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MOLECULAR AND EPIGENETIC CONTROL OF CNS TUMOUR AND PROGENITOR CELLS

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MOLECULAR AND EPIGENETIC CONTROL OF CNS TUMOUR AND PROGENITOR CELLS

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ABSTRACT

Despite advances in diagnosis and treatment, CNS tumours continue to result in high mortality and morbidity. Glioblastoma mutliforme (GBM), the most common primary brain tumour, is a recurrent tumour that despite advances in treatment, continues to demonstrate a median time of survival of 14.6 months. While GBM has a higher incidence in adults, medulloblastoma (MB) is more frequent in children. In this pediatric tumour, more recent therapeutic advances have improved the survival rate, however current treatments result in long lasting effects on cognitive development, subsequently leading to a poor quality of life for the patient. This emphasizes the need to better understand tumour mechanisms in this disease in order to design better treatments that will not only lead to destruction of the tumour, but also will not impact patient quality of life in the long term.

The heterogeneity of GBM cells is a huge drawback in current treatment, and can be attributed to the existence of a population of cancer stem cells (CSC). CSC share similar features with stem cells in that they can self renew and proliferate. In paper I, we describe the design and use of a novel system for detection of cancer stem cells involving fluorescent probes called luminescent conjugated oligothiophenes (LCO). We show that pHTMI, an LCO, is an improved cancer stem cell marker compared to existing markers. The role of the cytoskeleton in tumour malignancy is being explored as the cytoskeleton governs physical cellular features such as size, shape and regulates migration and invasiveness. In paper II we explore the cytoskeletal regulators of GBM cells and the effect on these regulators after oncology drugs. We identify a strong correlation between the cytoskeletal regulators and tumour malignancy that can help improve current GBM therapy.

Besides regulators of the cytoskeleton in the cytoplasm, the shape of the cell nucleus is affected by chromatin modifiers such as histone deacetylases (HDACs) and histone demethylases (HDMs) In paper III we elucidate the role of histone deacetylases specifically HDAC2 and HDAC3 in controlling differentiation of progenitor cells and the role of transcriptional regulators in overseeing these pathways. Previous studies have also identified the role of histone demethylases in regulating differentiation in progenitor cells. Since medulloblastoma is a developmental tumour, we explored this approach in medulloblastoma cells. In paper IV, we demonstrate the role of retinoic acid (RA) in regulating the expression of the histone demethylase *KDM6B/JMJD3* and neuronal genes. Further, we show that there is a cross-talk with KDM6B/JMJD3 and the TGFβ signaling mediator SMAD3 in medulloblastoma cells. A better molecular understanding of the mechanisms of these tumours could ensure improved diagnosis and treating molecular subtypes specifically.

POPULAR SCIENCE SUMMARY

Central nervous system tumours are one of the most well studied tumour types but continue to have a poor prognosis and can result in poor quality of life due to the effect of the treatment regimens currently applied that include surgery, radiation and chemotherapy. Subsequently, a deeper understanding of the components of these tumours, the environment that supports them and mechanisms of their progression and control, may aid in the development of better treatment regimens that not only improve the prognosis of the disease, but also help to decrease long-term treatment effects. This thesis discusses the heterogeneous components of tumours that include a small population of cells that can self renew and multiply, similar to so called stem cells. These cells have the potential to contribute to tumour recurrence, contributing to poor prognosis. We therefore suggest more selective treatment methods to detect and eliminate these cells, which could possibly be used in clinical intervention during surgery. Further, this study demonstrates the importance of understanding the structural framework of tumours in designing better chemotherapeutic agents. We suggest that existing chemotherapeutic regimens could be boosted with use of a combination of drugs specifically targeting different components of the tumour. We also studied pathways and components that regulate the development of cell types in the brain to better understand developmental CNS tumours and contribute to an improved diagnosis and treatment of these tumours.

LIST OF SCIENTIFIC PAPERS

I. ***Live detection of neural and glioma-derived stem cells by an oligothiophene derivative***
Ilkhanizadeh S*, **Gracias A***, Åslund A.K.O*, Bäck M, Simon R, Rraklli V, Migliori B, Kavanagh E, Nelander S, Westermark B, Uhrbom L, Forsberg-Nilsson K, Texeira A. I, Konradsson P, Uhlén P, Holmberg J, Joseph B, Hermanson O, Nilsson K. P.R.
Submitted (2017)

II. ***Understanding cytoskeleton regulators in glioblastoma multiforme for therapy design***
Masoumi S*, Harisankar A*, **Gracias A**, Bachinger F, Fufa T, Chandrasekar G, Gaunitz F, Walfridsson J, Kitambi SS.
Drug Design, Development and Therapy. 2016:10 2881-2897

III. ***Neural stem cell differentiation is dictated by distinct actions of nuclear receptor corepressors and histone deacetylases***
Castelo-Branco G, Lilja T, Wallenborg K, Falcão AM, Marques SC, **Gracias A**, Solum D, Paap R, Walfridsson J, Texeira AI, Rosenfeld MG, Jepsen K, Hermanson O.
Stem Cell Reports. 2014. 3:502-515

IV. ***Genome wide analysis reveals cross-talk between the retinoic acid-regulated H3K27 demethylase KDM6B/JMJD3 and the TGFβ signaling mediator SMAD3 in human medulloblastoma cells***
Gracias A, Hermanson O.
Manuscript (2017)

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LIST OF ABBREVIATIONS

ARAP1	Ankyrin repeat and PH domain 1
ARFIP2	ADP ribosylation factor interacting protein 2
ARPC2	Actin related proten 2/3 complex subunit 2
ATRA	All-trans retinoic acid
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
BTSC	Brain tumour stem cell
CIT	Citron Rho-interacting serine/threonine kinase
CLIP	CAP-GLY domain containing linker protein
CSC	Cancer stem cell
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DMSO	Dimethyl sulfoxide
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
FGF2	Fibroblast growth factor 2
GBM	Glioblastoma multiforme
GSC	Glioma-derived stem cell-like cells
GSN	Gelsolin
H3K4me3	Histone 3 lysine 4 trimethylation
H3K9me3	Histone 3 lysine 9 trimethylation
H3K27me2/3	Histone 3 lysine 27 di/ trimethylation
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HMT	Histone methyltransferase
HOX	Homeobox
IC_{50}	Half maximal inhibitory concentration
IDH	Isocitrate dehydrogenase
JmjC	Jumonji C
JMJD	Jumonji domain-containing protein
KDM	Lysine demethylase
LCO	Luminescent conjugated oligothiophene
LCP	Luminescent conjugated polythiophene
LIMK	LIM domain kinase
LSD	Lysine-specific demethylase
MB	Medulloblastoma

MBP	Myelin basic protein
MGMT	O6-methylguanine-DNA methyltransferase
MID1	Midline 1
MLL	Mixed-lineage leukemia
MSN	Moesin
MYCN	V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NCOR	Nuclear receptor co-repressor
NKX2.2	NK2 homeobox2
NSC	Neural stem cell
OL	Oligodendrocyte
p53/TP53	Tumour protein P53
p-FTAA	Pentameric form of formyl thiophene acetic acid
p-HTE-Ser	Penta-hydrogen thiophene ethanol serine
p-HTMI	Penta-hydrogen thiophene methyl imidazole
PI	Propidium iodide
PI3	Phosphoinositide 3
PLP	Proteolipid protein
PPP3CB	Protein phosphatase 3 catalytic subunit beta
PRC	Polycomb repressive complex
PROM1	Prominin 1
PTC	Patched
PTEN	Phosphatase and tensin homolog
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RXR	Retinoid X receptor
SHH	Sonic hedgehog
s-IBM	Sporadic inclusion body myositis
siRNA	Small (or short) interfering RNA
SMO	Smoothened
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
T3	Triiodothyronine hormone
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TMZ	Temozolomide
MYCN	V-myc avian myeloctomatosis viral oncogene neuroblastoma derived homolog
TSS	Transcription start site
UTX	Ubiquitously transcribed X chromosome tetratricopeptide repeat protein

UTY	Ubiquitously transcribed Y chromosome tetratricopeptide repeat protein
VPA	Valproic acid
WAS	Wiskott-Aldrich syndrome
WHO	World Health Organization

1 INTRODUCTION

1.1 CNS tumours

CNS tumours account for some of the most common cancers in children and adolescents (Main, et al. 2016; Siegel, et al. 2016). While less common in adults in comparison to other tumours, CNS tumours are associated with a high rate of morbidity and mortality (Siegel, et al. 2016). These diseases comprise of a diverse group of tumours classified based on their histology, demographics and clinical outcome (Louis, et al. 2007). As of 2016, the WHO introduced additional parameters to classify CNS tumours, including specific molecular patterns (Louis, et al. 2016). The hope is that these additional parameters will aid the accuracy of diagnosis and the development of more patient-specific treatment strategies.

The malignancy of the tumour is also regulated by other tumour components such as cytoskeletal properties that control the invasiveness of tumour cells and embryonic signatures (Quante, et al. 2011; Yilmaz and Christofori 2009; Yu, et al. 2011). Together with improved molecular classification of tumours, these components have the potential to be targets of cell therapy and/or drug development for improved treatment of CNS tumours.

1.1.1 Gliomas

Gliomas are the most common primary brain tumours in adults with a 70% rate of malignancy. The origin of these tumours is associated with neural stem cells, progenitor cells or de-differentiated mature neural cells that form cancer stem cells (Persson, et al. 2010; Singh, et al. 2004a; Stiles and Rowitch 2008). According to the 2016 WHO classification of tumours of the CNS, based on both phenotype and genotype, diffuse gliomas can be subgrouped into WHO grade II and III astrocytomas, grade II and III oligodendrogliomas and grade IV glioblastomas. A group of gliomas including ependymomas and a subgroup of astrocytomas exhibit a more restricted growth pattern compared to the diffuse gliomas and are grouped as WHO grade I CNS tumours (Louis, et al. 2016). These tumours are further grouped based on genetic parameters (Figure 1) such as (isocitrate dehydrogenase) *IDH* status, mutant or wild-type and 1p/19q chromosome arms, intact or co-deleted and grade I tumours that lack this mutation are eliminated from the classification (Claes, et al. 2007; Louis, et al. 2016). Glioblastoma Multiforme (GBM) is the most frequent and aggressive subtype of glioma. With the best possible treatment including surgery, chemotherapy and radiation, the prognosis is 14.6 months (Ray, et al. 2014). Some of the characteristics that contribute to recurrence of these tumours are the highly invasive and heterogenetic nature of the cells that makes it difficult to understand the origin of tumour cells and therefore it is difficult to develop therapies (Bayin, et al. 2014; Hoelzinger, et al. 2005; Louis, et al. 2007). Thus emphasizing the need to further understand the heterogeneity associated with GBM, which will in turn result in more specific and efficient treatment strategies.

