

**Conditional activation of Bmi1 in embryonic and postnatal
neural stem/progenitor cells in mouse models: Analysis of
its effect on their biological properties and tumourigenesis**

by

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Abstract

The Polycomb group protein Bmi1 is a key transcriptional regulator of self renewal of embryonic and adult neural stem and progenitor cells (NSPC) and its over-expression has been shown to occur in several types of brain tumour. In a Cre/LoxP-based conditional transgenic mouse model we show that fine-tuning of Bmi1 expression in embryonic NSPC is sufficient to increase their proliferation and self renewal potential *in vitro*. This is linked to downregulation of both the ink4a/ARF and the p21/Foxg1 axes. However, sustained over-expression of Bmi1 in differentiating progenitor cells leads to increased apoptosis of neuronal progenitor cells. Moreover, neurospheres derived from Bmi1 over-expressing NSPC contain more cells positive for glial markers such as NG2 and A2B5. Postnatally, however, increased self renewal capacity of NSPC is independent of Foxg1 and resistance to apoptosis is observed in neural progenitors derived from NSPC over-expressing Bmi1. No neoplastic transformation is seen in mice over-expressing Bmi1 in the SVZ stem cell compartment of transgenic mice (NestinCre;STOPFloxBmi1) aged up to 20 months. These studies provide strong evidence that fine tuning of Bmi1 expression is a viable tool to increase self renewal capacity of neural stem cells *in vitro* and that it does not elicit neoplastic transformation of these cells. However, activation of Bmi1 over-expression in the postnatal SVZ concomitantly with inactivation of p53, a common predisposing mutation to glial tumour development, leads to formation of low grade glioma in these mice. These data raise the possibility that genetic collaboration between Bmi1 and p53 may be a key event in the pathogenesis of low grade glial tumours.

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List of Abbreviations

AML	Myeloid leukaemia
APC	Adenomatous Polyposis Coli
Ara-C	Cytosine Arabinoside
Bcl-2	B cell lymphoma leukaemia 2 protein
bFGF	Basic Fibroblast growth factor
BLBP	Brain lipid-binding protein
Bmi1	B-cell-specific Moloney murine leukemia virus insertion site 1
BMPs	Bone morphogenic proteins
BTSC	Brain tumour stem cells
CMV	cytomegalovirus
CP	Cortical plate
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix molecules
EDTA	Ethylene disodium tetra acetate
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
ES	Embryonic stem
esc	Extra sex chromosome
EtBr	Ethidium bromide
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glumataate Aspartate Transporter
GM	Genetically modified
HBSS	Hanks balanced salt solution
IGL	Internal granular layer
IPCs	Intermediate progenitor cells

IRES	Internal ribosome entry site
kDa	kilodaltons
Kg	kilogram
LRP	Lipoprotein receptor-related protein
MA	Mantle
MAPK	Mitogen activated protein kinase
MEKK	Erk kinase kinase
MKK	MAP kinase kinase
ml	millilitre
NCFCA	Neural Cloney Forming Cell Assay
ng	nanogram
NG2	Type-1 transmembrane proteoglycan protein
NOD-SCID	Non-obese diabetic severe combined immunodeficiency
NS	Neurosphere(s)
NSC	Neural stem cell(s)
NSPC	Neural stem/progenitor cell(s)
O4	Oligodendrocyte marker (Myelin basic protein antibody)
OB	Olfactory Bulb
oIPC	Oligodendrocytic intermediate progenitor cell
Olig2	Oligodendrocyte lineage transcription factor 2
PBS	Phosphate buffered saline
PcG	Polycomb Group
PDGF	Platelet driven growth factor
PI3-K	Phosphatidylinositol-3 kinase
PIK	Phosphoinositide kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PRC1	Polycomb repressive complex 1

PRC2	Polycomb repressive complex 2
PSA-NCAM	Poly-sialated neural cell adhesion molecule
Ptch	Patched
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
R	Ratio
RACS	Replication competent avian leukosis
RG	Radial glia
RMS	Rostral Migratory Stream
RT	Room temperature
SCZ	Subcallosal zone
SGZ	Subgranular zone
Shh	Sonic hedgehog
shRNA	Short hairpin ribosomal nucleic acid
Smo	Smoothened
SVZ	Subventricular zone
TAE	Tis-Acetate-EDTA
TGF	Tumour Growth Factor
TrxG	Trithorax Group
UV	Ultraviolet
VZ	Ventricular zone
WHO	World Health Organisation

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List of publications

Yadirgi G, Leinster V., Acquati S., Bhagat H., Shakhova O. and Marino S.

Conditional activation of Bmi1 expression controls self renewal, migration and differentiation of neural stem/progenitor cells *in vitro* and *in vivo*.

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Robson LG, Zhang X, Radunovic A, **Yadirgi G, Bird K, Shakhova O and Marino S.**

The PcG gene Bmi1 modulates specification and maintenance of adult myogenic satellite cells.

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Yadirgi G, Marino S.

Adult neural stem cells and their role in brain pathology.

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Neural stem cells, tumour stem cells and brain tumours: dangerous relationships?

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Cole AR, Causeret F, **Yadirgi G, Hastie CJ, McLauchlan H, McManus EJ, Hernández F, Eickholt BJ, Nikolic M, Sutherland C.**

Distinct priming kinases contribute to differential regulation of collapsin response mediator proteins by glycogen synthase kinase-3 *in vivo*.

J Biol Chem. 2006 Jun 16;281(24):16591-8

Chapter 1— Introduction

1.1 Neural stem cells in the developing and postnatal mammalian brain

The peripheral and central nervous system (PNS and CNS respectively) arises from the neural plate, a sheet of cells, which originates from the ectoderm: one of the three structural primitive layers forming at gastrulation. Folding of the neural plate, through neurulation, transforms it into a tubular structure called the neural tube that has neural epithelial lining. The rostral part of neural epithelium forms the three brain vesicles (the forebrain (prosencephalon), midbrain and hindbrain). The forebrain vesicle gives rise to telencephalon and is related to the lateral ventricles; both regions are of interest to this study as the lining of the latter hold a particular subset of neuroepithelial cells, which act neural stem cells (NSC) during the embryonic development of the brain and will give rise cells with similar properties in the postnatal/adult brain (Merkle and Alvarez-Buylla, 2006, Corbin *et al.*, 2008). During embryonic cortical development, a primary proliferative zone called the ventricular zone (VZ) forms from the neuroepithelium and it is composed of radial glia cells (RG- see discussion below). A second proliferative zone called the subventricular zone (SVZ), which contains prenatal and postnatal NSC (see discussion below), emerges from the VZ during late embryonic development (Levison and Goldman, 1997).

1.1.1 From neuroepithelium to radial glial cells with stem cell capacity

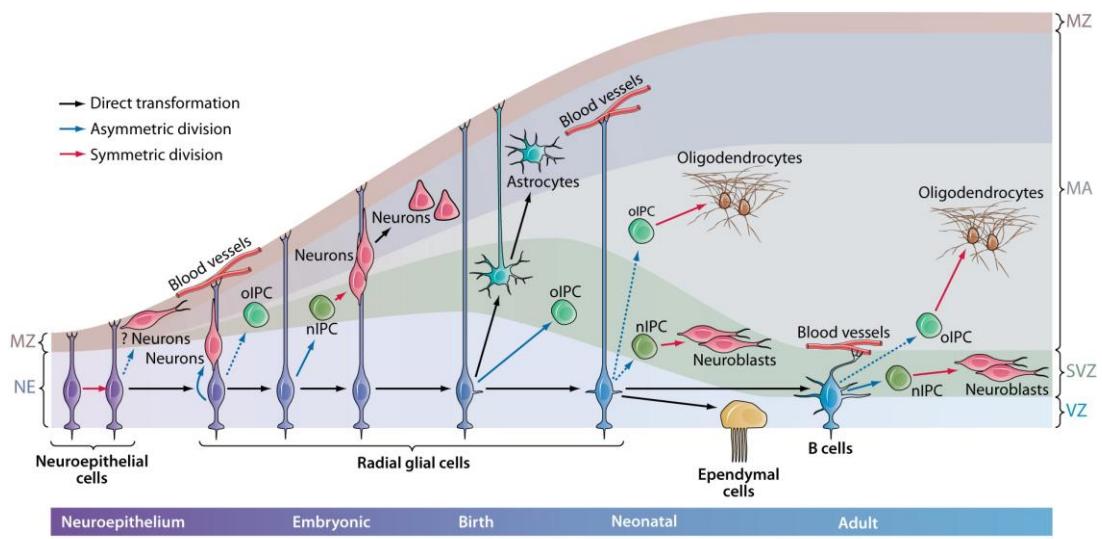
Development of mammalian brain entails generation of billions of neurons and many more of glial cells from just a few neuroepithelial cells, which form the neuroepithelium. Neuroepithelium, a single layer of cells, appears pseudostratified as

nuclei of neuroepithelial cells migrate up and down the basal-apical axis during a special kind of cell cycle termed interkinetic nuclear migration (INM), a property shared by radial glial (RG) cells and to a limited extent by basal progenitors (Takahashi *et al.*, 1993, Gotz and Huttner, 2005). Neuroepithelial cells undergo symmetric cell divisions to expand progenitor pools before the onset of neurogenesis (Knoblich, 2008). They do this by INM, a process that spans the entire apical-basal axis in neuroepithelial cells: in G1 phase of the cell cycle, the nucleus migrates to the basal side and stays there during S phase; during G2 phase, however, nucleus migrates back to apical side where mitosis occurs (Takahashi *et al.*, 1993, Gotz and Huttner, 2005). At embryonic day (E) 9-10 in mice, the neuroepithelial cells divide symmetrically to generate more neuroepithelial cells with the same developmental properties but also asymmetrically to give rise to two cells with different developmental properties, one of them acts as a potential source of neurons at this stage, though this is not supported by experimental data (Figure 1.1) (Kriegstein and Alvarez-Buylla, 2009). However, neuroepithelial cell transition to asymmetric divisions is associated with changes in their cellular identity. Indeed, with the onset and during the period of neurogenesis (from E14.5 to E16.5 in mice) neuroepithelial cells begins to acquire morphological and gene expression features generally associated with glial cells such as those found in the adult brain that have specialized functions and progenitor capacity (Merkle and Alvarez-Buylla, 2006, Conti and Cattaneo, 2010). These changes are associated with their transformation into Radial Glial (RG) cells. Also RG cells undergo asymmetrical and symmetrical mitotic divisions. The former kind of mitotic divisions leads to generation of intermediate progenitors (neurogenic progenitor cells, nIPCs; or oligodendrocytic pro-

genitor cells, oIPCs) that later terminally differentiate into neurons or oligodendrocytes (Figure1.1). On the other hand, symmetrical divisions increase the population of RG cells in the VZ (Figure1.1). Interestingly, INM is retained by RG cells though nuclear migration is confined to the portion of the cell in the VZ, where new neural progenitors and neurons are produced.

Moreover, the determinants of symmetric vs asymmetric divisions of RG cells have been implicated in regulation of their maintenance, cellular identity and products. For example, activation of Notch signalling promotes RG cell maintenance and identity, whereas its inactivation by mutations in Notch effector genes Hes1, Hes3 and Hes5 leads to premature neuronal differentiation and even depletion from most CNS regions (Hatakeyama *et al.*, 2004). Moreover, inactivation of Notch signalling through conditional disruption of Mindbomb1 (Mib1), a ubiquitin involved in endocytosis, leads to symmetric differentiation, whereby either two neurons or basal progenitors are produced and depletion of RG cells reached prematurely (Yoon *et al.*, 2008). Furthermore, RG cell spindle orientation has also been shown to be important for daughter cell fate determination: indeed, disruption of heterotrimeric G proteins or depletion of their binding partners/activators AGS-3, results in hyperdifferentiation of RG cells into neurons (Sanada and Tsai, 2005). In a recent study by Itaru Imayoshi *et al.*, (2010), inactivation of Notch signalling by conditional deletion of Rbpj, an intracellular signal mediator of all Notch receptors, results in complete loss of RG cells in telencephalon (Imayoshi *et al.*, 2010).

During these processes — neurogenesis and gliogenesis— that initiates a progressive thickening of the cortex, RG cell bodies remain in the VZ whereas their processes remain in contact with both pial and ventricular surfaces (Gadisseux and Evrard, 1985). These RG cell morphological changes are accompanied by acquisition of the expression of astroglial markers such as the glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST) and the brain lipid-binding protein (BLBP); and intermediate filament proteins such as nestin, vimentin, the RC1 and RC2 epitopes (Choi and Lapham, 1978, Choi, 1988, Campbell and Gotz, 2002, Mori *et al.*, 2005, Kriegstein and Alvarez-Buylla, 2009). In parallel, the tight junctional complexes between the neuroepithelial cells are replaced with adherence junctions (Aaku-Saraste *et al.*, 1997, Stoykova *et al.*, 1997). Moreover, the neuroepithelial cells start to make contact with endothelial cells of the developing vasculature, in a manner similar to those of astrocytes with progenitor capacity (Takahashi *et al.*, 1990, Mission *et al.*, 1991). These are the landmark changes that underline the transition of epithelial cells into RG cells, which are the precursors for both neuronal and glial cells, with specialized and progenitor capacity, of the CNS (Anthony *et al.*, 2004, Gotz and Huttner, 2005). Their capacity to generate all cell types of the brain was experimentally shown in a Cre/LoxP fate mapping experiment driven by promoter of BLBP, a protein expressed by RG cells throughout the developing brain (Hartfuss *et al.*, 2001), where neurons, interneurons and glial cells (with progenitor capacity) were labelled (Anthony *et al.*, 2004) in keeping with a RG origin.



AR Kriegstein A, Alvarez-Buylla A. 2009.
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Fig. 1.1 Transformation of neuroepithelial cells into embryonic and postnatal neural stem cells through generation and expansion of further layers such as VZ, SVZ and MA: Symmetrically dividing neuroepithelial cells generate more neuroepithelial cells and expand the epithelium. In parallel, the elongating neuroepithelial cells convert into radial glial (RG) cell, the neural stem cells of the embryonic stage of development. RG divide symmetrically and asymmetrically, give rise to neurons and oligodendrocytes through generation of intermediate progenitors (nIPC and oIPC). At the end of embryonic development some RG cells convert into astrocytes. Further, some RG cells convert into adult astrocytes that migrate into SVZ, where they reside in adult brain as adult neural stem cells (termed B cells in rodents), which continue to generate neurons and oligodendrocytes. This illustration was adapted from Kriegstein A., Alvarez-Buylla A. (2009) as it depicts well what is known about stem cells of the developing and adult brain, and puts in context and provides a framework to understand the developmental stages chosen to study compare and contrast neural stem cells in this study.

VZ, ventricular zone; SVZ, subventricular zone; MA, mantle; nIPC, neurogenic progenitor cell; oIPC, oligodendrocytic progenitor cell.

In vitro, however, a population of RG cells can easily generate all three major types of brain cells (neurons, oligodendrocytes and astrocytes), when allowed to differentiate, though only rarely can a single RG cell differentiate in all three cell types (Luskin *et al.*, 1988, Qian *et al.*, 2000). Moreover, the self-renewing RG cells of the VZ can give rise to IPCs that have no apical endfeet and ascending processes (Gotz and Huttner, 2005). Together these features; self-renewal capacity, generation of committed progenitors, and ability to differentiate into multiple cell types, suggest that RG cells act as the stem cells of the VZ.

1.1.2 Neural stem cells in the postnatal mammalian brain

In the last few years our view of the regenerative capacity of the adult mammalian brain has changed considerably. The traditional way of perceiving the adult brain as a static organ composed of postmitotic, fully differentiated cells has been challenged and several pivotal studies have shown how neurogenesis is retained during adult life, therefore conferring a certain degree of plasticity to the adult CNS.

Although, few reports in the 1960s (Messier *et al.*, 1958, Smart, 1961, Altman and Das, 1965) described mitotic activity and neurogenesis in the postnatal rodent brain, it has only recently been convincingly shown by seminal work of Luskin (Luskin, 1993) in rats and Lois and Alvarez-Buylla (Lois and Alvarez-Buylla, 1993) in mice, that neurogenesis occurs in the adult rodent SVZ.

Stem cells in several distinct anatomical brain regions persist throughout the life time of an organism. In the postnatal brain, multipotent stem cells are found not only in lateral walls of the lateral ventricles (SVZ) but also in the dentate gyrus of the hippocampus (subgranular zone, SGZ), between the hippocampus and the cor-

pus callosum (subcallosal zone, SCZ), and lastly at the boundary between internal granular layer (IGL) and white matter in the cerebellum (Doetsch *et al.*, 1999a, Seri *et al.*, 2001, Alvarez-Buylla *et al.*, 2002, Lee *et al.*, 2005, Seri *et al.*, 2006). Doetsch F (1999) and co-workers has shown that murine SVZ astrocytes act as NSC (they proliferate and re-populate olfactory bulb neurons *in vivo* and differentiate into glia and neurons *in vitro*) during normal cell turnover in the adult brain and in response to injury (Doetsch *et al.*, 1999a). Similar observations were noted for a subset of GFAP positive astrocytes located in the SGZ, where they proliferate and give rise to immature neurons (DCX+, PSA-, and NCAM+) *in vivo* (Seri *et al.*, 2001, Ming and Song, 2005). Moreover, a further subset of Sox2 positive SGZ cells, termed slowly proliferating NSC, has been shown to generate more rapidly dividing intermediate progenitor cells that generate migratory neuroblasts, which, in turn, differentiates into dentate gyrus granule neurons (Seri *et al.*, 2004, Johnston and Lim, 2010). However, some controversies still exist concerning the “stem cell” nature and the precise location of NSC of the SCZ and cerebellum. The self-renewal capacity of SCZ cells seems rather limited according to the experimental data available to date (Seri *et al.*, 2006): they form multipotent neurospheres *in vitro* and contribute to oligodendrocyte population in the corpus callosum *in vivo*. In the cerebellum, CD133+ cells from P7 mice have been shown to fullfill all criteria of NSC but only *in vitro* (Seri *et al.*, 2001, Lee *et al.*, 2005).

1.1.3 Neural stem cells in the SVZ

In the SVZ of the adult mammalian nervous system, NSC are termed B cells and they give rise to neurons, oligodendrocytes and astrocytes through generation of more committed transiently amplifying intermediate progenitors (termed C cells in the SVZ) (Figure 1.2; adapted from (Zhao *et al.*, 2008)), (Alvarez-Buylla and Garcia-Verdugo, 2002, Sutter *et al.*, 2007). During the process of neurogenesis in adult SVZ, type C cells give rise to neuroblasts (type A cells) that migrate tangentially through the rostral migratory stream to olfactory bulb, where they terminally differentiate into interneurons; granule and periglomerular neurons (Figure 1.2).

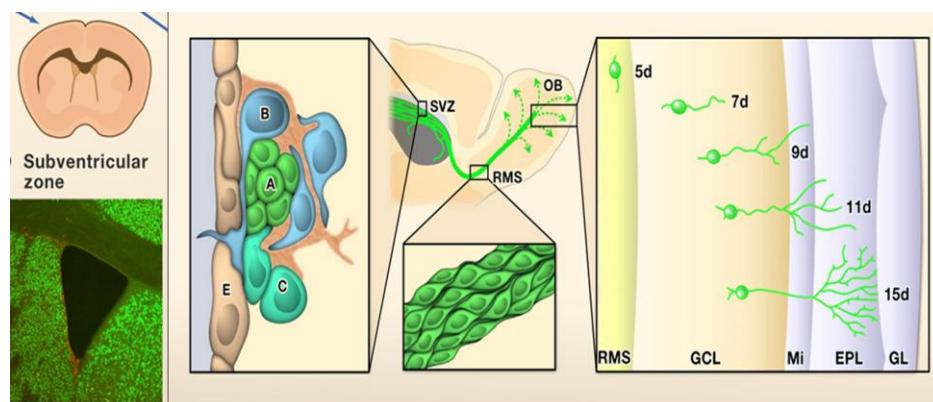


Fig. 1.2: Location, structure and function of adult subventricular zone in mice: The SVZ is separated from the lateral ventricle by the ependymal (E) cell lining. Mouse SVZ cells, located in the lateral wall of the lateral ventricles, are depicted as B (stem cells), C (intermediate progenitors) and A (migratory neuroblasts) cells. Neuroblasts form the rostral migratory stream (RMS) and migrate to the olfactory bulb (OB) where they terminally differentiate into interneurons. Adapted from Zhao *et al.*, 2008.

A landmark study by Doetsch *et al.*, 1997 has constructed a topological model for adult SVZ that enabled positive identification of not only type B cells but also C and

A cells (Doetsch *et al.*, 1997). F. Doetsch and co-workers have found that A cells (positive for PSA-NCAM, TuJ1 and nestin but negative for GFAP and vimentin) were ensheathed by B cells that are positive for GFAP and vimentin, whereas the type C cells (positive for nestin and Dlx2 (Doetsch *et al.*, 2002) but negative for other markers) formed focal clusters closely associated with A cells.

Several reports have conclusively shown that the postnatal SVZ NSC have an astrocytic nature and are neurogenic. This was shown by Doetsch *et al.*, 1999: mice were treated with cytosine- β -D-arabinofuranoside (Ara-C) for six days, a treatment that kills actively dividing type C and A cells but spares the quiescent type B cells. These cells were then activated and gave rise to C cells, which in turn differentiated into neuroblasts that repopulate the olfactory bulb interneuron compartment (Doetsch *et al.*, 1999a). The same authors have also shown that GFAP positive SVZ astrocytes can act as the stem cells under normal conditions. Here a transgenic mice (GFAP-tva), engineered to express the receptor (AP) for a replication-competent avian leukosis (RACS) retrovirus in SVZ astroglial cells (and their progeny) under the control of GFAP promoter (Holland and Varmus 1998), was used to label mitotically active GFAP-positive cells and follow their progeny (AP positive) in the brain. Indeed, the authors found that AP positive and GFAP expressing astrocytes give rise to AP positive type C cells and migrating neuroblasts in the RMS, and finally olfactory bulb interneurons (Doetsch *et al.*, 1999a). These findings suggest that the self-renewing and multipotent neural stem cells of the SVZ express GFAP and are part of a population of cells classically viewed as differentiated astrocytes.

A particularly crucial issue in stem cell research is the positive identification of stem cells *in vivo*. The most commonly used markers for identification and characterisation of type B NSC of the SVZ are GFAP, Musashi, Nestin, PDGFR- α and CD133 (Jackson and Alvarez-Buylla, 2008). These markers have contributed significantly to the characterisation of the anatomical location of these cells, however, they are by no means specific and there is no single marker available to identify adult SVZ stem cells.

1.1.4 Neural stem and progenitor cells *in vitro*

In culture, NSC can be maintained and expanded in non-adherent serum free culturing conditions supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF-2 (Reynolds and Weiss, 1992). Under these conditions, they form three-dimensional spatially distinct free-floating spheres termed neurospheres (NS), which can be subcloned several times upon dissociation. Conversely, they begin to differentiate upon addition of serum and withdrawal of mitogens (Reynolds and Weiss, 1992) and give rise to all main cell types of the adult brain, oligodendrocytes (10-20%), neurons (20-30%), and astrocytes (60-70%) (Ferron *et al.*, 2007). These assays are widely used to study the biological functions of NSC; the former method is used to assess their self-renewal capacity, whereas the latter their multipotency.

Recent findings have challenged the classical view that only SVZ type B cells can form multipotent neurospheres under *in vitro* conditions. A study by Doetsch *et al.*, 2002 showed that when the Dlx-2 positive transit-amplifying type C cells are treated with EGF, they can form multipotent neurospheres *in vitro* (Doetsch *et al.*,

2002). Similarly, stimulation of oligodendrocyte precursor cells with growth factors transforms them into multipotent neurosphere forming cells (Kondo and Raff 2000). It seems therefore more appropriate to define NS forming cells as NSPC.

In addition, some studies have shown that NS cannot be considered clonal entities; in fact, they are motile and can merge with each other, leading to misinterpretation of the relationship between frequency of stem cells and NS in culture (Singec *et al.*, 2006, Ferron *et al.*, 2007). Recently, Louis and co-workers (Louis *et al.*, 2008) have developed a new method that could address the problem of frequency of NSs and their relationship to number of NSPC *in vitro*. The method, named neural colony-forming cell assay (NCFCA), makes use of the extended proliferative potential of stem cells (which is rather limited in progenitors) to enrich for NSPC. In the NCFCA, high density NSPC are cultured in a collagen-based serum-free medium which inhibits cell migration and aggregation but promotes the formation of clonal colonies of variable sizes. Colonies >2 mm in diameter could generate neurons, astrocytes and oligodendrocytes upon differentiation and were, therefore, most likely originating from a single NSPC (Louis *et al.*, 2008).

Adherent and niche-independent cultures of NSC have also been established: Here, neurospheres are plated on gelatin-coated dishes and cultured in the presence of EGF and FGF-2 until bipolar cells expressing Nestin and RC2 are identified. This homogeneous population of cells can be maintained in an undifferentiated state or induced to differentiate, and are potentially synonymous with the NSC component of neurospheres (Conti *et al.*, 2005). This system has the advantage of allowing considerable expansion of a large quantity of these cells, which can be

rather relevant for purposes of regenerative medicine, where they have to be generated to test their contribution to brain repair.

Furthermore, adherent cultures of NSCs provide a homogeneous population of stem cells which can facilitate expression analysis and produce less variable results across laboratories (Conti *et al.*, 2005). However, it has been reported that prolonged culture of NSC in FGF-2 with the adherent method leads to loss of ability to differentiate into neurons, despite lack of evidence of genetic transformation (Qian *et al.*, 2000, Corti *et al.*, 2007). The NS assay and the NCFCA have the advantage of providing a more physiological environment, with a mixture of cells at various stages of differentiation, and this might be more appropriate for functional studies of NSC properties, such as self-renewal, proliferation, differentiation and survival.

1.1.5 Functional properties of NSC *in vitro*

During embryonic development and in adult life, the mammalian brain contains spatiotemporally defined, self-renewing and multipotent populations of neural stem/progenitor cells (NSPC), which generate neurons and glia cells of the developing brain and account for the limited regenerative potential of the adult brain respectively. Although different in their cellular and molecular characteristics, embryonic and postnatal NSPC share the peculiar role of maintaining a balance between self-renewal, where symmetrical cell divisions give rise to cells with equal developmental potential, and differentiation, where asymmetrical divisions produce fate-committed progeny, which terminally differentiate into the different

cells of the brain (Gage 2000; Temple 2001; Alvarez-Buylla and Lim 2004; Shen *et al.*, 2004; Shen *et al.*, 2008).

Much of our current understanding of biological, antigenic and functional properties of NSPC is derived from a combination of *in vivo* developmental and lineage tracing studies, and extensive use of a variety of *in vitro* assays. In particular the development of growth-factor-based (EGF and FGF2) protocols (Reynolds and Weiss 1992; Reynolds *et al.* 1992) for expansion and long term propagation of NSPC *in vitro* either as neurospheres or as adherent monolayer (Pollard *et al.*, 2006) have provided relatively simple, reproducible, fast and robust systems to study their biological and functional properties *in vitro* (Conti and Cattaneo 2010). Study of functional properties of NSPC *in vitro* is particularly significant especially because of a general lack of specific cell surface markers and/or distinct morphological properties to identify them *in vivo* (Louis *et al.*, 2008).

Moreover, studies have shown (Foroni *et al.*, 2007; Goffredo *et al.*, 2008; Sun *et al.*, 2008) that exposure to growth factors and serial passages do not significantly alter antigenic and biological properties of NSPC as they still exhibit cardinal stem cell features such as self-renewal over a long period of time and multilineage differentiation. However, other studies have casted doubts on the physiological relevance of NSPC cultured in a synthetic *in vitro* milieu and alterations in their spatial identity, differentiation potential as well as genetic and epigenetic statuses have been described (Gage 2000; Hitoshi *et al.*, 2002; Gabay *et al.*, 2003; Smith *et al.*, 2003; Pollard *et al.*, 2008).

In summary, although with all limitations of an *in vitro* system, these assays are useful; a) to provide a starting point for understanding physiologically relevant features of NSPC in a simplified context, b) to allow to expand these cells to obtain a large amount of material to enable biochemical and genetic studies c) to allow analysis of phenotypic changes upon genetic manipulation by delivery of siRNA or over-expression vectors and/or upon application of pharmacological agents or recombinant proteins, d) to provide a stable and reproducible system to study biological functions of NSPC obtained from genetically modified animals.

Self-renewal: The ability of NSPC to generate daughter cells with equal developmental potential, through mitotic cell divisions, over a long period of time is defined as self-renewal capacity, which can be measured by several assays. In culture, NSPC can be maintained and expanded in non-adherent serum free culturing conditions supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF-2) (Reynolds and Weiss, 1992). Under these conditions they give rise to three dimensional floating round cell clusters, named neurospheres, the formation of which is currently used as indication of stem cell property of the cultured cells (Deleyrolle and Reynolds 2009). When neurospheres are subcloned (that is dissociated and re-plated) only mitogen responsive NSPC will give rise to new neurospheres, while the majority of differentiated cells, which are also contained in the neurospheres, will die off (Reynolds and Rietze 2005).

Apoptosis: Apoptosis is defined as an irreversible, programmed process of autonomous cell death that does not elicit inflammation (Fink and Cookson, 2005). Indeed, apoptosis plays an important role in development, disease and tissue ho-

meostasis. A considerable number of studies have sought details of processes of cell death and apoptosis in neurospheres (Bez *et al.*, 2003, Milosevic *et al.*, 2004). Some of the mechanisms associated with initiation and execution of apoptosis include externalisation of phosphatidylserine proteins, activation of caspases that have proteolytic activity, expression of pro-apoptotic genes such as Bak, Bad and Blc-10 (Cory and Adams, 2002, Kang *et al.*, 2002, Rai *et al.*, 2005, Elmore, 2007). These and other genetic and biochemical changes eventually lead cells to apoptosis. It has been previously reported that neurospheres and neurosphere-forming cells undergo apoptosis *in vitro* and *in vivo* (Maric *et al.*, 2007, Galvin *et al.*, 2008). Details of the mechanisms and determination of the rate of apoptosis in NSPC and neurospheres can be studied with various assays based on different methodologies. Some of these are; a) TUNEL (Terminal dUTP Nick End-Labeling) method based on enzymatic labelling of DNA strand breaks, b) TEM (transmission electron microscopy) to visualise specific ultrastructural morphological characteristics, c) Immunohistochemistry or western blotting for detection of procaspases and active caspases, d) Fluorescent labelled Annexin V to detect phosphatidylserine residues on the outer membrane of cells (Kressel and Groscurth, 1994, Vermes *et al.*, 1995, Gurtu *et al.*, 1997, Bossy-Wetzel and Green, 2000, White and Cinti, 2004).

Differentiation: Neurospheres are morphologically and functionally heterogeneous structures; in fact they not only contain self-renewing NSPC (ca. 1-10% of the total number of cells) but also committed glial and neuronal progenitors as well as more differentiated cells and dead cells. Upon plating onto laminin-coated surface and withdrawal of growth factors, neurospheres attach to the substrate and cells start migrating radially from the NS body toward the periphery while they differen-

tiate into neurons (20-30%), astrocytes (60-70%) and oligodendrocytes (10-15%) (Doetsch *et al.*, 1999a, Singec *et al.*, 2006). It has been previously reported that as NSPC differentiate nestin expression is downregulated and expression of distinct neuronal and glial markers such as β III-tubulin, GFAP and O4 is activated (McKay, 1997).

The greatest hurdle with *in vitro* studies of NSPC differentiation has been the identification of substrates and culture conditions that are permissive to maintenance of differentiation properties similar to those *in vivo*. Indeed, the neurogenic versus gliogenic potential of NSPC declines with the number of *in vitro* passages. Various differentiation methods based on combination of factors such as removal of mitogens (EGF and FGF) and exposure to foetal bovine serum and/or specific substrates and cytokines have been developed to counteract this effect (Tropepe *et al.*, 1999, Garcion *et al.*, 2004, Chojnacki and Weiss, 2008). Yet, none of these methods have succeeded in achieving/preserving a percentage of neuronal differentiation greater than 20 percent of the population (Conti and Cattaneo, 2010). This discrepancy may be due to the differences between structural organisation of neurospheres compared with *in vivo* NSPC niches such as SVZ, which provides heterogeneity alongside spatial organization, specific cell-cell interactions, proximity to blood vessels and exposure to a plethora of cues that are absent from neurospheres (Conti *et al.*, 2005, Nelson *et al.*, 2008). Despite these differences inherent to the culture conditions, differentiation of NS is a useful assay to study both multipotency and differentiation properties of NSPC cells in a simplified *in vitro* context.

1.2 Molecular mechanisms controlling self renewal and differentiation of neural stem cells

Several signalling pathways have been shown to be involved in regulation of self-renewal and differentiation of embryonic, neonatal and adult NSPC, and not surprisingly some of these very same pathways have also been found aberrantly active in brain tumourigenesis. It is beyond the scope of this introduction to provide a comprehensive description of all signalling pathways involved in self-renewal and differentiation of NSC. Instead I will only focus on those pathways which are related to a) Bmi1 signaling pathway, or b) self-renewal and differentiation of forebrain NSC, particularly in mice, and c) transformation of normal NSC into BTSC.

1.2.1 Sonic hedgehog

In mammals, three secreted Hedgehog ligands (Sonic, Indian and Desert Hedgehog: Shh, Ihh, Dhh) have been identified. In the absence of these glycoproteins, the receptor Patched (Ptch) inhibits the activity of the transmembrane protein Smoothened (Smo). Upon binding of Shh with Ptch, inhibition of Smo is relieved, which in turn initiates a signalling cascade that results in dissociation of the transcription factors Gli1-3 from a multiprotein complex, the main components of which are a serine/threonine protein kinase, Fused (Fu), Suppressor of Fused (SuFu) and Protein Kinase A (PKA), anchored to the cytoskeleton (Taipale and Beachy, 2001). Activated Gli proteins are translocated into the nucleus (see schematic representation in Figure 1.3) and promote the transcription of genes, including cell cycle regulators (*cyclin D1*, *cyclin E*, *Myc*, *N-myc*) and growth factors and their receptors (*PDGF- α /PDGFR*, *EGF/EGFR*) (Pasca di Magliano and Hebrok, 2003,

Sanai *et al.*, 2005, Sutter *et al.*, 2007, Yadirgi and Marino, 2009). Shh has been shown to regulate self-renewal of NSCs in the SVZ and SGZ during late embryonic development (Ahn and Joyner, 2005) and postnatal life (Machold *et al.*, 2003, Palma *et al.*, 2005). Indeed, Gli1 is expressed by both GFAP-positive NSC (B cells) and GFAP-negative precursor cells (C cells) of the SVZ (Palma *et al.*, 2005).

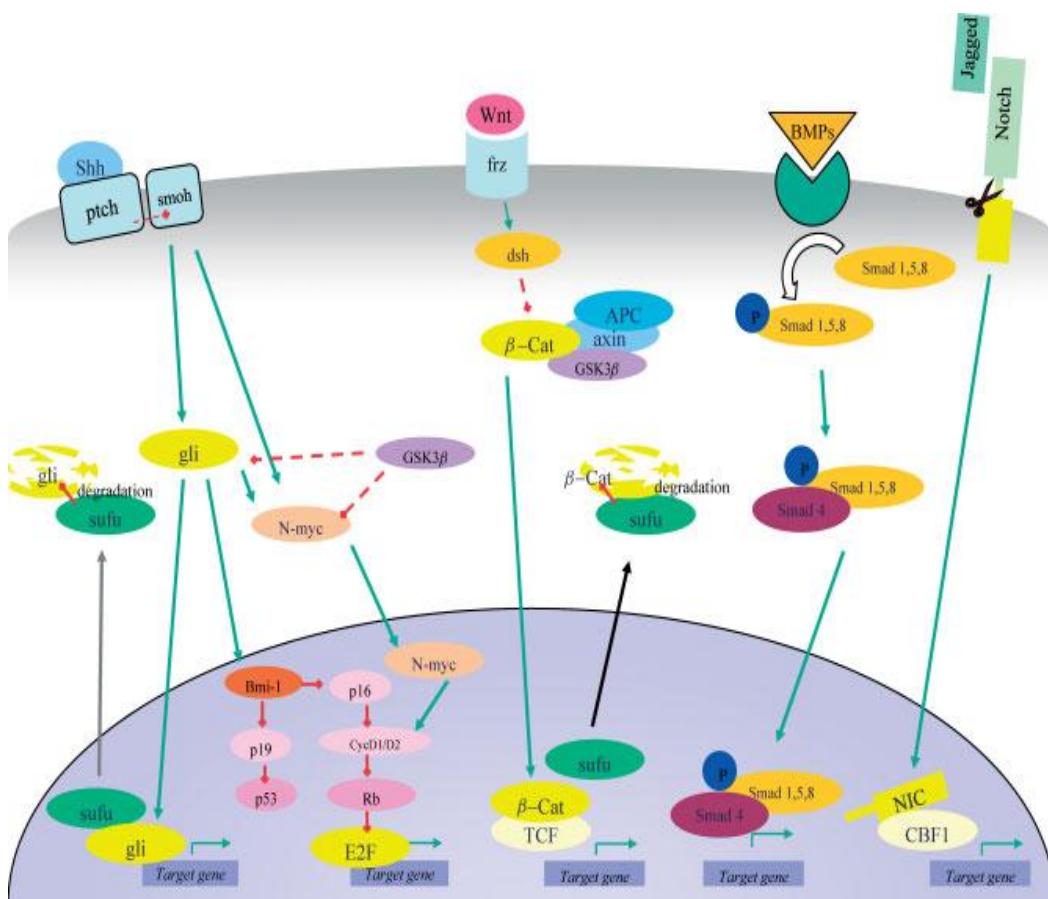


Fig. 1.3 Schematic representations of the main signalling pathways involved in the regulation of self-renewal and differentiation of NSPC (adapted from Yadirgi and Marino, 2009): effectors of these pathways, as expanded in the text below, include Bmi1, Shh, Wnt, BMPs, Notch, MAPKs, PI3K and PTEN.

These cells responded to endogenous Shh by increasing their proliferation, as assessed by BrdU incorporation *in vivo*, and self-renewal, as assessed by secondary neurosphere formation. Moreover, migrating neuroblasts in the SVZ and rostral migratory stream (RMS) express Ptch, and Shh has been shown to exert a chemoattractive activity on SVZ-derived neuronal progenitors *in vitro*, an effect blocked by the Smoothened antagonist named Cur61414.

Furthermore, experiments involving adenoviral transfer of Shh into the lateral ventricle or conversely Shh-blockade with physiological antagonist or neutralizing antibodies in the SVZ of adult mice, provided *in vivo* evidence that Shh can retain SVZ-derived neuroblasts (Angot *et al.*, 2008). A recent paper of the group of Alvarez-Buylla has shown that Shh is also essential for expanding the pool of granule neuron precursors in the SGZ to establish the adult stem cell population, and that this function of Shh is dependent on the integrity of the primary cilia located on the surface of both embryonic and postnatal NSC (Huangfu and Anderson, 2005, Han *et al.*, 2008). A study by Machold *et al.*, 2003 has shown that conditional ablation of Smo in nestin expressing NSPC, by using a NestinCre or an inducible Nestin-CreERT2, results in postnatal depletion of SVZ B and C cells (Machold *et al.*, 2003).

Interestingly, Shh-Gli signalling is also required for sustained glioma growth and survival, and to regulate self-renewal in CD133+ glioma stem cells (Clement *et al.*, 2007). Furthermore, activation of the EGF pathway is a well-known signature genetic abnormality in high-grade gliomas (Ekstrand *et al.*, 1991) and its recently described synergistic activity with Shh in mediating increased proliferation of SVZ

cells (Palma *et al.*, 2005) may provide a mechanism for understanding the formation of these tumours.

1.2.2 Notch

Notch signalling is initiated upon ligand (Delta-like 1-Delta-like 5) receptor (Notch1-Notch5) interactions between neighbouring cells, followed by a series of proteolytic events that results in nuclear translocation of the cleaved intracellular domain of Notch (NICD; see schematic representation in Figure 1.3). Upon association of NICD with nuclear protein mastermind (MAM) and transcriptional repressor protein complex CLS (components of which are RBP-Jκ, Suppressor of hairless and Lag-1) (Bray, 2006, Louvi and Artavanis-Tsakonas, 2006), activation of downstream transcriptional targets including the HES (hairy and enhancer of split paralogues) family genes *Hes1* and *Hes5*, *Hers1* and *Hers2* and the recently identified brain lipid-binding protein (BLBP) is achieved (Jarriault *et al.*, 1995, Hsieh *et al.*, 1996, Anthony *et al.*, 2005, Louvi and Artavanis-Tsakonas, 2006).

The Notch signalling pathway is required for maintaining the pool of multipotent NSCs in the developing and adult mouse brain, as well as in inducing differentiation of these cells (Tanigaki *et al.*, 2001, Hitoshi *et al.*, 2002, Nagao *et al.*, 2007). Indeed, mice deficient for *Notch1* or *RBP-Jκ*, a component of the pathway, showed a severe depletion of NSCs (Hitoshi *et al.*, 2002). Instead, NSCs could be obtained from embryonic stem cells deficient for *RBP-Jκ* (Hitoshi *et al.*, 2002). Moreover, neurospheres derived from E 14.5 mice lacking *Presenilin1*, a gene encoding a key regulator in Notch signalling, exhibit a skewed differentiation pattern dominated

by neurons ($MA2P^+$) and astrocytes ($GFAP^+$). Complementing these findings, a recent study by Androutsellis-Theotokis and colleagues (Androutsellis-Theotokis *et al.*, 2006) showed that fetal NSCs exposed to Notch ligands retain their multipotency and have better survival, possibly through activation of a survival cascade downstream of the insulin receptor, which promotes survival by activating the phosphatidylinositol-3-OH kinase PI(3)K and serine/threonine kinase Akt/PKB-mediated cytoplasmic phosphorylation events. Furthermore, activated Notch signalling supplemented with FGF2 increases the ratio of proliferating cells in the SVZ of rats with cerebral cortical ischaemia, in addition to increased regenerative response as measured by improved motor skills (Androutsellis-Theotokis *et al.*, 2006, Sutter *et al.*, 2007).

Activation of the Notch signalling pathway has been highlighted in glial and embryonal tumours (Pahlman *et al.*, 2004, Purow *et al.*, 2005, Fan *et al.*, 2006). Of particular interest is the study of Fan *et al.*, (2006) where pharmacological blockade of the Notch signalling pathway, through inhibitors of α -secretase, in medulloblastoma cell lines increased the frequency of differentiated and apoptotic cells but did not affect the growth potential of these cells. However, the ability of these cells to form tumour xenografts upon injection into mice was reduced and the number of CD133 positive neoplastic cells with stem-like characteristics was reduced, suggesting that Notch signaling is required for maintenance of BTSC in the tumour bulk (Fan *et al.*, 2006). In keeping with these findings, Notch signalling enhances nestin expression in gliomas (Shih and Holland, 2006), possibly implying an increase in the BTSC component in these neoplasms.

Interestingly, Hes1 was found to be a target of Sonic Hedgehog in mesodermal and neural stem cells and smoothened function was found to be necessary for the up-regulation of Hes1 in response to Sonic Hedgehog. However, the mechanism did not require γ -secretase-mediated cleavage of Notch receptors and appeared to involve transcription factors other than RBP-J κ , therefore implying a regulation of Hes1 in stem cells independent of canonical Notch signaling (Ingram *et al.*, 2008).

1.2.3 PTEN and PI3K

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumour suppressor gene which was shown to be commonly mutated/deleted in various malignancies including brain tumours such as glioblastoma (Li *et al.*, 1997, Stiles *et al.*, 2004).

The lipid phosphatase PTEN exerts its effects on the biological processes of cell proliferation, cell growth, cell migration and cell death mainly by antagonising the PI3K signalling through dephosphorylating one of its products namely phosphotidylinositol-3,4,5-triphosphate (PIP3) (Sun *et al.*, 1999). Activated PI3K converts the plasma membrane lipid phosphotidylinositol-4,5-triphosphate (PIP2) into PIP3 on the inner surface of the membrane, which, in turn, recruits Akt/PKB to the plasma membrane and alters its conformation to allow subsequent phosphorylation and thus activation by phosphoinositide-dependent kinase-1 (PDK-1) (Vazquez and Sellers, 2000, Song *et al.*, 2005). The downstream targets of Akt/PKB kinase include the cell cycle regulators MDM2, p27 and p21, proapoptotic factor BAD, caspases 3 and 9, and finally the kinase GSK-3 to name but a few among many important for

cellular events (Stiles *et al.*, 2004). Other signalling molecules activated by PIP3 include phosphatidylinositol-dependent kinases (PDKs), S6 kinases and mTOR, small GTPases Rac1 and Cdc42 (Vivanco and Sawyers, 2002).

