Chemical screening to uncover small molecules that modulate neural stem cell self-renewal and differentiation

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I, Bartlomiej Baranowski confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

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Abstract

In vitro expanded neural stem cells provide an important cellular model to explore mechanisms of neural development, for modelling of disease, and in the longer term may have applications in new types of stem cell-based therapies. However, our ability to steer neural stem (NS) cell lines into specific desired lineages *in vitro* remains limited. PDGFRα is one of the earliest markers of the transition of neural stem cells to oligodendrocyte progenitors. I established and characterised a novel set of mouse NS cell lines that report the activation of PDGFRα via expression of an H2B:GFP 'knock-in'. Three clonal 'PG1' cell lines were fully characterised. Under self-renewing conditions I found <1% of NS cell express the H2B:GFP reporter but this increases to ~15-20% following induction of differentiation. Using this cellular model system I carried out a high-content chemical screen of a diverse collection of 463 pharmacologically active small molecule modulators of 'stem cell pathways' and kinase inhibitors, to identify those capable of modulating NS cell self-renewal and differentiation. I did not uncover any small molecules capable of promoting OPC lineage specification. However, I found multiple HDAC inhibitors that were highly effective in blocking the activation of PDGFRα, a finding that mirrors published studies implicating HDAC inhibition in the later differentiation of OPCs to oligodendrocytes. Three further compounds, Nigericin (an ionophore), Withaferin (a steroidal lactone) and NFkB inhibitor also completely blocked OPC commitment. I focused on the specific cellular responses and downstream molecular events triggered by these molecules and tested their differentiation potential on human NS cells and malignant glioblastoma-derived NS cells.

Abbreviations

ALDH1	Aldehyde dehydrogenase 1
ASCL1	Achaete-scute homolog 1
ATP	Adenosine triphosphate
Bhlh	Basic helix-loop-helix
BMP	Bone morphogenetic protein
BMDC	Bone marrow-derived cell
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CIC-3	Chloride Channel-3
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DG	Dentate gyrus
DIPG	Diffuse intrinsic pontine glioma

DLL	Delta-like ligand
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
Dpi	Day post injection
DV	Dorsoventral
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GA	Gemistocytic astrocytoma
GBM	Glioblastoma multiforme

GBMO	Glioblastoma multiforme with oligodendroglial component
GBM-PNET	Glioblastoma multiforme with primitive neuroectodermal features
GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1
gBK	Glioma BK (gBK) channel
GCA	Granular cell astrocytoma
GC-GBM	Giant-cell glioblastoma multiforme
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GS	Gliosarcoma
GSC	Glioblastoma stem cell
HCA	High content analysis
HCG	Hypothalamic-chiasmatic-glioma
HCS	High content screening
HDACi	Histone deacetylase inhibitor
HIF-1	Hypoxia-inducible factor-1

HHV-6	Human herpesvirus-6
HRP	Horseradish peroxidise
HTS	High throughput screening
ID	DNA-binding protein inhibitor
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble
Ig	Immunoglobulin
IGFBP-2	Insulin-like growth factor binding protein-2
IGF-I	Insulin-like growth factor 1
iPS	Induced pluripotent stem cell
LOH	Loss of heterozygosity
MBP	Myelin basic protein
MDM-4	Mouse double minute 4 homolog
miRNA	MicroRNA
MGMT	O^6-methylguanine-DNA methyltransferase
MSH	MutS protein homolog

mTOR	Mechanistic target of rapamycin
NCX	Sodium Calcium exchanger
NEMO	NFkB essential modulator
NF1	Neurofibromatosis type 1
NG2	Neural/glial antigen 2
NIK	NFkB-inducing kinase
NLS	Nuclear localisation signal
NPC	Nasopharyngeal carcinoma
NRSE	Neuron-restrictive silencer element
NS	Neural stem
OB	Olfactory bulb
OPC	Oligodendrocyte progenitor cell
PAP	Prostatic acid phosphatise
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PCV	Procarbazine, CCNU, vincristine
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PEDF	Pigment epithelium-derived factor
PI	Propidium iodide
PI3K	Phosphatidylinositide 3-kinases
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha
PIP_2	Phosphatidylinositol biphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PLP	Proteolipid protein
PFA	Paraformaldehyde
PFS	Progression-free survival
PTEN	Phosphatase and tensin homolog
PTGS	Post transcriptional gene silencing
qHTS	Quantititative high-throughput screening

RA	Retinoic acid
RMS	Rostral migratory stream
RP	Roof plate
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAR	Structure-activity relationship
SCA	Small cell astrocytoma
SCID	Severe combined immunodeficiency
SDF-1	Stromal cell-derived factor 1
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
SGZ	Subgranular zone
SLC12A5	Solute Carrier Family 12 (Potassium/Chloride Transporter), Member 5
SV40	Simian virus 40
SVZ	Subventricular zone

SYT1	Synaptotagmin-1
T3	Triiodothyronine
TCF	T-cell Factor
TCGA	The Cancer Genome Atlas
TIC	Tumour initiating cell
TIMP-3	Metalloproteinase inhibitor 3
TGF	Transforming growth factor
TKI	Tyrosine-kinase inhibitor
TNS	Tumour neurosphere
TP53	Tumour protein p53
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UPL	Universal probe library
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter 1 Introduction

1.1 Development of the central nervous system

The formation of the nervous system is one of the most fascinating subjects of biology. The central nervous system (CNS) in vertebrates consists of the brain and the spinal cord. There are three major classes of cell types in the CNS: neurons, oligodendrocytes and astrocytes. The latter two are termed glial cells. Glial cells provide a physical support and protection for neurons. Oligodendrocytes insulate axons with a myelin sheath, which enhances saltatory conduction through the nodes of Ranvier. Astrocytes maintain extracellular ion balance and provide nutrients to neurons and are also an integral component of the blood-brain barrier protection, where they support endothelial cells biochemically through their projections called glial limitans.

There are known to be many hundreds, or even thousands, of different neuronal subtypes and it is increasingly clear that many distinct subtypes of glia exist. Most research effort has been devoted to understanding the generation and function of neurons, with glia receiving less attention. However, the total number of glial cells in CNS is at around 10-fold higher than neurons (Rowitch 2004). Macrophages, termed microglia, form the immune defence of the brain. They are usually not mentioned in the classical classification of CNS cells as they are formed in the bone marrow and then migrate to the brain as myeloid progenitor cells (Ritter, Banin et al. 2006).

The nervous system arises from the outermost tissue layer of the embryo, called the ectoderm, in a process called neural induction (Figure 1A). The ectoderm becomes specified

to give rise to epidermis and the neural plate, which requires formation of neuroectoderm induced by signalling from the mesoderm. At the onset of gastrulation mesodermal cells form a layer in between the endoderm and the ectoderm and go on to form a structure called the notochord. Notochord signalling to adjacent tissues leads the overlying ectodermal cells to acquire neuroectodermal character, with the remainder of the ectoderm giving rise to the epidermis.

During human development, the neuroectoderm is formed in the third week of development and it is responsible for the creation of the neural plate along the dorsal side of the embryo. In the fourth week of development a groove is formed in the neural plate allowing it to wrap in on itself to a make a hollow neural tube in the process called neurulation. Some cells stop dividing and differentiate into neurons and glial cells, the main cellular components of the brain. Proper development of the neural tube is crucial as it gives rise to the brain and spinal cord and it is a source of majority of neurons and glial cells in the mature human. Neurons are born at the earliest stages of CNS development and migrate to different parts of the developing brain, where they self-organise into different brain structures. The glial cells emerge at later embryonic time-points and postnatally.

1.2 Regulation of cell differentiation in the CNS

The nervous system is formed from an undifferentiated layer of embryonic neuroectodermal progenitor cells. The blockade of signalling of the bone morphogenetic proteins (BMPs) during gastrulation is sufficient to induce the neuralisation of the ectoderm in Xenopus embryos. Two antagonists of BMP activity include Noggin and Chordin, which are released by the organiser (node in mammals). Complex patterning of these neuroectodermal

progenitors occurs via different concentrations of signalling molecules (morphogens), such as Sonic hedgehog (Shh). Early embryological experiments identified somites and notochord to be responsible for the dorsoventral (DV) patterning. Shh is produced by the floor plate. It induces ventral neural cell types in a concentration-dependent manner. BMPs are produced by the roof plate. They are responsible for inducing dorsal neural cell types in a concentration-dependent manner. Retinoic acid is then used for motor neuron differentiation and to define motor neuron subtypes (Figure 1B) (Wilson and Maden 2005).

The acquisition of a specific neural cell fate depends on the initial spatial coordinates of a precursor cell within the neural plate, because of the environment created by signalling molecules, which progressively restrict the developmental potential. The spinal cord, which is structurally simpler than the brain, has provided the best studied example for defining mechanisms controlling the patterning along the dorsal-ventral (DV) axis. The spinal cord is divided into 11 domains of neural progenitors: 5 ventral domains called p3, pMN, p2, p1 and p1 (from ventral to dorsal) and 6 dorsal domains called dP1, dP2, dP3, dP4, dP5 and dP6 (from dorsal to ventral). These domains express characteristic transcription factors, which determine the type of neuronal cell type created. The competing patterning signals are secreted from the ventral floor plate and the dorsal roof plate. Sonic hedgehog composes the ventral floor plate signalling, opposed by members of the Wingless-type MMTV integration site (Wnt) and BMP families from the roof plate. Non-BMP members of the transforming growth factor-beta (TGF-beta) superfamily are also involved in this process.

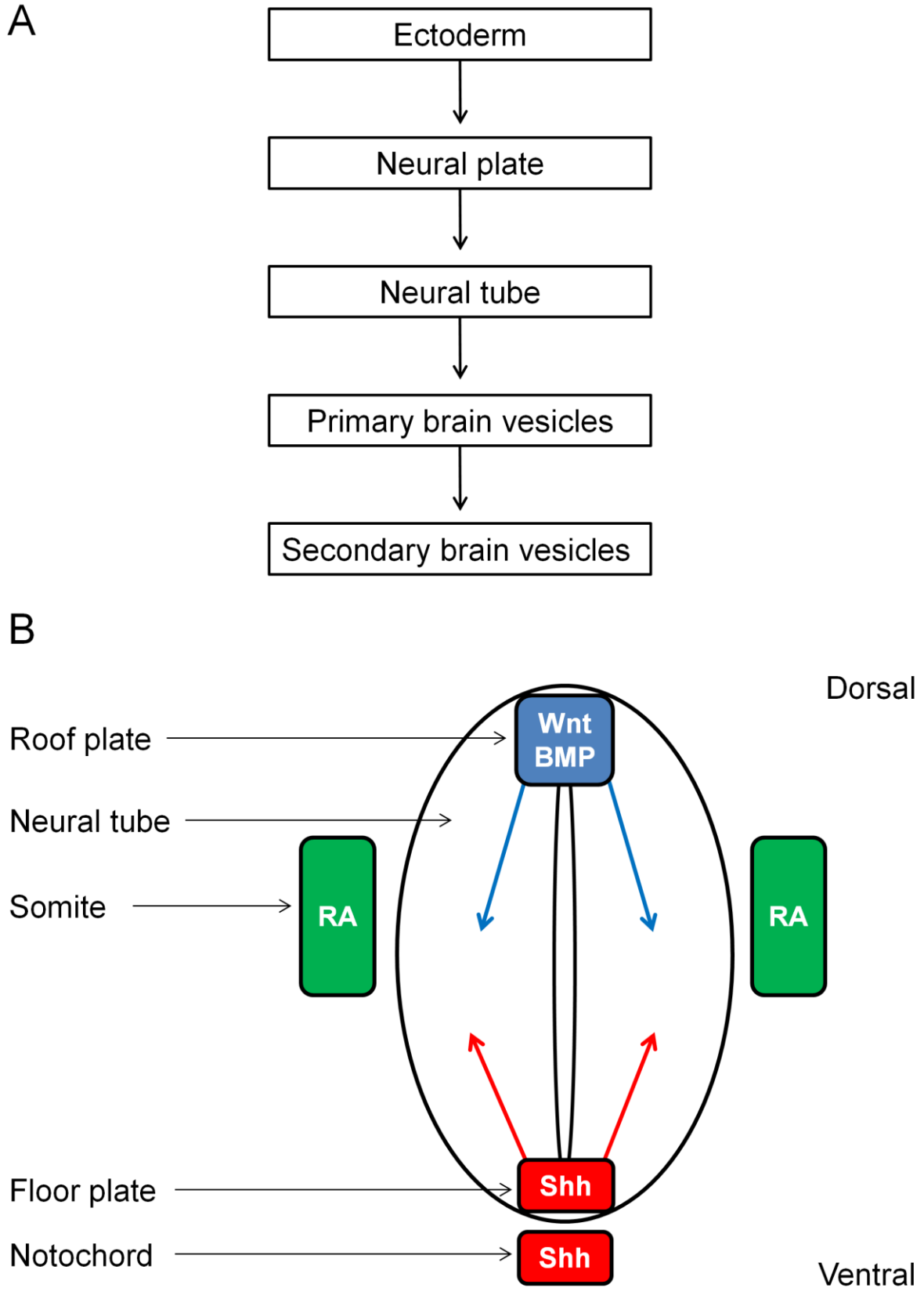


Figure 1. Key elements of the central nervous system development. (A) Schematic representing steps leading to the development of a brain; (B) Schematic showing key factors regulating the patterning of the nervous system;

The rostral caudal patterning involves fibroblast growth factors (FGFs) and retinoic acid mediated signalling. FGFs are produced by the caudal mesoderm, and must be down-regulated before the neural differentiation process can occur. FGF-8 downregulation is important for neuronal differentiation. Retinoic acid is a somatic signal, which opposes FGF-8 activity in neuroepithelium and paraxial mesoderm, where it controls somite boundary position. Retinoic acid is then further required for the expression of key ventral neural patterning genes (Diez del Corral, Olivera-Martinez et al. 2003, Novitch, Wichterle et al. 2003).

In the spinal cord, oligodendrocytes are firstly generated ventrally in the pMN domain and then later in the more ventral dP3-dP5 domains. The pMN domain initially gives rise to motoneurons and then to the majority of spinal cord OPCs. The identity of the pMN domain depends on the Shh gradient along the dorsoventral axis of the spinal cord, which results in the concentration-dependent induction of different transcription factors. Nkx2.2 is expressed only under the highest concentration of Shh in the most ventral region of the domain. On the other hand, Irx3 requires lower concentrations of Shh. Other closely related transcription factors like Nkx6.1 and Nkx6.2 can be induced under a wider range of Shh concentration, which allows them to be coexpressed with Nkx2.2 and Irx3 in their respective domains, but also in the domain between them. Expression of Olig2 gene is repressed by Nkx2.2 and Irx3, but it is a target gene for Nkx6.1 and Nkx6.2, thus it is expressed only between Nkx2.2 and Irx3 domains, which corresponds to the pMN domain. Therefore Olig2 is considered to be a marker for the pMN domain and its pluripotent neuroepithelial precursor cells. It is required for the formation of both oligodendrocytes and motoneurons (Kettenmann and Ransom 2013).

1.3 Neural stem cells

1.3.1 History of neural stem cell research

Neural stem cells are defined as multipotent, self-renewing cells, which can differentiate into neurons and glia (Figure 2). Such cells exist transiently during foetal development (and are termed radial glia), but in the 1990's it was also confirmed that they exist in the adult mammalian brain where they exist for the life-time of the animal. Adult neurogenesis was first observed in the 1960's following rat brain studies, where brain lesions were created, followed by an injection of thymidine-H(3); traumatized areas were characterized by labelling of glial cells, but also some neurons and neuroblasts, suggesting the proliferation of neurons in adult rats (Altman 1962). This led to further studies in the 1990's, which identified neural stem cells, within the ventricular zone of the lateral wall of the forebrain ventricle, as well as the hippocampus (Eriksson, Perfilieva et al. 1998). These cells contribute to tissue homeostasis and the production of new neurons in the adult central nervous system, within both the olfactory bulbs and hippocampus (Reynolds and Weiss 1992, Morshead, Reynolds et al. 1994). Adult neural stem cells were shown to be derived from foetal radial glia (Merkle, Tramontin et al. 2004).

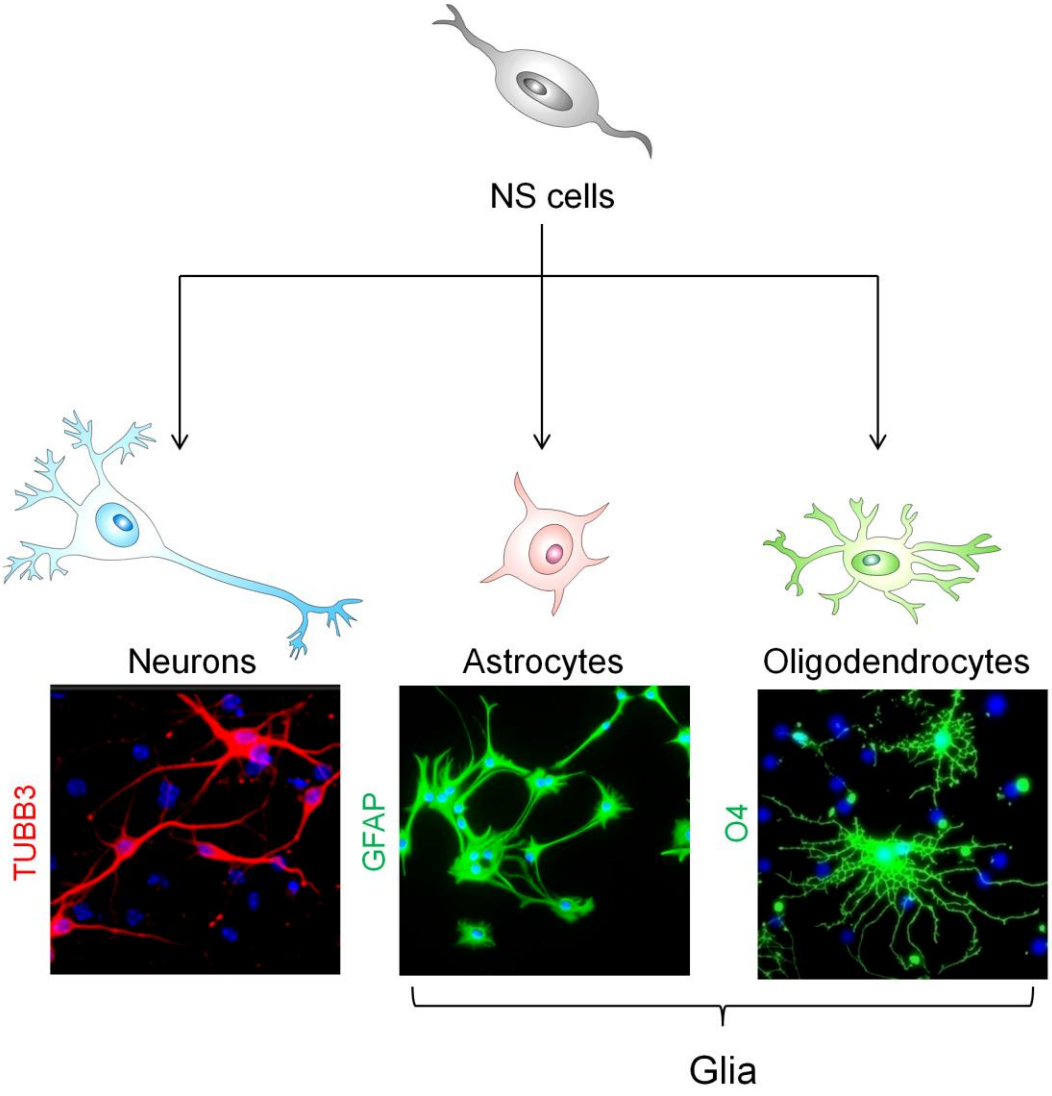


Figure 2. Neural stem cells can differentiate into mature neural cell types: neurons and glia, inclusive of astrocytes and oligodendrocytes. Images show neural cell types and their characteristic markers, TUBB3 for neurons, GFAP for astrocytes and O4 for oligodendrocytes; Image courtesy of Dr Steven Pollard, University of Edinburgh, UK;

1.3.2 Neural stem cell niche

Factors such as cell-cell interactions, vasculature, basal lamina and the extracellular matrix define a specific neural stem cell niche which has common features in both the SVZ and subgranular zone (SGZ) of the hippocampus (Doetsch 2003, Riquelme, Drapeau et al. 2008).

Adult SVZ NSCs have been shown to exist within a neurovascular niche consisting of two specific sub-localized compartments: the basal vasculature niche is a rich network of blood vessels and the apical ependymal niche lines the ventricles and is composed of stem cells intercalated with ciliated ependymal cells (Pastrana, Cheng et al. 2009). Alvarez-Buylla and Lim summarised the shared hallmarks of the neural stem cell niche as follows: (1) astrocytes serve as both stem cell and niche cell, (2) a basal lamina and concomitant vasculogenesis may be essential components of the niche, and (3) "embryonic" molecular morphogens and signals persist in these niches and play critical roles for adult neurogenesis. They termed these niches as "displaced" neuroepithelium, where cells and local signals preserve embryonic character to maintain neurogenesis for life (Alvarez-Buylla and Lim 2004).

Adult SVZ niche can be described in terms of three types of cells, called type A, -B, and –C cells (Figure 3). Type B cell have astrocytic features and are considered to be stem cells, which form migrating neuroblasts, also known as type A. These cells, via a rapidly dividing transit amplifying cell (type C) migrate rostrally to the olfactory bulb. Unlike type A cell, the type C divide rapidly, but remain stationary. Type B cells divide slowly and ensheath type A cells with cellular processes. Ependymal cells line wall of the lateral ventricle.

Intraventricular infusion of pigment epithelium-derived factor (PEDF) activates slowly dividing stem cells in the murine SVZ, while its blockade has an opposite effect, reducing their cycling. PEDF is secreted by components of the SVZ niche (Ramirez-Castillejo, Sanchez-Sanchez et al. 2006). Neural stem cells are stimulated by soluble factors released by endothelial cells, which stimulate self-renewal by activating Notch and Hes1, as endothelial coculture stimulates neuroepithelial cell contact (Shen, Goderie et al. 2004). Infusion of EGF into the lateral ventricle causes progenitor cells to proliferate and migrate towards the

ventricle. Fricker-Gates et al. transplanted *in vitro* expanded immature progenitor cells into the striatum of adult rats followed by a continuous infusion of EGF into the lateral ventricle and observed that the transplanted cells migrated towards the ventricle and proliferated, which was confirmed as cells incorporated bromodeoxyuridine (BrdU) (Fricker-Gates, Winkler et al. 2000). Kuhn et al. observed that both EGF and FGF-2 can expand the SVZ population of progenitor cells, but only FGF-2 was able to increase the number of newly born neurons in the olfactory bulb. EGF had an opposite effect, decreasing the amount of newborn neurons and instead increasing the generation of astrocytes in the olfactory bulb. Infusion of either of the growth factors did not affect the proliferation of the hippocampal progenitors (Kuhn, Winkler et al. 1997). Infusion of PDGF alone was also shown to have proliferative potential. PDGF arrests the formation of neuroblasts and induces proliferation of SVZ B cells, contributing to formation of large hyperplasias, which had some characteristics of gliomas (Jackson, Garcia-Verdugo et al. 2006).

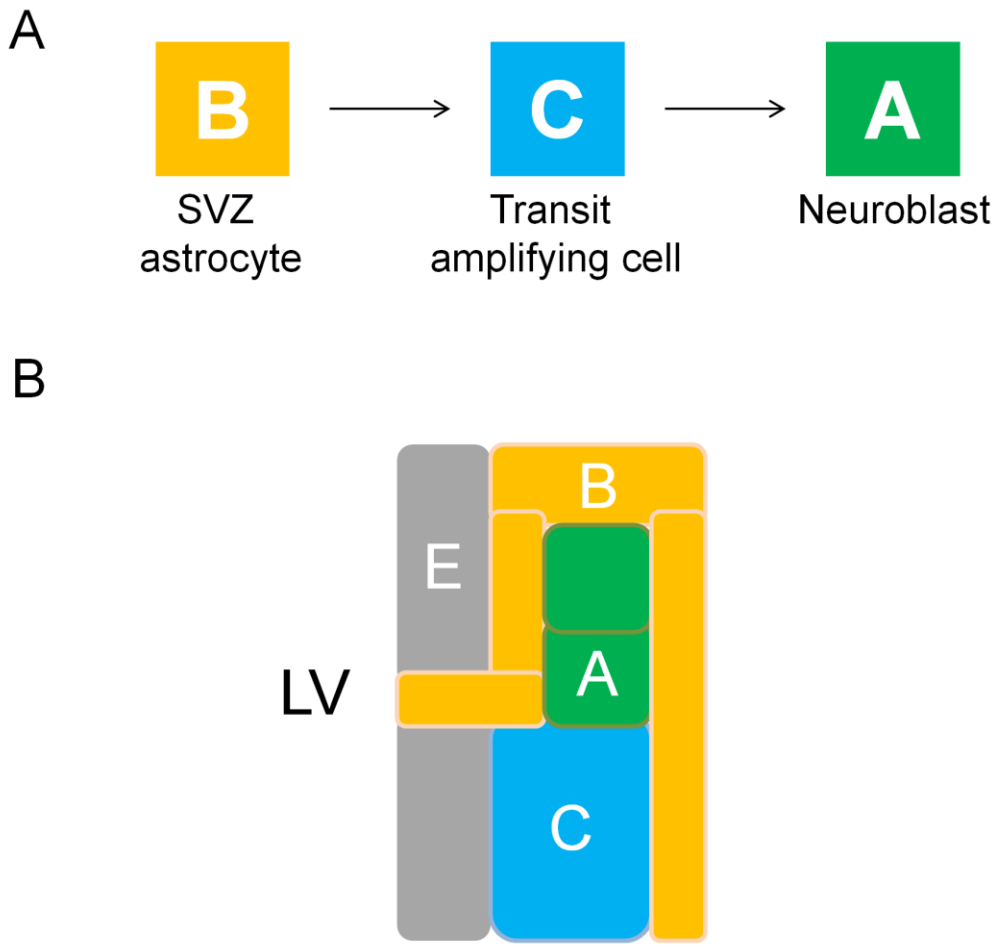


Figure 3. Cell types of the adult SVZ niche. (A) Schematic representing relationship between the key three cell types of the SVZ niche. Type B cells give rise to type A cells via a transit amplifying cell, called type C; (B) Schematic of frontal section of the adult mouse brain showing the subventricular zone adjacent to the lateral ventricle (LV). SVZ niche astrocytes in this region (B, yellow) are considered to be stem cells which can generate migrating neuroblasts (A, green). These cells are destined for the olfactory bulb via a transit-amplifying cell (C, blue). Ependymal cells (E, grey) line the walls of the lateral ventricle.

1.3.3 Molecular hallmarks and signalling pathways regulating neural stem cells

There has been a great deal of attention focussed on the identification of markers that can identify and enable prospective isolation of foetal and adult neural stem cells. One of the first markers to be identified was reported by Lendahl et al., who identified Nestin, a class VI intermediate filament protein, to be a molecule whose expression distinguishes stem cells

from the more differentiated cells in the neural tube (Lendahl, Zimmerman et al. 1990, Doetsch, Caille et al. 1999). The presence of Nestin was observed in adult neural stem cells, both *in vitro* and *in vivo* (Reynolds and Weiss 1992, Morshead, Reynolds et al. 1994).

One of the best markers, and known regulators of neural stem cell identity is Sox2. Sox2 is expressed by neural stem cells at embryonic and adult stage (Ellis, Fagan et al. 2004). It is required to maintain their proliferative ability by in part regulating Shh. In a study by Favaro et al. they showed that a deletion of Sox2 causes a loss in hippocampal neurogenesis, which was similar to the already observed loss due to Shh deletion. Chromatin precipitation identified Shh as a Sox2 target. They concluded that neural stem cells control their status through Sox2-dependent autocrine mechanisms (Favaro, Valotta et al. 2009). Mouse embryonic studies presented cerebral malformations, parenchymal loss and ventricle enlargement when Sox2 was deleted. Abnormalities in neurons, degeneration and cytoplasmic protein aggregates were observed at the cellular level (Ferri, Cavallaro et al. 2004).

Other frequently used markers include GFAP (only quiescent cells), Nestin, Musashi 1 and Musashi 2 with CD133 being the most controversial. CD133 was widely used as a marker of neural stem cell identity with some studies showing that it is possible to expand cell lines, which are originally negative for this marker (Sun, Kong et al. 2009, Chen, Guo et al. 2012).

Adult neural stem cells maintain a glial identity similar to embryonic radial glia (Doetsch 2003). It was suggested that two subpopulations of active and quiescent neural stem cells are maintained in the adult organism, with both having their cooperative function (Li and Clevers 2010). Recent attention has focussed on determining the phenotypic differences between active proliferating neural stem cells and those quiescent neural stem cells. CD133 may mark

a quiescent neural stem cell state (Beckervordersandforth, Tripathi et al. 2010). More recent studies have defined a key role for TLX (Qu, Sun et al. 2010) and NFI families (Martynoga, Mateo et al. 2013).

Extrinsic signals that control neural stem cell self-renewal and proliferation are numerous. BMP and Wnt signalling regulate function of adult neural stem cells throughout an individual's lifetime (Wexler, Paucer et al. 2009, Mira, Andreu et al. 2010), with Wnt/beta-catenin signalling involved in the proliferation of progenitor cells in the SVZ of the adult mouse brain (Hirabayashi, Itoh et al. 2004, Adachi, Mirzadeh et al. 2007). BMP on the other hand promotes cell cycle exit and causes differentiation of neural progenitors (Gross, Mehler et al. 1996), and likely in combination with FGF signalling is involved in sustaining the quiescent neural stem cell state (Mira, Andreu et al. 2010, Martynoga, Mateo et al. 2013) .

Other known extrinsic regulators include Notch and EGFR pathways (Alexson, Hitoshi et al. 2006, Aguirre, Rubio et al. 2010). Notch is responsible for regulation of identity and self-renewal of neural stem cells, while EGFR controls proliferation and migration of transiently amplifying progenitors and likely marks the transition from quiescent to 'active' proliferative neural stem cells (Pastrana, Cheng et al. 2009). Pigment epithelium-derived factor (PEDF), CXCL12 and FGF-2 are also involved in the self-maintenance of neural stem cells through non-cell-autonomous effects (Ramirez-Castillejo, Sanchez-Sanchez et al. 2006, Kokovay, Goderie et al. 2010).

Notch signalling plays role in regulating cell-to-cell communication, proliferation and differentiation, and apoptosis (Artavanis-Tsakonas, Rand et al. 1999). It regulates numbers and promotes survival of neural stem cells through induction of Hes3 and Shh through activation of cytoplasmic signals, including serine/threonine kinase Akt, STAT3 and mTOR.

When administered into brains of adult rats an increase in newly generated precursor cells can be observed after ischemic injury. Notch signalling is opposed by a control mechanism involving p38 mitogen-activated protein kinase (Androutsellis-Theotokis, Leker et al. 2006).

1.4 *In vitro* culture of neural stem cells

Large-scale sources of neural stem cells play an important role in basic research and have a potential to be used in novel treatments for neurological disorders. Oncogene-immortalised stem cells, neurospheres, and embryonic stem cell (ES)-derived neural stem cells have been widely used *in vitro* as sources of neural stem cells (Gottlieb 2002) – although how closely these relate to *in vivo* neural stem cells is unresolved (Pastrana, Silva-Vargas et al. 2011).

The neurosphere assay has been widely used to study neural stem cells *in vitro*. It is based on the ability of neural stem cells to self-renew in the presence of EGF and FGF-2 when cultured *in vitro* (Vescovi, Parati et al. 1999, Azari, Rahman et al. 2010). In this assay cells are grown in suspension, as floating cell clusters, termed neurospheres. One of the caveats of this method is the fact that the clusters are composed of stem, progenitor and differentiating cells, and often a large proportion of dying cells. Another caveat is the possibility of sphere formation by transient-amplifying progenitor cells (Reynolds and Rietze 2005, Marshall, Reynolds et al. 2007, Ahmed 2009). Neurospheres formed by the SVZ cells cultured *in vitro* in the presence of EGF were shown to be derived from highly mitotic, Dlx2-positive, transit-amplifying C cells and not from the quiescent stem cells *in vivo* (Doetsch, Petreanu et al. 2002). EGF causes C cells to downregulate Dlx2 and arrest neuronal production, making these cells highly proliferative and invasive. A neural colony forming cell assay was designed to discriminate between the bona fide neural stem cells and neural progenitor cells (Deleyrolle and Reynolds 2009, Azari, Rahman et al. 2010). In this collagen based semi-solid

assay, it is possible to discriminate stem from progenitor cells, because of their long-term proliferative potential.

Neural stem cells can be also grown as adherent NS cell lines, which show a multi-lineage differentiation ability and self-renewal potential (Conti, Pollard et al. 2005, Pollard, Conti et al. 2006, Pollard 2013). The term 'NS cells' was used to highlight the similar experimental attributes compared with ES cells. Initially, the NS cell lines were derived from the ES cells differentiated into heterogeneous neuroepithelial progenitors cultured as an adherent monolayer. After a number of passages cells lost the heterogeneous morphology and uniformly expressed Sox2 and Nestin. The process was facilitated by FGF-2 and EGF present in a defined basal media (Ying, Stavridis et al. 2003).

Adherent culture conditions offer a number of advantages over the neurosphere assay and other methods. NS cell line propagation *in vitro* reduces the need for a constant supply of embryos. All cells in the layer are equally exposed to EGF and FGF-2. This allows for a suppression of differentiation and apoptosis, which can occur in the neurosphere assay, enabling propagation of homogenous populations. NS cell lines have proven useful in defining transcriptional targets of key transcriptional regulators: Mash1 (Gohlke, Armant et al. 2008) and REST (Johnson, Teh et al. 2008), and were also exploited in studies of reprogramming (Stricker, Feber et al. 2013, Stricker and Pollard 2014). NS cell lines can be easily expanded, which makes them suitable for a number of applications including genome manipulations, gene expression profiling and biochemical analysis. Moreover, the adherent culture is well suited for chemical screening, because it is possible to monitor and track individual cells (Pollard, Clarke et al. 2009). Clonal cell lines can easily be generated and 'picked' – avoiding the need for elaborate semi-solid suspension culture regimes.