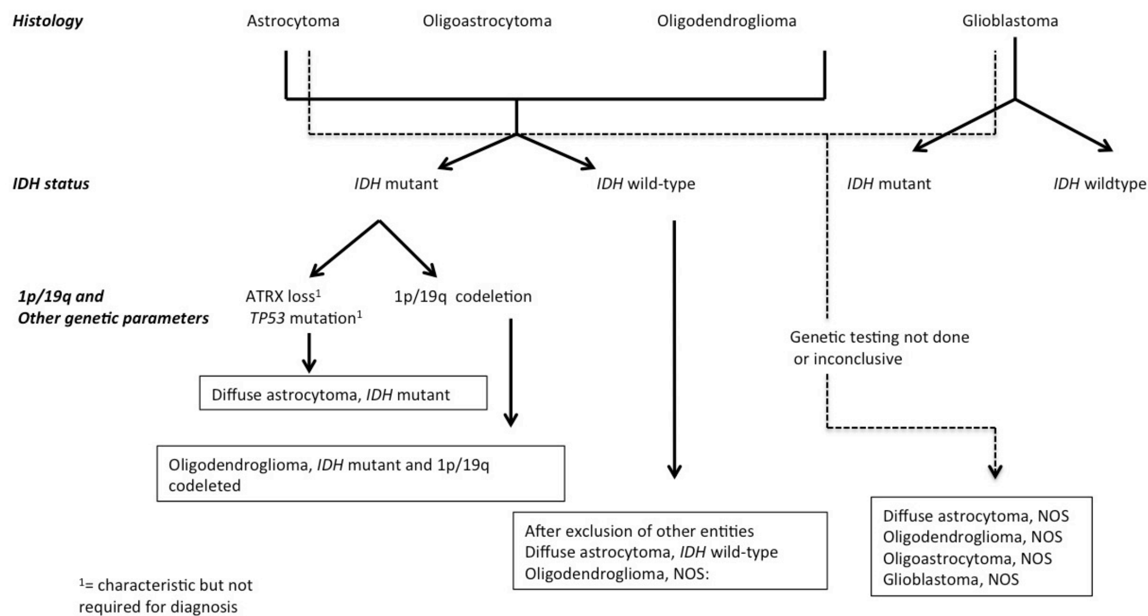

Figure1. Classification of diffuse gliomas based on genetic features. Figure adapted from (Louis, et al. 2016).

1.1.2 Medulloblastoma

Medulloblastoma is the most common malignant brain tumour in children, representing about 20% of CNS tumours with a higher incidence rate in males (Massimino, et al. 2016). With current treatment forms the 5-year survival rate is over 70% (Massimino, et al. 2016). Removal of medullablastoma tumours leads to developmental and cognitive deficits due to the cerebellar location of these tumours resulting in a poor quality of life. This creates a need to understand the molecular basis of these tumours with embryonic origin (Giordana, et al. 1999) in addition to the histological classification, to assist in specific diagnosis and personalized treatment. The histological classification includes, variants with desmoplastic/nodular, medulloblastoma with extensive nodularity, larger cell and anaplastic features (Louis, et al. 2007). This need for a molecular classification led to four subgroups of MB tumours: WNT-activated, sonic hedgehog (SHH)-activated, Group 3 and Group 4 (Figure 2) (Jones, et al. 2012). The 2016 WHO classification of CNS tumours narrowed down the subgroups further, adding subtypes such as SHH-activated and *TP53*-mutant and SHH-activated and *TP53*-wildtype (Louis, et al. 2016). While some of these subgroups have defined signaling pathways that are drivers of the mutation, other subgroups have a less certain molecular signature and epigenetic mechanisms have been suggested (Jones, et al. 2012; Robinson, et al. 2012). It is important to understand these molecular classifications with regards to mutations to assist in diagnosis and treating these tumours.

WNT subgroup tumours are the least common tumours amongst the medulloblastoma subgroups and have the best prognosis. The diagnosis of these tumours is well-established using immunohistochemistry (Massimino, et al. 2016). This subgroup involves disregulation in the WNT signaling pathway due to a mutation leading to overexpression of β-catenin resulting in increased cell proliferation (Gilbertson 2004; Massimino, et al. 2016). SHH

subgroup tumours account for about 30% of cases with an intermediate diagnosis (Massimino, et al. 2016). SHH pathway is crucial in the development of the cerebellum where the SHH ligand induces proliferation of neural precursor cells (Wechsler-Reya and Scott 1999). Inhibition of the SHH signaling pathway as a form of treatment has been explored and SHH inhibitors are in different stages of development (Taipale, et al. 2000). Initial studies using SHH inhibitors have shown signs of skeletal and CNS developmental toxicities (Samkari, et al. 2015). Since SHH-medulloblastomas are more prevalent in infants, using these SHH inhibitors can result in developmental toxicity (Kimura, et al. 2008). Another challenge with treatment of SHH subtype tumours is the molecular hetererogeneity observed. Different gene mutations have been associated with different age groups hence it is essential to understand the signaling pathways better to develop specifically targeted therapies towards these tumours (Kool, et al. 2014) Group 3 and Group 4 tumours are less well characterized with regards to underlying mutations and thus the pathogenesis as a result of the mutations (Massimino, et al. 2016). Group 3 subgroup accounts for 25-28 % of tumours and is known to have the poorest patient prognosis of the medulloblastoma subtypes (Massimino, et al. 2016). Pre-clinical studies using chemotherapeutic targets and inhibitors of the PI3 kinase pathway are being assessed for possible treatment of these tumours (Pei, et al. 2012; Petronis, et al. 2003). Group 4 tumours are the most commonly occurring subtype, and patients with these tumours have an intermediate prognosis with conventional chemotherapy and radiation therapy (Massimino, et al. 2016).

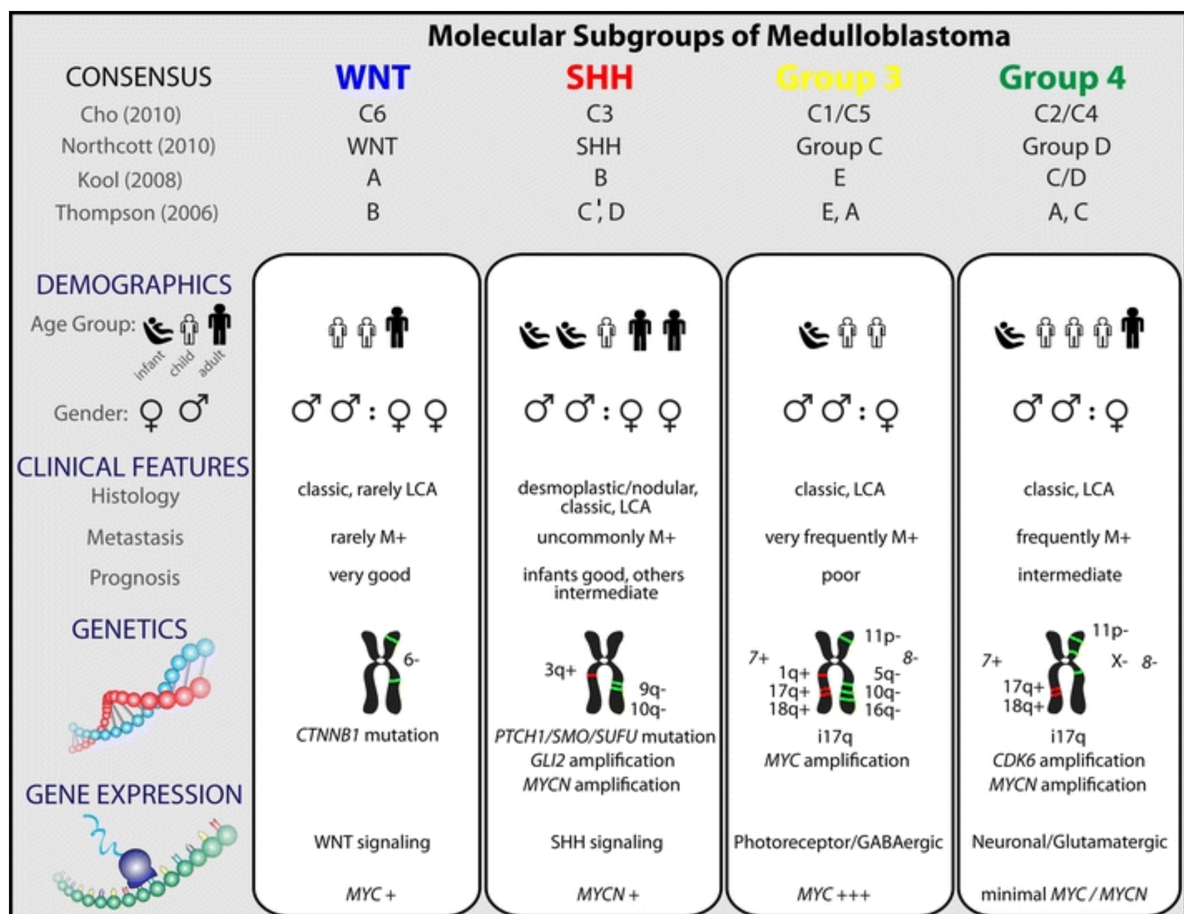

Figure 2. Molecular classification of Medulloblastoma (Taylor, et al. 2012).

1.2 MECHANISTIC CONTROL OF NEURAL PROGENITOR CELLS

Neural stem cells (NSCs) are multipotent cells with the potential to self- renew and are capable of developing into major CNS cell types (Figure 3). Neural stem and progenitor cells share similar cellular processes with CNS tumour cells including cell proliferation, expression of certain genes and signaling pathways. (Reya, et al. 2001). The knowledge of molecular mechanisms involved in the regulation of these cells is critical to the understanding of CNS cancers. Transcription factors are key regulators in the maintenance of cells or differentiation into specific cell types. In the maintenance of stem and progenitor cell fate, the Sox family of transcription factors is well studied and continues to draw interest. Sox2 is a well-established transcription factor in the development of the CNS due to its role in controlling stem cell state and fate (Masui, et al. 2007). The formation of neurons is disrupted in cultures of neural progenitor cells that are depleted of Sox2 and the absence of *Sox2* in mice brain resulted in loss of GFAP and nestin expression (Ferri, et al. 2004). In the context of gliomas, expression of Sox2 in association with TGFβ, a cytokine involved in regulating cellular functions, was shown to maintain tumour-initiating cells in glioma (Ikushima, et al. 2009). Sox proteins are mainly involved in transcriptional activation however Sox9 and Sox10, other members of the same Sox family of transcription factors can function as transcriptional repressors (Lee, et al. 2012).

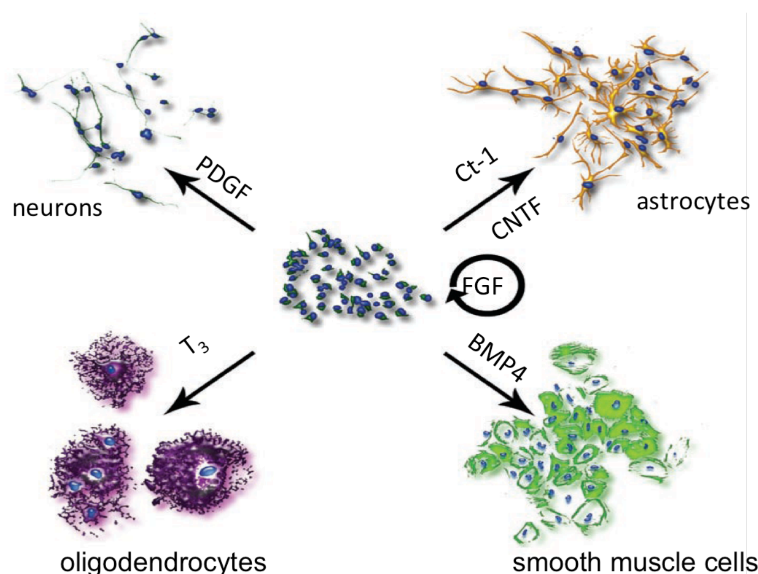

Figure 3. NSCs can develop into different cell types *in vitro* in the presence of media supplemented with factors (Teixeira, et al. 2007).

