Investigation of the anticancer activity and molecular mechanisms of Disulfiram in Glioblastoma Multiforme

VINODH KANNAPPAN

(Student Number: 0821312)

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Research Institute in Healthcare Science
Faculty of Science and Engineering
University of Wolverhampton

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Author's Declaration

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Vinodh Kannappan

Date
Greater joy than at his birth, a mother feels, when she hears, her son being hailed as a good man by people.

The good which a father can do to his son, is to prepare him, for a prominent role in the assembly of the learned.

“I dedicate this thesis to my beloved Dad and Mom who made this possible”
Glioblastoma Multiforme (GBM) is the most common lethal brain tumour associated with dismal survival rate. GBM is considered to be an incurable malignancy as these tumours evade all intricate attempts of therapy and no contemporary chemotherapeutic regimen is effective. Although the existence of cancer stem cells (CSCs) is still debatable, it is widely accepted that GBM has a small population of cells expressing CSC markers (~1%) that are highly resistant to chemo-radiation therapy. Recent evidence indicates that hypoxia induces cancer stem cell (CSC) phenotypes via epithelial-to-mesenchymal transition (EMT) that promote therapeutic resistance in solid tumours. Given that GBMs are extensively hypooxygenated heterogenous tumours, understanding the molecular relationship between hypoxia, biology of CSCs, EMT and chemoresistance would be invaluable for development of drugs that can target CSCs. Evidence suggests that hypoxia inducible factors (HIFs), NF-κB and aldehyde dehydrogenase (ALDH) together orchestrate the stemness and chemoresistance in hypoxia induced CSCs. But the insights on the mechanisms still remain obscure. In this study we used an in vitro GBM CSC and hypoxia model along with NF-κB-p65 and HIF transfected GBM cell lines to investigate the relationship between HIFs, NF-κB activation and ALDH activity and their role in chemoresistance.
The findings of this study demonstrated that GBM cells grown as spheres consist of a vast proportion of hypoxic cells with elevated CSC and EMT markers suggesting hypoxia induced EMT. GBM-CSCs are chemoresistant and displayed increased levels of HIFs, NF-κB and ALDH activity. It was also observed that stable transfection of GBM cells with NF-κB-p65 or HIFs induced CSC and EMT markers indicating their essential role in maintaining CSC phenotypes. The study also highlighted the importance of NF-κB and ALDH in driving chemoresistance and the potential role of NF-κB as the master regulator of hypoxia induced stemness in GBM cells.

In this study, we used Disulfiram (DS), an anti-alcoholism drug, in combination with copper (Cu) to target the hypoxia-NF-κB axis and inhibit ALDH activity to reverse chemoresistance in GBM CSCs. We showed that DS/Cu is cytotoxic to GBM cells and completely eradicated the resistant CSC population at low nanomolar levels in vitro. We also demonstrated that DS/Cu effectively inhibited GBM in vivo using newly formulated PLGA-DS nanoparticles. DS is an FDA approved drug with low/no toxicity to normal tissues and can freely pass through the blood brain barrier (BBB). Further study may lead to quick translation of DS into clinical trials.
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<tbody>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>TCR</td>
<td>T-Cell Receptor</td>
</tr>
<tr>
<td>AAG</td>
<td>Alkyladenine DNA Glycosylase</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>AGT</td>
<td>O(^6)-Alkylguanine-DNA Alkyltransferase</td>
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<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
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<td>AKT3</td>
<td>Murine Thymoma Viral Oncogene Homologue 3</td>
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<td>Aldehyde dehydrogenase</td>
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<td>ATT-NOR</td>
<td>Attached normoxia culture</td>
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<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
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<td>Blood brain barrier</td>
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<td>CAF</td>
<td>Cancer Associated Fibroblast</td>
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<td>Abbreviation</td>
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<td>CAM</td>
<td>Cancer Associated Macrophage</td>
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<td>DDC</td>
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<tr>
<td>DMEM</td>
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<tr>
<td>EBRT</td>
<td>External Beam Radiation Therapy</td>
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<td>Enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
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<td>Endothelial PAS Domain Protein 1</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal related kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>Poly HEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
</tr>
<tr>
<td>hFGF</td>
<td>Human fibroblastic growth factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HP</td>
<td>Hypoxyprobe</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia Response Element</td>
</tr>
<tr>
<td>HSCs</td>
<td>Haematopoietic stem cells</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IκBα</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
</tr>
<tr>
<td>IkBβ</td>
<td>IκB kinase β</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun N-Terminal Kinases</td>
</tr>
<tr>
<td>KPS</td>
<td>Karnofsky Performance Scale</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple drug resistance</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal epithelial transition</td>
</tr>
<tr>
<td>MGMT</td>
<td>O⁶-Methyl Guanine Methyl Transferase</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molar</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRP</td>
<td>Multi-Drug Resistance Associated Protein</td>
</tr>
<tr>
<td>MTIC</td>
<td>3-Methyl-(Triazen-1-Yl) Imidazole-4-Carboxamide</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NANOG</td>
<td>Homeobox protein NANOG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NHA</td>
<td>Normal human astrocytes</td>
</tr>
<tr>
<td>nM</td>
<td>Nano molar</td>
</tr>
<tr>
<td>NP</td>
<td>Nano particle</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NS</td>
<td>Neurosphere</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAC</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PHD</td>
<td>prolyl-hydroxylases</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>PTPRD</td>
<td>Protein Tyrosine Phosphatase, Receptor Type, D</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Control Trial</td>
</tr>
<tr>
<td>REL A</td>
<td>v-rel avian reticuloendotheliosis viral oncogene homolog A</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel Homology Domain</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Luciferase unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SART1</td>
<td>Squamous Cell Carcinoma Antigen Recognized By T Cells</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SLC</td>
<td>Stem-Like Cell</td>
</tr>
<tr>
<td>SLUG</td>
<td>Snail Family Zinc Finger 2</td>
</tr>
<tr>
<td>SNAI1</td>
<td>Snail Family Zinc Finger 1</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>SUS</td>
<td>Suspension</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline - Tween</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIC</td>
<td>Tumour Initiating Cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TWIST</td>
<td>Twist Family bHLH Transcription Factor</td>
</tr>
<tr>
<td>VCR</td>
<td>Vincristine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
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Chapter 1