Similarly to neurosphere culture, adherent NS cell lines also have limitations. Cells grown this way display changes in dorsal-ventral positional identity, which can limit their differentiation potential to specific type of neurons or glial cells (Conti and Cattaneo 2010) – although anterior-posterior markers such as Hox gene expression seems to be preserved (Sun, Pollard et al. 2008). The sustained exposure to FGF-2 results in upregulation of ventral markers such as Olig2 and Mash1, which in turn limit neuronal differentiation potential to GABAergic interneurons (Gabay, Lowell et al. 2003). Culture conditions could also affect temporal identity of NS cells as FGF-2 induces expression of EGFR, which is usually found in later foetal stages (Pollard, Wallbank et al. 2008). Thus, *in vitro* propagated neural stem cells share features with late foetal gliogenic progenitors and radial glia, and most likely do not have potential to generate all neuronal and glial cell types of the nervous system.

Adherent culture of neural stem cells is particularly well suited to high content drug screening, where the ability to identify and track individual cells using automated software is crucial (see Chapter 4).

1.5 Oligodendrocyte differentiation

Much progress is made in our understanding of the origins of the oligodendrocytes during development, and identification of some key regulatory genes and useful markers (Richardson, Kessaris et al. 2006). In the early developing nervous system gradients of activity of Shh and BMP are responsible for the initial induction of lineage-specific transcription factors that specify the oligodendrocyte lineage (e.g. Olig2 and Nkx2.2).

Oligodendrocyte progenitor cells (OPCs) initially emerge from the ventral neural tube (Rowitch 2004). The critical downstream transcriptional regulators that are required for

promoting the oligodendrocyte differentiation program include Olig2, Nkx2.2, and Sox10. Sox10 is also crucial for glial development in the peripheral nervous system and is restricted to myelin-forming oligodendroglial cells of the central nervous system, providing a useful marker and functionally important regulator of oligodendrocyte differentiation (Stolt, Rehberg et al. 2002). Dephosphorylation of Olig2 at serine 147 triggers the switch from motor neuron production to oligodendrocyte precursors in the embryonic spinal cord (Li, de Faria et al. 2011). Transcription factors Nkx2.2 and Nkx6.2 are co-expressed in myelinating oligodendrocytes suggesting their involvement in maintenance and creation of the myelin sheath (Cai, Zhu et al. 2010).

Oligodendrocytes develop postnatally from PDGFRα-positive OPCs that emerge during foetal development in the pMN domain. OPCs depend on PDGF-A binding to PDGFRα for continued proliferation and migration. PDGF-A controls the number of OPCs both in embryo and in adult brain (van Heyningen, Calver et al. 2001, Woodruff, Fruttiger et al. 2004). PDGF-A deficient mice, which survive postnatally suffer from severe hypomyelination (Calver, Hall et al. 1998, Fruttiger, Karlsson et al. 1999). SVZ cells stimulated with PDGF-A differentiate towards PDGFRα-positive cells leading towards oligodendroglial differentiation (Jackson, Garcia-Verdugo et al. 2006, Menn, Garcia-Verdugo et al. 2006). PDGF-A induces embryonic Nestin-positive neural progenitor cells to become NG2-positive oligodendrocyte progenitor cells (Hu, Fu et al. 2008). PDGF and PDGFR is also known to be involved in many other developmental processes, from lung alveolar myofibroblast development and alveogenesis (Bostrom, Willetts et al. 1996) to cardiovascular and renal development (Leveen, Pekny et al. 1994, Soriano 1994). In particular, PDGFRα is required for the development of the neural crest and for patterning of the somites (Soriano 1997).

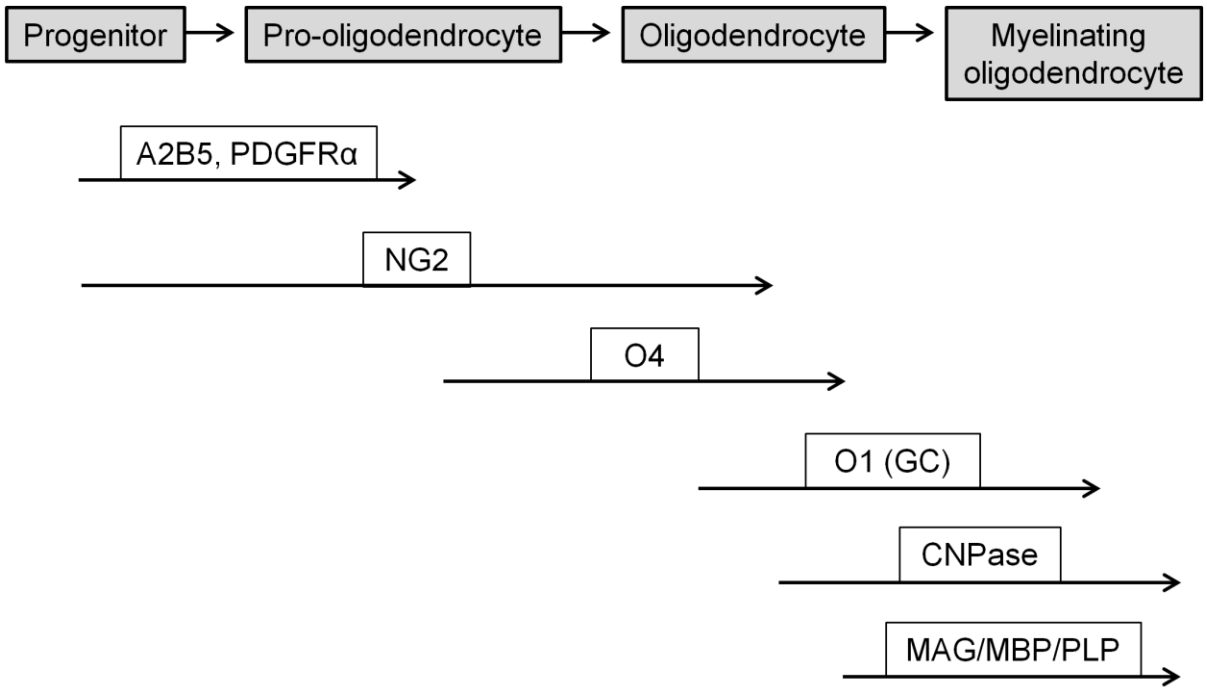


Figure 4. Markers of oligodendrocyte differentiation. Adapted from (Zhang 2001). Differentiation of oligodendrocytes is a stepwise process from bipolar progenitors to pre-oligodendrocytes with multiple processes, followed by membrane-sheath-bearing oligodendrocytes and myelinating oligodendrocytes.

Full differentiation of OPCs into oligodendrocytes occurs between postnatal days 4-12 (P4-P12). Mature markers include 2′,3′-cyclic nucleotide 3’-phosphohydrolase (CNPase), myelin basic protein (MBP), proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) (Patel, McCandless et al. 2010) (Figure 4).

In vitro studies of rat oligodendrocyte progenitors through the 1980s and 1990s led to the description of two types of astrocytes called type 1 and type 2 astrocytes. Type 1 astrocytes were defined as cells with fibroblast-like morphology, the ability to proliferate in the presence of EGF and were A2B5-negative. Type 2 astrocytes divided infrequently in culture and bound tetanus toxin and A2B5 antibody (Raff, Miller et al. 1983). Studies performed on rat optic nerve identified cells with an ability to differentiate into type 2 astrocytes if cultured

in the presence of fetal calf serum, and into oligodendrocytes in its absence, suggesting the A2B5-positive cells as progenitors for both oligodendrocyte and type 2 astrocyte lineages (the 'O2-A' progenitors) (Raff, Miller et al. 1983). How the O2-A progenitor relates to the radial glia and other progenitors within the developing mouse CNS remains unclear (Rowitch and Kriegstein 2010).

In vitro differentiation of OPCs into functional oligodendrocytes can be triggered by a withdrawal of PDGF-AA and an addition of T3, thyroid hormone (Barres, Lazar et al. 1994, Ahlgren, Wallace et al. 1997, Baas, Bourbeau et al. 1997). While NS cell lines, from both mouse and human, generate mature oligodendrocytes using similar protocols, this is highly inefficient (1-10%). Improved understanding of the molecular mechanisms controlling NS cell lineage choice *in vitro* may elucidate principles of cell differentiation control *in vivo*. NS cell conversion to oligodendrocytes *in vitro* is likely to mirror the transition from NS cell through oligodendrocyte progenitor cell (OPC) to a mature oligodendrocyte *in vivo*. Efficient production of human oligodendrocytes in the laboratory may open up new opportunities for cell transplantation medicine, for example in the treatment of demyelinating disorders such as multiple sclerosis.

1.6 Glioma and glioblastoma

1.6.1 Background

Glioma is a type of a brain tumour that arises most frequently in the forebrain. Gliomas make up 80% of all malignant brain tumour cases. They can be characterised according to the histological appearance and include: ependymoma, oligodendrolioma, and most frequently astrocytoma. However, gliomas can be highly heterogeneous both between patients and

within individual tumours – where mixtures of astrocyte-like cells, oligodendrocyte and immature progenitors exist. An additional form of classification includes their grade, according to the pathologic evaluation of the extent of aggressive features. Low grade gliomas are well differentiated and have a better prognosis for patients. They can grow slowly over a period of years and can remain unidentified until symptoms arise. High grade gliomas are anaplastic, malignant and have a worse prognosis. They are highly vascular with a tendency to infiltrate, making surgical resection of the tumour impossible. They are characterised by extensive areas of necrosis and hypoxia.

High grade gliomas are called glioblastoma (GBM) or high grade astrocytoma, and these tumours are highly malignant and typically grow back after the removal surgery. About half of GBM cases are bilateral or occupy more than one lobe of a hemisphere. Prognosis is a dismal 14-month median survival. Symptoms of brain gliomas include headaches, nausea, seizures and vomiting. An increase in intracranial pressure can also lead to cranial nerve disorders. Cell type, grade of malignancy and location of a tumour can influence treatment possibilities. Standard treatment includes resection, followed by a chemo- and radiotherapy (Adamson, Kanu et al. 2009).

GBM is the most common and most malignant primary brain tumour in adults (Ohgaki, Dessen et al. 2004, Ohgaki and Kleihues 2005, Louis, Ohgaki et al. 2007). It typically contains cells with neural stem cell and glial progenitor characteristics (Murat, Migliavacca et al. 2008). GBM is characterised by a presence of hyperplastic blood vessels and small areas of necrotizing tissues surrounded by anaplastic cells. Usually formed in the cerebral white matter, GBMs grow quickly before symptoms are observed.

The causes of gliomas are not completely understood, with only a minority of cases being hereditary genetic disorders, such as tuberous sclerosis complex and neurofibromatoses (type 1 and 2) being a predictor of their development. Genetic mutations frequently associated with gliomas include mutations in tumour suppressor protein 53 (*TRP53*), phosphatase and tensin homolog (*PTEN*) and activating mutations in epidermal growth factor receptor (*EGFR*).

A range of subtypes of GBM have been described and various other disease variants include fibrillary/epithelial GBM, small cell astrocytoma (SCA), GBM with oligodendroglial component (GBMO), GBM with primitive neuroectodermal features (GBM-PNET), gemistocytic astrocytoma (GA), granular cell astrocytoma (GCA), paediatric high grade glioma (HCG) as well as diffuse intrinsic pontine glioma (DIPG) (Karsy, Gelbman et al. 2012). The current WHO classification system recognizes three distinct GBM variants, classic GBM, gliosarcoma (GS) and giant-cell GBM (GC-GBM) (Louis, Ohgaki et al. 2007). The giant-cell GBM is characterised by a prevalence of multinucleated (more than 20 nuclei), giant cells with diameter up to 400 um. Gliosarcoma is rare accounting for approximately 2.1% of all GBMs and contains sarcomatous components, malignant cells of mesenchymal origin (Ayadi, Charfi et al. 2010). How these distinct diseases emerge and the molecular underpinnings of GBM subtypes are only now being uncovered.

1.6.2 Genetics of human glioblastoma

GBM can be defined as ‘primary GBM’, which presents acutely as a high grade disease with mutations in EGFR, PTEN and INK4A/ARF (CDKN2A) and the secondary GBM subtype evolving from the slow progression of low grade disease with PDGF and TP53 events (Kleihues and Ohgaki 1999). The vast majority of GBMs develop rapidly after a short clinical history and without evidence of a less malignant precursor lesion. Over 60% of

patients with primary GBM have a clinical history of less than 3 months (Ohgaki, Dessen et al. 2004). Secondary GBMs are more common in younger patients with mean age of 45 versus 62 for primary GBM and account for 10% of cases (Ohgaki and Kleihues 2009). These tumours grow slower through a progression from low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). The mean time of progression from these tumours to GBM was estimated at 5.3 years and 1.4 years respectively. The genomic classification of GBM includes four subtypes: classical, proneural, neural, and mesenchymal (Verhaak, Hoadley et al. 2010) (Figure 5). Classical subtype contains extra copies of *EGFR* gene with rarely mutated *TP53* (Hayden 2010). Proneural has high rates of TP53, PDGFRA and IDH1 alterations. Mesenchymal subtype contains mutations in NF1, a gene encoding Neurofibromatosis type 1, and less alterations and lower expression levels of EGFR than any other subtype (Kuehn 2010). The neural subtype expresses neuron markers, such as NEFL, GABRA1, SYT1 and SLC12A5 (Verhaak, Hoadley et al. 2010).

Large scale sequencing of GBM genome led to discovery of novel signalling pathways and genetic alterations, which could create a new direction in GBM treatment (Cancer Genome Atlas Research 2008, Parsons, Jones et al. 2008). Despite being histologically indistinguishable, the mRNA and protein expression profiles of primary and secondary GBMs are significantly different (Ohgaki and Kleihues 2007).

The malignant transformation of GBM cancer cells, as in case of other malignancies, could be due to multiple chromosomal aberrations, nucleotide substitutions and epigenetic modifications. The large-scale multi-dimensional analysis of these molecular characteristics in human cancer is aim of The Cancer Genome Atlas (TCGA) project.

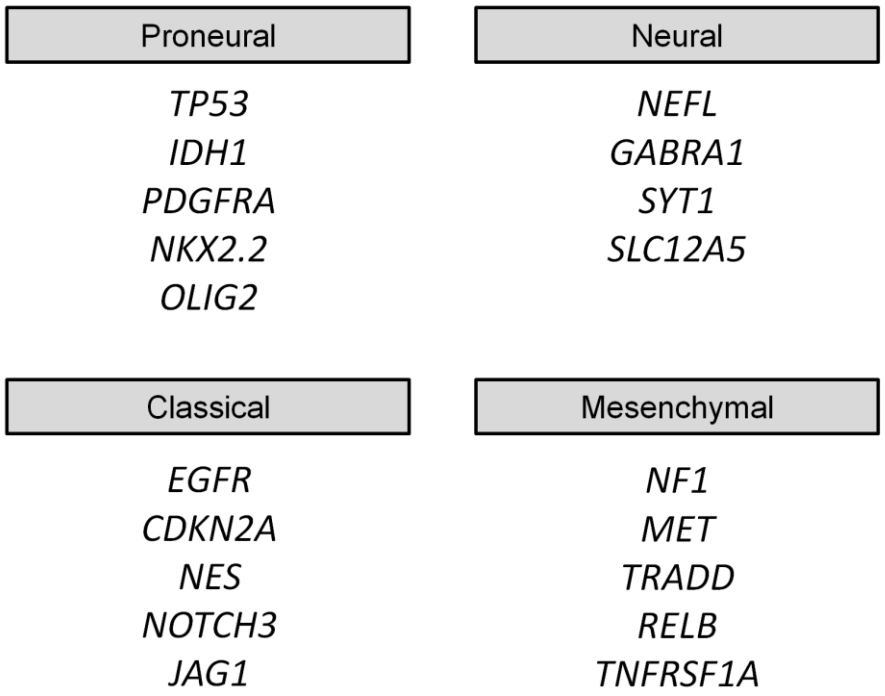


Figure 5. Summary of key genomic alterations across GBM subtypes. Adapted from (Verhaak, Hoadley et al. 2010).

The data from TCGA analyses of GBM samples provided new insights into the frequency of ERBB2, NF1 and TP53, frequent mutations of the phosphatidylinositol-3-OH kinase regulatory subunit gene PIK3R1 and a network view of the pathways altered in GBM development. Integrated analysis of mutation, DNA methylation and clinical treatment data revealed a correlation between O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and a hypermutator phenotype consequent to a mismatch repair deficiency in treated GBMs (Cancer Genome Atlas Research 2008). MGMT removes promutagenic alkyl groups from O^6-methylguanine (Pegg 2000). This molecule causes G:C -> A:T mutations during DNA replication (Margison, Santibanez Koref et al. 2002, Kaina 2004). A methylation-specific PCR approach showed a correlation between the MGMT promoter methylation status and long-term patient survival with higher methylation rate

associated with long-term survival GBM type (Martinez, Schackert et al. 2007). Epigenetic silencing of the MGMT DNA-repair gene using promoter methylation compromised DNA repair and was shown to be associated with longer survival in GBM patients, irrespective of treatment used, with patients treated with temozolomide and radiotherapy with a significantly longer survival (Hegi, Diserens et al. 2005, Criniere, Kaloshi et al. 2007).

The analysis of protein-coding genes in GBMs led to identification of IDH1 mutations (Parsons, Jones et al. 2008). Subsequent studies identified IDH1 mutations to be more frequent in secondary than in primary GBMs (Balss, Meyer et al. 2008, Watanabe, Nobusawa et al. 2009, Yan, Parsons et al. 2009, Bleeker, Molenaar et al. 2012). This is contrasted by rare IDH1 mutations in primary GBMs, suggesting different cells of origin (Ohgaki and Kleihues 2009). IDH1 is localised on 2q33.3 (Narahara, Kimura et al. 1985). The IDH1 protein catalyses oxidative carboxylation of isocitrate to α-ketoglutarate, which results in the production of NADPH in the citric acid cycle (Geisbrecht and Gould 1999). Unlike other IDH molecules which are present in mitochondria, the IDH1 is present in the cytosol. Mutations of IDH1 in GBMs cause formation of inactive heterodimers, which impair the enzyme's affinity for its substrate and lead to increased levels of hypoxia-inducible factor subunit HIF-1α, a transcription factor which can facilitate tumour growth (Yan, Parsons et al. 2009). IDH1 mutations are currently considered the only genetic alterations frequent among oligodendrogliomas, astrocytomas and oligoastrocytomas suggesting a critical role of IDH pathways in initial tumour formation (Watanabe, Nobusawa et al. 2009).

Approximately 40% of primary GBMs carry EGFR amplification. This however, is very rare in secondary GBMs (Ekstrand, Sugawa et al. 1992, Watanabe, Tachibana et al. 1996, Ohgaki, Dessen et al. 2004). EGFR mutation called EGFRvIII, characterised by deletion of exons 2-7

is the most common EGFR variant in GBM patients. It shows a constitutive activation of the receptor and exerts mitogenic effects (Huang, Nagane et al. 1997). Growth factor receptors like EGFR and PDGFR are activated when their respective ligands bind to their extracellular domain. This leads to the activation of intracellular signalling cascades, one of which is PI3K pathway. Following membrane receptor activation, phosphatidylinositol 3-kinase (PI3K) is recruited to the cell membrane. Composed of a catalytically active protein p110a and a regulatory protein p85a, the PI3K complex phosphorylates phosphatidynositiol-4,5-bisphosphate (PIP_2) to the respective 3-phosphate (PIP_3), which activates downstream effector molecules like AKT (protein kinase B) and mTOR. This signalling cascade results in cell proliferation and increased cell survival due to the blockade of apoptosis. The regulatory molecule, PTEN, is capable of inhibiting the PIP_3 signal and inhibiting the proliferation process (Mellinghoff, Wang et al. 2005). Similarly to EGFR, the PTEN gene is frequently mutated in primary GBMs (15-40% cases), but rare in secondary GBM (Tohma, Gratas et al. 1998, Knobbe, Merlo et al. 2002, Ohgaki, Dessen et al. 2004). Mutations of PIK3CA, the gene encoding catalytically active component (p110α) of PI3K complex are rare and account for 5% in primary and 13% in secondary GBMs (Kita, Yonekawa et al. 2007).

Findings from TCGA estimated overall frequency of alterations in the EGFR/ RAS/ NF1/ PTEN/ PI3K pathway in 88% of GBMs with additional findings like NF1 mutations/ homozygous deletions to be present in 18% of cases and PIK3R1 in 10% (Cancer Genome Atlas Research 2008). Interestingly, mRNA expression of PDGFR was observed in astrocytic tumours (all grades), however, amplification of the gene was seen only in a small subset of GBMs (Hermanson, Funa et al. 1992). NF1 tumour suppressor gene encodes neurofibromin, which acts primarily as a RAS negative regulator, but it is also involved in adenylate cyclase and AKT-mTOR mediated pathways (Lee and Stephenson 2007).

Mutations in TP53 occur more frequently in secondary GBMs (65% vs 28% in primary GBMs) (Ohgaki, Dessen et al. 2004, Ohgaki and Kleihues 2005). MDM2 is induced by wildtype TP53 (Barak, Gottlieb et al. 1994, Zauberman, Flusberg et al. 1995). In turn, MDM2 binds to mutant and wildtype TP53 inhibiting its ability to activate transcription (Momand, Zambetti et al. 1992, Oliner, Kinzler et al. 1992). MDM2 amplification appears to be exclusive to primary GBMs, which lack TP53 mutation (Reifenberger, Liu et al. 1993, Biernat, Kleihues et al. 1997), however it is not frequent, occurring in less than 15% of cases (Nakamura, Watanabe et al. 2001). There appears to be a significant inverse correlation between $p14^{ARF}$ alterations and TP53 mutations and also between MDM2 amplification and TP53 mutations (Zawlik, Kita et al. 2009). The results of the TCGA study on GBMs estimated the overall frequency of genetic alterations in the TP53/ MDM2/ MDM4/ $p14^{ARF}$ pathway to be 87%, through $p14^{ARF}$ homozygous deletion or mutation (49%), TP53 mutations or homozygous deletions (35%), MDM2 amplification (14%) and MDM4 amplification (7%) (Cancer Genome Atlas Research 2008).

A CNS-specific genetic deletion of *tp53* and *pten* in mice showed a development of a penetrant acute-onset high-grade malignant phenotype with a close resemblance to primary GBM in humans. It has been established that a dual inactivation of p53 and PTEN leads to an undifferentiated state with high renewal potential and drives increased Myc protein levels, which could be responsible for impaired differentiation and enhanced renewal of NSCs doubly null for p53 and PTEN and tumour neurospheres (TNSs) derived from this model (Zheng, Ying et al. 2008).

RB1 protein is responsible for the control of progression through G_1 into the S-phase of the cell cycle. The CDK4/ cyclin D1 complex phosphorylates RB1, leading to activation of E2F

transcription factor, responsible for activation of genes involved in the progression from G1 into S-phase (Sherr and Roberts 1999). Progression from one phase into the next one can be inhibited by $p16^{INK4a}$ which binds to CDK4 and inhibits the CDK4/ cyclin D1 complex (Sherr and Roberts 1999). The CDK4 amplification, loss of RB1 and homozygous deletion of $p16^{INK4a}$ are largely mutually exclusive and put together they are present in half of primary GBMs and approximately 40% secondary GBMs (Biernat, Tohma et al. 1997).

GBMs also harbour structural genetic changes. Most frequent are loss of heterozygosity (LOH) on chromosome 10 is a common genetic alteration, which occurs in up to 80% of GBMs (Karlbom, James et al. 1993, Rasheed, McLendon et al. 1995, Ichimura, Schmidt et al. 1998, Fujisawa, Reis et al. 2000, Ohgaki, Dessen et al. 2004). The deleted loci include 10p14-15, 10q23-24 (PTEN) and 10q25-pter (Karlbom, James et al. 1993, Rasheed, McLendon et al. 1995, Fults, Pedone et al. 1998). Frequently deleted in GBMs is the chromosomal region at 9p23-24.1. It encodes a gene called PTPRD, a receptor protein tyrosine phosphatise with tumour suppressor function (Solomon, Kim et al. 2008, Veeriah, Brennan et al. 2009). LOH 22q occurs in 82% of secondary GBMs and it is characterised by a small 957 kb deletion of a region encoding tissue inhibitor of metalloproteinases-3 (TIMP-3) (Nakamura, Ishida et al. 2005).

1.6.3 Molecular pathways that drive primary glioblastoma

Inhibitor of DNA binding 2 (Id2), a transcription factor known to regulate proliferation and differentiation of stem and progenitor cells, is de-repressed in adult tissue neural stem cells (NSCs) lacking TP53 and modulates their proliferation. Constitutive expression of Id2 in differentiating NSCs results in maturation resistant oligodendroglial precursor cells (OPCs), a cell population implicated in the initiation of glioma. Id2 overexpression leads to the

inhibition of the Notch effector Hey1, a bHLH transcription factor, a direct transcriptional repressor of the oligodendroglial lineage determinant Olig2. *In vivo* experiments with Id2-enhanced NSCs in a murine model engineered to express platelet-derived growth factor in the CNS resulted in glioma. These findings implicated a mechanism of altered NSC differentiation in glioma development and showed a possible explanation behind a proneural subtype of GBM. These data also support the concept that cellular and molecular characteristics of tumour cells are linked to the transformation of distinct subsets of adult tissue progenitors (Havrda, Paolella et al. 2014).

A statistical approach to identify potential driver mutations in GBM was established to narrow a gap between known genetic changes, which occur in GBM and the unknown consequences of many of these mutations. The integrated analysis of copy number variations and somatic mutations unravelled the landscape of in-frame gene fusions in GBM. Mutations and deletions lead to disruptions of LZTR1, encoding an adaptor of CUL3-containing E3 ligase complexes. LZTR1 restrains the self-renewal and growth of glioma spheres that retain stem cell features. Loss-of-function mutations in CTNND2 are associated with the transformation of glioma cells along the mesenchymal phenotype. Recurrent translocations fusing the coding sequence of EGFR to several partners were also reported. EGFR-SEPT14 gene fusion is the most frequent functional gene fusion in human glioblastoma. It leads to STAT3 signalling activation and confers mitogen independence and sensitivity to EGFR inhibition (Frattini, Trifonov et al. 2013).

The PDGF family of proteins was extensively studied (Westermark and Wasteson 1976, Andrae, Gallini et al. 2008). Human PDGF-AB was isolated from platelets by Carl-Henrik Heldin in 1979 (Heldin, Westermark et al. 1979). It includes a number of PDGF proteins,

PDGF-A, -B, -C, -D. These molecules function as dimers, thanks to the presence of PDGF/VEGF homology domain, which forms intra- and inter-molecule disulphite bridges. Five dimers were observed: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and –DD. (Fredriksson, Li et al. 2004). They act through two cell surface tyrosine kinase (RTK) receptors, named PDGFRα and PDGFRβ, which have a common domain structure including a split intracellular tyrosine kinase domain and five extracellular immunoglobulin (Ig)-like domains (Claesson-Welsh 1994). When a receptor binds its respective ligand a hetero- or homodimerization of receptors occurs, which leads to transphosphorylation of the intracellular domains and activation of the receptor. Intracellular mediators then dock to phosphotyrosine residues in the receptor, causing activation of downstream signalling pathways.

Presence of PDGF was confirmed in human clonal glioma cell lines (Nister, Heldin et al. 1982, Nister, Heldin et al. 1984). Experiments using U-343 MGa glioma cell line showed clonal variation in PDGF secretion and in capacity of glioma cells to bind 125I-PDGF (Nister, Heldin et al. 1986), which is a radioiodinated purified platelet-derived growth factor (Bowen-Pope and Ross 1982). Interestingly, the growth rate correlated with the amount of PDGF produced, with immature cells secreting highest level of this ligand and glia-like cells showing highest level of 125I-PDGF binding capacity.

Expression of PDGF and its receptors suggests the presence of autocrine and paracrine stimulatory loops in human gliomas (Hermanson, Funa et al. 1996, Lokker, Sullivan et al. 2002).Infusion of PDGF was sufficient to arrest production of neuroblasts and induce proliferation of B cells in the SVZ, which led to formation of hyperplasias with some characteristics of gliomas (Jackson, Garcia-Verdugo et al. 2006). PDGF and its respective

receptors are frequently co-expressed in gliomas suggesting an autocrine loop mechanism. A study using retrovirus coding for the PDGF B-chain showed its ability to induce brain tumours in mice. The resulting tumours coexpressed PDGF B-chain and PDGFRα mRNA, explained by the autocrine mechanism of transformation, and showed consistent expression of Nestin, suggesting that they were derived from immature neuroglial progenitors (Uhrbom, Hesselager et al. 1998). When treated with CGP 57148B, a specific PDGFR tyrosine kinase inhibitor, the autocrine receptor activation was blocked, almost completely inhibiting cell proliferation (Uhrbom, Hesselager et al. 2000).

Primary brain tumours can be induced by murine intracerebral injection with avian recombinant retroviruses, which contain different oncogenes, into mice which are genetically engineered to be susceptible to retroviral gene transfer. This approach targets specific cell types in the brain and allows for a creation of tumour models, which are histologically similar to human tumours (Uhrbom and Holland 2001). This system was used to investigate the effect of PDGF-B when combined with other proteins, including INK4a-ARF and p53. When either of the tumour suppressor genes was deleted together with retrovirally expressed PDGF-B, a decrease in PTEN levels and increased Akt phosphorylation were observed (Hesselager, Uhrbom et al. 2003).

1.6.4 Current treatment

First line adjuvant treatment for GBMs is still dominated by cytostatic agents and radiotherapy. New approaches using targeted intervention with inhibitors of neo-angiogenesis and growth factor receptors gain recognition. Temozolomide (TMZ), an alkylating agent, and a concurrent radiotherapy compose a first-line adjuvant therapy for GBM patients (Hunter, Smith et al. 2006, Stupp, Hegi et al. 2007). Addition of temozolomide to radiotherapy

improves survival of newly diagnosed GBM patients, causing minimal additional toxicity (Stupp, Mason et al. 2005, Chamberlain, Glantz et al. 2007, Dall'oglio, D'Amico et al. 2008).

First generation of TKIs did not bring expected results, as *in vitro* growth of glioma lines was not affected. Newer drugs seem to struggle in the clinical trial setting (Dresemann, Weller et al. 2010). Analysis of tumour explants identified two different types of glioma cultures, type A and type B. Type B cultures had mesenchymal features and responded to mono-treatment with imatinib, a PDGFR inhibitor. Type A showed stem cell-like features, self-renewal *in vitro,* formation of neurospheres and *in vivo* tumour growth and did not respond to mono-treatment. Resistance of type A cells was correlated with expression of SOX2 (Hagerstrand, He et al. 2011). Expression of SOX2, OCT4, KLF4 and NANOG, transcription factors able to induce pluripotent stem cells (iPS cells) (Takahashi and Yamanaka 2006), was shown in high-grade human glioma tissues (Holmberg, He et al. 2011). This could possibly explain resistance to TKIs and give a new direction in treatment, targeting stem cell-like compartment of the tumour, either by killing it, or leading to its differentiation and sensitising to other agents, which can kill more mature cells.

Infiltrative nature of GBM poses one of the biggest hurdles for the therapy prognosis. Tumour cells interact with the surrounding normal tissue leading to expression of CD95 ligand. Binding of CD95 ligand to CD95 on GBM cells recruits the Src family member Yes and the p85 subunit of phosphatidylinositol 3-kinase to CD95, which signal invasion via the glycogen synthase kinase 3-beta pathway and subsequent expression of matrix metalloproteinases. A blockade of CD95 activity reduces the number of invading GBM cells (Kleber, Sancho-Martinez et al. 2008). Studies involving CD95 inhibitor APG101, which is a glycosylated fusion protein consisting of the extracellular domain of human CD95 and the Fc

domain of human IgG1, showed a good safety and tolerability profile in a clinical trial with healthy volunteers. APG101 blocks the interaction between CD95 and its cognate ligand CD95L (Tuettenberg, Seiz et al. 2012).

1.6.5 Cancer stem cells and differentiation therapy

GBM tumours are composed of phenotypically heterogeneous populations of cells, which show variation in morphology, proliferation potential and ability to initiate tumour growth (Dick 2008). Heterogeneity of GBMs was recognised and is a subject of discussion (Shackleton, Quintana et al. 2009, Bonavia, Inda et al. 2011). Two models have been proposed to explain tumour heterogeneity. The hierarchical model predicts that tumours arise from stem cell-like precursor cells, which in the process of normal developmental/differentiation pathways generate non-malignant progeny. The stochastic model states that tumours arise as a biologically homogenous group of cells i.e. all cells are equally malignant. In the stochastic model, random fluctuations in gene expression or acquisition of genetic differences are expected to underlie tumour heterogeneity. Thus, any cell in this model has a potential to initiate tumour formation due to accumulation of DNA mutations (Dick 2009). It is quite likely that different models apply to different cancers, and even within the same patient at different stages of tumour progression, as for example melanoma has high levels of CSCs (Quintana, Shackleton et al. 2008).

The idea of cancer stem cells gained recognition when a study of human acute myeloid leukaemia identified a population of CD34-positive CD38-negative cells, which drive proliferation of the tumour (Lapidot, Sirard et al. 1994). Interestingly, they showed that CD34-positive/CD38-positive and CD34-negative cells do not possess this ability. CSCs in other tumours were then also identified. A limiting dilution assay identified a population of

CD44-positive/CD24-negative/lin-negative cells, which when engrafted in SCID mice and showed tumorigenic potential (Al-Hajj, Wicha et al. 2003). Similar studies were performed for colorectal cancer (Dalerba, Dylla et al. 2007), colon cancer (O'Brien, Pollett et al. 2007), pancreatic cancer (Li, Heidt et al. 2007), liver cancer (Yang, Ho et al. 2008), prostate cancer (Wang, Kruithof-de Julio et al. 2009), and other cancer types. Interestingly, a study in human pancreatic cancer showed potential to distinguish distinct CSCs populations responsible for tumour growth and metastatic activity (Hermann, Huber et al. 2007).