A study by Fan *et al.*, 2009, has found that nuclear PTEN functions are mediated by ways other than its phosphatase activity, at least in prostate cancer cell lines such as DU145 (Fan *et al.*, 2009). Interestingly, though, the study showed that BMI1 co-localizes with nuclear PTEN in primary prostate cancer tissue and that this attenuates BMI1 function and reciprocally increases PI3K activity (due to lack of its inhibition by PTEN) (Fan *et al.*, 2009).

Conditional deletion of PTEN in nestin-positive progenitor cells resulted in increased cell proliferation, enlarged cell size and decreased cell death in the embryonic ventricular zone of the telencephalon, leading to mice with enlarged and morphologically abnormal brains (Groszer *et al.*, 2001). In these mice, PTEN deficiency increased neural stem cell proliferation and self-renewal without disrupting their capacity to differentiate into the three lineages of neurons, astrocytes and oligodendrocytes (Groszer *et al.*, 2001, Groszer *et al.*, 2006). Further analysis has shown increased expression of cell cycle and DNA replication related genes such as cyclin B1, cyclin B2, cyclin D1, cyclin E1, Ki-67 and DNA primases in these cells. Interestingly, fewer PTEN null NSC were shown to be in the quiescent (G_0/G_{1A}) phase of the cell cycle compared with controls, suggesting that PTEN negatively regulates NSC self-renewal potential by modulating G_0 - G_1 cell cycle entry (Groszer *et al.*, 2006).

1.2.4 MAPKs

The mitogen-activated protein kinases (MAPKs) are a family of intracellular signaling molecules that play an essential role in a wide variety of cellular processes including survival, growth, proliferation, differentiation, maintenance and self-renewal (Tibbles and Woodgett, 1999). In mammals, three members of the MAPK family have been identified: extracellular signal-regulated kinase (ERK, including ERK1/2 and ERK5), p38 (including p38 α , p38 β , p38 γ , and p38 δ), and c-Jun N-terminal kinase (JNK, including JNK1, JNK2, and JNK3) (Widmann *et al.*, 1999, Johnson and Lapadat, 2002, White *et al.*, 2007). Activation of ERK, p38 and JNK by phosphorylation through MAPK kinases (MEKs or MKKs), which are activated by MAPK kinase kinases (MEKKs), initiates a signaling cascade that transduce extracellular stimuli into intracellular responses of transcriptional and non-transcriptional cell function regulation (Hazzalin and Mahadevan, 2002).

MAPKs play an important role in connecting changes in cell surface receptors, such as integrins, to intracellular signals that may be important for maintenance of stem cells. A previous study has shown that NSPC express a number of β 1 integrins (Jacques *et al.*, 1999). Recently, a study by Campos *et al.*, 2004 has shown that activation of MAPK signaling, through activation of β 1 integrin by ECM molecules, is required for maintenance of NSPC as assessed by neurosphere formation assay (Campos *et al.*, 2004). A recent study has suggested that a downstream target of MAPKs may regulate activity of P^cG gene family members, particularly of Bmi1 (Voncken *et al.*, 2005). The study by Voncken *et al.*, (2005) has shown that 3pK, also known as MAPK-activated protein kinase 3 (MAPKAPK3), acts as a kinase for

Bmi1 *in vivo* and *in vitro* and that phosphorylation of Bmi1 by 3pK leads to P_cG/protein dissociation from chromatin and de-repression of the Ink4a/Arf locus; thereby leading to increased p14ARF expression.

1.3 Role of P_cG genes in the regulation of stem cell functions

The first Polycomb group (P_cG) proteins were identified in Drosophila (fruitfly) as repressors of Hox genes (a family of transcription factors that are expressed in sequence along the anterior-posterior axis and involved in determination of axial identity), through studies of mutant phenotypes that had *extra sex combs* (*esc*) on the second and third legs of males rather only on the first leg (Ringrose and Paro, 2004). In vertebrates, too, Polycomb mutants exhibit posterior transformation of body segments, where the identity of one body segment is transformed into the identity of another (van der Lugt *et al.*, 1994). Moreover, the P_cG proteins have been shown to regulate other cellular processes such as cell cycle, cellular senescence and actin polymerization (Guney and Sedivy, 2006, Martinez and Cavalli, 2006). More recently, however, some of the P_cG proteins have been shown to be involved in regulation of proliferation, self-renewal and differentiation of specific stem cell types, including neural stem cells. In the next section I will describe the current findings about mode of action of P_cG proteins and their involvement in regulation of neural stem cell self-renewal, maintenance and differentiation, with particular attention to the role of Bmi1.

1.3.1 P_cG proteins are epigenetic regulators

The term epigenetics is broadly used to describe mechanisms that regulate heritable establishment and maintenance of transcriptional states and thus cellular iden-

tity, beyond the primary DNA sequence of a genome. The gene expression profile of a single genome may therefore be altered allowing for cellular and functional diversity (Richards, 2006, Bernstein *et al.*, 2007).

In eukaryotic cells the genetic information is packed into chromosomes, which are composed of lower order structures called chromatins. In turn, chromatins are formed by folding of lower order structures termed core histones wrapped with DNA helix; viewed in a sequence forming a structure referred to as ‘beads-on-a-string’ (Figure 1.4). Core histones have ‘tails’ with specific amino acid sequences that when modified alter chromatin accessibility, which has important consequences for many cellular processes ranging from chromosome segregation to DNA repair and transcription (Sparmann and van Lohuizen, 2006).

Two of the most studied epigenetic regulatory factors, P_cG group and Trithorax group (TrxG), act antagonistically in epigenetic regulation of cellular processes, where the former represses and the latter promotes gene expression through regulation of specific amino acid modifications in histone tails (Fisher and Merkenschlager, 2002). Histone tails contain specific amino acid sequences that are targets of covalent modifications such as acetylation, phosphorylation, poly(ADP)-ribosylation, ubiquitylation and methylation (Figure 1.4). Whereas trimethylation of lysine 4 (H3K4me³) or acetylation of lysine 14 of histone H3 (H3K14ac) are associated with actively transcribed regions, the H3K9me³ or H3K27me³ are indicative of repressed chromatin state (Strahl and Allis, 2000, Sparmann and van Lohuizen, 2006).

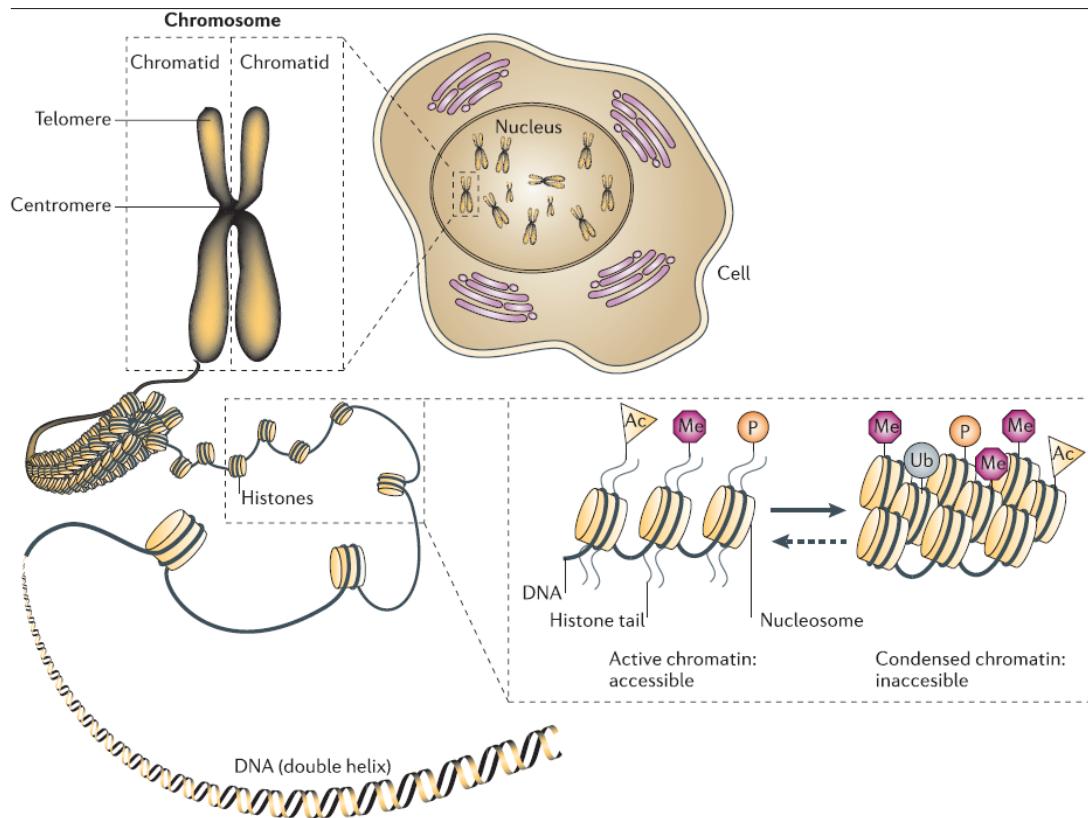


Fig. 1.4 Organisation of genetic information in eukaryotic cells is shown at various levels such as chromosomes, chromatin and histones: Epigenetic modifications to the N-terminal of histone tails influence chromatin accessibility to transcription factors that regulate gene expression. The figure was taken from Anke Sparmann and Marteen van Lohuizen (2006) Nat. Rev. Cancer.

1.3.2 Polycomb Repressive Complexes and epigenetic modifications

In the nucleosome, DNA helix complexes loop around core histones and form higher order organisation termed chromatin, which undergo dynamic structural changes ensuing modifications to specific sequences in histone tails (Fisher and Merkenschlager, 2002). Biochemical purification and functional genetic studies have assigned the P^cG components into one of the two functionally distinct mul-

imeric complexes termed the Polycomb repressive complex 1 or 2 (PRC1 or PRC2) (van Lohuizen, 1998). The PRC2 consists of the mammalian homologues of the *Drosophila melanogaster* proteins and are named as EED, EZH1, EZH2 and SUZ12 in mice (Figure 1.5 for a full list of vertebrate PRC2 members). These core components initiate gene repression mainly by trimethylation of lysine 27 and lysine 9 of H3 (H3K27me³ and H3K9me³, respectively). The core components of the PRC1 are CBX2, 4 and 8 (mammalian homologues of *Drosophila melanogaster* chromodomain component named *Polycomb (PC)*); EDR1, 2 and 3; BMI1, MEL18 and ZNF134, and can recognise the H3K27me³ mark at promoter regions of genes and other sites on the DNA (Figure 1.5 for a full list of vertebrate PRC1 members). Binding of the PRC1 complex to recognition sites maintains a repressive state of transcription. This simplified description of hierarchical gene silencing by way of “initiation” and “maintenance” is being challenged by recent studies that showed continuation of P_cG group proteins mediated gene silencing even in absence of N-terminal histone tails and depletion of specific binding sites (Francis *et al.*, 2004, Papp and Muller, 2006). Nevertheless, maintenance of stable gene silencing involves monoubiquitination of H2A (H2Aub1) recognition site by PRC1. Interestingly the latter modification is stimulated by Bmi-1, a component of the PRC1 (Buchwald *et al.*, 2006). Together these alterations contribute to a repressive chromatin state, which is thought to be important for stem cell maintenance and differentiation.

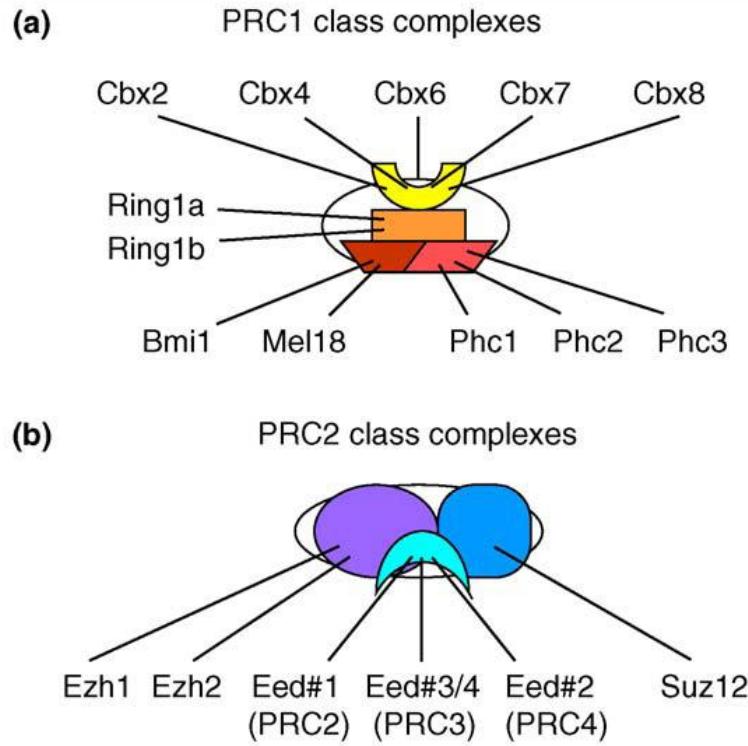


Fig. 1.5 Representation of vertebrate components, not necessarily found in all cell and tissues, of PRC1 (a) and PRC2 (b): PRC1 components include Bmi1, Mel18, Phc1 (2 and 3), and Cbx2 (4, 6, 7 and 8) whereas PRC2 is composed of Ezh1 (and 2), Eed1 (2, 3, and 4). Diagram was adopted from Tom K. Kerppola Trends in Cell Biology 2009.

1.3.3 P_cG genes and NSC maintenance and differentiation

Several recent studies have highlighted a significant role for P_cG proteins in maintenance of cellular identity, self-renewal and differentiation of not only embryonic but also postnatal and adult stem cells. Mice deficient in various components of P_cG proteins were shown to have defects in embryonic stem (ES) cells (O'Carroll *et al.*, 2001, Boyer *et al.*, 2006), NSC (Molofsky *et al.*, 2003), cerebellar progenitors (Leung *et al.*, 2004), haematopoietic stem cells (HSC) (Lessard *et al.*, 1999, Park *et al.*, 2003a, Kim *et al.*, 2004), and neural crest cells (Molofsky *et al.*, 2003).

Much of our understanding of role of P_cG genes in stem cells, and particularly of Bmi1, derives from knockout studies. For example, the study by Park I.K. and co-workers (2003) has shown that proto-oncogene Bmi1 is expressed by fetal and adult mouse and adult human HSC. It was noted that the number of HSC in the fetal liver of Bmi1 deficient mice was comparable to normal levels. However, in the postnatal mice the number of HSC was markedly reduced and transplantation of fetal bone marrow and liver cells obtained from Bmi1 deficient mice were unable to restore haematopoiesis. In terms of the changes in gene expression they noted over-expression of Bmi1 targets such as p16Ink4a and p19Arf (Park *et al.*, 2003a). Other studies have shown that the role of Bmi1 in regulating haematopoiesis in mice changes in relation to its interactions with other P_cG genes. A study showed that the self-renewal capacity of murine HSC is severely impaired in absence of P_cG genes Rae28 and Bmi1, while increased proliferation of HSC was observed in Eed deficiency, and also in absence of both Eed and Bmi1 (Lessard *et al.*, 1999). This study suggests that Bmi1 is epistatic to Eed in regulating HSC proliferation in mice. Interestingly, these changes were not associated with changes in gene expression of p16Ink4a and p19Arf, further suggesting that a hierarchical mode of action exists between components of P_cG genes.

ES cells can be maintained in culture in a pluripotent state, and are imbued with self-renewal and multilineage differentiation capacity. These two opposing properties require on one hand a tight regulation of transcriptional state to maintain self-renewal and on the other flexibility to allow differentiation during development. To determine the possible role of P_cG proteins in regulating these functions in mouse ES cells, several studies have looked at the location of P_cG proteins on large

segments of genome (Boyer *et al.*, 2006, Bracken *et al.*, 2006). PcG protein binding sites were enriched for homeodomain-containing transcription factors of Dlx, Irx, Lhx and Pax gene families, which are involved in regulation of neurogenesis, haematopoiesis and axial patterning (Hombria and Lovegrove, 2003). Among the targets were also the genes encoding for components of signalling pathways (TGF β , BMP, Wnt and FGF) that were previously shown to be required for maintenance and proliferation as well as differentiation of various tissue stem cells, including neural stem cells (Boyer *et al.*, 2006, Bracken *et al.*, 2007). With regards to differentiation of NSC during cortical development, it was shown that inactivation of PcG genes Ring1B or Ezh2 or Eed, which repress the promoter of the proneural gene neurogenin1, prolonged the neurogenic phase and delayed the onset of the astrogenic phase in mice (Hirabayashi *et al.*, 2009).

1.3.4 Bmi-1 and the ink4a/arf locus

Bmi-1 (B cell-specific Moloney murine leukaemia virus integration site 1) is a member of the Polycomb group (PcG) gene family of chromatin modifiers and transcriptional repressors (van Lohuizen *et al.*, 1991). The *murine* Bmi-1 gene is located in chromosome two; consists of ten exons and encodes for a nuclear protein of 324 amino acids that take a position of fourty five-fourty seven kilodalton (kD) on SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) (Haupt *et al.*, 1991, van Lohuizen *et al.*, 1991, Alkema *et al.*, 1993). Low levels of Bmi-1 mRNA are ubiquitously expressed in all tissues but higher levels are particularly detected in thymus, heart muscle, brain, testis, ES cells, embryonic and post-

natal CNS and peripheral nervous system (PNS) NSC throughout life (van der Lugt *et al.*, 1994, Molofsky *et al.*, 2003).

The recent observation that *Bmi-1* is highly expressed in NSC and is necessary for their self-renewal in both adult peripheral nervous system (PNS) and CNS suggests that *Bmi-1* signalling plays a major role in their maintenance (Molofsky *et al.*, 2003). Indeed, reduced proliferation of *Bmi-1*-deficient SVZ NSC (quiescent) leads to their postnatal depletion, albeit the proliferation of neural forebrain progenitors is unaffected (Molofsky *et al.*, 2003). However, inactivation of *Bmi1* impairs proliferation of postnatal granule cell progenitors leading to depletion of granule neurons and consequently to ataxia (van der Lugt *et al.*, 1994, Leung *et al.*, 2004).

The best-characterized downstream mechanism of *Bmi1* function is through inhibition of the tumour suppressor locus *Ink4a/Arf* (Figure 1.6) (Jacobs *et al.*, 1999a, Bruggeman *et al.*, 2005). The mouse *Ink4a/Arf* locus encodes for two protein products, $p16^{ink4a}$ and $p19^{arf}$ ($p16^{INK4a}$ and $p14^{ARF}$ in human), both of which are involved in cell cycle regulation (Quelle *et al.*, 1995, Sherr, 2001). In the absence of *Bmi-1*, both products of the *Ink4a/Arf* locus are highly expressed (Quelle *et al.*, 1995). During the G_1 phase of the cell cycle, $p16^{ink4a}$ directly inhibits cooperation of cyclin D1 and cyclin D-dependent kinases 4/6 (Cdk4/6), which, when unblocked, form a complex that phosphorylates retinoblastoma protein (pRB) (Figure 1.6) (Sharpless and DePinho, 1999). Phosphorylated RB is unable to suppress E2F transcription factors (E2Fs), the target genes of which (*DNA polymerase II*, *cyclin E*, *p19 myb* and *dihydrofolate reductase*) are important for cell cycle progression (i.e. G_1 -S transition) (Vernell *et al.*, 2003). In contrast, $p19^{ink4a}$ expression leads to p53-

mediated cell cycle arrest and apoptosis (Figure 1.6) (Park *et al.*, 2004). Association of p19^{ink4a} with mouse double minute 2 (Mdm2) inhibits Mdm2-mediated ubiquitin-dependent degradation of p53, which, when accumulating in the cell, leads to the induction of genes such as *Wig1* and *p21* that are involved in cell cycle arrest and apoptosis (Honda and Yasuda, 1999, Sutter *et al.*, 2007). Taking into account that *Bmi1* has been demonstrated to be downstream of *Shh* (Leung *et al.*, 2004); this mechanism could at least in part mediate Shh function on NSC properties.

Moreover, in primary cultures of mouse embryonic fibroblasts, induction of p16^{Ink4a} and p19^{ARF} leads to senescence whereas Bmi-1 over-expression prevents it by suppressing the Ink4a/Arf locus (Jacobs *et al.*, 1999a, Dimri *et al.*, 2002). In ageing mice, the decrease in SVZ NSC self-renewal and progenitor proliferation has been shown to correlate with physiological p16^{ink4a} expression, and this effect could significantly be reversed by p16^{ink4a} suppression alone (Molofsky *et al.*, 2005). As p16^{ink4a} deficiency predisposes ageing mice to cancer (Sharpless *et al.*, 2001), it is intriguing to think that loss of p16^{ink4a} expression noted in human glioblastoma can represent a powerful mechanism by which the NSC escape senescence and possibly give rise to tumours.

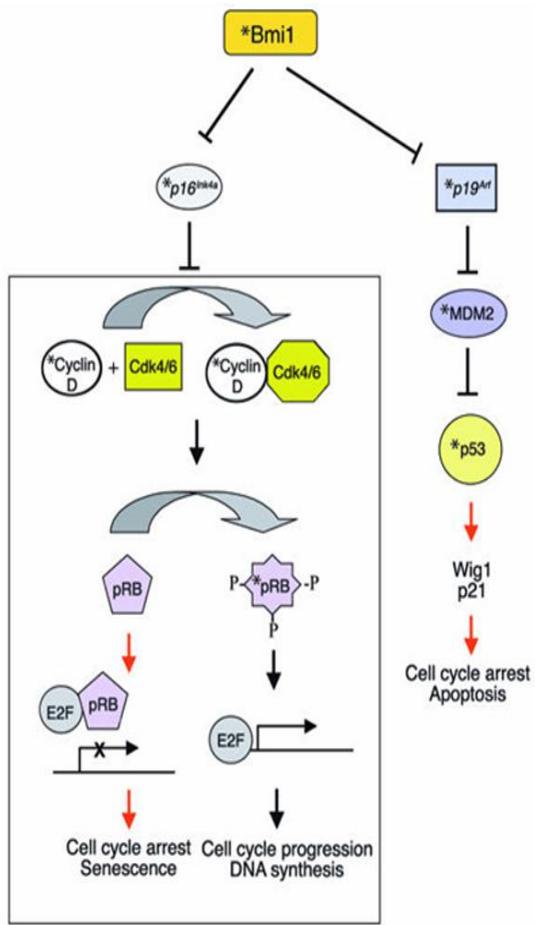


Fig. 1.6 “Canonical” downstream targets of Bmi1: The red arrows indicate the possible cellular processes that may be affected in absence of Bmi1 whereas the black arrows indicate the possible pathways activated and cellular processes that may occur when Bmi1 represses the Ink4a/Arf locus. The diagram was adapted from Park *et al.*, 2004.

1.3.5 New roles and targets of Bmi1 beyond *Ink4a/Arf* locus

Role of Bmi1 in regulating self-renewal and differentiation of NSC, during development and postnatal time points in mice, through regulated repression of Ink4a/Arf locus has been well established (Molofsky *et al.*, 2003, Leung *et al.*,

2004). However, concomitant deletion/suppression of either $p16^{Ink4a}$ or $p19^{ARF}$ or both have reversed only partially the aforementioned defects in self-renewal of NSC and proliferation of granule cell progenitors derived from Bmi1^{-/-} mice (Bruggeman *et al.*, 2005, Molofsky *et al.*, 2005). These observations suggest that whereas *Ink4a/Arf locus* is an important downstream target of Bmi1, other downstream target and mechanism may exist. Several studies have identified new targets and mechanisms of Bmi1 in regulating biological functions of NSC independent of the *Ink4a/Arf locus*.

Adhesion and migration properties of SVZ NSC has been associated with specific expression and interaction pattern of extracellular matrix proteins and their receptors such as collagens and Beta-1 integrin receptors (Campos, 2004, 2005). Bruggeman *et al.*, 2009 has shown that Bmi1 deficient mouse NSC secrete a Collagen-like extracellular matrix compound that they strongly adhere to in culture, independently of *Ink4a/Arf locus*. Moreover, Bmi1 has been shown to regulate self renewal of embryonic NSC located in VZ of the developing forebrain through inhibition of a different cell cycle inhibitor, namely *p21WAF1/Cip1* (Fasano *et al.*, 2007). Recently, a study by Fasano *et al.*, (2009) has shown that Bmi1 cooperates with Foxg1, a forebrain specific transcription factor, in regulating self-renewal of NSC *in vivo* (Fasano *et al.*, 2009). In this context it is interesting to suggest that Bmi1 acts in a developmental-stage-dependent manner: where suppression of *Ink4a/Arf locus* is more important for postnatal NSC as opposed to inhibition of p21 and/or cooperation with Foxg1 during development.

Lack of Bmi1 in HSC and thymocytes results in increased levels of reactive oxygen species (ROS), through de-repression of targets genes (other than Ink4a/Arf) involved in ROS generation or regulation of mitochondrial function (Liu *et al.*, 2009). Premature death or hypersensitivity to neurotoxic agents of Bmi1 deficient neurons has been associated with increased p19^{Arf}/p53 level (Chatoo *et al.*, 2009). Indeed, Chatoo *et al.*, (2009) has shown that in Bmi1 deficient neurons, the accumulation of p53 and co-repressors at promoter regions of antioxidant genes, results in a repressed chromatin state and antioxidant gene down-regulation that in turn leads to increased ROS levels and cell death.

Furthermore, Bmi1 has recently been highlighted for its role in maintenance of GBM-initiating CD133+ stem cells, as well as in conferring resistance to apoptosis and radiotherapy of normal and cancerous NSC (Abdouh *et al.*, 2009, Facchino *et al.*, 2010). The study by Abdouh and coworkers (2009) has shown that ablation of BMI1 by shRNA, inhibits growth and clonogenic potential of GBM cell lines— effects that were independent of INK4A/ARF locus but rather related with depletion of CD133+ cells possibly through enhanced apoptosis and differentiation. A new role for BMI1 in DNA damage response has been proposed by the same group in the study by Sabrina Facchino *et al.*, (2010), where the authors has shown that resistance of tumour-initiating CD133+ NSC was due to BMI1 over-expression mediated “recruitment and activation” of DNA double-strand break response machinery, including the ataxia-telangiectasia-mutated (ATM) kinase, and proteins of the non-homologous end joining (NHEJ) repair pathway such as histone H1.

1.4 Role of neural stem cells in the pathogenesis of glial tumours

Traditionally, cancer has been viewed as a disease whereby differentiated cells acquire mutations, leading to activation of proto-oncogenes and inactivation of tumour-suppressor genes, together resulting in uncontrolled growth. However, it was puzzling that brain tumours with morphological features often recapitulating undifferentiated neural progenitor cells could arise from fully differentiated post-mitotic cells, which could hardly accumulate enough genetic mutations to undergo neoplastic transformation. Much as the involvement of proto-oncogene activation in combination with tumour-suppressor gene inactivation may have significant contribution to tumourigenesis, our understanding about which cells can be transformed to form tumours has reached a new level with experiments showing that normal NSC can give rise to brain tumours (Zheng *et al.*, 2008, Jacques *et al.*, 2009).

The study by Zheng H *et al.*, (2008) has shown that GFAP-cre-mediated inactivation of PTEN and p53 in stem and progenitor cells during development results in formation of malignant astrocytomas and GBM in adulthood. More recently, Jacques *et al.*, (2009) has provided interesting evidence that deletion of PTEN and p53 in GFAP positive adult SVZ NSC results in formation of gliomas. Studies by other groups has shown that inactivation/deletion of PTEN and p53, Nf1 or p53 in adult nestin-expressing progenitor cells results in formation of malignant gliomas (Alcantara Llaguno *et al.*, 2009). Together these findings suggest that tumour-suppressor gene inactivations in neural stem/progenitor cells are sufficient for their transformation into tumour inducing and maintaining cells.

In the next section I will i) describe the different types of brain tumour paying particular attention to gliomas; ii) describe possible cells of origin of brain tumours iii) highlight the similarities between normal and cancer stem cells in terms of biological functions and molecular properties.

1.4.1 CNS neoplasms

Brain tumours are the second most common neoplasms in children and their incidence is also relatively high in the adult population, especially in elderly people. In the world, 2-3 % of all benign and malignant primary tumours are those of the CNS. Their prevalence has been estimated to be 69 patients per 100,000 population, whereas in the Western countries annual incidence is approximately 15 patients per 100,000 population (Ohgaki and Kleihues, 2005). The perception of brain tumours has changed substantially over the last two decades and significant new insights into the molecular mechanisms involved in their pathogenesis have been gained. However, little has changed in the clinical approach towards these neoplasms and they are still associated with very high morbidity and mortality.

The most recent classification of brain tumours is the WHO classification of tumours of the nervous system published in 2007, which is based on the consensus recommendations of an international WHO working group of experts (Kleihues and Cavenee, 2000, Louis *et al.*, 2007). This classification provides criteria to distinguish between the various intrinsic brain tumours of neuroepithelial origin and several other groups of intracranial neoplasms not originating from the brain tissue itself. The WHO grading system provides histopathological criteria to estimate the biological behaviour of these tumours and represents a scale of malignancy to predict

the prognosis of a tumour. It ranges from grade I to IV, where slowly growing tumours are grade I or II, grade III tumours grow faster and show anaplastic foci with atypical nuclei and mitotic activity, and grade IV tumours are characterised by anaplasia in a large portion of the tumour or the entire mass, microvascular proliferations and areas of necrosis (Kleihues *et al.*, 2002, Riemenschneider and Reifenberger, 2009a).

In the last decade animal models have contributed significantly to our understanding of the pathogenesis of human brain tumours. Genetically modified mice representing the common genetic alterations that occur in human tumours have been engineered using transgenic, conventional and conditional tissue-specific knockout technologies (Reilly and Jacks, 2001, Gutmann *et al.*, 2003). Animal models are currently available for most of the major CNS tumour types, including glioma, oligodendrogloma, medulloblastoma, and meningioma (Weiss *et al.*, 2002).

Gliomas are the most common type of intrinsic tumours affecting the CNS and they comprise a variety of different tumour forms. Astrocytic tumours (WHO grade I to IV) are by far the most common and they comprise pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma and glioblastoma. Glioblastoma, the most common glioma is a WHO grade IV tumour and can arise either *de novo* (primary glioblastoma) or as a result of malignant progression of a low-grade glioma (Behin *et al.*, 2003). The highest incidence is in the older population and the five-year survival rate is only 3% (McCarthy *et al.*, 2002). Primary and secondary glioblastomas carry mutations in multiple chromosomes and exhibit aberrations in different genetic pathways. Molecular and genetic analyses of the past years have

identified frequent aberrations in two different pathways that allowed distinguishing of primary from secondary glioblastomas (Ohgaki and Kleihues, 2007). Accordingly, primary glioblastomas exhibit frequent *EGFR* amplification, homozygous deletion of *CDKN2A* and *p14^{ARF}*, *CDK4* amplification, *MDM2* or *MDM4* amplification, *RB1* mutation/homozygous deletion, over-expression of epigenetic silencing gene *NDGR2*, monosomy 10 and *PTEN* mutation; whereas secondary glioblastomas arises from low-grade precursor lesions that typically carry mutations in *TP53* and *IDH1* (isocitrate dehydrogenase 1), promoter hypermethylation of the *RB1* gene, and overexpression of *PDGFR-α* and epigenetic silencing genes *MGMT* and *EMP3* (Nakamura *et al.*, 2001, Parsons *et al.*, 2008, Riemenschneider and Reifenberger, 2009b). Interestingly, though, the different molecular aberrations found in primary and secondary glioblastomas results in alterations in the same signalling pathways, namely p53, pRb1, PTEN/PI3K/AKT and MAPK (Riemenschneider and Reifenberger, 2009a). This may explain why both types of glioblastomas share comparable histological features and poor outcome.

1.4.2 Are NSC the cell of origin of brain tumours?

A central issue in cancer biology is to understand the molecular mechanisms of tumour formation and the directly linked ontogeny of tumours. Over the past 80 years, brain tumours were thought to originate from the cell types of the adult brain that they resembled most as assessed by comparison of histological features: for example, astrocytomas were thought to arise from astrocytes; oligodendroglomas from oligodendrocytes; ependymoma from ependymal cells etc. (Louis *et al.*, 2001, Louis, 2006). However, this concept is being increasingly challenged and

the role of NSC in brain tumour formation is currently being investigated, with results from several groups indicating both stem and progenitors as cells of origin of brain tumours.

Radial glia cell-like stem cells defined by the expression of various markers CD133, Nestin, RC2 and BLBP have been shown to give rise to ependymomas, a subtype of glial tumours arising from different locations of the ventricular system along the craniospinal axis (Taylor *et al.*, 2005). Despite the histological similarity of the tumours originating from different locations, site specific alterations in signalling pathways such as upregulation of NOTCH in supratentorial and HOX family transcription factors in spinal ependymomas, were shown. These data raise the possibility that different populations of NSC are susceptible to neoplastic transformation through different molecular changes (Taylor *et al.*, 2005).

Two recently published papers have shown that medulloblastomas can also arise from stem cells when the Shh pathway was aberrantly activated in these cells during embryonic development. However, they claim that a commitment to a granule cell lineage is needed for medulloblastoma to develop (Schuller *et al.*, 2008, Yang *et al.*, 2008). This is an interesting hypothesis, which however does not explain the occurrence of medulloblastoma in adult life when progenitors of granule cell lineage are no longer present.

Moreover, Jackson *et al.*, (2006) have shown that PDGFR- α expressing quiescent adult rodent SVZ B cells, became highly proliferative and contributed to formation of atypical hyperplasia with features of gliomas when stimulated with surplus PDGF ligand (Jackson *et al.*, 2006). This observation correlates well with the clinical

observation that over-expression of PDGF ligand and receptor is a common early change in low-grade gliomas (Behin *et al.*, 2003) and suggested that SVZ NSC might be involved in the formation of these tumours.

CNS-specific deletion of p53 and an allele of Pten in GFAP expressing neural stem and progenitor cells, using the transgenic mouse model *hGFAP-Cre⁺;p53^{lox/lox};Pten^{lox/+}*, results in formation of gliomas (anaplastic astrocytomas) between 15 to 40 weeks of ages (Zheng *et al.*, 2008). In this study, it could not be fully determined whether the tumours originated from NSC *per se*, but the similarities in biological properties (self-renewal) and expression profile (positive for Nestin, GFAP and Olig2 but negative for mature neuronal and oligodendrocyte markers such as NeuN and Mbp) between the gliomas and NSC was suggestive. However, the GFAP-Cre model used in this study targets all GFAP expressing cells during development and cells arising thereby.

In a more recent study by Jacques *et al.*, specific targeting of adult GFAP positive cells confined to periventricular region was achieved, using a transgenic mouse model bearing conditional alleles of PTEN and p53 (Jacques *et al.*, 2009). Concomitant deletion of PTEN and p53 in GFAP-expressing adult stem cells (type B cells) gave rise to gliomas (anaplastic astrocytomas).

Recently, somatic cells have been reprogrammed into pluripotent stem (iPS) cells through viral transduction of four transcription factors, c-Myc, Oct4, Sox-2 and Klf4 (Takahashi and Yamanaka, 2006, Nakagawa *et al.*, 2008) and more recently even by means of expression of only Oct-4 and either Klf-4 or c-Myc in NSC (Kim *et al.*, 2008). These are exciting findings opening tremendous opportunities for future

stem cell-based therapies, but they also raise the possibility that terminally differentiated cells such as glia and neurons can revert to a more undifferentiated state through genetic mutations and contribute to tumour formation. Interestingly, Moon *et al.*, showed that mouse astrocytes cultured under NSC conditions upon overexpression of Bmi1 gave rise to NSC-like cells (NSCLC) (Moon *et al.*, 2008). These NSCLC were shown to express NSC marker genes including nestin, CD133 and Sox2; could self renew and, differentiate into neuronal and glial lineage *in vitro* (Moon *et al.*, 2008). Whether these cells could initiate tumourigenesis is to date unknown. However, BMI1 copy numbers are increased in gliomas, especially in high-grade astrocytomas, suggesting that overexpression of BMI1 may contribute to brain tumour pathogenesis (Hayry *et al.*, 2008a).

1.4.3 NSC and BTSC: What do they have in common?

A subpopulation of cells termed BTSC has been isolated from several tumours including gliomas (Ignatova *et al.*, 2002, Hemmati *et al.*, 2003, Lee *et al.*, 2006), medulloblastomas, astrocytomas (Hemmati *et al.*, 2003, Singh *et al.*, 2003) and ependymomas (Taylor *et al.*, 2005). These cells express stem cell markers, among others Musashi-1, Sox2, nestin, MELK and CD133 (Galli *et al.*, 2004, Singh *et al.*, 2004a) and were shown to be able to re-initiate tumourigenesis; in fact as few as 100 of these cells gave rise to tumours when injected into immunodeficient mice and retained their tumour phenotype upon serial transplantation (Singh *et al.*, 2004a).

In the light of these findings, a brain tumour can be viewed as a renewing system with a defined cell hierarchy, in which quiescent but indefinitely self-renewing

BTSC give rise to a limited number of transit-amplifying neoplastic progenitors that have a limited proliferation potential, which eventually will give rise to terminally differentiated neoplastic cells (Alvarez-Buylla and Temple, 1998, Reya *et al.*, 2001). This hypothesis, so called hierarchical model of tumourigenesis, predicts that the bulk of the tumour is composed of functionally heterogeneous cells; some with a limited proliferative capacity; others with the ability to proliferate extensively and initiate new tumours.

The similarities between normal NCS and BTSC has led to the hypothesis that a causal link might exist between the two and that endogenous NSC might give rise to brain tumours possibly through transformation into BTSC (Figure 1.7). Furthermore, it is also conceivable that BTSC are tumour cells regaining stem cell-like properties without being directly derived from NSC (Figure 1.7).

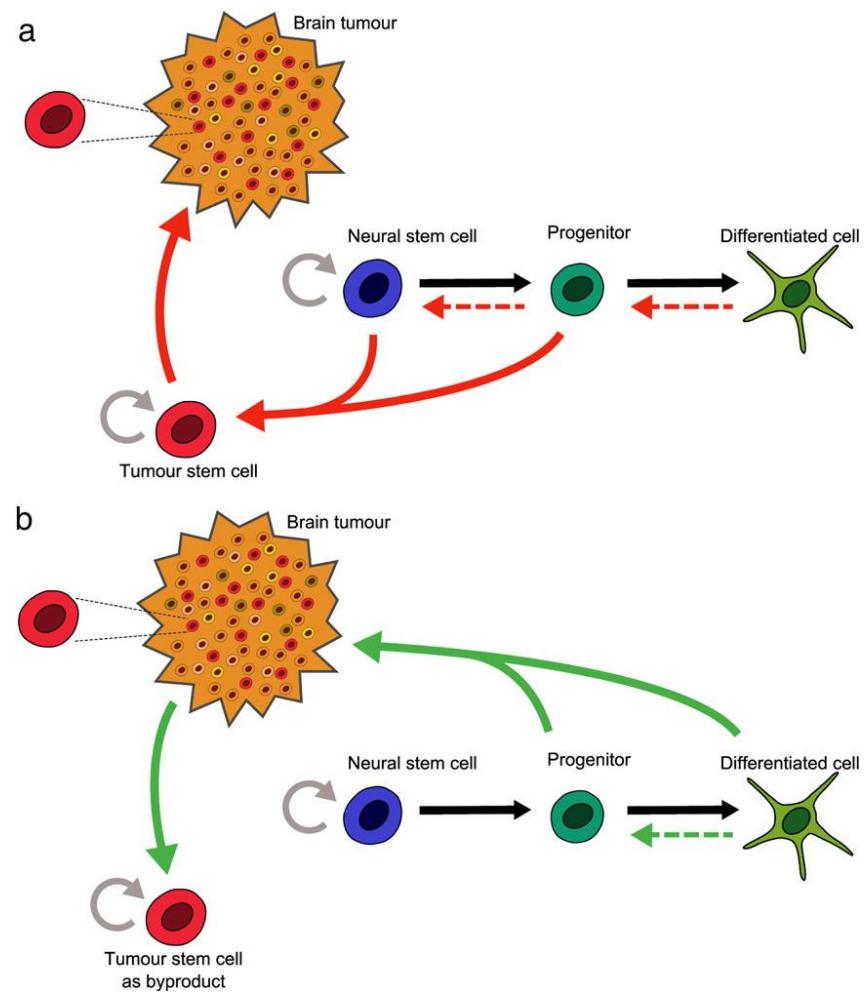


Fig. 1.7 Schematic representation to show the relationship between NSPC and BTSC, and brain tumour formation: a) Transformed NSC give rise to BTSC, which give rise to and maintain tumours; b) Progenitors or differentiated cells may be the origin of brain tumours that harbour BTSC. Dotted arrows indicate possible de-differentiation of cells that may occur before tumourgenesis whereas grey semi-circles signify self-renewal capacity of NSC and BTSC alike. The figure was adapted from Sutter *et al.*, *Biochim. Biophys. Acta*. 2007.

1.5 Bmi1, neural stem cells and brain tumour pathogenesis

The PcG gene Bmi1 has been demonstrated to be required for stem cell self-renewal and maintenance in mice. In addition, Bmi1 over-expression has been shown in human gliomas. Here I will describe the landmark findings that have shaped a role for Bmi1 in regulating neural stem cell functions and that have suggested a role for this gene in brain tumour pathogenesis.

1.5.1 Bmi1 and neural stem cells

Bmi1 is required for self-renewal and maintenance of peripheral and central nervous system NSC, and that the extend of its requirement increases over time, both *in vitro* and *in vivo* (Molofsky *et al.*, 2003). NSC isolated from Bmi1 deficient mice at developmental time points of E14.5, P0 and P30 have been shown to form significantly less and smaller neurospheres (Molofsky *et al.*, 2003). Moreover, to assess the effects of Bmi1 deficiency on proliferation of NSC, the authors have examined the rate of BrdU incorporation of SVZ cells at P0 and P30. They concluded that the rate of proliferation of Bmi1 deficient SVZ cells was significantly lower compared with normal mice. In addition, the reduced self-renewal capacity of Bmi1 deficient SVZ NSC leads to their postnatal depletion. Reduction in the rate of proliferation and self-renewal of Bmi1 deficient NSC were attributed in part to increased expression of the cyclin-dependent kinase inhibitor gene p16^{ink4a} as its inhibition partly reversed the previously noted self-renewal defects. Further, it was suggested that Bmi1 deficiency does not perturb differentiation and survival of PNS and CNS NSC. A study by Bruggeman *et al.*, (2005) has looked at the relative contribution of de-repression of p16^{ink4a} and p19^{Arf} in various cell types of Bmi1 de-

ficient mice, particularly the NSC (Bruggeman *et al.*, 2005). Importantly, this study has found that not only inhibition of p16^{ink4a} but also that of p19^{Arf} in Bmi1 deficient NSC was sufficient to partly reverse proliferation and self-renewal defects observed at various time points studied. Moreover, restoration of long-term self-renewal capacity (five of weekly passages- in this study) of Bmi1 deficient NSC was achieved through concomitant deletion of either p19^{Arf} alone or in combination with p16^{ink4a} in these cells. In addition to previously stated importance of p16^{ink4a} in conveying Bmi1 function, the significance of p19^{Arf} as a downstream target of Bmi1 in regulating proliferation and self-renewal of NSC was therefore stressed by this study (Bruggeman *et al.*, 2005). Moreover, Bruggeman *et al.*, (2005) has also highlighted that Bmi1 deficient neurospheres are multipotent but with a reduced efficiency compared with control spheres.

In contrast to the effects of Bmi1 deficiency on the proliferation and self-renewal of postnatal NSC through repression of Ink4a/Arf locus, it was noted that embryonic NSC did not seem to depend on Ink4a/Arf locus repression (Fasano *et al.*, 2007). Together these findings and observations did show the possibility that Bmi1 function in neural stem cells must be mediated through other developmental time point dependent downstream targets. Indeed, Fasano *et al.*, (2007) have identified repression of p21-Rb cell cycle regulatory pathway by Bmi1 as an important mechanism for regulation of proliferation and self-renewal during embryonic development. In their study Fasano *et al.*, (2007) have used lentiviral-mediated RNA interference at different developmental stages *in vivo* and *in vitro*. One striking observation was that acute reduction of Bmi1 expression by way of shRNA interference resulted in impairment of proliferation and self-renewal, in addition to in-

creased apoptosis of cortical NSPC as early as E11, and at E14 and E16. Interestingly, the expression levels of p16^{ink4a} and p19^{Arf} were unchanged at 48 hours post-treatment. Their analysis showed up-regulation of a member of the Cip/Kip family of cyclin-depended kinase inhibitors namely, p21/Clip1 upon shRNA mediated silencing of Bmi1 expression in cortical NSPC. Further gene expression analysis of these cells, particularly at E11, has shown that Bmi1 knockdown leads to changes in expression of genes such as Tcf4 and Id2, which can affect Rb function (Tetsu and McCormick, 1999, Lasorella *et al.*, 2005). The authors have thus concluded that acute knockdown of Bmi1 in cortical NSPC induces apoptosis, reduces proliferation and self-renewal by altering the p21/Rb pathway, in a developmental stage specific manner (Fasano *et al.*, 2007).