A signaling pathway that plays a prominent role in CNS development is the Shh pathway that mediates proliferation of progenitor cells. This signaling pathway includes players such as Smoothened (Smo) and Patched (Ptc) that mediate Gli-related genes (Figure 4). Knockdown of Gli2 inhibited the expression of Sox2 and other genes maintaining stemness in NSCs (Takanaga, et al. 2009). Genetic alterations in genes associated with the Shh-Gli pathway are implicated in CNS tumours. (Samkari, et al. 2015; Xie, et al. 2013). These studies provide

evidence that neural progenitor cells share control mechanisms with tumour cells highlighting the importance of an improved understanding of these mechanisms. Epigenetic mechanisms have also shown to play a role in the control of neural progenitors and this will be discussed further in this thesis.

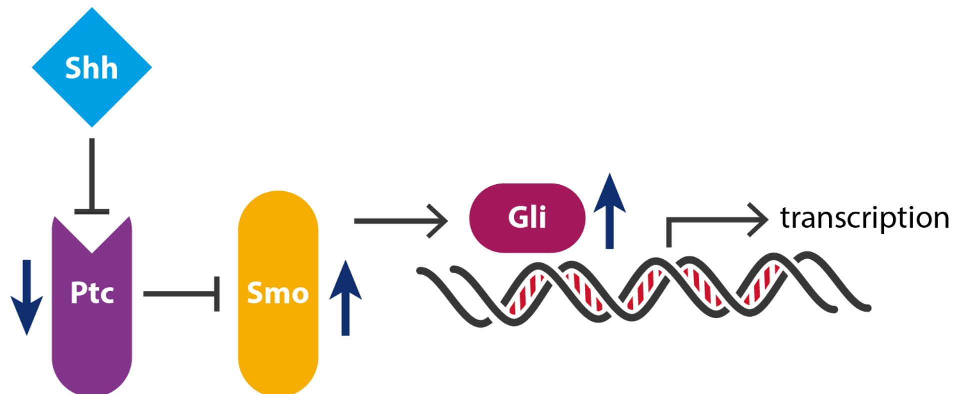

Figure 4. Shh inhibits the repression of Smo by Ptc leading to the regulation of Gli proteins mediating transcription. Figure adapted from (Ruiz i Altaba, et al. 2002) Figure illustrated by Jakub Lewicki.

1.3 CANCER STEM CELLS

One of the reasons for the poor prognosis of glioblastoma is the recurrence of this disease. This recurrence can be due to the heterogeneity of the tumour cells making complete resection difficult to estimate, or the resistance of a population of tumour cells to radiation and chemotherapy (Cho, et al. 2013). These heterogeneous and highly resistant populations of cells have been identified as cancer stem cells (CSC). According to these findings, two existing models to understand tumour expansion have been identified: the clonal expansion model and the cancer stem cell model. According to the clonal expansion model, the tumour consists of a heterogeneous population of cells that have the potential to proliferate extensively whereas in the CSC model, the tumour cells have limited proliferative capacity and only a small subset of the the tumour population, the CSC, can self renew and proliferate (Reya, et al. 2001) . The percentage of CSCs varies between tumour types and is controversial between studies (Bao, et al. 2013; Cho and Clarke 2008). Failure to treat tumours given the basis of the clonal expansion model suggests that the cancer stem cell model may be more accurate in understanding the heterogeneity of the tumor (Figure 5).

Normal stem cells possess unique properties of self-renewal, proliferation and the ability to differentiate into cell types. The cancer stem cell is defined by its stem cell-like properties of self-renewal and ability to propagate. These cells can divide indefinitely, producing cell types that contribute to tumour growth. The difference between the cancer stem cell in comparison to the normal stem cell is the lack of homeostasis in cancer stem cells due to their uncontrolled cell proliferation (Tan, et al. 2006).

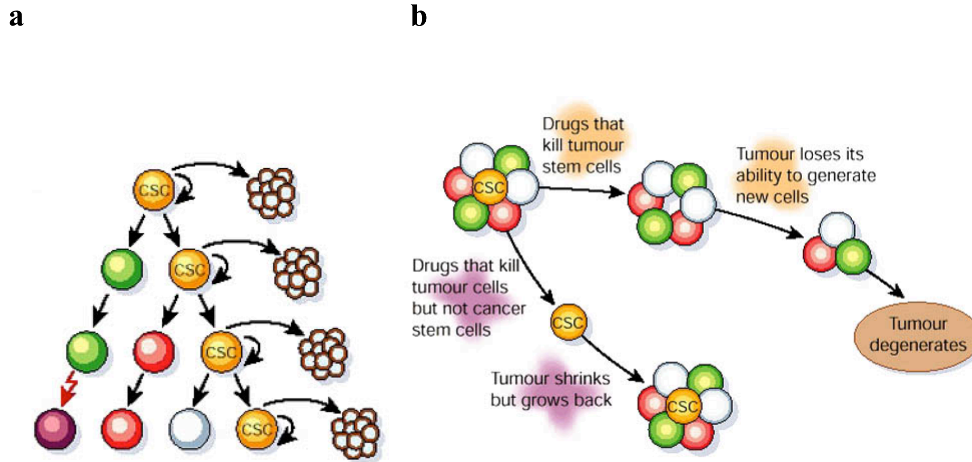

Figure 5. a) Small subset of cells possess the ability to form new tumours, b) Cancer stem cells are less sensitive to therapy (Reya, et al. 2001).

The exact origin of the brain tumour cells is unknown however there are multiple hypotheses. Neural stem cells and brain tumours share certain signaling pathways such as Notch, Wnt and Shh signaling, suggesting the neuroglial lineage of brain tumour stem cells (BTSCs) (Reya, et al. 2001; Singh, et al. 2003). Neurogenic niches have been identified in the subventricular zone along the wall of the lateral ventricles and the subgranular zone along the dentate gyrus within the hippocampus (Eriksson, et al. 1998; Sanai, et al. 2004). Since NSCs and progenitor cells from these neurogenic niches continue to exist in adults, they have been suggested as candidates for the origin of brain tumour stem cells (Modrek, et al. 2014). The other possibility is that differentiated cells dedifferentiate to form brain tumour stem cells (Modrek, et al. 2014; Sanai, et al. 2005). A majority of gliomas occur in adults and are thus not included in the group of developmental tumours, suggesting that molecular mechanisms involving the transformation of adult cells must cause the tumour (Nduom, et al. 2012). The dedifferentiation is due to the ability of genetically modified differentiated cells to form more progenitor or stem cell-like cells that contribute to gliomagenesis (Friedmann-Morvinski, et al. 2012).

Existence of BTSCs was first observed by Singh et al, where BTSC were isolated from tumour samples based on the expression of CD133 (Prominin1/PROM1) (Singh, et al. 2003). To be characterized as a BTSC, the cell needs to possess self-renewal properties, must be multipotent and be able to initiate tumours in animal models (Bayin, et al. 2014; Singh, et al. 2003). To ensure self-renewal *in vitro*, these cells are grown in suspension and their consistent sphere-forming ability is an indicator of long-term self renewal (Lee, et al. 2007). Self-renewal *in vivo* is assessed by introducing BTSC in xenograft models and evaluating tumour formation (Singh, et al. 2003; Singh, et al. 2004b). Identifying elements of the tumour lineage can provide insights on the multipotency potential of BTSCs (Rampazzo, et al. 2013).

1.3.1 Putative markers to detect CSC

In order to better understand cancer stem cells, it is necessary to identify methods to specifically detect this population of cells within a tumour and thus develop cancer stem cell-targeted therapy. Markers currently used to detect CSCs are those that are used to detect NSCs. CD133 (also called Prominin-1), is a five transmembrane domain, cell surface glycoprotein that was first isolated from neuroepithelial stem cells in mice and later was discovered in human hematopoietic stem and progenitor cells (Weigmann, et al. 1997; Yin, et al. 1997). The possibility of a small population of CD133+ cells to induce tumours in immune-deficient mice suggested the possibility of using CSC markers in therapy (Singh, et al. 2004a; Singh, et al. 2004b). This marker has been identified as a CSC marker in various cancers including colorectal, gastric, pancreatic, breast, prostate, liver, lung and head and neck squamous cell carcinoma (Boman and Wicha 2008). Clinically, the presence of CD133+ cells has been negatively associated with patient survival in those suffering from colorectal cancer and gliomas (Horst, et al. 2008; Wu, et al. 2015). In a study on patients with pancreatic adenocarcinoma, poor prognosis was linked to CD133+ samples, specifically in biopsies where the number of CD133+ cells greatly outnumbered the CD133-population (Maeda, et al. 2008). Also, the CD133+ population showed a higher level of lymphatic invasion, indicating a role in cellular migration (Maeda, et al. 2008). Despite evidence that supports CD133 as a CSC marker, there have also been studies that prove the possibility of both CD133+ and CD133- cells to be involved in clonogenecity, self- renewal and tumorigenic capacity (Joo, et al. 2008; Wang, et al. 2008).

Another marker that has been used in the detection of CSC is CD44. CD44 is a transmembrane, cell surface glycoprotein that is involved in several cellular processes including proliferation, differentiation, migration and angiogenesis, and is as well a co-receptor for cytokines and growth factors (Naor, et al. 2002). CD44 expression was shown to distinguish neural stem cells from embryonic stem cells with < 4% positive CD44+ population in embryonic stem cells compared to < 95% positive in neural stem cells (Pollard, et al. 2008). It has been used as a prognostic marker in several tumours, including lung, colorectal, breast, hepatocellular, head, neck and hypopharyngeal squamous cell carcinoma (Yan, et al. 2015). The role of CD44 in cell adhesion and migration suggests that it is involved in tumour formation (Naor, et al. 2002). In mice with gastric cancer, CD44 expression was associated with tumorigenesis where $CD44^{+/+}$ developed larger tumours than $CD44^{-/-}$ mice, suggesting a role for CD44 in tumour initiation and progression (Ishimoto, et al. 2011). In mice with glioma, $CD44^{+/+}$ mice developed high-grade gliomas with shorter survival time as compared to $CD44^{-/-}$ mice suggesting that CD44 is implicated in glioma growth (Pietras, et al. 2014). Downstream CD44 signaling after ligand binding promoted stem cell characteristics in glioma cells suggesting this glycoprotein regulates cell stemness. (Pietras, et al. 2014).