Introduction
Cancer is a major public health problem and a leading cause of disease in the world. It is also the major cause of death worldwide surpassing coronary heart disease and stroke (WHO.int, 2015; Ferlay et al., 2014). According to the latest World cancer report 2014 published by the World Health Organization’s International Agency for Cancer Research (WHO-IARC), the global burden of cancer is growing at an alarming rate with an estimated 14.1 million new cases reported in 2012. With the current trend, this figure is expected to increase by 68% in 2030 to reach 23.6 million new cases every year. Over the same time the number of deaths caused by cancer will increase from 8.2 million in 2012 to 13 million per year (Bray et al., 2012; IARC- World cancer report, 2014; IARC.FR, 2015). Lung cancer (13%), breast cancer (11.9%) and bowel cancer (9.7%) are the most commonly diagnosed cancers and the majority of cancer deaths are due to lung, liver, stomach, colorectal and female breast cancers. Most cancer deaths occur in less developed countries due to high population, lack of early diagnosis and access to treatment (Gulland, 2014; IARC- World cancer report, 2014; CRUK, Cancer Stats, 2015).

1.1 Brain Tumours

Among all the cancers, tumours occurring in the brain and central nervous system (CNS) are relatively rare and constitute only 2% of total cancer cases in the world (256,000 cases in 2012) (Ferlay et al., 2014). There are more than 130 different types of tumours that can occur in the brain and other parts of CNS. They can be either primary (those which originate in the brain) or secondary (other cancers that metastasize to brain) brain tumours (CRUK,
Brain, other CNS and intracranial tumours key statistics, 2015). Though small in number, they are considered to be the worst type of tumours due to the sensitive location, lack of successful therapeutic measures and very high mortality rates. Primary brain tumours are those, which develop intracranially from the brain parenchyma to its surrounding structures. They are a heterogeneous group of benign or malignant tumours that are considered to be an important cause of cancer related deaths in adults and children (de Robles et al., 2014). Primary brain tumours account for 2.3% of cancer related deaths with an estimated 189,000 reported deaths worldwide in 2012 (CRUK, Cancer statistics, 2014; Ferlay et al., 2014).