The same authors have shown the role of Foxg1 in mediating p21 dependent role of Bmi1 in embryonic NSC. The transcription factor Foxg1 is a member of the winged-helix gene family that is expressed incrementally in forebrain progenitors from development to adulthood, and is thought to be important for regional maintenance of NSC (Dou *et al.*, 1999, Shen *et al.*, 2006). In the study by Fasano *et al.*, (2009) Bmi1 was over-expressed in SVZ NSPC by means of transduction with lentiviral vector (Bmi1 Over), *in vitro* and *in vivo*. NSPC isolated at E11, E14, E16, P0 and adult were transduced with either Bmi1 Over or empty (EV) lentiviral vector. Then, plated in neurosphere-forming conditions and assessed for secondary neurosphere formation. They found that secondary neurosphere-formation frequency between Bmi1 Over and EV transduced NSPC were increased with advancing developmental stages, and with each passage (Fasano *et al.*, 2009). Further, the authors examined whether Bmi1 over-expression *in vivo* by injecting Bmi1 Over into E14 mouse lat-

eral ventricles *in utero* and electroporating into the developing cortex would stimulate progenitor proliferation. Whereas the Ev control transduced cells had followed the normal pattern of development with most cells migrating to cortical plate (CP) and acquiring a neuronal morphology, those transduced with Bmi1 Over remained in VZ and SVZ and a fewer had migrated into CP. These findings suggest that Bmi1 over-expression *in vivo* increases proliferation of VZ and SVZ NSPC and inhibit production of migration and neuronal differentiation, at least in comparison with the normal developmental pattern observed in controls (Fasano *et al.*, 2009). Expression analysis of Bmi1 Over transduced LeX positive cells (a surface marker that labels NSC and proliferative progenitors but not differentiated cells (Capela and Temple, 2006)) showed that Foxg1 expression is significantly increased. Conversely, knockdown of Foxg1 by shRNA reduces secondary neurosphere formation potential of Bmi1 Over transduced NSPC, suggesting that Foxg1 expression increased in parallel to Bmi1 expression and that it is required for Bmi1 regulated self-renewal of stem cells (Fasano *et al.*, 2009).

1.5.2 Bmi1 and brain tumour pathogenesis

The first line of evidence that identified Bmi1 as an oncogene came from studies of E μ -Bmi1 transgenic mice, which developed T and B cell lymphomas (Haupt *et al.*, 1993). Development of lymphomas was accelerated by concomitant over-expression of c-Myc mainly due to inhibition of Myc-mediated induction of p19^{Arf} and apoptosis, thus highlighting Bmi1 as an oncogene and establishing collaboration between the two oncogenes (Jacobs *et al.*, 1999b).

Studies in acute myeloid leukaemia (AML) have reinforced the role of Bmi1 as an oncogene and its role in maintenance of the cancer stem cells in these tumours. Lessard *et al.*, showed that BMI1 is required for self-renewal and proliferation of leukaemic stem cells, which is the fraction of the tumour that retains the ability to generate AML in the primary host in absence of BMI1, but fails to initiate tumour in secondary recipient without expression of BMI1 (Lessard and Sauvageau, 2003).

Interestingly, Bmi1 over-expression or/and gene amplification has been reported in several brain tumours such as medulloblastomas and gliomas (Leung *et al.*, 2004, Phillips *et al.*, 2006, Hayry *et al.*, 2008b). Over-expression refers to increased expression of a gene beyond physiological levels whereas amplification is a genetic aberration manifesting itself as increased copy number in the DNA. The study by Leung C *et al.*, (2004) showed that Bmi1 is over-expressed in cerebellar precursor cells during postnatal expansion of external granular layer (EGL) in mice and human. Moreover, BMI1 over-expression was noted in a substantial number of primary human medulloblastomas, a tumour thought to originate from aberrantly proliferating progenitor cells (Leung *et al.*, 2004).

A study by Hayry V *et al.*, (2008) investigated the genetic status of 100 low and high grade primary or recurrent gliomas for expression patterns of BMI1 using chromogenic *in situ* hybridisation (Hayry *et al.*, 2008a). Interestingly some of the tumours had BMI1 deletion but most particularly astrocytomas had increased copy number (3-5 copies). The authors reported that there were no significant differences in prognosis of cases where BMI1 gene was deleted or amplified. More recent studies have shown that BMI1 is required for maintenance and self-renewal

of GBM stem cells (Abdouh *et al.*, 2009). The study by Abdouh *et al.*, (2009) showed that CD133 positive stem cells isolated from glioblastomas expresses high levels of BMI1 and EZH2. Moreover, ablation of either of these oncogenes in GBM cell lines cultured as neurospheres inhibited their proliferation and self-renewal potential, independent of INK4A/ARF locus. Indeed, gene expression analysis of the shRNA treated GBM cell lines (using replication-incompetent lentiviruses) showed significant reduction in expression of stem cell associated genes such as CD133, MUSASHI, SOX2 and LHX2. Moreover, some of the transduced cell lines exhibited extensive apoptosis and differentiation, suggesting further that BMI1 is required for maintenance of CD133 positive GBM stem cells, through mechanisms independent of INK4A/ARF locus (Abdouh *et al.*, 2009).

A study by Bruggeman *et al.*, (2007) examined the role of Bmi1 in development and phenotype determination of gliomas, using an orthotopic model for gliomas where deficiency for Ink4a/Arf in combination with constitutively active mutant of EGF receptor (*EGFR) was induced either in NSPC or astrocytes (Bruggeman *et al.*, 2007). Primary astrocytes transduced with Ink4a/Arf^{-/-} *EGFR vectors produced tumours immediately when stereotactically intracranially injected into mice brain, where Bmi1^{-/-};Ink4a/Arf^{-/-} *EGFR cells went through a lag phase. Both tumours resembled human gliomas and developed into grade III-IV (oligo)astrocytomas or GBM. However, there were differences in the cellular composition and differentiation of tumours: the majority of Ink4a/Arf^{-/-} tumours were positive for progenitor marker Nestin, neuronal marker TuJ1, and astrocytic marker GFAP, whereas only half of Bmi1^{-/-};Ink4a/Arf^{-/-} tumours were positive for Nestin and only one expressed TuJ1 (Bruggeman *et al.*, 2007). These observations suggest that Bmi1 controls de-

velopment and composition of differentiated cells in gliomas through mechanisms independent of Ink4a/Arf locus. On the other hand, NSPC transduced with *EGFR and Bmi1^{-/-};Ink4a/Arf^{-/-} did not produce any GBM but only low grade gliomas. While all Ink4a/Arf^{-/-} tumours were positive for Nestin, only few of the Bmi1^{-/-};Ink4a/Arf^{-/-} tumours stained positive for Nestin (Bruggeman *et al.*, 2007). Together these findings suggest that malignant transformation of NSPC depends on Bmi1.

CD133+ BTSC isolated from GBM are resistant to all known therapies including irradiation, mainly because of DNA repair mechanisms through activation of DNA double strand response machinery (e.g., ATM) and nonhomologous end joining proteins (e.g., histone gamma-H2AX). The study by Facchino *et al.*, (2010) has shown that BMI1 co-localises with DNA damage response components in CD133+ BTSC, especially after gamma irradiation, thus boosting their resistance to therapy (Facchino *et al.*, 2010). Conversely, inactivation of BMI1 in these cells renders them more radiosensitive. These results may suggest that down-regulation of Bmi1 expression may be a viable approach to increase radiosensitivity of BTSC.

1.6 Transgenic mouse models and conditional control of gene expression

Developments in molecular biology have provided us with powerful tools to turn genes on and off at our discretion. Ability to alter genomic loci or insert foreign transgenes into mouse genome has facilitated the study of many complex biological processes such as embryogenesis and cancer. In the next section I will describe some of the strategies used to generate transgenic mouse models, and highlight the advantages of the Cre/loxP based system we have used in this study to analyse roles of Bmi1 in biological functions of embryonic and postnatal NSPC. We have

also used previously generated tumour prone conditional transgenic mice ($\text{PTEN}^{\text{LoxP}/\text{LoxP}}$ and $\text{p53}^{\text{LoxP}/\text{LoxP}}$), in combination with Bmi1 over-expression, to study effects of Bmi1 over-expression in brain tumour formation.

Conditioned by the availability of the technologies and purpose of study, different kinds of transgenic animals (knockout, conditional knockout, knock-in etc.) can be generated. A knockout mouse is derived from embryonic stem (ES) cells that have integrated a knockout construct (non-functional DNA sequence of the targeted gene) by homologous recombination, which is a method in which the native and foreign nucleotide sequences are exchanged. The construct is cloned into ES cells by using the recombinant DNA technology. With this technology the targeting construct replaces a specific segment of DNA in the ES cells, thereby replacing the functional allele. The knockout ES cells are then microinjected into normal mouse blastocysts where they mix with cells from the normal embryo to form a chimeric mouse which contains cells derived from both the host embryo and knockout ES cells. In principle, up to 100 percent of the resulting mouse chimera can be formed from cells derived from knockout ES cells. However, it is sufficient if the gonads of the chimeric mice are derived from knockout ES cells, as in this case mating the chimeric mice with wild-type mice would produce offspring with all cells heterozygous for the homologous recombination. Thereafter the knockout allele can be transmitted in a Mendelian fashion. However, not all biological processes can be studied by gene knockout or transgene expression strategies as many genes have different roles during embryogenesis and adulthood, and may result in embryonic lethality and preclude an analysis of its function in late development or adulthood. Moreover, it becomes impossible to determine roles of specific genetic alterations

on tissues and cells of interest as the resultant phenotypes from such genetic modifications could be due to cell autonomous, non-cell autonomous or systemic effects in the whole animal; or even a combination of these at various degrees of importance. One way to overcome these limitations is to use a binary genetic system, in which gene expression is controlled by the interactions of an ‘effector’ transgene and ‘target’ transgene. Such binary system is based on Cre/loxP, where Cre enzyme acts as the effector and the loxP recognition sequences are on the target transgene. This system allows for spatial and temporal control of gene activation in transgenic mice. In conditional transgenic mice, the conditional control of a particular gene is based on Cre enzyme, whose expression can be controlled by cell specific promoters.

1.6.1 Adenovirus-mediated Cre delivery is an efficient method to recombine loxP sites in NSPC *in vitro*

Since its discovery as a part of normal life cycle of P1 bacteriophage by Henderson and Hamilton (Sternberg and Hamilton, 1981, Sauer and Henderson, 1988), the Cre/loxP system has been extensively used to manipulate the cellular genome in cell culture systems, mice and other organisms. The Cre/loxP system consists of two components; the Cre enzyme and the loxP sites. The latter are introduced into the DNA of the organism of interest by conventional cloning strategies followed by electroporation or calcium precipitation into ES cells where homologous recombination will integrate the modified genetic sequence into the host DNA (Nagy, 2000). The Cre gene encodes for a 38 kD protein, Cre recombinase, that catalyses recombination between two loxP sites. loxP sites consists of a specific 34-base pair

(bp) sequences that can be divided into three functional regions; a central 8bp sequence, where recombination takes place, and two flanking palindromic 13 bp sequences (Nagy, 2000). At the molecular level, a single recombinase binds to palindromic halves of a loxP site and forms a tetramer by bringing two loxP sites together, mediating recombination at the central 8bp sequence (Figure 1.8) (Voziyanov *et al.*, 1999, Yu and Bradley, 2001). Whether this recombination results in a deletion, inversion or chromosomal translocation of intertwining sequences is determined by the orientation, location and copy number of loxP sites (Figure 1.9) (<http://cre.jax.org/introduction.html>).

Delivery of Cre into the cells of interest can be achieved by several means both *in vivo* and *in vitro*. Previous studies have shown that Cre/loxP system functions efficiently in eukaryotic organisms and mammalian cells (Smith *et al.*, 1995). Among other systems, adenoviral vectors constructed to express Cre recombinase have previously been used to mediate loxP-dependent recombination in various tissues and cells *in vitro* (Anton and Graham, 1995). Adenoviruses with deleted E1 and therefore defective in replication could be propagated in Human Embryonic Kidney 293 (Hek293) cells and grown to high titres, and yet retain their properties to infect a wide variety of cells (Addison *et al.*, 1997). These recombinant Adenoviruses can infect both proliferating and quiescent cells and once in the cytoplasm, release their virions, which are transported into nucleus and replicate using the host's replication machinery (Anton and Graham, 1995).

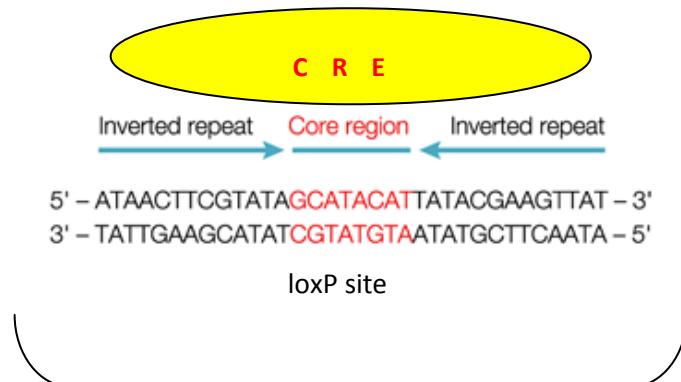


Fig 1.8 Schematic representation of Cre/loxP system: One Cre recombinase binds to 13 bp inverted repeat sequences of LoxP sites and catalyses recombination at the core region. The modified figure was originally adopted from Yuejin Yu & Allan Bradley Nat. Rev. Genetics, 2001.

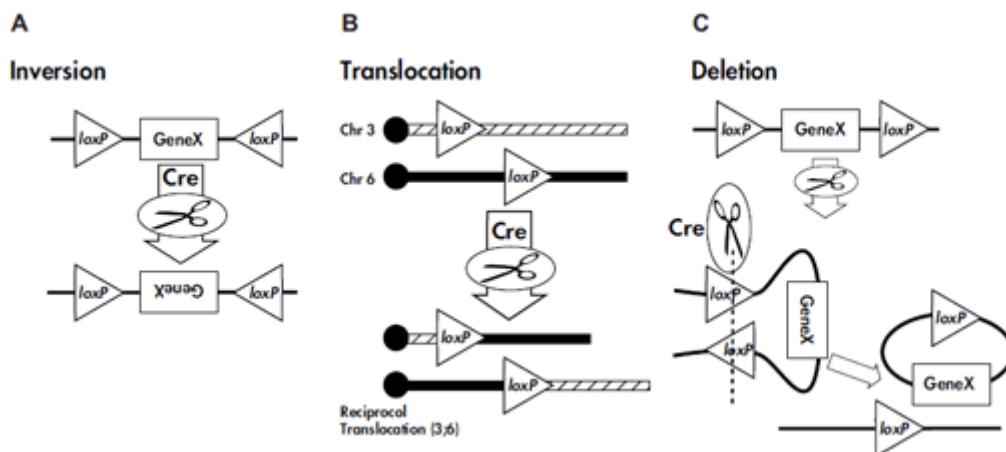


Fig 1.9 Three possible outcomes of a Cre-lox recombination: A) Inversion of the floxed segment, when the LoxP sites are oriented in opposite directions, B) Chromosomal translocation when the LoxP sites are located on different chromosomes, C) Deletion of the floxed segment when the LoxP sites are oriented in the same direction on a chromosome segment. Diagram was adopted from the site, <http://cre.jax.org/introduction.html>, in July 2010.

1.7 Hypothesis

Bmi1 is a member of the Polycomb group (PcG) gene family of chromatin modifiers and transcriptional repressors, the essential role of which in development and homeostasis of the CNS was first highlighted by studies on knockout mice. *Bmi1*-/- mice displayed impaired self-renewal and maintenance of SVZ NSC leading to their postnatal depletion. The molecular mechanisms of Bmi1 function in postnatal NSC are mediated in part by transcriptional repression of the *ink4a* locus. This locus encodes two cell cycle inhibitors, *p16^{Ink4a}* and *p19^{ARF}*, the activities of which increase with postnatal age and have been linked to cellular senescence. However, concomitant deletion/suppression of *p16^{Ink4a}* or *p19^{ARF}* or both does not completely rescue the above mentioned defects in self-renewal of NSC derived from *Bmi1*-/- mice.

Another cell cycle inhibitor, namely *p21^{WAF1/Cip1}*, contributes to mediating Bmi1 function in developmental contexts. Other NSC functions, such as migration, have also been shown to be controlled by Bmi1 in an *ink4a* independent manner.

Increased expression of BMI1 has been demonstrated in humans, not only in haematological malignancies, such as high grade lymphomas, but also in neural tumours, e.g. medulloblastomas and neuroblastomas. A tight regulation of Bmi1 expression might therefore not only be crucial for maintaining a balance between self renewal and differentiation or senescence of NSCs, but it may also play a role in CNS tumourigenesis, when deregulated.

By taking into account the essential roles of Bmi1 in NSC, I hypothesised the following:

- 1) Over-expression of Bmi1 in NSC will increase their proliferation and self-renewal capacity through suppression of cell cycle inhibitors such as $p16^{Ink4a}$, $p19^{ARF}$ and $p21^{WAF1/Cip1}$.
- 2) Over-expression of Bmi1 in NSC will confer resistance to apoptosis by reducing Mdm2-mediated ubiquitin dependent degradation of p53 (possibly because of reduced $p19^{ARF}$ expression).
- 3) Bmi1 over-expression mediated increased proliferation and self-renewal together with resistance to apoptosis makes NSC a potential origin of brain tumour(s) in mice.
- 4) Bmi1 over-expression in NSC will reveal novel gene targets involved in regulation of stem cell function *in vitro* and *in vivo*.

1.8 Aims

- To determine whether Bmi1 over-expression in NSPC at different developmental time points would alter their biological properties such as self-renewal, apoptosis and differentiation.
- To identify molecular changes associated with Bmi1 over-expression in NSPC.
- To determine whether Bmi1 over-expression in SVZ NSC is sufficient for tumourigenesis in mice.

Chapter 2— Materials and Methods

2.1 Generation, maintenance, breeding and genotyping of genetically modified mice

2.1.1 Conditional Transgenic Mouse Model with Double Reporter System

The Cre/loxP system takes advantage of the P1 bacteriophage Cre recombinase, a 38 kDa enzyme that catalyses specific excision of DNA between 34 base pair (bp) recognition sequences called loxP and consecutive ligation of the remaining DNA (Hamilton and Abremski, 1984, Nagy, 2000).

A modified version of the pccall2 construct (Lobe *et al.*, 1999), where the human placental alkaline phosphatase gene was replaced with enhanced green fluorescent protein (EGFP) (pCCALL2 vector (Novak *et al.*, 2000), was chosen to drive conditional expression of Bmi1. The pCCALL2-Bmi1 plasmid contains pCCAG promoter region (Niwa *et al.*, 1991) composed of CMV (cytomegalovirus) enhancer and β -actin promoter sequences; the first reporter β geo (a fusion of LacZ and neomycin coding sequences) followed by three copies of the SV40 polyadenylation sequence, which are flanked by LoxP sites; the cloning site, which is separated from the second reporter EGFP by the intermediate internal ribosome entry site (IRES) sequence, followed by three copies of the SV40 polyadenylation sequence (Figure 2.1). The full-length murine Bmi-1 cDNA was cloned into the cloning site of this modified version of the pCCALL plasmid.

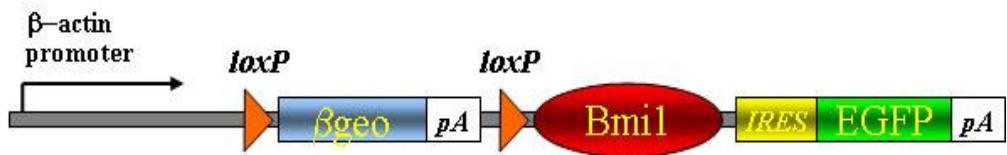


Fig. 2.1 Schematic representation of the pCCALL2-Bmi1 construct:
The STOPFloxBmi1 mouse line was generated by electroporation of this construct into TC-1 mouse embryonic stem cells.

Briefly, the pCCALL2-Bmi1 vector was electroporated into TC-1 mouse embryonic stem cells. Selection for neomycin resistance was applied and resistant clones were selected and expanded (Figure 2.2, A). Clones with high level LacZ expression were further screened for single transgene integration using Southern Blot analysis (Figure 2.2, B), a method widely used to detect specific DNA sequences in DNA samples. Four ES cell clones that had single copy integration were selected for further analysis and electroporated with a Cre recombinase expression vector (pOG231) carrying a puromycin resistance gene to test whether Bmi1 overexpression could be induced. Upon antibiotic selection, resistant clones were chosen, amplified and further screened for β -galactosidase activity. LacZ negative clones were further analysed by Western blot and the level of Bmi1 expression was assessed as compared to the baseline level prior to recombination and to the expression level of a medulloblastoma sample (Figure 2.2, C).

Clones IB5 and IE1 were selected for injection into blastocysts to generate chimeric mice. Germline transmission and line establishment was achieved for the clone IB5, from now on defined STOPFloxBmi1.

The expression pattern of STOPFloxBmi1 mice was assessed by LacZ expression analysis both in embryonic and postnatal brains. At embryonic day 16.5 (E16.5) robust LacZ expressions was seen in most tissues (Figure 2.2, D), but interestingly in all layers of the developing neocortex, in the external granular layer of the cerebellum and in scattered cells in the deep cerebral and cerebellar white matter (Figure 2.2, E). At postnatal day 7 (pd7) and p20 (not shown), LacZ expression was seen in the cerebral cortex, in the hippocampus, in the basal ganglia and in the subventricular zone (SVZ) (Figure 2.2, F). LacZ expression in the ventricular zone (VZ) at E16.5 of STOPFloxBmi1 was also analysed by X-gal staining (Figure 2.2, G). And that crossing between NestinCre and STOPFloxBmi1 mice generates double transgenic embryos (NestinCre;STOPFloxBmi1) that have no LacZ expression in the VZ at E16.5; indicating successful recombination of lox-P sites and loss of the first reporter gene (Figure 2.2, H).

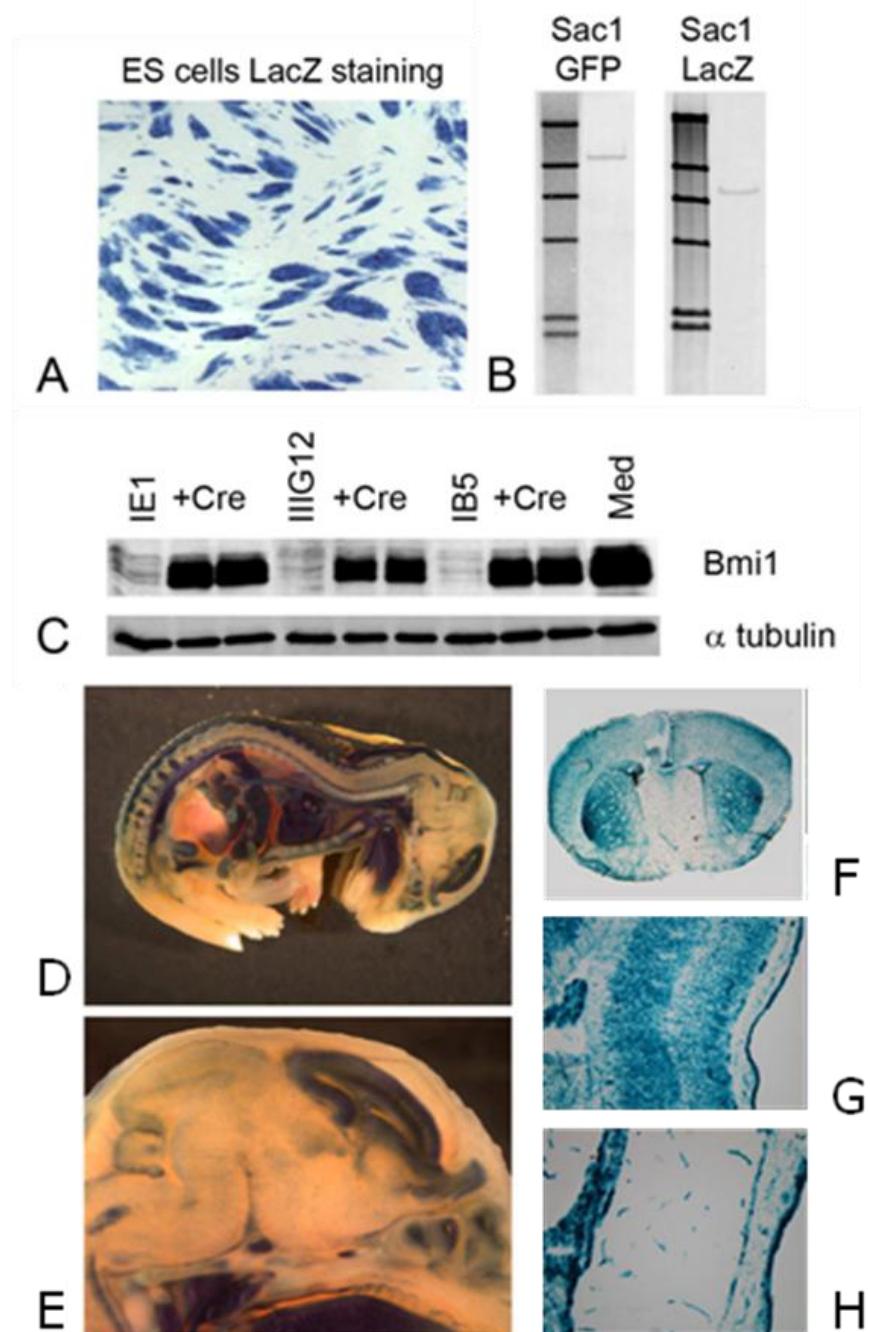


Fig. 2.2 Verification of the pCCALL2-Bmi1 vector integration into ES cells, and assessment of its functionality *in vitro* and *in vivo*: A) Neomycin resistant ES cells with LacZ expression B) Confirmation of single copy integration (Southern Blot) C) Over-expression of Bmi1 in ES cells upon Cre-mediated recombination (Western Blot). D) LacZ expression in all tissues of the STOPFloxBmi1 whole embryo cross section at E16.5 E) LacZ expression throughout the developing cortex and VZ at E16.5 F) LacZ expression in the SVZ at P7 G) High magnification showing LacZ expression in the VZ of STOPFloxBmi1 embryo at E16.5 H) Loss of LacZ expression in the VZ of NestinCre;STOPFloxBmi1 embryo at E16.5. These experiments were performed by Olga Shakhova.

2.1.1.1 Nestin Cre mediated recombination of LoxP sites in NSPC

The Cre/loxP system has been used in transgenic mouse lines to enable targeted/conditional gene activation or deletion in specific tissues and cells *in vivo* taking advantage of the specificity of a given promoter (Ray *et al.*, 2000, Lewandoski, 2001, Dubois *et al.*, 2006). Briefly, the system requires cross-breeding between two transgenic animal lines: one line carries an allele of interest flanked at the 5' and 3' ends by loxP sequences whereas the other line carries a Cre transgene, the expression of which is regulated by a heterologous promoter. Recombination of the loxP sites in the littermates is determined by the expression pattern of the Cre transgene.

To activate Bmi1 expression in NSPC *in vivo* we have used a Nestin-Cre transgenic mouse line where Cre mediated recombination of loxP sites occurs in all cells expressing nestin (Dubois *et al.*, 2006). Nestin is an intermediate filament protein first identified in neuroepithelial stem cells, which is widely used as a marker for NSPC during development and adult tissues (Wiese *et al.*, 2004). The transgenic construct (Nes-Cre) used to generate this line consists of the rat nestin gene excluding the transcriptional start site, followed by modified Cre gene carrying a nuclear localization signal and actin-polyA sequence, and finally the complete intron 2 of the rat nestin gene (Figure 2.3, Dubois *et al.*, 2006).

In order to study the efficiency and spatiotemporal expression pattern of Nes-Cre, Dubois and co-workers crossed Nestin-Cre males with R26R females (Dubois *et al.*, 2006). The latter line is a commonly used reporter line, where the LacZ gene is expressed upon Cre mediated deletion of a floxed stop cassette. During embryonic

development, Cre expression and therefore recombination was detected in the neuroectoderm as early as day E7; at E12 it became detectable in the VZ where it reaches its peak by day E14.5 and E15.5 (Figure 2.4) (Kawaguchi *et al.*, 2001, Dubois *et al.*, 2006). Similarly, expression of Cre driven by the Nestin promoter was shown from E11.5 onwards in the developing neocortex (Dubois *et al.*, 2006).

Indeed, although nestin is expressed in a variety of cell types during development and in adult tissues, it is widely recognised as a marker for NSPC (Wiese *et al.*, 2004). The second intron of both rat and human nestin gene has been shown to be sufficient for both midbrain and general CNS expression (Dubois *et al.*, 2006).

In this study, this line will be used to generate embryonic and postnatal NSPC which have been exposed to over-expression of Bmi1 since E11.5 of embryonic development.



Fig. 2.3 Schematic representation of the Nes-Cre construct: The mouse line termed Nestin-Cre, which was used in this study to activate over-expression of Bmi1 in STOP^{Flox}Bmi1 transgenic line, carry this construct. (Adopted from Dubois *et al.*, 2006)

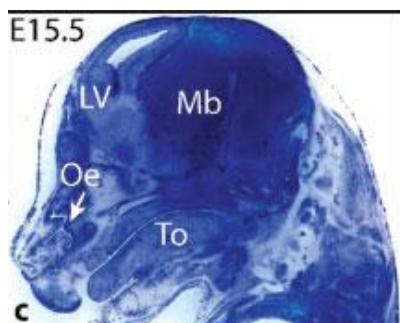


Fig. 2.4 Nes-Cre mediated recombination pattern during late embryogenesis: Compound hemizygous embryos carrying paternally inherited Nes-Cre and Z/AP (the latter has the expression of LacZ before, and alkaline phosphatase (AP) after loxP recombination (Lobe *et al.*, 1999). Whole mount AP staining of embryo section shows that Nes-Cre mediated recombination of LoxP sites occur in lateral ventricles (LV) and midbrain (Mb) in addition to other sites (Adopted from Dubois *et al.*, 2006).

2.1.2 Maintenance of Mice

The full range of genetically modified (GM) mouse lines and normal mouse bred for use in in-vitro and in-vivo experiments throughout this project include; STOP-FloxBmi1, Nestin-Cre, Rosa26Lac-Z, PTEN^{LoxP/LoxP}, p53^{LoxP/LoxP}, NOD/SCID and normal C57Bl/6 mice. All mice were maintained at the Biological Sciences Unit (BSU), and animal care and procedures were carried out in accordance with guidelines and protocols approved by the United Kingdom Home Office. Standard polymerase chain reaction (PCR) methods were used to determine the genotype of compound litter mates/embryos produced from different crossings made between the above mentioned mouse lines.

The genetic background of the mice selected for breeding is always important especially in the case of knockout and transgenic mice. For genetic experiments, as in the case in this study, failure to choose breeders of the appropriate genetic background would eventually result in unwanted although often subtle changes in the characteristics of the mice. A strain is defined as inbred if it was obtained by sibling matings for more than 20 generations, after which all offspring are considered genetically identical. With inbred mice, the goal is to preserve genetic uniformity in the colony. With most strains, this involves nothing more than mating males and females of the same strain. The only precaution is to avoid generation of a sub-strain or genetic drift, which occurs when a closed colony of inbred mice is isolated for many generations. A substrain may differ genetically from the original inbred strain and may yield different results when used in research. To avoid this, it is sufficient to introduce outsiders from the same inbred strain on a regular basis.

C57BL/6 is the most widely used inbred strain. It is commonly used as a general purpose strain and background strain for generation of transgenic mice. Although this strain is refractory to many tumours, it provides a permissive background for maximal expression of most mutations. C57BL/6 mice breed well, are long-lived and are used in a variety of research areas including developmental biology, diabetes and obesity, immunology and neurobiology. For convenience and advantages aforementioned C57BL/6 mice were used for production of STOPFloxBmi1 conditional transgenic mice.

2.1.3 Breeding and Time mating

The following compound genotypes were used: NestinCre/STOPFloxBmi1; PTEN^{LoxP/LoxP}/STOPFloxBmi1 and p53^{LoxP/LoxP}/STOPFloxBmi1. Compound mice were kept homozygous for all alleles and in-bred within the same colony.

Depending on the project's progress, mice from E16.5, p7 and p70 were used for experiments. Timed mating between NestinCre male and STOPFloxBmi1 female mice was set up to obtain embryos at the desired developmental timepoint. Every morning each female was examined for presence of a vaginal plug, which is composed of coagulated secretions from male sex glands, and indicates that coitus and ejaculation had occurred. Then the female is placed in a separate cage and considered pregnant for 0.5 day. 16 days later, female mouse is sacrificed and embryos removed; decapitated and brains placed in ice cold HIB buffer, while tail tipping performed for isolation of genomic DNA. Brains were individually treated: meninges removed and whole telencephelon processed for isolation of neural stem and progenitor cells, which were plated at clonal density in culture medium.

Meanwhile, PCR is performed on genomic DNA using primers for EGFP and Cre to identify compound and single transgenic embryos. This allowed for selection of the cultures to be used in our experiments. While NSPC from single transgenic embryos (STOPFloxBmi1 or Nestin-Cre) were used as control, those of compound transgenic (NestinCre;STOPFloxBmi1) were treated as experimental material.

2.1.4 Genotyping: From DNA Isolation to Amplification of Gene of Interest

A local anaesthetic, EMLA cream (supplied by the BSU), was applied to tails of P5 litter mates, or ears of P70 mice for 15 minutes before they were excised (approximately 0.5 millimetres) using a sculpture for the former and a sharp scissors for the latter, whereas for E16.5 embryos the tail samples were taken post-decapitation. As their processing and use in the rest of the procedure is the same, tail or ear samples will be referred to as tissue samples hereafter.

Tissue samples were digested in 500 microlitres (μ l) of lysis buffer (50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS) and Proteinase K (Biolabs) on a thermomixer compact (Eppendorf), for two-three hours at 55° C and mixing at 1000 units per minute (upm). Upon full digestion, samples were centrifuged (Thermo Scientific, Heraeus Fresco 17) at 13000 (13k) revolutions per minute (rpm) for ten minutes at room temperature (RT), and the supernatant (SN) was transferred into correspondingly labelled eppendorfs tubes containing 500 μ l isopropanol-2 (Fisher Scientific) to precipitate deoxyribonucleic acid (DNA). Samples were then centrifuged again under the same conditions as above, supernatants decanted and tubes drained inverted on a paper towel. To remove salts away from the pelleted DNA, a final wash with 70% ethanol was performed before DNA was

dissolved in 50-100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), on the thermomixer compact for 30 minutes at 55° C and mixing at 1000 upm. Dissolved DNA was stored at 4° C.

Standard PCR methods based on denaturation, annealing and extension were used to amplify DNA segment(s) of interest (amplicon), so that genetically modified mice could be distinguished from non transgenic littermates.

Table 2.1 provides predominantly used pipetting scheme and range of final concentration(s) for Taq polymerase based PCR procedure, though minor changes were applied whenever required:

Table 2.1: Pipetting scheme for PCR-assay for genotyping

Reagent	Quantity (µl), 25µl reaction volume	Final concentration
H ₂ O	13.65 -14.15	
Enzyme Buffer Thermo Pol (10 x conc.)	2.5	1X
dNTP mix (4mM)	2.5	0.4 mM of each
Upstream primer (10 pM/µl)	2.5	0.1 µM
Downstream primer (10 pM/µl)	2.5	0.1 µM
Taq DNA polymerase (5 u/ml)	0.35	1.75 U
Template DNA (µg/µl)	0.5 - 1	

A list of primer sequences used is given below in Table 2.2, some of which were commercially available, whereas others were designed (*) *de novo*. Primers were designed using both the mouse genomic sequence provided online at

http://www.ensembl.org/Mus_musculus/index.html and the program named primer3 available online at <http://frodo.wi.mit.edu/>. Criteria used for primer selection were based on length, sequence and melting temperature: the primer length between 18-24 nucleotides; the Guanine (G)/Cystine (C) content ratio between 50% and 50%; the 3' ends free of secondary structures, repetitive sequences, complementary sequences, palindromes and highly degenerate sequences; and similar melting temperatures between 55° C and 65° C.

Table 2.2: Primer sequences used for genomic DNA

Company	Name	Sequence (5'-3')	Species	°C (T _m)	bp
MWG-Biotech	LZ 1*	-CGTCACACTACGTCTGAACG-	<i>Mus musculus</i>	58	500
MWG-Biotech	LZ 2*	-CGACCAGATGATCACACTCG-	<i>Mus musculus</i>	58	500
MWG-Biotech	EGFP* Fwd	-CCTACGGCGTGCAGTGCTTCAGC-	<i>Mus musculus</i>	65	300
MWG-Biotech	EGFP* Rev	-CGCGAGCTGCACGCTGCGTCCTC-	<i>Mus musculus</i>	65	300
MWG-Biotech	TAG-83F	-GGAGGAGAGAGACCCCGTGAAA-	<i>Mus musculus</i>	65	500
MWG-Biotech	TAG-82B	-ACACGAAGTGACGCCATCCGT-	<i>Mus musculus</i>	65	500
MWG-Biotech	Cre Fwd*	-CTATCCAGCAACATTGGGCCAGC-	<i>Mus musculus</i>	65	700
MWG-Biotech	Cre Rev*	-CCAGGTTACGGATATAAGTCATGAC-	<i>Mus musculus</i>	65	700

Table 2.3: PCR programs used for genotyping

PCR Programs Optimised for Primers	Denaturation (° C)	Primer annealing (° C)	Extension (° C)	Number of Cycles	Final Extension (° C)
EGFP	94 for 5 min	65 for 5 min	72 for 1 min	35	72 for 5 min
CRE	94 for 5 min	65 for 5 min	72 for 1 min	35	72 for 5 min
TAG	94 for 5 min	65 for 5 min	72 for 1 min	40	72 for 5 min
LZ	94 for 5 min	58 for 5 min	72 for 50 sec	35	72 for 5 min

The PCR amplification products were run according to standard agarose electrophoresis methods and visualised by ultraviolet (UV) light: 1.5 µl 4X loading dye (Sigma) were added to each reaction, they were run on a 2 % TAE (Tris-Acetate-EDTA) agarose gel containing 0.01 % ethidium bromide (EtBr) at 120 volts for 30 minutes. Furthermore, the PCR programs given above in Table 2.3 were optimised for each primer and reactions were run on a standard PCR machine (eppendorf).

2.2. Isolation and culturing of murine embryonic and postnatal NSPC

The isolation and culturing of NSPC was undertaken essentially in accordance with previously described methodologies (Lois and Alvarez-Buylla, 1993) (Gritti *et al.*, 1999). Here, I will describe essential details of how NSPC were isolated, cultured

and expanded in preparation for functional assays to characterise their growth and differentiation properties *in vitro*.

2.2.1 Dissection of the developing neocortex at E16.5 and of the subventricular zone at P7 and P70.

E16.5 embryos and P7/P70 mice were culled by decapitation and heads placed immediately in a Petri dish containing ice cold HIP buffer. For E16.5 embryos, the fibrocollagenous skull structures were removed with forceps, the brain was released and the neocortex was dissected and placed in a Petri dish containing ice cold HIP buffer. For P7/P70 mice, the skin was removed from the head using small scissors. A superficial coronal cut was made at the junction between the nasal and frontal bones using small scissors. Curved scissors were used to gain access to the brain, via a longitudinal cut through the skull along the sagittal suture. The skull was then peeled off using curved forceps. The head was inverted and, optic and trigeminal nerves as well as hypophysis/hypothalamus stalk were cut using forceps to allow the brain to be detached. The brain was then placed in a Petri dish containing ice cold HIP buffer. An approximately 2-3 mm thick frontoparietal slice was obtained, as depicted in Figure 2.5, A and B. In brief, the olfactory bulbs rostral to incision (a) were removed as well as the brain regions caudal to incision (b). The SVZ, which is located in the lateral wall of the lateral ventricles from the crossing of the anterior commissure to the rostral opening of the third ventricle, was isolated by cutting a strip of tissue approximately 1-2 mm wide and 2-4 mm long along the lateral wall of the lateral ventricle underneath the corpus callosum.

The tissue of interest, embryonic neocortex or SVZ region, were then homogenised using a tissue chopper before further processing.

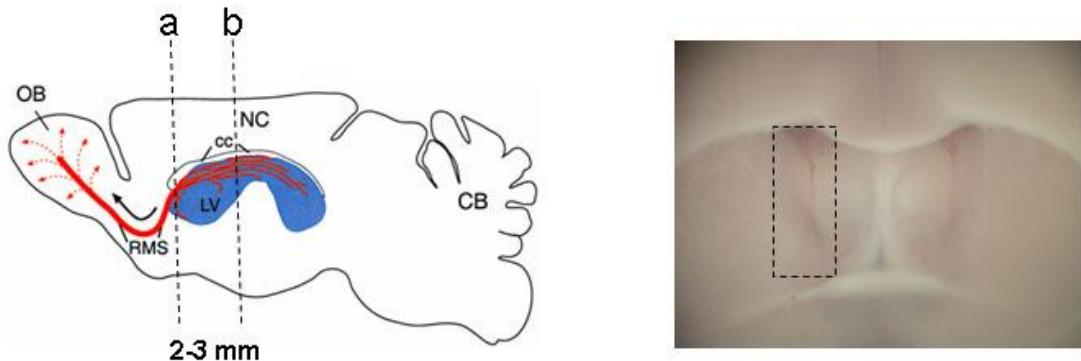


Fig 2.5 Dissection of the SVZ from postnatal brains A) Schematic representation of the cut up strategy to isolate SVZ from the rest of the brain. B) Macroscopic view of the adult brain slice obtained, dotted square indicate the area used for NSPC preparation.

2.2.2 Isolation of NSPC

The Papain Dissociation System (PDS) (Worthington) based on the proteolytic properties of the enzyme papain (sulphydryl protease from *Carica papaya* latex), was used to isolate NSPC from the brain homogenate. Tissue homogenate was placed in a Petri dish containing papain solution at 37° C for 40 minutes (embryonic neocortex) or 1 hour (postnatal SVZ). The homogenate mixture was further dissociated into single cell suspension by trituration using a 1 ml syringe with 21 gauge (21G) needle. The cell suspension was transferred into a 15 ml falcon tube and centrifuged at 600 rpm for 6 minutes. Supernatant was removed and pellet

immediately re-suspended in DNase dilute albumin-inhibitor solution which, in turn, was carefully transferred into a 15 ml tube containing albumin-inhibitor solution that formed the discontinuous density gradient. Following centrifugation for five minutes at 500 rpm, dissociated NSPC and other cells formed the pellet, whereas the membrane fragments and debris remained at the interface between the two layers of the gradient. Supernatant was removed and pellet re-suspended in 1 ml DMEM/F12 (Invitrogen) supplemented with 1X B27 (Invitrogen), and the mitogens epidermal growth factor (EGF, 20 ng/ml) and fibroblast growth factor (FGF, 20 ng/ml).

2.2.3 Trypan Blue exclusion test to determine number of cells in the cell suspension

Number of cells was determined by Neubauer's improved counting chamber (VWR). 10 μ l sample of the cell suspension obtained was mixed with 10 μ l of Trypan Blue Solution (dye). 10 μ l of this mixture was applied to Neubauer's improved counting chamber, and all viable cells (trypan blue negative) in each of the outer primary squares ($4 \times 1\text{mm}^2$) were counted at the light microscope.

Cell number was determined using the formula below:

$$\text{Number of cells in suspension} = \text{mean no. of cell count} \times 2 \text{ (dilution factor)} \times 10^4 \text{ ml}^{-1}$$

$2 \times 10^4 \text{ ml}^{-1}$ cells (postnatal SVZ) and $3 \times 10^4 \text{ ml}^{-1}$ cells (embryonic neocortex) were seeded in each well of a 6-well plate (9.6 cm^2) in two ml culture medium. The relationship between the growth area and plating densities for postnatal and embry-

onic NSPC is then 4000 and 6000 per cm² respectively, in keeping with what commonly described in the literature (Ferron *et al.*, 2007).

2.2.4 Culturing of NSPC with the neurosphere assay.

In agreement with previously described work (Reynolds and Weiss, 1992), free-floating clusters of stem cells and progeny (so called neurospheres) arose between 5 and 7 days in culture (DIC) in growth factor supplemented medium. These primary neurospheres (approximately 180-200 micrometers in diameter) were transferred into a 15 ml falcon tube and centrifuged at 500 rpm for 3 minutes. Supernatant was removed, and the pellet was re-suspended in 1 ml fresh culture medium. Neurospheres were dissociated by gentle trituration approximately 20 times using a 1 ml syringe with 21G needle. Cell number was determined as described above and cells were plated at the same density under the same culture conditions to allow for formation of secondary neurospheres. Similar procedure was used for dissociation and replating of secondary NS to tertiary NS, etc. (sequential passages).