CD44 is thus a promising target in many tumours and, due to its properties regulating CSCs, can be used to therapeutically target these cells. However, the fact that it is expressed in

several different tumour cells and exists on normal cells as well could prove to be a challenge in using this marker therapeutically (Yan, et al. 2015). Thus there is a need to identify selective and specific CSC markers for effective therapeutic detection of these cells.

1.4 CYTOSKELETON REGULATORS

Together with the knowledge of specific cells contributing to the tumour as well as mechanisms involved in their control, it is important to understand the cytoskeletal framework that supports the tumour cells. Cytoskeletal components provide more than just a structural framework for the cell; these components are also involved in the regulation of growth of normal and transformed cells (Pawlak and Helfman 2001). The cytoskeletal framework assists the cell in responding to different environmental stimuli (Fletcher and Mullins 2010). Such cytoskeletal components include microtubules, microfilaments and intermediate filaments (Figure 6). The protein tubulin is the building block for microtubules, actin for microfilaments, and intermediate filaments are formed of several different subunit proteins. During the malignant transformation of cells, one of the features observed is the alteration of actin filaments in cells due to the influence of oncogenes (Pawlak and Helfman 2001). PTEN and p53 are common mutations in GBM that are associated with cell migration (Muller, et al. 2011; Tamura, et al. 1998). There is evidence that the absence of both PTEN and p53 maybe implicated in the cell invasion process seen in GBM (Djuzenova, et al. 2015). Cytoskeletal properties are also crucial during metastasis, where cell-cell and cell-matrix contact is disrupted (Yilmaz and Christofori 2009). An example of a cytoskeletal modulator in GBM is cofilin, a regulator of the actin cytoskeleton (Yamaguchi and Condeelis 2007). Control of the expression of cofilin, regulated tumour cell invasion suggesting that cytoskeletal activity should be taken into account during GBM therapy. Cytoskeletal components are victims of cell stress during therapy as they control signaling pathways involved in molecule transport, regulate protein-protein interactions and influence enzyme activity. (Parker, et al. 2014). A better understanding of cytoskeleton regulators and functions they mediate in cell responses could be useful in developing treatment strategies for GBM.

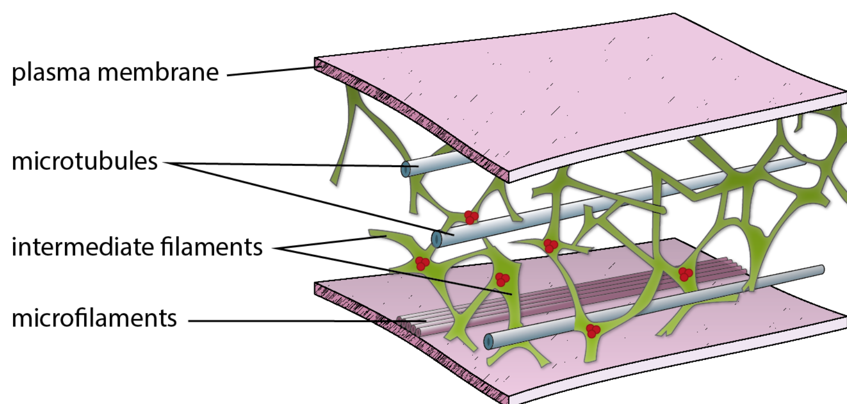

Figure 6. Cytoskeletal components. Figure illustrated by Jakub Lewicki.

One of the drawbacks of therapy against GBM is that the cells often become resistant to the chemotherapeutic applied (Lefranc, et al. 2005). One such commonly used example is temozolomide (TMZ), an oral alkylating agent that acts by interfering with DNA replication forming cytotoxic methyl adducts which trigger apoptosis (Roos, et al. 2007). However, high levels of the protein O6-methylguanine-DNA methyltransferase (MGMT) is associated with resistance to TMZ leading to tumour progression (Roos, et al. 2007). A study complementing TMZ with a modified cytoskeletal regulator showed that this improved tumour cell sensitivity to TMZ and increased apoptosis (Kislin, et al. 2009). Various cytoskeletal-targeting agents are being explored for GBM therapy. An example is Patupilone, a novel tubulin-binding agent, which has proven to act against CNS malignancies when combined with radiation (Fogh, et al. 2010; Oehler, et al. 2012). This drug stabilizes the microtubule, has anti-angiogenic effects and is independent of efflux pumps that are abundant in tumour cells (Oehler, et al. 2012). The co-treatment of cytoskeletal regulators with existing drugs such as TMZ needs to be explored for future GBM therapies. The success of small molecule probes in the enhancement GBM treatment paves the way for the further development of novel probes that could improve current GBM treatment strategies.

1.5 LUMINESCENT MOLECULAR PROBES

1.5.1 Properties of conjugated oligomers and polymers

Monomers are molecular units that can form complex molecules. An oligomer consists of a small number of monomers and a polymer can contain an unlimited number of monomers. Conjugated oligomers and polymers are characterized by alternating single and double bonds on their oligomer and polymer backbones (Figure 7). Unlike polymers such as the commonly used polyethylene and polystyrene, which are insulators and are colourless, the conjugated polymers are semiconductors and interact with light. Due to their chemical structure, they have interesting electronic and optical properties. These oligomers and polymers are used in LEDs due to their ability to emit light after induction with electric current, an effect known as electroluminescence. They have also been used in solar cells transistors (Strobl 2007).

One of the important aspects in the design of conjugated polymers are the side chains, which play a role in improving the solubility and intermolecular interactions. Due to the large structure of polymers, many are insoluble and can be very challenging to study their properties (Zade, et al. 2011). The most studied conjugated oligomers and polymers are those with the thiophene subunit (Zade, et al. 2011). Luminescent conjugated oligo- and polythiophenes (LCOs and LCPs) have proved to be useful thiophene polymers for various biological applications. The thiophene-based polymers are capable of identifying protein aggregates from a broader spectrum of diseases in comparison to conventionally used protein markers (Klingstedt, et al. 2013b). The optical properties of these thiophene-based polymers are particularly dependent on the number of thiophene units and the positioning of side chains

(Klingstedt, et al. 2013b). LCOs are a smaller group of LCPs consisting of a pentameric backbone and a less ionic side chain substitution than LCPs (Aslund, et al. 2009).

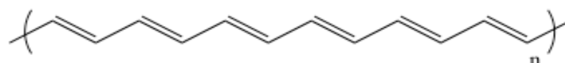

Figure 7. Conjugated polymer

1.5.2 Conjugated oligomers and polymers for biological applications

Due to their optical properties, conjugated polymers have been used as probes to detect peptide conformation (Nilsson, et al. 2003; Nilsson, et al. 2004). A charge specific LCP derivative was shown to distinguish between native and fibrillar amyloids *in vitro,* thus providing a fast, non-covalent based interaction method to detect fibrillar proteins (Herland, et al. 2005; Nilsson, et al. 2005). The LCPs have also been used in the detection of amyloid deposits associated with Alzheimer's disease in different ex vivo tissue samples, wherein these probes emit different coloured light depending on which amyloid deposit it binds to. Thus, these probes can be used as a sensitive method to decipher between protein conformations based on different wavelengths of light, thus providing a potential method for early detection of diseases such as Alzheimer's (Nilsson, et al. 2006). Although LCPs are useful in identifying fibrillar deposits *in vitro*, their use *in vivo* has not proven to be as successful (Aslund, et al. 2009).

The shorter backbone and less ionic side chain of LCOs enable these LCOs to overcome challenges faced by LCPs. The LCOs exhibited selectivity to protein aggregates and some of them were able to cross the BBB (Aslund, et al. 2009). LCPs can only detect fibrillar but not pre-fibrillar deposits (Aslund, et al. 2009). One of the LCOs, p-FTAA (penta-formlythiophene acetic acid) was able to detect pre-fibrillar deposits, proving these molecules can be useful tools in the diagnostics of protein aggregates (Aslund, et al. 2009). Recently, it was shown that not only can this particular LCO detect pre-fibrillar structures, but it has the potential to induce a structural transformation in the toxic protein aggregates into non-toxic and insoluble fibrillar structures that are resistant to proteolytic degradation, suggesting a therapeutic use for this LCO (Civitelli, et al. 2016). The specificity of these molecular probes extends to detecting protein aggregates in skeletal muscle fibres in patients with sporadic inclusion body myositis (s-IBM), with p-FTAA, detecting aggregates in these tissues that were shown to be negative with conventional markers (Klingstedt, et al. 2013a).

Not only are the molecular probes such as LCOs and LCPs highly selective markers, they are also non-toxic making them optimal markers for cellular staining (Palama, et al. 2011). LCP derivatives were used in non-transformed cells such as fibroblasts, myoblasts, leukocytes and macrophages, where they detected lysosome-related acidic vacuoles (Bjork, et al. 2007). This specificity, however, was not observed in transformed, malignant cells, indicating selectivity of this marker to non-transformed cells (Bjork, et al. 2007). Several LCOs stained different cell types with a preference to some cancer cells. The fluorescent intensity was dependent on

the side chain group to the thiophene backbone, where LCOs with imidazole moieties were preferred over amino acid side chains (Cieslar-Pobuda, et al. 2014). Labeling with two of the LCO derivatives in a cancer cell line showed a small population of cells with higher intensity of fluorescence, suggesting the presence of a side population of cells that could potentially be the cancer stem cell population, however more evidence is needed to confirm the identity of this labeled cell type (Cieslar-Pobuda, et al. 2014). Thus LCOs may provide promising alternatives to conventional markers in the detection of biological targets besides protein aggregates.

1.6 EPIGENETICS

Epigenetics involves the study of processes that alter gene activity and can be inherited without changes in the underlying DNA sequence (Wu and Morris 2001). Epigenetic factors function alongside the genome to determine various cellular processes. Epigenetic reprogramming can be seen as early as embryonic development and continues throughout the cell's lifespan giving it a unique epigenetic signature (Morgan, et al. 2005). Some of the epigenetic modifications include DNA methylation, chromatin modification, RNA silencing (Weinhold 2006). These epigenetic mechanisms are implicated in processes regulating development and tumour formation.

Historically according to the histone code hypothesis, transcription of the genome is governed by several histone modification patterns (Allis and Jenuwein 2016; Strahl and Allis 2000). The N-terminal of histone tails can be modified via post-translational processes, leading to epigenetic regulation of transcription. Histone modification results in creating either transcriptionally favourable regions, or unfavourable regions that lead to gene repression (Jenuwein and Allis 2001). Histone acetylation and methylation are the two major forms of histone modifications. The enzymes responsible for these covalent modifications are dependent on the position of specific amino acids (Strahl and Allis 2000) (Figure 8).