1.2 Gliomas

Among different histological subtypes of primary brain tumours gliomas are the most frequent malignant entities that arise in the glial tissue. Glial cells are the most common group of cells in the brain that maintain the blood brain barrier and provide the nerve cells with protection, support, energy and nutrients. Different glial cells have different functions, for example, astrocytes hold the neurons in place and supply them with nutrients, oligodendrocytes provide insulation to the neurons, microglia process dead neurons and ependymal cells secrete cerebrospinal fluid and line the ventricular regions (Kandel, 2003). Gliomas represent 81% of malignant brain tumours and they develop as astrocytomas, oligoastrocytomas, oligodendrocytomas or ependymomas depending on the cell type or can be a mix of two cell types. They are classified according to the grade of malignancy established by the WHO as grade I-IV.
Oligodendrogliomas and oligoastrocytomas fall under grade II and grade III respectively. Astrocytomas are of four types: pilocytic, grade I; diffuse, grade II; anaplastic, grade III; and glioblastoma multiforme (GBM), grade IV (Louis et al., 2007). The most common type of gliomas are the astrocytomas which account for 76% of all gliomas, the subtype glioblastoma multiforme alone accounting for more than 54% of all gliomas. The other less frequent types of gliomas are anaplastic astrocytomas (10-15%), anaplastic Oligodendrogliomas and anaplastic oligoastrocytomas (10%). The remaining 5% belong to rare glioma types, such as anaplastic ependymomas and anaplastic gangliogliomas (Wen and Kesari, 2008).

1.3 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumour in adults (Dolecek et al., 2012; Filippini, 2012). Glioblastomas consist of a heterogeneous group of tumours that differ both genetically and phenotypically and are classified as WHO grade IV astrocytomas, the most malignant grade. Compared with other gliomas, GBMs are highly invasive, proliferative and angiogenic in nature (Karcher et al., 2006). Based on clinical presentation GBMs are of two subtypes: i) de novo primary tumours, which accounts for more than 85% cases occur commonly in patients older than 60yrs and develop by multistep tumourigenesis of normal glial cells ii) The remaining 10-15% of cases present as secondary GBMs where low grade gliomas like diffuse or anaplastic astrocytoma can slowly progress or are transformed to GBM over a period of 4-5 years (Tso, 2006). This type of GBM is quite rare and
is very common in patients below the age of 45. Despite remarkable achievements in oncology in recent years, treatment and management of GBM patients still remain very challenging.

Table 1.1 WHO classifications of gliomas (Louis et al., 2007).

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Grade IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytic tumours</td>
<td>Pilocytic astrocytoma</td>
<td>Pilomyxoid astrocytoma, Diffuse astrocytoma</td>
<td>Anaplastic astrocytoma</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>Oligodendroglial tumours</td>
<td></td>
<td>Oligodendroglioma</td>
<td>Anaplastic oligodendroglioma</td>
<td></td>
</tr>
<tr>
<td>Ependymal tumours</td>
<td>Subependymoma</td>
<td>Ependymoma</td>
<td>Anaplastic ependymoma</td>
<td></td>
</tr>
<tr>
<td>Mixed and other common types of glioma</td>
<td>Angioblastic glioma, pilocyticoma</td>
<td>Oligoastrocytoma</td>
<td>Anaplastic oligoastrocytoma</td>
<td></td>
</tr>
</tbody>
</table>

**Fig 1.1** Distribution of all primary gliomas in the CNS (Adamson et al., 2009)
1.3.1 Historical Overview of Glioblastoma multiforme

Glioblastomas were originally known as ‘fungus medullare’ during the 1800s. GBM was first identified as tumours originating from glial cells through macroscopic and microscopic observations done by Dr. Rudolf Virchow in 1863 and hence he coined the term ‘glioma’. Later in 1920, Dr. Walter Dandy reported the differences between GBM and other gliomas by observing the extensive infiltrative nature of GBM and named it as ‘spongioblastoma’ (Fleming, 1926). In 1926 two neurosurgeon/neuropathologists Bailey and Cushing thoroughly studied the microscopic features and clinical history of over 400 glioma samples. Cushing introduced the term glioblastoma multiforme in the second half of the nineteenth century. The term ‘multiforme’ reflects the heterogeneous nature in clinical presentation, genetic characteristics, pathology and responsiveness to treatment of this type of tumour (Bailey and Cushing, 1926).

1.3.2 Epidemiology of Glioblastomas

Cancer registry data from Europe and North America show that the number of recorded GBM cases is 3.1 per 100 000 adults each year, and is more common in men in comparison to women at the ratio of 1.26: 1 (Crocetti et al., 2012; Jemal et al., 2010; Omuro, 2013). Around 87% of the cases occur in adults at a median age of 65yrs at diagnosis. Though GBM is less common in children, it contributes to 15% of all childhood brain tumours (Arora et al., 2008). There are no differences in morphology or histology between adult and childhood GBM. It
is also reported that GBM is more common in the Caucasian population than in other ethnic origins (Filippini, 2012; Wen and Kesari, 2008). Despite aggressive treatment like surgery, radiotherapy and chemotherapy the median survival period for GBM patients is only 14.6 months (Stupp et al., 2005; Darefsky et al., 2011). Population based studies show that only less than 30% of newly diagnosed GBM patients live for more than 1 year and only 3-5% of GBM patients live for more than 2 years. This is one of the worst survival rates among all cancers (Ostrom et al., 2014; Sant et al., 2011; Jung et al., 2012; Lamborn et al., 2008; Arrigo et al., 2011; Mirimanoff et al., 2007). Recurrent GBMs have much worse prognosis with a median survival between 3-5 months. Childhood GBMs have a very poor prognosis. Secondary GBMs in women are known for their worse median survival rate (Krex et al., 2007; Henriksson et al., 2011).