Both embryonic and postnatal NSPC were dissociated and replated for at least two passages before assessment of cellular properties were carried out to ensure elimination of cellular debris and of differentiated cells.

2.3 Adenoviral Vectors and the induction of Bmi-1 Overexpression *in vitro*

Two different replication-defective adenoviruses were used: one expressing green fluorescent protein (GFP) and other expressing Cre recombinase. Both viruses

Adeno-GFP (A-GFP) and Adeno-CRE (A-CRE) were gifts of Professor Anton Berns, Molecular Genetics, The Netherlands Cancer Institute, Amsterdam.

2.3.1 Amplification and titre determination of Adenoviruses

The batches of adenovirus that were available in the laboratory (stock Adeno-CRE 1×10^9 pfu/ml and Adeno-GFP 1×10^8 pfu/ml) could be expanded using Human Embryonic Kidney 293 cells (Hek-293) (Clonetech #C3202-1). Hek-293 cells were plated in 150 cm^2 Petri dishes, in DMEM (high glucose)/10% FCS/1% penicillin/streptomycin/2 mM Glutamine, 24 hours before infection at a density to ensure that the monolayer of cells reach 50-70% confluent on the day of infection. Cell number has been estimated to be 2×10^7 per dish. Cells were infected with M.O.I. > 5 of both viruses. Cells were then incubated for 90 minutes at 37° C , 5% CO_2 , and dishes were briefly swirled every 30 minutes to ensure maximum contact between virus and cells. Virus-containing media was then discarded in a bottle containing 1% virkon, and 20 ml fresh growth media added. Cells were incubated for 2-3 days at 37° C , 5% CO_2 until cytopathic effect (detachment of approximately 50% of cells) was observed. The rest of the cells were dislodged by gentle agitation and all were pulled in 50 ml conical tubes. Cell suspension was centrifuged at 1500 g, for 5 minutes at RT. Pellet was re-suspended in 1 ml PBS and cells were lysed with 3 consecutive freeze-thaw cycles. After the third cycle, cells were briefly centrifuged to pellet debris, and aliquots of suspension containing virus was prepared.

Next, Plaque Assay was performed to determine the plaque forming unit (pfu) of each virus. For this, Hek-293 cells were plated in 6 well plates at a density of 4×10^5 cells/ml, 24 hours before the procedure. 5 % agarose was prepared by dissolving

2.5 gram Seaplaque Agarose in 50 ml Dulbecco's Phosphate Buffered Saline (DPBS) (pH 7). Serial dilutions, from 10^{-1} to 10^{-10} , of the virus were prepared with sterile DPBS. When the cell density had reached 80-90 % confluence, growth media was removed and 0.2 ml adenovirus dilutions of interest (10^{-3} to 10^{-10}) were added to wells. Cells were incubated for 1 hour at 37° C, 5% CO₂ to allow viral infection. Meanwhile, 0.5 % agarose solution was prepared by mixing 45 ml pre-warmed growth media with 5 ml of 5 % stock agarose. The virus inoculum was removed and 2 ml of 0.5 % agarose solution was added to each well. Agarose was allowed to set for about 5-10 minutes at RT before the plate was incubated for 5-7 days at 37° C, 5% CO₂. Then, 1 ml of neutral red solution (0.03 %) was added to each well, and the plate was incubated for 2-3 hours. Neutral red is taken up by the healthy cells, and therefore plaques appear as clear circles against red or pink background. Number of plaques for each well was counted using the aid of a custom made grid. The viral titre, a quantitative biological activity of the virus expressed as pfu/ml is calculated using the following formula:

Pfu/ml= no of plaques/ (dilution factor) x (volume of virus dilution added to each well)

The plaque forming unit (pfu), which is a measure of the strength or concentration of a component/particle in a given volume, was calculated to be 2.5×10^9 pfu/ml and 1.5×10^8 pfu/ml for A-CRE and A-GFP respectively.

2.3.2 Determining the Multiplicity of Infection (MOI) of virus needed for optimal lox-P recombination in NSPC

The plaque forming unit (pfu), which is a measure of the strength or concentration of a component/particle in a given volume, was estimated to be 2.5×10^9 pfu/ml and 1.5×10^8 pfu/ml for Adeno-Cre and Adeno-GFP respectively.

NSPC were infected on the next day following dissociation of primary neurospheres either with A-CRE, to enable Bmi1 over-expression by Cre-mediated recombination or A-GFP, to provide a control for infection. Optimization tests for use of adenoviruses were performed to obtain both highest infection efficiency and viability. The following formula was used to calculate amount of virus needed to infect a given number of cells to achieve the desired MOI:

$$\text{Amount of virus (\mu l)} = \text{Cell number} \times \text{MOI of interest} / \text{Virus (pfu/\mu l)}.$$

Our trials demonstrated that the optimal multiplicity of infection (MOI), defined as the average number of phage particles that infect a single bacterial cell in a specific experiment, and therefore referring to the infectious efficiency of a virus, was eight for both Adeno-Cre and Adeno-GFP viruses.

2.4. *In vitro* NSPC assays

I have chosen and optimised several assays to assess effects of Bmi1 over-expression on NSPC properties such as proliferation, self-renewal, differentiation and apoptosis. In the next section, I will describe the experimental procedures of the assays used in this study to explore NSPC properties *in vitro*.

2.4.1 Serial dilution assay for self-renewal

Tertiary neurospheres were dissociated by trituration and 4000 cells were suspended in a 15 ml falcon tube containing 2 ml culture media. Serial dilutions (1:2) were prepared from this as follows; 1 ml of cell suspension from the initial tube was transferred to another 15 ml tube, which already contained 1 ml of media. From the second tube 1 ml of cell suspension was transferred to yet another tube containing 1 ml media. This operation was repeated for the next 4 tubes sequentially producing a range of cell concentrations from 2000 (first tube) to 64 cells (sixth tube). Then each tube was supplemented with 1 ml culture media to obtain a total volume of 2 ml in each tube. From these tubes 500 µl was transferred into four wells of a 24-well plate, which had then received 500 µl of culture media next day. This was repeated for each tube in the series producing four replicas for a serial dilution of a range of cell concentrations from 500 ml^{-1} to 16 ml^{-1} . After 6-8 days the newly formed neurospheres (with diameter greater than 40 µm) were counted in each well and the mean of four technical replicas was plotted against the initial number of cells.

2.4.2 Long-term propagation of NSPC

Another way of assessing self-renewal capacity is *in vitro* long-term propagation of NSPC. Primary neurospheres were mechanically dissociated and 125 cells/well were plated in technical quadruplicates in 96-well plates. The number of neurospheres arising in each well was counted within 4-6 days using a light microscope. Neurospheres were dissociated by trituration and re-plated under the same conditions. These two procedures were repeated for 10 passages. The number of neuro-

spheres arising from experimental and control NSPC was plotted against number of passages, providing an independent measure of ability of self-renewal over time.

2.4.3 Proliferation

Proliferation ability of NSPC from postnatal and embryonic mice was measured by quantitation of Alamar Blue (a dye which is reduced by metabolic intermediates produced in proliferating cells) reduction in normal culture conditions. Cells (1.0×10^4) were seeded in replicas in wells of a 96-well flat bottom culture plate. Alamar Blue solution (10% final concentration v/v) was added to each well (except the blank/medium only wells) and the plate was incubated for 2 hours at 37°C before the first measurement was taken. Dye reduction was measured at 2 different wavelengths (570nm and 595nm) using a standard spectrophotometer. Measurements were taken at 12 hour intervals over 72 hours.

In addition, in an independent experiment, actual size of neurospheres was determined as an indirect measure of proliferation (data not shown- as there was no difference between the experimental and control neurospheres).

2.4.4 Click-iT™ EdU based proliferation assay

The Click-iT™ EdU assay is a more accurate method for assessment of proliferation as it directly measures DNA synthesis. Tertiary neurospheres we dissociated 16 hours prior to incubation to ensure that differentiated and progenitor cells are eliminated before addition of EdU, allowing measurement of proliferation of neural stem cells. A 2X working solution of EdU was prepared in the culture media

from the 10 mM stock solution. NSC were incubated with 10 μ M EdU solution for 3 hours at 37°C, 5% CO₂. After incubation, media was removed and 100 μ l/well of 3.7% formaldehyde in PBS added for 15 minutes at RT. The fixative was removed and cells were washed twice with 3% BSA in PBS (100 μ l/well). Wash solution was removed. And 0.5% Triton X-100 in PBS (100 μ l/well) was added for 20 minutes at RT. The Triton X-100 was removed and cells were incubated with 1X Click-iT™ EdU reaction cocktail (1X Click-iT™ reaction buffer, CuSO₄, Click-iT™ Alexa Fluor 488, and Reaction buffer additive) for 30 minutes, protected from light. Reaction cocktail was removed and cells were washed once with 3% BSA in PBS (100 μ l/well). Propidium Iodide (PI- excitation wavelength 647 nm) (20 μ g/ml⁻¹) was added to cells, and assessment of proliferation and cell cycle was performed using LSR II (Becton Dickinson) flow cytometer. Cells from the same population without the EdU treatment was used as negative staining control. Thirty thousand cells were recorded in each analysis.

2.4.5 Apoptosis and Live Cell Cycle Analysis

Neurospheres were incubated with Hoechst 33342 (10 μ g/ml⁻¹) for 45 minutes at 37°C in advance for cell cycle analysis. They were then dissociated into single cells, collected by centrifugation at 700 rpm for five minutes, washed once with PBS, and collected in an eppendorf by centrifugation at 300 \times g for 10 minutes. Supernatant was removed and approximately 1 \times 10⁶ cells per assay were re-suspended in 100 μ L 1X Annexin Binding Buffer (BioSource™). And then Hoechst 33342 (10 μ g/ml⁻¹) was added again, after 5 minutes supplemented with Annexin V-Alexa 647 conjugate (2.5 μ g/ml⁻¹) for 10 minutes at RT. For NSC that had been infected with

A-GFP, 4-6-Diamidino-2-phenylindole (DAPI; 200ng/ml⁻¹) was then added. However, for NSC from E16.5, PI (20μ/ml⁻¹) was used. All apoptosis and cell cycle (G₁, S and G₂m phases) experiments were analysed by LSR II (Becton Dickinson) flow cytometer. Thirty thousand cells were recorded in each analysis.

2.4.6 Neurosphere differentiation

Neurospheres were plated onto cover slips coated with 25μg/ml laminin in serum free Neurobasal medium containing 1X B27 to differentiate over 5, 7 or 9 days. Differentiated neurospheres were fixed in 4% PFA for 15 minutes at room temperature and immunostained with primary antibodies including mouse monoclonal anti-βIII-tub (1:500, Abcam); rabbit polyclonal anti-GFAP (1: 500, DakoCytomation); mouse monoclonal anti-O4 (1:500, Chemicon International); mouse monoclonal anti-Nestin (1:500, Chemicon); rabbit polyclonal anti-NG2 (1:100, Chemicon); mouse monoclonal anti-A2B5 (1:200; Chemicon); rabbit polyclonal anti-Musashi-1 (1:500, Chemicon); mouse monoclonal antibody raised against Bmi-1 (1:400, Upstate); mouse monoclonal/rabbit polyclonal antibody raised against glial fibrillary acidic protein (GFAP, 1:500, Molecular Probes); mouse monoclonal antibody recognizing β-tubulin Isotype III (1: 500, Abcam); mouse monoclonal antibody raised against O4 (1:500, Chemicon International), rabbit polyclonal antibody recognizing Green Fluorescent Protein (GFP, 1 :400; Abcam). Secondary antibodies used were Alexa Fluor 596-conjugated anti-rabbit IgG or anti-mouse IgG (1:500, Invitrogen); Alexa Fluor 488-conjugated anti-mouse IgG or anti-rabbit IgG (1:500, Invitrogen).

The ratio between positive cells and the total number of cells (DAPI) were computed from 10 successive fields chosen around the epicentre of the neurospheres.

2.4.7 Expression analysis in neurospheres

Neurospheres from control and transgenic animals were studied for expression of a variety of genes and reporter genes, antigens and proteins. In the next section I will describe the methods and materials used for these assays.

2.4.8 X-gal Staining

For X-gal staining, neurospheres from STOPfloxBmi1 mice were fixed in 4 % formaldehyde (PFA)/PBS for 10 minutes before they were incubated at the ratio 1:25 with 25 mg/ml X-gal/PBS (Calbiochem) and X-gal staining solution comprising 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 2 mM MgCl₂, at 37° C and overnight. After staining, neurospheres were post-fixed in 70 % ethanol/PBS and stored at 4°C.

2.4.9 Immunocytochemistry on cells and neurospheres

10-15 neurospheres were plated onto Laminin (20 ng/ml¹) coated cover glass (18 mm) in 12 well plates, and incubated in Neurobasal medium without EGF or FGF but B27. The cells were differentiated for 6 days and then fixed in 4 % PFA for 15 minutes at RT. Following rinses with PBS and permeabilisation with 0.5% Triton X-100 for 30 minutes at RT, the cultures were blocked with 5 % Normal Goat Serum (NGS) for 40 min at RT. Primary antibodies (see below) were diluted in NGS (5 %) / Triton X-100 (0.5 %) and applied overnight at 4°C or 2 hours at RT. After repeated rinses in PBS, they were further incubated with secondary antibodies diluted in PBS (see below) for 2 hours, in dark and at RT. Following further rinses in PBS, cells were counterstained with DAPI (200ng/mL) for 10 minutes, in dark and RT, and mounted on slide using DAKO fluorescence mounting medium. Controls with omis-

sion of primary antibodies were made, with no positive signals detected. The primary antibodies used were: mouse monoclonal antibody raised against Bmi-1 (1:400, Upstate); mouse monoclonal/rabbit polyclonal antibody raised against glial fibrillary acidic protein (GFAP, 1:500, Molecular Probes); mouse monoclonal antibody recognizing β -tubulin Isotype III (1: 500, Abcam); mouse monoclonal antibody raised against O4 (1:500, Chemicon International), rabbit polyclonal antibody recognizing Green Fluorescent Protein (GFP, 1 :400; Abcam). Secondary antibodies used were Alexa Fluor 596-conjugated anti-rabbit IgG or anti-mouse IgG (1:500, Invitrogen); Alexa Fluor 488-conjugated anti-mouse IgG or anti-rabbit IgG (1:500, Invitrogen).

To study neurosphere composition using immunohistochemistry, 12 μ m cryostat sections were prepared from pre-fixed (in 4% paraformaldehyde overnight at 4° C) and cryo-protected (in 30 % sucrose solution (w/v)) tertiary neurospheres embedded in OCT (TissueTek-Sakura). Sections were dried overnight at room temperature and used for immunohistochemistry: blocked in PBS (0.06% Triton X-100 or 0.1% Tween-20) containing 5% normal goat serum and incubated overnight with primary antibodies (rabbit polyclonal anti-GFAP (1:500, DakoCytomation); mouse monoclonal anti-GFAP (1:500, Chemicon International); rabbit polyclonal anti-NG2 (1:100, Chemicon); mouse monoclonal anti-A2B5 (1:200, Chemicon International); rabbit polyclonal anti-Dlx2 (1:200, Chemicon International) at room temperature. The application of appropriate secondary antibodies and counter staining with DAPI (2 μ g/ml, Sigma) was followed by picture acquisition using a Zeiss LSM 510 confocal microscope.

2.4.10 Neurosphere composition and flow cytometry.

To study neurosphere heterogeneity using flow cytometry, neurospheres were harvested by centrifugation, mechanically dissociated into single cells and washed once with PBS. The cell suspension for experimental and control samples were parted in two, where one part was incubated (1 hour, RT) with the primary antibodies (anti-GFAP, 1:500; anti-NG2, 1:100; anti-A2B5, 1:200 or combination of these) and other with control antibodies to obtain background fluorescence (rabbit IgG for GFAP and NG2; mouse IgG for GFAP; IgM for A2B5) both antibodies diluted in 1 % PBS/ 0.5 % BSA. Cells were washed twice with 1 % PBS/ 0.5 % BSA. Both cell parts were then incubated (30 minutes, RT) with secondary antibodies (goat anti-rabbit IgG Alexa Flour 488, 1:500; goat anti-mouse IgG Alexa Flour 488, 1:500; goat anti-mouse IgM Alexa Flour 488, 1:500; goat anti-rabbit IgG Alexa Flour 596, 1:500). Cells were washed once with 1 % PBS/ 0.5 % BSA and re-suspended in PI ($20\mu\text{ml}^{-1}$) before being analysed the LSR II (Becton Dickinson) flow cytometer.

2.4.11 Western blotting

Proteins were extracted using Ripa buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Complete mini, Roche Diagnosis). Protein concentration was determined using the BCA assay. Equal amount of protein (10-20 μg) for each condition was loaded into a 12% BisTris gel, separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Proteigene). Membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature and incubated overnight, in 2% milk/Tris-buffered saline containing 0.1% Tween-20 (TBST), with the following primary antibodies at

4°C : mouse monoclonal anti-Bmi-1(1:200; Millipore); rabbit polyclonal anti-p16 (M-156) (1:200; Santa Cruz Biotechnology); rabbit polyclonal anti-p19ARF (1:500, abcam); mouse monoclonal anti-p21 (1:200; Calbiochem); and mouse monoclonal anti- α -Tubulin (1:5000; Sigma). Membranes were incubated with appropriate peroxidise-conjugated secondary antibodies at 1:5000 for one hour at room temperature prior to visualization of immunoreactive proteins on a film using the ECL kit (Amersham).

2.4.12 RNA extraction and cDNA synthesis

Large quantities of fresh or frozen tertiary neurospheres were suspended in Trizol® Reagent (Invitrogen, Paisley, UK) and vigorously shaken and incubated for 5 minutes at room temperature (RT) to permit a complete dissociation of nucleoprotein complexes. Subsequently 0.2 ml of chloroform was added per 1ml of Trizol reagent. After vigorous shaking for 15 seconds the samples were incubated at RT for 3 minutes and centrifuged at 12,000 x g for 5 minutes. Following centrifugation the colourless upper aqueous phase was transferred into a new tube which was mixed with 0.5 ml of Isopropyl alcohol per 1 ml of Trizol reagent. Then, after incubation at RT for 10 minutes, the RNA samples were centrifuged for 10 minutes at 13,000 x g at RT. Following the phenol-chloroform RNA extraction, isopropanol precipitation and the RNA pellet formation by centrifugation, the RNA samples were washed using 300 ml of 75% ethanol and dissolved in RNase-free water. The RNA concentration was determined spectrophotometrically at 260 nm, and the quality of RNA was assessed based on the ratio of absorbance at 260 and 280 nm with a NanoDrop (ND-1000, NanoDrop Technologies, USA). 1-2 μ g of RNA was imme-

diately treated with DNaseI (Invitrogen) to minimize genomic contamination. 1ug of DNase-treated RNA was then retrotranscribed by SuperScript III (Invitrogen) and resultant cDNA used as template for quantitative real time PCR.

2.4.13 Real-Time quantitative PCR and data analysis

SYBR green reactions were performed with SYBR Green Jump Start Taq Ready Mix (Sigma Aldrich) and run on ABI PRISM7500 Sequence Detection System. Expression of genes of interest was determined following normalization to the level of a housekeeping gene, GAPDH, in each sample. A standard curve was generated by real-time PCR analysis from triplicates of five ten-fold dilutions of cDNA generated from 1 ng of control sample RNA. As a negative control mRNA measurements were performed on samples treated with DNase but not with reverse transcriptase enzyme on each single run. The relative expression level of the gene of interest was computed from each experimental sample by calculating the ratio between the gene of interest (ng/reaction) and the GAPDH gene (ng/reaction).

Taqman (Invitrogen) assays were performed with FAM labeled probes in 96 well plates using the Applied Biosystems 7500 RT PCR machine. Amplification conditions for both Taqman and SYBR Green assays were as follows: 95° C for 15 min followed by 40 cycles of 95° C for 10 seconds; 60° C for 15 seconds; 72° C 20 seconds. The cDNA content was normalized against the expression of the housekeeping gene GAPDH. All results represent the mean of at least three biological samples and each reaction has been performed in triplicates.

Primers sequences designed to analyze mRNA expression of selected genes by real-time PCR are shown in Table 2.4:

Table 2.4: Primer sequences of selected genes

Genes	Sequence (5'-3')
GAPDH	F: GCACAGTCAAGGCCGAGAAT R: GCCTTCTCCATGGTGGTGAA
Col1a1	F: GACCCAAGGCCCGTGGT R: GACCCTTATGCCTCTGTCA
Col 9a1	F: TACCAAAACTGGGCAAAGC R: ATTGGCTAAAGAGAAATTG
Neurog2	F: CAATTACATCTGGCGCTC R: ACGCCGGCTGTTGGTGCAG
Foxg1	F: GTGAGGATTGTGATCTGTGT R: ACGAGTGGTAGCTTGTAAATT
Bcan	F: CCGAGGTAGGCAGCATAGAG R: AAGGTCAAAGGGTCGTCTT
Neurocan	F: TCAGAGGCCCTAACGTGCTGT R: GTGGGACTGGGTCATATTG
Ncam1	F: GGTGACCCCTGATTAGAAA R: GGATGGAGAAGACGGTGTGT
Cspg4 (NG2)	F: GAAGATGATGCGAGGTGGAT R: AGCTGATGCTGGAGGTGTCT
Col4a1	F: CCAAAGGATCAGTTGGAGGA R: CTCTTGGCTCCCTCTCT
Col4a2	F: ACCAGATGGCTCCTGTTG R: ACATTGGCAGTCTCCAGCTT

2.4.14 Administration of adenoviruses

Viral infection of SVZ NSPC was achieved by unilateral stereotaxic injections of 5 µl of A-Cre and A-GFP (2.5 x 10⁹ pfu/ml and 1.5 x 10⁸ pfu/ml respectively) into anaesthetized adult mice placed in a stereotaxic frame. Viruses were administered with a Hamilton syringe (26G), using coordinates of 0.5 mm lateral to coronal suture and a depth of 2mm.

2.5 Statistics

Statistical analysis was performed using SPSS 15.0 software. Statistical significance was determined by Student's t-test, paired test. All results in the graphs and tables, where it applies, are indicated as the mean ± standard deviation of the mean (SD) where p < 0.05 is considered as statistical significance. Degree of significance is denoted by asterisks where * indicates p < 0.05; ** indicates p < 0.01 and *** indicates p < 0.001. The Welch correction is used to provide a valid t-test in the presence of small sample size and unequal population variances.

CHAPTER 3 — RESULTS

3.1 *In vitro* assays to assess self-renewal, proliferation, apoptosis and differentiation of neural stem/progenitor cells.

The availability of robust *in vitro* culturing conditions for NSPC provided an experimental platform to develop a variety of experimental procedures to study different aspects of their biological properties such as self-renewal, proliferation, differentiation and apoptosis (Conti and Cattaneo 2010). Here, I will describe; 1) the optimisation of the assays I have chosen to study the biological properties of these cells *in vitro* and 2) the experimental approach I have taken to efficiently deliver Cre recombinase to NSPC *in vitro*.

3.1.1 Self-renewal

I have chosen to study self-renewal capacity of NSPC using; 1) a serial dilution method (Campos *et al.*, 2004) and 2) a repeated passaging method (Hitoshi *et al.*, 2002; Delyrolle and Reynolds 2009).

Serial dilution method: This method allowed us to assess the number of cells contained in a neurosphere which was capable of giving rise to neurospheres upon dissociation. The initial experiments were designed to determine the most appropriate well size and the most appropriate number of cells to be plated in a given volume of culture media to obtain a number of neurospheres which could be accurately and reproducibly counted with a light microscope. I have chosen to use 24 well plates and a range of concentrations between 16 cells ml⁻¹ and 500 cells ml⁻¹ in each well. The number of neurospheres originating from any given concentra-

tion was assessed with a light microscope and the aid of a custom made grid at 6-7 days *in vitro* (DIV). In our hands, 16 cells per well would generate a range of 4 to 12 neurospheres while 500 cells per well would produce between 104 and 269 (Figure 3.1A). This method allowed us to assess clonogenicity of NSPC while taking into account the notion that neurospheres are motile and can merge when plated at high density (Singec *et al.*, 2006). Moreover, this method allowed us to simultaneously monitor the effects of cell density on the self-renewal capacity of NSPC.

Repeated passaging method: A cardinal feature of stem cells is that they can be passaged over a long period of time. To test self-renewal ability of NSPC over an extended period of time and its effects on neurosphere forming capacity with each passage, I employed the repeated passaging method. Briefly, primary neurospheres were mechanically dissociated and 125 cells/well were plated in technical quadruplicates in 96 well plates. Before each subculture passage (every 4-6 days for 10 passages) total number of neurospheres was counted using 5x objective of a light microscope. On average there were found to be between 20 and 59 neurospheres at each passage (Figure 3.1B).

In addition, at each subculture passage the total number of live cells from combined neurospheres of technical replicates were counted using Trypan blue exclusion method. In combination, the number of neurospheres and the combined cell number obtained at each subculture passage provided us an indication of the growth characteristics of NSPC contained within neurospheres and of their self-renewal capacity.

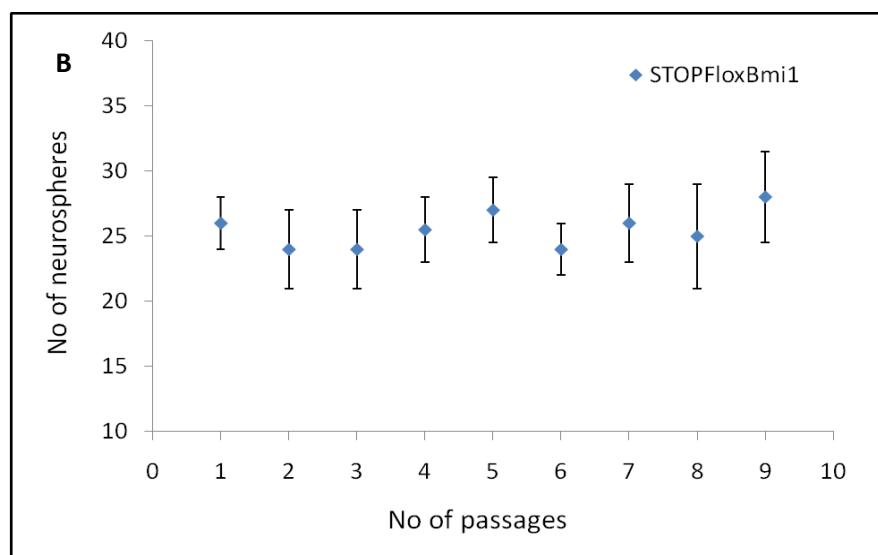
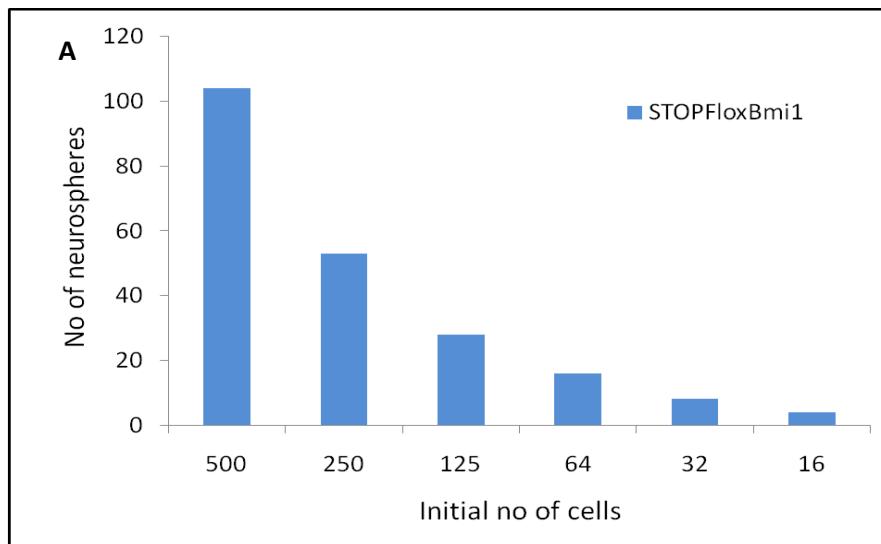


Fig. 3.1 A) Assessment of self-renewal capacity of control NSPC using serial dilution method: The column chart exemplifies the typical number of neurospheres arising from a range of NSPC cultured for 6-7 DIV in media containing mitogens EGF and bFGF-2, and B27 supplement. **B) Assessment of self-renewal capacity of control NSPC using repeated passaging method:** The scatter chart shows the typical number of neurospheres arising from 125 NSPC cultured for 4-6 DIV.

3.1.2 Proliferation

Briefly, tertiary neurospheres were dissociated into single cells 24 hours before the procedure and then replated in normal culture conditions. The following day, cells were incubated with 10 mg/ml of Click-iT™ EdU for 2 hours –as recommended by the manufacturer’s protocol- or for 4 hours –to account for the predicted slow proliferation rate of NSPC. The number of EdU positive cells was assessed by means of flow cytometry. The 3 hours incubation time was clearly superioras it consistently and reproducibly allowed me to detect a proliferation rate of circa 5-10 % in the cultures as compared to 1-3 % I detected when the cells were incubated with EdU only for 2 hours.

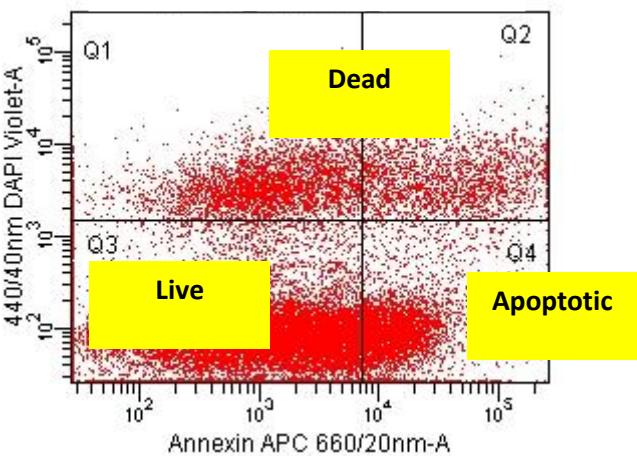
Alamar Blue assay: This is a dye which is widely used to quantify both proliferation and viability (Ahmed *et al.*, 1994) of cells *in vitro*. Alamar blue is reduced by metabolic intermediates produced in metabolically active cells, and the rate of reduction as measured spectrophotometrically at 2 different wavelengths (565nm and 595nm) is proportional to the rate of proliferation and viability of the cells. Initial experiments were designed to determine the smallest number of cells required to detect a detectable change in Alamar Blue reduction over time.

Having tried a range of cells from 1000 to 30 000 per/well, we found that the changes in proliferation were best observed if we plated 10 000 cells per well of a 96 well plate. Tertiary neurospheres were dissociated by trituration and cells were plated in media containing non-toxic water soluble Alamar Blue dye. Measurements were taken with a spectrophotometer at 12 hour intervals over 3 days. This timescale was chosen to ensure death of more differentiated cells (up to 90 per-

cent of progenitors and other cells die off 24 hours after plating (Reynolds and Rietze, 2005) and formation of small neurospheres from proliferating NSPC. Technical and biological replicas confirmed consistency and reproducibility of this assay. Differences in Alamar Blue reduction by experimental and control cells could be due to innate differences in proliferation and apoptosis (or other types of cell death) or their combinations at different rates; features that cannot be distinguished from spectrophotometric readout. Therefore, use of Alamar Blue alone does not provide sufficient evidence to clearly distinguish between changes in culture that could arise from differences in proliferation and/or viability/cell death. Nonetheless, quantitative assessment of proliferation and viability over time provides invaluable insight into dynamics of neurosphere formation, which is tightly associated with proliferation from a heterogeneous population of cells.

3.1.3 Apoptosis

Tertiary neurospheres were dissociated by trituration. This was followed by addition of Annexin Binding Buffer and conjugated Annexin V. DAPI or PI was used in parallel to exclude dead cells. Percentage of Annexin V positive cells were assessed by means of flow cytometric analysis (Figure 3.2). This analysis allowed me to quantify the rate of apoptotic cell population contained in the neurospheres.



Population	#Events	%Parent	%Total
All Events	30,000		100.0
P1	29,854	99.5	99.5
Q1	3,881	13.0	12.9
Q2	2,221	7.4	7.4
Q3	19,241	64.5	64.1
Q4	4,511	15.1	15.0

Fig. 3.2 Quadrant dot plot analysis of control neurospheres with Annexin V and DAPI: The table provides quantitative information about the quadrants of the dot plot, where sum of Q1 and Q2 represents the dead cells (20.4 %), Q3 live cells (64.5 %) and Q4 apoptotic cells (15.1 %).

3.1.4. Differentiation

For these studies, I have used the *in vitro* differentiation method for NSPC essentially as described by Doetsch and co-workers (1999). NS were plated onto laminin coated coverslips and cultured in Neurobasal medium containing B27 supplement while growth factors EGF and FGF were withdrawn. We found that 5-6 DIV differentiation under these conditions is sufficient to obtain cells that are positive for β III Tub (neurons), O4 (oligodendrocytes) and GFAP (astrocytes) (Figure 3.3), at a ratio comparable to those commonly quoted in the field (Singec *et al.*, 2006). To study changes in nestin expression NS were allowed to differentiate for 9-10 DIV.

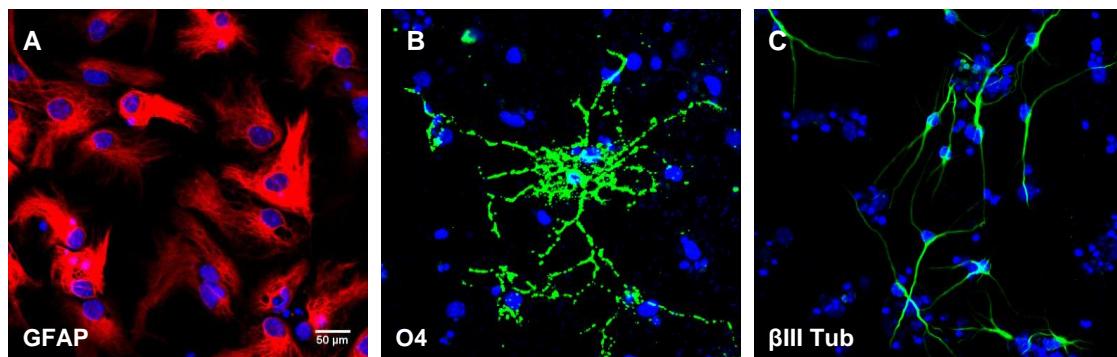


Fig 3.3 Assessment of differentiation capacity of control neurospheres in absence of mitogens on laminin coated coverglass for 6-7 DIV. Neurospheres contain multipotent NSPC as GFAP + astrocytes, O4 + oligodendrocytes, and β III Tub + neurons are generated upon differentiation. Images were taken with Zeiss LSM510; Magnifications 40X, and scale bar (50 μ m) is indicated on image A.

3.1.5 Cre-mediated recombination of loxP sites in NSPC

NSPC from postnatal control mice were cultured, as previously described. After two passages NSPC were infected with Adeno-GFP at a range of MOI (2-12) and were allowed to form neurospheres. Neurospheres were examined using a fluo-

rescent microscopy. The efficiency of infection, as assessed by GFP positivity in the neurospheres, was almost 100% (Figure 3.4).

Next, I set out to determine what MOI of Adeno-CRE virus would be most efficient to recombine loxP sites in NSPC. In order to test this, I took advantage of the established Cre-loxP system in R26R mice; where cells of the R26R mice permanently express LacZ upon Cre-mediated recombination. Briefly, SVZ NSPC of adult R26R mice were isolated and cultured for two passages. Secondary neurospheres were mechanically dissociated and re-plated overnight before infection with Adeno-CRE virus using a range of MOI (2-12). X-gal staining was performed on tertiary neurospheres and they were analyzed using a light microscopy. Results for the MOI of Adeno-CRE virus that produced the most efficient recombination in terms of least toxicity and greatest spread of LacZ staining in all cells of the neurospheres are shown in Figure 3.5.

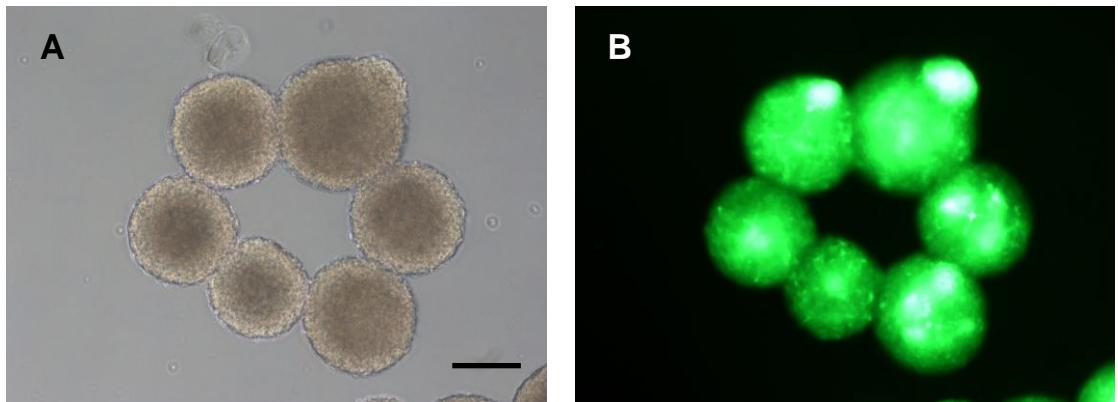


Fig 3.4 Testing efficiency of infection of Adenoviruses in NSPC: A) Light microscopy image of the Adeno-GFP infected neurospheres. B) High infection efficiency in neurospheres arising from Adeno-GFP infected NSPC is visible under a fluorescent microscopy. Note that almost all cells of all neurospheres are green. Magnifications 40X. Scale bar 200 μ m.

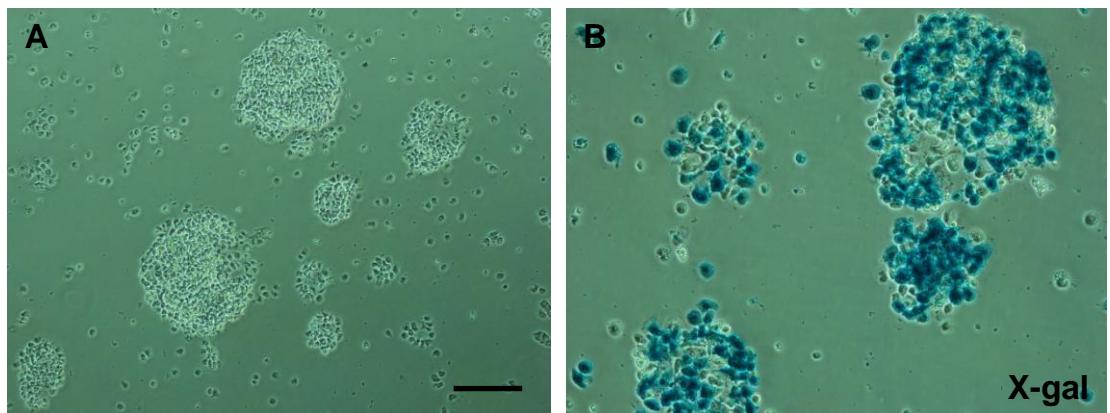


Fig 3.5 Testing efficiency of infection of Adenoviruses in NSPC: A) Control neurospheres are not positive for LacZ. B) High infection efficiency in neurospheres arising from Adeno-CRE infected R26R NSPC. All cells of all neurospheres were blue. Magnifications 20X (A) and 40X (B). Scale bar 200 μ m in (A), and 100 μ m in (B).

3.1.5.1 Adenovirus mediated expression of GFP and CRE in NSPC has similar effects on their biological properties

To control for non specific effects of infection and over-expression of any exogenous proteins, NSPC were isolated from control (non transgenic) littermates and infected in parallel with Adeno-CRE and Adeno-GFP. Self renewal assays, proliferation and apoptotic assays as well as induction of differentiation were carried out as previously described on these cultures. I carried out the experiments for the self renewal and apoptotic assays while a colleague in the laboratory, Dr. Reto Sutter, carried out the proliferation and differentiation assays.

Self-renewal

Normal (non transgenic) NSPC from SVZ of postnatal mice was isolated and cultured for three passages. Tertiary neurospheres were mechanically dissociated into single cells and infected with either Adeno-GFP (MOI 8) or Adeno-CRE (MOI 8), and serially diluted from 500-16 cells per well. The neurospheres arising from normal NSPC infected with different viruses were counted 6 days later, and results are shown in Figure 3.6.

In conclusion, we found that NSPC infected with either Adeno-CRE or Adeno-GFP viruses gave rise to similar number of neurospheres (Fig 3.6). The experiment was performed twice and two technical replicas were analysed for each experiment.

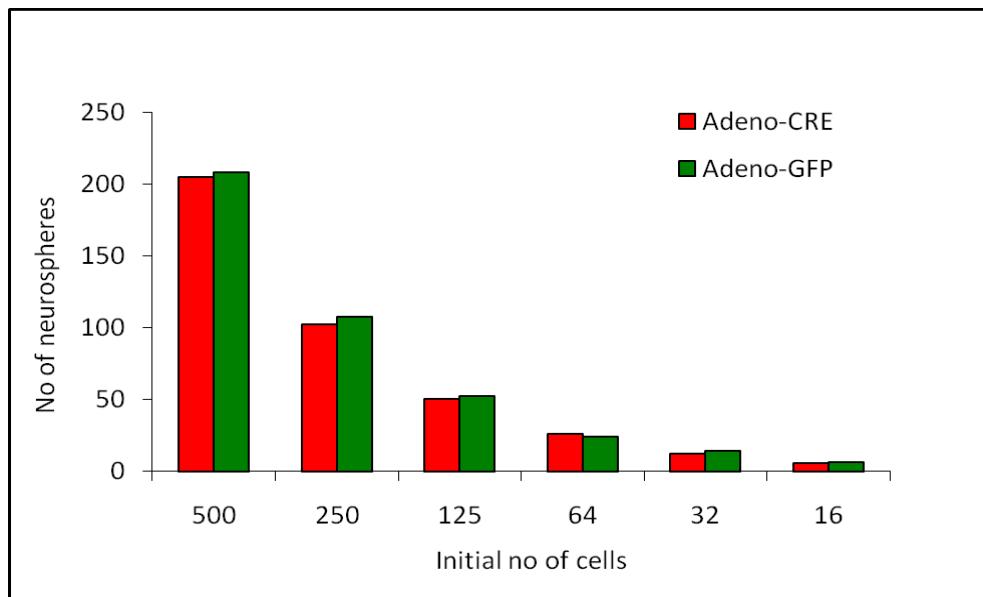


Fig 3.6 Testing possible differential effects of adenoviruses on self-renewal of normal NSPC: Clonogenic assay was performed on normal NSPC infected with either Adeno-CRE or Adeno-GFP viruses. Note that similar number of NS originate from equal number of plated cells (n=2).

Apoptosis

A small population of cells are known to undergo apoptosis in neurospheres. Whether Adeno-CRE and Adeno-GFP viruses differentially contribute to induction of apoptosis in the NS is unknown and we set out to analyse this as follow. Normal tertiary neurospheres arising from cells infected either with Adeno-CRE or Adeno-GFP were mechanically dissociated and processed for Annexin V based apoptosis test. The number of Annexin V positive cells was assessed by FACS analysis, while DAPI positive dead cells were excluded from the analysis. No difference in the

apoptotic rate of these two cultures was observed (Figure 3.7, Q4) and a specific toxicity of either of the Adeno-viruses used could be excluded.

Similarly, comparable results were found in proliferation and differentiation assays carried out on cultures infected either with Adeno-CRE or Adeno-GFP (data not shown).