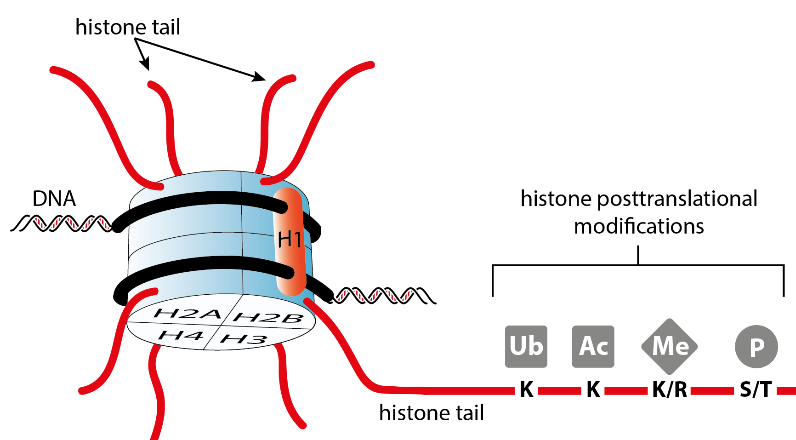

Figure 8. Histone tail modifications such as ubiquitination on the lysine residue, acetylation on the lysine residue, methylation on the lysine or arginine residue, phosphorylation on the serine/threonine residue. Figure adapted from (Fullgrabe, et al. 2010). Figure illustrated by Jakub Lewicki.

1.6.1 HISTONE DEACETYLASES

Histone acetylation is one of the most common modifications of histones and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), both of which control the acetylation of lysine residues on the N-terminus of histones. Histone acetylation is implicated in transcriptional activation and is controlled by the balance of the activity of HATs and HDACs (Struhl 1998). HDACs belong to a large superfamily of deacetylases and can be further classified into subgroups including class I, IIa, IIb and IV based on the highly conserved deacetylase domain. Class I HDACs consist of HDAC1, 2, 3 and 8 and class II HDACs include HDAC4, 5, 6, 7, 9 and 10 (de Ruijter, et al. 2003). For the purpose of this thesis, the Class 1 HDACs will be discussed in further detail.

Class I HDACS are known to be ubiquitously expressed and localized to the nucleus, however studies have also reported the existence of HDAC3 in the cytoplasm (Yang, et al. 2002). Wang et al demonstrated that HDACs play a role in gene activation as well as in repression. They showed that class I HDACs were enriched at gene promoters and gene regions of active genes, in contrast to reports suggesting their sole involvement in repression of gene transcription (Wang, et al. 2009; Xu, et al. 1999). HDACs have been shown to interact with transcription factors to mediate proliferation and cell fate determination. The silencing mediator for retinoid and thyroid receptors (SMRT) and the nuclear receptor corepressor (N-CoR) are two corepressors related to HDACs and are involved in transcriptional regulation of NSC differentiation as they repress gene expression and are required for cell fate determination (Guenther, et al. 2001; Hermanson, et al. 2002; Jepsen, et al. 2007; Lilja, et al. 2013; Miller and Gauthier 2007).

1.6.2 HISTONE DEMETHYLASES

Histone methyltransferases (HMTs) and histone demethylases (HDMs) catalyze the histone methylation process, leading to silencing or expression of genes respectively, depending on the specific lysine or arginine residues present (Strahl and Allis 2000). Histone methylation is a reversible process and has been shown to exist on histones H3, H4 and the linker histone H1.1 (Labbe, et al. 2013; Shi, et al. 2004). The lysine residues can be mono-, di- or tri-methylated. The addition or removal of these methyl groups leads to repression or expression of genes. For instance, tri- methylation of lysine 4 on histone H3 (H3K4me3) leads to activation of gene expression, whereas di and tri-methylation of lysine 27 residue on histone 3 (H3K27me2/3) and tri-methylation of lysine 9 on histone 3 (H3K9me3) (Cao, et al. 2002; Peters, et al. 2002) results in repression of gene expression (Santos-Rosa, et al. 2002).

The concept of histone demethylases was developed by the functional characterization of LSD1 (lysine-specific demethylase 1), (Shi, et al. 2004) also known as (lysine-specific histone demethylase 1 (KDM1) (Allis, et al. 2007). LSD1 (KDM1A) and LSD2 (KDM1B) are two classes of histone demethylases that are structurally similar (Karytinos, et al. 2009). Both can demethylate mono- and di- methyl groups from lysine residues but not tri-methyl

groups (Labbe, et al. 2013; Metzger, et al. 2005; Shi, et al. 2004). With the exception of KDM1A and KDM1B, the other KDMs include the Jumonji C (JmjC) protein domain and these demethylases have the potential to demethylate mono -, di-, and tri-methyl groups on lysine residues with the help of cofactors such as Fe (II), α-ketoglutarate and molecular oxygen (Tu, et al. 2007).

Two such lysine demethylases that contain homologous JmjC domains are the Jumonji domain-containing protein D3 (JMJD3) also called KDM6B, and the ubiquitously transcribed tetratricopeptide (TPR) gene on the X chromosome (UTX), also known as KDM6A (Swigut and Wysocka 2007). These two histone demethylases have been shown to participate in gene regulation and development (Agger, et al. 2007; Jepsen, et al. 2007; Lan, et al. 2007). UTY is a paralog of UTX and belongs to the KDM6 family of histone demethylases, however its demethylase activity is significantly lower than UTX and JMJD3 due to a structural change in the JmjC catalytic domain (Shpargel, et al. 2012; Walport, et al. 2014). Gene regulation by JMJD3 and UTX is achieved by specifically demethylating the dimethyl and trimethyl groups on H3K27 (Hong, et al. 2007; Xiang, et al. 2007). In the di- and tri-methylated state, H3K27 is associated with gene inactivation, however demethylation to the mono-methylated state leads to gene activation (Agger, et al. 2007; Barski, et al. 2007; Burgold, et al. 2008).

These studies provide evidence of the association of HDACs and HDMs in controlling gene expression, signaling pathways and cell fate specification, however a better understanding of the functions of these factors would enable understanding the mechanisms of diseases that involve misregulation of these factors.

1.6.3 MOLECULAR ROLES OF JMJD3 AND UTX

JMJD3 and UTX are implicated in epigenetic processes, regulation of gene expression and cellular reprogramming. The polycomb repressive complex 2 (PRC2) is a prominent player in epigenetic regulation of differentiation and cell fate (Ringrose and Paro 2007). A catalytic subunit of PRC2 can di- and tri- methylate H3K27, resulting in gene silencing (Schwartz and Pirrotta 2007). During embryogenesis however, JMJD3 and UTX play a role in reducing the repression by demethylating H3K27, which leads to the activation of HOX genes. These genes are important in regulating development (Agger, et al. 2007). Retinoic acid- induced differentiation of neural stem cells is controlled by SMRT/NCOR and JMJD3. In this case SMRT/NCOR prevents neuronal differentiation by repressing *Jmjd3* (Jepsen, et al. 2007) (Figure 9a). JMJD3 is also known to mediate development through interaction with SMAD3 at the transcriptional start site (TSS) of TGFβ-associated genes, and depletion of JMJD3 affects the expression of TGF-β-induced genes (Estaras, et al. 2012) (Figure 9b). The same study also showed the developmental role of JMJD3 in the chick spinal cord, as JMJD3 was essential for SMAD3-induced neuronal differentiation.

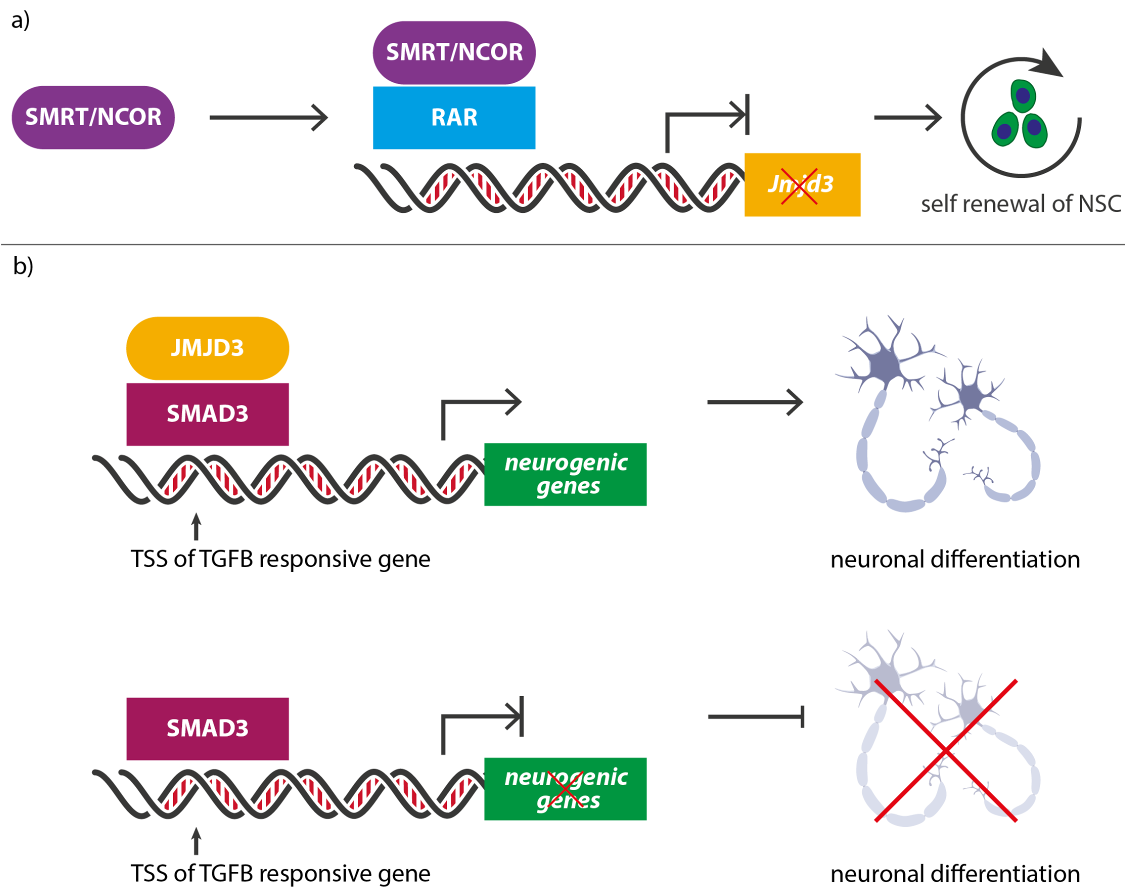

Figure 9. a) SMRT/NCOR interact with retinoic acid receptor and represses JMJD3 leading to self-renewal of NSCs. b) JMJD3 and SMAD3 colocalize at the TSS of TGFβ responsive genes in NSC resulting in neuronal differentiation. Figure adapted from (Estaras, et al. 2012) Figure illustrated by Jakub Lewicki.

1.6.4 EPIGENETICS AND CNS TUMOURS

Epigenetic control mechanisms have been implicated in glioblastoma and in medulloblastoma. In a specific subtype of glioblastoma (classical) methylation of the DNA-repair-gene O6-methylguanine-DNA methyltransferase (MGMT) results in gene silencing. This makes the tumour more prone to damage by chemotherapeutics due to the inability to repair DNA damage by the methylated MGMT resulting in better patient response to chemotherapy, and subsequently better patient prognosis (Brennan, et al. 2013; Hegi, et al. 2005). In pediatric gliomas, mutations in the gene coding for the histone H3.3 at K27 and G34 residues result in different molecular signatures (Bjerke, et al. 2013; Schwartzentruber, et al. 2012). These mutations are located on the histone tail making them accessible to epigenetic modifications including methylation and acetylation influencing gene expression (Huang and Weiss 2013).