The cancer stem cell hypothesis states that only a small fraction of tumour cells has the potential to self-renew and initiate tumours when transplanted into immunocompromised mice. Cancer stem cells are thought to be responsible for maintenance and origin of solid malignancies, as they share properties of stem cells, the ability to self-renew and to differentiate into mature cell types (Sell 2004, Clarke and Fuller 2006, Vescovi, Galli et al. 2006). This idea was reinforced by a breast cancer study, where tumorigenic cells were identified based on their marker expression (Al-Hajj, Wicha et al. 2003) and in brain tumours (Galli, Binda et al. 2004, Huntly and Gilliland 2005) showing that stem cell characteristics of tumour cells can be induced by growth conditions (Ignatova, Kukekov et al. 2002).

The origin of cancer stem cells and their relation to normal tissue stem cells remains under investigation (Sanai, Alvarez-Buylla et al. 2005). Brain cancer stem cells were shown to express a cell surface marker CD133, which was initially associated with neural stem cells (Singh, Clarke et al. 2003, Singh, Hawkins et al. 2004). Glial progenitors in adult white matter with overexpressed PDGF form tumours, which closely resemble human GBMs suggesting their potential role in tumour development and maintenance. SVZ progenitors were infected with a retrovirus expressing both PDGF and GFP. At day three post injection

(dpi), proliferation of PDGFRα-positive progenitors was seen in the SVZ and the white matter around the injection site. By day ten, rats developed diffusely infilitrating tumours, which resembled GBMs. Both infected and uninfected PDGFRα-positive cells were proliferating, suggesting that PDGF acted via both autocrine and paracrine signalling (Assanah, Lochhead et al. 2006). *In vivo* transplantation assays demonstrated that full progressed tumours require PDGF-B to overcome cell-cell contact inhibition and to confer *in vivo* infilitrating potential on tumour cells (Calzolari, Appolloni et al. 2008).

CD133, also known as prominin, is considered a stem cell marker in both normal and cancer tissues. Cells positive for CD133 appear to have potential to self-renew, proliferate and differentiate (Singh, Clarke et al. 2003). *In vivo* studies investigating the tumour-initiating potential of CD133-positive cells showed mixed results with some stating that only these cells are capable of tumour initiation (Singh, Hawkins et al. 2004) with others showing similar potential in CD133-negative cells (Joo, Kim et al. 2008). CD133-positive cells show resistance to radiotherapy and chemotherapy (Bao, Wu et al. 2006, Liu, Yuan et al. 2006). When isolated, the CD133-positive and CD133-negative populations have differential molecular profiles and growth characteristics (Beier, Hau et al. 2007). Interestingly, CD133 expression is associated with neurosphere formation only in primary GBMs and not in secondary GBMs suggesting possible different cells of origin in these tumours (Beier, Hau et al. 2007). CD133-positive GBM cancer stem cells are radioresistant and preferentially expanded in hypoxic conditions. Possible use of radiosensitizers in combination with therapies targeting this characteristic of GMB cancer cells could lead to improvements in PFS (Sheehan, Shaffrey et al. 2010).

Glioma stem cells contribute to radioresistance of the tumour mass by an increase in DNA repair capacity and preferential activation of the DNA damage checkpoint response. The CD133-positive cells survive ionizing radiation better when compared to the tumour mass, both *in vitro* and in brains of immunocompromised mice. Interestingly, the radioresistance of these cells can be reversed by inhibition of Chk1 and Chk2 checkpoint kinases (Bao, Wu et al. 2006).

If GBMs are driven by cell with stem cell characteristics, then activation of specific differentiation pathways in cancer stem cells could lead to a loss of their stem cell properties, making tumours more sensitive to cytotoxic treatment or simply preventing their further growth. Bone morphogenetic proteins were shown to inhibit the tumorigenic potential of human brain tumour-initiating cells (Piccirillo, Reynolds et al. 2006). In particular, BMP-4 shows the strongest effect, reducing the number of tumour-initiating cells in human GBMs, through activation of Smad signalling cascade. BMPs are able to increase expression of neural differentiation, without affecting cell viability, which was presented as a decrease in CD133-positive population size and reduction in clonogenic ability (Piccirillo, Reynolds et al. 2006).

Brain CSCs, similarly to NSCs are located in a proximity of blood vessels, also in hypoxic areas (Seidel, Garvalov et al. 2010) and this may provide an important niche to sustain immature features (Gilbertson and Rich 2007). A neuronal cell adhesion molecule, called L1CAM, is expressed in gliomas. When CD133-positive L1CAM-positive cells were cosegregated and targeted with lentiviral-mediated short hairpin RNA (shRNA) the neurosphere formation was disrupted and proliferation reduced. L1CAM knockdown reduced

expression of Olig2 and increased levels of p21 (WAF1/CIP1) in CD133-positive glioma cells (Bao, Wu et al. 2008).

1.6.6 *In vitro* culture of GBM stem cells

A link between neural stem cells and their malignant counterparts (cancer stem cells) was hypothesised as a hierarchical process of differentiation was suggested for GBM and other solid tumours, where a subpopulation of stem cell-like or tumour initiating cells (TICs) are responsible for the maintenance of the tumour. Only by targeting TICs would it be possible to avoid disease recurrence following debulking of the tumour mass. Even if not necessarily arising from neural stem cells, the TICs are reminiscent of neural stem cells in their biology and their role in maintaining the tissue of a tumour. Forcing differentiation of brain cancer stem cells may therefore have therapeutic value. *In vitro* expansion of putative GBM cancer stem cells could be a valuable tool to understand processes behind their cellular behaviour and to create novel cancer treatments.

A combination of EGF and FGF-2 on an adherent substrate is important for propagation of both mouse and human NS cells without spontaneous differentiation or cell death (Conti, Pollard et al. 2005, Sun, Pollard et al. 2008). A similar approach was used to establish glioma stem cell lines (Pollard, Yoshikawa et al. 2009). Primary cultures presented diversity of cellular phenotypes, being a mixture of putative stem cells, progenitors and differentiated cells. The heterogeneity was, however, reduced after 2-3 passages. A number of cell lines from histopathologically distinct types of tumour were established, including GBM (G144, G166, and GliNS2), GC-GBM (G179) and an anaplastic oligoastrocytoma (G174). The established lines express markers of NS cell/neural progenitors, like Vimentin, Sox2, Nestin,

3CB2. However, some variation between the lines was observed with G144 and G179 lines being highly motile and showing dynamic changes in cell shape.

The adherent GNS lines offer a number of advantages like increased proliferative expansion of glioma stem cells when compared side-by-side to the neurosphere culture. EGF and FGF-2 fully suppress differentiation of these cells to oligodendrocytes (O4-positive cells), or neurons (Tuj-1-positive cells). In the absence of growth factors G144 and G179 GNS begin to differentiate, with G144 being characterised by a significant number of O4-positive or CNPase-positive oligodendrocyte-like cells within one week. On the other hand, G179 mostly generates Tuj-1-positive cells. Interestingly, G166 line continues to proliferate in the absence of growth factors without any apparent differentiation.

In the presence of BMP-4 GNS lines G144 and G179 change morphology within seven days of treatment. Cells express high levels of GFAP, with a small population of Doublecortin-positive neuronal-like cells, which is also observed in human foetal NS cells. G166 line has a lower frequency of GFAP expression showing that a capacity to differentiate, efficiency and lineage choice vary between the cell lines (Pollard, Yoshikawa et al. 2009).

Human GNS lines are suitable for a chemical screening application. They offer a number of advantages over the neurosphere drug screening. Most importantly, it is possible to quantify cell proliferation, which is more difficult in the suspension culture, because of variable cell death. Fusion of neurospheres can interfere with analyses based on the sphere number or their size. Also, a real-time monitoring of cellular activity is possible only in the adherent GNS culture. Pollard et al. used GNS lines to screen 450 compounds from the NIH Clinical collection using G144, G166 and G179 GNS lines and identified 38 compounds which had a significant cytotoxic or cytostatic effect on at least one line. Line-specific effects were

observed for 15 compounds, which was consistent with the individualized phenotypes of GNS cells (Pollard, Yoshikawa et al. 2009). Danovi et al. used GNS lines to screen a library of 160 small molecule kinase inhibitors and identified a compound called JNJ-10198409, which induced a mitotic arrest at prometaphase in GNS cells, but not in human NS cells (Danovi, Folarin et al. 2013). These studies validated adherent GNS culture as a tool for chemical screening application.

1.7 High-content chemical screening

1.7.1 Background

Two terms, high content screening (HCS) and high throughput screening (HTS) are frequently confused. While in HCS a rich phenotypic information is extracted from either fixed or live cells, the HTS allows for assessment of hundreds of thousands of compounds using a single biochemical assay. HCS includes any method used to analyse cells with a simultaneous readout of multiple phenotypic parameters (Gasparri 2009). Widely used in drug discovery, but also in biological research, HCS allows for the observation of morphological changes and variations in the production of phenotypic markers, which could be measured with the use of immunostaining or, if transgenic reporters are used, a direct measure of fluorescence reporters e.g. green fluorescent protein (GFP) in live cells.

There are two main goals, which every HCS method aims to achieve. First, acquisition of spatially or temporally resolved information on an event and second, its automatic segmentation and quantification. In order to achieve these goals HCS incorporates automated microscopes (spatially resolved instruments) and fluorescent measurements (temporal resolution). Use of automated systems and software allows for rapid and unbiased analysis of

data. The image analysis software can be supplied with instruments needed for the experiments, however it is not uncommon to standardise multiple experiments and instruments to use a single platform.

Considering the large scale of the analysis it is common to identify multiple potentially interesting molecules based on their effect on cell phenotype, which could be described as desired e.g. increase in protein expression, undesired e.g. cytotoxicity or unexpected e.g. change in morphology or complete inhibition of protein expression.

Increasingly the use of liquid handling devices combined with automated image acquisition and processing are crucial for the efficient data collection and analysis and can now be carried out in individual academic laboratories. Technological advances that led to the development of high-throughput microscopy spurred the concept of HCS as a tool bridging depth and throughput of biological experiments. Hardware improvements, like auto-focusing and sample positioning and development of image analysis software, position HCS as one of the new crucial methods in biological research and a mainstream technology in pharmaceutical industry.

1.7.2 Chemical versus genetic screening

HCS is an alternative to a genetic approach to analyse biological systems. The genetic approach entails determining the phenotypic consequences of mutations in genes and ordering genes into functional pathways (Stockwell, Haggarty et al. 1999). This approach is widely used in a number of study organisms including fruit flies, nematodes, yeast, plants, zebrafish and mice (Nadeau and Dunn 1998). With whole organism models like mice there are, however, some limitations, which can include the cost of space, animals, their long

generation time, small litter size and the difficulty inherent in identifying and mapping recessive mutations. In addition, many gene products can be essential, redundant or expressed in a tissue-specific or a temporal manner. Some of these problems can be minimised by the use of tissue culture, which can still provide a useful tool for a study for understanding of physiological and developmental pathways in a mammalian system (Xiong, Battaglino et al. 1998).

Use of libraries of small molecules, which can alter protein function directly, has a potential to overcome limitations of genetic analysis. Small molecule approach in cell-based assays can lead to a specific functional alteration in all copies of gene product, making it analogous to an inducible dominant or homozygous recessive mutation. Another similarity lies in possibility to identify target sequences. As mutation sites can reveal functionally relevant gene coding sequences, it is possible to identify functionally relevant protein residues based on small molecule mechanism of interaction (Fenteany, Standaert et al. 1995).

One of the most important differences between small molecules and mutations as a source of phenotypic variation is the fact that small molecule-induced changes, unlike genetic mutations, are usually not hereditary. The perturbations from small molecules are then usually reversible and conditional, as small molecules can be added or removed from the experiment at will.

1.7.3 Target identification

The goal of the small molecule screening is the identification of a compound which would elicit its effects at low nM concentrations, meaning high specificity and affinity. Unfortunately, this is rarely the case with low uM concentrations considered to be a good

outcome. In order to improve potency, chemical analysis is employed leading to modifications in compound structure. However, this process can be expensive and requires time, making it harder to rival natural products which are readily available. Identification of target pathways or influenced molecules is a rate-limiting step in HCS. In the past targets of small molecules were identified by affinity methods or candidate-based approaches. Thus, identification of a 'hit' or 'lead' compound is only the first step in drug development pipelines.

There are multiple new methods to identify small molecule molecular targets. One of the methods involves preparation of radiolabeled derivatives of the small molecule and determining the molecular targets that are labelled by these radioactive probes (Kwon, Owa et al. 1998). Another approach is to use a 'three-hybrid' transcriptional activation system. It anchors a derivative of the active ligand for display against a library of cDNAs, which are fused to a transcription activation domain (Licitra and Liu 1996, Borchardt, Liberles et al. 1997) Also, expression cloning can be used to find target within a small pool of proteins (King, Lustig et al. 1997). In affinity methods small molecules are tethered to a solid support with cell extracts being passed over them, looking for a specific small molecule-protein binding.

One way to circumvent the difficulties of defining targets of novel small molecules is to work with panel of compounds with known activities and targets/pathways. Many libraries of known inhibitors/agonist compounds are commercially available, with some laboratories creating their own diverse libraries with compound annotations and effect measured scoring system to identify commonly known and new potentially interesting molecules (Root, Flaherty et al. 2003).

A combined approach of comparing small molecule and RNAi phenotypes can also provide valuable information (Eggert and Mitchison 2006). This method is based on the assumption that if RNAi and small molecule treatments give a similar phenotype it is likely that a protein target is the same. In a study of proteins involved in cytokinesis, a genome-wide RNAi and small molecule screens were performed in parallel allowing for identification of a small molecule inhibitor of the Aurora B kinase pathway (Eggert, Kiger et al. 2004). It is important to mention that this approach also has some limitations as the phenotype of small molecule inhibition might be different from an RNAi phenotype, where protein is depleted from the cell (Eggert, Field et al. 2006). Two additional approaches to identification of small molecules which are biologically active include design of small molecules to interact with active sites of proteins, which structures are understood (Blundell 1996), and screening methods developed to identify novel protein-small molecule interactions (Borchardt, Liberles et al. 1997, Huang and Schreiber 1997).

1.7.4 HCS applications

HCS has applications in a wide range of study fields. Kinetic HCS studies usually measure calcium fluxes, cytotoxicity, and receptor internalization, while studies of cellular processes like proliferation, differentiation or motility are predominantly performed as fixed endpoint assays. This however, is changing with the development of new tools allowing cell tracking and 'learning' software capable of identifying different cell types and states, like progenitors or differentiated cells. Turning standard biological methods into HCS can be also seen. The wound-healing assay measuring epithelial cell motility was scaled up to 384-well format to screen for cell migration defects (Yarrow, Perlman et al. 2004, Yarrow, Totsukawa et al. 2005). In this case HCS allowed for a simultaneous measurement of rate changes during

closure of an individual wound, motility and relative morphology of cells and trailing cell sheet.

The applications of HCS include drug discovery where tens of thousands of molecules are analysed in search of clinically relevant drugs. Field of chemical genetics uses HCS to study genome and gene function by identifying small molecules that regulate gene activity. This could be very beneficial, leading to targeted chemical knockout of proteins, in case knockout mice cannot be made because of protein involvement in development and growth, causing lethality in knockout embryos. Studies using chemical knockout could further our understanding of gene functionality in development and adult organisms.

HCS is used in a variety of study models, including primary cells and whole organisms e.g. zebrafish (Zon and Peterson 2005) and *C. elegans* nematodes (Fraser, Kamath et al. 2000). Use of whole organisms allows for identification of functional data, which can show direct promise in curing some of the diseases e.g. preventing cardiovascular defects caused by a genetic mutation as it was shown in the zebrafish model (Peterson, Shaw et al. 2004). A genome-wide deletion mutant screen in yeast identified nine missing components of the phosphate-responsive signal transduction PHO pathway (Huang and O'Shea 2005). Another example is a large-scale automated screening of a mammalian U2OS osteosarcoma cell line, where individual overexpression of 7364 microarrayed cDNAs led to identification of genes involved in cell proliferation under a variety of growth conditions (Harada, Bower et al. 2005).

HCS applications can be seen as both fixed endpoint assays and time-lapse assays, with a fixed point approach being potentially a high throughput screening method. Depending on the goal of the experiment, it is possible to prepare a whole time-course by preparing multiple

plates processed over time or define half time of cellular processes of interest in the beginning of an experiment and then set the fixation time accordingly (Giuliano, Haskins et al. 2003). Incorporation of an environmental chamber into the HCS reader system allows for live HCS applications. This can be accomplished with the use of specific devices, e.g. a live cell imaging system called Incucyte, or by applying add-ons to an endpoint HCS device (Giuliano, Haskins et al. 2003, Taylor and Giuliano 2005). In this case, full kinetic measurements can be made, before, during and after the treatment, providing a full range of biological information (Abraham, Taylor et al. 2004).

HCS contrasts with traditional cell-based methods like plate-based reporter assays, where a readout observed is an average of thousands of cells, as HCS acquires morphometric and functional information from collections of individual cells. This allows for a more in-depth analysis and observations of variation within cell populations in situations when only a subpopulation of cells is affected. This could be due to heterogeneity of the cell population, or due to e.g. variable transfection efficiency.

The most common methods include labelling proteins with fluorescent tags, e.g. GFP fused to endogenous protein or the use of fluorescent antibody, followed by automated image analysis allowing to understand the effect of a substance of interest on a given protein level. It is possible to tag multiple proteins at once thanks to their different absorption and emission maxima and measure different cell characteristics at once. Another less common method involves use of small molecule fluorescent probes.

1.7.5 Technical aspects of HCS

A range of parameters need to be optimised in order for successful HCS to be carried out. A typical mammalian cell, which is attached and spread on a microplate bottom can have a size of 20-50 um across and a nucleus of about 5-10 um in diameter. One hundred of these cells, as a confluent layer, occupy an area of about 400 um on a side. This however, depends on the cell type, plating density, and the amount of spreading. HCS platform design has to allow for acquisition of images with sufficient contrast, resolution, and signal-to-noise ratio, which will enable image algorithms to extract features of interest. Parameters like maximum cell density have to be considered, e.g. a cell-spreading assay requires subconfluent cells, whereas high confluency is better for receptor activation assays. In order to achieve an adequate level of statistical significance it might be necessary to analyse 100 or up to 1000 or more cells, which occupy an area as large as several square millimetres. For most applications an HCS system with a field of view of several square millimetres and a resolution around 1 um is considered acceptable (Gough and Johnston 2007).

A new emerging field of HCS using time-lapse movies of cells expressing GFP-tagged proteins faces its own challenges. Currently, a storage and analysis of movies is the limiting step for this approach. A limited number of studies were performed, where a systematic annotation of experimental videos was used to develop a phenotypic profiling system, which showed a high correlation with cellular processes and biochemical pathways enabling for prediction of new functions of previously uncharacterised genes (Sonnichsen, Koski et al. 2005).

One of the most essential aspects of a HCS system is a reliable autofocus system. A slightly soft focus even 1% of the time can affect screening results. For the HCS application a

reliable autofocus has to deal with a number of challenges. It has to be fast, as even 1s/well adds more than 6 minutes to the scan time on a 384-well plate, and offer precise focus within a few microns, depending on the objective used. Another challenge is microplates, which bottoms are universally bowed, typically by more than a hundred microns corner to centre. This can affect the z-position of the well, leaving a variation from one well to another as big as 50um or more. Microplates, their thickness and surface quality have to be also considered, with glass-bottom plates providing a much smoother and uniformly thick substrate. However, their bottom is still bowed and the plates are still considered too expensive for a routine used in most laboratories.

Software is an important component of any HCS application. The automated imaging platform software must offer a possibility of visual data mining (Berlage 2005). A level of confidence has to be established in the beginning of every run to validate that all systems are functioning properly. The software must be able to monitor and display the progress of the run in real time, e.g. checking progress on a plate level, plate views with some defined representation in a form of confluency or a heat map and image views of fields already captured. The software has to be interactive enough to allow user to assess the quality of the experiment and programmed analysis.

Every HCS imaging system has to meet a minimum of requirements like sufficient resolution and sensitivity to capture and analyze the cellular features of interest, spectral channels to distinguish multiple fluorescent labels, a field of view large enough to image multiple cells, adequate speed to meet the needs of the planned screening volume and flexibility to address a wide range of assay requirements. However, the optimum design for a particular assay

requires an understanding of the experimental goals and the relationships between these requirements (Gough and Johnston 2007).

One of the biggest challenges of image-based screenings is the analysis of data. A typical screening will generate gigabytes or even terabytes of data, which have to be extracted, saved, analysed and understood. Cell profiler and Cell profiler analyst, software produced by the Broad Institute, is open-source software commonly used for high content screening applications (Carpenter, Jones et al. 2006, Jones, Kang et al. 2008, Logan and Carpenter 2010). It enables to quantitatively measure phenotypes from thousands of images automatically. A simplified interface presents advanced algorithms in a form of individual modules which, when placed in a sequential order, form a pipeline. The software can measure multiple characteristics including: cell number, percentage of dye- or fluorescent tag-positive cells, cell size, texture and fluorescence intensity. The alternative to Cell profiler is java-based software called ImageJ, which can handle small scale processing and analysis. This tool is suitable for novice users allowing for basic segmentation and cell quantification, but it does not yet provide scalability necessary for HCS.

Currently, relational databases are used to facilitate image analysis, although storing only meta-information about images in a database with actual images being stored as ordinary files is considered to be an efficient method. Open Microscopy Initiative (OME) and open-source academic consortium provide databases for image handling.

Quantitative phenotypic information has to be extracted from the images, which typically requires interaction of screeners with experts in the field of image analysis. Typical analysis starts by identification of individual cells and regions of interest, e.g. nuclei or organelles in case subcellular information is needed in order to understand the biological function.

Subsequently, other parameters like fluorescence intensity, shape, size and texture are measured. It is possible to take over ten measurements for a single object (Perlman, Slack et al. 2004).

1.8 Goal of the project

The specific aim of this project was to identify small molecules that can direct or stimulate differentiation of neural stem cells. The long-term goal is to make use of this knowledge and reagents to force differentiation of human GBM stem cells with a view to develop new types of differentiation therapy. Oligodendrocyte differentiation is often observed in primary GBM and is therefore an attractive lineage to focus on.

A transgenic PDGFRα 'knock-in' reporter strain of mice (B6.129S4-*Pdgfra*$^{tm11(EGFP)Sor}$/J) was obtained courtesy of Dr Philippe Soriano (Hamilton, Klinghoffer et al. 2003). These mice express the H2B-eGFP fusion gene from the endogenous PDGFRα locus (Figure 6). Homozygotes for this knock-in targeted mutation have an embryonic lethal phenotype and fail to survive beyond embryonic day 15.5, exhibiting abnormal placenta development and vasculature. In this project I made use of these reporter mice to establish a novel panel of mouse NS cell reporter lines, use these in HCS and identify some of the key molecular events controlling the process of NS cell lineage choice and differentiation, and associated small molecule inhibitors that modulate NS cell differentiation.

The main objective of this work was the identification of cell permeable small molecules that can direct the differentiation of NS cells. There are four major goals:

1) Establish and validate PDGFRα-GFP reporter NS cell lines.

2) Use these reporter cells lines in chemical screens (HCS).
3) Identification and validation of small molecules that influence NS cell differentiation.
4) Assessment of whether these agents influence GBM-derived NS cells self-renewal and differentiation.

It is anticipated that the use of *in vitro* screening platform can provide insights into molecular mechanisms underlying NS cell differentiation and broaden our understanding of the differentiation process. Longer term we hope our findings will impact clinical treatment of GBM through identification of new therapeutic agents and pathways. Furthermore, a direct and efficient differentiation protocol could allow for improved control of NS cell differentiation in the laboratory for applications in new types of regenerative medicine.

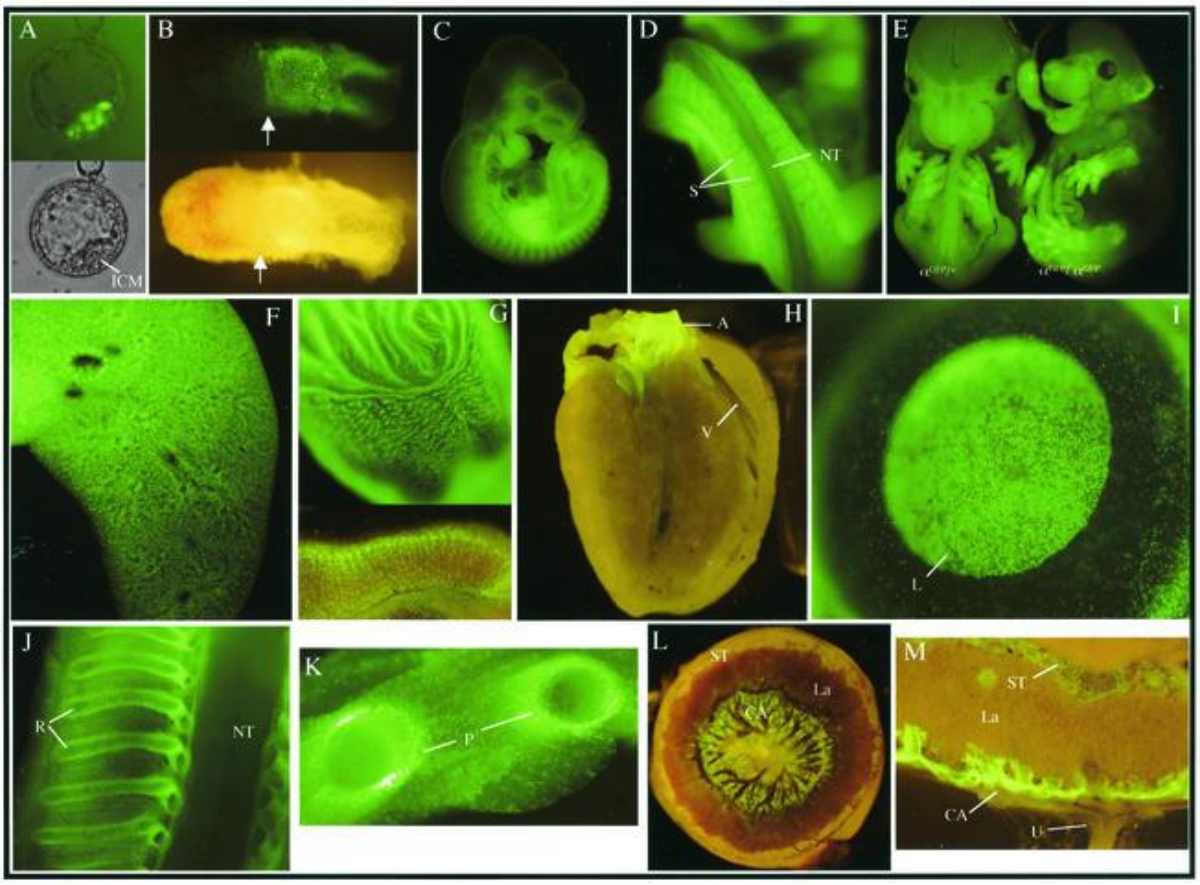


Figure 6. Expression patterns of PDGFRα (α^{GFP}) in embryonic and adult tissues. Adapted from (Hamilton, Klinghoffer et al. 2003); (A) Expression in polar trophectoderm of E4.5 blastocysts. Bottom panel, visible light; top panel, UV. (B) Expression in extraembryonic ectoderm of an E6.5 embryo. Top panel, UV; bottom panel, visible light. Arrows denote the boundary of embryonic and extraembryonic tissues, and Reichert's membrane was not removed. (C) Whole-mount E10.5 embryo $\alpha^{GFP/+}$. (D) Magnification of the caudal somite. (E) E13.5 littermates. Left embryo, $\alpha^{GFP/+}$; right embryo, $\alpha^{GFP}/\alpha^{GFP}$. Expression in whole-mount E18.5 organs, lung (F), stomach (G, top portion), and transverse section of adult stomach in the glandular region (G, lower portion). (H) Sagittal section of adult heart. (I) Whole-mount adult eye. (J) Sagittal section of ribs and spinal cord of an E16.5 $\alpha^{GFP/+}$embryo. (K) E18.5 $\alpha^{GFP/+}$ transverse section through ribs. Whole-mount (L) and transverse section (M) of an E14.5 $\alpha^{GFP/+}$ placenta visualized under fluorescence. ICM, inner cell mass; NT, neural tube; S, somites; V, ventricle; A, aorta; L, lens; R, ribs; P, perichondrium; La, labyrinth; ST, spongiotrophoblast layer; CA, chorioallantoic layer; U, umbilical cord.

Chapter 2 Methodology

2.1 Culture of NS cells and differentiation protocols

2.1.1 Mouse NS cell lines

Mouse primary NS cell cultures were previously established from E17.5 forebrain from an established transgenic B6.129S4-Pdgfra$^{tm11(EGFP)Sor}$/J mouse strain (Hamilton, Klinghoffer et al. 2003) and were termed PG1 (H2B:GFP expressed from PDGFRα locus). I established twelve different clonal cell lines from these initial cultures (termed, PG1-1 through 12). Previously characterised wild-type NS cells, Cor3-4, were used as a control (Pollard, Conti et al. 2006).

2.1.2 Proliferation

Cells were cultured in homemade NS cell 'complete media' consisting of DMEM/F-12 supplemented with glucose, NEAA, Pen/Strep, HEPES, 75 mg/ml BSA solution, 2-mercapthoethanol, B27 and N2 supplements. A sterility check was performed using Tryptose solution 1:1. Cells were expanded by addition of 10 ng/ml EGF and 10 ng/ml FGF-2. Adhesion of cells to the plastic surface of flasks was promoted by the addition of Laminin (1 μg/ml) (Sigma) to the culture media. Cells were typically grown to ~80-90% confluence, dissociated using Accutase (PAA) and split 1:3 or 1:5 during passaging. I routinely used cultures <25 passage to avoid risks of accumulation of karyotypic abnormalities.

2.1.3 NS cell differentiation

Mouse foetal NS cells were cultured in homemade NS cell media (see above) with 10 ng/ml FGF-2, 10 ng/ml PDGF-AA and laminin (EGF withdrawal). This triggers the process of oligodendroglial differentiation. Cells were incubated for 3-4 days in this condition at which point cells undergo morphological and molecular changes associated with differentiation. To promote full differentiation the culture media was replaced with NS cell media without any growth factors for the following 3-4 days (FGF-2 and PDGF withdrawal). To promote astrocytic differentiation mouse foetal NS cells were cultured in homemade NS cell media (see above) supplemented with 10 ng/ml BMP-4, leading to a consistent formation of GFAP-positive cell population with characteristic spread morphology.

2.2 Quantitative RT-PCR

Qiagen RNeasy kit (Qiagen) was used for total mRNA extraction and cDNA was synthesised using Superscript III according to manufacturer's instructions (Invitrogen). A LightCycler 480 (Roche) was used for the quantitative RT-PCR. Probe-based PCRs were performed using the Universal Probe library (Roche). Normalisation of samples was carried out using 18S ribosomal RNA as a loading control. Experiments were performed in technical duplicates or triplicates depending on sample availability. Fold change calculations were made relative to undifferentiated cells. The Universal Probe Library Assay Design Center (Roche) was used for primer design.

Table 1. Primer pairs for RT-PCR

Gene	Forward primer	Reverse primer	UPL Probe Number
H2B:GFP	Gaagcgcgatcacatggt	ccatgccgagagtgatcc	67
PDGFRα (#1)	Gaaggcaggcacatttacatc	ccagtttgatggatgggagt	25
PDGFRα (#2)	Gtcgttgacctgcagtgga	ccagcatggtgatacctttgt	80
OLIG2	Agaccgagccaacaccag	aagctctcgaatgatccttcttt	21
SOX10	Atgtcagatgggaacccaga	gtctttggggtggttggag	21
FOXG1	Gaaggcctccacagaacg	ggcaaggcatgtagcaaaag	4
NG2	Cagaagggaccagctagagg	gggcttctcaacgagaacat	16
NKX2.2	Gcagcgacaacccctaca	atttggagctcgagtcttgg	20
GFAP	Tcgagatcgccacctacag	gtctgtacaggaatggtgatgc	67
TUBB3	Gcgcatcagcgtatactacaa	ttccaagtccaccagaatgg	104

2.3 Western blotting

Lysis buffer comprised NaCl (150 mM), NP-40 (0.25%), Tris-HCl (20 mM), EDTA (2 mM) and NaF (1mM). Protease inhibitors were added to the lysis buffer: AEB5F (1:200), Leupeptin (1:1000), DTT (1:1000) and benzamidine (1:1000). A 12% gel was used for the protein separation and the blotting was performed using the following antibodies: rabbit monoclonal PDGFRα antibody (1:500, Cell Signaling Technologies), mouse monoclonal GFP antibody (1:500, Sigma) and rat hybridoma anti-tubulin antibody (1:1000) as a loading control. ECL system (Amersham) was used for protein detection.

2.4 Flow cytometry analysis of PDGFRα activation

Proliferating PG1 cells were used as a negative control for GFP expression and for setting gates. Differentiating mouse wild-type COR3-4 cells were used as a negative control to set the gates as there are shifts due to differences in cell shape/size and autofluorescence during the differentiation process. Cells were incubated in differentiating conditions for the period of 4 days, then dissociated with accutase and harvested. The pellet was resuspended in 'wash media' (DMEM/F12) at $2x10^6$ cells/ml. 5 μg/ml Propidium Iodine (PI, Sigma-Aldrich) was added to cells ten minutes prior to the analysis. Cells were analysed on Cyan ADP Analyzer (Beckman Coulter) or sorted on MoFlo XDP Cell Sorter (Beckman Coulter). The flow cytometry data was analysed using Kaluza Analysis Software (Beckman Coulter). $1x10^4$ cells per condition were analysed.