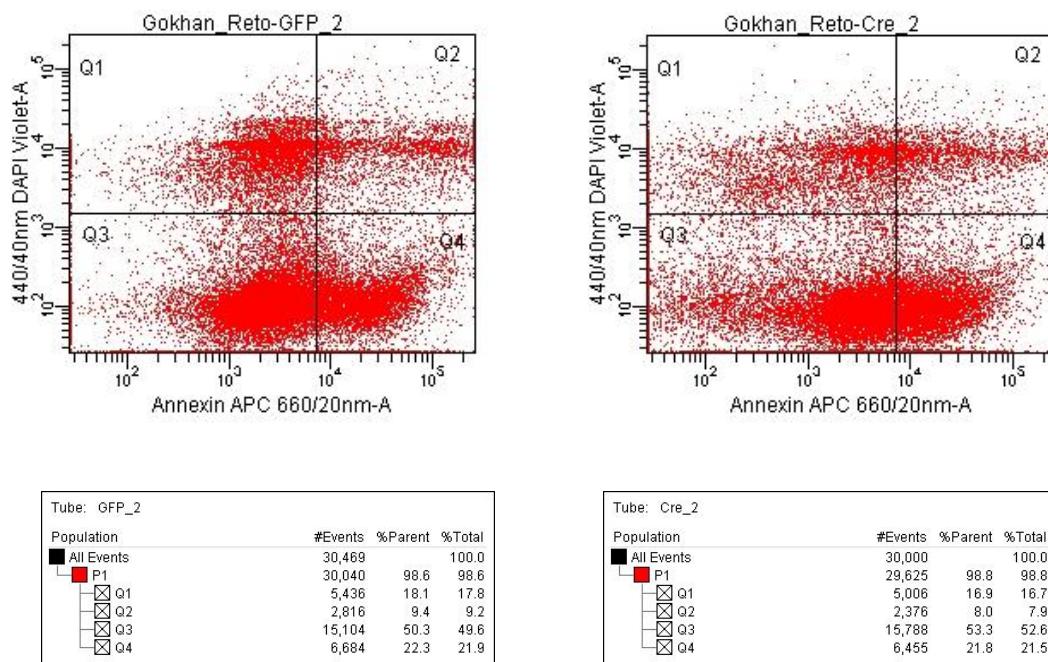


Fig 3.7 Assessment the effect of adenoviruses on apoptotic properties of neurosphere cells: The experiment was performed on normal NSPC cells infected with either Adeno-GFP (left panel) or Adeno-CRE (right panel). For each experiment 30 000 events were recorded and the quadrant 4 (Q4) both in the table and the dot plot represents Annexin V positive cells, that is apoptotic cells. The number of Annexin V positive cells in both experiments is approximately the same; 22.3 % for Adeno-GFP infected cells and 21.8 % for Adeno-CRE infected cells.

n= 2

3.2.1 Analysis of expression of the transgenic construct in embryonic and postnatal NSPC

The first reporter gene contained in the construct -the LacZ gene- encodes for the β -galactosidase enzyme. The enzymatic activity of this protein can be exploited to trace cells expressing the construct both *in vitro* and *in vivo*. Indeed, if the promoter is active in a specific cell, β -galactosidase enzyme will be synthesised and the cells will turn blue when incubated with X-gal, a dye which is converted into an insoluble blue product by the β -galactosidase enzyme. We therefore set out to analyse whether the transgene was expressed in embryonic and postnatal NSPC obtained from STOPFloxBmi1 by means of X-gal staining.

NSPC were isolated from the developing neocortex at E16.5 or from the SVZ at P7 or P70 and cultured as neurospheres in the presence of B27 and mitogens (EGF and FGF2b) for three passages as previously described. We found strong and homogeneous blue staining in all cells contained within the neurospheres at 5-6 DIV (Figure 3.8, B), as compared to a complete lack of β -galactosidase activity in control neurospheres (Figure 3.8, A).

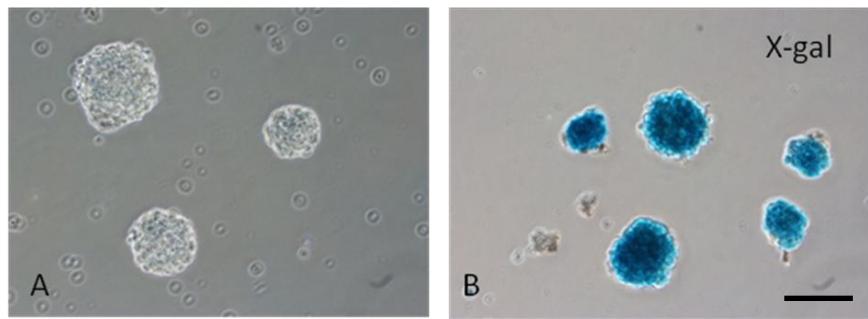


Fig 3.8 Assessment of the transgene activity in NSPC isolated from STOPFloxBmi1 mice embryos. A) Neurospheres derived from control (wt) NSPC do not stain for X-gal. B) X-gal staining showing expression of transgene in all cells of neurospheres derived from NSPC of STOP-FloxBmi1 mice at E16.5. Magnifications 20X. Scale bar is 200 µm. n=2

Next, we set out to analyse whether infection with Adeno-CRE would efficiently recombine the floxed sequence of the construct and therefore remove the LacZ gene. Embryonic and postnatal NSPC were infected on the next day following dissociation of tertiary neurospheres with Adeno-CRE and X-gal staining was performed on NS at 5-7 DIV (Figure 3.9).

Lack of X-gal positivity demonstrated efficient Cre mediated recombination of the floxed LacZ/neomycin fusion gene.

We therefore conclude that the transgene is active in NSPC and in all cells derived thereof regardless of age of isolation and that Adeno-CRE infection of the cultures efficiently removes the floxed sequence from the STOPFloxBmi1 construct.

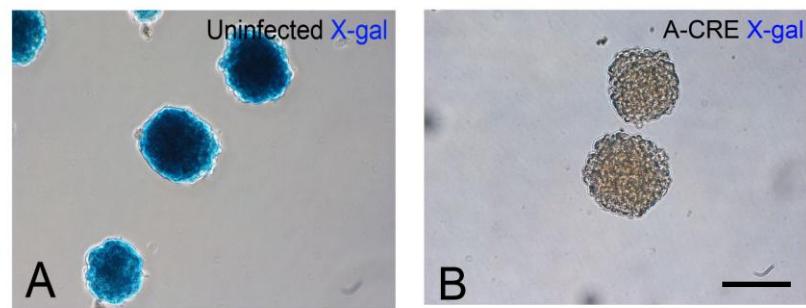


Fig 3.9 Assessment of the efficiency of A-CRE mediated recombination of the LoxP sites using X-gal staining: A) Neurospheres derived from STOPFloxBmi1 NSPC (control) are blue, indicating activity of the reporter gene. B) Loss of X-gal positivity in all cells of neurospheres derived from A-CRE infected NSPC demonstrates permanent recombination of Lox-P sites. Magnifications 20X. Scale bar is 200 μ m. n=2

3.2.2 Activation of Bmi1 over-expression in NSPC

Two experimental settings were used to activate Bmi1 expression in NSPC:

- A) Adenovirus mediated Cre delivery in NSPC isolated from STOPFloxBmi1 at E16.5, P7 or P70, from now on referred to as “acute activation of Bmi1 expression”
- B) Nestin promoter driven Cre expression in the developing neocortex of STOP-FloxBmi1 mice followed by isolation of NSPC from double transgenic embryos at E16.5, from now on referred to “chronic activation of Bmi1 expression”.

We reasoned that the combination of these two approaches would allow us to discriminate between the impacts of an acute up-regulation of Bmi1 expression on NSPC properties versus the impact of a protracted over-expression of Bmi1 in these cells.

3.2.2.1 Acute activation of Bmi1 expression

Tertiary neurospheres originating from E16.5 NSPC were mechanically dissociated, replated at clonal density and infected on the following day with Adeno-CRE, to enable activation of Bmi1 over-expression. To assess whether adenoviral mediated Cre recombination of the STOPFloxBmi1 construct resulted in Bmi1 over-expression, immunofluorescent staining for Bmi1 was carried out on NS originating thereof. Confocal analysis of this staining showed higher levels of Bmi1 expression in Adeno-CRE infected neurospheres as compared with non infected controls (Figure 3.10). As it is difficult to estimate an upregulation on the baseline expression of an endogenous protein with an immunohistochemical staining, which is essentially a non quantitative method, we set out to assess also expression of GFP, the sec-

ond reporter gene which should be expressed only upon activation of the construct. We show that GFP is also expressed in the experimental neurospheres as compared to controls. Co-localisation of the two markers is shown in double immunofluorescent staining analysed with a confocal microscope.

Similar results were obtained also in experiments on NSPC isolated from the postnatal subventricular zone (data not shown). We therefore conclude that adenovirus mediated Cre delivery is highly efficient and resulted in recombination of loxP sites with removal of β -galactosidase expression and activation of Bmi1 expression driven by the chicken β -actin promoter.

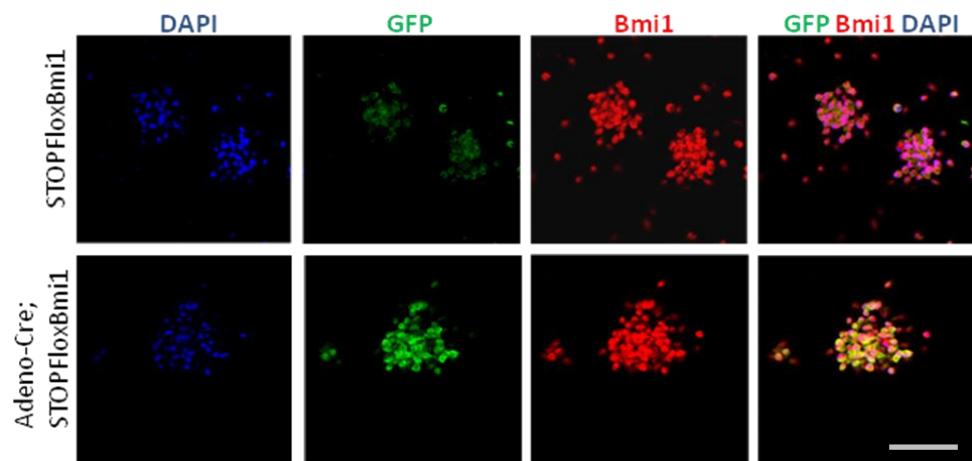


Fig 3.10 Activation of Bmi1 expression *in vitro*: Assessment of Bmi1 over-expression in neurospheres derived from tertiary NSPC infected with Adeno-Cre (bottom panel). Note that upper panel does not express EGFP (green) and has lower level of Bmi1 (red). Scale bar is 200 μ m. n=2

3.2.2.2 Chronic Activation of Bmi1 expression

NSPC were isolated from the developing neocortex of single transgenic embryos (STOPFloxBmi1 or Nestin-Cre) and of compound transgenic mice (Nestin-Cre;STOPFloxBmi1) and cultured as previously described .

X-gal staining of tertiary neurospheres derived from compound transgenic NSPC revealed lack of β -galactosidase activity as compared to controls isolated from STOPFloxBmi1 mice (Figure 3.11).

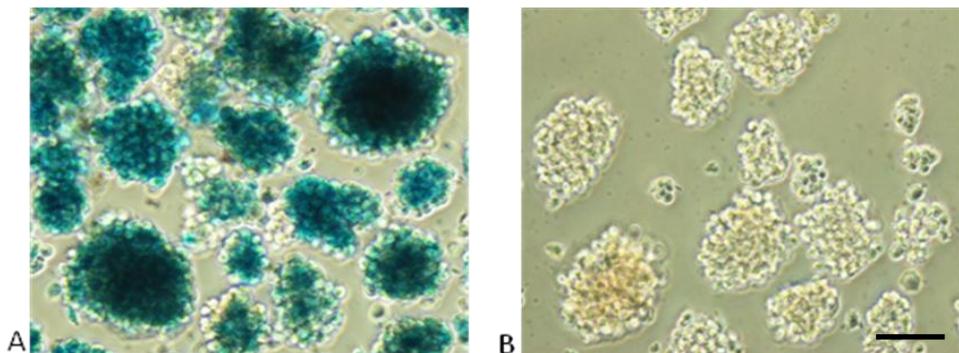


Fig 3.11 Successful *in vivo* recombination of loxP sites and removal of β -geo expression in NestinCre;STOPFloxBmi1 compound mice: A) X-gal positive neurospheres from NSPC of STOPFloxBmi1 mice (control). B) *In vivo* loss of floxed LacZ/neomycin fusion gene is permanent as NSPC from compound mice give rise to neurospheres that are negative for X-gal staining. Magnifications 20X. Scale bar is 200 m μ . n=2

These data show that Nestin-driven Cre expression is an efficient method to recombine loxP sites in embryonic NSPC.

Next we set out to assess and quantify the level of Bmi1 expression upon Nestin-Cre induced recombination. NSPC were isolated from E16.5 embryos of Nestin-

Cre;STOPFloxBmi1 and STOPFloxBmi1, and cultured as previously described. Protein lysate and RNA were isolated from tertiary neurospheres and Western blotting or SYBR green RT-PCR were performed to assess Bmi1 over-expression.

Reverse-transcription of the RNA into cDNA was simultaneously performed for the control and experimental samples. SYBR green RT-PCR was performed and expression of Bmi1 was determined for each sample following normalisation to the level of housekeeping gene, ribosomal 18S. All experiments have shown that Nestin-Cre;STOPFloxBmi1 samples express approximately 1.8 fold more of Bmi1 mRNA compared with controls (Figure 3.12).

Next, I performed Western blotting experiments to determine whether the increased mRNA expression in NestinCre;STOPFloxBmi1 samples is translated into protein. All Western blotting experiments performed showed a two fold increase in Bmi1 protein level in NestinCre;STOPFloxBmi1 neurospheres as compared to single transgenic cultures. Equal protein loading was assessed by reprobing the membrane with an antibody against tubulin, while specificity of the antibody was confirmed by lack of staining in the negative control lane which contained protein homogenates extracted from Bmi1-/ brain (Figure 3.13).

In conclusion, I found that efficient activation of the transgene is achieved by both Adeno-Cre infection and nestin driven cre expression in NSPC and their progeny isolated from STOPFloxBmi1 transgenic line.

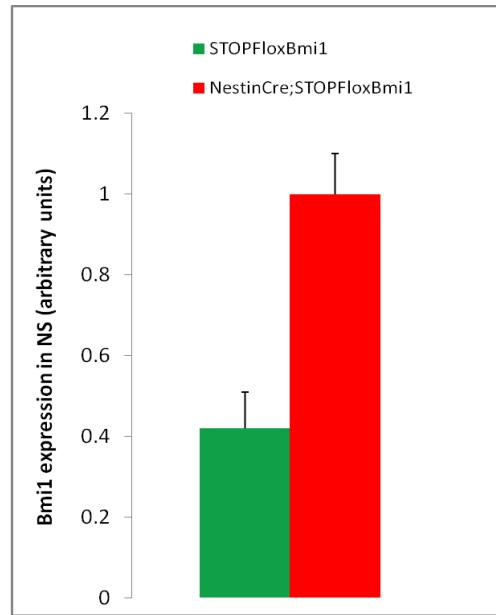


Fig 3.12 SYBR green RT-PCR measurement of mRNA for Bmi1: Measurements expressed in arbitrary units shows that Bmi1 expression is increased approximately by two fold in the NestinCre;STOPFloxBmi1 neurospheres, compared with those of control (STOPFloxBmi1). n=2

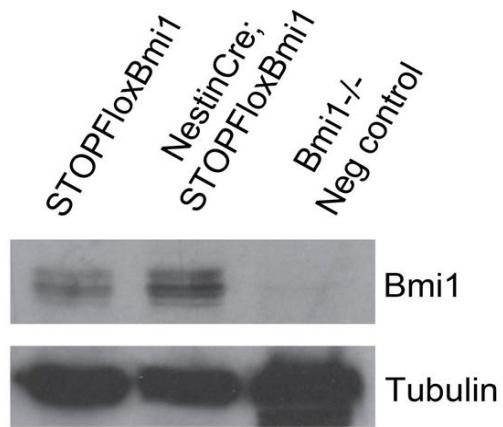


Fig 3.13 Assessment of activation of Bmi1 over-expression using Western blotting: monoclonal antibody against Bmi1 shows approximately two fold increase in expression in the Nestin-Cre;STOPFloxBmi1 neurospheres (middle lane), compared with those of control (left panel). Tubulin was used as loading control. n=2

3.3 Analysis of the impact of Bmi1 over-expression on cellular properties of NSPC *in vitro*

3.3.1 Bmi-1 over-expression increases self-renewal of NSPC

A cardinal feature of stem cells is their ability to self-renew by either symmetrical or/and asymmetrical cell division (Lois and Alvarez-Buylla, 1994). Previous studies on Bmi-1 null mice had shown that Bmi-1 is an essential component of neural stem cells' self-renewal machinery (Park *et al.*, 2003b). We set out to determine whether Bmi1 over-expression increases NSPC self-renewal capacity using two methods: 1) assessment of the number of neurospheres arising from serially diluted NSPC (Campos *et al.*, 2004) and 2) assessment of long term passaging (Reynolds and Rietze, 2005). These two methods complement each other in the assessment of self-renewal as the former measures the percentage of cells with the ability to give rise to neurospheres in a given culture at a given time point whereas the latter determines the capability to give rise to neurospheres over time.

3.3.1.1 Serial Dilution

NSPC were isolated from STOPFloxBmi1 and NestinCre;STOPFloxBmi1 telencephalon at E16.5 and subcloned three times before they were serially diluted from 500 to 16 cells per well. After 7 DIV the neurospheres in each well were counted at 20x magnification using a grid for accuracy and the results are shown in Figure 3.14. The number of neurospheres arising from cells plated across the density range from 16, 32, 64, 125, 250 and 500 in experimental and control samples is 12 vs 4, 23 vs 8, 42 vs 16, 79 vs 27, 149 vs 52, 269 vs 104, respectively. Two-tail Student's t-

test with Welch correction revealed that there is a significant difference in self-renewal capacity between Bmi-1 over-expressing and controls cells at all cell densities used (p values in order for cell densities from 16 to 500 are 0.001, 0.001, 0.001, 0.001, 0.001, 0.001).

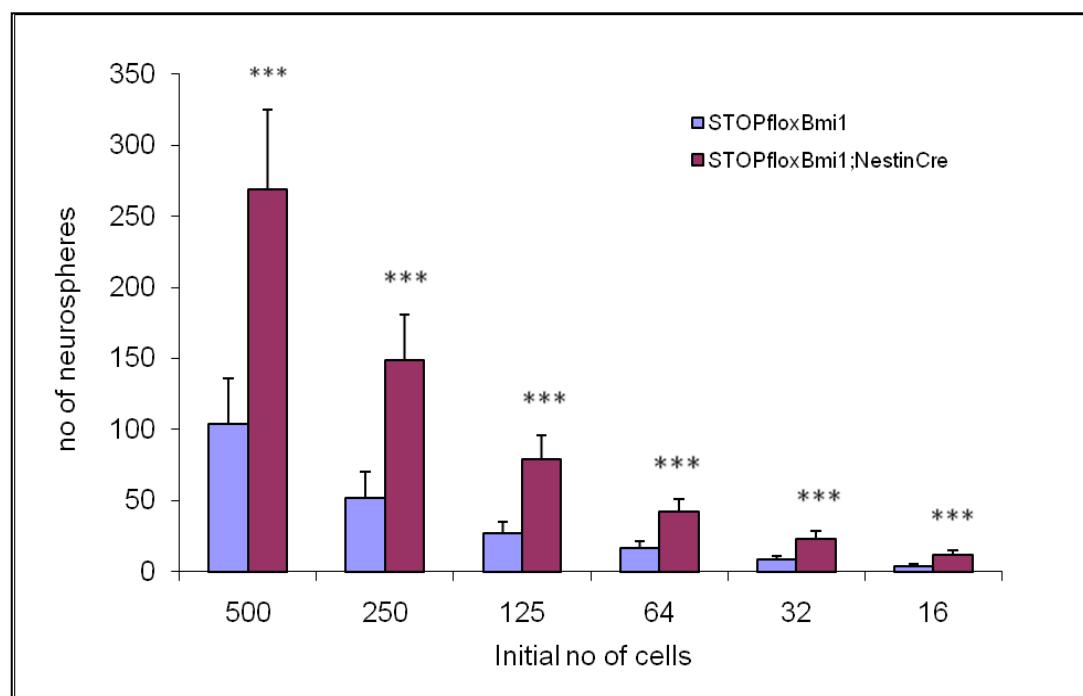


Fig 3.14 Comparison of self-renewal capacity between the Bmi1 over-expressing tertiary telencephalic NSPC derived from STOPFlox-Bmi1;NestinCre and control STOPFloxBmi1 mice at E16.5: Note that more neurospheres are produced from Bmi1 over-expressing NSPC at all densities tested. ***= p<0.001, n= 4.

Next, we wanted to determine whether a similar difference in self-renewal was also achievable when activation of Bmi-1 over-expression was induced acutely by adenoviral infection. We repeated the same experiments and took the same measurements using NSPC cells infected with Adeno-CRE or Adeno-GFP. The re-

sults are shown in Figure 3.15. Similarly, activation of Bmi1 expression in tertiary NSPC cells resulted in formation of significantly more number of neurospheres at all densities plated from 16 to 500 per well (*p* values are 0.001, 0.001, 0.001, 0.001, 0.001 and 0.001 respectively).

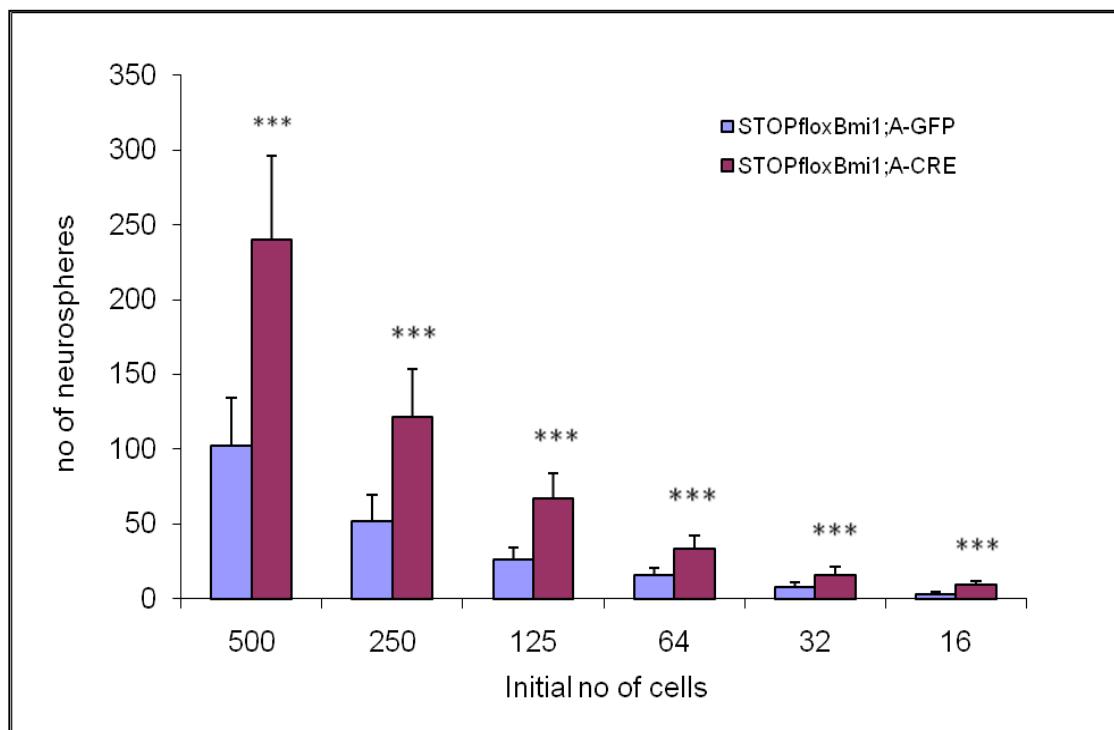


Fig 3.15 Acute activation of Bmi1 expression in STOPFloxBmi1 NSPC increases their self-renewal capacity. ***= *p*<0.001, *n*= 4.

These results show that over-expression of Bmi1 in NSPC maintain a higher proportion of cells in a developmental stage where they can give rise to neurospheres, regardless of the route of over-expression; ‘acute’ induction of Bmi-1 expression by Adeno-Cre infection or ‘chronic’ by crossing STOPfloxBmi-1 mice with Nestin-CRE mice. To determine whether cell density would have an impact on Bmi1-

induced clonogenic activity, we compared the ratio (Adeno-Cre/Adeno-GFP; NestinCreSTOPFloxBmi1/STOPFloxBmi1) of the neurospheres formed at each “cell dilution plated” for both acute and chronic activation of Bmi1 expression (Table 3.1).

RATIO (experimental/control)						
Cell density	500	250	125	64	32	16
STOPFloxBmi1;A-GFP/STOPfloxBmi1;A-CRE	2.3	2.3	2.5	2.2	1.9	3.3
STOPFloxBmi1/STOPfloxBmi1;NestinCre	2.6	2.8	2.9	2.6	2.8	3.3

Table 3.1 Comparison of the R of neurospheres obtained from plating different number of NSPC: Cell density or route of activation of Bmi1 in NSPC does not influence the increment in ratio of spheres generated, compared with controls, in 5-7 DIV.

The ratio of the neurospheres arising from plating of 500 NSPC infected with Adeno-GFP and Adeno-Cre is 1:2.3, whereas that of single and compound transgenic cells is 1:2.6. Similar results were obtained at all cell densities (see Table 3.1), in keeping with the interpretation that Bmi1 over-expressing NSPC give rise to more neurospheres independently of their original cellular density and route of activation of Bmi1 over-expression.

Next, I set out to study effects of activation of Bmi-1 expression by means of Cre-mediated recombination, on the biological properties of postnatal and adult NSPC, in a similar fashion to that of embryonic cells.

NSPC obtained upon dissociation of tertiary NS derived from SVZ of P7 and P70 STOPFloxBmi1 mice were infected with Adeno-CRE or Adeno-GFP. The resulting neurospheres were dissociated and plated as previously described. Neurospheres arising from each of these were counted and the results are shown in Figure 3.16.

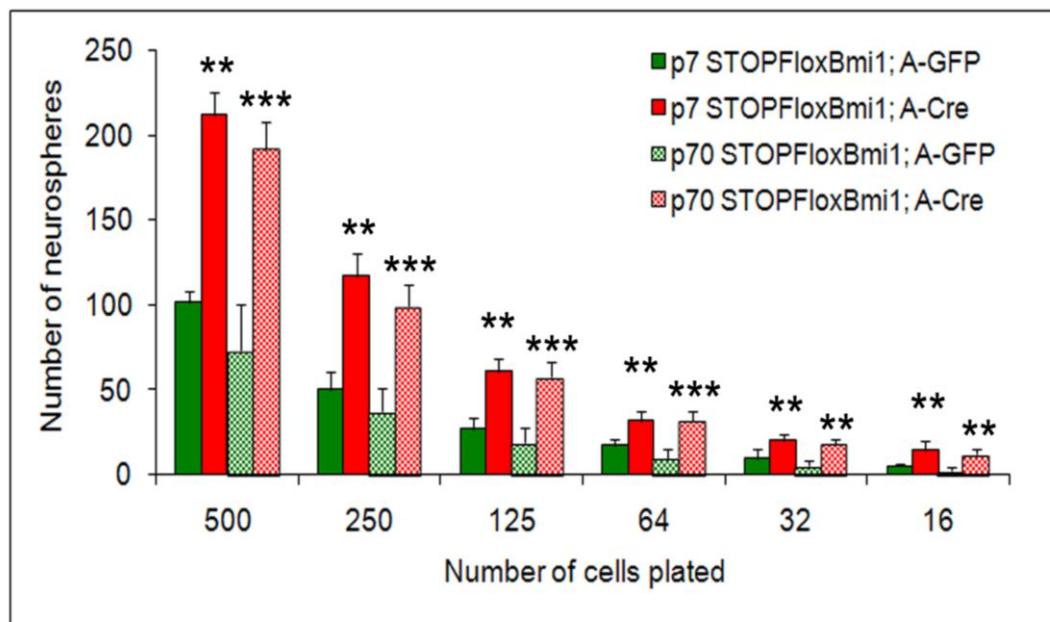


Fig 3.16 Serial dilution based assay showing that Bmi1 over-expression in postnatal NSPC by means of “acute” activation leads to a greater clonogenic activity. **= p<0.01, ***= p<0.001, n=3.

As seen in Figure 3.16, “acute” activation of Bmi-1 over-expression in postnatal NSPC has similar effects as observed in embryonic NSPC.

In conclusion, these results show that overexpression of Bmi1 in NSPC increases self renewal/clonogenic activity of these cells *in vitro* independently of whether

the cells were isolated from the developing neocortex during embryonic development or the postnatal SVZ.

3.3.1.2 Long-term passaging

NSPC can be perpetuated for a long time in culture, a property which is used as a measure to assess their self-renewal capacity (Reynolds and Rietze, 2005).

Therefore, I set out to further assess the impact of Bmi1 overexpression on the self-renewal capacity of NSPC by repeatedly dissociating neurospheres and replating cells for 10 rounds. Tertiary NS of E16.5 NestinCre;STOPFloxBmi1 mice were dissociated, 125 cells were plated in 96 well dishes and cultured for 4-6 days until small neurospheres of the size of 80 μ m diameter originated (data not shown). These NS were counted as previously described and results plotted into a graph (Figure 3.17). The number of neurospheres arising from equal number of plated cells for nine sequential passages is 36 vs 24, 28 vs 21, 37 vs 24, 33 vs 18, 35 vs 23, 30 vs 23, 31 vs 21, 32 vs 20, 32 vs 21 and 32 vs 21 in NestinCre;STOPFloxBmi1 mice and controls respectively (Figure 3.18). The same experiment was performed for P7 NSPC where Bmi1 expression was activated through infection with Adeno-Cre and similar results were obtained. The number of neurospheres arising from equal number of plated cells for nine sequential passages 44 vs 28, 42 vs 27, 43 vs 27, 44 vs 26, 42 vs 29, 43 vs 26, 43 vs 27, 43 vs 27, 42 vs 29, 43 vs 29 and 42 vs 28 respectively.

These experiments provided an independent confirmation of the increased self renewal of NSPC overexpressing Bmi1, independently of the developmental time-point analysed.

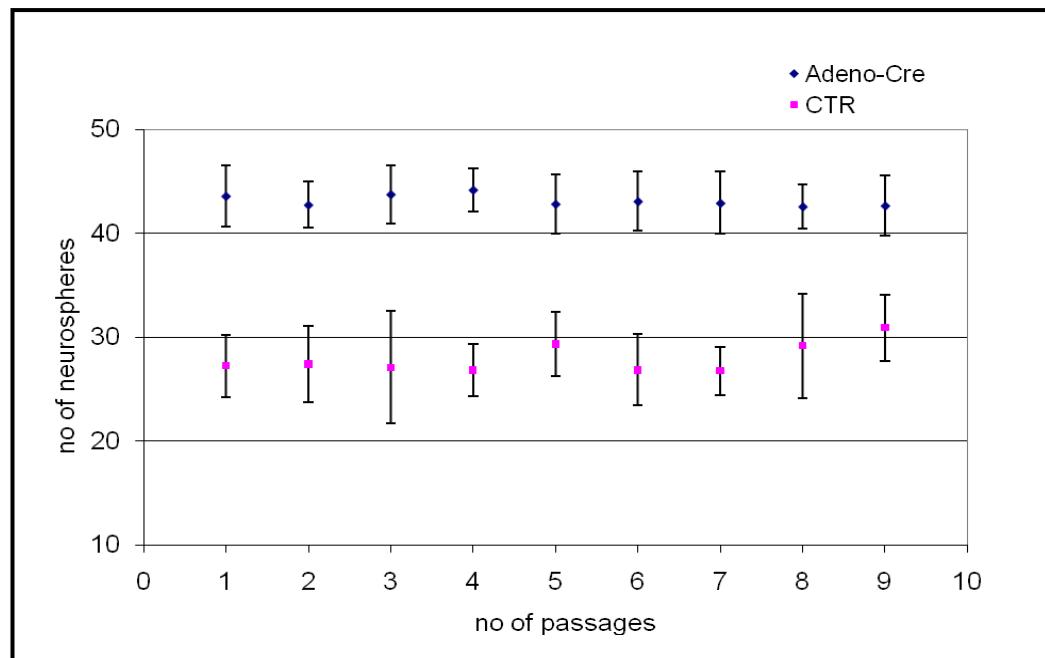


Fig 3.17 Repeated passaging assay showing that Bmi1 over-expression in postnatal NSPC by means of “acute” activation leads to a greater clonogenic activity. n=3

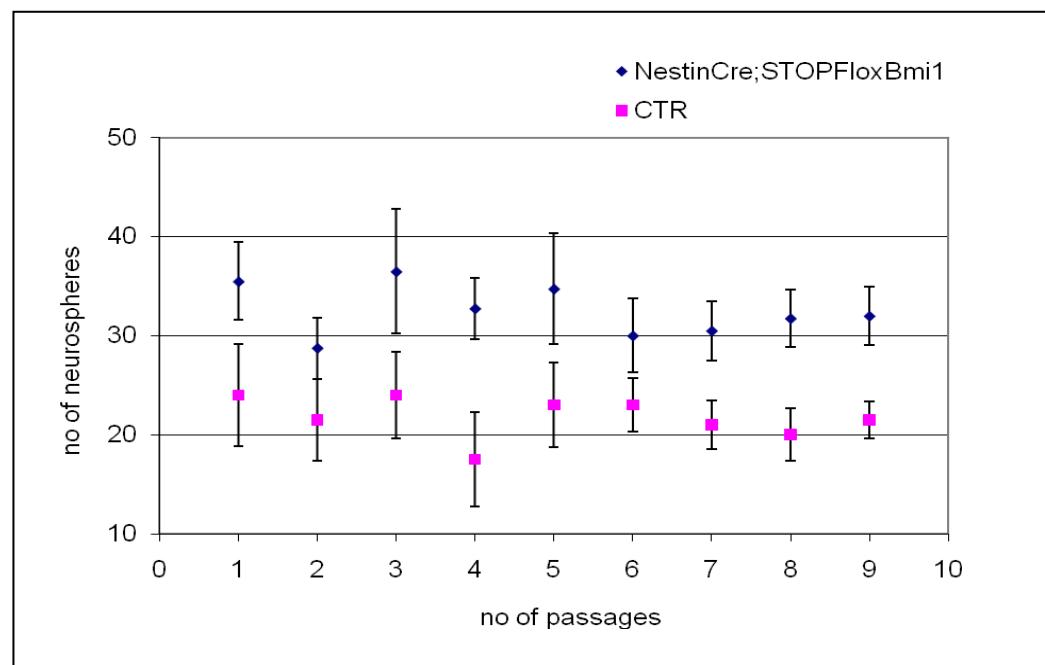


Fig 3.18 Repeated passaging assay showing that Bmi1 over-expression in embryonic NSPC by means of “chronic” activation leads to a greater clonogenic activity. n=3

3.3.2 Over-expression of Bmi1 increases proliferation and viability of NSPC

NSPC are thought to have both a low proliferation rate (Morshead *et al.*, 1994) and a finite number of mitotic divisions (Shay and Wright, 2000). Their proliferation is regulated by several pathways (Sutter *et al.*, 2007) including Bmi1 mediated inhibition of Ink4a/Arf locus. In ageing mice, a decrease of both the self-renewal and proliferation potential of the SVZ NSPC have been shown to correlate with increased p16ink4a expression, and that these effects could significantly be reversed by suppression of p16ink4a (Molofsky *et al.*, 2006). Given the role of Bmi-1 in repression of p16ink4a expression, I set out to assess whether Bmi-1 over-expression induces increased proliferation of NSPC. Assessment of proliferation was performed by means of a colorimetric assay- Alamar Blue- and EdU (5-ethynyl-2'-deoxyuridine) incorporation and detection, as previously described.

Tertiary neurospheres arising from NSPC isolated from Nestin-Cre;STOPFloxBmi1 and STOPFloxBmi1 were mechanically dissociated and 10 000 cells were plated in each well. Measurements of Alamar Blue reduction were carried out at 12 hour intervals for 72 hours at 2 different wavelengths (570nm and 595nm) using a spectrophotometer (BioTek SIAFR Synergy HT microplate reader with KC4 v3.4 software). Percentage of Alamar Blue reduction over time is plotted in a line graph for experimental cells and controls (Figure 3.19).

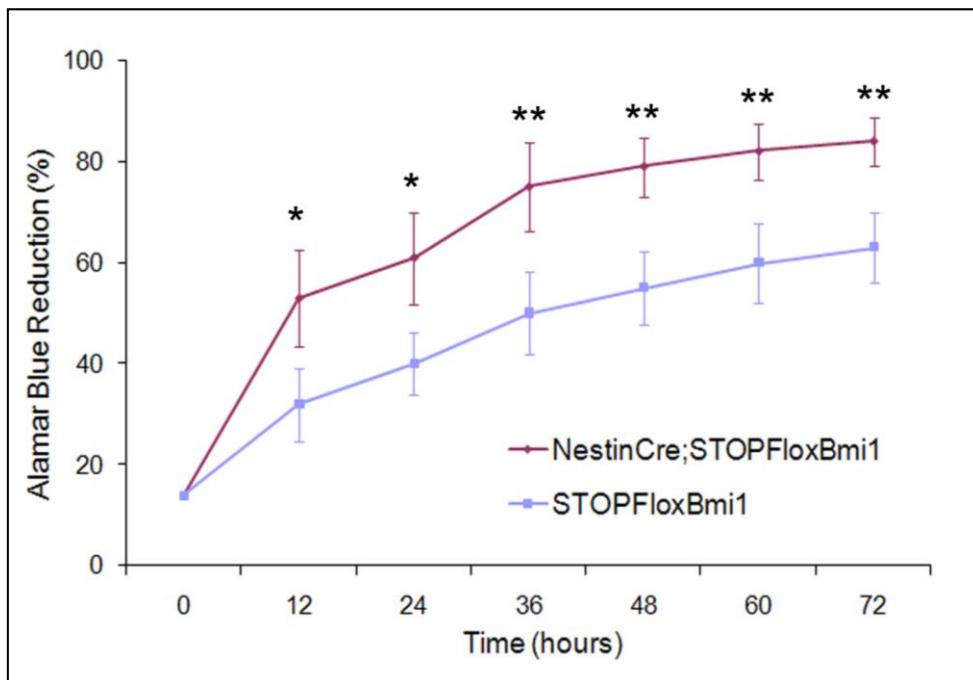


Fig 3.19 Alamar Blue dye based assessment of proliferation/viability of E16.5 NSPC. Increased proliferation/viability is seen in NestinCre;STOPFloxBmi1 NSPC as compared with controls, at all time points analysed. * = $p < 0.05$, ** = $p < 0.01$, n=4.

We found increased proliferation/ viability in NSPC overexpressing Bmi1. It is important to note that the proliferation rate of Bmi-1 over-expressing NSPC reaches its peak at 36 hours with 75% reduction of Alamar Blue, followed by a phase of plateaux when reduction of the Alamar Blue reaches 87%. The proliferation rate of control cells reaches a plateaux when Alamar Blue is reduced by approximately 60%. Indeed proliferation/viability of NSPC could be impaired by the size of the neurospheres they are imbedded in and by exhaustion of mitogens and nutrients in the culture media (Mori *et al.*, 2006).

These results suggest that Bmi1 over-expression confers increased viability to NSPC as assessed by their increased metabolic rate. This, in turn, raises the possibility that over-expression of Bmi1 increases the proliferation of these cells. To provide further support to this interpretation I used a complementary approach to assess proliferation of NSPC, the EdU incorporation and detection assay (Figure 3.20).

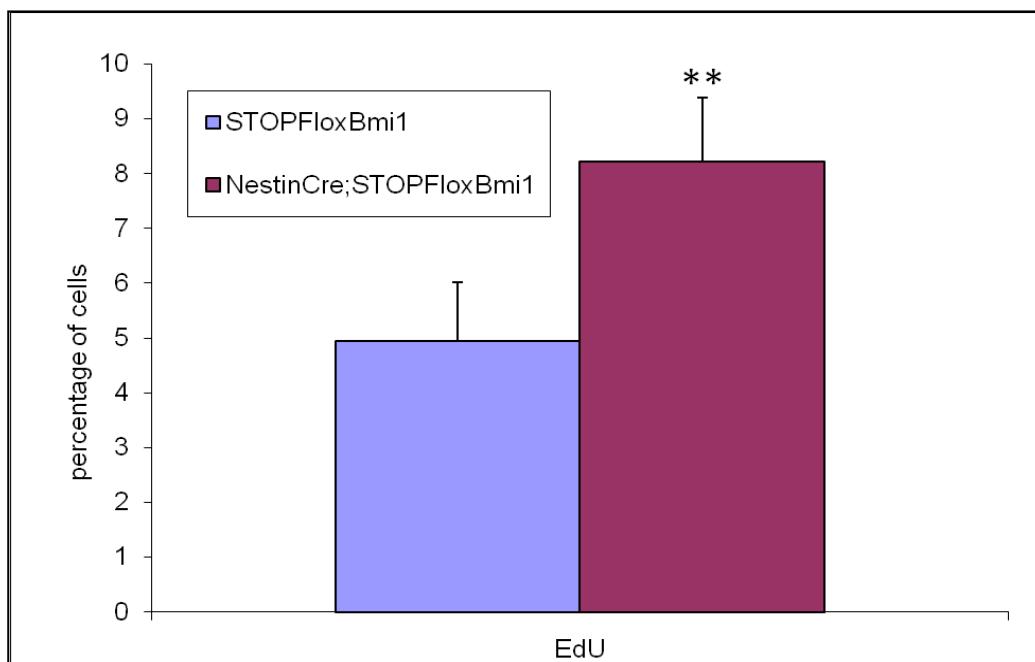


Fig 3.20 EdU based assessment of proliferation of E16.5 NSPC revealed increased rate of DNA synthesis in NestinCre;STOPFloxBmi1 samples. **= p<0.01 n=3

The average percentage of experimental NSPC incorporating EdU, e.i. actively synthesising DNA, is 8.3, whereas that of control is 4.9. The difference is significant at 95% significance level.

The Alamar Blue dye showed that activation of Bmi1 expression in embryonic NSPC increases their metabolic rate, an event which may be linked either to increased proliferation or to increased cellular viability. However, assessment of proliferation by means of EdU incorporation and detection conclusively shows that activation of Bmi1 expression in NSPC results in an increase of their proliferation.

Similar results were obtained when these experiments were performed on P7 NSPC (Figure 3.21), in keeping with this being an effect of Bmi1 overexpression independent of the developmental timepoint analysed.

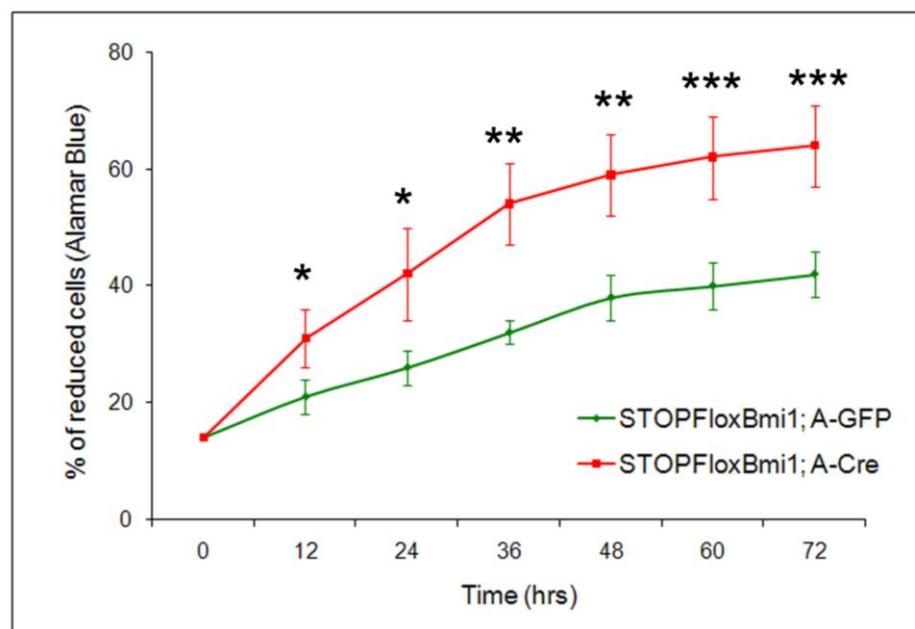


Fig 3.21 Alamar Blue assay shows increased proliferation of P7 NSPC upon activation of Bmi1 expression by Adeno-Cre as compared to controls. *= p<0.05 **= p<0.01, ***= p<0.001, n=3.

3.3.3 Bmi1 over-expression differentially affects apoptosis of progenitor cells contained in neurospheres in a temporal dependent manner.

Bmi1 is involved in regulation of apoptosis of NSC via direct inhibition of the Ink4a/Arf tumour suppressor locus, particularly of the protein product p19^{ink4a} (Sutter *et al.*, 2007). It was therefore conceivable that Bmi1 over-expression could induce resistance to apoptosis in NSPC. We set out to test this hypothesis by studying the apoptotic rate upon induction of Bmi1 over-expression.

Tertiary neurospheres derived from E16.5 NSPC of NestinCre;STOPFloxBmi1 and STOPFloxBmi1 mice were mechanically dissociated into single cells at 6-7 DIV and processed for Annexin V apoptosis assay, as previously described. Flow cytometry analysis showed increased apoptosis in Bmi-1 over-expressing cultures (mean= 24.9%, n=4) as compared with control (mean=16.6%, (Figure 3.22)).

As the vast majority of progenitor cells contained in the neurospheres are committed progenitors, these findings suggest that these cells are more susceptible to apoptotic death induced by protracted culturing upon activation of Bmi1 expression.

Intriguingly, when I performed the same experiment on neurospheres derived from NSPC isolated from the postnatal SVZ at P7, different results were obtained.