In cases where the tumour expresses a G34 mutation, upregulation of MYCN gene, which is an oncogene, is observed. This provides a means to therapeutically target patients with this mutation by inhibiting effects of MYCN (Bjerke, et al. 2013). A link between histone methyltransferase (MLL) genes and medulloblastoma has been suggested (Parsons, et al. 2011). MLL-family genes are associated with development similar to Wnt and Shh signaling pathways, both of which are associated with tumorigenesis in medulloblastoma (Parsons, et al. 2011). *MLL2/MLL3* are epigenome modifying genes and mutations in these genes were seen to occur across all histologic subtypes and molecular subgroups associated with medulloblastoma (Dubuc, et al. 2013; Parsons, et al. 2011). The histone demethylase UTX is a binding partner of MLL2, and mutations in this gene are observed in the Group 4 medulloblastoma subgroup (Dubuc, et al. 2013). More than 50% of Group 4 tumours are associated with decreased expression of UTX and JMJD3 (Dubuc, et al. 2013). Thus, epigenetic regulation is a likely a prominent player in tumorigenesis in CNS-associated cancers.

Due to the involvement of histone demethylases in cancers, the development of histone demethylase inhibitors is being explored as a therapeutic strategy. Recently a selective inhibitor of UTX and JMJD3, GSK-J4 was developed (Kruidenier, et al. 2012). In CNS tumours, particularly in pediatric gliomas with the K27 mutation on histone H3.3, a decrease in H3K27me3 was observed (Venneti, et al. 2013). GSK-J4 was used as a treatment in GBM with the K27 mutation (Hashizume, et al. 2014). The inhibitory effects on histone demethylase function led to increased H3K27 methylation, and inhibited glioma cell growth *in vitro* and *in vivo* (Hashizume, et al. 2014). This inhibitory effect on the proliferation of cells was JMJD3 specific because the effect was no longer observed when JMJD3 was depleted (Hashizume, et al. 2014).

1.7 MOLECULAR ROLES OF ALL-TRANS RETINOIC ACID

All-trans Retinoic acid (ATRA, will be referred to as RA) is a biologically active metabolite of vitamin A. Along with other metabolized products including, β-carotene, retinol, retinal, isotretinoin, 9-cis retinoic acid, and 13-cis retinoic acid it serves as a ligand triggering several molecular pathways as it binds the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Connolly, et al. 2013; Nakayama, et al. 2001). These receptors function as heterodimers, and modulate transcription by binding to RA response elements (RAREs) on the DNA (Gillespie and Gudas 2007). RA plays a pivotal role in controlling epigenetic processes by modulating histones marks. For instance the presence of RA causes an increase in activation marks such as H3K4me3 and H3ac, however it can also cause a decrease in the repressive mark H3K27me3 (Kashyap, et al. 2011). The co-existence of both gene activating and repressive marks associated with promoters is called the bivalent domain or a poised state (Azuara, et al. 2006; Bernstein, et al. 2006). RA-inducible genes have been shown to be associated with bivalent domains where they modulate levels of H3K27me3 and H3K4me3, thus regulating gene expression (Kashyap, et al. 2011; Kashyap, et al. 2013).

The presence of RA ligands dictates the role of these RARs in controlling the differentiated state of tissues (Mahony, et al. 2011). In the absence of RA ligand, the corepressor complex SMRT or NCOR bind to the ligand-binding domain of RAR and this is crucial in regulating the expression of neuronal genes thus controlling developmental pathways (Jepsen, et al. 2007). Pathways involved in CNS tumour development are similar to pathways regulating neuronal genes, suggesting the role of RA in tumour development and maintenance needs to be explored.

2 AIMS OF THE THESIS

The overall aim of this thesis was to understand the molecular characterization of glioblastoma, medulloblastoma and progenitor cells, and utilize this knowledge to design molecular probes and investigate oncogenic drugs to aid in improved treatment options.

Paper I: To investigate the use of an oligothiophene derivative, to detect human glioma-derived stem cells and contrast this with other putative CSC markers.

Paper II: To evaluate the effects of oncogenic drugs on cytoskeletal modulators.

Paper III: To investigate the role for class I histone deacetylases and associated corepressors in neural stem cell differentiation.

Paper IV: To explore putative roles of the histone demethylase KDM6B/JMJD3 in medulloblastoma cells.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Improved detection of stem and cancer stem cells is necessary for better diagnosis and treatment of diseases (Sagar, et al. 2007). The use of molecular probes has been successful in recording biological events. An example includes the luminescent conjugated oligothiophenes and polythiophenes (LCOs and LCPs). These thiophene-based conjugated polymers exhibit fluorescence when binding to biological motifs such as protein aggregates and cell types (Cieslar-Pobuda, et al. 2014; Herland, et al. 2005; Nilsson, et al. 2003; Nilsson, et al. 2004). We explore the possibility of using LCOs for the detection of embryonic neural stem cells (NSCs), which could be applied for treatment of various CNS tumours.

NSCs derived from E15.5 rat embryo cortex were grown on plates coated with poly-L ornithine and fibronectin along with medium containing the fibroblast growth factor (FGF2) (Teixeira, et al. 2007). Differentiated cell types were derived by exposing the isolated NSCs to soluble factors and withdrawing fibroblast growth factor 2 (FGF2). For example, CNTF induced astrocytic differentiation, smooth muscle cells were observed in the presence of FBS and a combination of BMP4/Wnt3a induced neuronal and astrocytic differentiation. Several LCO and LCP variants were screened using both undifferentiated and differentiated cell types, and one screen identified the LCO p-HTMI as a potential marker for NSC cultures. In this case, 10 minutes after treatment with p-HTMI, the cytoplasm of NSCs fluoresced at a wavelength similar to green fluorescence proteins. This fluorescence was unique to NSCs as differentiated cells displayed either very low, or no signal. The LCO variant, p-HTE-Ser was not selective to undifferentiated cells, but displayed a weak staining in fully differentiated smooth muscle cells and mature astrocytes. Since the optical properties of thiophene-based poylmers depends on the positioning of side chains (Klingstedt, et al. 2013b), we thought to investigate whether the methylated imidazole moieties of p-HTMI played a role in the specificity to NSCs, pentameric LCOs lacking the methylated imidazole moiety were generated and tested on cells, however no fluorescent detection was observed. This highlights the role of the methylated imidazole moiety in specific detection of NSCs.

Flow cytometry is commonly used to quantify fluorescent signals in a population of cells. FGF2-treated NSCs were stained with p-HTMI or p-HTE-Ser, and analyzed using flow cytometry. Fluorescence was only observed in p-HTMI stained cells and not in p-HTE-Ser stained cells. To control for cell-cell leakage of the LCOs, cells were separately stained with p-HTMI and p-HTE-Ser, then combined and analyzed using flow cytometry. This identified two separate fluorescence peaks eliminating the possibility of cross contamination of the LCOs. Thus p-HTMI is a selective marker for undifferentiated NSCs *in vitro* but not differentiated cells. The selectivity of p-HTMI is specific for NSCs only and not embryonic stem cells alone.

In order to test the application of LCOs in tumour cells, we used the rat C6 glioma as an experimental model to study GBM (Grobben, et al. 2002). To explore the differentiation potential of C6 glioma cells, these cells were grown with media supplemented with FBS and containing soluble factors such as FGF2, CNTF, and valproic acid (VPA). Immunochemistry confirmed the lack of differentiation potential of these cells. Previous studies have shown that glioma stem cell lines can be obtained from gliomas when these tumour cells were incubated with media-supplemented with growth factors including N2, B27, EGF and FGF-2 (Pollard, et al. 2009). Thus, we generated C6-derived NSCs using the protocol to culture NSCs. C6-derived NSCs treated with FGF2, formed nestin-positive cultures, treatment with CNTF resulted in increased astrocytic differentiation and treatment with VPA resulted in TUJ1-positive cells indicating a neuronal identity. Validation by qPCR demonstrated that mRNA levels of *Gfap* and *Tubb3* were increased after treatment with CNTF and VPA respectively. These results provided evidence that C6 glioma cells, grown as NSCs in the presence of external factors acquire characteristics of neural stem cells.

The percentage of cancer stem cells varies between studies and tumour types (Bao, et al. 2013; Cho and Clarke 2008). The C6 glioma model was shown to have 1-4% of CSC(Kondo, et al. 2004). When p-HTMI was introduced to C6 glioma cells, approximately 1-2% of the cells stained for p-HTMI, in contrast p-HTE-Ser stained a majority, about >95% of the C6 glioma cells. The C6-derived NSCs also displayed a distinct green fluorescence when treated with p-HTMI, however no staining was observed with p-HTE-Ser treatment. These results were confirmed using flow cytometry analysis validating the selectivity of p-HTMI to a subset of C6 glioma cells.

Given the selectivity of p-HTMI to a subpopulation of C6 glioma cells, we sought to understand the properties of cells detected by p-HTMI. Previously characterized cells from three patients with GBM were used for this study(Xie, et al. 2015). These GBM-derived stem cell-like cells, (GSCs) were subject to flow cytometry analysis after introduction of p-HTMI. 70-90% of cells in these three cultures showed a strong green fluorescence within 10 minutes of application of p-HTMI. To verify the effect of p-HTMI on cell survival, cell death was examined by treatment with propidium iodide (PI) and Annexin V, but no significant difference was observed between control and p-HTMI- treated cells, indicating that this marker does not impact cell survival. Comparison with putative markers to detect stem-like cells, such as CD133 and CD44, indicated that CD133 stained approximately 20-70% of cells in comparison to the 70-90% p-HTMI stained cells. To verify the specificity of p-HTMI over CD133, we compared p-HTMI and CD133-treated U87 cells that have previously been shown to contain a very small subpopulation of GSCs in culture (Heldring, et al. 2014). CD133 and p-HTMI detected a small percentage of cells however CD133 detected more cells than p-HTMI, suggesting that p-HTMI is a more sensitive marker for the detection of GSCs. Since CD44 has been suggested as a marker for CSC (Pietras, et al. 2014; Yuan, et al. 2011), the three GSC cultures were treated with CD44. A majority of the cells were labeled with this marker, suggesting lack of specificity for CSCs compared to p-HTMI. CD271, or nerve growth

receptor factor has been shown to be a prominent marker for neural progenitor cells (NPCs) (van Strien, et al. 2014). To further elucidate the characteristics of these GSCs we co- stained the GSC cultures with CD271 and p-HTMI, followed by flow cytometry analysis. The p-HTMI and CD271 positive cells overlapped, confirming a neural progenitor origin in these cells.

Besides the ease of use and immediate result advantage that LCOs have over other markers, they have been shown to be applicable *in vivo* for the detection of amyloid aggregates in patients with Alzheimer's disease (Klingstedt and Nilsson 2012). To investigate the possibility of using p-HTMI in an *in vivo* context, we injected human GBM- derived stem cell-like cells into the right striatum of mice brains (Heldring, et al. 2014; Kitambi, et al. 2014; Xie, et al. 2015). The animals were subject to daily observation for neurological symptoms. Neurological symptoms such as motor dysfunction, piloerection and behavioural symptoms were observed 16-20 weeks after cell transplantation. After a terminal dose of avertin,
p-HTMI was injected into the original site of cell transplantation. Following sectioning of the brain, tissue was analyzed and a fluorescent staining was detected in a subpopulation of transplanted cells whereas non-injected tumour tissue was unstained although the perfusion solution contained p-HTMI. This verified the *in vivo* applicability of p-HTMI but that it does not cross the blood-brain barrier and needs to be applied in the tumour region of interest.