Age is an important prognostic factor in GBM where the survival rate is clearly higher for younger patients while elderly patients have a median survival rate of only 4.5 months (Reardon and Arvold, 2014; Iwamoto et al., 2008). Epidemiological data suggest that GBM incidence rates are rapidly increasing especially with patients aged over 65 years (Reardon and Arvold, 2014). Among this age cohort the incidence rate has doubled from 5.1 per 100,000 in the 1970s to 11 per 100,000 in the 2000s. No contemporary treatment regimen is shown to improve the survival rates despite 3 decades of advancements in oncology and long term survival for GBM patients is exceptional (Perez-Larraya et al., 2011; Wick et al., 2012).
1.3.3 Etiology of Glioblastoma multiforme

The etiology of GBM has not been fully elucidated and the predisposing factors are poorly understood. Although various risk factors like ionizing radiation, pesticides, polycyclic aromatic compounds, solvents, rubber industry, petrochemical industry and electromagnetic fields are described in medical history, GBM is widely considered to be a spontaneous tumour (Spinelli et al., 2010). About 1% of GBM patients represent familial aggregations; however, the genetic background for development of this type of GBM is different from spontaneous ones (Karim et al., 2011). Some genetic diseases like tuberous sclerosis, Turcot syndrome, and other genetic disturbances like the loss of genetic material in chromosome 10q, amplification of genes like EGFR, FGFR2, IRS2, AKT3 and mutations in genes like PTEN, TP16, TP53, PARK2, PTPRD and NF1 are also attributed to the development of GBM (McLendon et al., 2008; Urbańska et al., 2014). Recently, investigators believe that human cytomegalovirus (HCMV), is also among the etiologic agents for the development of GBM. Although various contributing factors and risk factors for GBM are mentioned in the literature, the true etiologic agent is still undetermined.

1.3.4 Biological and morphological features

GBM mainly form in the hemispheres of the brain or very rarely in subtentorial brain stem or cerebellum. It is characterized by infiltrating growth and typically contains a mix of tumour cells and stromal cells, therefore it is difficult to clearly distinguish tumour mass from the normal tissue. The tumour consists of
signature characteristics like poorly differentiated neoplastic astrocytes with brisk mitotic activity, polymorphism, anisokaryosis, neoangiogenesis and necrosis (Robson, 2001). GBM cells also show nuclear pleomorphism (increased nuclear to cytoplasmic ratio) and intranuclear inclusions (Schultz et al., 2005). Neovascularization is a characteristic feature of GBM that distinguishes it from low grade gliomas and contain irregular layer of pericytes and have vascular thrombosis (Rojiani and Dorovini-Zis, 1996; Di leva et al., 2011). Presence of necrotic foci is one of the most distinctive characteristics of GBM. Histologically, two types of necrosis have been observed in GBM (Robson, 2001). The first type has a large central necrotic region in the tumour, which is formed because of insufficient blood supply, and the second type contains multiple, small, irregular pseudopalisading necrotic foci, which are created by radially, oriented glial cells. The zone of pseudopalisades contains the rapidly dividing neoplastic cells that have undergone central necrosis; a population resistant to apoptosis; inflammatory cells around these necrotic regions; migratory cells and stem like cells (Brat, 2004). This is the reason why GBMs are inherently aggressive and resistant tumours with increased malignancy and infiltration that makes it almost impossible to remove, treat or cure this malignancy. Despite the aggressive and proangiogenic nature of GBM, metastasis of this neoplasm outside CNS is extremely rare. It is assumed that the barrier created by cerebral meninges, absence of lymphatic vessels and short life span of patients are key reasons behind the low metastatic potential of GBM (Lun et al., 2011; Robert and Wastie, 2008).
1.3.5 Molecular subtypes of GBM

Recent insights into the genomic profiling of GBM have allowed clinicians to categorize GBM into four subtypes based on distinguishing genetic alterations and molecular profiles. Using integrated genomic approach researchers described an expression-based molecular classification of GBM into classical, mesenchymal, proneural, and neural with specific molecular signature for each subtype as shown in the figure below (Verhaak et al., 2010; Brennan et al., 2009; Network, 2013; Phillips et al., 2006).