2.5 Immunocytochemistry

Cells were washed twice with PBS and fixed in 4% Paraformaldehyde for 10 minutes at room temperature. Fixed cells were washed with PBST (PBS +0.1% Triton) followed by 10-30 minutes incubation in a blocking solution (0.1% BSA + 3% goat serum). Primary antibodies diluted in the blocking solution were added to cells and incubated overnight at 4^{o} C. Wash steps for 3 x 5 minutes followed by 2 x 15 minutes incubations with PBST were used to remove unbound antibody. Secondary goat anti-mouse or rabbit antibodies conjugated to Alexa flours (488, 594, or 647) (Molecular probes, 1:1000) were diluted in blocking solution and incubated with cells for 1 hour in the dark at room temperature. The removal of secondary antibodies was performed exactly as for primary antibodies followed by 5 minutes incubation of cells with DAPI for nuclear counterstaining (Sigma, 1:2500, diluted in PBST). Observation and image collection was performed under the inverted microscope Zeiss Axio observer Z1 (Zeiss). Images were acquired using AxioVision 4.8.2.0 software.

2.6 Karyotyping

Cells were washed twice with PBS, dissociated using Accutase (PAA) and the cell pellet was mixed with 5 ml of 0.56% (0.075M) KCl solution for 20 minutes at room temperature causing cells to swell. This incubation was followed by addition of 0.5 ml of MeOH/Acetic Acid (3:1) (FIX solution) in which cells were spun for 5 minutes at 1100 rpm. The pellet was then resuspended in 1 ml of FIX and kept on ice for 15 minutes. Metaphase spreads were performed by dropping 250 ul of cells on a glass slide from a height of around 30 cm. The slides were then rapidly dried and incubated with DAPI (diluted in PBS, 1:2500, Sigma). After 10 minutes incubation slides were mounted with hardmount (Vector labs) and

coverslips. Observation was performed using Zeiss Z1 microscope at 40X magnification. The modal chromosomal number was determined by counting ~40 cells in metaphase per cell line.

2.7 High Content Chemical Screening

Cells were plated in 96-well plates (Iwaki) using CyBi- SELMA (CyBio) semi-automatic 96-fold liquid handling station at $6x10^3$ cells/well in differentiating conditions (as outlined above). The same device was used for the addition of StemSelect™ and InhibitorSelect™ libraries to the plates. For the screening a 1 in 10,000 dilution (for StemSelect) or 100 nM dilution (InhibitorSelect) of the original library stock was used. Cells were incubated at 37°C in INCUCYTE™ Live-Cell Imaging System for the period of 3 days with images acquired every 3 hours. The screening experiment was repeated 4 times over a period of 6 month. Data was processed and analysed using CellProfiler (http://www.cellprofiler.org/) and R 2.11 software (http://www.r-project.org/). Presented heat maps show result of a combined analysis of the 4 biological replicates.

2.8 Transfection

Cells were transfected with the pcDNF5 x-EGFPstop plasmid (NFkB GFP reporter), or dsRed plasmid (for mouse brain explants studies) using Lipofectamine LTX Reagent (Invitrogen) or Neon® Transfection System (Life Technologies) according to manufacturer's instructions. Cells were 70-90% confluent on the day of transfection. In case of Neon transfection, $2.5x10^6$ cells were resuspended in 100 ul Buffer R and gently mixed with 2ug DNA. For optimal transfection efficiency Neon device settings were adjusted to a Single

Pulse with a Voltage of 1600 and Width of 20. Stable transformants were selected using flow cytometry based on their expression of GFP reporter.

2.9 Statistical Analysis

Data are presented as mean ±SD. The statistical analysis was performed using Student T-Test Calculator available at http://www.socscistatistics.com/tests/studentttest/Default2.aspx . A p-value equal to or less than 0.05 was considered as statistically significant. Statistical significance was evaluated by double-sided t-test.

Chapter 3 Establishment and characterisation of a PDGFRα H2B:GFP reporter NS cell line

3.1 Introduction

Identification of cells with self-renewing and multipotent differentiation potential was first demonstrated using *in vitro* assays from the embryonic mouse brain (Temple 1989). Stem cells in the adult mammalian brain reside in two distinct anatomical sites: the subventricular zone (SVZ) along the lateral wall of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (Conover and Notti 2008).

Cell culture of cells derived from mouse developing or adult SVZ shows that FGF-2 and/or EGF enables the continuous *in vitro* expansion of cells that retain neural stem cell characteristics (Reynolds and Weiss 1992, Vescovi, Reynolds et al. 1993). Neural stem cells *in vitro* give rise to all three neural lineages: oligodendrocytes, astrocytes and neurons (Reynolds and Weiss 1992). Such *in vitro* models provide a useful cellular model system to explore neural stem cell self-renewal and differentiation.

Neural stem cells can also be expanded continuously as adherent cell lines that display self-renewal and multi-lineage differentiation ability (Conti, Pollard et al. 2005, Pollard, Conti et al. 2006, Pollard 2013). In this thesis I use the term 'NS cell' to refer specifically to these adherent *in vitro* cell lines. A particular advantage of using adherent monolayer is the suppression of differentiation and apoptosis, enabling propagation of more pure populations of stem cells, and facilitating cellular phenotyping. This allowed assigning radial glia identity to the neurosphere-forming stem cell population with RC2, BLBP and GLAST markers expressed uniformly. Adherent culture is also well suited for chemical screening as individual

cells can be monitored and tracked (Pollard, Clarke et al. 2009). These defined culture conditions and homogenous populations of NS cells provide a useful cellular model system to explore those pathways that control self-renewal and differentiation.

PDGFRα is one of the earliest markers of the transition from radial glia/neural progenitor to OPCs (Pringle and Richardson 1993). Expression of PDGFRα is necessary for this lineage progression (Hall, Giese et al. 1996). PDGFRα is a cell surface tyrosine kinase receptor, which interacts with the members of a platelet-derived growth factor family, and is implicated in diverse cellular processes including cell proliferation, differentiation and development. OPCs express PDGFRα, and expression is subsequently extinguished as cells transit to become mature oligodendrocytes (Ellison and de Vellis 1994).

My first goal was to establish a useful live cell reporter to monitor the transition of NS cells to OPCs. This could be extremely useful for differentiation studies, with the ability to track OPC lineage specification through reporter activation in case of oligodendrocyte differentiation or inhibition indicating some alternative fate.

3.2 Results

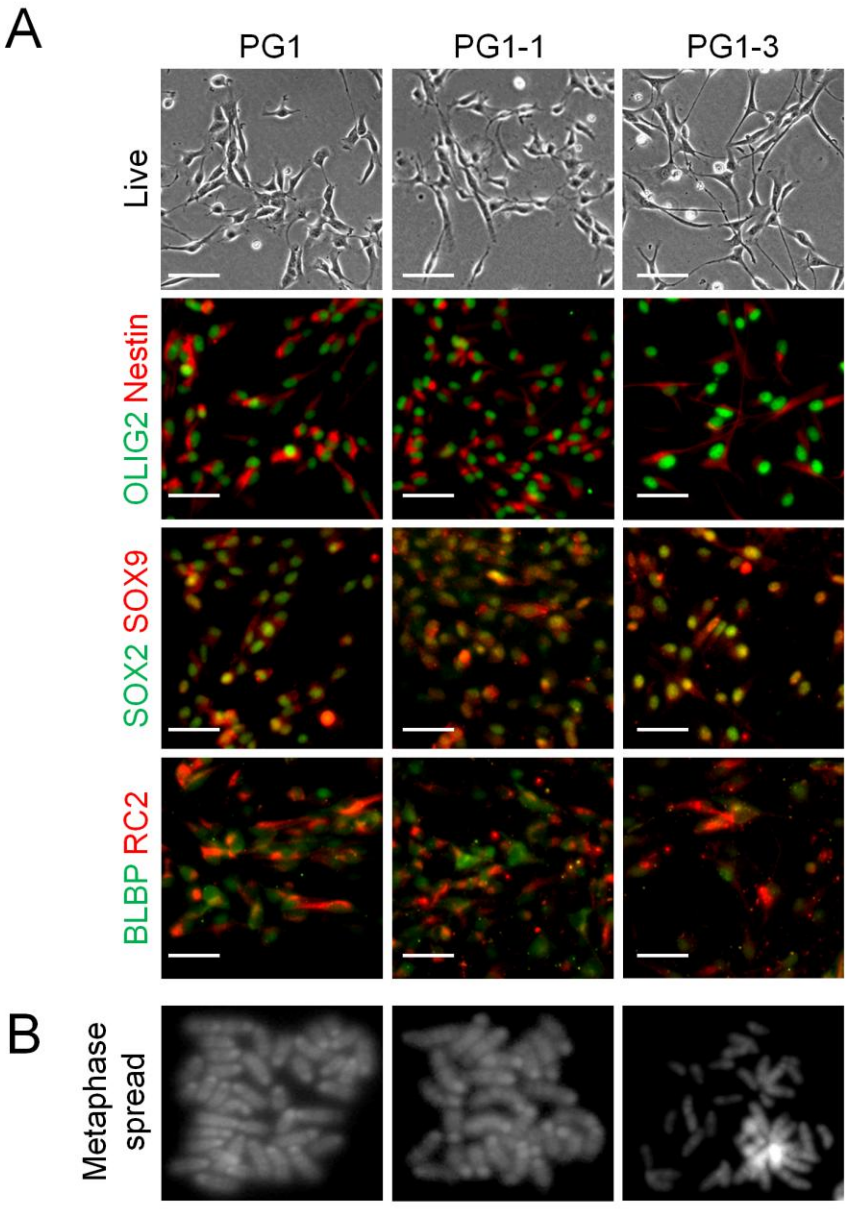
3.2.1 Derivation of PG1 clonal cell lines

The PDGFRα GFP reporter mouse strain B6.129S4-*Pdgfra*$^{tm11(EGFP)Sor}$/J was used and E17.5 forebrain was obtained and used to establish a primary NS cell culture (S. Pollard, unpublished). I expanded these primary cultures (termed PG1 for PDGFRα-GFP) further before plating cells at clonal density to generate NS cell colonies (typically 5000 cell plated and left for 10-14 days for colonies to emerge). Colonies were picked into 24 well plates and

expanded further. 12 independent clonal NS cell lines were established (termed, PG1-1 to PG1-12). Three clonal lines (PG1-1, PG1-3, and PG1-5) were selected based on optimal adhesion to the substrate and were expanded further and characterised. Each cell line expressed the NS cell markers: Nestin, RC2, BLBP, OLIG2, SOX2, SOX9 with morphology of neural stem cells (Figure 7A). However, following metaphase spreads to determine the karyotype we eliminated PG1-5 from further analysis as it was aneuploid (Figure 7B).

Differentiation potential of PG1 cells was also analysed. When treated with BMP-4 or 5% Foetal Calf Serum (FCS), PG1 cells expressed higher levels of GFAP, a marker of astrocytic lineage, consistent with previously reported NS cells. Activation of GFAP was also observed in PDGF and FGF-2 treated cells, but at a much lower frequency. Levels of immature neural progenitor markers Nestin and NG2 were downregulated using either of the differentiation protocols. SOX2, which was expressed by proliferating cells, appeared to be expressed by cells treated with PDGF and FGF-2, but not BMP-4 suggesting the BMP treated cultures were driven towards a differentiated state (Figure 8A).

It has been reported that PDGFRα expression marks quiescent astrocyte stem cells in the forebrain. We tested PG1 cells with a combination of BMP-4 and other growth factors, including EGF, FGF-2, and PDGF led to identification of BMP-4 and FGF-2 to determine whether GFAP and GFP were ever co-expressed. We find that less than 0.15% cells were expressing these two molecules, which was confirmed in a immunofluorescent analysis of over 2,000 cells (Figure 8B and C). Therefore, the majority of GFAP cells induced in PG1 cultures with BMP are likely post-mitotic astrocytes.



Cell line	Cell count	Chromosomal number
PG1	38	40
PG1-1	37	40
PG1-3	40	40
PG1-5	60	38

Figure 7. PG1 cell lines show characteristics of NS cells. (A) Live phase-contrast images and immunostaining reveal neural stem cell morphology and expression of neural stem cell markers in NS cell lines; both PG1 and two clonal line PG1-1 and -3 expressed OLIG2, Nestin, SOX2, SOX9, BLBP, and RC2; scale bars 50µm; (B) Example images of metaphase spreads from PG1 and two clonal lines and a table summarising karyotypes of cell lines tested. One clonal cell line (PG1-5) showed abnormal chromosomal number and was removed from further analysis.

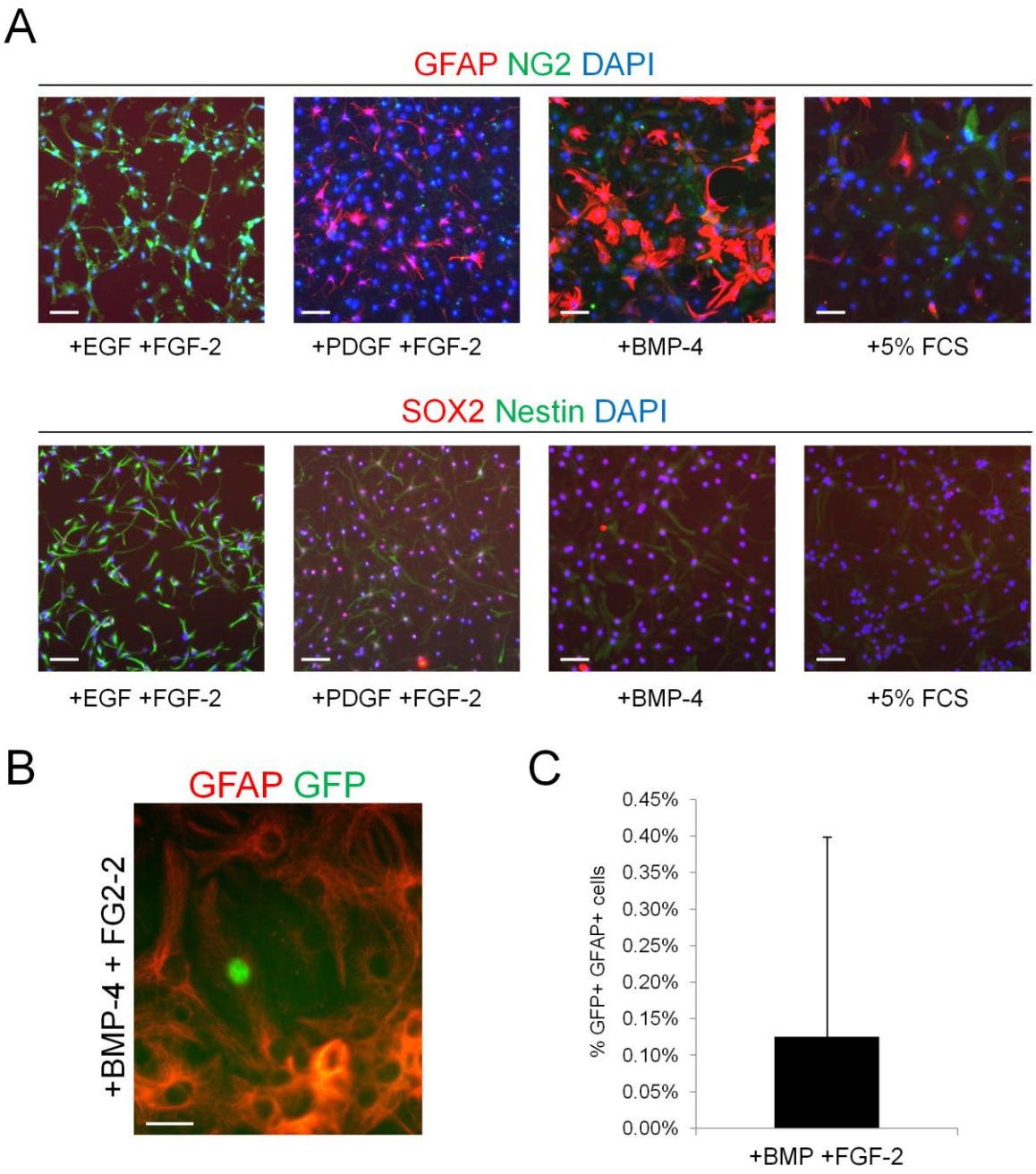


Figure 8. NS cells *in vitro* can self-renew and differentiate into neural cell types. (A) Cells treated with a combination of growth factors: EGF and FGF-2 (proliferation conditions), PDGF and FGF-2 (oligodendroglial differentiation conditions), BMP-4 and 5% FCS (astrocytic differentiation conditions); Proliferating cells express higher levels of NS cell markers: NG2, SOX2 and Nestin, while BMP-4 treatment leads to higher expression of GFAP, which is a marker of astrocytes; Scale bars, 100µm; (B, C) Less than 0.5% of cells treated with BMP-4 and FGF-2 are double-positive for GFAP and GFP (PDGFRα) potentially marking type B cells *in vitro*; Scale bar, 25 µm;

3.2.2 The GFP reporter is activated in PG1 cells in OPCs

The kinetics of activation of the GFP reporter was next analysed each day over the course of a week using PG1-1 cultures (Figure 9A). Using flow cytometry I found that GFP-positive cells are present at <1% in the proliferating cultures. Following plating of cells in PDGF and FGF (removal of EGF) I observed emergence of 30-50% GFP-expressing cells between days 3 and 6, with a drop at day 7 (Figure 9A). This drop in GFP may be related to the cell death and likely selection that occurs following removal of growth factors (PDGF and FGF) on day 4 and so increases may be explained by selection. However, through days 1-4 there is little cell death. Western immunoblotting and immunofluorescence microscopy, confirmed expression of GFP reporter at day 4 of differentiation (Figure 9B and D). GFP expressing cells were noted to have the characteristic bipolar morphology of OPCs, and contrasted with nearby flat, spread morphology of GFP-negative cells – likely astrocyte cells (Figure 9C). The differences between cell lines in the efficiency of PDGFRα activation seem stable as PG1-3 typically gave lower GFP induction (Figure 9E). Variability between different clonal NS cell lines in efficiency and consistency of differentiations was noted previously in a wide range of different NS cell lines. The explanation for this remains unclear.

To confirm the OPC identity of GFP expressing cells, I next harvested GFP-expressing cells at day 4 of differentiation using fluorescence activated cell sorting (FACS) (Figure 10A). mRNA was harvested and qRT-PCR analysis performed to assess expression of PDGFRα as well as a series of differentiation markers in GFP-positive and -negative populations. PDGFRα and GFP mRNAs were enriched in the GFP-positive sorted fraction. Encouragingly further markers of OPCs were highly enriched in the GFP subpopulation, namely: OLIG2, NKX2.2 and SOX10 (Figure 10B).

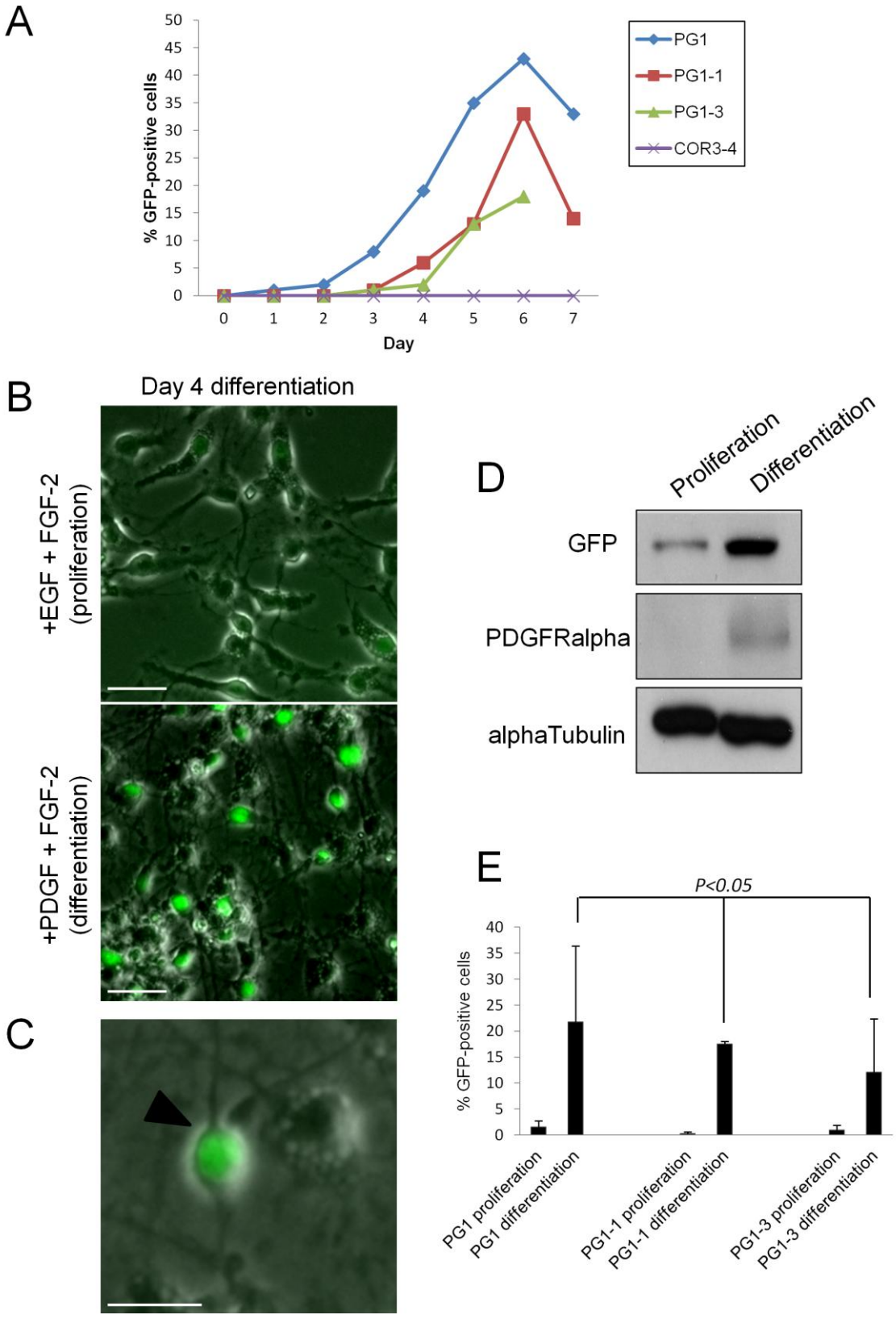


Figure 9. PG1 NS cells can differentiate into PDGFRα-positive oligodendrocyte progenitor cells *in vitro*. (A) Time-course flow cytometry analysis of GFP expression shows that PDGFRα-positive cells emerge on day 3-4 of differentiation with a peak of expression on day 6; analysis performed as a single technical experiment on PG1 and two clonal lines (PG1-1 and -3), COR3-4 was used as a GFP-negative NS cell line (B,C) Images showing GFP activation during oligodendroglial differentiation (EGF withdrawal); Scale bars, 50µm (C) Oligodendrocyte progenitor cell (black arrow) has a characteristic bipolar morphology; Scale bar, 25µm; (D) Western blotting analysis showing expression of PDGFRα and GFP on day 4 of differentiation; (E) Flow cytometry analysis on day 4 of differentiation shows a variable expression of GFP between parental PG1 and two clonal lines (PG1-1 and PG1-3); the difference between PG1 and PG1-1, and PG1 and PG1-3 was statistically significant at $p<0.05$ (independent t-test; plotted mean ±SD; 2 biological replicates for each line);

When differentiated further, cells started expressing O4, a marker of oligodendrocyte lineage, on day 7 of differentiation and all O4 expressing cells were GFP-positive. However, not all GFP-positive cells were O4-positive, suggesting some limiting factor/signal in promotion of oligodendrocyte differentiation in our cultures (Figure 10C). Immunocytochemistry for OLIG2 and SOX10 confirmed that all OPCs and O4 cells co-expressed these two key regulators (Figure 10C and D).

In summary, these data suggest that: 1) the PG1-1 and PG1-3 clonal NS cell lines express characteristic NS cell markers and are karyotypically normal. 2) GFP reporter is not expressed in undifferentiated NS cells but is activated following induction of differentiation. 3) GFP expressing cells are enriched in a range of OPC markers and display OPC morphology. 4) A subset of GFP-expressing OPCs can differentiate into O4-positive cells and express markers of oligodendrocyte lineage. Thus, PG1-1 and PG1-3 represent a useful cellular model for studies of PDGFRα activation during NS cell differentiation.

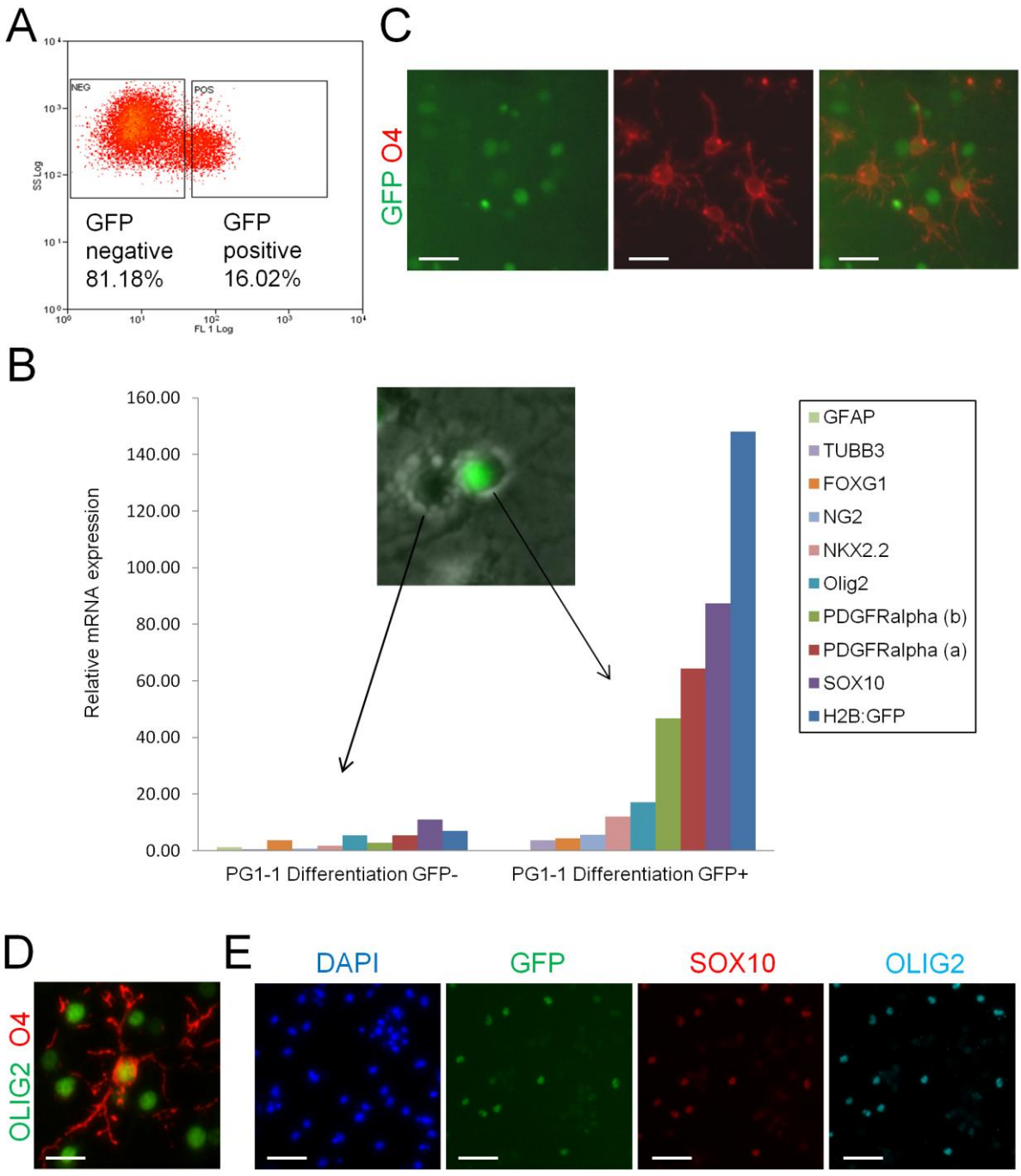
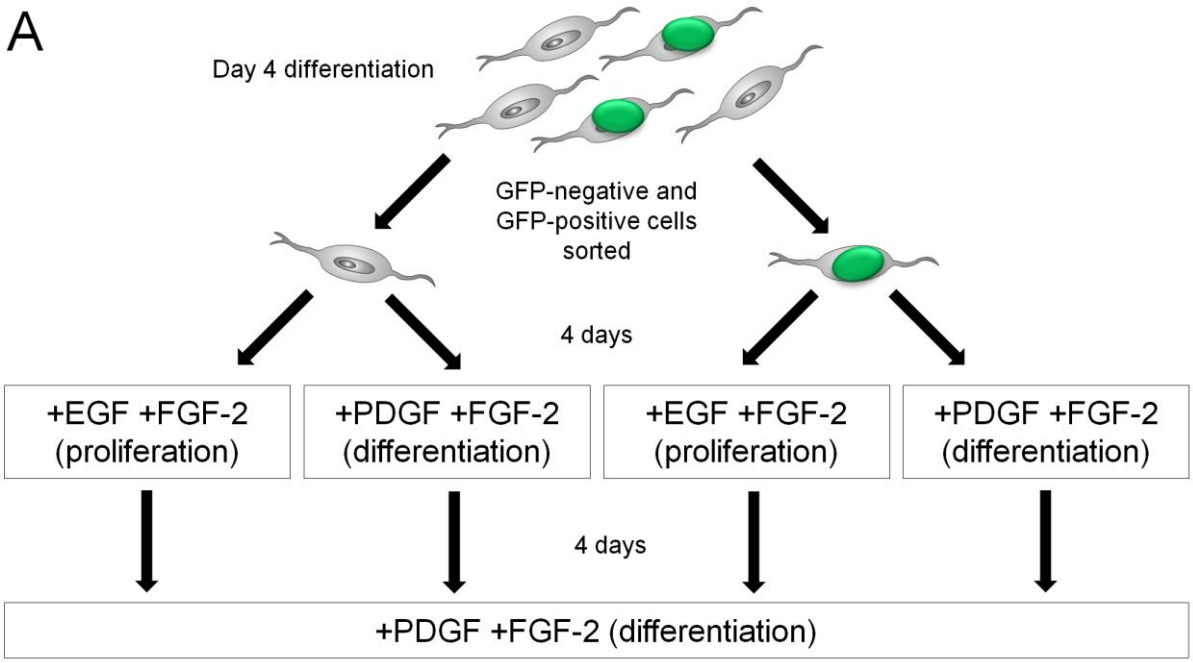


Figure 10. GFP-positive PG1-1 cell are enriched in markers of oligodendrocyte lineage. (A) Typical plot of flow cytometry and gating strategy for the isolation of GFP-positive and GFP-negative cells used in qPCR analysis; (B) qPCR analysis reveals an enrichment of oligodendrocyte lineage markers (including PDGFRα and SOX10) in GFP-positive cells; (C) GFP-positive OPCs are able to differentiate into O4-positive cells; Scale bars, 50µm; (D,E) Only a subset of cells positive for OLIG2, GFP and SOX10 become O4-positive; Scale bars, 20µm (D), 75µm (E);

3.2.3 Acquisition of PDGFRα does not indicate commitment to differentiation

The PG1 cells provide a useful tool to investigate the process of differentiation commitment. I therefore investigated whether acquisition of GFP/PDGFRα expression indicated a loss of responsiveness to EGF. NS cells were differentiated for 4 days (described in Methodology) and GFP-negative and GFP-positive cell populations were sorted. These isolated subpopulations were re-plated with either EGF and FGF-2 (self-renewal) or PDGF and FGF-2 (differentiation) for 4 days (Figure 11A). I found that GFP-positive cells were able to proliferate in response to re-exposure to EGF and extinguished expression of GFP after 4 days. Cells plated in PDGF and FGF-2 could not be expanded continuously and rapidly exited cell cycle (Figure 11B). Interestingly, GFP-negative cells can activate the reporter after 4 days in proliferating condition, followed by the differentiation period, at a level similar to the original GFP-positive population, suggesting that GFP negative cells are not committed to an alternative fate, but can also revert to the NS cell state (Figure 11B).

My results indicate that activation of the GFP-positive state is reversible as cells can readily extinguish expression of the reporter and re-enter the cell cycle when reexposed to EGF and FGF-2.



