways regulating the diverse functions of astrocytes *in vivo* as precursors, support cells, repair cells and cells involved in neuronal information processing.

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6 Appendix

6.1 Acknowledgements

This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that helped to get this done, but it will be tough to thank them enough.

In the first place I would like to express my gratitude to Prof. Magdalena Götz for her supervision, advice and guidance from the very early stage of my research career and sharing her extraordinary experiences with me through out the work. Above all and the most needed, she provided me unflinching encouragement and support in various ways. Her extraordinary scientific motivation, dedication, stimulating ideas and passion in science were a source of inspiration as a student and young researcher and scientist. I am indebted to her more than she knows.

I owe my special thanks to Prof. Benedikt Grothe for inviting me to begin my career as a scientist and introducing me to Magdalena.

My sincere thanks go out to Annalisa Buffo with whom I started my work and also collaborated during my PhD research. Her conceptual and technical insights into my thesis work have been invaluable.

I would like to thank in particular to Leanne and Ruth, for their ‘collective’ help in reading and correcting my thesis and giving useful criticism and advice.

Also, I would like to thank to all my present and former lab members and technicians. GSF has been a source of friendships as well as good advice and collaboration. I am especially grateful to Dilek, Silvia, Barbara and Costa for being so great friends in the lab. I want to give my special thanks to Andrea and Angelika for always being so supportive and caring.

I made some real special friends during my stay in Munich. Both Pankaj and Jyoti are part of my family now and have especial place in my heart. Pankaj (who is more than a friend and like an elder brother to me) and Jyoti were always supportive and caring with

my work and health during my hard time.

Last but not least I would like to thank Partho (better half of my life) and both our parents and my star brother shashank for long lasting source of energy during this exhaustive research. Really my special thanks go to Partho who always made me feel special. He helped me to concentrate on completing this dissertation and supported mentally during the course of this work. Without his help and encouragement, this study would not have been completed. Partho, papa & mummy and my sweet little brother always supported me, taught me, and loved me. To them I dedicate this thesis.

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