On average, Annexin V positive cells in neurospheres obtained from postnatal NSPC constituted 7.9% of the total whereas they represented 13% in the controls (Figure 3.23).

We conclude that activation of Bmi1 expression confers resistance to apoptosis in progenitor cells contained within neurospheres originating from postnatal NSP in our culture conditions.

Taken together these data imply that intrinsic, developmental stage dependent factors play a crucial role in determining the apoptotic response of Bmi1 over-expressing NSPC.

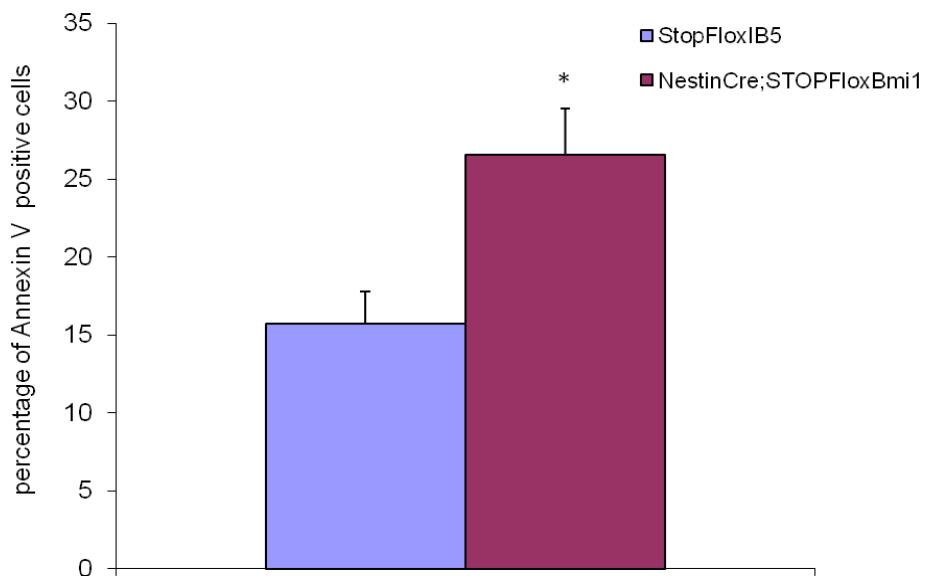


Fig 3.22 Increased apoptosis in Bmi1 over-expressing neuro-spheres as detected by Annexin V assay. *= p<0.05, n=4

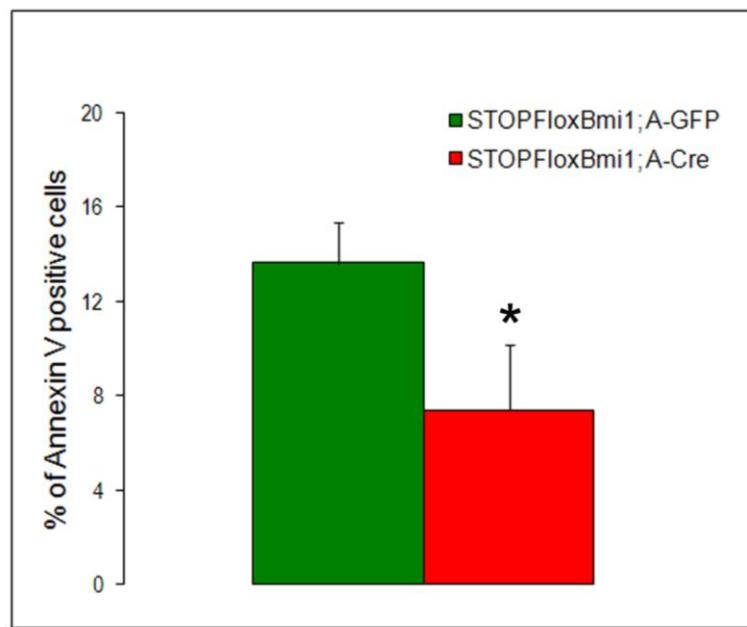


Fig 3.23 Reduced apoptosis in NSPC upon “acute” Bmi-1 over-expression at P7. *= p<0.05, n=4.

3.3.4 Effects of Bmi-1 over-expression on differentiation

3.3.4.1 Analysis of the cellular composition of neurospheres over-expressing

Bmi1

Neurospheres consists of a heterogeneous population of cells. In fact, only a small number of cells of the neurosphere are capable of stem cell properties, such as self-renewal. To begin to characterise the impact of activation of Bmi1 over-expression on the differentiation potential of NSPC, I analysed the cellular composition of NS over-expressing Bmi1 as compared with controls. I have chosen to evaluate cellular composition of neurospheres using previously reported neural stem and progenitor cell markers namely glial fibrillary acid protein (GFAP), type-1 transmembrane proteoglycan protein (NG2), and ganglioside epitope (A2B5). GFAP is a marker commonly associated with astrocytes and yet most of NSPC, isolated from neurogenic sites of postnatal and adult mouse brain, express it. A previous study has demonstrated that GFAP positive SVZ cells give rise to neurons terminating at the olfactory bulb in mice (Doetsch *et al.*, 1999a). Another study by Doetsch *et al.*, has shown that upon deletion of transit amplifying cells and neuroblasts in the SVZ of mice with the arabinofuranoside (Ara C), GFAP positive astrocytes/NSPC, which are spared by Ara C, were sufficient for regeneration of the SVZ (Doetsch *et al.*, 1999b). Moreover, primary GFAP positive astrocytes cultured with media containing EGF and FGF-2, form neurospheres and act as multipotent NSPC. However, whether GFAP-positive cells within neurospheres represent NSPC and/or astrocytes is unclear.

The NG2 proteoglycan is a type 1 transmembrane protein, previously considered to be expressed in oligodendrocyte precursor cells that exclusively gave rise to oligodendrocytes. Recent findings have shown that NG2 expressing cells generate not only oligodendrocytes but also protoplasmic astrocytes and in some instances neurons *in vivo*. Moreover, NG2 positive cells are thought to contribute to gliomas formation and glioma angiogenesis (Chekenya *et al.*, 2002a, Chekenya *et al.*, 2002b). Recently, NG2 positive neurosphere forming cells were isolated from embryonic murine cortex, striatum and adult spinal cord (Chekenya and Pilkington, 2002, Dromard *et al.*, 2008). Interestingly, the same study has found that NG2 negative embryonic spinal cord cells gave rise to neurospheres containing NG2 positive cells. Moreover, NG2-positive cells isolated from neurospheres by flow cytometry and plated in media containing EGF and FGF2, were capable of generating neurospheres (Dromard *et al.*, 2008). Interestingly, these cells have been shown also to be positive for A2B5, another marker classically thought to be exclusively expressed in oligodendrocyte precursors *in vivo*.

A) Flow cytometry analysis

To dissect the cellular composition of NS, simultaneous detection of multiple antigens by means of incubation with fluorescence labelled specific antibodies followed by flow cytometric quantification was used (as in Figure 3.24).

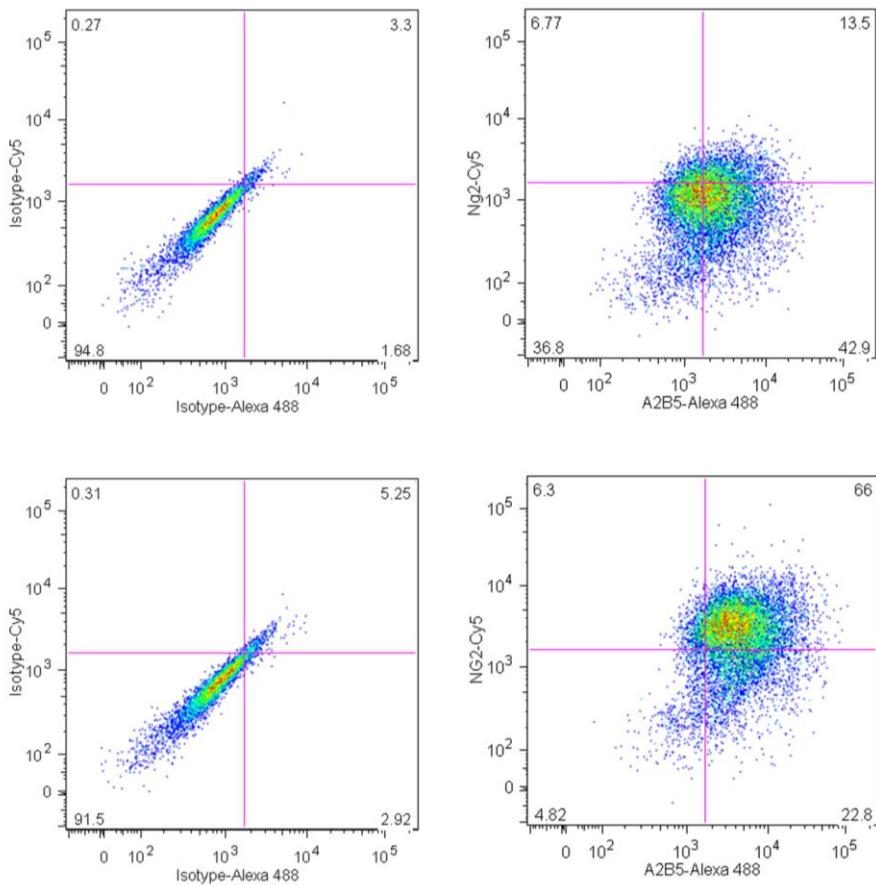


Fig 3.24 FACS dot plots showing the percentage of A2B5 (x-axis) and NG2 (y-axis) positive cells, and their non-immune controls (top and bottom left), control (top panel) and experimental (bottom panel) neurospheres. The upper right quadrants, of the right top and bottom plots respectively, show that experimental NS had 66% double positive cells as compared to 13.5% in control NS. n=3

NS derived from Bmi-1 over-expressing NSPC displayed a significantly higher percentage of cells co-expressing GFAP and NG2 (79%), GFAP and A2B5 (73%), NG2 and A2B5 (54%) compared with control neurospheres, respectively 41%, 13% and 17% (Figure 3.24 p<0.001 and p<0.01). Quantification of three independent biological replicas is plotted below in Figure 3.25.

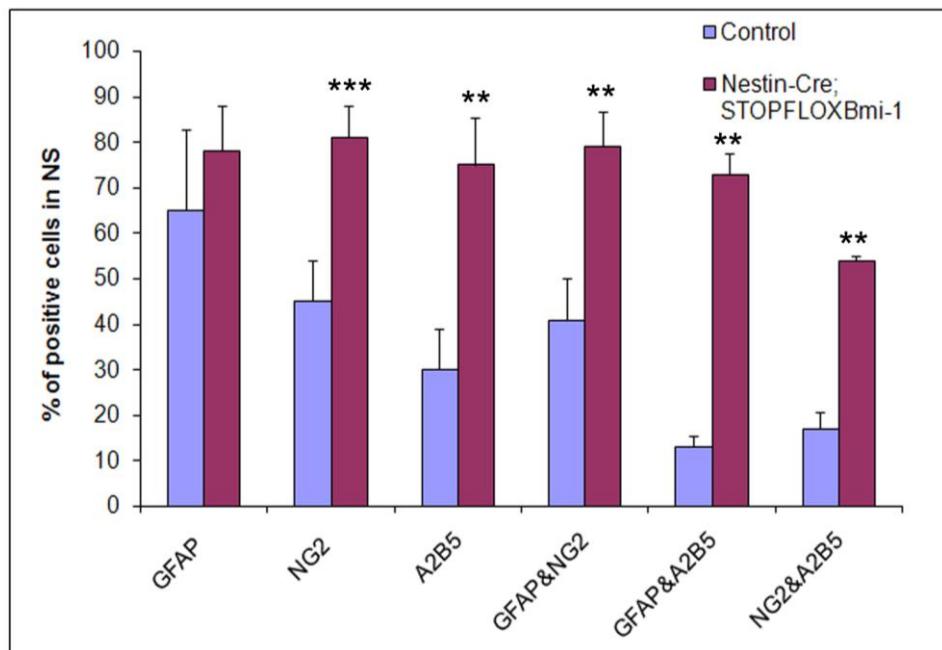


Fig 3.25 Bmi1 over-expressing cells contain more cells positive for early progenitor markers such as NG2, A2B5, GFAP and NG2, GFAP and A2B5, and NG2 and A2B5. **= p<0.01, ***= p<0.001, n=3.

Cells expressing NG2+ and A2B5+ in neurospheres have previously been shown to be self-renewing and multipotent (Dromard *et al.*, 2008). Our data show that over-expression of Bmi-1 in NSPC results in expansion of the pool of self-renewing and NS-forming cells *in vitro* and they are compatible with the results we obtained with the self renewal assays.

B) Immunohistochemical analysis

In order to verify these findings with an independent method, neurospheres derived from NestinCre;STOPFloxBmi1 and controls NSPC were embedded in OCT,

cryo-sectioned and immunostained against either non immune controls (IgG or/and IgM) or with anti-NG2, anti-A2B5, anti-GFAP or combinations of these antibodies and analysed by confocal microscopy. Bmi1 over-expressing NSPC give rise to NS that have a greater number of cells positive for NG2 and A2B5 (bottom left- see inset), GFAP and NG2 (bottom middle), and more GFAP and A2B5 positive cells (bottom right- see inset), all compared with control NS (Figure 3.26). Note that the results seen in Figure 3.26 are in agreement with those obtained from flow cytometric analysis.

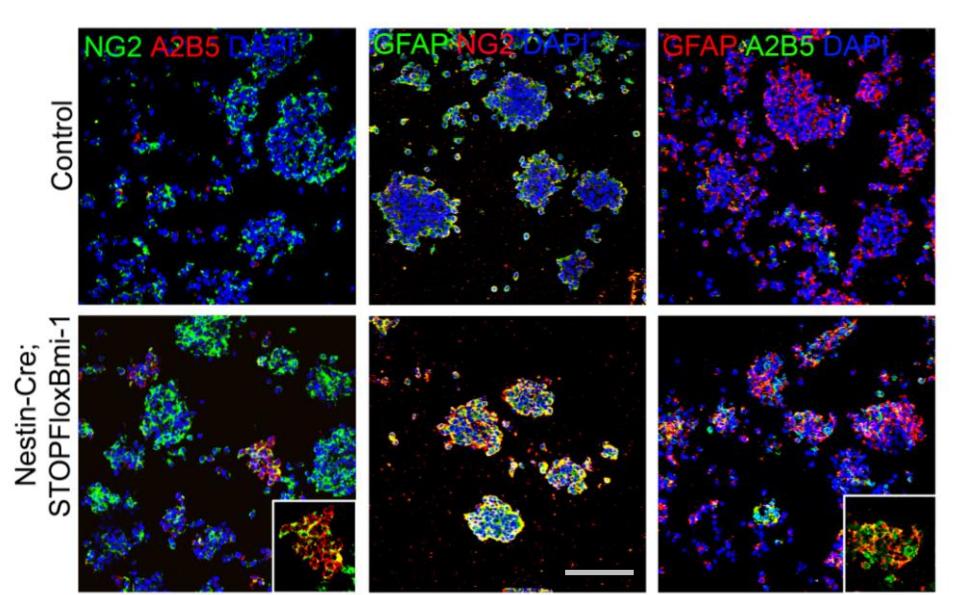


Fig 3.26 OCT sections showing the cellular composition of NS:
Note that Bmi1 over-expressing neurospheres have more double positive cells for all antigens studied compared with controls. DAPI was used for counterstaining. Magnifications 20X, insets 40X, Scale bar is 200 μ m, n=3.

We have further analysed Bmi1 over-expressing neurospheres for expression of markers associated with neuronal differentiation such as the homeobox gene Dlx2. This marker was chosen as its role in the acquisition of neuronal fate has been described in the developing forebrain (Petryniak *et al.*, 2007). Immunolabelling of OCT embedded NS revealed a reduced number of cells expressing Dlx2 in Bmi1 overexpressing cultures compared with controls (Figure 3.27).

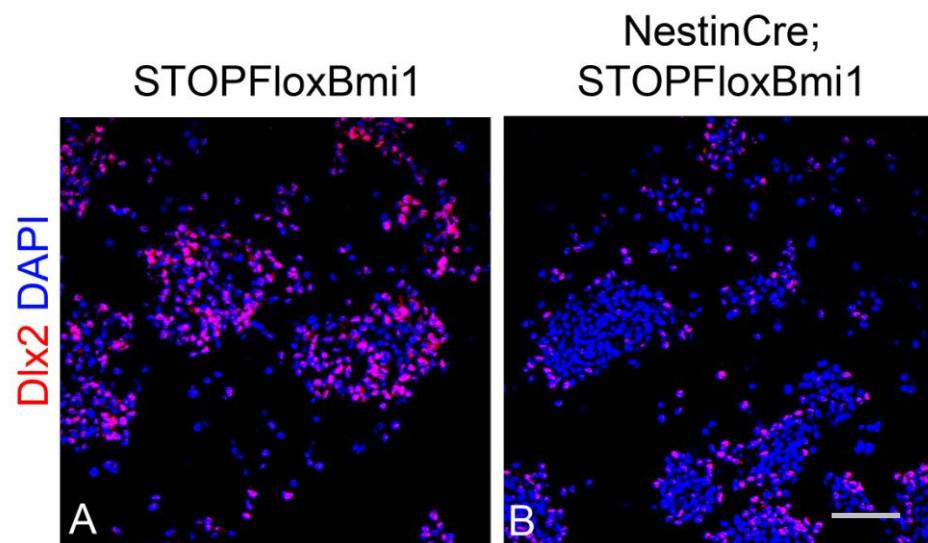


Fig 3.27 Immunostaining for Dlx2 on OCT sections of NS: A) Control NS contain Dlx2 positive cells both in the centre and the periphery, B) Bmi1 over-expressing NS have less cells positive for Dlx2 and they are focused mainly on the periphery of the sphere. DAPI was used for counterstaining. Magnifications 40X, Scale bar is 100 μ m, n=3.

These results are in keeping with the data of Petryniak *et al.*, (2007) who have shown that deletion of Dlx1 and Dlx2 homeobox transcription factors in oligodendrocyte progenitor cells results in inhibition of neuronal differentiation and pro-

motion of myelinating oligodendrocyte differentiation *in vivo*. Together, these results suggest that over-expression of Bmi1 in NSPC increases the number of undifferentiated cells contained within NS, and that neuronal differentiation may be impaired.

3.3.4.2 Analysis of the impact of Bmi1 overexpression on the differentiation potential of progenitor cells.

Next, I analysed the impact of increased Bmi1 expression on the differentiation capacity of NSPC contained in NS derived from Nestin-Cre;STOPFloxBmi1 compared with controls. Induction of differentiation was carried out as previously described. Fluorescent immunolabelling against specific antigens as detailed below was performed at DIV5 and DIV9 and DAPI was used for counterstaining. For each experiment 10 random image fields were taken at 40X magnification using a confocal microscopy, and cells were counted by image2 software.

A) Neuronal differentiation is delayed at DIV5

The percentage of cells expressing nestin is higher (36% vs 13%) at DIV5 in Bmi1 over-expressing cultures (Fig 3.28 A and F, p<0.01). Moreover, the expression of Musashi, another marker of undifferentiated/uncommitted NSPC (Kaneko *et al.*, 2000), was retained in a significantly higher number of cells (50% vs 28%) in the transgenic cultures (Fig 3.28 B and G p<0.001). While the percentage of GFAP positive astrocytes and O4 positive oligodendrocytes was similar in both cultures, a significant reduction in the number of βIII-tubulin positive neurons was noted in

Nestin-Cre;STOPFloxBmi1 cultures (Fig. 3.28 E and J, p<0.01). The percentage of cells expressing markers of immature glial and oligodendroglial progenitors such as A2B5 (28% vs 10%) and NG2 (19% vs 10.9%) as well as co-expressing GFAP and A2B5 (20% vs 3.5 %) and GFAP and NG2 (17% vs 10.5%) was significantly higher in cultures over-expressing Bmi1 (Fig.3.28 C and H and D and I, p<0.001 or p<0.01). The results for immunostaining experiment are shown in Figure 3.28. Their quantification is shown in Figure 3.29.

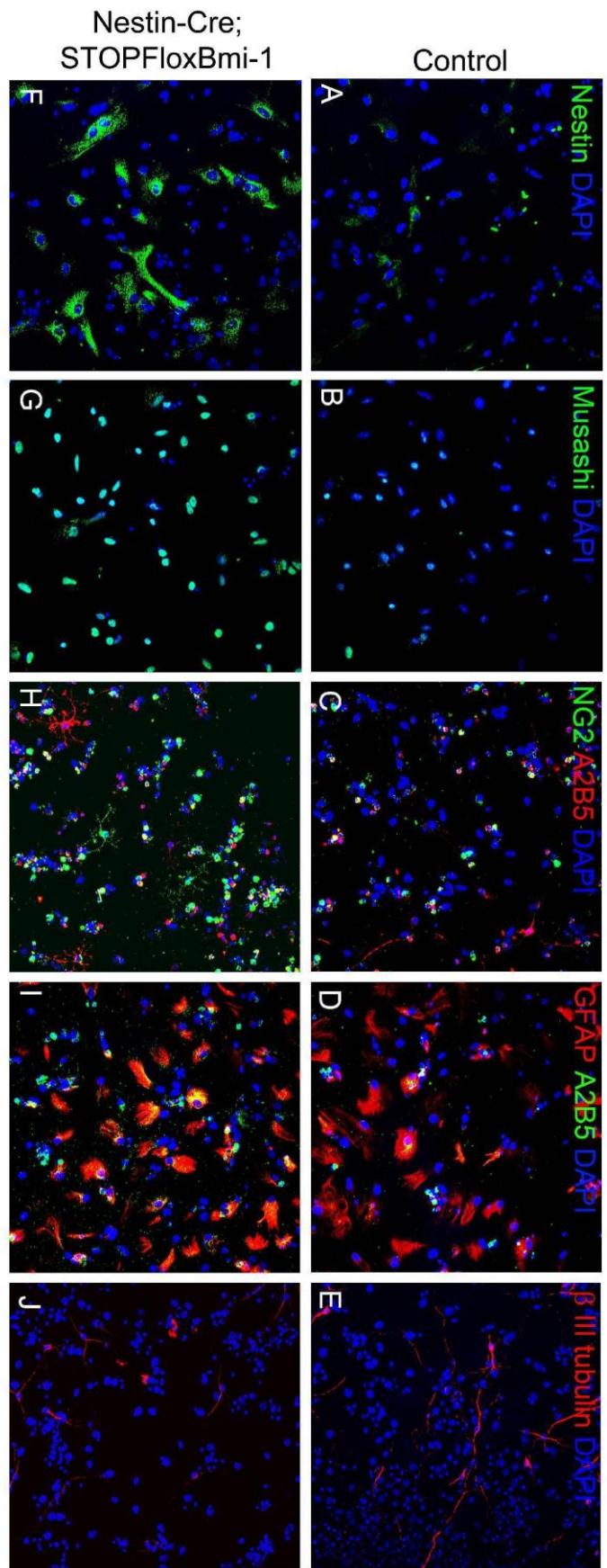


Fig 3.28 Differentiation of tertiary neurospheres on laminin coated glass coverslips for 5 DIV: Increased number of nestin+ and musashi+ as well as NG2/A2B5+ cells in NestinCre;STOPFloxBmi1 neurosphere at 5DIV. A&F) Nestin expression is maintained by more cells of the experimental culture. B&G) Bmi1 over-expressing cells continue to express Musashi at 5 DIV differentiation. C&H) Bmi1 over-expressing cells generates more of NG2 and A2B5 positive oligodendroglial progenitors. D&I) More GFAP and A2B5 double positive cells are generated from Bmi1 over-expressing NSPC. E&J) Bmi1 over-expressing NSPC generate less neurons. Magnifications 40X, n=3.

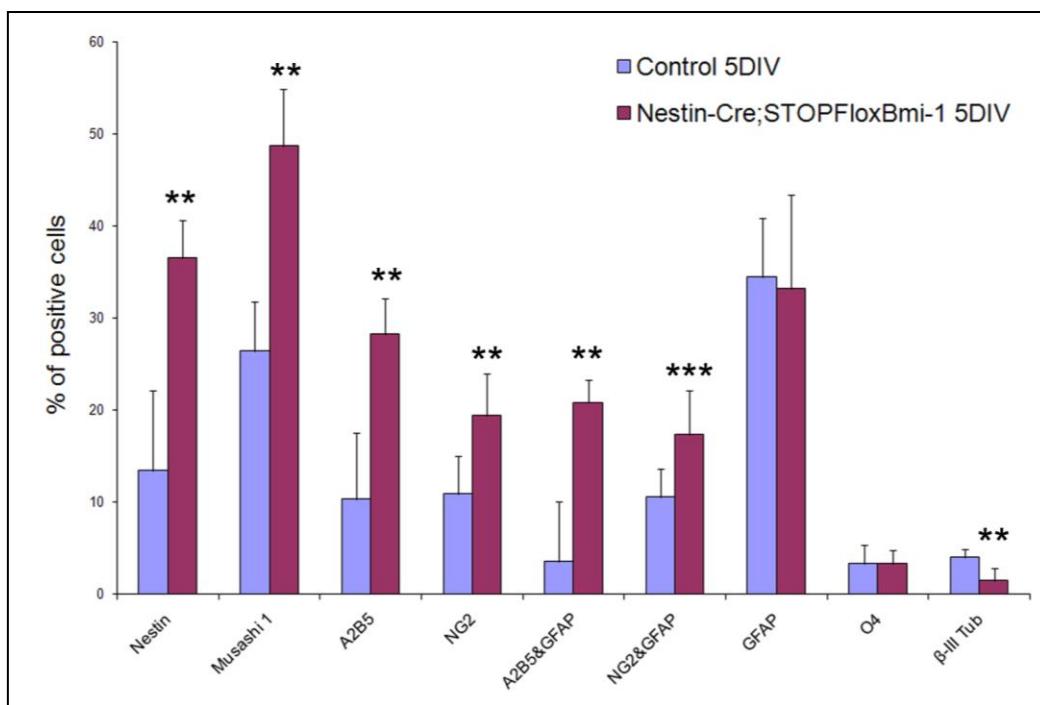


Fig 3.29 Quantification of the immunocytochemistry analysis of differentiation properties Bmi1 over-expressing NSPC compared with controls: Increased number of undifferentiated (Nestin and Musashi positive cells) and glial differentiated progenitors (GFAP and NG2; GFAP and A2B5 positive cells) and reduced number of β III Tubulin positive neurons. Note that there is no difference in the number of O4 positive oligodendrocytes. **= p<0.01, n=3.

The reduced number of neuronally differentiated cells is surprising and may suggest that increased levels of Bmi1 expression in differentiating neural progenitors maintains cells in an undifferentiated state or alternatively favours glial versus neuronal differentiation.

B) Complete differentiation is achieved at later stages with an increased number of neurons and oligodendrocytes produced from Bmi1 over-expressing NSPC

Similar analysis performed at DIV9 showed higher number of O4 positive oligodendrocytes (Fig 3.30, A&E, 10% vs 6.5%), but also a higher number of β III-tubulin positive neurons (Figure 3.36 B&F, 8.5% and 5.4%) in Bmi1 over-expressing cultures compared with controls (Figure 3.30).

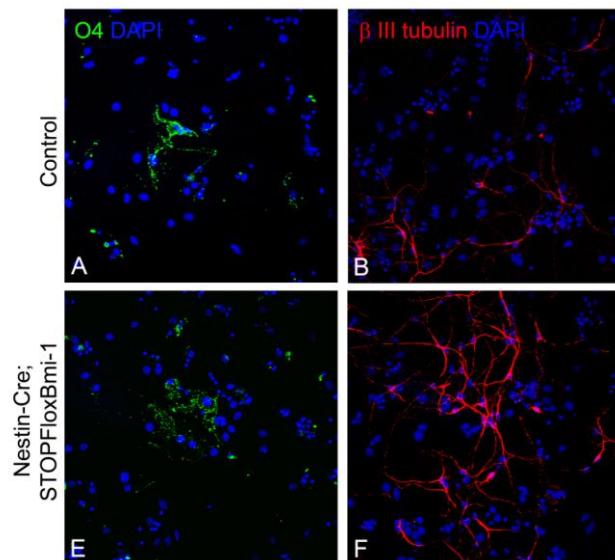


Fig 3.30 Neurosphere differentiation on laminin coated coverslips for 9DIV, and immunostaining with antibodies against antigens β III tubulin and O4 to identify neurons and oligodendrocytes respectively: Bmi1 over-expressing NS give rise to more neurons and oligodendrocytes after 9 DIV differentiation. n=3

In addition, I have performed co-immunostaining labelling against antigens GFAP and A2B5, GFAP and NG2 and lastly NG2 and A2B5, following 9 DIV differentiation of neurospheres (Figure 3.31). Note that there are no NG2 and A2B5 positive cells in the control cultures. Interestingly, the percentage of differentiated cells generated from Bmi1 over-expressing NS compared with controls, that is positive for A2B5 and GFAP (20.5 % vs 10.4 %), NG2 and GFAP (9.1 % vs 2.9 %), NG2 (11 % vs 3.2%) were significantly different (Figure 3.32 for quantification of immunostaining).

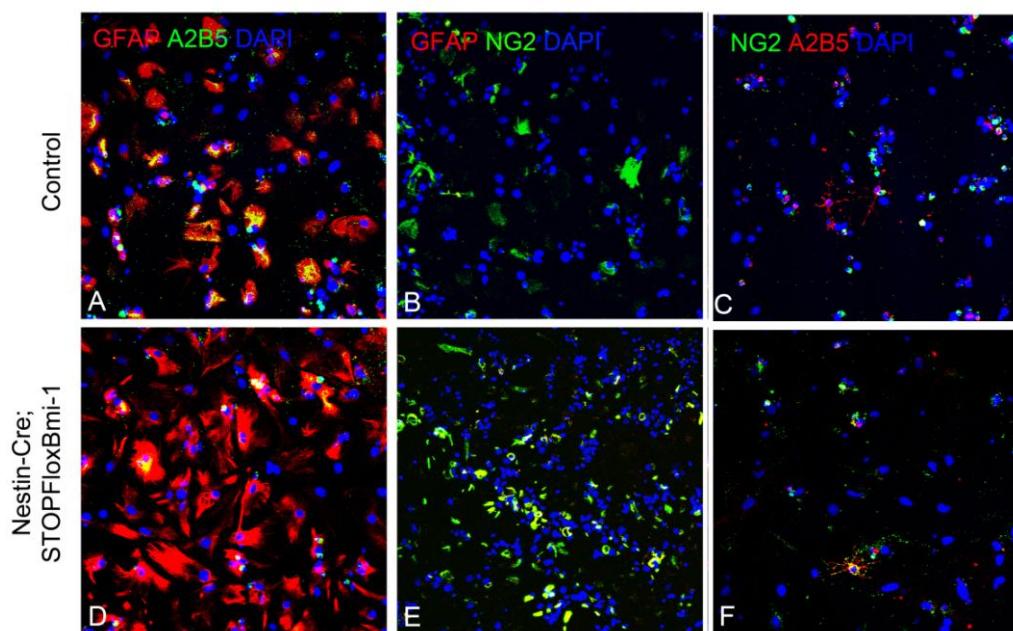


Fig 3.31 Neurosphere differentiation on laminin coated coverslips for 9DIV, and co-immunostaining with antibodies against antigens GFAP and A2B5, GFAP and NG2, and NG2 and A2B5 for glial cell progenitors: More undifferentiated and early progenitor cells are seen in Bmi1 over-expressing cultures even after 9 DIV differentiation. n=3

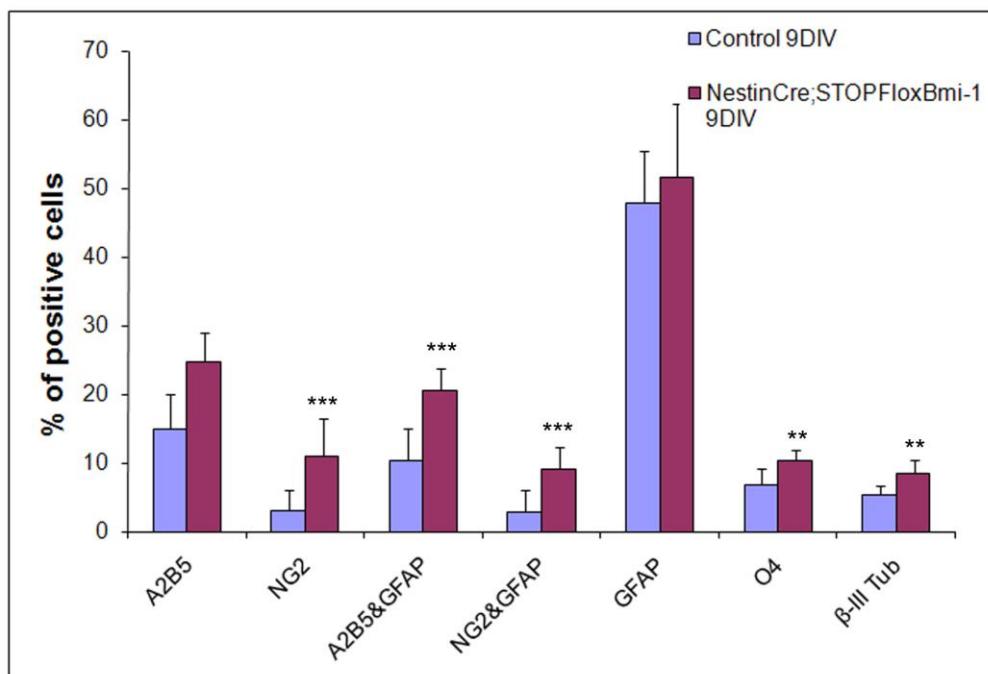


Fig 3.32 Quantification of 9 DIV differentiation assay: Neurosphere differentiation on laminin coated coverslips for 9DIV, and co-immunostaining with antibodies against antigens GFAP and A2B5, GFAP and NG2, and NG2 and A2B5 for glial cell progenitors: More undifferentiated and early progenitor cells as well as more neurons and oligodendrocytes are seen in Bmi1 over-expressing cultures. **= p<0.01, ***=p<0.001, n=3.

This analysis of the effects of activation of Bmi1 expression on differentiation suggests that Bmi1 maintains NSPC in an undifferentiated state longer. This is evident from the reduced number of terminally differentiated cells and the high number of progenitor cells in Bmi1 over-expressing cultures at DIV5. However, at 9 DIV a higher number of βIII-tubulin positive neurons and O4 positive oligodendrocytes were seen. Taken together these findings suggest that Bmi1 over-expression delays but does not hamper trilineage differentiation of progenitor cells.

Similar experiments carried out on postnatal NSPC and NS derived thereoff demonstrate that Bmi-1 overexpression in postnatal progenitors does not alter their differentiation potential (Figure 3.33), as they can give rise to neurons (β IIIITub), oligodendrocytes (O4) and astrocytes (GFAP). Moreover, it shows that their morphology is unaffected. Next, I tested whether Bmi-1 overexpression changed the relative distribution of these cell types in our culture conditions. However no difference was found in their relative distribution in comparison with control cells and the obtained figures were comparable to the figures commonly quoted in the field (Singec *et al.*, 2006), (Figure 3.34 for quantification of data).

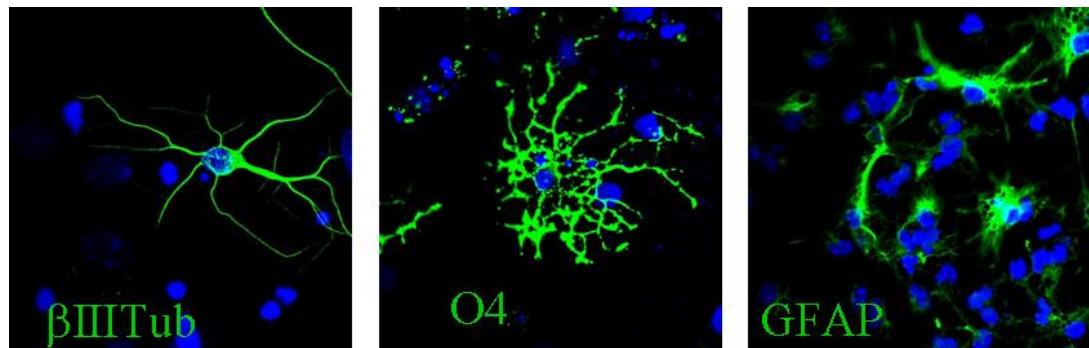


Fig 3.33 Bmi-1 over-expression does not alter postnatal NSPC differentiation potential and the morphology of their progeny, n=3.

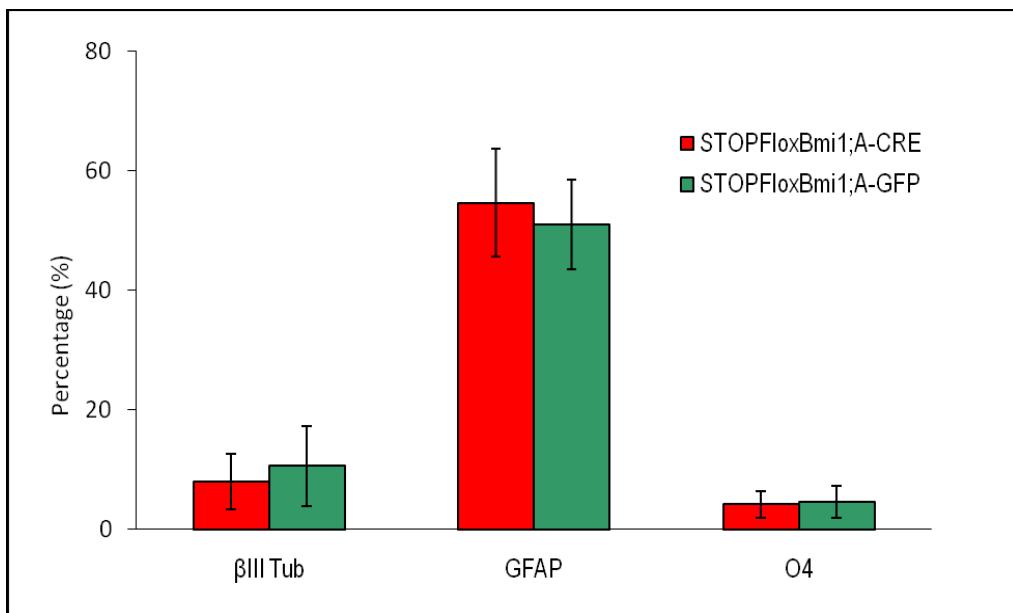


Fig 3.34 Quantification of differentiation assays performed on Bmi-1 over-expressing and control NS: relative distribution of cell types (neurons 8.1 % vs 10.6 %, astrocytes 54.6 % vs 51.1 %, oligodendrocytes 4.2 % vs 4.6 %) are similar to those commonly quoted in the literature. n=3.

In summary, the impact of Bmi1 over-expression on the differentiation potential of NSPC differs at different developmental timepoints.

3.3.5 Analysis of the molecular mechanisms mediating the observed effects of Bmi-1 over-expression on the biological properties of NSPC *in vitro*.

3.3.5.1 Canonical downstream targets of Bmi1.

Bmi1-/- mice displayed impaired self-renewal and maintenance of SVZ NSC leading to their postnatal depletion (Molofsky *et al.*, 2003, Park *et al.*, 2003a). The molecular mechanisms of Bmi1 function have been shown to be mediated in part by transcriptional repression of the *ink4a* locus (Jacobs *et al.*, 1999a). This locus encodes two cell cycle inhibitors, $p16^{Ink4a}$ and $p19^{ARF}$ (Sherr, 2001), the activities of which increase with postnatal age and have been linked to cellular senescence (Molofsky *et al.*, 2003).

Recently Bmi1 has been shown to regulate self renewal of embryonic NSC located in ventricular zone (VZ) of the developing forebrain through inhibition of a different cell cycle inhibitor, namely $p21^{WAF1/Cip1}$ (Fasano *et al.*, 2007). A more recent paper by the same group has extended our knowledge of Bmi-1 and $p21^{WAF1/Cip1}$ association in regulation of embryonic NSPC self-renewal, by including into the equation also Foxg1. Foxg1 is winged-helix gene family member a gene previously known to be expressed by forebrain progenitor cells during development and throughout adulthood (Dou *et al.*, 1999, Shen *et al.*, 2006).

The respective contribution of the Ink4aArf and Foxg1-p21 axes to mediation of Bmi1 function in the biological properties of embryonic and postnatal NSPC is still unclear. We set out to analyse how overexpression of Bmi1 in NSPC would influence the expression of key members of these two molecular axes. To this end we

perform protein analysis by means of Western blotting for $p16^{Ink4a}$, $p19^{ARF}$, $p21^{WAF1/Cip1}$, and quantitative RT-PCR for Foxg1.

We set out by testing whether activation of Bmi1 over-expression by “acute” and “chronic” methods would make a difference in functions of Bmi1 on suppression of $p16^{Ink4a}$, $p19^{ARF}$, $p21^{WAF1/Cip1}$ (Figure 3.35).

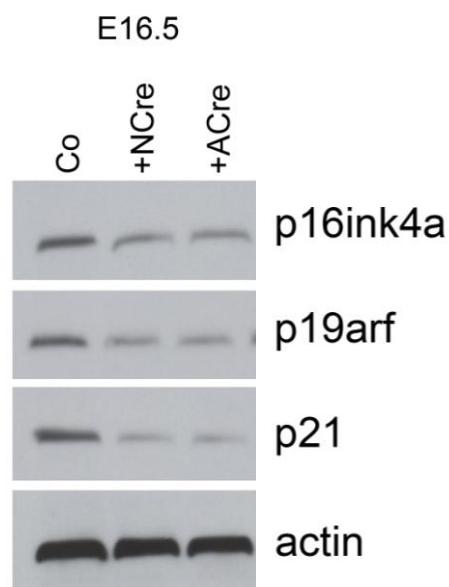


Fig 3.35 Western blotting showing the effects of “acute” and “chronic” activation of Bmi1 over-expression on expression of “canonical” downstream effectors of Bmi1. Suppression of expression of p16, p19 and p21 are achieved in both systems. Protein lysate obtained from STOPFloxBmi1 neurospheres was used as control. Actin was used as loading control. n=3

Reduced levels of $p16^{Ink4a}$, $p19^{ARF}$, $p21^{WAF1/Cip1}$ was seen in neurospheres derived from NestinCre;STOPFloxBmi1 or STOPFloxBmi1;A-Cre as compared to controls. This finding suggests that Bmi1 represses expression of these cell cycle inhibitors simultaneously and independent of whether Bmi1 expression was activated chronically during embryonic development *in vivo* or acutely *in vitro*.

Western Blotting analysis was performed also on neurospheres originating from NSPC isolated at p7 and p70 and similar results were obtained (Figure 3.36).

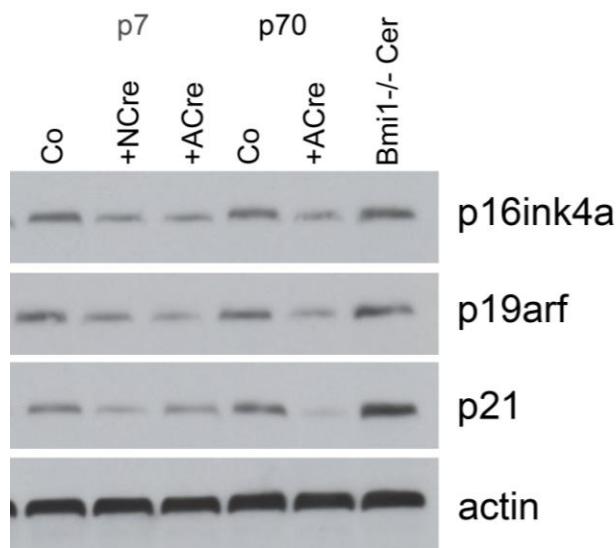


Fig 3.36 Western blotting performed on protein isolated from postnatal and adult NSPC showing the effects of “acute” and “chronic” activation of Bmi1 over-expression on expression of “canonical” downstream effectors of Bmi1. Suppression of expression of p16, p19 and p21 are achieved in both systems. Protein lysate obtained from STOPFloxBmi1 neurospheres was used as control. Actin was used as loading control. n=3

Furthermore, in keeping with recent data supporting a correlation between the expression of Bmi1 and Foxg1 (Fasano *et al.*, 2009) we also show increased levels of Foxg1 ($p<0.01$), independently of whether Bmi1 activation occurred during embryonic development *in vivo* or acutely *in vitro*.

In keeping with published literature (and that of Fasano *et al.*, 2009) overexpression of Bmi1 in embryonic neurospheres revealed downregulation of the expression of Foxg1, independently on whether Bmi1 activation was induced during embryonic development *in vivo* or acutely *in vitro* (Figure 3.37). However, no changes in the expression of this gene were found in P7 and P70 neurospheres.