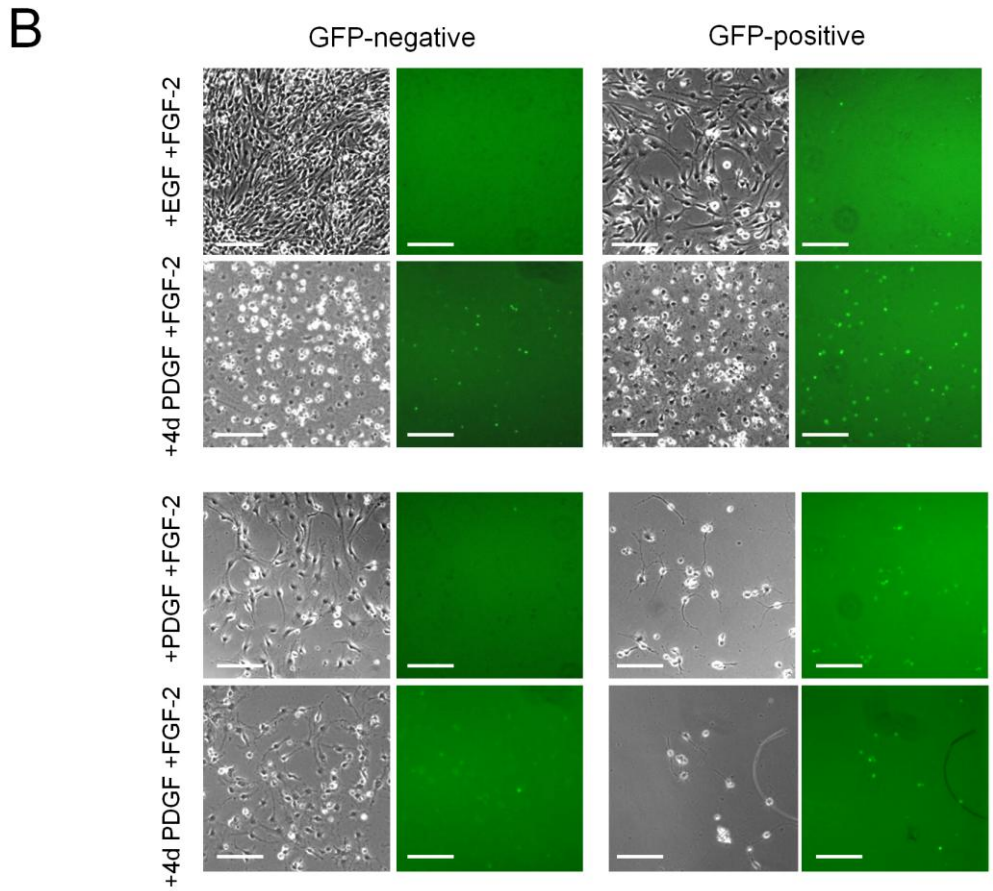


Figure 11. GFP-positive OPCs can be reverted to NS cell state. (A) Experimental design to establish the reversibility potential of the PDGFRα-positive state; (B) Microphotographs of sorted GFP-/+ cells incubated in proliferating or differentiating conditions to evaluate reversibility potential of OPCs show that PDGFRα expression is reversible with the EGF treatment and GFP status does not mean cell commitment to oligodendrocyte lineage; Scale bars, 150 µm;

3.2.4 Cell-cell contact affects activation of PDGFRα

In addition to line-to-line variation in the proportion of GFP activation, it is also a feature of NS cell differentiation that there is variability between experiments in the differentiation efficiency. One potential explanation for this is the effect of cells' density. To explore this further and to establish the optimal plating density for future chemical screens based on PG1 cells I tested different plating densities and assessed GFP activation.

Cells were counted and plated in identical numbers, but in variable vessels sizes, leading to a range of distinct densities (ranging from a 24-well plate to a T-25 flask) (Table 2). I scaled the volume of media in each vessel to ensure any secreted signals would be diluted to a similar final concentration. Thus, the cell density was the only variable between each condition.

Table 2. Experimental setup for assessing the effect of cell density of PDGFRα activation

	24-well plate	**12-well plate**	**6-well plate**	**T-25 flask**
Surface area (cm^2)	2	3.8	9.4	25
Cells/cm^2	100,000	52,600	21,300	8,000

Cells were incubated in differentiating conditions for 4 days and flow cytometry analysis was performed (Figure 12A). It was possible to observe a direct correlation between the plating density and the percentage of GFP-positive cells, with higher density increasing GFP

activation. A similar trend was observed in all three cell lines with GFP expression doubling from the lowest (8,000/cm^2) to the highest (100,000/cm^2) plating density (Figure 12B&C). This suggests that activation of PDGFRα is dependent on appropriate cell-cell signaling.

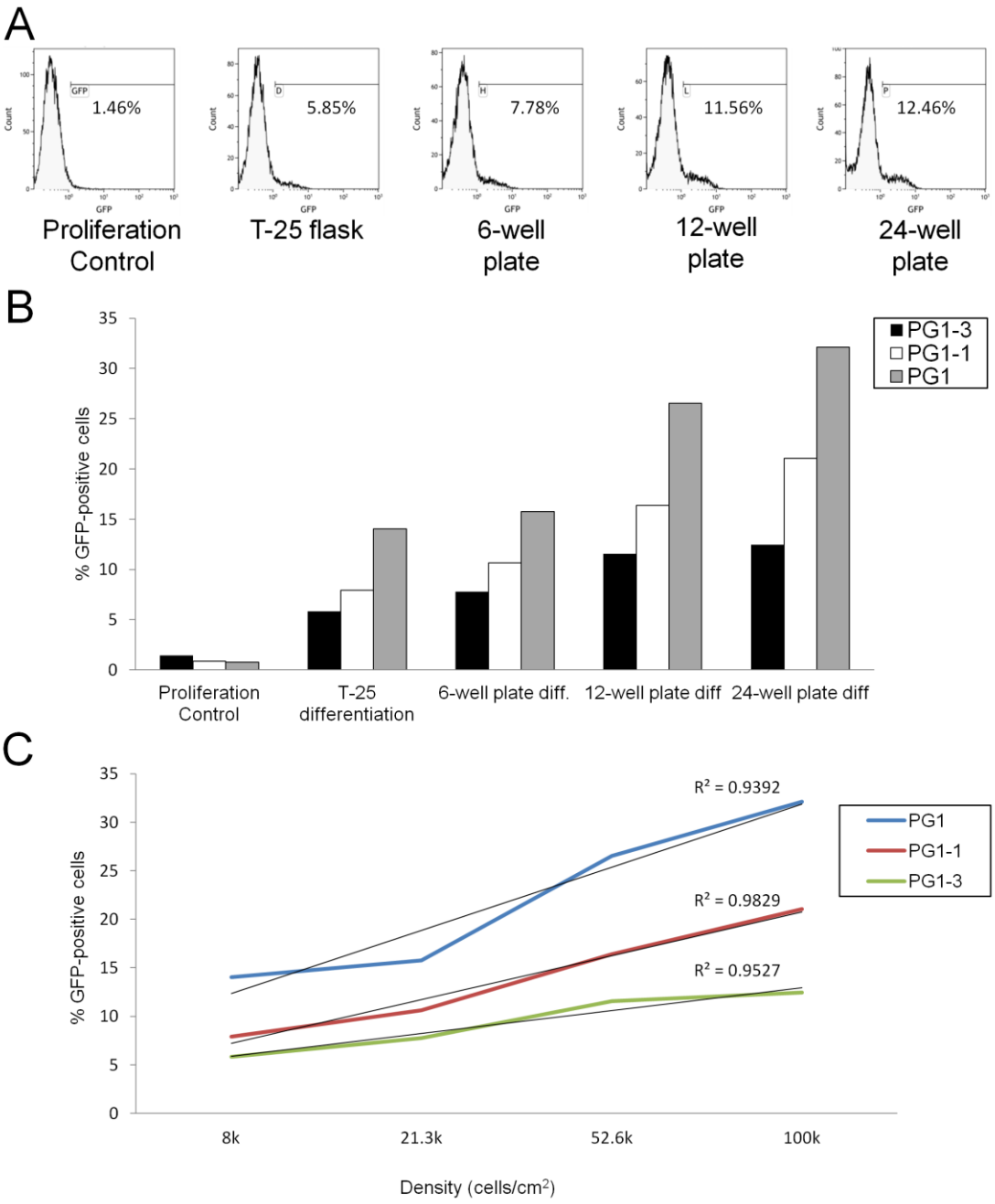


Figure 12. Cell-to-cell contact increases the activation of PDGFRα. (A) Flow cytometry plots from analysis performed on PG1-3 on day 4 differentiation show higher levels of GFP expression associated with smaller vessel (higher cell plating densities). (B) Results of flow cytometry analysis from three cell line show a similar trend regardless of the cell line used; Higher plating density translates into higher expression of PDGFRα (GFP); Analysis performed as a single technical experiment on three independent cell lines; (C) Correlation between density and GFP expression can be established as a similar trend was observed in all three cell lines tested (R^2 analysis performed);

3.2.5 Establishment of mouse brain explants cultures

We next assessed whether the PG1 cells could engraft and begin differentiation when placed into a brain slice culture. Explant cultures have a potential to reduce the need for animal testing and to bridge the gap between *in vitro* and *in vivo* studies. They can more accurately mimic the natural differentiation environment of neural cells, allowing for cell-to-cell contact interactions and signalling. They can also reduce the time needed to validate small molecule treatments as brain explants cultures infiltrated by cancer cells can be established in a matter of hours, requiring only enough time for attachment and infiltration of the explant layer by cancer cells.

I therefore aimed to establish in the Pollard laboratory CNS slice cultures as a useful *ex vivo* tool to explore PG1 cell differentiation. ~100 um slices were generated using a vibratome and placed onto a cell culture insert. Interestingly, I observed high expression levels of Nestin in slices containing ventricles, marking the niche of neural stem cells (Figure 13A). I generated PG1 cells expressing constitutively the dsRed reporter under the control of a CAG-expression plasmid. PG1-dsRED cells were able to engraft into the brain slices and survive long term in this artificial environment (Figure 13B). When treated with PDGF and FGF-2, the PG1 cells were able to differentiate into OPCs and express GFP reporter (Figure 13C). G144, a human GBM cell line, was used to test the potential for GBM studies using this model. Again, I was able to observe the infiltration of the explant layer for up to one week (Figure 13D). These data confirm that GFP reporter expression can be observed in cells plated into brain slices. However, we did not pursue further differentiation of cells or study their ability to generate mature oligodendrocytes.

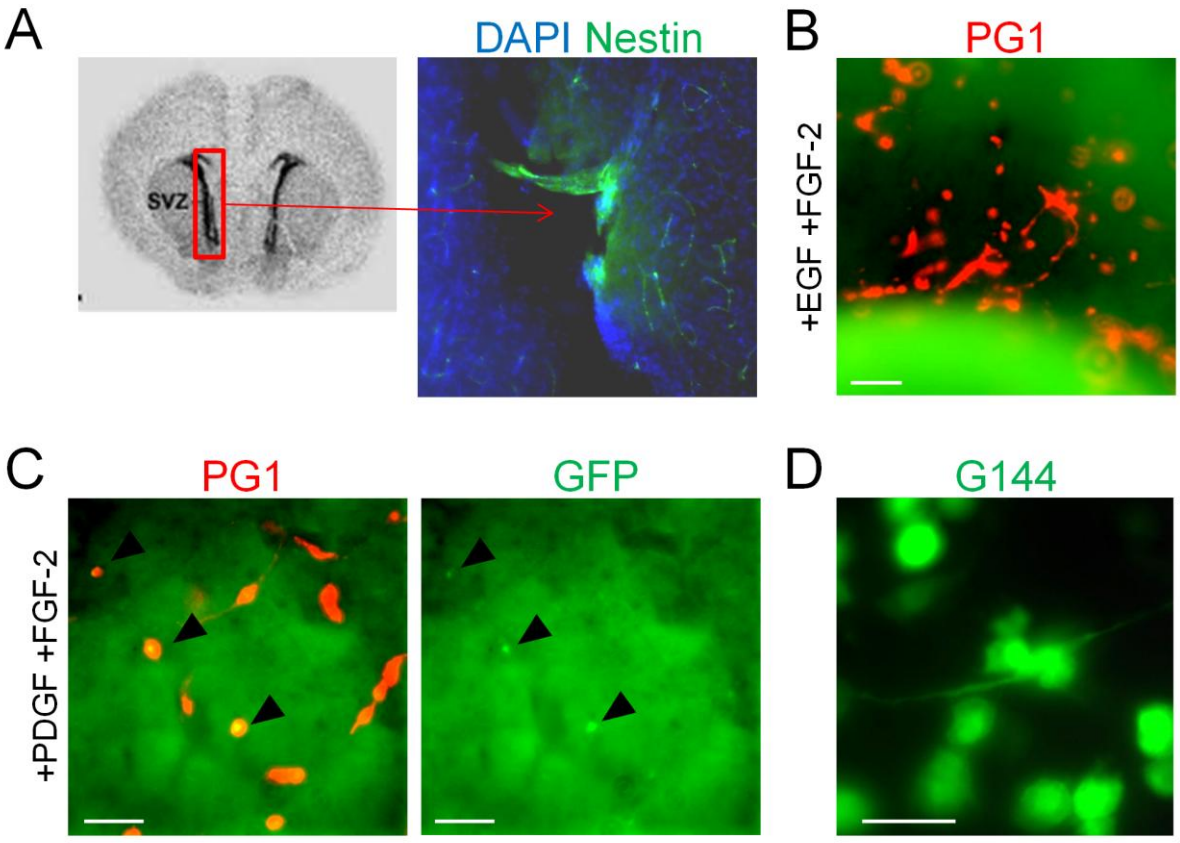


Figure 13. Mouse brain explant culture as a tool for oligodendrocyte differentiation studies. (A) Brain explant culture containing layers of cells from SVZ shows expression of Nestin marking SVZ stem cells with nuclei in blue (DAPI staining); (B) PG1 cells infiltrate the explant culture and (C) can differentiate into OPCs when EGF is withdrawn; Scale bars, 75µm; GFP expression marked with black arrows); (D) Human GBM cell line G144 tested also infiltrates the explant culture; Scale bar, 20µm;

3.3 Discussion

PG1 mouse NS cell lines express typical markers of neural stem cells and they are responsive to growth factors known to influence proliferation (EGF/FGF) or astrocyte differentiation. The normal karyotype for the mouse genome was observed in NS cells (n=40) and the generation of clonal and karyotypically normal NS cell lines was important, as these cell lines are later used in chemical screening experiments (Chapter 4).

The generation of GFAP-positive GFP-positive type B cells was not observed in the GFAP cells indicating these are unlikely to be quiescent astrocyte stem cells (Jackson, Garcia-Verdugo et al. 2006) consistent with the downregulation of Sox2. I tested combinations of BMP-4 with other key regulators of NS behaviour, EGF, FGF-2 and PDGF. Only when BMP-4 was delivered with FGF-2 did we observe any indication of GFAP and GFP co-expressing cells. This is potentially interesting to pursue in future studies, as recent reports have suggested BMP and FGF treated NS cell lines may indeed have type B – quiescent stem cell characteristics (Martynoga, Mateo et al. 2013). *In vitro* expansion of these early, undifferentiated cells would be of interest to further delineate neural processes and to understand processes leading to their activation.

Oligodendrocyte differentiation using PDGF and FGF-2 remains suboptimal, leading to only 15-20% induction of OPCs in culture. We found no effects of included Forskolin in the differentiation protocol (not shown) (Raible and McMorris 1990, Joubert, Foucault et al. 2010). Chemical screening could therefore be valuable in order to identify molecules and pathways that increase the efficiency of this process.

Not all PDGFRα-positive cells become O4-positive *in vitro,* which marks a possibility for other processes and factors being involved, but also defining different stages of differentiation. In a study by Ellison and de Vellis they recognised three different populations of cells expressing PDGFRα: GD3-positive, GD3-/O4-positive and O4-positive, which marked progenitor and preoligodendrocyte cells in development (Ellison and de Vellis 1994). As oligodendrocytes mature they lose expression of PDGFRα and NG2 and start expressing O4 antigen (Nishiyama, Komitova et al. 2009). This could mean that NS cells in my culture are a heterogeneous non-synchronised population at different stage of development.

We find that activation of PDGFRα expression does not mean that cells are committed towards oligodendrocyte lineage, as GFP-positive OPCs rapidly lost GFP expression when re-plated with the EGF and FGF-2 present in the media. Moreover, they were able to proliferate and presented a characteristic NS cell morphology. The fact that sorted GFP-negative cells were able to activate GFP when replated with PDGF and FGF-2 in the media suggests that this process could be dependent on some intrinsically determined mechanism (such as transcription factor heterogeneity), with 15% GFP-positive cells at any given time following induction of differentiation. Further analysis is required to validate this finding.

Cell-to-cell contact plays an important role in the activation of PDGFRα. I observed a direct correlation between the cell plating density and the percentage of GFP-positive cells, which suggests possible involvement of both autocrine and paracrine signalling. Plating density is important for the survival of OPCs when cultured on their own, either as a result of shakedown of a flask and replating or by FACS. High-density cultures yield the highest percentage of O4-positive oligodendrocytes, while low-density cultures remain at the NG2-positive progenitor stage (Yang, Watanabe et al. 2005). The high-density is associated with increased levels of a cell cycle inhibitor p27(Kip1), reduced expression of cyclin A and changes in phosphorylation levels of Rb. Interestingly, these changes are reversible, when cells are replated at a lower density (Nakatsuji and Miller 2001). This observation could somehow explain the lack of commitment of sorted PDGFRα-positive cells, as possibly they were not plated at a high enough density.

Brain slice culture more closely mimics the *in vivo* environment than *in vitro* differentiation protocols. There are two challenges identified in the explant culture. Firstly, only a small proportion of cells actually infiltrates the explants layers. Secondly, it can be difficult to find

a focal plane to image and track the infiltrative cells as they are positioned between layers of other explants cells. Brain slice culture is therefore of value in determining the maturation and function of cell populations, but is less suitable for higher throughput studies such as chemical screen and mechanistic studies.

3.4 Summary

In summary, these results confirm that I have generated a novel and useful cell lines for studies of the transition from neural stem cell/radial glia to an oligodendrocyte specified progenitor. These cell lines are ideally suited for chemical and genetic screening.

Chapter 4 Chemical screening for modulators of NS cell differentiation

4.1 Introduction

High content screening (HCS) refers to the phenotypic screening of cells in a manner in which extensive molecular and morphological information is extracted from responses of cells to experimental treatments (either chemicals or genetic perturbations). Chemical screens might include small pharmacologically active molecules, peptides, or antibodies. Genetic screen encompass RNA molecules leading to inhibition of gene expression, a process known as RNA interference (RNAi) (Taylor, Haskins et al. 2007, Haney 2008) and more recently the use of synthetic nucleases such as the CRISPR/Cas system (Shalem, Sanjana et al. 2014, Wang, Wei et al. 2014).

Chemical genetics has been used extensively over the past two decades as a method for identifying potentially interesting compounds and signalling pathways using small molecule libraries (Schreiber 1998, Crews and Splittgerber 1999). To cause a specific phenotypic change, a small molecule has to be able to cross the cell membrane or target cell surface molecules and must display some specificity in its activity (Eggert and Mitchison 2006).

Using HCS it is possible to define in an unbiased manner those pathways which control specific biological processes, and this ultimately provides the foundation for developing new drugs that can be used in medicine. Two strategies can be employed, either screening of compound libraries with known biological targets, or screening of new chemical entities generated through chemical synthesis. The latter is more typically used in the pharmaceutical industries and uses more high throughput, as many compounds will have poor drug-like qualities such as cell permeability. Target identification is considered to be the rate limiting

step in chemical genetics/high-content screening (Eggert and Mitchison 2006). Small molecules with known targets therefore can overcome this bottleneck in defining what the specific molecular target and pathway that has affected the cells.

HCS is considered a discovery tool in stem cell biology, where the goal is to uncover specific effects on cell fate, e.g. differentiation, death, proliferation, senescence and where cellular heterogeneity in the differentiating cell population is commonplace. HCS allows scientists to track non-uniform and asynchronous differentiation of stem cells *in vitro* over time and to monitor the behaviour of transient intermediate progenitor cells, a clear advantage over endpoint assays (Danovi, Folarin et al. 2012). HCS is also implemented in studies of cancer-derived stem cells. Small molecules with potent biological effects on the fate of these cells represent useful research tools and new drug leads for regenerative medicine and oncology (Danovi, Folarin et al. 2012).

Small molecules that can modulate the lineage choice and differentiation of NS cells may have value as research tools, for use in regenerative medicine or for use in new types of differentiation therapy for brain cancer. To search for small molecules capable of modulating differentiation of NS cells and to dissect pathways modulating this process, I set up a chemical screening of commercially available known small molecule inhibitors of 'classic' stem cell and developmental pathways, as well as known kinase inhibitors. A total of 463 compounds from three distinct libraries of commercially available small molecules were used ('StemSelect', 'InhibitorSelect I' and 'InhibitorSelect II'; obtained from EMD Calbiochem Merck Millipore. The StemSelect library consisted of 303 pharmacologically active small molecules previously implicated in regulation of development and stem cell pathways. The InhibitorSelect libraries consisted of 160 kinase inhibitors giving a total of 463 screened

molecules. Our choice of high content screening platform was the Incucyte FLR device, which enable image acquisition of phase images (10x) and GFP fluorescence in live cells in an automated manner directly in the tissue culture incubator. 6 x 96 well plates can be screened with this device, and this is compatible throughput for the small libraries we were screening.

4.2 Results

4.2.1 Optimisation of Incucyte FLR live cell screening conditions

Our goal was to search for compounds that could influence (increase or decrease) the numbers of OPCs (GFP-positive) that emerge following differentiation of NS cells. A pre-requisite of any cell based screening assay is the optimisation of culture conditions and parameters for the differentiation and assessment of the sensitivity of the assay. Initial studies of GFP activation showed that complete medium in which NS cells are grown has high autofluorescence levels, which are detrimental in an imaging-based screening, increasing background and reducing the signal to noise. To solve this problem I compared our standard complete NS cell media (DMEM-based) with that using EMEM. EMEM contains lower levels of vitamin B12 (Riboflavin), which is responsible to the autofluorescence in the 488nm region. Exchange of DMEM with EMEM did not affect cell viability or differentiation potential, but we indeed found a much better signal in the GFP expressing OPCs in this condition (Figure 14A).

Another important factor was to assess if the amount of medium used could be reduced without compromising the consistency of differentiation. I found that cells struggled to survive with lower volumes for the required time of 3-4 days and increased non-specific

death was seen at day 2. This was possible due to reduced concentrations of growth factors and other molecules, which were at that point used up by cells in culture (Figure 14B). As the chemical screening experiment was planned to last for an uninterrupted period of 3 days I had to find the media volume which would not affect cell survival and at the same time allowed for a correct drug concentration in every well. I observed that 50uL of media in a well of a 96-well plate was the minimum volume to satisfy both requirements when $6x10^3$ PG1-1 cells/well were used (data not shown).

I also tested a range of different cell culture plastics/substrates (no coating versus, gelatin or laminin). Only laminin coating enabling sufficient cell attachment to the bottom of the vessel. The test of three different concentrations of laminin showed that only the standard concentration (described in Methodology) provided fully attached cells, with no sign of cell clumping, which was important for the chemical screening as I was able to easily distinguish individual cells, improving accuracy of automated CellProfiler analysis of the chemical screening (Figure 14C). Ibidi supply tissue culture plastics that is optimised for high resolution imaging studies and we tested these plasticware. Although image quality was slightly increased we found significant clumping/detachment with this plasticware.

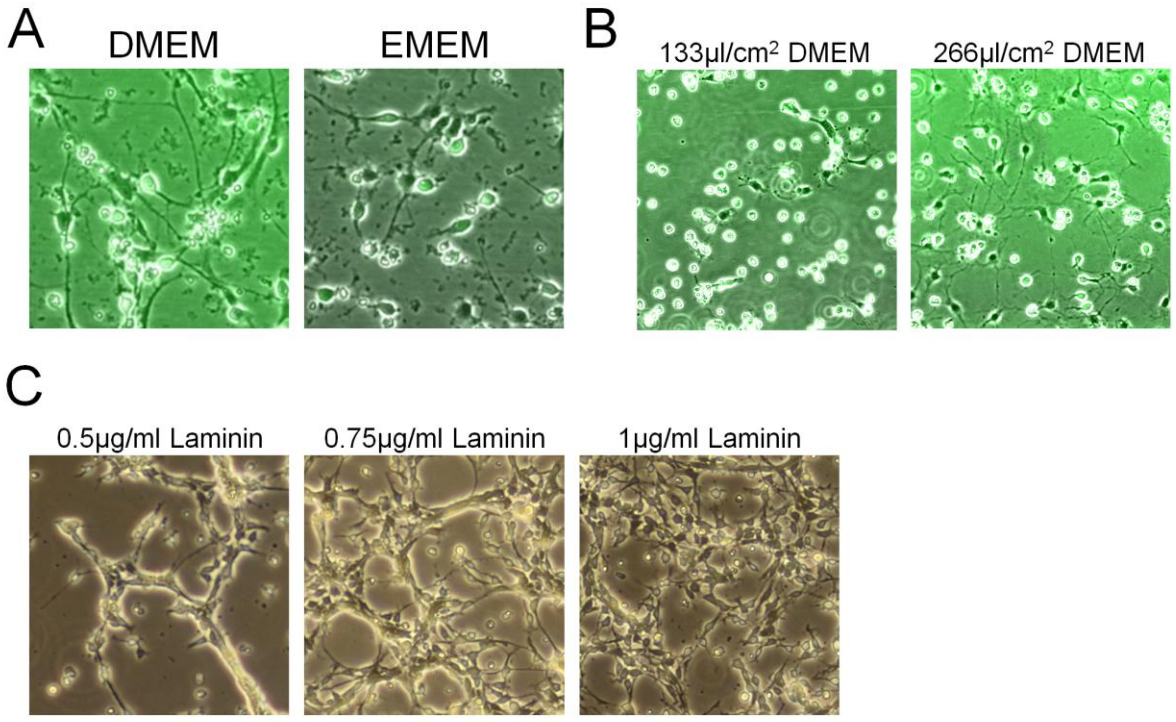


Figure 14. Optimisation of Incucyte FLR live cell screening conditions. (A) Reduction in media autofluorescence was achieved by switching the basal media from DMEM to EMEM; (B) Media volume affects cell survival over time, with higher volumes promoting an overall increase in cell survival; (C) Reduction in laminin concentration causes cell clumping, which can affect analysis of High Content Screening data;

Mouse NS cells were deposited into 96-well plates under differentiation conditions (see Methodology) and compounds were added while cells were still in suspension using the Cybi Selma 96-head liquid handling device (CyBio). Plates were then transferred to a live-cell imaging device (Incucyte, Essen BioSciences) and each well was imaged over the course of 66 hours at 3 hours intervals. More frequent imaging to achieve a finer time-course of differentiation was explored, however there was significant non-specific cell death due to phototoxicity at 1 and 2 hour intervals (not shown). Data were then processed and analysed using CellProfiler and R 2.11 software, with dedicated analysis pipelines established similarly to previous screens from our laboratory (Danovi, Folarin et al. 2012) (Figure 15).

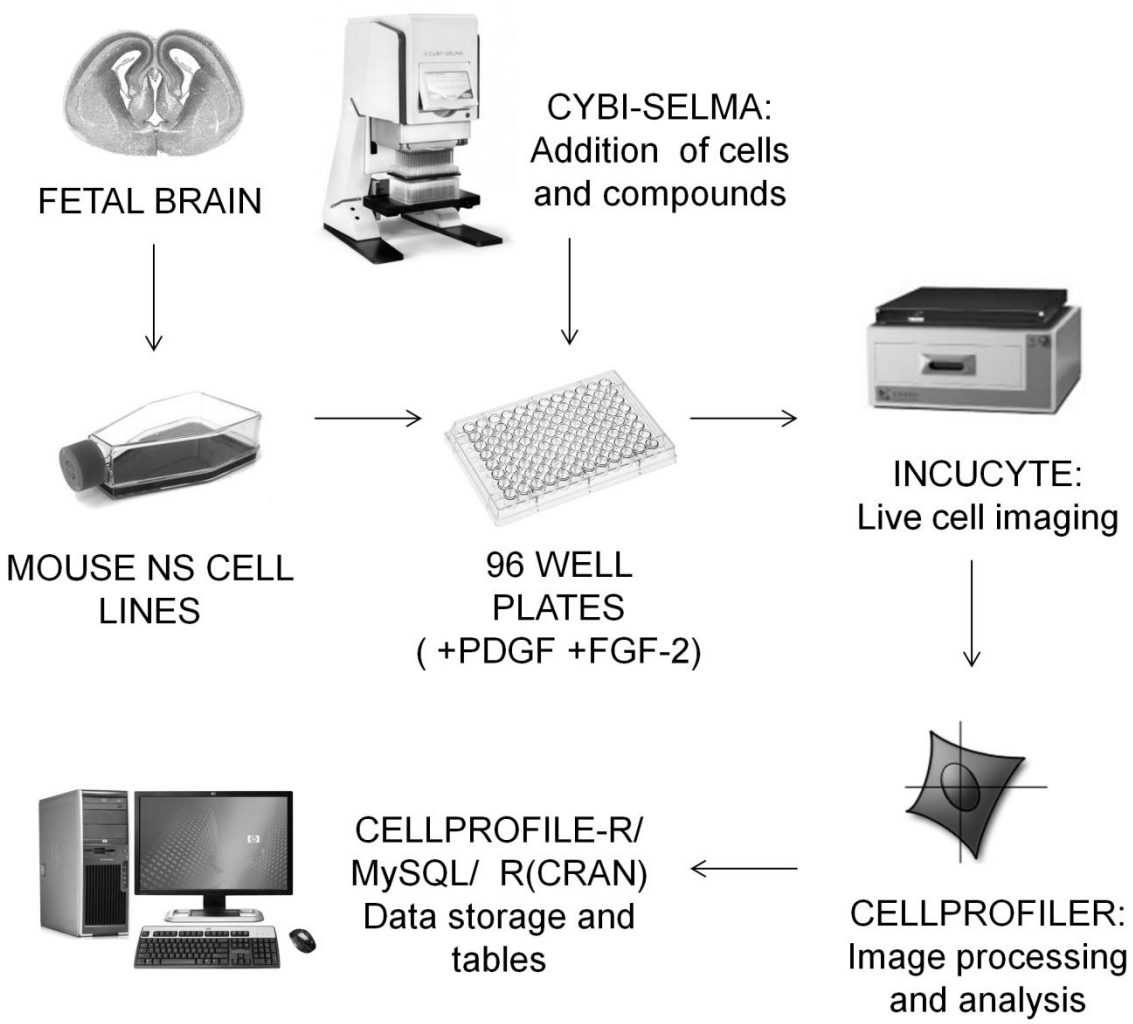


Figure 15. Chemical screening process pipeline. Cells are isolated from the mouse foetal forebrain and NS cells are culture as primary stem cell lines *in vitro*. Compounds and cells are added to 96-well plates using a semi-automated liquid handling device (CYBI-SELMA). Live cell imaging system, Incucyte, is used to take images of each well of a 96 well plate at the established intervals. CellProfiler software is then used for image processing and analysis, followed by data storage and creation of tables for analysis using CellProfile-R, MySQL and R(CRAN).

4.2.2 Screening of StemSelect Library for small molecules that modulate OPC lineage commitment

To find modulators of NS cell differentiation I firstly set out to screen the StemSelect Library. This library contains 303 selected small molecules known from previous literature to influence stem cell pathways. I screened this library using PG1-1 cell line in four independent chemical screenings performed over a period of six months. The results were normalised to account for differences between experiments. Criteria for selection for further study were either complete inhibition of GFP expression or any increase in GFP expression as observed using generated heat maps. As it is common in a chemical screening to identify 'false' positive compounds we decided to use flow cytometry as a method to accurately evaluate the potential of molecules which met our criteria of GFP inhibition or increase in activity.

No compounds within this library were able to increase the frequency of GFP cells compared to control wells with 18 false-positive compounds observed (data not shown). However, 12 compounds that could suppress GFP expression without effects on general cell viability were identified (Table 3). These molecules were further validated using flow cytometry. Most notably, compounds classified as Histone Deacetylase Inhibitors (HDACi) appeared to be the most effective GFP blockers, as 5 (Apicidin, HDACi III, HDACi VI, Scriptaid and HDACi IV) out of 12 selected molecules belong to this protein family. Figure 16 presents results of the high content screening analysis, which was composed of investigation of generated heat maps showing absolute and relative change in GFP expression.