The use of LCOs as markers for protein aggregates has been well studied (Civitelli, et al. 2016; Klingstedt, et al. 2013b). More recently LCOs were used in the detection of cell types and some of the LCOs showed a preferential staining to a small population of cancer cells (Cieslar-Pobuda, et al. 2014). This study introduces a novel marker, pHTMI, for live detection of neural stem cells and glioma-derived stem cell-like cells. This marker demonstrates a higher selectivity compared to stem cell markers such as CD133 and CD44 to detect GSCs (Pietras, et al. 2014; Singh, et al. 2003). Due to the ability of this marker to be visualized in vivo, a possible clinical use in the detection of CSC can be suggested during surgical resection of GBM along with existing markers currently being used to detect tumour cells (Stummer, et al. 2014), but further studies need to be performed to define the clinical use of pHTMI.

3.2 PAPER II

The cellular cytoskeleton is crucial to the regulation of cell morphology and dynamics, especially in the transformation of cells during malignancy. Recently, various small molecules were identified that are subsequently being investigated for the use in cancer therapy in order to increase efficacy of existing treatments(Oehler, et al. 2012). In this study we established an overview of GBM cytoskeleton regulators available in the literature. 85 genes were listed and classified into cytoskeleton subgroups based on their function such as actin modulators, cortical cytoskeleton modulators, microtubule modulators, calmodulins and

calcenurins, G-protein signaling members, cellular projections, cell shape/ size modulators, cell motility, cell cycle and cytoskeleton adaptors, kinases and phosphatases. The expression of these genes was compared between patient-derived GBM tumour tissue and non-tumour tissue from existing transcriptomic data, and the results were depicted as a heat map. Comparative transcriptomic analysis revealed that, in most cytoskeletal subgroups, there was a significant difference in expression (both increases and decreases) of these genes between the two tissues. This suggested that a better understanding of the cell cycle regulators could be useful in GBM therapy. Genetic alterations are known to be prevalent in GBM (Parsons, et al. 2008). To investigate the extent to which genetic alterations associated with the cytoskeletal genes prevailed in GBM cases, 291 GBM patient cases from the Cancer Genome Atlas (TCGA) were used, and it was found that nearly all the genes of interest involved in the structure and function of the cytoskeleton showed genetic alterations. Fourteen genes were identified with 5% or higher gene alteration, and of these fourteen genes; CLIP2 exhibited the highest genetic alteration (14%). These results suggest that cytoskeletal modulators are indeed affected in GBM and developing therapies targeting these genetic alterations could have a significant impact for treating this disease.

To further elucidate the role of cytoskeleton regulators in GBM, CD133-positive and CD133-negative cells from GBM tumour and NSCs were analyzed as well as GBM-derived stem-like cells before and after differentiation were compared. Similar to the expression changes discussed previously, microtubule modulators showed the highest difference in expression compared to other cytoskeleton regulators studied. To understand if this differential gene expression could have an impact on patient survival, survival plots were generated for each cytoskeleton regulator studied which in turn led to the identification of twelve genes that correlated directly with patient survival. Reduced expression of ARF1P2, ARPC2, ARAP1, CLIP2, MID1, WAS, GSN, CLIP1, LIMK1 and MSN correlated with a significant increase in patient survival, whereas increased expression of CIT and PPP3CB correlated with increased patient survival. Given our *in silico* analyses of gene expression, gene alteration and better patient survival, six genes CLIP1, CLIP2, MID1, ARAP1, ARF1P2 and MSN, were chosen for further study. Since these genes are crucial in tumour development, it was important to investigate the effect of FDA-approved oncology drugs on these modulators.

To select the most potent drugs, GBM cells were tested with a consistent concentration of 10 μM of 125 oncology drugs, and cell viability was measured by quantifying ATP production after 4 days of exposure to the drugs. The drugs were classified into 7 groups based on their mechanism of action. The screen demonstrated that a majority of the drugs that decreased GBM cell viability were kinase inhibitors (>50%), antimetabolite and alkylating agents (>40%). One drug from each of the seven groups that severely affected cell viability was selected and compared to TMZ. TMZ had little effect on cell viability compared to all the FDA-approved oncology drugs tested in this study. This suggests that it may be possible to complement other, more potent, drugs with TMZ to increase the efficacy of these drugs and obtain a better prognosis for patients. A log-dilution series of concentrations of TMZ ranging from 1mM to 100μM was performed in order to understand the cell viability effects of TMZ

on GBM cells. It was shown that a high TMZ dose with a half maximal inhibitory concentration (IC_{50}) value of 269μM at day 4 could be tolerated in these cells.

To analyze the effects of the 7 selected oncology drugs on the cytoskeleton, cells were treated with 10μM of each compound and 269 μM of TMZ. Following 2 days of exposure, cells were fixed and immunostained for acetylated-tubulin, phalloidin and DAPI to visualize cell morphology. Fluorescent imaging identified clear nuclear fragmentation in cells treated with mitomycin, ixabepilone, enzalutamide, and cisplatin, while cells treated with topotecan hydrochloride, TMZ, mitoxantrone, omacetaxine displayed fewer cells with nuclear fragmentation. Cells treated with mitomycin, ixabepilone, mitoxantrone, enzalutamide and omacetaxine showed pronounced clumping of the actin cytoskeleton whereas in cells treated with topotecan hydrochloride, TMZ, and cisplatin no visible cytoskeleton clumping was observed. Treatment with ixabepilone caused the most severe effect on cell morphology amongst the drugs tested. TMZ-treated cells displayed little effect on cell morphology compared to drugs tested. These drugs target different features of the cells suggesting a combination of drugs would be more efficient in treatment. Some compounds used in this study such as topotecan hydrochloride, ixabepilone and mitoxantrone are already in the testing stages for combination therapy with TMZ for GBM (Boiardi, et al. 2008; Bruce, et al. 2011; Kaiser, et al. 2013).

We next assessed gene expression of cytoskeleton regulators (CLIP1, CLIP2, MSN, ARIFP2, ARAP2 and MID1) that correlated with longer patient survival when cells were treated with both selected oncology drugs and TMZ. GBM cells were treated with the respective compounds for 2 days and mRNA expression levels were quantified. The effect of each compound on the cytoskeleton regulators differed with some compounds causing an increase, while others caused a decrease in the expression of cytoskeleton regulators. One of the more pronounced effects was observed when cells were treated with mitoxantrone. Treatment with this drug resulted in decreased expression of CLIP1 and ARAP1. In addition, expression of CLIP2 was dramatically increased following treatment with omacetaxine. CLIP1 and CLIP2 are known to play a role in microtubule interaction with membranous organelles (Lewkowicz, et al. 2008). High expression of CLIP1 has shown to be a mediator in sensitivity of chemotherapeutic agents such as paclitaxel (Sun, et al. 2012). Although all drugs tested led to a decreased expression of CLIP1, a few drugs, including toptecan hydrochloride, mitomycin, omacetaxine and TMZ displayed less of a dramatic decrease in the expression of CLIP1 compared to other drugs. The survival curve data demonstrated that, low expression of CLIP2 correlated with increase in patient survival. The compounds tested revealed an increased expression of CLIP2 but treatment with drugs such as TMZ, enzalutamide, mitoxanthrone and cisplatin resulted in a less prominent increase. TMZ was the common drug here with a more favourable outcome in CLIP1 and CLIP2 expression. TCGA-derived GBM patient survival curve analysis described earlier revealed that overall survival of GBM patients decreased with high expression of cytoskeleton regulators such as MSN, ARFIP2, ARAP1 and MID1. Treatment with TMZ decreased expression of these cytoskeleton regulators involved in cell migration, membrane ruffling, receptor trafficking and

microtubule functioning (Aranda-Orgilles, et al. 2008; Daniele, et al. 2008; Van Aelst, et al. 1996; Wu, et al. 2013).

This study highlights the importance of a better understanding of the cytoskeletal regulators in GBM. Cytoskeletal regulators respond to environmental cues and control cellular dynamics (Fletcher and Mullins 2010). Our results demonstrated that cytoskeletal regulators in particular expression of microtubule regulators is increased in tumour tissue suggesting that manipulating the microtubule can be utilized for GBM therapy. Patupilone is a tubulin-binding agent that is being explored in GBM treatment and initial studies with this small molecule have been successful when complemented with radiation (Fogh, et al. 2010; Oehler, et al. 2012). Genetic alterations in cytoskeletal regulators were identified in our study, suggesting that such alterations could be targets for GBM therapy (Fife, et al. 2014). Moreover the study elucidates the advantages of using TMZ, in combination with cytoskeleton regulators to increase chemosensitivity. Drugs targeting cytoskeletal regulators may be candidates for co-administration with TMZ in order to improve GBM therapy. With the knowledge of the function of cytoskeleton regulators and compounds influencing them, this will enable selection of compounds for combination therapy of GBM.

3.3 PAPER III

Neural stem cell proliferation and differentiation is an intricately defined process with several signaling molecules and transcription factors regulating this process. Some of these regulators include the histone HDACs, NCoR/Ncor1 and SMRT/Ncor2. In this study we shed light on the specific function of transcriptional repressor checkpoints controlling differentiation using genome-wide and single-gene analysis of these regulators. Since HDAC2 and HDAC3 are known to be prevalent along with genes involved in the transcriptional regulation of differentiation, a chromatin immunoprecipitation-sequencing (ChIP-Seq) and single gene ChIP analysis in NSCs was performed in order to understand their functions. A validation of the ChIP-Seq results using ChIP-qPCR revealed that HDAC2 and HDAC3 were enriched at promoters of the Rhox-family of genes known to be involved in development(Maclean, et al. 2005). Also, HDAC2 and HDAC3 were also enriched at the promoters of transcription factors *Pax6* and *Sox8*, both of which are involved in neuronal differentiation. In this case, HDAC3 was significantly enriched at the promoter of *Pax6* and *Sox8*, however HDAC2 demonstrated an increased enrichment only at the promoter of *Sox8*.

To further investigate the specific roles of these HDACs in NSCs, we knocked down *Hdac2* and *Hdac3* mRNA in NSCs using specific small interference RNA (siRNA). In comparison with a control, knockdown of *Hdac2* and both *Hdac2* and *Hdac3* resulted in a global hyperacetylation of H3K9, significant increase of TuJ1-positive cells, and increased H3K9 acetylation on the *Bdnf*IV promoter (Koppel and Timmusk 2013). Single knockdown of *Hdac2* did not have any effect on these genes. qRT-PCR revealed that siHDAC3, but not siHDAC2, induced a significant increase in *Bdnf* expression. This result highlighted the role

of HDAC3 alone in H3K9 acetylation, *Bdnf* expression and neuronal differentiation in progenitor cells. Previous studies have shown that the HDAC-associated corepressors NCoR and SMRT, contribute to the neuronal differentiation in NSCs where spontaneous neuronal differentiation was observed in NSCs from *Smrt*-deficient mice, but not in *Ncor*-deficient or *Ncor* and *Smrt* deficient NSCs. (Hermanson, et al. 2002; Jepsen, et al. 2007). We analyzed the expression of *Bdnf* in *Smr*t$^{-/-}$ and *Ncor*$^{-/-}$ NSCs and it was observed that *Smrt*$^{-/-}$ but not *Ncor*$^{-/-}$ demonstrated a significant (>4 fold) increase in *Bdnf* mRNA levels compared to the control in NSCs.