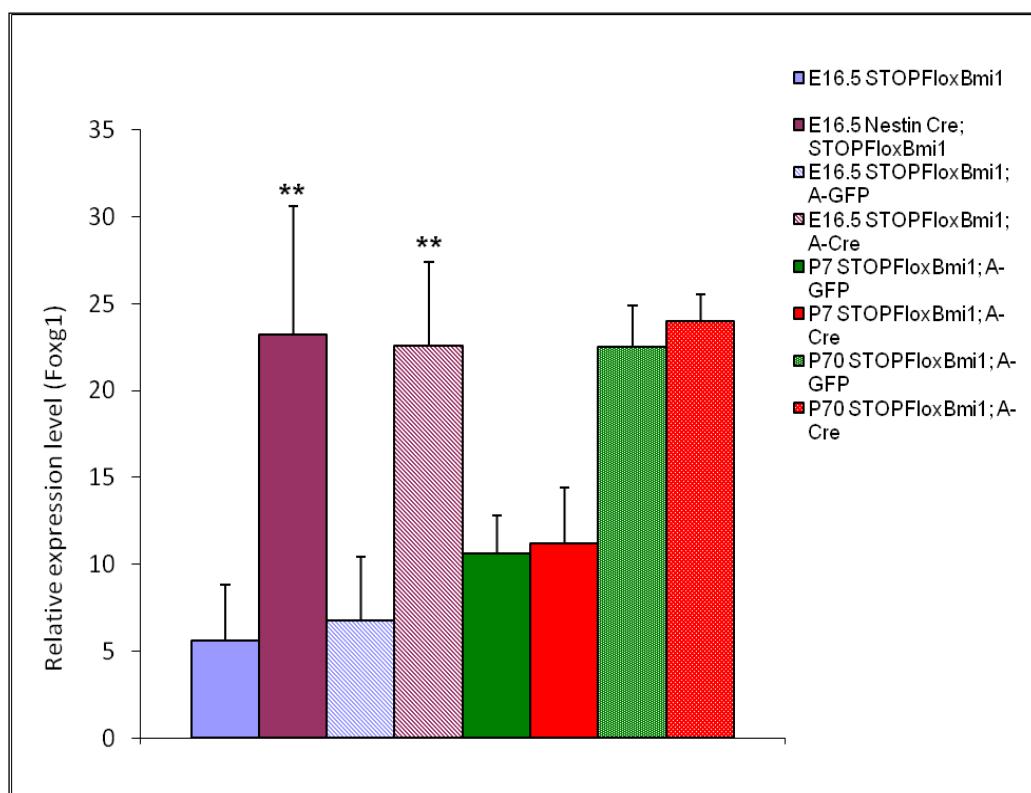


Fig 3.37 Showing the relative expression of Foxg1 gene in Bmi1 over-expressing and control neurospheres: Expression of Foxg1 gene in Bmi1 over-expressing embryonic neurospheres is approximately 5 fold greater than those of control. There is no difference in expression of Foxg1 in postnatal and adult neurospheres. **= $p<0.01$, n=3.

3.3.5.2 Gene expression profiling using microarray technology

So far, we have shown that Bmi1 over-expression in embryonic NSPC increases their proliferation and apoptosis as well as their self renewal capacity while it delays differentiation in neurospheres, and that these changes are associated with down-regulation of Bmi1's known/recently identified downstream targets. The molecular mechanisms of Bmi1 function in all biological properties of NSPC examined above have been shown to be mediated in part by transcriptional repression of the *ink4a* locus (Jacobs *et al.*, 1999b).

However, concomitant deletion/suppression of either $p16^{Ink4a}$ or $p19^{ARF}$ or both did not completely rescue the above mentioned defects in self-renewal of NSC derived from Bmi1-/- mice (Molofsky *et al.*, 2003, Bruggeman *et al.*, 2005, Sparmann and van Lohuizen, 2006). Other NSPC functions, such as for example migration, have also been shown to be controlled by Bmi1 in an *ink4a* independent manner (Bruggeman *et al.*, 2009). Moreover, it is interesting that the developmental stage of NSPC influences the preferential downstream targets of Bmi1. Indeed, Bmi1 has been shown to regulate self renewal of embryonic NSC located in ventricular zone (VZ) of the developing forebrain through inhibition of a different cell cycle inhibitor, namely $p21^{WAF1/Cip1}$ (Fasano *et al.*, 2007).

In this context, we set out to study which other novel genes might be involved in the changes noted post Bmi-1 over-expression in biological functions of NSPC.

3.3.5.2.1 Illumina Expression Array analysis

To establish genome-wide expression profiles of NestinCre;STOPFloxBmi1 and control neurospheres, total RNA was isolated from tertiary neurospheres (n=2). Gene expression profiling was carried out at the Genome Centre in Charterhouse Square. The Illumina BeadChip arrays, which are high-density photolithographic oligonucleotide microarrays, were used. This system allows accurate measurement of relative concentration of an RNA sequences in a complex mixture of nucleic acids. Each platform represents approximately 46 000 genes (Illumina Expression Array).

We initially checked whether the control genes are present on the scatter plot, which shows the overview and clustering of gene expression. Indeed, we could see CRE on one side of the distribution and CV40 and NeoR on the other, as well as most of the genes of experimental and control samples clustered closely together suggesting that the number of differentially expressed genes was low (Figure 3.38).

NestinCre;STOPFloxBmi1 vs. STOPFloxBmi1

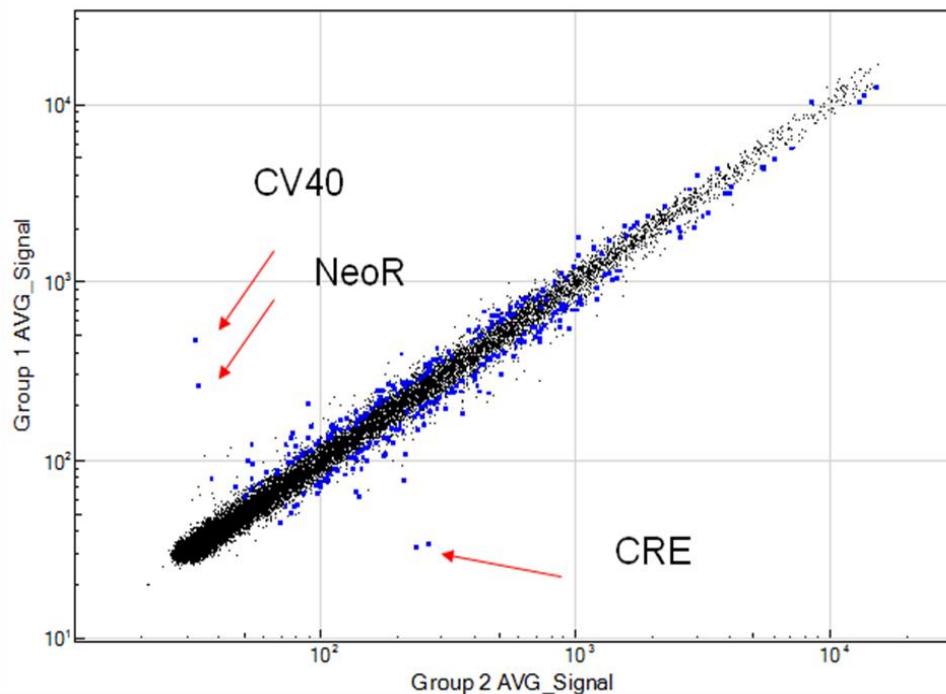


Fig 3.38 The scatter plot showing the distribution of gene expression of NestinCre;STOPFloxBmi1 and STOPFloxBmi1 neurospheres: Note that most of the genes are closely distributed except the reporter genes for samples (arrows).

Next, we set a cutting point at 95% significance for selection of most differentially expressed genes between experimental and control samples. This application was performed on the BeadStudio (Illumina) software. Statistical analysis revealed that 1600 genes were significantly ($P<0.05$) differentially expressed between the experimental and control samples. However, only 700 of these genes were annotated (Figure 3.39).

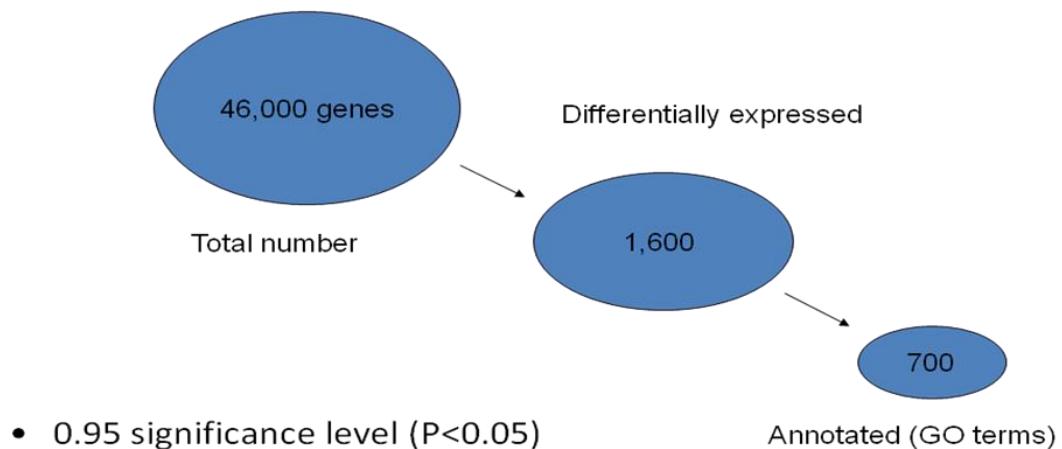


Fig 3.39 Selection process for differentially expressed genes that are further studied using MetaCore or RT-PCR

3.3.5.2.2 Validation of Target Genes Using Real-Time Quantitative PCR- embryonic

Genes with highest expression profiles were selected for verification processes. These genes included transcription factors (Dlx2, Dbx2, and Irx2) and others (Ng2, Ncan, Ncam and Bcan) potentially involved in regulation of neurogenesis and differentiation, and cell adhesion molecules (Col9a1, Col1a1, Col4a1 and Col4a2) regulating cell to cell contact, as well as other cellular processes, in neurospheres.

Both TaqMan probe based-assays and SYBR Green Jump Start Taq Ready Mix assays were performed and run respectively on Applied Biosystems 7500 RT PCR and ABI PRISM7500 Sequence Detection System machines.

qPCR expression profiling of NS isolated from Nestin-Cre;STOPFloxBmi1 and control mice revealed significant differences in the expression of transcription factors involved in neuronal specification – Dbx2, Neurog2, Dlx2 and Irx2 ($p<0.001$ or $p<0.01$ or $p<0.05$) (Figure 3.40). Moreover, several members of the collagen family-Collagen Ia1, IVa1 and IVa2 and IXa1- as well as cell adhesion molecules Ng2, Ncam, Ncan and Bcan were deregulated in the double transgenic NS ($p<0.001$ or $p<0.01$ or $p<0.05$) (Figure 3.40).

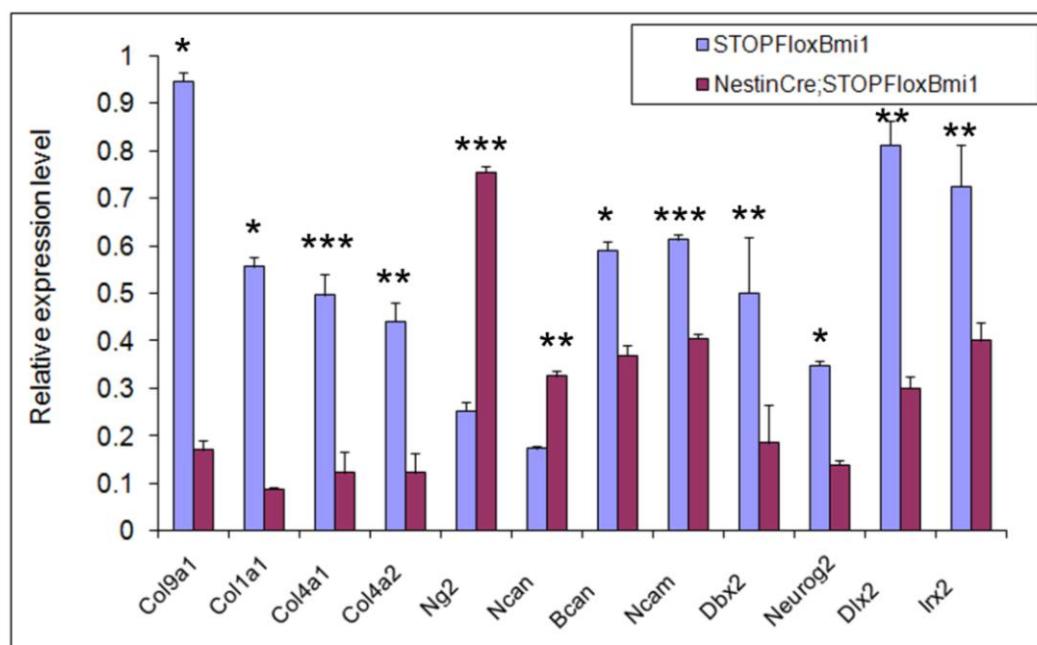


Fig 3.40 qRT-PCR arrays results show deregulation of cell adhesion molecules and down-regulation of transcription factors involved in neurogenesis in NS over-expressing Bmi1. * $= p< 0.05$; ** $= p<0.01$; *** $= p<0.001$; n=3.

Increased expression of Bmi1 results in upregulation of the expression of several members of the collagen family cell adhesion molecules such as Collagen Ia1, IVa1 and IVa2 and IXa1, as well as cell adhesion molecules Ng2, Ncam, Ncan and Bcan. Note that reduced expression of members of the Collagen IV family and changes in expression of cell adhesion molecules may have functional consequences on migration of NSPC as Bmi1 has been previously shown to be regulating this process *in vitro* (Bruggeman *et al.*, 2009). Moreover, upregulation of NG2 in Bmi1 over-expressing neurospheres may account for increased number of oligodendroglial progenitors identified upon induction of differentiation. Reduced expression of Dlx2 in Bmi1 over-expressing neurospheres is in keeping with previous immunohistochemistry studies. Moreover, Bmi1 over-expressing neurospheres have significantly lower expression of a series of transcription factors - Dbx2, Neurogenin2, Irx2 (and Dlx2)- all of which play an important role in neuronal fate commitment (Ahn *et al.*, 2004, Lacin *et al.*, 2009, Roybon *et al.*, 2009). Dlx2 in particular is known to regulate the balance between neurogenesis and oligodendrogenesis in the developing forebrain (Petryniak *et al.*, 2007). A Bmi1- mediated downregulation of Dlx2 may therefore explain the delayed neuronal differentiation and increased number of oligodendroglial progenitors observed in Bmi1 overexpressing cultures.

3.3.5.2.3 Validation of differentially expressed genes by quantitative real-time PCR-in postnatal and adult NSPC

Next we set out to study changes in gene expression profile of P7 and P70 (latter for only expression of Foxg1) neurospheres derived from tertiary NS/P cells infected with Adeno-Cre virus. Briefly, we wanted to test whether “acute” activation of Bmi1 expression in postnatal NS/P cells would produce similar changes in gene expression profile observed in embryonic stage.

To perform this, SVZ NS/P cells were isolated from P7 and P70 STOPFloxBmi1 mice and cultured for three passages before infection with Adeno-Cre virus. RNA was isolated from tertiary neurospheres and cDNA was derived as previously described and used as template for quantitative RT-PCR analysis performed using SYBR Green Jump Start Taq Ready Mix and Taqman assays.

We have assessed expression of transcription factors Dlx2 and Irx2, genes which are potentially involved in neurogenesis, and Ng2, Col4a1, Col4a2, Ncan, Bcan and Ncam, which are members of collagen family and cell adhesion molecules potentially involved in self-renewal and cell-to-cell contact in neurospheres- some of those that we had studied in embryonic neurospheres.

Both TaqMan probe based-assays and SYBR Green Jump Start Taq Ready Mix assays were performed and run respectively on Applied Biosystems 7500 RT PCR and ABI PRISM7500 Sequence Detection System machines. I found that only Col4a1 significantly differentially expressed by the NSPC over-expressing Bmi1. All other genes; Col4a2, Ng2, Ncan, Bcan, Ncam, Dlx-2, Foxg1 and Irx-2, that were differentially expressed in the embryonic NSPC were not significant at the postnatal time point (Figure 3.41).

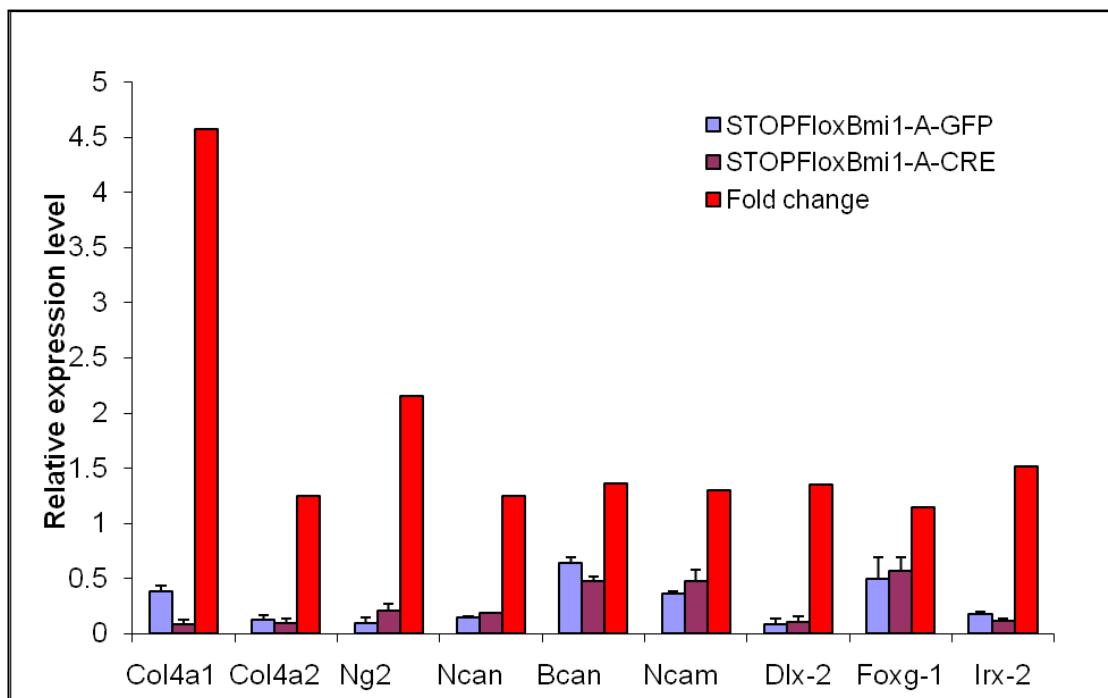


Fig 3.41 qRT-PCR results showing that only Col4a1 gene is differentially expressed ($p<0.001$) upon Bmi1 over-expression. $n=3$.

Given that we have found similarities and in some cases differences in the biological properties of embryonic compared with postnatal Bmi1 over-expressing NS/P cells as assessed by functional tests for self-renewal, proliferation, differentiation

and apoptosis, it seemed reasonable to investigate and determine the degree of difference in gene expression levels in postnatal NSPC compared with those of embryonic. In terms of changes in biological properties we have found that Bmi-1 over-expression in postnatal and adult NSPC leads to increased resistance to apoptosis, makes no difference in differentiation potential, however self-renewal and proliferation are increased. In a similar fashion, the changes in gene expression profile in the Bmi1 over-expressing postnatal NSPC, as presented above in Figure 3.41, points to a differential affect of acute Bmi1 over-expression between embryonic and postnatal time points studied. Indeed, in the Bmi1 over-expressing postnatal neurospheres only one gene namely, Col4a1 is significantly differentially expressed. These findings suggest that Bmi1 acts through different sets of downstream targets in regulation of NSPC properties in the postnatal time point compared with that of embryonic.

3.3.5.2.4 KEGG PATHWAY

We then took two approaches in analysing these genes further. First, we uploaded these genes into KEGG PATHWAY database, which is a regularly updated collection of pathway maps and their interactions, to have a fast and reliable glance at possible pathways and cellular processes that may be altered upon Bmi-1 over-expression. Indeed we found that several pathways, their components and the cellular processes that they are most likely to alter in the experimental samples were significantly affected. These included MAPK signalling pathway, Insulin signalling pathway and Wnt signalling pathway. On the other hand, several core cellular processes and functions were also affected. These included focal adhesion, regulation of actin cytoskeleton, ECM-receptor interactions, Cell adhesion molecules (CAMs), Adherens junctions, and axon guidance (Table 3.2).

Table 3.2 showing changes in the cellular processes and functional pathways observed in Bmi1 over-expressing NSPC.

<u>mmu04010</u> <u>MAPK signaling pathway</u>	14182 Fgfr1; fibroblast growth factor receptor 1 14186 Fgfr4; fibroblast growth factor receptor 4 17762 Mapt; microtubule-associated protein tau 18218 Dusp8; dual specificity phosphatase 8 19042 Ppm1a; protein phosphatase 1A, magnesium dependent, alpha isoform 192176 Flna; filamin, alpha [SP:FLNA_MOUSE] 19252 Dusp1; dual specificity phosphatase 1
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	20130 Rras; Harvey rat sarcoma oncogene, subgroup R
	23882 Gadd45g; growth arrest and DNA-damage-inducible 45 gamma
	240168 Rasgrp3; RAS, guanyl releasing protein 3
	26414 Mapk10; mitogen-activated protein kinase 10
	26921 Map4k4; mitogen-activated protein kinase kinase kinase 4
	26971 Pla2g2f; phospholipase A2, group IIF
	56613 Rps6ka4; ribosomal protein S6 kinase, polypeptide 4
	70686 Dusp16; dual specificity phosphatase 16
<u>mmu04512 ECM-receptor interaction</u>	11603 Agrn; agrin
	12826 Col4a1; collagen, type IV, alpha 1
	12827 Col4a2; collagen, type IV, alpha 2
	15529 Sdc2; syndecan 2
	16403 Itga6; integrin alpha 6
	16780 Lamb3; laminin, beta 3
	243816 Gp6; glycoprotein 6 (platelet)
<u>mmu01430 Cell Communication</u>	11465 Actg1; actin, gamma, cytoplasmic 1
	12826 Col4a1; collagen, type IV, alpha 1
	12827 Col4a2; collagen, type IV, alpha 2
	16403 Itga6; integrin alpha 6
	16780 Lamb3; laminin, beta 3
<u>mmu04514 Cell adhesion mole- cules (CAMs)</u>	15000 H2-DMb2; histocompatibility 2, class II, locus Mb2
	15529 Sdc2; syndecan 2
	16403 Itga6; integrin alpha 6
	18417 Cldn11; claudin 11
	56863 Cldn9; claudin 9

<u>mmu04520 Ad-herens junction</u>	11465 Actg1; actin, gamma, cytoplasmic 1 14182 Fgfr1; fibroblast growth factor receptor 1 16001 Igf1r; insulin-like growth factor I receptor 17127 Smad3; MAD homolog 3 (Drosophila) 19263 Ptprb; protein tyrosine phosphatase, receptor type, B
<u>mmu04360 Axon guidance</u>	12631 Cfl1; cofilin 1, non-muscle 13845 Ephb3; Eph receptor B3 18481 Pak3; p21 (CDKN1A)-activated kinase 3 235611 Plxnb1; plexin B1
<u>mmu04910 Insulin signaling pathway</u>	13684 Eif4e; eukaryotic translation initiation factor 4E 18709 Pik3r2; phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2 (p85 beta) 18746 Pkm2; pyruvate kinase, muscle [EC:2.7.1.40] 18762 Prkc ζ ; protein kinase C, zeta 26414 Mapk10; mitogen-activated protein kinase 10
<u>mmu04310 Wnt signaling pathway</u>	17127 Smad3; MAD homolog 3 (Drosophila) 20319 Sfrp2; secreted frizzled-related protein 2 20377 Sfrp1; secreted frizzled-related protein 1 22415 Wnt3; wingless-related MMTV integration site 3 26414 Mapk10; mitogen-activated protein kinase 10 93960 Nkd1; naked cuticle 1 homolog (Drosophila)

Of these pathways and processes that are altered upon Bmi-1 over-expression the most significantly and highly altered was that of MAPK signalling pathway, as assessed by the sheer number of components that are altered (15) and their fold difference in expression being more than two fold. Moreover, the MAPK signalling pathway has been highlighted as an important component of NSPC self-renewal regulation, and proliferation and apoptosis in other cell types. The similarities and correlations drawn between roles of MAPK signalling pathway portrayed in the literature and the changes we have observed in NSPC functions upon Bmi-1 over-expression has made a strong case for us to study changes in MAPK signalling pathway components in our experimental and control neurospheres.

In order to study expression of MAPK signaling pathway components protein lysate was isolated from experimental and control tertiary neurospheres. 10ng/ μ l protein was used to perform Western blotting experiments.

We initially looked at the total MAPK (ERK1/2) levels using antibodies against ERK and P-ERK, and found that there is no significant difference in protein levels between experimental and control neurospheres (Figure 3.42).

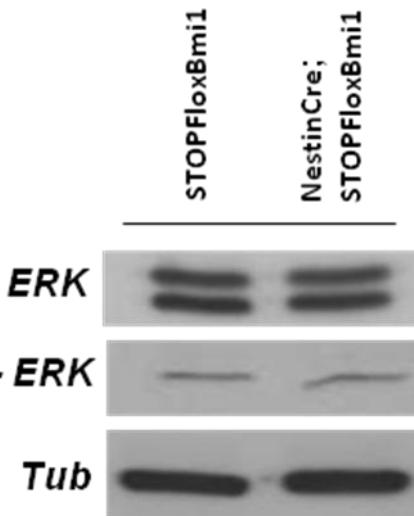


Fig 3.42 Western Blot analyses reveal that Bmi1 over-expression does not change levels of ERK and P-ERK in neurospheres. Tubulin was used as loading control. n=3

This finding suggested that the changes in expression of components of MAPK pathway could be more subtle and requires a more specific examination of the pathway. Independent studies of the other three main branches of the pathway, namely p38, SAPK/JNK and Jun were carried out. Indeed, the MAPK pathway related differentially expressed genes seen in the KEGG PATHWAY database analysis were pointing towards a possible change in the p38 branch of the pathway. Western Blotting analysis was performed for p38, SAPK/JNK and Jun branches of the MAPK pathway and the results are shown in Figure 3.43.

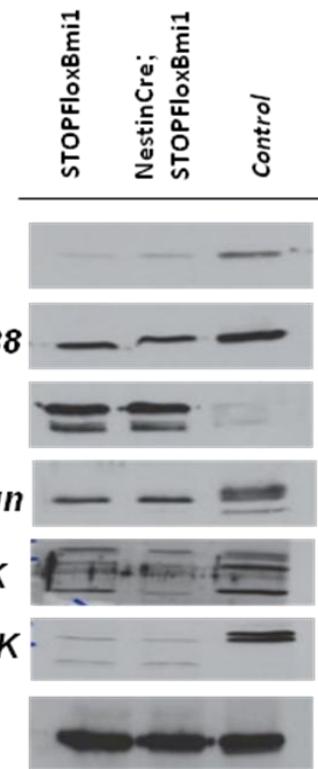


Fig 3.43 Western Blot analyses show that three main branches of MAPK pathway do not alter in neurospheres over-expressing Bmi1. Tubulin was used as loading control. n=3

No changes in protein levels of neither of the branches of MAPK pathway were found in Bmi1 over-expressing neurospheres.

3.3.5.2.5 MetaCore

In the next stage of microarray data analysis, we applied a further filter, which in principle oblige a minimum of 1.5 fold difference, as a requirement for accepting a gene as a potential target for advanced analysis. This application reduced the total number of differentially expressed genes to 450, some of which were either down-regulated more than 2 fold or up-regulated more than 2 fold, though a majority of the gene population in the list were clustered around a two fold expression difference.

Here we used MetaCore, a manually curated software suite, which is based on regularly updated database of protein-protein, protein-DNA and protein-RNA interactions published in peer reviewed journals, transcription factors, signalling and metabolic pathways and the effects of bioactive interactions. MetaCore is used for functional analysis of microarray data using enrichment analysis. Briefly, enrichment analysis consists of matching gene IDs of possible targets for the "common", "similar" and "unique" sets with gene IDs in functional ontologies in MetaCore. The probability of a random intersection between a set of IDs the size of target list with ontology entities is estimated in p-value of hypergeometric intersection. The lower p-value means higher relevance of the entity to the dataset, which shows in higher rating for the entity. The ontologies include GeneGo Pathway Maps, GeneGo Process Networks, GO Processes, GeneGo Diseases (by Biomarkers). The degree of relevance to different categories for the uploaded datasets is defined by p-values, so that the lower p-value gets higher priority.

Differentially expressed genes ($P<0.05$ and expression > 1.5 fold) were uploaded to begin the analysis. Metacore allowed us to visualise what pathways, networks and biological processes our differentially expressed genes in Bmi1 overexpressing neurospheres relates to and involved in, in the up-to-date knowledge about protein-protein interactions and their pathways.

Canonical pathway maps represent a set of about 650 signaling and metabolic maps covering human biology (signaling and metabolism) in a comprehensive way. All maps are drawn from scratch by GeneGo annotators and manually curated and edited. Experimental data is visualized on the maps as blue (for downregulation) and red (upregulation) histograms. The height of the histogram corresponds to the relative expression value for a particular gene/protein.

These are about 110 cellular and molecular processes whose content is defined and annotated by GeneGo. Each process represents a pre-set network of protein interactions characteristic for the process. Experimental data is mapped on the processes and shown as red (up-regulation) and blue (down-regulation) circles of different intensity. Relative intensity corresponds to the expression value.

See below for a set of results drawn as list of processes, pathways and their maps, and list of pathologies/malignancies from the 700 genes uploaded (though 450 are accepted as significantly differentially expressed) into MetaCore software.

GeneGo Pathway Maps

Canonical pathway maps represent a set of about 650 signaling and metabolic maps covering human biology (signaling and metabolism) in a comprehensive way.

All maps are drawn from scratch by GeneGo annotators and manually curated & edited. Experimental data is visualized on the maps as blue (for downregulation) and red (upregulation) histograms. The height of the histogram corresponds to the relative expression value for a particular gene/protein (Figure 3.44).

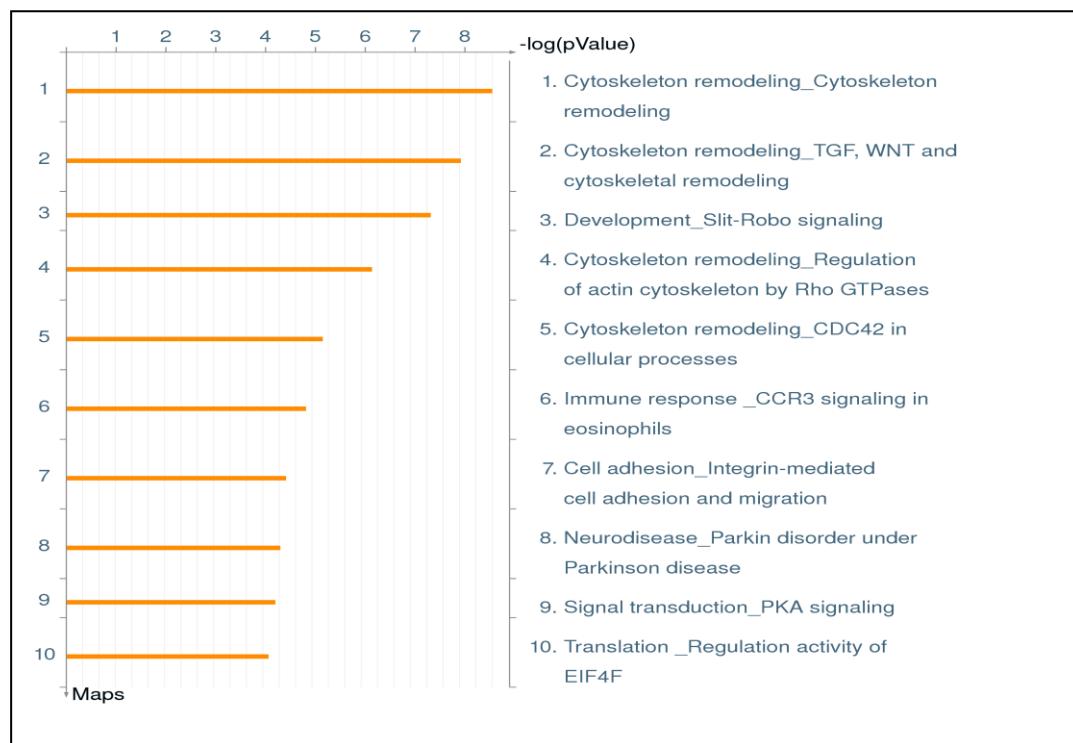
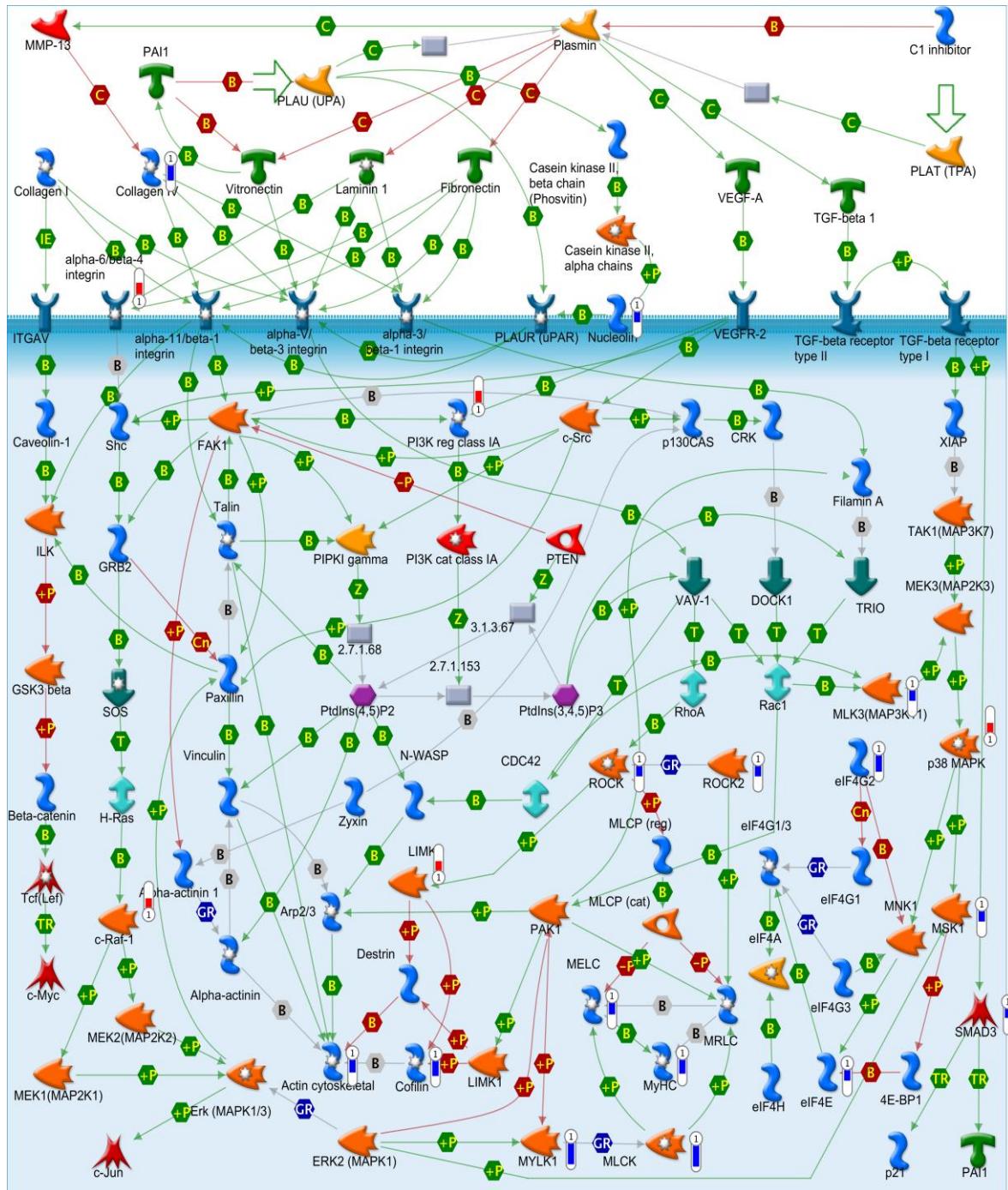


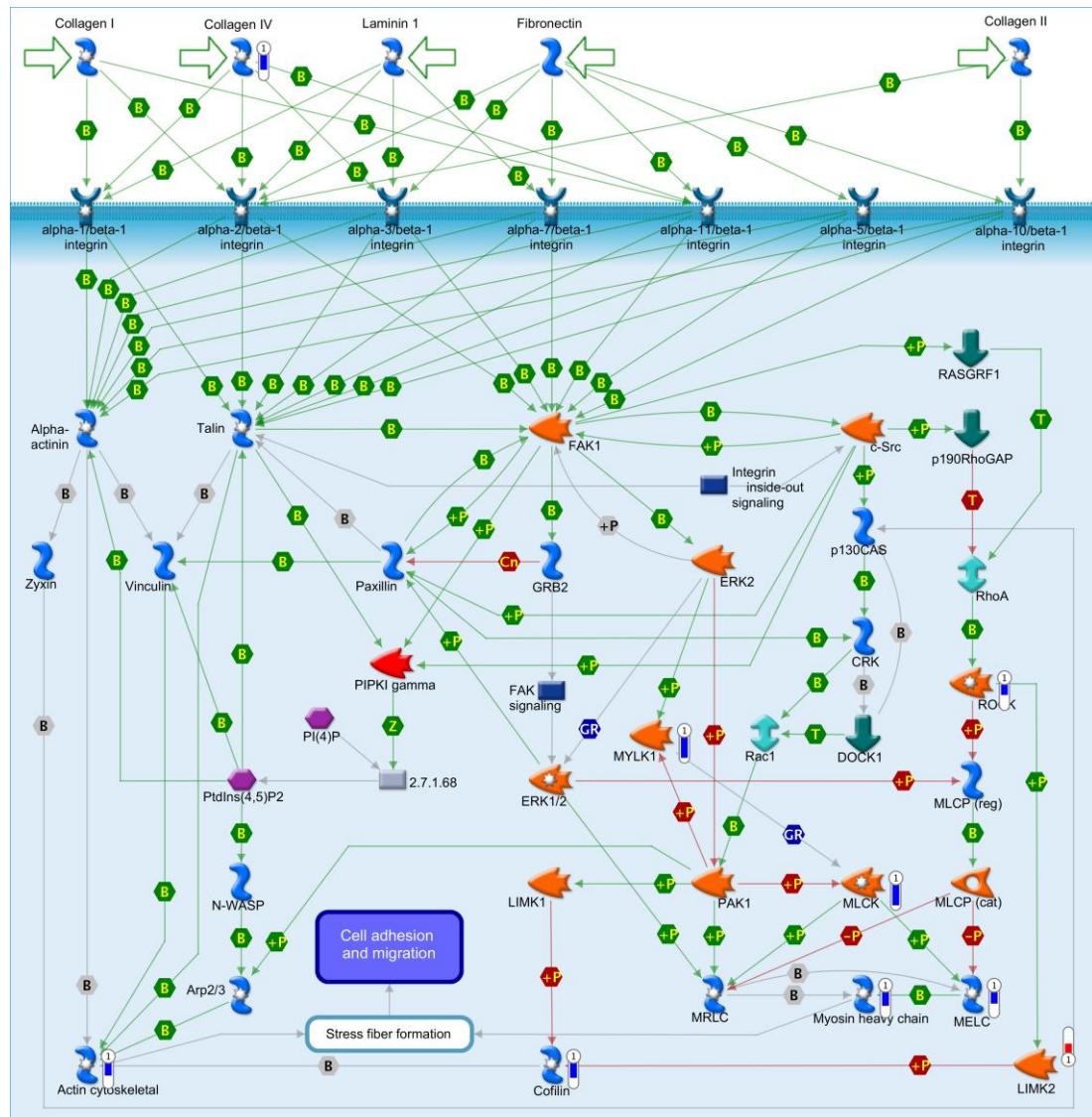
Fig 3.44 GeneGo Pathway Maps. Sorting is done for the 'Statistically significant Maps' set.

Below are two examples of the maps generated from MetaCore analysis:

1. **Cytoskeleton remodelling:** up-regulated genes shown in red bars are (PI3K reg class IA, LIMK, alpha-6/beta-4 integrin and c-Raf-1) and down-regulated genes shown in blue bars include (Collagen IV, Nucleolin, SMAD3).



7. Cell adhesion_ integrin-mediated cell adhesion and migration: the only up-regulated gene shown in red bar is (LIMK2) and down-regulated genes shown in blue bars include (Collagen IV, MYLK1, ROCK, Nucleolin, Myosin heavy chain and Actin cytoskeletal).



In addition to the maps, a series of networks that relate to cellular processes are also generated automatically by the MetaCore software. A table summarising these processes based on the protein-protein interactions is provided below in Figure 3.45.

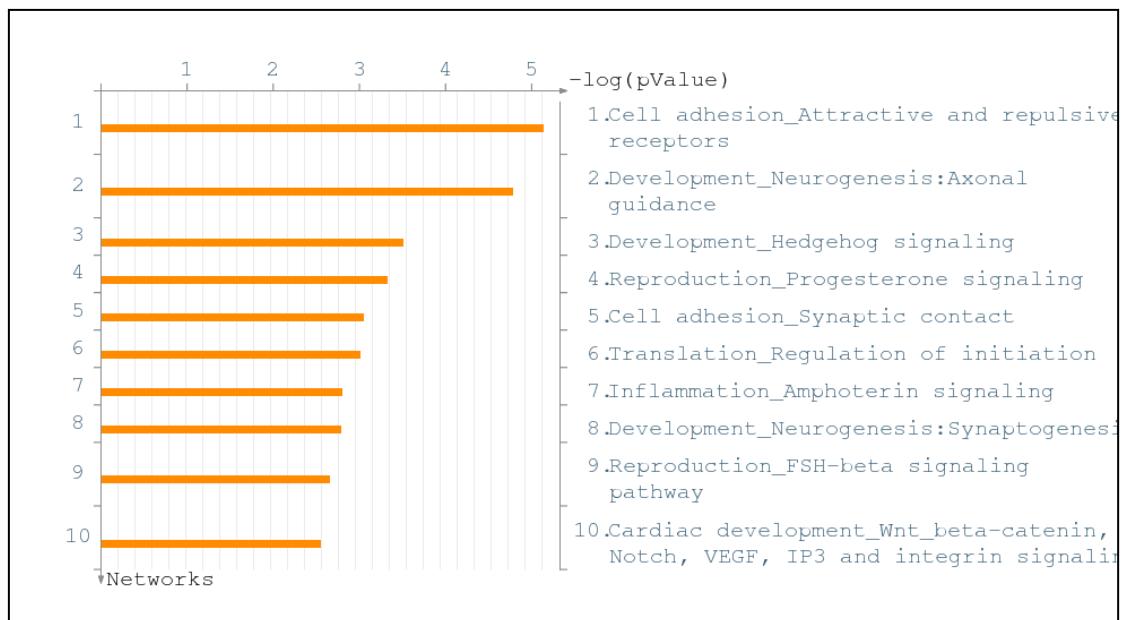
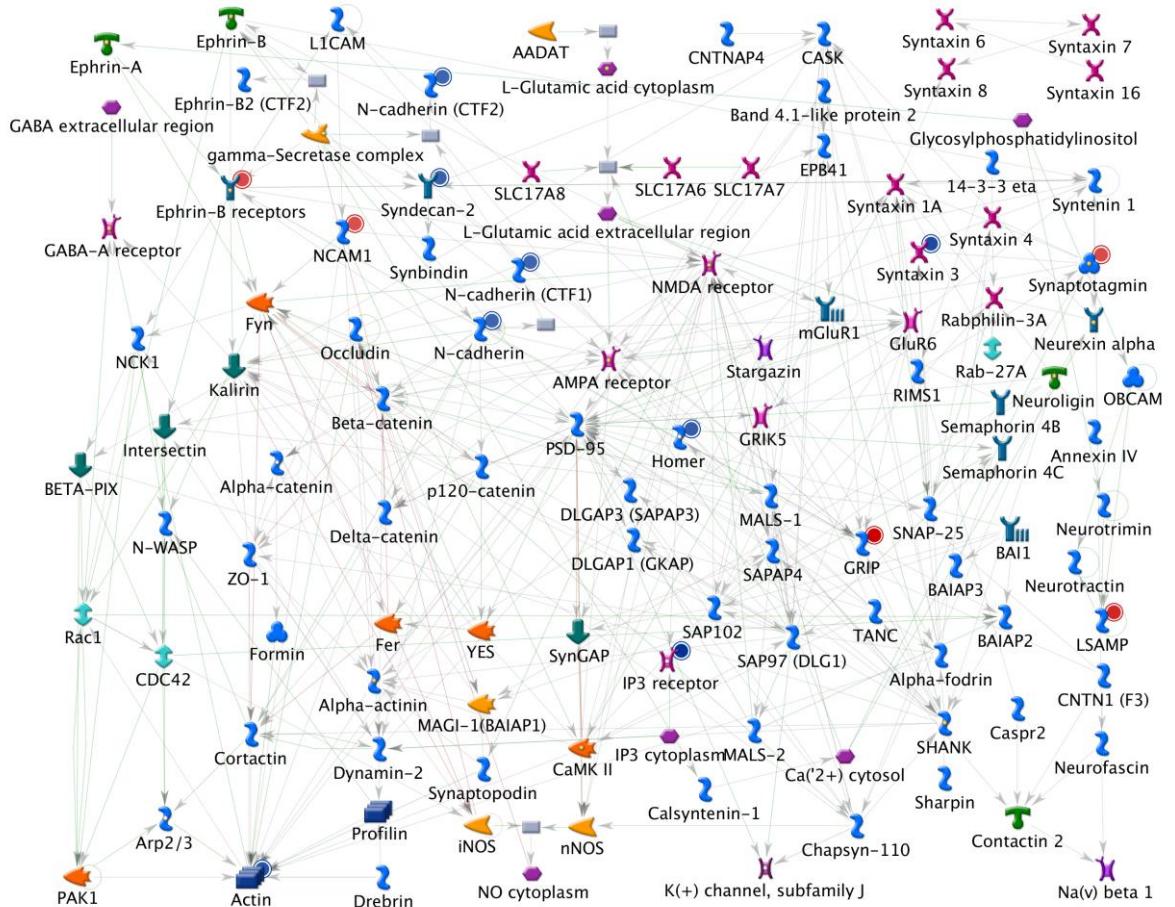


Fig 3.45 GeneGo Process Networks. Sorting is done for the 'Statistically significant Networks' set.