Table 3. Summary of GFP inhibitors identified in the chemical screening. Twelve compounds were identified as potential GFP inhibitors, which required further validation; Relative mean change in GFP expression was compared to DMSO controls at the last experimental time-point (66 hours);

GFP inhibitor	**Relative mean change in GFP expression**	**Standard deviation**
Apicidin	0.08	0.08
Corticosterone	0.53	0.08
Dexamethasone	0.58	0.27
ACA	0.65	0.18
HDACi III	0.2	0.18
HDACi IV	0.51	0.1
HDACi VI	0.41	0.16
Nemadipine-A	0.41	0.14
Scriptaid	0.4	0.1
Nigericin	0.2	0.14
Valproic acid	0.64	0.25
Withaferin A	0.31	0.07

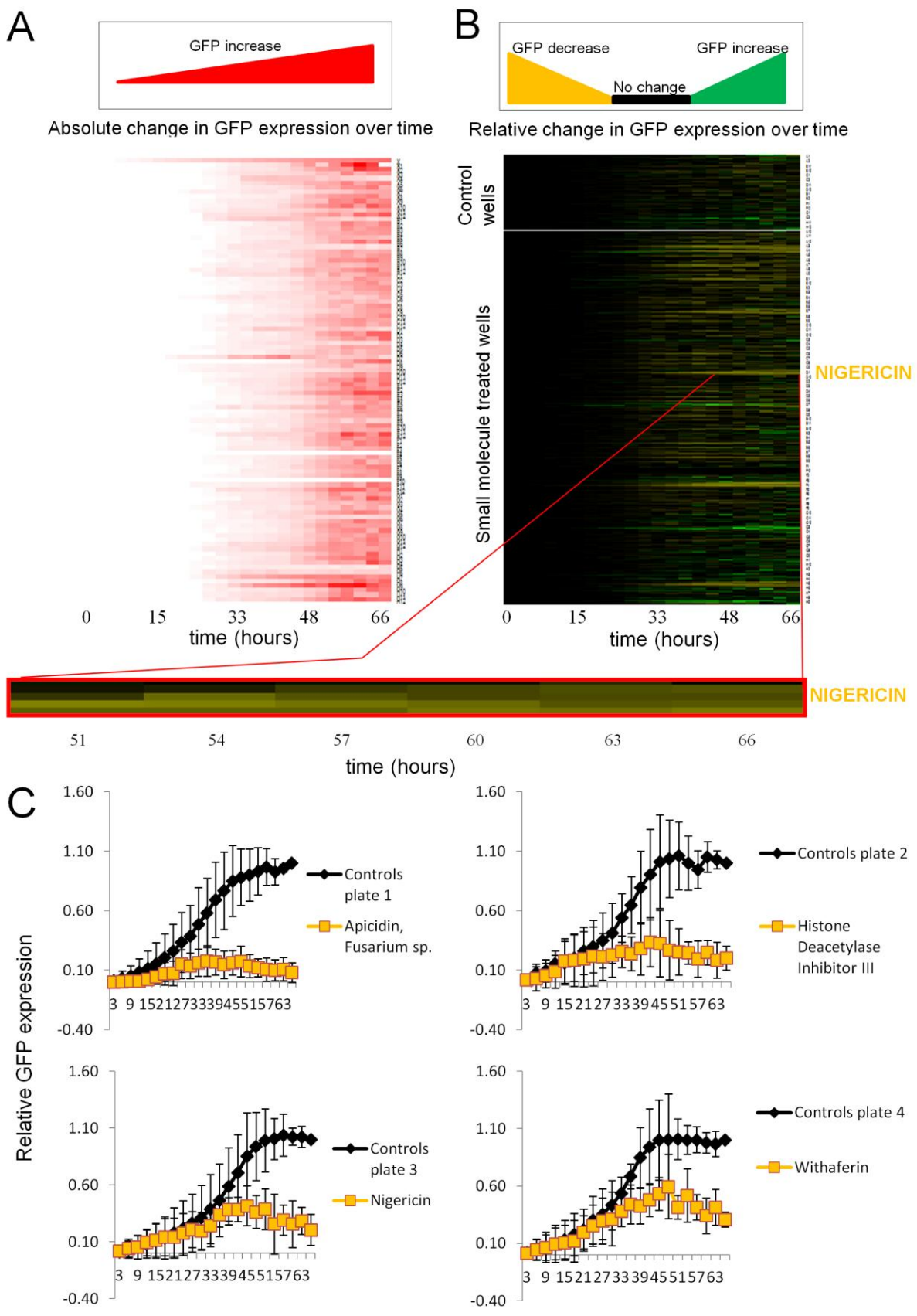


Figure 16. Representation of chemical screening results in a form of heat maps. (A) Absolute change in GFP expression over time in all drug-treated wells of 96-well plates; (B) The same data as A presented as relative change in GFP expression, compared to average GFP expression in control wells at each time-point with Nigericin showing reduction in GFP expression (marked with yellow colour) on day 3 of differentiation; Both heat maps show combined results from 4 independent screenings as described in Methodology; (C) Results of the 4 most effective GFP inhibitors identified in the screening shown as a relative change in GFP expression compared to control wells (all graphs);

To validate the identified hits from the initial screen flow cytometry analysis was used in an independent experiment with the same compounds. This confirmed the activity of the family of Histone Deacetylase Inhibitors (HDACi) in blocking activation of the PDGFRα reporter (Figure 17A). The four most potent inhibitors identified were: Apicidin, Histone Deacetylase Inhibitor III (both HDACi), Withaferin (NFkB Activation Inhibitor) and Nigericin (a polyether antibiotic and ionophore) were then used for the further analysis as they appeared effective in a complete inhibition of GFP expression. I validated these four molecules using independent experiments and quantitation of GFP using flow cytometry allowed for a more precise analysis of GFP expression. A lack of GFP expression due to the small molecule treatment suggested that cells were blocked from becoming oligodendrocyte progenitor cells. (Figure 17B). Morphology of the treated cells also suggested that the characteristic bipolar morphology of oligodendrocyte progenitor cells did not emerge in the presence of each of these compounds (Figure 17C).

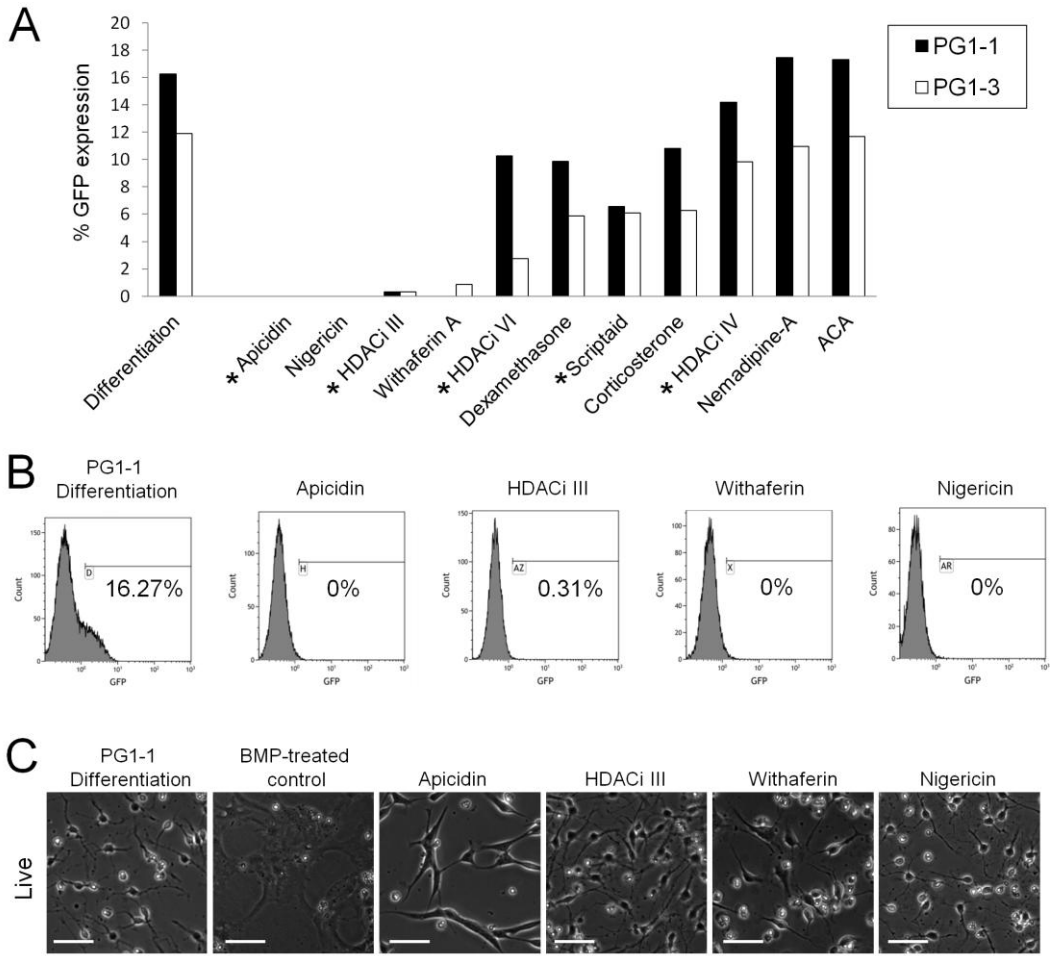


Figure 17. Four molecules were selected as GFP inhibitors for post-screening analysis. (A) Results of a flow cytometry validation of small molecules identified in the chemical screening as GFP inhibitors; HDACi are marked with *; Validation performed as a single experiment on two clonal cell lines; (B) The four most effective GFP inhibitors can completely block GFP activation – but without any effect on cell viability; (C) Live phase-contrast images showing a change in morphology in drug-treated cells; Scale bars, 40µm;

4.2.3 Screening of InhibitorSelect Libraries for molecules that modulate OPC lineage

As no compounds that stimulated OPC specification were identified, we next assessed the phenotypic consequences of another small compound library called InhibitorSelect library which comprises 160 known kinase inhibitors. Cells were plated in 96-well plates and compounds were added while cells were still in suspension (as outlined in Methodology). As this was an additional chemical screening to the screening of the StemSelect library, it was performed as a single experiment with three technical replicates.

I was not able to identify any compounds that could induce increased numbers of GFP-positive cells based on the analysis of images generated during the screening. However, 9 GFP inhibitors were observed (K-252a, NFkBi, Staurosporine, IC261, SB 203580, Akt Inhibitor IV, Fascaplysin, CDK4 Inhibitor III, SU9516).

The validation was performed at three different concentrations of kinase inhibitors: 10 nM, 100 nM and 1 uM (Figure 18A). Most notably, two compounds: K-252a (Staurosporine analog) and NFkB Activation Inhibitor (known as 545380-34-5), appeared to block GFP expression at a level similar to previously identified ‘hits’ from StemSelect screening (Figure 18B).

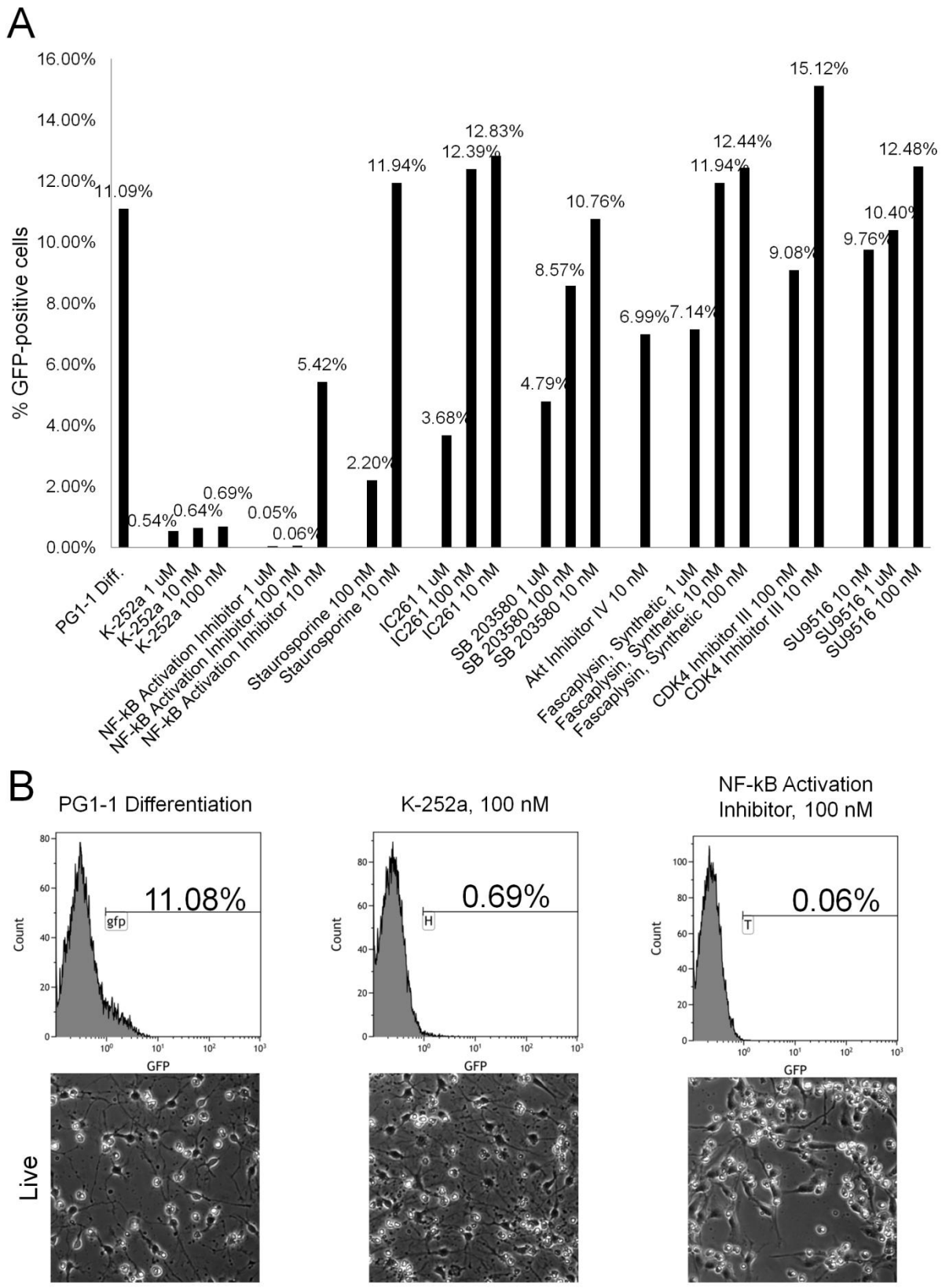


Figure 18. Validation of GFP inhibitors identified in the screening of the InhibitorSelect library. (A) Flow cytometry validation of compounds identified as GFP inhibitors (cytotoxic drug concentrations not shown) (B) Flow cytometry graphs and live phase-contrast microphotographs of the two most effective GFP inhibitors: K-252a and NFkBi;

4.2.4 Chemical screening for agents that drive OPC differentiation to O4-positive oligodendrocytes

As my original chemical screening did not identify compounds that could enhance OPC specification I next pursued a modified screening strategy in which we searched for agents that might act on the 20% of OPC-GFP positive cells and drive their differentiation/maturation into oligodendrocytes expressing O4. For this we used the same set of chemical libraries and assessed them by visual inspection looking for cells displaying characteristic oligodendrocyte morphology (Figure 19A).

Quabain and gamma-Secretase Inhibitor IX were identified using these assays as compounds that resulted in increased proportion of O4 cells (described in Methodology). Quabain appeared to effectively improve cell survival, showing a reduced number of apoptotic cells, when compared to DMSO control. Gamma-Secretase Inhibitor IX most likely acted as a selective inhibitor of GFP-negative cells, leaving the majority of GFP-positive cells in culture (Figure 19B). The hits from this screen were not pursued further as there was no compound identified that increased the absolute numbers of GFP cells.

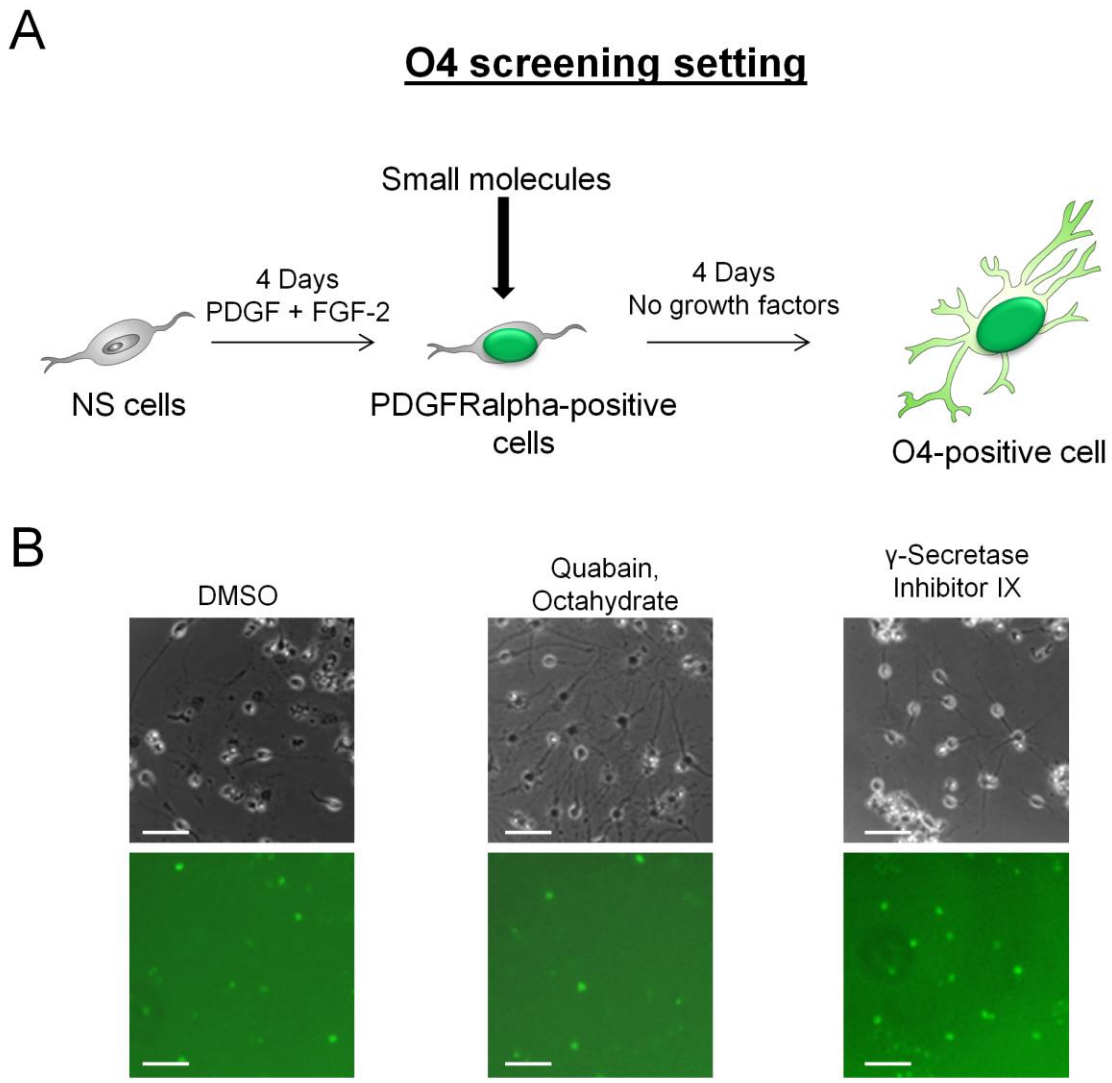


Figure 19. Chemical screening of StemSelect and InhibitorSelect compound libraries to uncover molecules promoting formation of O4-positive cells. (A) A schematic of the O4 screening experimental design. NS cells were allowed to differentiate into GFP-positive OPCs, at which point drugs were added. After an additional four days of differentiation cells were fixed and analysed using light microscopy; (B) Microphotographs of 4% PFA-fixed cells showing compounds of interest, selected on the basis of improved cell survival (Quabain) and selection of GFP-positive cells (gamma-Secretase Inhibitor IX); GFP expression showed in the green channel; Scale bars, 40µm;

4.3 Discussion

Mouse NS cell lines are a valuable cellular model for studies of transcriptional and signalling pathways that influence stem cell self-renewal, lineage commitment and differentiation. We reasoned that a HCS approach with mouse NS PDGFRα-GFP reporter cell lines might allow us to identify compounds and associated pathways that would improve the differentiation process *in vitro* and might have value in the treatment of human brain cancer, or for directed differentiation of human stem cells for use in regenerative medicine.

These experiments have demonstrated the value of adherent culture in enabling identification of compounds that can modulate neural stem cell fate – with many members of the HDAC inhibitors blocking oligodendrocyte lineage specification. Such a screen would be more challenging if culturing the stem cells using the more widely used 'neurosphere' culture regime.

Autofluorescence can cause a problem in reporter-based assays and in HCS in particular, as it is crucial for the software to recognise background from the fluorescent reporter protein. Understanding of the limitations and specifications of devices used can be helpful, as the contrast can be improved by using reporter with the emission different than the source of autofluorescence, but this is possible only if the device can analyse the given light spectrum. Riboflavin was identified in a number of studies as a key factor interfering with GFP imaging analysis (Surribas, Resina et al. 2007, Bogdanov, Bogdanova et al. 2009). Creation of media composition with reduced riboflavin concentration can address this issue.

One of the drawbacks of the NS cell culture *in vitro* as a monolayer is the use of laminin or collagen as agents promoting adherence of cells to the vessel surface. These molecules could

potentially alter the expression of cell surface receptors responsible for adhesion, migration, proliferation and differentiation (Ariza, McHugh et al. 2010, Jurga, Dainiak et al. 2011). Interestingly, use of hydrogel-based 3D-scaffold with laminin, showed enhanced neuronal differentiation and survival rate, when compared to 2D-cultures (Ortinau, Schmich et al. 2010). Although laminin has its limitation, it still outweighs the use of neurosphere culture in the HCS setting.

The four selected compounds from the StemSelect library were identified in four independent chemical screening experiments, which shows that the chemical screening can offer reproducible results. Moreover, compounds classified as HDACi clustered together despite being part of different 96-well plates, showing the importance of their activity in oligodendrocyte differentiation. This finding is discussed further in the General Discussion. .

A chemical screening of the InhibitorSelect library identified another molecule, which was able to inhibit GFP expression at a level similar to NFkBi, called K-252a. It was not further analysed as it is a Staurosporine analog. Staurosporine is an alkaloid isolated from bacterium Streptomyces staurosporeus. It is a potent, but not specific inhibitor of protein kinases (Karaman, Herrgard et al. 2008), commonly used to induce apoptosis, thus it is not surprising that it did not lead to oligodendrocyte differentiation.

Quabain and gamma-Secretase Inhibitor IX were the two compounds identified in the chemical screening for agents that drive OPC differentiation into O4-positive oligodendrocytes. Quabain improved cell survival, showing reduced number of apoptotic cells, when compared to DMSO control. Gamma-Secretase Inhibitor IX acted as a selective inhibitor of GFP-negative cells, leaving the majority of GFP-positive cells in culture. Quabain, also known as ouabain or g-strophanthin is a cardiac glycoside. It binds to and

inhibits activity of the plasma membrane Na^+/K^+-ATPase. It also indirectly affects handling of calcium ions by sodium calcium exchanger (NCX). Quabain, at nanomolar or lower concentrations can stimulate the Na^+/K^+-ATPase (Gao, Wymore et al. 2002). As the chemical screening was performed at 1uM concentration it would suggest that quabain acted as an inhibitor of Na^+/K^+-ATPase. Interestingly, similar to my findings quabain was shown to increase survival of retinal ganglion cells *in vitro*, though at the significantly lower concentration of 3nM. This was associated with activity of protein kinase C, which when inhibited blocked the effect of quabain (de Rezende Correa, Araujo dos Santos et al. 2005).

Gamma-Secretase is a key regulator of myelination. Gamma-Secretase inhibitors enhance differentiation and ensheathment of oligodendrocytes. Differentiation of OPCs is inhibited by Notch1 signaling (Wang, Sdrulla et al. 1998). Treatment of OPC cultures with DAPT, a Gamma-Secretase inhibitor, increases number of MBP-positive cells. It is then possible that Gamma-Secretase Inhibitor IX identified in the chemical screening acts by inhibition of Notch signalling and promotes differentiation of OPCs at the cost of other GFP-negative cells, which do not survive in the absence of growth factors. Although these compounds were not explored further in this study, in future it would be worth exploring whether combinations of Quabain and Gamma-Secretase might identify an improved protocol for OPC generation from NS cells or pluripotent stem cells. Further studies would be needed to establish the role of the identified small molecules and their impact on cell proliferation, differentiation ability and survival.

4.4 Summary

We have demonstrated the utility of PG1-1 cells for live cell image-based chemical screening. Conditions for screening were optimised and 463 different known

pharmacologically active compounds were assessed for their ability to influence OPC lineage specification. Five molecules effective at blocking activation of PDGFRα: two HDACi (Apicidin, HDACi III), two NFkB inhibitors (Withaferin, NFkBi) and a polyether antibiotic and ionophore (Nigericin) were identified. Chemical screening for agents that drive OPC differentiation to O4-positive oligodendrocytes identified two molecules which could be investigated further, quabain (increased cell survival) and gamma-Secretase Inhibitor IX (selective survival of OPCs). No compounds were identified that promoted OPC specification.

Chapter 5 Characterisation of the cellular responses of newly identified small molecule modulators of NS cell differentiation

5.1 Introduction

Compounds identified following the screening of the StemSelect and InhibitorSelect chemical libraries targeted a range of different biochemical pathways/processes. Apicidin and HDACi III act as histone deacetylase inhibitors. Withaferin and NFkBi block NFkB pathway (Withaferin has also been shown to disrupt intermediate filaments such as Vimentin), while Nigericin is an ionophore and a polyether antibiotic.

I investigated the role of molecular pathways affected by the identified small molecules, with the focus on neural development, cell and cancer biology. This allowed me to further understand what happens to mouse neural stem cells at different stages of neural differentiation, but also to put it into perspective for GBM differentiation studies.

5.1.1 HDAC signalling

HDACi, block histone deacetylation, and together with histone acetyl transferases (HATs) alter the packaging of DNA into chromatin. The reversible modification of core histones' terminal process is a major epigenetic mechanism used to regulate gene transcription in eukaryotic cells. HATs acetylate lysine residues in core histones, which leads to a more open, transcriptionally active chromatin. HDACs remove the acetyl groups from the lysine residues, which results in a more condensed, and therefore less transcriptionally active chromatin. HDACi can block this process, locking histones into a permanently hyperacetylated and hence active state (Dokmanovic, Clarke et al. 2007). Several HDACi

have been shown to act as cytostatic agents inhibiting proliferation of tumour cells, triggering cell cycle arrest, apoptosis or differentiation. Currently there are 18 HDACi known to human. They are categorised into four classes, which are based on their homology of accessory domains to yeast HDACs.

The process of histone acetylation and de-acetylation therefore indirectly affects non-histone proteins, including transcription factors by altering their ability to be turned on or off. The effect on activity can vary with enhanced activation or repression being a possibility. Moreover, acetylation can affect non-histone transcription factors in a direct manner as these molecules are known to be modified by acetylation (Yang and Seto 2007). A well known example of a non-histone protein that is regulated by acetylation is p53. Therefore interpretations of the phenotypic responses of cells to HDACi are not straightforward in terms of the specific underlying molecular pathway.

A sequential activity of specific HDACs could be important for the differentiation of distinct populations of glia and neurons. Neural stem cells and progenitors have been shown to switch expression of HDAC1 and HDAC2 as they commit to a neurogenic lineage. Administration of the HDACi valproic acid (VPA) decreases the production and differentiation of cells in the dentate gyrus, rostral migratory stream and the olfactory bulb (Foti, Chou et al. 2013). Deletion of HDAC1 or HDAC2 in developing neurons results in severe hippocampal abnormalities and lethality by postnatal day 7 (Montgomery, Hsieh et al. 2009). Interestingly, another study showed that VPA induced neuronal differentiation of adult hippocampal neural progenitors and inhibited differentiation of astrocytes and oligodendrocytes. Hsieh et al. observed upregulation of neuron-specific genes, including NeuroD, a neurogenic basic helix-loop-helix transcription factor (Hsieh, Nakashima et al. 2004).

5.1.2 NFkB signalling

NFkB is a protein complex controlling transcription of DNA. It forms a cellular response to stimuli like stress, free radicals, bacterial and viral antigens, and cytokines (Gilmore 2006). There are five mammalian NFkB family members, which are divided into two classes. They share a structural homology with the retroviral oncoprotein v-Rel (Rel homology domain in the N-terminus) and hence they can be classified as NFkB/Rel proteins (Gilmore 2006). Class II NFkB, including RelA, RelB and c-Rel, have a transactivation domain in the C-termini. Class I molecules are synthesised as large precursors, called p105 and p100, which are then processed to form mature NFkB subunits, p50 and p52. This process is mediated by the ubiquitin/proteasome pathway, where ankyrin repeats of the C-terminal are selectively degraded (Karin and Ben-Neriah 2000). Moreover, class I molecules are considered to be transcriptional repressors as they have no intrinsic ability to activate transcription.

NFkB belongs to the family of "rapid-acting" primary transcription factors, which are present in cells in an inactive state and become activated without a need for a new protein synthesis. The signalling pathway can be activated by a variety of stimuli, such as tumour necrosis factor α (TNFα), reactive oxygen species (ROS) and cocaine (Chandel, Trzyna et al. 2000).

NFkB dimers in unstimulated cells are bound by Inhibitors of kB (IkBs), which contain multiple copies of ankyrin repeats. IkBs mask nuclear localisation signals (NLS) of NFkB and keep them inactive in the cytoplasm (Jacobs and Harrison 1998). Activation of a IkB kinase (IKK) leads to degradation of IkB proteins and release of the NFkB complex. IKK is composed of a heterodimer of catalytic subunits IKKα and IKKβ with a regulatory protein called NFkB essential modulator (NEMO), also known as IKKγ.

IKK phosphorylates two serine residues in IkB regulatory domain, leading to their ubiquitination, followed by degradation in a proteasome. Once released, the NFkB complex is free to enter the nucleus and activate expression of genes, which can then trigger the physiological response. Moreover, the NFkB triggers expression of its own repressor, IkBα leading to reinhibition of NFkB and its inhibition in the cytoplasm (Nelson, Ihekwaba et al. 2004).

Activation of the NFkB/RelB:p52 dimer, described as a non-canonical pathway, can be initiated by some differentiating or developmental stimuli, including B-cell activating factor (BAFF), which leads to activation of signals needed for formation and maintenance of B cells or lymphotoxin B, which leads to activation of an inflammatory response. This pathway uses an IKK complex that comprises the two α subunits, but not NEMO. Ligand-induced activation of NFkB-inducing kinase (NIK) leads to phosphorylation and activation of IKKα complex by NIK. IKKα complex can then phosphorylate p100 leading to the processing and liberation of the p52/RelB heterodimer, which can actively regulate gene expression in the nucleus (Bonizzi, Bebien et al. 2004). In conclusion, the non-canonical pathway depends on NIK processing of p100 into p52, while the canonical pathway relies upon NEMO-IKK degradation of IkB molecules.

Members of the NFkB family are expressed postnatally in the subventricular zone and in the rostral migratory stream. RelA and p50 are expressed in migrating neuron precursors, radial glial cells and a population belonging to the astrocytic lineage. RelB component of the pathway is expressed only in migrating neuron precursors (Denis-Donini, Caprini et al. 2005). NFkB pathway is constitutively activated in glutamatergic neurons (Kaltschmidt and Kaltschmidt 2009). In Schwann cells NFkB is involved in the myelination process. It is

highly expressed in pre-Schwann cells and then progressively declines as cells differentiate (Nickols, Valentine et al. 2003).

5.1.3 Ionophore signalling

Ionophores are lipid-soluble molecules used to transport ions across the lipid bilayer of the cell membrane. There are two classes of ionophores, chemical compounds and channel formers. Chemical compounds bind to a particular ion and shield its charge from the surrounding environment and facilitate the crossing of the hydrophobic interior of the lipid membrane. Channel formers introduce a hydrophilic pore into the cell membrane and allow ions to pass through without the contact with the membrane's hydrophobic interior.

Antibiotic properties of ionophores come from the fact that they can disrupt the transmembrane ion concentration gradients, which are crucial for the survival and proper functioning of microorganisms. A number of ionophores were identified and described, including Gramicidin D, acting on H^+, Na^+ and K^+, which was obtained from the soil bacterial species Bacillus brevis. This molecule is active against Gram-positive bacteria, except the Gram-positive bacilli and against select Gram-negative bacteria.

Nigericin is derived from Streptomyces hygroscopicus and is structurally and functionally similar to another antibiotic known as monensin. Monensin is isolated from Streptomyces cinnamonensis. It preferentially forms complexes with monovalent cations like Li^+, Na^+ and K^+ and can transport them across lipid membrane in an electroneural (non-depolarizing) exchange (Pinkerton and Steinrauf 1970). Sodium ions can be transported in both electrogenic and electroneural manner (Huczynski, Janczak et al. 2012).

Nigericin acts as a H^+, K^+, Pb^{2+} ionophore, mostly as an antiporter of H^+ and K^+. It is active against the Gram-positive bacteria and inhibits the Golgi function in eukaryotic cells. Of great interest to my studies, Nigericin was identified in a high-throughput chemical screen as one of the molecules inhibiting growth of breast cancer stem cells (Gupta, Onder et al. 2009). It inhibits mammary tumour growth *in vivo* and induces increased epithelial differentiation of tumour cells. The ionophore is also effective in the treatment of nasopharyngeal carcinoma (NPC). A study using cell lines with high and low proportion of CSCs showed that Nigericin selectively targets CSCs and makes them sensitised to cisplatin, a widely used clinical drug (Deng, Liang et al. 2013). Nigericin was shown to effectively sensitize glioma cell lines to TRAIL-mediated apoptosis via ER stress, CHOP-mediated DR5 upregulation and c-FLIP downregulation (Yoon, Kang et al. 2013).

In a study of chronic lymphocytic leukemic Nigericin was identified as a potent inhibitor of the Wnt signalling cascade (Lu, Choi et al. 2011). In Wnt overexpressing HEK293 cells, Nigericin blocked the phosphorylation of the lipoprotein receptor-related protein 6 (LRP6) and induced its degradation. Expression of Wnt target genes including LEF1, cyclin D1 and fibronectin was down-regulated, while normal human peripheral blood lymphocytes resisted the toxicity (Lu, Choi et al. 2011). Canonical Wnt signalling through beta-catenin pathway prevents oligodendrocyte differentiation from progenitor to an immature state. Using a Wnt antagonist Shimizu et al. observed an increase in the number of immature oligodendrocytes in the spinal cord explant culture (Shimizu, Kagawa et al. 2005).

5.2 Results

5.2.1 Investigation of drug concentrations

The chemical screening was performed at only one drug concentration (1uM for StemSelect and 100nM for InhibitorSelect). I further tested a range of concentrations: 0.05uM-10uM for compounds identified from the StemSelect library and 1nM-1uM for the NFkBi.

I used both live cell imaging and viable cell count to determine the effect of different drug concentrations on cell survival. The process of viable cell count was automated, to reduce potential user bias. Cells were counted using ViCell Cell Viability Analyzer (Beckman Coulter) (Figure 20A and B).

Concentration >1uM led to cell death, <1uM did not affect the morphology and GFP inhibition to the same extent as the original screening concentration. Therefore, I used 1uM as a concentration used for validation of Apicidin, HDACi III, Nigericin and Withaferin. For NFkBi I picked two concentrations: 100nM and 1uM whenever possible.