Microarray analysis of *NCoR*$^{-/-}$ NSCs demonstrated a prominent upregulation of oligodendrocyte (OL)-associated genes such as myelin-basic protein (*Mbp*), myelin proteolipid protein (*Plp*), *beta tubulin 4* and *Nkx2.2* and glial markers such as *s100β*. This upregulation was not, however, observed in neuronal genes. The OL-associated gene upregulation was restricted to *Ncor*$^{-/-}$ NSCs but not *Smrt*$^{-/-}$ NSCs or NSCs exposed to the HDAC inhibitor valproic acid (VPA). *Ncor*$^{-/-}$-related upregulation of OL-associated genes was verified by immunochemistry and qRT-PCR analysis. These results suggest the role of NCoR in mediating differentiation of NSCs into oligodendrocytes. Triiodothyronine (T3) is known to be important in OL development (Billon, et al. 2002). Due to the influence of T3 stimulation on NCoR, which then regulates transcription (Astapova, et al. 2008), we decided to investigate the effect of T3 on NSCs. When NSCs were treated with T3, this resulted in OL differentiation. To further explore the correlation between NCoR and HDACs in regulating NSC differentiation, NSCs were treated with T3 and VPA alone as well in in combination. VPA treatment alone did not affect OL gene expression but combined treatment with T3 and VPA resulted in an increased expression of OL-associated genes. An increase in late versus early markers of OL differentiation was also observed. This was confirmed by observing morphological features after immunochemistry.

Since our ChIP-Seq analysis identified that HDAC2 was enriched at the promoter of *Sox8*, a gene involved in oligodendrocyte differentiation, this suggested the influence of HDAC2 in the differentiation process of oligodendrocytes. NSCs, both with and without T3 were transfected with siHDAC2 and/or siHDAC3. Knockdown of *Hdac*s resulted in a significant increase in OL-associated genes in the cells differentiated with T3. In the cell lines Oli-neu (Jung, et al. 1995) and CG4 (Louis, et al. 1992), knockdown of *Hdac2* resulted in an increased expression of OL-associated genes. These results indicate that the previously described effect of VPA on T3 differentiated OL is partially dependent on HDAC2. Gene expression profiling of NSCs treated with T3, VPA or VPA and T3, identified an increase in the OL-associated transcription factor *Sox10* following VPA treatment, but it was unaffected by T3. Also *Sox10* expression was increased with siHDAC2 and T3 treatment but not with siHDAC3. Transfection with siRNA against *Sox10* partially prevented the upregulation of OL-associated genes and terminal OL differentiation was blocked following treatment with VPA and T3. These findings emphasize the role of SOX10 in controlling OL differentiation in NSCs when treated with VPA/siHDAC2 and T3.

To further understand the role of transcriptional regulators in OL differentiation, the enrichment of HDAC2 and HDAC3 was investigated in the *Mbp* and *Sox10* gene regulatory regions. This demonstrated that both HDAC2 and HDAC3 were enriched in the regulatory regions of *Mbp* but in *Sox10* regulatory regions, only HDAC2 enrichment was observed. Since SOX2 has been reported to be a binding partner of HDACs and nuclear co-repressors (Engelen, et al. 2011) the relevance of the HDAC2-mediated repression of *Sox10* with SOX2 was questioned. It was found that SOX2 was present in the regulatory regions of *Sox10* suggesting a SOX2-mediated HDAC2 repression of Sox10 influencing OL differentiation. Knockdown of *Sox10* also resulted in an increase in the expression of stem cell related genes such as *Sox2* and *Sox9*. Thus it can be concluded that *Sox10* plays a crucial role in OL differentiation especially in the late stages of differentiation and also represses stem cell related genes.

3.4 PAPER IV

Medulloblastoma tumours demonstrate significant molecular heterogeneity, and a better understanding of the factors that govern this heterogeneity could assist in improved diagnosis and tumor-specific treatments. These tumours have been classified into 4 subgroups based on genetic alterations and signaling pathways. The subgroups include, WNT, SHH, Group 3 and Group 4 (Taylor, et al. 2012). Histone demethylases such as KDM6B/JMJD3 associate with the WNT and SHH signaling pathways to regulate developmental processes (Burchfield, et al. 2015). Studies have reported the role of KDM6B/JMJD3 in the presence of RA, in mediating neuronal differentiation programs (Jepsen, et al. 2007).

We treated the DAOY medulloblastoma cell line with RA for 6h and 24h and observed a transient upregulation of *KDM6B/JMJD3* at 6h. Given the role of KDM6B/JMJD3 in development, we examined the expression of certain neuronal genes and transcriptional regulators after treatment with RA. A slight upregulation of *TUBB3* and *NIF3L1* was observed amongst other developmental genes investigated. To validate whether the transient upregulation observed with RA treatment is linked to *KDM6B/JMJD3*, this gene was knockdown using siRNA. However the RA-mediated upregulation of *TUBB3* and *NIF3L1* was no longer prevalent. The expression of *TUBB3* and *NIF3L1* was decreased in the RA-treated samples in 3 out of 4 experiments, which could suggest a potential role for *KDM6B/JMJD3* in the maintenance of neuronal gene expression in the presence of RA. However, the results did not provide evidence for an essential role for KDM6B/JMJD3 in the RA-mediated upregulation of these genes.

These findings were further validated by performing a gene expression analysis of DAOY treated with RA/DMSO for 6h. This experiment identified *SMAD3* as a RA target. Previous studies have associated the interaction of KDM6B/JMJD3 with SMAD3 in regulation of TGFβ- induced neuronal differentiation (Estaras, et al. 2012). Since medulloblastoma is a developmental tumour, investigating the association of SMAD3 with KDM6B/JMJD3

could be useful in understanding signaling pathways associated with medulloblastoma. Cells transfected with siRNA-targeting *KDM6B/JMJD3* resulted in a diminished expression of *SMAD3* in the presence of RA. In the presence of siRNA-targeting *SMAD3*, the upregulation of *KDM6B/JMJD3* was affected. This result provides evidence of the association of KDM6B/JMJD3 with SMAD3 in this cell line.

To further investigate the association of KDM6B/JMJD3 and SMAD3, we performed a genome-wide sequencing study to identify potential binding sites of SMAD3 and KDM6B/JMJD3 in the presence of RA/DMSO. Through this study it was evident that SMAD3 and KDM6B/JMJD3 co-occupy genomic loci upstream of the *KDM6B/JMJD3* gene locus independent of RA. This suggests that the RA-mediated effect of KDM6B/JMJD3 is either independent of this site, or that RA could mediate the recruitment of other factors to regulate KDM6B/JMJD3. The results also demonstrated that RA regulates the SMAD3 genomic recruitment as many binding targets were enriched in the presence of RA compared to genomic recruitment by SMAD3 in the presence of DMSO or genomic recruitment by KDM6B/JMJD3 in the presence of DMSO/RA. The analysis also demonstrated co-occupancy of SMAD3 and KDM6B/JMJD3 irrespective of treatment, suggesting a cross talk between SMAD3 and KDM6B/JMJD3.

This study describes the molecular interactions in medulloblastoma particularly the regulation of *KDM6B/JMJD3* by RA. Jepsen et al proposed a model of upregulation of *KDM6B/JMJD3* in the presence of RA, in neural stem cells (Jepsen, et al. 2007). SMAD3 has been identified as an indicator of pathogenesis of medulloblastoma (Aref, et al. 2013). Our results identify that SMAD3 and KDM6B/JMJD3 co-occupy the genomic loci upstream of the KDM6B/JMJD3 gene, and SMAD3 showed highest occupancy after RA treatment. Thus, there is a cross talk between SMAD3 and KDM6B/JMJD3 and further experiments are needed to characterize the RA-mediated increase in SMAD3 genomic interactions in medulloblastoma.

4 CONCLUSIONS

Through the studies outlined in this thesis, we provide evidence leading to a better understanding of the molecular characteristics of glioblastoma and medulloblastoma. We introduce a novel approach for specific detection of neural stem and cancer stem cells using a fluorescent probe. In addition, we have also determined a great deal about mechanisms involved in neural progenitor cells, which contribute to tumour formation in these CNS tumours.

The ability to identify specific cell types is useful in understanding the function of these cells in pathways implicated in diseases. Current methods of cell detection, such as immunochemistry, involve using a combination of markers in order to detect different cell types. While this method can offer some level of specificity, immunological markers can still detect a range of cell types, indicating the need for a more specific marker. In paper I, we introduce a novel molecular probe, p-HTMI that can be used on live cells for quick and easy detection of neural stem cells. Further, comparison studies using putative markers of cancer stem cells demonstrate increased specificity of this molecular probe compared to existing immunological markers. p-HTMI did not impact cell survival, indicating it could be applied *in vivo,* and further work would need be done to determine if the probe could be applied in combination with other chemotherapeutics to act as a more efficient GBM therapy. The *in vivo* applicability of this molecular probe suggests a possible use in surgical intervention, however further studies need to be performed to validate this use.

Regulators of the cytoskeleton are important determinants of tumour growth and survival. Understanding the impact of chemotherapy on cytoskeletal integrity could aid in the development of more efficient GBM therapy. In paper II, we demonstrate the difference in expression of cytoskeleton regulators in tumour and non-tumour tissue, and correlate the effect of this expression on patient survival. Our findings suggest that cytoskeleton regulators could offer attractive targets for GBM in combination with existing chemotherapeutics such as TMZ. Since each drug targets different cell characteristics, combination therapy could ensure a more effective treatment.

Differentiation of neural progenitor cells is controlled by several signaling factors and control mechanisms. Elucidating the roles of factors involved in the neural differentiation process could provide cues as to how these processes are involved in disease. Paper III identifies the function of HDACs in controlling differentiation of neuronal and oligodendrocyte-related genes in neural stem cells. The function of HDACs is mediated by external factors such as T3, RA, as well as transcription factors SMRT and NCOR. These control mechanisms in differentiation of neural stem cells highlights the need for various checkpoints controlling development of neural progenitors.

Paper IV provides an insight into the signaling pathways involved in medulloblastoma. We show that upregulation of genes involved in neuronal development, such as *KDM6B/JMJD3*, is based on transient treatment with RA. This gene regulation was shown to be associated

with a binding partner SMAD3. Genome-wide analysis of binding sites revealed that KDM6B/JMJD3 and SMAD3 co-occupy the region upstream of the KDM6B/JMJD3 gene locus regardless of RA, and the since SMAD3 showed highest genomic interactions in the presence of RA, future studies need to be performed to investigate these genomic interactions with SMAD3 mediated by RA and how this may influence the crosstalk between genes implicated in medulloblastoma.

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