Below is one example of GeneGo process networks:

1. Cell adhesion_Attractive and repulsive receptors:



Moreover, we could obtain a list of human diseases, whose gene content is annotated by GeneGo, that are related to differentially expressed genes upon Bmi1 over-expression in neurospheres. Disease folders are organized into a hierarchical tree. Gene content may vary greatly between such complex diseases as cancers and some Mendelian diseases. Also, coverage of different diseases in literature is

skewed. These two factors may affect p-value prioritization for diseases. The list of diseases associated with our differentially expressed genes is shown in Figure 3.46.

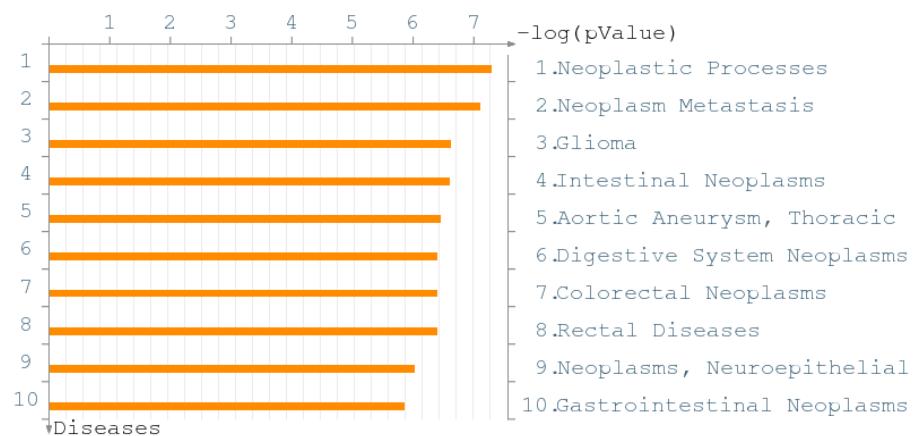


Fig 3.46 GeneGo Diseases (by Biomarkers). Sorting is done for the 'Statistically significant Diseases' set.

3.4 Assessment of tumourigenicity of NSPC overexpressing Bmi1 *in vivo*.

Background

A central issue in cancer biology is to understand the molecular mechanisms of tumour formation and whether “normal stem cells” can be involved in the pathogenesis of tumours. Particularly in the context of the adult brain, where the majority of cells are terminally differentiated and post-mitotic, stem cells are an attractive cell of origin for brain tumours.

Over the past 80 years, brain tumours were thought to originate from the cell types of the adult brain that they resembled most as assessed by comparison of histological features: for example,; astrocytomas were thought to arise from astrocytes; oligodendrogiomas from oligodendrocytes; ependymoma from ependymal cells (Louis *et al.*, 2001, Louis, 2006). However, this concept is being increasingly challenged and the role of NSPC in brain tumour development is currently being investigated.

Moreover, cells with stem cell properties have been isolated from paediatric and adult human brain tumours. These cells, so called “brain tumour stem cells” (BTSC), were shown to express stem cell markers Musashi-1, Sox2, nestin, MELK and CD133 (Hemmati *et al.*, 2003, Galli *et al.*, 2004, Singh *et al.*, 2004b, Jackson *et al.*, 2006), to have self-renewal capacity (as assessed by neurosphere formation, *in vitro*) and to differentiate into cells that express glial and neuronal markers (Ignatova *et al.*, 2002). Moreover, these cells were found to be tumourigenic; in fact they gave rise to tumours when injected into immunodeficient mice and retained their tumour phenotype upon serial transplantation (Singh *et al.*, 2004b).

This concept is intriguing and might imply that signalling pathways involved in the regulation of the balance between self-renewal and differentiation of adult NSPC could contribute as well to tumour initiation. Indeed, Jackson *et al.*, has shown that deregulation of proliferation in B-type neural stem cells of the SVZ by stimulating them through activation of their PDGFR α results in formation of hyperplasia resembling oligodendrogiomas (Jackson *et al.*, 2006). Moreover, combination of deletions or mutations of tumour suppressor genes (Nf1, Pten and p53) in GFAP positive NSPC/astrocytes or nestin expressing NSPC *in vivo* resulted in formation of brain tumours. For example, GFAP promoter dependent Cre activation mediates somatic inactivation of Nf1 and p53 results in formation of malignant astrocytomas in a mouse model (Zhu *et al.*, 2005). Additional haploinsufficiency of PTEN accelerates formation of astrocytomas in this mouse model (Kwon *et al.*, 2008). GFAP-Cre mediated inactivation of p53 and PTEN also led to malignant astrocytomas (Zheng *et al.*, 2008). These studies cannot determine whether tumours arose from NSPC as GFAP is also expressed by white matter astrocytes. Interestingly, targeting inactivation of Nf1, p53 or Nf1, p53 and PTEN in adult NSPC by means of nestin driven Cre expression results in formation of malignant gliomas (Alcantara Llaguno *et al.*, 2009), suggesting that cell of origin determines the phenotype of the tumours.

Bmi1 and brain tumour stem cells

Elevated Bmi1 expression has been shown to induce tumourigenesis in the haemato poetic system in transgenic mice (Alkema *et al.*, 1997a). Increased expression of BMI1 has been demonstrated in humans, not only in haematological malignancies, such as high grade lymphomas (Bea *et al.*, 2001), but also in neural tumours, e.g.

medulloblastomas and neuroblastomas (Leung *et al.*, 2004, Cui *et al.*, 2007). A study based on orthotopic transplantation model of glioma showed that Bmi1 regulates development and phenotype of low-grade diffuse astrocytomas independently of Ink4a/Arf tumour locus (Bruggeman *et al.*, 2007). Moreover, neurospheres derived from human tumours showed high levels of BMI1 expression both during proliferative stem cell state and upon differentiation (Hemmati *et al.*, 2003).

Recent studies have shown that BTSC express high levels of Bmi1 (Abdouh *et al.*, 2009), are particularly resistant to apoptosis (Bao *et al.*, 2006, Ghods *et al.*, 2007) and express A2B5 (Rebetz *et al.*, 2008). Similarly, in my studies, I found that Bmi1 over-expression in postnatal NSPC leads to increased proliferation and self-renewal, confers resilience to apoptosis to progenitors originating from these NSPC and increases the number of A2B5 positive cells in these neurospheres.

It was therefore interesting to assess whether Bmi1 over-expression in NSPC would lead to glioma formation *in vivo*.

We set out to investigate i) whether subcutaneous injection of tertiary neurospheres, derived from Bmi-1 over-expressing NSPC would give rise to tumours ii) whether over-expression of Bmi1 specifically in nestin positive SVZ NSPC would initiate glioma formation (NestinCre;STOPFloxBmi1 mice), iii) whether postnatal activation of Bmi-1 over-expression in SVZ NSPC would result in brain tumours (intra-ventricular injection of Adeno-Cre virus in STOPFloxBmi1 mice).

3.4.1 No tumours upon subcutaneous injection of neurospheres overexpressing

Bmi1 in NOD-SCID mice

Tertiary NS were dissociated and NSPC were infected with Adeno-Cre virus, as previously described. They were then cultured for 5 DIV until moderate size neurospheres (100-120 μ m) formed. NS were pulled, resuspended in matrigel, which is a viscous substrate that provides nutritious environment for neurospheres and contribute to avoid their dispersion upon injection (BD Biosciences) and injected subcutaneously in both flanks of five of 2 months old NOD-SCID mice.

The mice were kept under observation for 6 months, and all sites of injection were analysed at the time the experiment was terminated. No tumours or tumour like lesions were found (Table 3.3).

3.4.2 Overexpression of Bmi1 in Nestin positive NSPC does not induce brain tumour formation.

In order to generate compound mice (NestinCre;STOPFloxBmi1) overexpressing Bmi1 in nestin positive NSPC, crossings between NestinCre male (Dubois *et al.*, 2006) and STOPFloxBmi1 females were set up. Note that only paternally inherited NestinCre allele ensures strong loxp recombination in neural tissues including NSPC (Dubois *et al.*, 2006).

A cohort of 17 compound mice was aged for up to 8 months. Of these 5 were sacrificed at age 6 months to check for possible abnormal growth or hyperplastic lesions. The remaining 11 mice were kept until 8 months and then sacrificed for fur-

ther analysis. Neither gross examinations nor histochemical analysis revealed any tumour like lesion in the brains of these mice (Table 3.3).

We concluded that prolonged over-expression of Bmi1 in nestin expressing SVZ NSPC does not induce brain tumour formation.

3.4.3 Bmi1 over-expression in SVZ of STOPFLOXBmi1 mice by adenovirus mediated Cre activation does not trigger tumour formation

We observed a dual effect of Bmi1 over-expression on apoptosis in NS, depending on whether the cultures were obtained from embryonic or postnatal cells with postnatal NS showing a lower apoptotic rate as measured by Annexin V positivity, suggesting a resistance to apoptosis. Therefore, we reasoned that activation of Bmi1 over-expression postnatally could be more likely to elicit tumour formation.

We set out to test whether intraventricular injection of Adeno-CRE would efficiently target SVZ NSPC. RosaLac-Z reporter mice (R26R) were used to test efficiency and spread of Adeno-Cre mediated recombination of loxP sites in NSPC lining the lateral walls of lateral ventricles *in vivo*. Two months old R26R were intraventricularly injected with different amounts of 10^9 plaque-forming units Adeno-Cre virus, or with equal volume of PBS as control, to determine amount of virus needed to achieve optimal recombination at the site of interest. Mice were culled 5 days post-injection and brains were processed for X-gal staining. Whole mount X-gal staining reveals that recombination had occurred in the medial, lateral and dorsolateral walls of the lateral ventricles, where NSPC reside (Figure 3.47). We found that 5 μ l of 10^9 plaque-forming units Adeno-Cre virus was sufficient to produce recombination of Lox-P sites at the lateral ventricles (Figure 3.47). Similar

analysis was performed by Jacques *et al.*, (2010), where they used Adeno-GFP virus to identify the type of cells infected in the lateral ventricles; and their report shows that the nestin and GFAP positive lateral ventricular cells are targeted by this virus.

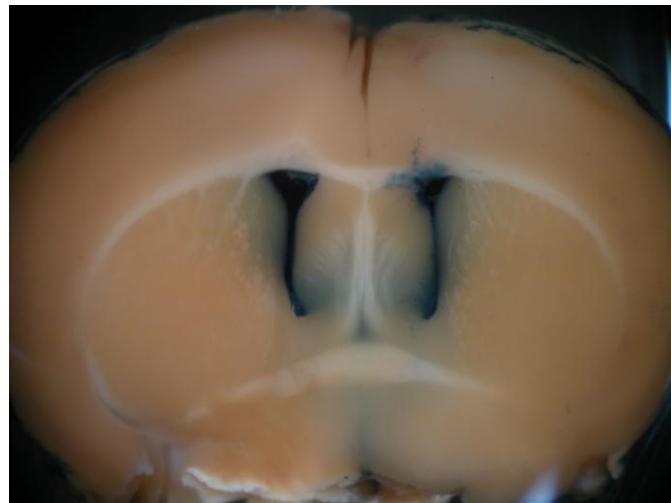


Fig 3.47 X-gal staining on coronal section of ROSA26^{Lox} mouse 5 days after intra-ventricular infection with Adeno-Cre virus: Recombined cells seen as blue are confined to thin periventricular region where NSPC reside.

Next, a cohort of 15 STOPFLOXBmi1 mice was injected intraventricularly with 5 µl Adeno-Cre and kept under observation for 20 months. All brains were analysed histologically at the termination of the experiment, no brain tumours were observed (Table 3.3).

3.4.4 Induction of low grade astrocytoma in compound mutant mice over-expressing Bmi1 and lacking p53 in SVZ NSPC *in vivo*

Next, we set out to determine whether inactivation of key tumour suppressor genes such as PTEN (Marino *et al.*, 2002) and P53 (Marino *et al.*, 2000) in addition to over-expression of Bmi1 in SVZ NSPC would give rise to gliomas. We have chosen to target these tumour suppressor genes -PTEN and p53- as they are frequently altered in brain tumours of glial origin (Collins, 2002).

While mice lacking PTEN^{LoxP/LoxP} and p53^{LoxP/LoxP} in NSPC do not develop brain tumours, previous reports have shown that GFAP-Cre-mediated conditional inactivation of these tumour suppressor genes in NSPC of SVZ results in formation of gliomas (Zheng *et al.*, 2008, Jacques *et al.*, 2009).

To assess whether Bmi1 overexpression would induce glioma formation in combination with other genetic mutations known to play a role in glioma pathogenesis in mice and human, compound mice were generated where Bmi1 overexpression was induced while either p53 or PTEN were deleted. To this end crossings were set up between PTEN^{LoxP/LoxP} and STOPFloxBmi1 and between p53^{LoxP/LoxP} and STOPFloxBmi1. Intermediate genotypes were further intercrossed to generate PTEN-Floxhom;STOPFloxBmi1 and p53Floxhom;STOPFloxBmi1.

Two recent studies have shown that concomitant deletion of PTEN and p53 tumour suppressor genes in CNS specific NSCP results in the formation of gliomas (Zheng *et al.*, 2008, Jacques *et al.*, 2009). Here PTENFloxhom;p53Floxhom mice were also generated for use as positive control for the procedure. Adult mice were injected intra-ventricularly with Adeno-Cre to induce recombination of LoxP sites

leading to inactivation of PTEN, p53 and activation of Bmi1 in NSPC of the SVZ of adult mice, as previously described.

A cohort of 21 compound (PTEN^{Floxhom};STOPFLOXBmi1) and 22 control (PTEN-^{Floxhom}) mice was generated and injected intraventricularly with Adeno-Cre to test the tumourigenicity of Bmi1 over-expression in combination with deletion of PTEN in NSPC lining the SVZ (Table 3.3). A cohort of 28 compound (P53^{Floxhom}; STOPFLOXBmi1) and 21 control (P53^{Floxhom}) mice was treated as above to test the tumourigenicity of Bmi1 over-expression in combination of deletion of p53 in NSPC lining the SVZ (Table 3.3).

A cohort of 12 PTEN^{Floxhom};p53^{Floxhom} compound mice was also injected intraventricularly with Adeno-Cre virus as positive controls (see Table 3.3).

Table 3.3

Genotype/treatment	Age (months)	Brains analyzed	CNS tumours
NOD-SCID (injection of NS)	1-12	5	0
	17-24	0	0
NestinCre;STOPFloxBmi1	1-12	5	0
	17-24	16	0
STOPFloxBmi1 (Adeno-Cre)	1-12	11	0
	17-24	6	0
PTEN ^{Floxhom} ;STOP ^{FLOXBmi1} (Adeno-Cre)	1-12	5	0
	17-24	16	ongoing
p53 ^{Floxhom} ;STOP ^{FLOXBmi1} (Adeno-Cre)	1-12	5	2
	17-24	23	ongoing
PTENp53RosaLacZF ^{Floxhom} (Adeno-Cre)	1-12	To be analysed	To be analysed
	17-24	0	0

Table 3.3 A summary of all *in vivo* studies to test the role of Bmi1 in tumorigenesis. The column entitled Genotype/treatment indicated the mouse models (compound conditional/transgenic) used, whereas the Age (months) indicates two categories of time points when the brains were examined. Note that activation of Bmi1 over-expression is initiated by intraventricular injection of Adeno-Cre virus. And that Bmi1 over-expression combined with deletion of p53 results in brain tumour formation.

These mice are on tumour watch since summer 2009. Interestingly, one brain tumour with histological features of a low grade glial tumour has developed in a p53^{Floxhom}; STOPFLOXBmi1 nine months after Adeno-Cre injection (Figure 3.48). The neoplasm showed increased cellularity, mild nuclear atypia, fibrillary cytoplasm and infiltrative growth pattern. Immunostaining for GFAP confirmed the glial nature of this tumour (data not shown).

Although these are early days to draw any conclusion from these experiments - indeed we plan to continue the tumour watch for at least 6-8 months- it is possible to speculate that a p53 deficient background may provide a permissive environment for overexpression of Bmi1 to induce glioma formation in the mouse.

Brain tumours following intraventricular Adeno-Cre virus injection

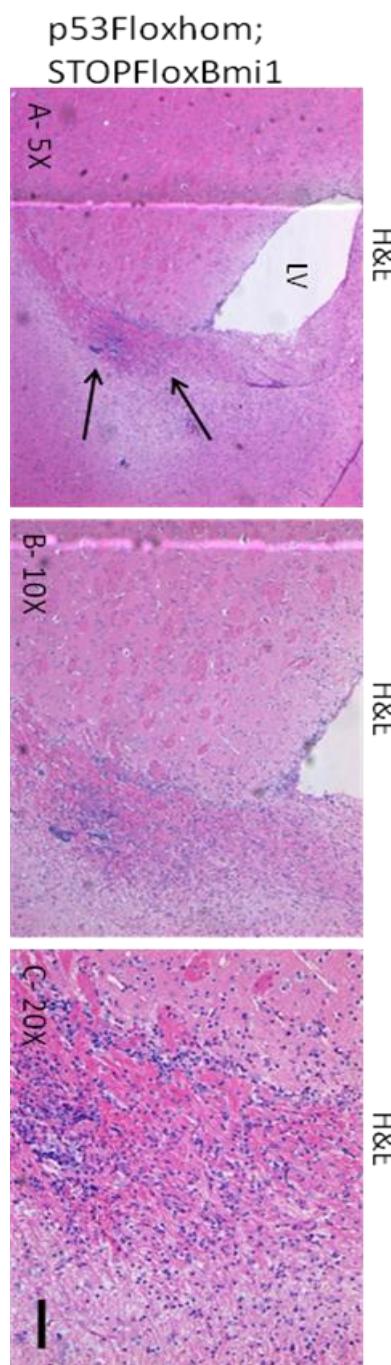


Fig 3.48 Haematoxylin eosin staining on a coronal section of p53^{Floxhom};STOP^{Flox}Bmi1 mouse injected intraventricularly with Adeno-Cre virus: Arrows in (A) indicate the tumour whereas high magnification (B and C) shows increased cellularity, mild nuclear atypia and diffuse infiltrative growth pattern in keeping with a low grade glial tumour. LV= lateral ventricle. Scale bar is 200 μ m.

Chapter 4- General Discussion

4.1 Evaluation of the effects of Bmi1 over-expression on biological properties of NSPC using a conditional transgenic mouse model

This study set out to study the role of Bmi1 in NSPC, using a Cre/loxP conditional transgenic mouse model where Bmi1 is conditionally over-expressed, in order to identify i) functional changes in biological properties of NSPC and ii) changes in NSPC gene expression profile which may hint at novel genes and pathways regulated by Bmi1 in these cells as well as iii) whether Bmi1 over-expression in NSPC is sufficient to initiate brain tumour formation.

To achieve *in vitro* activation of the transgene Adeno-CRE virus infection was used whereas for *in vivo* activation either intraventricular injection of Adeno-Cre or compound STOPFloxBmi1 and Nestin-Cre mice were used. My experiments have shown that approximately 2 fold over-expression of Bmi1 is achievable in both embryonic and postnatal NSPC after activation of the transgene.

4.1.1 Proliferation and Self-renewal

Previously it has been shown that disruption of Bmi1 expression in mice leads to posterior transformation, neurological abnormalities and severe proliferative defects in lymphoid cells (van der Lugt *et al.*, 1994) whereas Bmi1 over-expression induces lymphomas (Alkema *et al.*, 1997b). In the postnatal brain, Bmi1 is required for proliferation and self-renewal of SVZ NSC and cerebellar granule cell progenitors (Molofsky *et al.*, 2003, Leung *et al.*, 2004). To study the role of Bmi1 over-expression on the biological properties of SVZ NSPC *in vitro* and *in vivo*, the Marino

laboratory had generated a conditional transgenic mouse model to activate Bmi1 expression in a cell specific and time controlled fashion by means of Cre-mediated recombination of LoxP sites. I took advantage of this system to study the effects of Bmi1 over-expression in NSPC at different developmental time points, and by means of acute and chronic/protracted activation of the transgene by Adeno-Cre infection *in vitro* or by crossing with NestinCre mice *in vivo* respectively. Initially, I looked at the impact of increased Bmi1 expression on the proliferation and self-renewal capacity of NSPC *in vitro* using Alamar Blue and EdU based assays for proliferation and serial dilution and long-term passaging for self-renewal. Overexpression of Bmi1 in embryonic NSPC leads to increased self renewal and proliferation *in vitro*. These effects are independent of whether a protracted activation of Bmi1 expression was induced *in vivo* by means of nestin driven Cre expression or whether acute induction of Bmi1 expression was achieved *in vitro* by adenoviral mediated Cre delivery. These findings are well in keeping with independent *in vivo* studies, performed by a postdoc in the lab, in which the number of proliferating RG cells were shown to be increased upon overexpression of Bmi1 (pH3 and RC2 immuno-labelling on E12.5 and E16.5 embryos). Similarly, activation of Bmi1 expression in postnatal NSPC leads to increased proliferation (as assessed by Alamar Blue assay) and self-renewal (as assessed by serial dilution and long-term passaging). Interestingly, the cell density did not alter the effects of Bmi1 over-expression on NSPC self-renewal, raising the possibility that cell intrinsic changes are playing a crucial role in this context.

These data support and extend a recent report where lentiviral mediated over-expression of Bmi1 by intraventricular injection at E14 led to similar results, al-

though the *in vivo* observation in this study was limited to 3 days after injection (Fasano *et al.*, 2009). On the contrary, over-expression of Bmi1 in nestin positive progenitor cells did not elicit a similar effect *in vivo*, albeit inducing increased self renewal *in vitro* (He *et al.*, 2009). Although it is currently unclear why this is the case, it is conceivable that different levels of Bmi1 expression may elicit different effects on self renewal/proliferation of embryonic NSPC.

4.1.2 Apoptosis

Next, I analysed the effects of Bmi1 over-expression on apoptotic death of cells contained within neurospheres. I found that the percentage of Annexin V positive cells was higher in neurospheres derived from Bmi1 over-expressing embryonic NSPC. In keeping with my *in vitro* data, experiments performed by a colleague in the lab have shown that Bmi1 over-expression *in vivo* during embryonic cortical development results in apoptosis of intermediate neuronal committed progenitors, a phenomenon that could account for the reduced brain size of adult double mutant mice. However, NSC located in the VZ of the developing neocortex did not undergo apoptosis despite higher levels of Bmi1 expression. In keeping with these findings, no increased apoptosis was found in NSC cultured as a homogeneous monolayer under adherent conditions. These data show that the *in vitro* assays used are faithfully reproducing the phenotype observed *in vivo* and they are therefore a useful tool to gain further understanding on the cellular and molecular mechanisms underlying the role of Bmi1 overexpression in NSPC.

Interestingly, the apoptotic rate of cells contained in neurospheres arising from NSPC isolated from postnatal SVZ was lower than in control. These data allow us to

conclude that while the role of Bmi1 in self renewal and proliferation of NSPC is independent of the developmental timepoint analysed, its role in induction of apoptosis is dual and developmental timepoint-related.

The study by Chatoo W. *et al.*, (2009) has shown that Bmi1 expression is required for regulation of antioxidant defences, in neurons, through repression of p53 dependent pro-oxidant activity (Chatoo *et al.*, 2009). Although a recent study has shown that lack of Bmi1 is associated with impaired mitochondrial function evident from increased intracellular levels of reactive oxygen species (ROS) and activation of DNA damage response pathway in HSC (Liu *et al.*, 2009), no previous study to date has linked Bmi1 over-expression with regulation of apoptosis in forebrain NSPC.

4.1.3 Evaluation of changes in neurosphere composition upon Bmi1 over-expression

Neurospheres arise from clonal expansion of NSPC under conditions that favour proliferation and self-renewal of these cells. They are widely used to study self-renewal and multipotency of NSPC *in vitro*. Moreover, neurospheres have been studied as a possible source of new cells for therapies of human nervous system diseases such as multiple sclerosis, Parkinson's and spinal cord lesions (Pluchino *et al.*, 2003). Recently, the value of this culture system in drug testing on spheres derived from individual glioblastoma cells has also been tested (Gal *et al.*, 2007).

To gain insight into the cellular composition of neurospheres derived from NSPC over-expressing Bmi1, I used both flow cytometry for quantification and immunohistochemistry for localisation of specific cellular markers.

Interestingly, we found that tertiary neurospheres derived from Bmi1 over-expressing NSPC contain higher percentage of cells that are double positive for NG2 and A2B5. Cells expressing NG2+ and A2B5+ in neurospheres have previously been shown to be self-renewing and multipotent (Dromard *et al.*, 2008). These data may therefore support our previous observation that over-expression of Bmi1 in NSPC results in expansion of the pool of self-renewing and NS-forming cells *in vitro*. However, as it is at present unclear how NG2 and A2B5 positive cells relate to Nestin, Sox2, Musashi positive cells, it is also conceivable that over-expression of Bmi1 skews the identity of cells contained within neurospheres toward a glial fate, indeed we also found more cells that are positive for GFAP and A2B5, GFAP and NG2. This conclusion is in keeping with data obtained by a colleague in the lab. She has shown that the number of neuronal committed progenitors arising within neurospheres over-expressing Bmi1 is lower, therefore supporting the notion that Bmi1 influences fate specification at least *in vitro*.

4.1.4 Differentiation

In mammals, symmetric cell divisions are thought to maintain the size of the NSC pool at any given time (Potten and Loeffler, 1990). In contrast, NSC may undergo asymmetric divisions to maintain the balance between self renewal and differentiation (Reya *et al.*, 2001). Our data show that overexpression of Bmi1 increases the pool of NSC both *in vivo* and *in vitro* and that this is linked to an increased proliferation of these cells.

A previous study showed that lack of Bmi1 leads to increased population of astroglial cells capable of proliferation, and Bmi1 deficient mice have more astrocytes at

birth (Zencak *et al.*, 2005). The same study has shown that Bmi1 is expressed in the germinal zones *in vivo* and in NSPC *in vitro*, but not in differentiated cells, suggesting that down-regulation of Bmi1 is necessary for differentiation of NSPC (Zencak *et al.*, 2005). However, no previous report has associated Bmi1 over-expression with NSPC differentiation. Moreover, our own data obtained with the analysis of neurospheres composition had hinted at a potential role of Bmi1 in fate specification.

To evaluate the effects of Bmi1 over-expression on differentiation of E16.5 NSPC, I cultured several neurospheres on laminin coated coverslips for 5DIV and 9DIV. Upon induction of differentiation at 5DIV, a significant reduction of β III-tubulin positive neurons was noted in Bmi1 overexpressing cultures, although the percentage of GFAP+ astrocytes and O4+ oligodendrocytes were similar to control. This suggests that neuronal fate specification is linked to attenuation of Bmi1 expression. The increased number of Musashi positive cells noted in differentiating NSPC over-expressing Bmi1 at DIV5 gives additional support to this interpretation. Musashi promotes self-renewal and maintenance of NSC through transcriptional inhibition of p21^{WAF1/Cip1} and it is downregulated during neuronal differentiation (Okano *et al.*, 2005, Battelli *et al.*, 2006).

4.2 Bmi1 acts through different downstream targets depending on the developmental time point

Previous findings (He *et al.*, 2009) showed that upregulation of Bmi1 expression led to reduced expression of p16^{Ink4a} and p19^{Arf} in a developmental stage-independent fashion. Indeed we also observe this effect both in embryonic and

postnatal NSPC. Importantly, downregulation of the expression of these genes was observed both when Bmi1 activation was achieved during in a chronic/protracted way by means of nestin-driven Cre expression and acutely *in vitro* by adenoviral mediated Cre delivery. Downregulation of p21^{WAF1/Cip1} expression was also observed at all time points analysed and both upon chronic and acute overexpression of Bmi1. Whilst we could confirm upregulation of Foxg1 in embryonic NSC/NPC upon activation of Bmi1, as recently reported by Fasano *et al.*, (2009), we could not detect a similar upregulation at both postnatal timepoints analysed (Fasano *et al.*, 2009). We cannot exclude that this might reflect a dosage effect. Our single copy gene approach allows us to obtain a tightly controlled 1.8 fold increase of Bmi1 expression compared with a potentially greater upregulation (approximately 8 fold) obtained using a lentiviral transduction approach of Fasano *et al.* (Fasano *et al.*, 2009). This observation may be interesting to explore further, due to the differences in the level of expression between our model and that of Fasano', as it suggest that Bmi1 may act in a "concentration dependent manner". However, it is also conceivable that downregulation of p21^{WAF1/Cip1}, either directly through Bmi1 or indirectly in the context of p19^{Arf} downregulation, contributes to self renewal/proliferation control of postnatal NSPC independently of Foxg1 upregulation.

4.3 Microarray based analysis of gene expression of Bmi1 over-expressing neurospheres reveal novel targets involved in stem cell function regulation

Bmi1 is known to control biological properties of NSPC partly by transcriptional repression of the *ink4a* locus (Jacobs *et al.*, 1999a), and downregulation of p21^{WAF1/Cip1} during embryonic development (Fasano *et al.*, 2007), or in association with Foxg1 upregulation (Fasano *et al.*, 2009).

However, concomitant deletion/suppression of either *p16^{Ink4a}* or *p19^{ARF}* or both did not completely rescue the proliferation and self-renewal defects of NSPC derived from Bmi1-/ mice (Molofsky *et al.*, 2003, Bruggeman *et al.*, 2005). Other NSPC functions, such as migration, have also been shown to be controlled by Bmi1 in an *ink4a* independent manner (Bruggeman *et al.*, 2009). Our experiments have shown that the developmental stage of NSPC influence the effect of Bmi1 overexpression on some of their properties such as for example on their susceptibility to apoptotic death. However, downregulation of the “canonical” downstream targets of Bmi1 has been seen at all developmental timepoints. It is therefore likely that additional unknown target genes of Bmi1 exist. Full transcriptome screening by means of Illumina BeadChip arrays was performed to try and identify novel genes and pathways, which may be deregulated upon over-expression of Bmi1.

From a list of 700 significantly differentially expressed genes in experimental samples, we have chosen to independently verify and further study a selection of genes that are most relevant to the biological changes observed in NSPC upon Bmi1 over-expression. As Bmi1 is a transcriptional repressor, we focused in first

instance on genes that showed reduced expression. We have chosen transcription factors (Dlx2, Dbx2, and Irx2) and other genes (Ng2, Ncan, Ncam and Bcan) known to play a role in regulation of neurogenesis and differentiation, as well as cell adhesion molecules (Col9a1, Col1a1, Col4a1 and Col4a2). We reasoned that these genes would be the most appropriate for further studies as our functional studies on Bmi1 over-expressing neurospheres have shown that differentiation is delayed, proliferation and self-renewal are increased, and migration is impaired (the latter data has not been described as it was performed by a colleague).

We show, using quantitative RT-PCR, that increased expression of Bmi1 results in downregulation of the expression of several members of the collagen family cell adhesion molecules such as Collagen Ia1, IVa1 and IVa2 and IXa1, as well as cell adhesion molecules Ncan and Bcan, whereas NG2 and Ncam were upregulated. Note that reduced expression of members of the Collagen IV family and changes in expression of cell adhesion molecules may have functional consequences on migration of NSPC as Bmi1 has been previously shown to be regulating this process *in vitro* by Bruggeman *et al.*, (2009). Therefore, it is also conceivable that Bmi1 regulates migration of NSPC through modulation of cell adhesion (Bruggeman *et al.*, 2009), through suppressing the expression of Collagens I, IV and IX in these cultures.

Moreover, upregulation of NG2 in Bmi1 over-expressing neurospheres may account for the increased number of intermediate glial progenitors identified upon induction of differentiation. Reduced expression of Dlx2 in Bmi1 over-expressing neurospheres is in keeping with previous immunohistochemistry studies. More-

over, Bmi1 over-expressing neurospheres have significantly lower expression of a series of transcription factors - Dbx2, Neurogenin2, Irx2 (and Dlx2)- all of which play an important role in neuronal fate commitment (Ahn *et al.*, 2004; Lacin *et al.*, 2009; Roybon *et al.*, 2009). Dlx2 in particular is known to regulate the balance between neurogenesis and oligodendrogenesis in the developing forebrain (Petryniak *et al.*, 2007). We can therefore speculate that a Bmi1- mediated down-regulation of Dlx2 may explain the delayed neuronal differentiation and increased number of oligodendroglial progenitors observed in Bmi1 overexpressing cultures.

Next, I set out to determine whether similar changes in gene expression were also induced in the postnatal NSPC upon activation of Bmi1 over-expression. Similar QPCR assays were performed for cDNA obtained from postnatal NS for the most differentially expressed genes identified at embryonic time point: Col4a1, Col4a2, Ng2, Ncan, Bcan, Ncam, Dlx-2, Foxg1 and Irx-2. To our surprise only Col4a1 was significantly differentially expressed by Bmi1 over-expressing postnatal NSPC. It is therefore possible to speculate that these differences observed in the expression profile of these genes may be involved in the differential role Bmi1 plays in the embryonic and postnatal NSPC context.

We were able to draw a list of pathways, network processes and disease that are associated with differentially expressed genes, using multifunctional MetaCore software. As the manually curated Metacore software relies on the accuracy of the differentially expressed genes in building pathways and networks that could be related to a biological phenomenon, independent confirmation of most significantly differentially expressed genes gains importance. For most cases the results

from RT-PCR studies were compatible with array findings. However, in the case of Ncam, we found a discrepancy between what the array data (1.3 fold up-regulation in the Bmi1 over-expressing cells) and RT-PCR results (1.5 fold down-regulation). Note that the discrepancy is not confined to the magnitude of Ncam expression (which would have been a quantitative difference due to statistical parameters or power of detection etc.) but to a qualitative shift from induction to repression, which could lead to false interpretation of biological processes. Of course, the discrepant result could be due to sequences used for amplification of the gene of interest or simply be a false readout. Hence the importance of using quantitative RT-PCR that is sensitive and linear in gene expression measurement validation.

Moreover, it is worth mentioning that the interpretation of the data obtained with Metacore analysis warrant some caution and independent validation of the most significantly deregulated pathways and network would be needed. In fact while KEGG pathway analysis, which uses a similar software platform, revealed that several components of MAPK pathway were altered upon Bmi1 over-expression, these findings could not be confirmed with assay aiming at assessing pathway activity. Indeed, when we performed Western Blotting to confirm that MAPK pathway was altered at the level of protein phosphorylation, we were surprised to find out that the phosphorylation of both MAPK and of its specific components such as p38, c-Jun or JNK was unaltered. Although we cannot exclude that this may be due to protein turn over, it is also well known that differences at the RNA level may not translate into functional difference of the pathway.

4.4 Brain tumours and Bmi1 over-expression

Cells with stem cell properties, so called brain tumour stem cells (BTSC), are thought to play a crucial role in initiation and maintenance of brain tumours (reviewed in (Stiles and Rowitch, 2008). These cells express high levels of Bmi1 (Abdouh *et al.*, 2009), are particularly resistant to apoptosis (Bao *et al.*, 2006, Ghods *et al.*, 2007) and have recently been shown to be A2B5 positive (Rebetz *et al.*, 2008).

Overexpression of Bmi1 in postnatal SVZ NSC/NPC *in vitro* led to increased self renewal capacity of both embryonic and postnatal NSC/NPC. However, resilience to apoptosis was noted in NS originating from postnatal NSC/NPC overexpressing Bmi1. Moreover, we observed increased number of A2B5 positive undifferentiated, glial progenitor cells, in these NS. It was therefore interesting to assess whether Bmi1 overexpression in NSC/NPC *in vivo* led to tumour formation. We show that both embryonic and postnatal activation of Bmi1 overexpression in stem cells did not lead to brain tumour formation in mice kept under observation for up to 20 months. This is in agreement with the study of He *et al.*, (2009) where tumours were not seen when Bmi1 overexpression was induced in nestin positive progenitor cells. Fasano *et al.*, (2009), however, reports that overexpression of Bmi1 by means of intraventricular lentiviral injections led to tumour formation in the first week after birth, although no further details were given on the type of tumour these mice developed. More studies are clearly needed to elucidate these differences. It is important to mention that He *et al.* (2009) and potentially also Fasano *et al.* (2009) achieved higher expression levels of Bmi1 in their systems as

compared with our single copy gene approach. Moreover, the genetic background of the mice used in the experiments might account for some of these apparently contradictory results. It is noteworthy that both our study and the one of He were carried out in C57Bl6 genetic background while Fasano *et al.* used Swiss Webster mice for their experiments.

Finally, I set out to test whether Bmi1 over-expression in SVZ NSPC concomitantly with deletion of PTEN or p53 would be sufficient to induce tumourigenesis. PTEN and P53 are the most frequently mutated/deleted tumour-suppressor genes in human cancers. Combination of genetic mutations rather than single deletions/mutations provides a more favourable environment for *de novo* formation of tumours. It is well established that induction of haploinsufficiency of PTEN in Nf1/p53 astrocytoma mouse model accelerates formation of grade III astrocytomas, whereas deletion of PTEN with activation of Akt pathway leads to grade IV tumours (Kwon *et al.*, 2008). Moreover, activation of Ras pathway in p53-deficient astrocytes renders them more prone to transformation into self-renewing, multipotent cells that are positive for nestin and CD133 (Lee *et al.*, 2008). Deletion of p53 and PTEN in GFAP positive SVZ NSC leads to formation of high grade gliomas (Jacques *et al.*, 2009).

A colony of 40 mice for each conditional transgenic genotype (STOPFloxBmi1; *p53*^{lox/lox} or STOPFloxBmi1; *Pten*^{lox/lox}) was activated by intraventricular injection of A-Cre virus. Intraventricular injection of virus ensures activation of Bmi1 expression and deletion of homozygous floxed alleles of Pten or p53 in a thin layer of periventricular cells. The experiment is still ongoing and it is premature to draw

any conclusion at the present time, however it is encouraging that a low grade astrocytoma was seen in one STOP^{Flox}Bmi1; *p53*^{lox/lox} mouse.

In conclusion we show that fine tuning of the expression level of the PcG gene Bmi1 is a viable tool to increase self renewal capacity of both embryonic and postnatal NSC/NPC in the mouse and this approach does not impair the long term differentiation capacity of these cells. However, sustained over-expression of Bmi1 in differentiating progenitor cells leads to increased apoptosis and delayed neuronal differentiation. Upregulation of Bmi1 expression during embryonic development and in the postnatal SVZ does not lead to neoplastic transformation of these cells. However, Bmi1 over-expression in combination with p53 deletion in periventricular cells of adult mice results in formation of low grade gliomas.

4.5 Statement regarding the hypotheses

In this study I have showed that Bmi1 over-expression in NSC increases their proliferation and self-renewal *in vitro*. Changes in these properties of NSC are associated with reduced expression of cell cycle inhibitors, namely *p16/INK4a*, *p19^{ARF}* and *p21^{WAF1/Cip1}*, which are previously shown to be targets of Bmi1. I showed that expression of the above mentioned cell cycle inhibitors are reduced upon Bmi1 over-expression, at all developmental time points studied.

I found that induction of Bmi1 over-expression in embryonic NSC (E16.5) increased their apoptosis *in vitro*—a diametrically opposite result to my hypothesis. This could possibly be due to changes in expression of anti-apoptotic genes such as survivin. Further studies are needed to elucidate this. However, in the postnatal and adult NSC, Bmi1 over-expression confers resistance to apoptosis; a finding in line

with my hypothesis. Interestingly, taken together, these partially conflicting results suggest that Bmi1 regulates apoptosis in NSC through different genes and pathways at different developmental time points.

Bmi1 over-expression alone is insufficient for transformation of NSC into tumour initiating cells. However, in combination with deletion of p53, Bmi1 over-expression in NSPC is sufficient for initiation of low grade glioma in mice.

Over-expression of Bmi1 in NSC leads to changes in expression of 700 genes some of which I have selected on the basis of possible significance in NSC functions and verified using RT-QPCR. These include transcription factors (Dlx2, Dbx2, and Irx2) and other genes (Ng2, Ncan, Ncam and Bcan) known to play a role in regulation of neurogenesis and differentiation, as well as cell adhesion molecules (Col9a1, Col1a1, Col4a1 and Col4a2). Further studies are needed to elucidate a direct and functional connection between Bmi1 and these targets.

Chapter 5—Future Studies

In this thesis, I have shown that sustained over-expression of Bmi1 in neurospheres delays neuronal differentiation at 5DIV, possibly by counteracting repression of Nestin and musashi-1 expression and/or by repressing proneural genes such as Dlx2. This effect was transient and at the later days of differentiation (9DIV), more neurons and oligodendrocytes developed. It was previously shown that absence of Bmi1 leads to depletion of postnatal NSC, highlighting Bmi1 as a transcription factor required for maintenance of stem cell in adult mice. However, it was not shown whether sustained over-expression of Bmi1 would counteract differentiation of NSPC, when placed in conditions promoting differentiation. These new lines of evidence, besides endowing Bmi1 with a strong role in differentiation of NSPC, also deserved further direct studies to clarify the nature of possible interaction between Bmi1, Dlx2, nestin and Musashi-1. Co-immunohistochemistry and co-immunoprecipitation assays could be used for example to check for any direct interaction between Bmi1 and Dlx2.

In this thesis, I have shown that Bmi1 over-expression has a different impact on apoptosis of NSPC contained within NS depending on the developmental stage. These results are in line with *in vivo* studies where a colleague in the lab has found that sustained Bmi1 over-expression in neuronal committed progenitor cells during neocortical development induces their apoptotic death. Resistance to apoptosis in postnatal NSPC could be tested further by application of glutamate or other apoptosis inducing agents to cultures. Given that Bmi1 has been recently associated with recruitment of DNA damage response machinery (Facchino *et al.*,

2010) in both normal and cancerous neural stem cells positive for CD133, it would be interesting to investigate this feature further. Our conditional transgenic mouse would be a good model to test whether Bmi1 over-expression would confer greater resistance to apoptosis or other forms of cell death. A better understanding of the mechanisms involved in Bmi1 mediated resistance to apoptosis would help designing more targeted treatments for cancer patients.

Moreover, in my thesis I showed by gene expression analysis, and validation assays by RT-PCR, that Bmi1 over-expression induces significant changes to the expression of genes involved in a variety of cellular processes: these genes included transcription factors (Dlx2, Dbx2, and Irx2) and others (Ng2, Ncan, Ncam and Bcan) potentially involved in regulation of neurogenesis and differentiation, as well as cell adhesion molecules (Col9a1, Col1a1, Col4a1 and Col4a2). In order to further characterise the role of these genes in inducing the phenotype observed in my studies it would be interesting to reconstitute the expression of downregulated genes and/or knockdown the expression of upregulated genes by shRNA and study the functional consequences of this manipulation.

Lastly, in this thesis I have provided preliminary data showing that Bmi1 over-expression in SVZ NSPC in mice is not sufficient to induce tumourigenesis. However, in combination with p53 deficiency, Bmi1 over-expression is sufficient to produce low grade glioma. These are very preliminary data, the mice are still on tumour watch and we currently do not know whether the incidence of the tumours will be high enough to justify further studies. However further characterisation of the morphological characteristics of these tumours, including marker ex-

pression analysis as well as studies of early neoplastic lesions and cell of origin could be carried out to try and gain more insights into the collaboration Bmi1-p53 in gliomas pathogenesis.

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