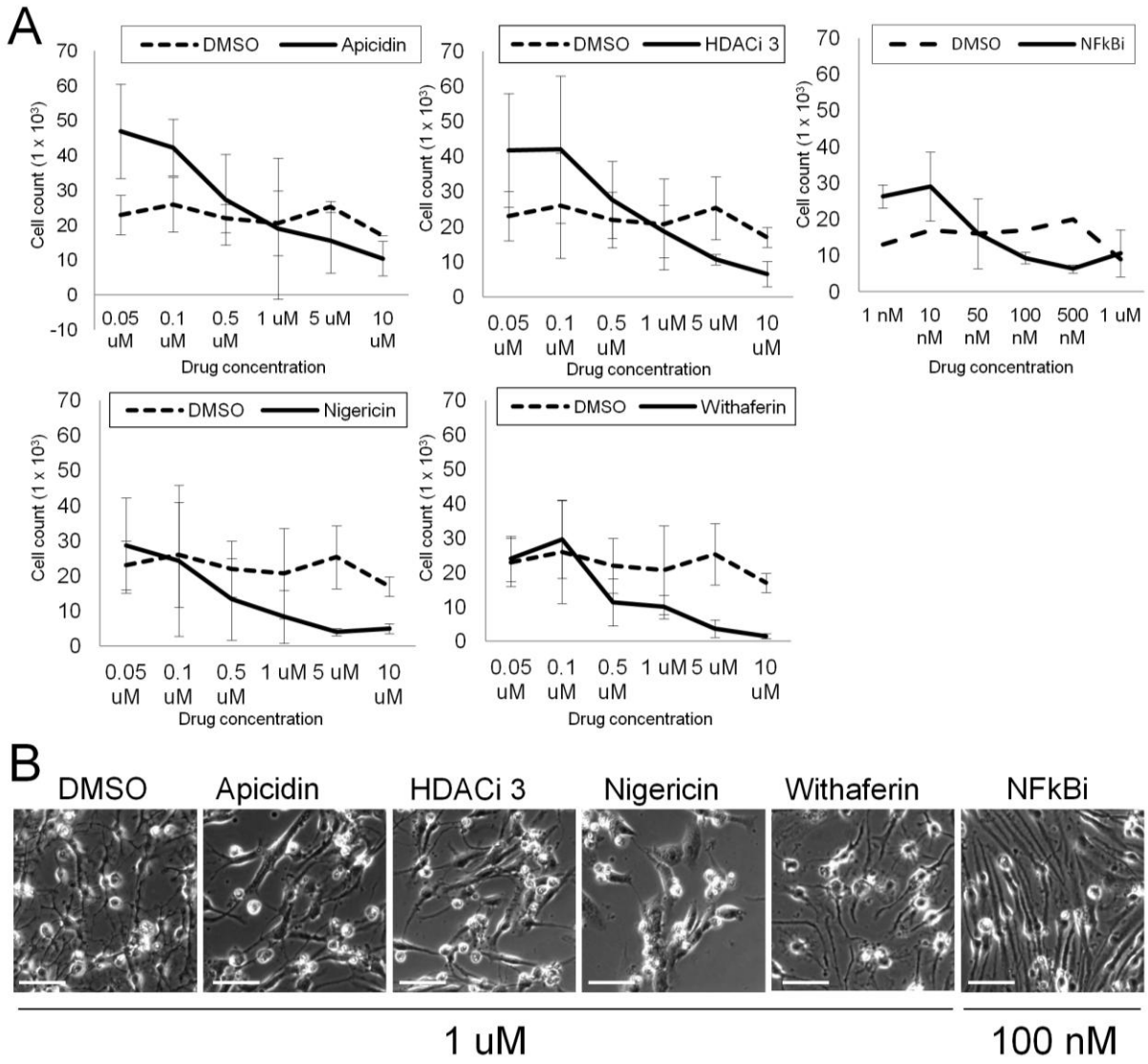


Figure 20. Higher concentrations of small molecules affect viability of NS cells. (A) Compounds showed a cytotoxic effect on cell viability especially at doses >1uM; (B) Microphotographs showing live, drug-treated cells at the concentration used in the chemical screening; Scale bars, 40μm;

5.2.2 Is the emergence of GFP-positive OPCs ablated due to blockade of induction or selective killing of OPCs?

As compounds identified in the chemical screening were effective at blocking activation of GFP reporter I tried to determine the explanation for loss of GFP. At least two possibilities could account for lack of GFP: induction of PDGFRα gene expression may fail to occur, or alternatively GFP reporter induction may occur, but cells are then rapidly be killed. To explore these two possibilities I first investigated whether GFP-positive cells are selectively killed by the inhibitors. It was possible to answer this question by firstly differentiating NS cells into GFP-positive OPCs, followed by addition of the compounds (Figure 21B). This contrasts the original chemical screening design, where NS cells triggered to differentiate in the presence of inhibitors (Figure 21A).

After adding drugs at day 3 to cultures containing ~15% GFP cells, I observed that although there is a reduction in GFP numbers following exposure to inhibitors, there was not a complete ablation of GFP as observed when inhibitors were given at the onset of differentiation (Figure 22). Compounds belonging to the HDACi family did appear to reduce percentage of GFP-positive cells after 48 (day 6 total) and 72 hours (day 7 total) and therefore both a blockade in induction, and some sensitivity of GFP cells to the presence of HDACi may occur. NFkBi in both instances caused unspecific cell cytotoxicity and cell death and I was not able to validate its potential. Nigericin appeared to act similarly to NFkBi, causing cytotoxicity, with reduced percentage of GFP-positive cells (Figure 21C). The subject of drug cytotoxicity and possible explanations are discussed further in the Discussion part of this thesis.

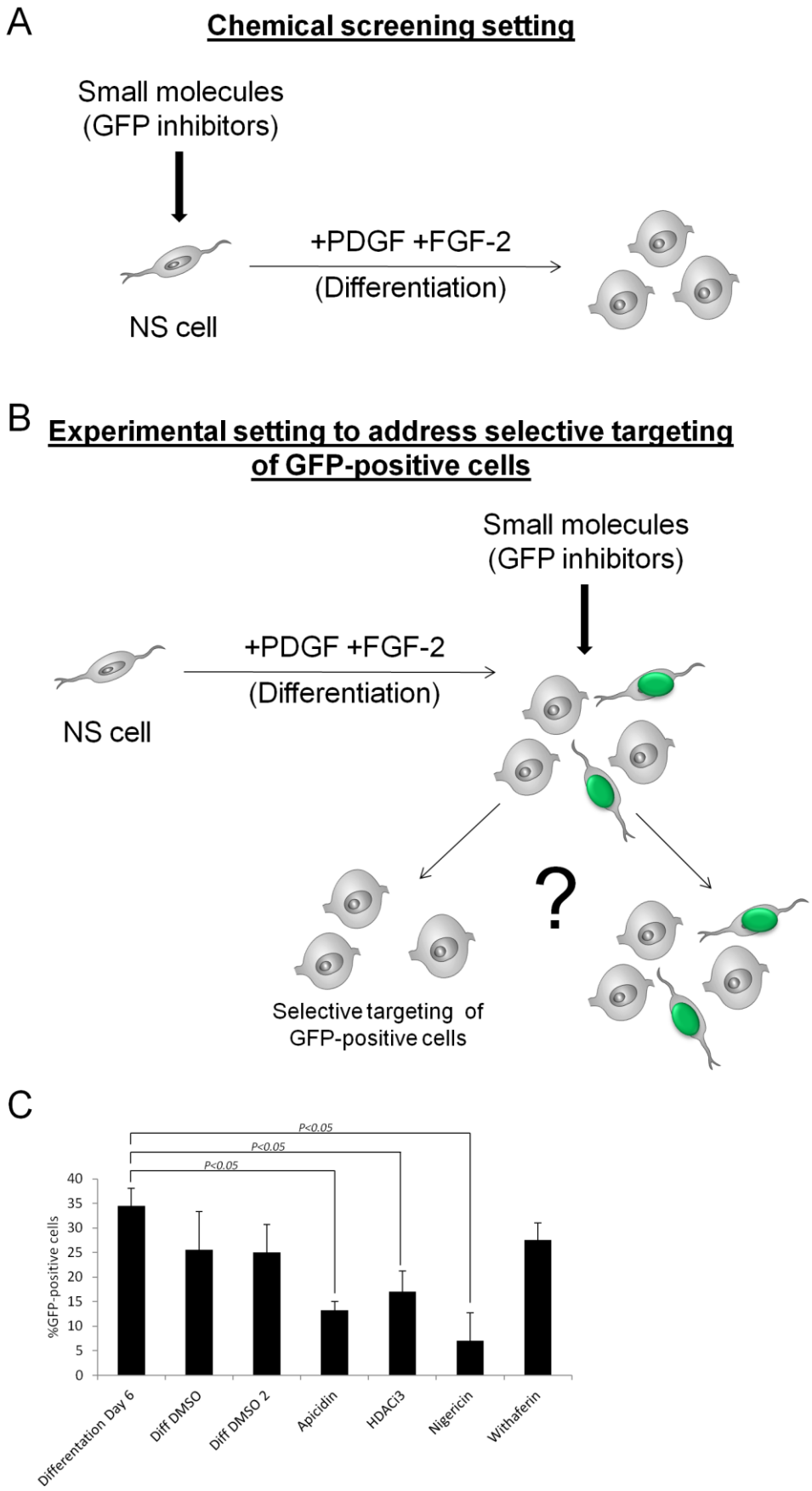


Figure 21. Small molecules can selectively reduce the number of GFP-positive cells. (A) Chemical screening setting; Drugs were added to the NS cells at the induction of differentiation; (B) Experimental setting to address selective targetting of GFP-positive cells; Cells were allowed to differentiate into GFP-positive OPCs at which point drugs were added to estimate their potential in selective targeting of these cells; (C) Results of a flow cytometry analysis showing that Apicidin, HDACi III, and Nigericin selectively reduced the number of GFP-positive cells when compared to a control (Differentiation Day 6; $p<0.05$; double-sided t-test);

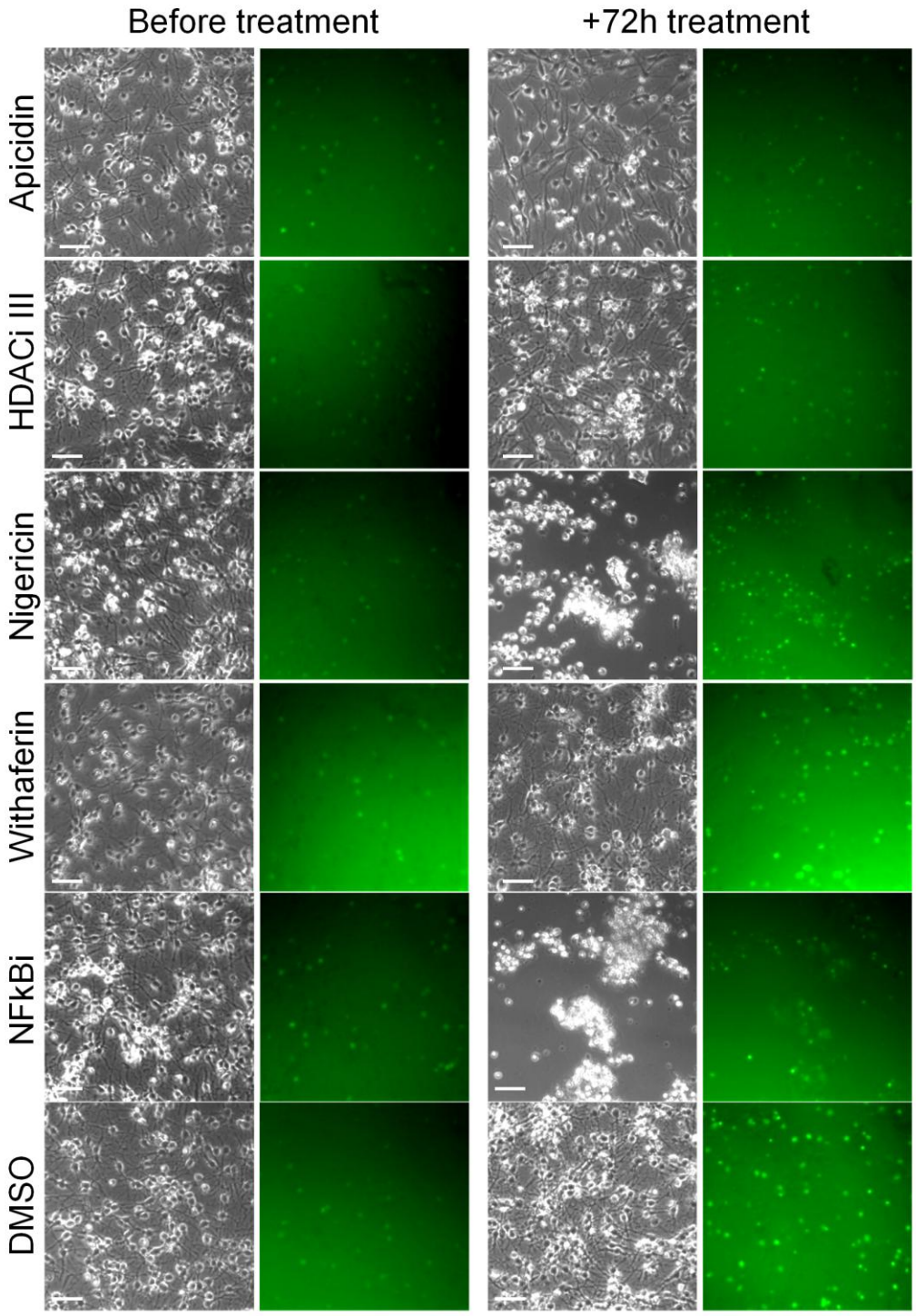


Figure 22. Selective targeting of GFP-positive cells was not observed using live cell imaging. Nigericin and NFkBi showed unspecific cytotoxic effect on cell proliferation; Scale bars, 40µm;

5.2.3 Differentiation potential of GFP inhibitors

Loss of GFP may be explained by cells being forced to become terminally differentiated O4-expressing oligodendrocytes (hence PDGFRα-negative). We ruled out this possibility, as no GFP cells were activated at early stages in the screen, and the morphology of the inhibitor-treated cells did not indicate oligodendrocytes. Alternative possibilities to explain lack of GFP induction would be that either cells are 'stalled' in an immature NS cell state (albeit no-proliferative), or that they are forced to differentiate along alternative lineages, either astrocyte and/or neuronal (Figure 23). Time-lapse movies of the differentiating cells suggested neuronal differentiation was unlikely, and that astrocyte differentiation may be occurring. Therefore we stained the inhibitor treated differentiating cultures with neuronal and astrocyte markers (TuJ1, GFAP). Alongside experiments in the differentiation setting we investigated the role of the small molecules in the proliferating setting with EGF present in the media to understand whether the effect we observed was 1) related to the culture conditions or 2) independent of the growth factors used.

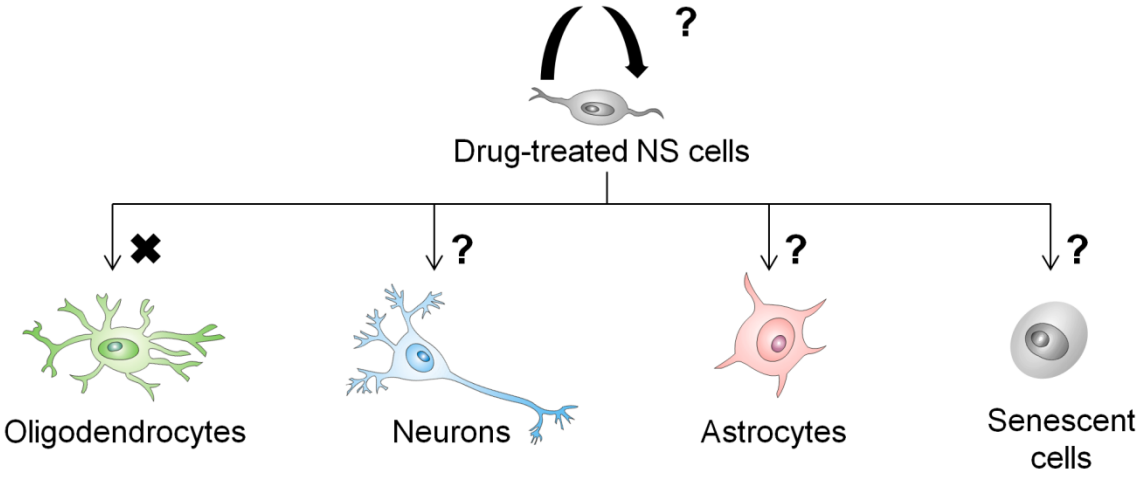


Figure 23. A schematic showing possible fate of drug-treated NS cells. GFP inhibitors block activation of oligodendrocyte differentiation program. These cells could self-renew as NS cells, become senescent or differentiate into other neural cell types: neurons or astrocytes;

We found that inhibitor-treated cells did not differentiate into astrocytes or neurons, as shown by expression levels of GFAP (astrocytic marker) lower than in DMSO-controls and BMP-4-treated cells (positive control). Expression of TUBB3 (detected by TuJ1 immunostaining), a neuronal marker, was not observed (Figure 24A and B). Two different concentrations of DMSO were used in these experiments. DMSO control called "DMSO 2" corresponds to the 1uM concentration of NFkBi, which has 10 times the concentration of DMSO used in the chemical screening. These data suggested that inhibitors were not blocking OPC induction by diverting cells to an alternative lineage. Also, these compounds did not trigger differentiation when added to proliferating NS cells in the presence of EGF and FGF-2 (proliferation conditions) (Figure 24C).

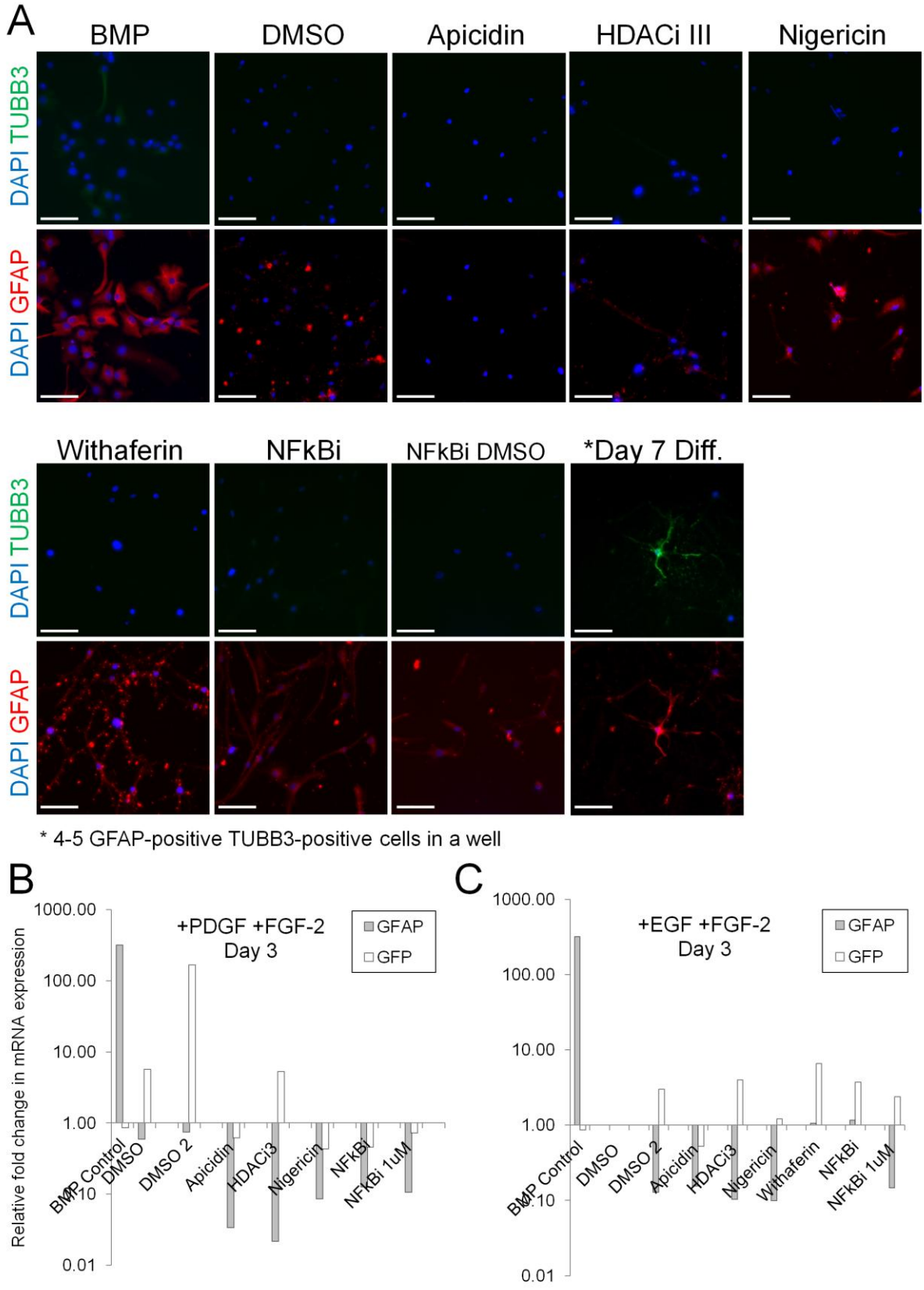


Figure 24. Drug treatments do not lead to neural differentiation.(A,B) Immunofluorescence and qPCR analysis do not show increase in astrocytic (GFAP) or neuronal (TUBB3) markers during drug treatments when compared to GFAP (astrocytic marker) or TUBB3 (neuronal marker); Scale bars, 60µm; (C) Drug treatments do not lead to astrocytic differentiation in proliferating conditions (+EGF, +FGF-2);

An explanation for the inhibitor block could be that cells are arrested in a senescent NS cell state. To explore this further I next analysed expression of p21 and p53 in both proliferating and differentiating drug-treated cells (Figure 25A and B). I observed upregulation of p21 and p53 with all drug-treatments, irrespective of growth factors used. This data suggests cells trigger stress response pathways in response to inhibitors and that this may interfere with OPC lineage commitment. I did not observe any change in the expression of Olig2, BLBP and Nestin, compared to normal differentiating controls, suggesting that cells can begin to exit the NS cell state, but fail to upregulate lineage programs (Figure 25C&D).

Increased levels of p21 led me to investigate whether there was an accompanying increase in beta-galactosidase levels, a marker often used to indicate senescence (most often in fibroblast cultures). Cells were differentiated for a period of five days to allow enough time for the cellular response and activation of intracellular pathways. Although I observed that the NFkBi in particular triggered increased frequency of beta-gal cells, suggesting it promotes cell senescence in a subset of cells, the other compounds showed only modest or no increases in beta-gal (Figure 25E). Cell viability showed that cells are able to recover from the drug treatment and were able to start doubling again when re-exposed to self-renewal conditions (EGF and FGF), indicating that a significant proportion of the cells were not permanently arrested/senescent (Figure 25F). Altogether these data indicate that the effects of identified inhibitors in blocking OPC induction occur via an arrest of differentiating cells at early stages of differentiation and upregulation of p21, which effectively blocks differentiation to neuronal or glial fates. Immunofluorescence analysis showed that HDACi can effectively reduce the number of proliferating cells, observed as a reduction in Ki67, even in the presence of a potent mitogen like EGF (Figure 25G).

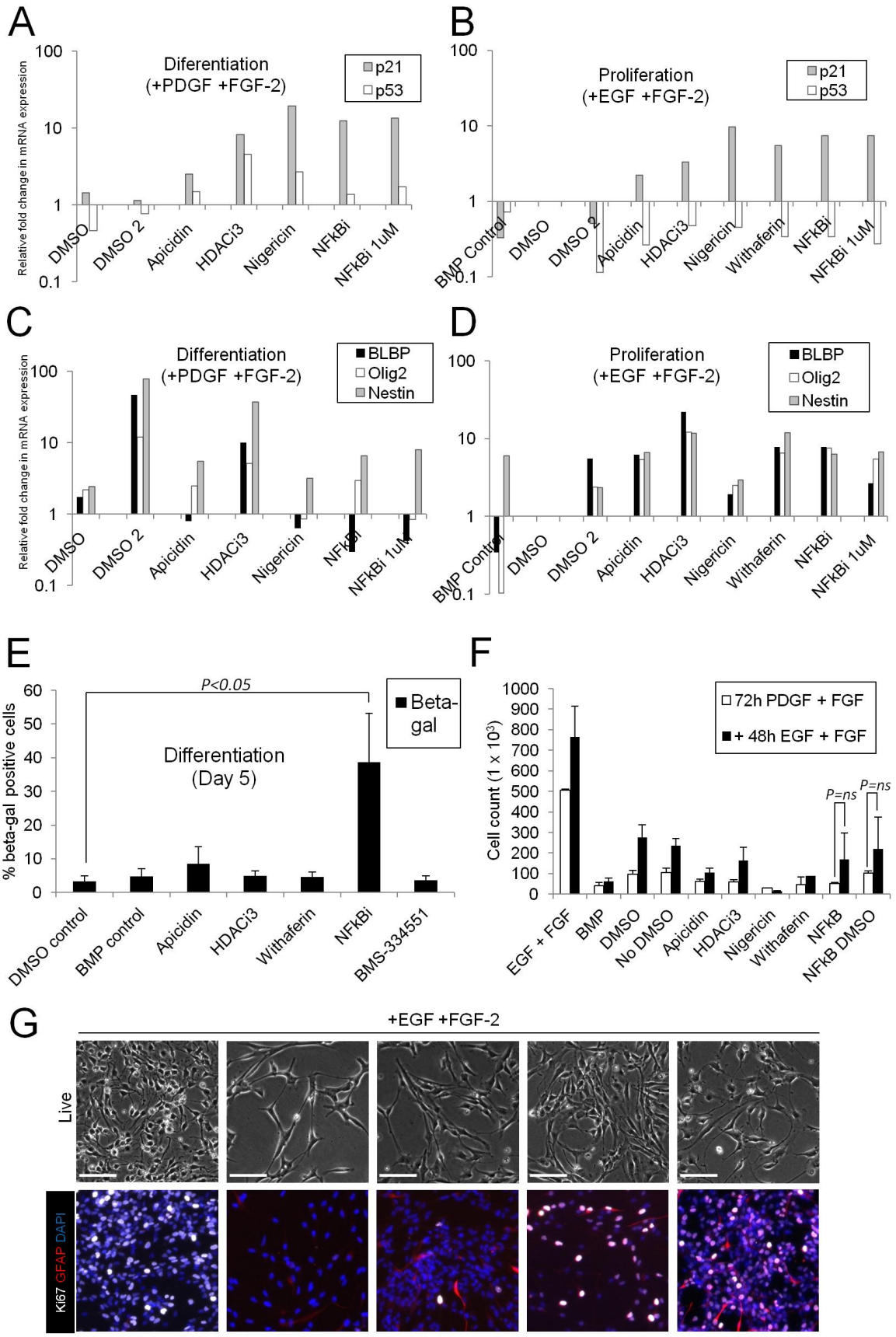


Figure 25. Drug treatments cause cellular stress. (A,B) Levels of p21 were increased in all drug treatments, in both proliferating and differentiating conditions; p53 expression was increase in differentiating conditions, but not in proliferation; (C,D) Levels of NS cell markers remain stable in proliferating and differentiating conditions; (E) NFkBi causes significant cell senescence as indicated by beta-gal levels ($p<0.05$; double-sided t-test); (F) Drug treated cells can recover and continue proliferating when EGF is reintroduced in the media, however the increase in cell proliferation was not significant for both NFkBi and the DMSO control (double-sided t-test); (G) HDACi (Apicidin, HDACi III) can reduce the number of proliferating NS cells despite the presence of EGF as indicated by the reduction in Ki67 expression; Scale bars, 75µm;

5.2.4 Validation of NFkBi and its potential to trigger cell senescence

It is not uncommon in the field of chemical screening to identify compounds, which show their activity through a non-specific 'off target' effect. This is an often inherent feature of the shared features of enzymatic domains (e.g. kinases). Limited knowledge about the given compound and its action through pathways may therefore undermine efforts to validate the molecular target and pathway of newly identified candidates from HCS.

The NFkB inhibitor (NFkBi, also known as 545380-34-5, PubChem ID 509554) identified from the InhibitorSelect chemical screen may have promoted cellular senescence, based on the dramatic increase in beta gal staining. NFkB signalling is important in a range of human diseases, and therefore over the years many NFKB inhibitors have been developed, including some which have been explored in the clinic.

We therefore compared responses of the identified NFkBi with a frequently used inhibitor of NFkB pathway; a molecule known as BMS-345541 (BMS). To monitor NFkB signalling I obtained an NFkB-GFP signalling reporter, which comprises multiple repeats of the target sequence upstream of GFP. PG1-1 cells were transfected (using lipofection) with the NFkB-GFP reporter and then exposed to the two inhibitors. I analysed cells, which were treated as an adherent cell culture, but also cells which were in suspension. This was done for two reasons, firstly to investigate whether NFkBi affects cell attachment and secondly to compare its effect on cells in suspension, which tend to clump and form neurospheres, reducing exposure of the drug to the more internalised cell layers.

I first demonstrated that the reporter construct was working appropriate by transfecting control cells, and indeed I observed substantial numbers of GFP expression (from the NFkB promoter). This GFP expression was reduced in the presence of BMS, confirming that all GFP signal is the result of NFkB signalling. Importantly, however, when treated with the NFkBi identified in our screen, I did not see any reduction in the GFP levels, suggesting that NFkB is not the molecular target of the inhibitor molecule described in the InhibitorSelect library as NFkBi (Figure 26A&B). Therefore the specific molecular target and pathway for NFkBi is likely not operating through the NFkB signalling pathway to mediate the effects on GFP induction. The molecular target was not pursued further given the time constraints.

Consistent with this, while the NFkBi was effective at reducing the number of proliferating PG1-1 cells, both in suspension and as an adherent cell layer, after 48 and 72 hours of treatment (Figure 26C-F), we did not observe similar effects for BMS-345541 activity. In summary, the NFkB signalling pathway, while active in proliferating NS cells, does not therefore seem to be the important molecular pathways targeted by the NFkBi.

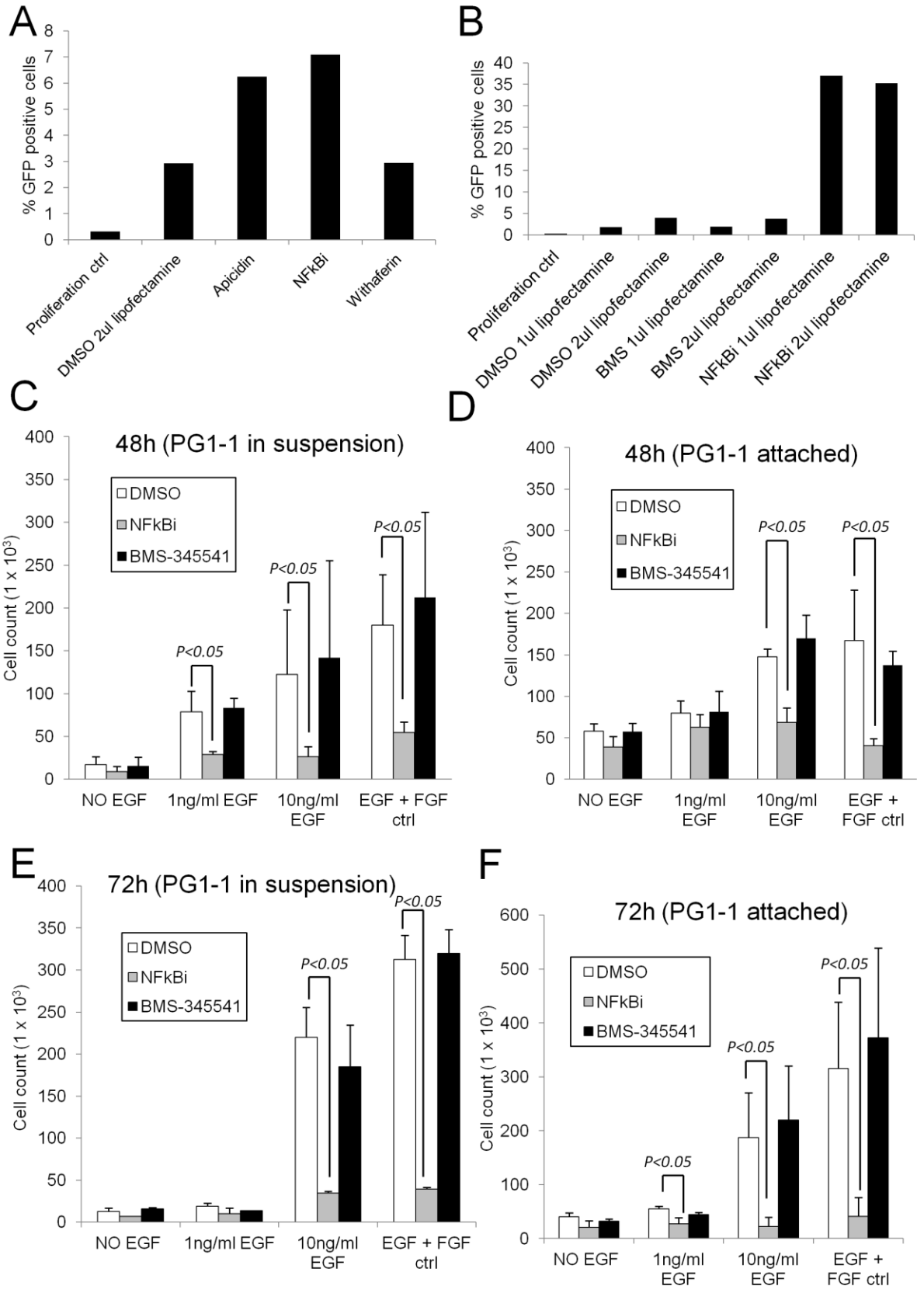


Figure 26. Validation of NFkBi potential and molecular target. (A,B) Cells with overexpressed NFkB were treated with Apicidin (negative control), Withaferin and BMS-345541 (positive controls) and NFkBi identified in the chemical screening; Reduction in NFkB levels was not observed in the NFkBi-treated cells; (C,D,E,F) NFkBi significantly reduces the number of proliferating cells after 48 and 72 hours of treatment ($p<0.05$; double-sided t-test); The process is not affected by culture conditions (attached cells vs cells in suspension); The response of BMS-345541 and NFkBi show no correlation;

5.2.5 Effects of inhibitors on human neural stem (NS) cells and glioblastoma-derived NS cells (GNS)

The experimental goal of this project was to identify compounds, which could be used in the treatment of GBM. The selected compounds did not show potential to be used in a differentiation therapy, but they had a cytostatic effect on mouse cells and in case of NFkBi appeared to trigger cell senescence. Thus, we decided to investigate the effect of these small molecules on human GNS in hope that the GNS lines would be more sensitive to the treatment than control human NS cells. We focused on two lines, called G7 and G144, which were derived from primary human glioblastomas.

When NFkBi was used to treat GNS cell lines I observed a similar trend as in the mouse NS cell line. In particular, G7 cell line appeared to be more responsive to the NFkBi treatment in the presence of EGF and FGF-2 (Figure 27A-D). The activity was dependent on the presence of the growth factors as none of the lines tested were responsive to the treatment in their absence. I did not observe an increased efficacy with 1uM concentration of NFkBi, which could validate 100nM concentration as a minimum effective dose of this drug. This however requires additional validation.

Phase images of live cells show that cells did not change the morphology and there was no sign of rounded apoptotic cells. However, I observed a reduction in proliferation of the control human foetal cell line, named CB660 (Figure 27E&F).

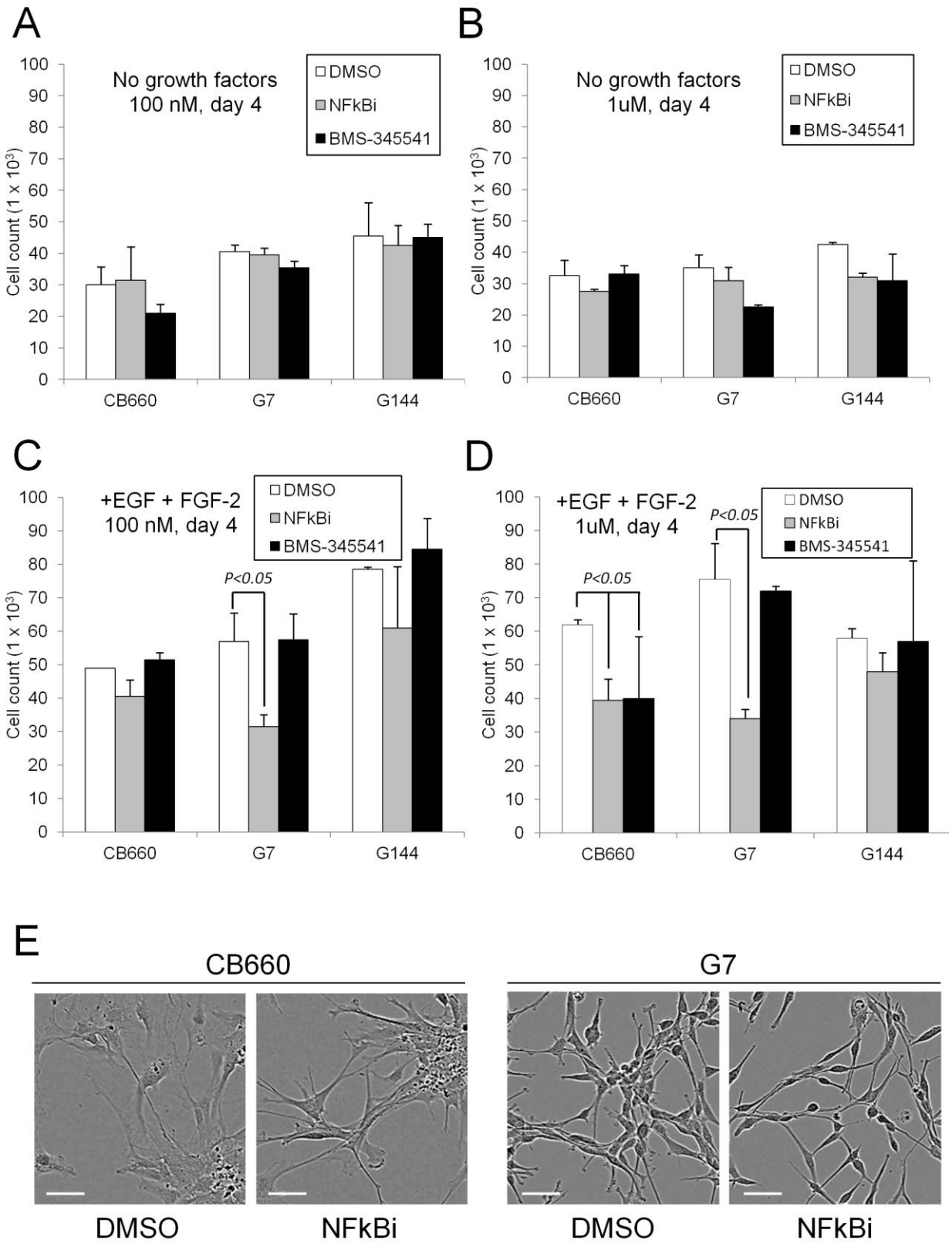


Figure 27. NFkBi reduces proliferation of GBM cell lines. (A,B) In the absence of growth factors, GBM cells show decreased level of proliferation; (C,D) NFkBi at 100nM can reduce the number of proliferating GBM cells (G7 and G144), while NFkBi 1uM affects proliferation of the control human foetal cell line (CB660) ($p<0.05$, double-sided t-test); (E) NFkBi does not lead to cell death as shown in the microphotographs of live cells; Scale bars, 40μm;

I made a similar observation at a later time point (day 7) (Figure 28A-D). CB660 cell line was not proliferative, so I decided to use another foetal line, named U5, which showed that NFkBi at 100nM is able to inhibit the proliferation of G7 GNS cell line without cell death in the U5 line. The concentration of 1uM caused inhibition of foetal lines (Figure 28E and F).

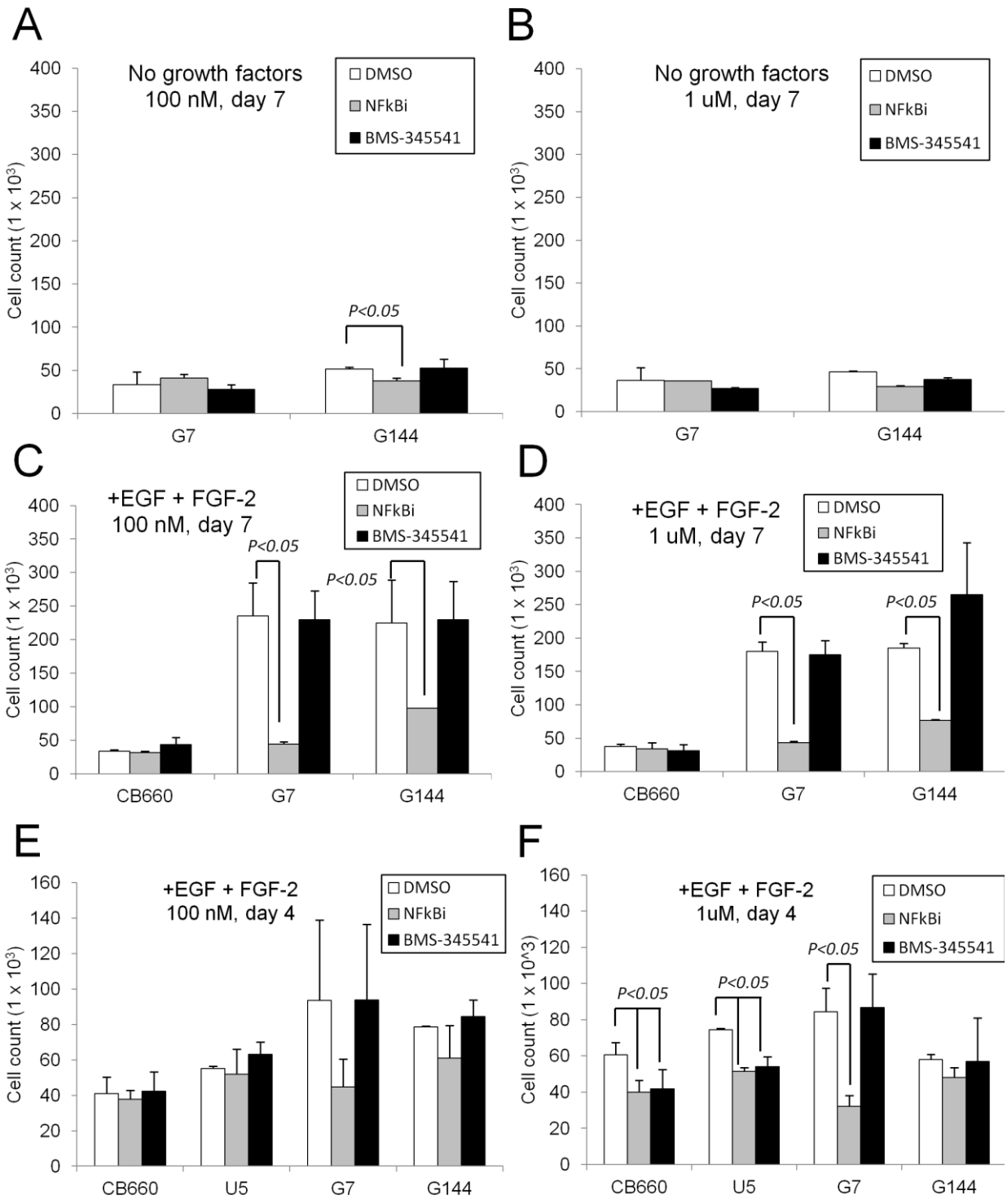


Figure 28. 100Nm NFkBi reduces proliferation of GBM, but not control cell lines in culture. (A,B) On day 7 of differentiation GBM and control lines showed reduced proliferation in the absence of growth factors; (C,D) G7 and G144 GBM cell lines showed reduced proliferation on day 7 of the drug treatment with NFkBi; (E,F) Control human foetal cell line (U5) is not affected by the 100nM NFkBi treatment, however it shows a reduced proliferation at the higher NFkBi concentration ($p<0.05$; two-sided t-test performed);

Interestingly, when other identified inhibitors identified from the screen were tested on human cell lines I observed that two molecules, Apicidin and Nigericin are effective inhibitors of proliferation. Unfortunately, however, they also inhibited proliferation of CB660 cells with similar potency, suggesting they might not have any specific value in targetting glioma stem cells (Figure 29A and B).

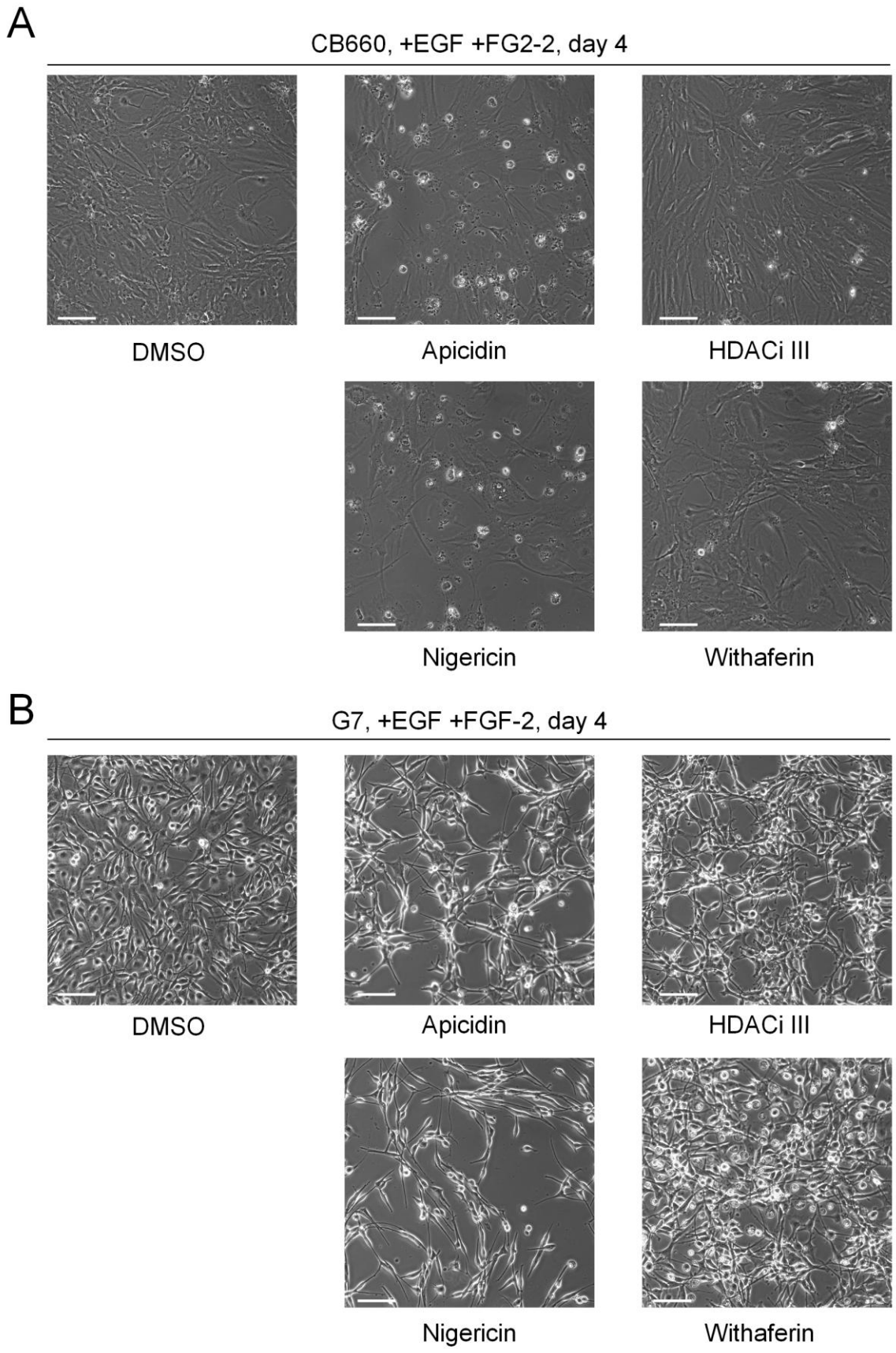


Figure 29. Effect of GFP inhibitors from the StemSelect library on the proliferation of human foetal and GBM cell lines. Apicidin and Nigericin reduce the number of proliferating GBM cells (B; G7), but they also affect proliferation of the control human foetal cell line (A; CB660); Scale bars, 80μm;

5.3 Discussion

Validation of inhibitors identified in the chemical screening shows the importance of a clear objective of the work, as molecules perform differently depending on cell lines used (mouse vs human), growth factors and readout measurements, e.g. Ki67 levels or confluency measurements.

I observed that HDACi can selectively reduce the percentage of GFP-positive cells, though not completely. As my experiments show, the PDGFRα activation does not indicate commitment to oligodendrocyte lineage. Thus, HDACi could reverse the PDGFRα status of cells and turn them back into NS cells. HDACs are important for oligodendrocyte lineage progression. A deletion of both HDAC1 and HDAC2 results in stabilisation and nuclear translocation of beta-catenin, which represses Olig2 expression and oligodendrocyte lineage progression (Ye, Chen et al. 2009). Unspecific cytotoxicity of NFkBi is further discussed in General Discussion.

Identified molecules did not lead to progression of any of the neural lineages. Instead they caused cell stress, as increase in level of p21 was observed. Apicidin was shown to activate p21 in human prostate carcinoma cells. It causes accumulation of acetylated histones H3 and H4 which leads to p21 transcription (Kim, Lee et al. 2001). In a similar setting of prostate cancer cells also Withaferin was shown to upregulate p21 and cause G2/M cell cycle arrest (Roy, Suman et al. 2013).

Activity of NFkBi identified from the InhibitorSelect library does not correspond with the one of BMS-345541 (BMS), a well established, highly selective inhibitor of IKK. By blocking the IKK, BMS leads to reduction in NFkB activity (Burke, Pattoli et al. 2003). This

suggests a different molecular target for the compound identified and described as NFkB inhibitor in the chemical library. As literature describing this molecule is sparse other comparative methods of validation could be used to identify the molecular target of the drug. NFkBi is the most promising compound identified in the chemical screening as it showed specificity against human GBM cells, even though initial experiments using mouse NS cells did not show reduction in Ki67 expression.

Effect of HDACi was consistent in both mouse and human cell lines, as cells exited cell cycle. However, a reduction in proliferation of control human cell line was observed. This could be still beneficial in a GBM treatment as no apoptosis in control lines was observed. Two HDACi, vorinostat and depsipeptide, were approved for the treatment of refractory cutaneous T cell lymphoma. Another HDACi, valproic acid, was tested in GBM treatment along with temozolomide and radiation therapy (Shabason, Tofilon et al. 2011). Another study showed that a combination of bortezomib, a proteasome inhibitor, with HDACi has a synergistic cell killing effect on primary and conventional GBM cell lines (Asklund, Kvarnbrink et al. 2012). This could modify the way a chemical screening is performed, looking for a combination of an established treatment molecule, e.g. temozolomide, with chemical libraries of compounds in search of a synergistic effect and a better patient outcome.

5.4 Summary

Validation of the compounds identified in the chemical screenings was performed. Apicidin and HDACi III selectively target GFP-positive cells and reduce their proportion in culture. None of the compounds leads to neural differentiation programs, causing cell stress as shown by upregulated levels of p21 in proliferating and differentiating conditions and p53 in

proliferating conditions. Drug treatments do not affect levels of NS cell markers including BLBP, Olig2 and Nestin. NFkBi causes cell senescence as indicated by beta-Gal levels. HDACi reduce the number of proliferating NS cells as indicated by reduced number of Ki67-positive cells. A comparative analysis of NFkBi and BMS-345541, a well-established NFkBi inhibitor, showed no correlation in activity, suggesting a different mode of action for the compound described as 'NFkBi' in the InhibitorSelect library. 100nM NFkBi was effective at reducing the number of proliferating NS and GNS cell lines with a minimal effect on control human NS cell lines (U5, CB660). Apicidin and Nigericin can inhibit proliferation of GNS cells, but they also reduce proliferation of the control line.

Chapter 6 General Discussion

6.1 Mouse NS cell lines as a tool to model oligodendrocyte differentiation

Studies of developmental biology provide us with a wealth of information, allowing us to identify, describe and understand the function of the key genetic controls that underpin the formation of the central nervous system. However, *in vitro* studies provide a powerful complementary experimental platform that is well suited to defining cell intrinsic mechanisms of lineage choice and differentiation, and enable simplified biochemical analysis of underlying mechanisms. Knowledge of how to manipulate stem and progenitor cells *in vitro* will also be essential for cell based human disease modelling, and cell based therapies. This knowledge is also highly applicable to studies of human cancer.

Protocols have been developed to generate oligodendrocyte progenitors from multipotent neural stem cells, through oligodendrocyte progenitor cells and immature oligodendrocytes to fully functional oligodendrocytes expressing myelin basic protein. However, these protocols are highly variable in efficiency and typically unreliable. Here I have demonstrated that a differentiation protocol using PDGF and FGF-2 allows for a development of ~15% PDGFRα-positive OPCs. In a study using retroviruses to drive PDGF expression Assanah et al. observed that less than 20% of cells in a formed tumour mass express the GFP reporter, which corresponds with my findings of PDGFRα activation when NS cells are cultured *in vitro* in the presence of PDGF (Assanah, Lochhead et al. 2006). Therefore, both *in vitro* and in the context of cancer models, OPC specification seems to be restricted. In search of molecular pathways involved in OPC specification I have generated and validated in a useful PDGFRα-GFP reporter cell line and carried out chemical screening.

My results suggest that cell plating density is one of the most important factors affecting progression of NS cells into OPCs, and that this can be influenced by both autocrine and paracrine signalling. I observed a direct positive correlation between number of cells plated and percentage of OPCs in culture, higher plating density meaning higher percentage of OPCs in culture. My observation expands on the findings of Yang et al. where they showed the importance of density for maturation of oligodendrocytes as I show its relevance for the initial step of lineage progression, the activation of PDGFRα. Similarly to my findings, Nakatsuji and Miller also observed that the state of an OPC is reversible. Despite cell cycle exit and activation of p27(Kip1) when replated OPCs, I observed that cells can re-enter the cell cycle and revert to an NS cell state. In future studies it would be of interest to determine whether commitment to differentiation occurs at the later time point when O4 is activated and PDGFRα expression extinguished. O4-positive cells could be sorted using FACS and plated at different densities, in a presence of EGF. This could allow to answer a number of questions: 1) Do O4-positive cells respond to the plating density and EGF in a way similar to PDGFRα-positive OPCs? 2) Does the plating density affect the differentiation potential of O4-positive cells? 3) Do O4-positive cells divide in the presence of EGF? 4) Is the O4-positive state reversible, or does it represent a point of lineage commitment?

If results were promising, this would stimulate interest in using the O4 marker as an endpoint in chemical screening to identify molecules driving this process or by modifying the differentiation protocol, something which I have only explored briefly in this project. Use of FACS allows for selection of distinct cell populations.

The commitment to the oligodendrocyte lineage is particularly important from the perspective of the GBM differentiation therapy, where tumour cells could be potentially

locked into this more differentiated and less aggressive state. Cells would then become irresponsive to mitogenic growth factors like EGF. Thus, both progression of NS cells into OPCs and their commitment to the lineage are important to the success of the differentiation therapy. Indeed spontaneous differentiation into oligodendrocytes is a feature of subtypes of GBM – although it remains unclear whether these are truly postmitotic.

The plating density effect on the efficiency of OPC specification is likely to be related to the Notch signalling pathway. A neurosphere study by Grandbarbe et al. showed that a lack of Notch signalling decreased a number of PDGFRα-positive cells, leading to MAP2-positive neuronal cells (Grandbarbe, Bouissac et al. 2003). The Notch pathway defines cell fate decisions by interactions between cellular neighbours. A validation of Notch signalling in my *in vitro* system could be performed by firstly analysing the expression levels of Notch and its ligand proteins Jagged and Delta in both proliferating and differentiating conditions, followed by a similar analysis in the presence of a Notch inhibitor molecule.

Interestingly, the compound identified in the screening for O4 cells called a gamma-Secretase Inhibitor IX inhibits Notch signalling and promotes differentiation of OPCs, most likely at the cost of other GFP-negative cells. Further focus on the gamma-Secretase Inhibitor IX is clearly an area of interest in future studies.

6.2 Chemical screening – technical considerations

The chemical screening performed in this thesis was carried out as four independent experiments, performed over a 6-month span. Therefore, the identified molecules, particularly the multiple HDAC inhibitors that were identified, represent a clear and significant finding from my thesis. HDAC inhibitors clearly affect the ability of the

oligodendrocyte specification program to emerge. Although we did not identify chemicals that could stimulate increased OPC production, the progress that we have made in establishing the reporter cell lines and optimisation of the screening protocols and analysis pipeline now means that we could extend the assay to other libraries of compounds.

The HCS method described here allows for a screening of hundreds, or even thousands of compounds with a wide variety of functions ranging from inducers, activators and agonists to antagonists of cellular pathways and other modulators of cell fate, which can be used in studies of cellular survival, proliferation, migration, invasion, differentiation and fate determination. Potential improvement and future directions for the screening strategy could include adaptation to the 384-well plate system, which would make the whole process more time-efficient, as the time handling cells, plates and chemical libraries could be significantly reduced. Moreover, the 384-well plates require less volume, which in turn reduces the volumes of drugs used. Another practical advantage would be freeing of space in the imaging device, as one 384-well plate would replace four 96-well plates. This would offer an opportunity to perform more technical replicates of experiments at the same time.

For my studies I used a mouse NS reporter cell line expressing GFP from the endogenous locus of PDGFRα, which marks oligodendrocyte lineage progression. This system using adherent NS cell lines could be further developed as new lines e.g. MBP-GFP would offer a valuable insight into processes driving oligodendrocyte lineage progression. Moreover, we could investigate other neural lineages, establishing reporter lines for neuronal and astrocytic differentiation using markers like Tuj1 or GFAP. A powerful future step would be to include multiple reporters using distinct fluorescent proteins within the same cell line, enabling multiplex monitoring of fate choice.

In the chemical screening I identified a number of false-positive molecules, acting as both inhibitors and inducers of GFP activation. These molecules, when tested using flow cytometry, did not show the expected results. A common problem was compound autofluorescence and its accumulation inside of cells. In the validation step of the five selected small molecules I observed a differential response to drug treatment when replicates of experiments were performed. Small molecules in some instances showed a lack of effect on GFP expression or a cytotoxic effect. This could be due to decreased drug stability in solution, which is crucial for a success of any chemical screening-based study.

The chemical stability of compounds in chemical libraries can be affected by environmental conditions during a long-term storage. Other factors, which can affect compound stability include compound concentration, presence of reactive contaminants, intrinsic compound stability, storage-related factors: container, sealing, storage time, temperature, humidity and freeze-thaw cycles (Blaxill, Holland-Crimmin et al. 2009). Most compounds in chemical libraries are dissolved in DMSO, which is hygroscopic and quickly absorbs water from the atmosphere. Water can accelerate degradation and precipitation of compounds, if present in DMSO compound solutions. Exposure of the library to the laboratory environment and its humidity levels were shown to play role in this process (Ellson, Stearns et al. 2005).

Small molecules analysed in this thesis were diluted from the original stock concentration in order to achieve the final screening concentration of 1uM. As 1% DMSO was cytotoxic to cells, the final drug dilution was obtained by using phosphate buffered saline (PBS) instead of DMSO to reduce the final DMSO concentration. PBS is a water-based salt solution, which could explain the reduced activity of small molecules in some experimental replicates. A good practice would be to separately purchase compounds selected for further investigation

and store them in small aliquots, which could be used and discarded after a single experiment without a need for a freeze/thaw cycle. Moreover, this would reduce the need to use the chemical library stock, limiting its exposure to harmful factors and promoting drug stability, which can improve consistency of outcomes in experimental replicates.

6.3 Chemical screening – findings

The aim of this work was to isolate compounds modulating lineage choice of neural stem cells and thus to dissect pathways responsible for the process of neural differentiation. This knowledge could be then used in the field of GBM research, leading cancer stem cells to a post-mitotic differentiated state. Moreover, efficient differentiation of NS cells *in vitro* could allow for their use in regenerative medicine applications. Five molecules identified in the chemical screening were validated for their ability to block GFP expression and further characterised.

The observation of HDACi as the most prominent family of compounds is of importance. It is necessary to consider that these molecules inhibit HDACs, which reversibly modify core histones terminals, a process considered to be a major epigenetic mechanism used to regulated gene expression in cells. The process can affect histones, but also other non-histone proteins like transcription factors, which function can be modified by acetylation. HDAC1 is involved in a regulation of the Notch signalling pathway together with another co-repressor called SMART. The SMART/HDAC1 complex can turn off transcription of Notch target genes (Kao, Ordentlich et al. 1998). HES5, which is a downstream target of Notch signalling, inhibits expression of Sox10 and MBP (Liu, Li et al. 2006).

HDACs can also affect the Wnt pathway, which is involved in inhibition of the oligodendrocyte differentiation (Shimizu, Kagawa et al. 2005). This is possible due to their recruitment by Groucho-related proteins GRO, TLE and GRG (Sekiya and Zaret 2007). In this relation, HDACs could act together with Groucho-related proteins to inhibit Wnt and in turn activate the oligodendrocyte differentiation process.

Interestingly, Foti et al. used VPA, one of the HDACi identified in the StemSelect screening, to show that neural stem cells switch expression of HDAC1 and HDAC2 as they commit to a neurogenic lineage in the SVZ and DG. VPA reduced the number and differentiation of cells in the DG, RMS and OB (Foti, Chou et al. 2013). Ye at al. reported that a deletion of both HDAC1 and HDAC2 results in the stabilisation and nuclear translocation of beta-catenin, which represses Olig2 expression and oligodendrocyte lineage progression. Double knockout animals died around P14. Markers of the oligodendrocyte lineage, Olig2 and PDGFRα were lost at E15.5, with no markers of mature oligodendrocytes at P4. Moreover, when primary cells from double-null mice were cultured *in vitro* they did not differentiate into oligodendrocytes (Ye, Chen et al. 2009).

Shen et al. showed that histone deacetylation is essential for the correct timing of oligodendrocyte differentiation during development *in vivo*. Inhibition of histone deacetylase activity during the first 10 postnatal days resulted in hypomyelination and delayed expression of differentiation markers. VPA administration after myelination onset did not affect the myelin gene expression (Shen, Li et al. 2005). In a later study, Shen et al. found that an increased activity of histone deacetylases correlates with the ability to remyelinate demyelinated lesions in young animals (Shen, Sandoval et al. 2008).

Our finding therefore builds on the literature that HDACs play a critical role in the oligodendrocyte specification. Our future studies should therefore focus mechanistically on how HDACi affect the Olig2 and the links between Olig2 and PDGFRα transcriptional activation. The cell lines we have developed and characterised clearly provide a convenient model to explore the specific mechanistic basis further.

Apicidin was shown to inhibit class II HDAC4 in a study of human ovarian cancer cell migration (Ahn, Kang et al. 2012), while another study showed its specificity for HDAC2 and HDAC3, both class I molecules (Khan, Jeffers et al. 2008). HDACi III, also known as m344, causes increase in hyperacetylated histone H4 and shows selectivity for HDAC6, a class IIB HDAC. It is worth mentioning that HDACs 2 and 3 are localised in the nucleus, while HDAC6 is mostly in the cytoplasm and shuttles in and out of the nucleus depending on the signalling. The fact that the two HDACi block different histone deacetylases suggests that the effect on oligodendrocyte differentiation is unspecific and related more to the overall cell cycle exit and gene regulation action of HDACs than a specific cellular mechanism.

My observation is that HDACi are important not only for the timing of oligodendrocyte differentiation or their myelination potential, but also for the initial lineage specification from a neural stem cell/radial glia progenitor to a PDGFRα-expressing OPC. Chemical screening focused on families of HDAC inhibitors could prove to be very beneficial to expand on this and previously reported studies. We could also explore further how the role of different HDACs changes over time, in proliferation and after induction of differentiation. This way we could also control cell populations, regulating their lineage progression by treatment using specific HDACs. Ultimately the genetic loss of function will be required to define whether there are specific HDAC family members that are required for the NS cell to OPC transition.

Nigericin is able to completely block expression of PDGFRα. However, this effect could be due to cytotoxicity, as Salinomycin, a closely related ionophore was shown to be cytotoxic against the neural cells. This effect is due to elevated cytosolic concentration of sodium ions, which in turn cause increase in cytosolic calcium ions, because of the sodium/calcium exchangers' activity (NCXs). Elevated levels of Ca^{2+} lead to calpain activation and caspase-dependent apoptosis (Boehmerle and Endres 2011). This could explain why cells did not differentiate into OPCs, but instead retained the morphology of the NS cells, or were apoptotic in case of PDGFRα-positive cells treated with Nigericin.

Withaferin A has been reported to inhibit the NFkB activity. Described as a steroidal lactone, Withaferin can also bind to and inhibit Vimentin, causing the filaments to aggregate *in vitro*. It has a potent dominant-negative effect on F-actin, which requires Vimentin expression and induces apoptosis (Bargagna-Mohan, Hamza et al. 2007). Withaferin acts as a potent inhibitor of angiogenesis through a process associated with inhibition of cyclin D1 expression (Mohan, Hammers et al. 2004). Withaferin downregulates expression of GFAP by covalently binding it at cysteine 294 and causes G0/G1 cell cycle arrest (Bargagna-Mohan, Paranthan et al. 2010). It is then possible that the lack of PDGFRα activation is due to the cell cycle arrest at an early stage of NS cell, which does not allow for a progression to an OPC.

6.4 Validation of NFkBi

NFkBi was the most promising candidate compound identified in the chemical screening as we found that it could reduce proliferation of GBM cell lines in the presence of EGF and FGF. However, we failed to recapitulate the same response with the widely used and potent NFkB inhibitor BMS-345541, suggesting the NFkBi is operating through some other

pathway or target. Furthermore PG1 cells that were lipofected with an NFkB GFP reporter plasmid indicated no reduction in GFP expression in NFkBi treated cells.

The NFkBi compound was created using structure-activity relationship (SAR) studies. It is based on quinazoline derivative 6a, which elucidates requirements for NFkB inhibition (Tobe, Isobe et al. 2003). In a study by Tobe et al this compound is described as 11q. It contains a 4-phenoxyphenethyl moiety at the C(4)-position and showed strong inhibitory effects on both NFkB transcriptional activation and TNFα production in the original study using Jurkat cells. The target protein for the activity of NFkBi was not identified in that study, thus it was not immediately possible to answer whether the difference between NFkBi and BMS-345541 can be explained by a different molecular target.

Some of the drugs approved for patient treatments have no known primary molecular target and lack a well-defined mechanism of action. Bioinformatics tools are used to address this problem. Based on chemical similarities between drugs and structurally known ligands it is possible to predict their interactions (Keiser, Setola et al. 2009). A similar approach could help to identify the molecular target of the NFkBi. Alternatively, affinity methods could be used. NFkBi would be then tethered to a solid support and exposed to a cell extract. Small molecule-protein binding interaction could be observed and the target confirmed. These directions are a major undertaking and beyond the scope of this thesis.

6.5 Chemical screening as a tool for differentiation therapy

Our knowledge gained from the differentiation studies *in vitro* could be useful in the field of GBM research, leading cancer stem cells to a post-mitotic differentiated state. Moreover, efficient differentiation of NS cells *in vitro* could allow for their use in regenerative medicine

applications. With the use of differentiation therapy cancer stem cells could lose their stem cell properties, making them more susceptible to cytotoxic treatment and preventing their growth.

Mouse NS cells are easier to work with than human NS and GNS cells, as they proliferate more rapidly and are easily genetically manipulated. This is the reason why in this study I focussed on mouse NS cells and only then moved to test molecules for their effects on human GNS cells. Unfortunately, we failed to uncover small molecules that potently drove the process of differentiation – either to oligodendrocyte or neuronal and astrocyte lineages. However, future chemical screening could be performed directly on GNS lines. With the advent of improved technologies for genome editing directly in human NS and GNS cells it has become feasible to engineer live cell GFP reporters, and this will open up new opportunities for HCS of human stem cells.

In conclusion, I have developed tools and assays that enable live cell screening for modulation of neural stem cell fate. Screening of further chemical libraries may provide new insights into molecular mechanisms underlying NS cell differentiation and reveal small molecules that can be used as chemical 'tools' to study basic mechanisms. Although unsuccessful in our attempts to identify agents that can efficiently promote differentiation, the search for such compounds is important and may lead to new types of differentiation therapy for brain tumours.

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