Figure 1.2 Molecular Subtypes of GBM. (A) Genetic and chromosomal alterations in primary and secondary GBM. (B) Recent sub classification of primary GBM in four further distinct subtypes through integrated genomic analysis. (Adapted from Agnihotri et al., 2013).
1.4 Treatment for GBM

Treatment of GBM has a very challenging and a long history with more than a hundred different types of clinical trials reported in the literature even dating back to 1960s. This was mainly attributed to the brain itself as a sensitive organ controlling multiple functions; hence, any damage caused to it by the tumour or treatment strategies would lead to poor quality of life and very short survival period. Over many years, the challenges in developing effective treatment for GBM were met only by numerous Phase III randomized control trials (RCTs). The current standard therapy for newly diagnosed GBM includes maximal safe surgical resection, followed by parallel radiation therapy to the resection site and chemotherapy with adjuvant Temozolomide (Stupp et al., 2005). This procedure which is also known as Stupp protocol emerged as the standard of care for GBM after the seminal work done by Stupp and colleagues that increased the survival rate from 12.1 months to 14.6 months.

1.4.1 Surgical resection

Surgery remains a cornerstone in the treatment of GBM. The primary aim of surgery is to achieve gross total resection of the tumour without affecting neurological functions (Anton et al., 2012). In addition to cytoreduction, surgery provides samples for histological and molecular analysis to confirm the diagnosis and to identify the subtypes that lays foundation for further treatments and prognosis (Ryken et al., 2014). Surgery is also considered as a therapeutic measure to reduce the intracranial pressure thereby leading to recovery of lost neurological function. However, on the other hand cytoreductive surgery leads to loss of vital brain tissue that will further cause immediate neurological deficits.
Hence, many neurosurgeons argue against surgery as a treatment attempt because of the anticipated worst quality of life (Ryken et al., 2014). However, it has to be noted that many retrospective studies and RCTs in the past has shown that a safe maximal surgical resection of the tumour >98% will significantly improve the survival rate helps patients respond better to radiotherapy and chemotherapy after surgery (Lacroix et al., 2001). In contrast, elderly patients above the age of 70 have no benefit from surgery as the overall median survival rate is increased only by a month (from 4.5 months to 5.7 months) with the additional burden of poor quality of life (Harter et al., 2014; Ryken et al., 2014). GBM is diffuse, highly invasive, infiltrates adjacent tissue and have ill-defined borders, so a complete resection is impossible. In addition, involvement of eloquent structures or both hemispheres will make it a very difficult decision for the neurosurgeon to call for surgery. When the tumour is reduced partially, the residual tumour behaves in a very aggressive and malignant manner with massive oedema and acute neurological effects. The Surgeons generally avoid surgery strictly if the patients have a poor performance status (Karnofsky performance scale [KPS] <70), very old age, complicated location or involvement of both cerebral hemispheres (Harter et al., 2014). Advanced surgical imaging techniques like intraoperative magnetic resonance imaging (MRI), diffusion tensor imaging, awake craniotomy, cortical mapping, stereotactic guidance, and fluorescent guided resection, have made it easier for neurosurgeons to visualize a defined border for the tumour for maximal resection (Hoover et al., 2010). However with all these advancements surgery appears to improve survival only for a short term and there is no change in the 2 years disease free survival (Ryken et al., 2014).
1.4.2 Radiotherapy

The use of adjuvant radiotherapy (RT) following surgical resection has been a historical gold standard for management of GBM patients. The current standard for RT in GBM is postoperative fractionated focal external beam radiation therapy (EBRT). About 60Gy of RT is administered as 30 fractions (2Gy/fraction) over a period of 6 weeks to the surgical resection cavity and 2cm ring of surrounding tissue beyond the rim of the cavity. Dose higher than 60Gy has no survival benefit due to increased toxicity. RT is usually given after 2 weeks of surgery to provide adequate time for wound healing. In some cases like multifocal GBM, whole brain RT is used (Leibel and Sheline, 1987). Phase III RCTs have shown that RT clearly doubles survival rates after surgery from 4-5 months to 9-10 months. RT is shown to be the best treatment choice for elderly patients aged over 70yrs where surgery is not an available option for them. Given that RT along with supportive care alone provides a survival advantage in elderly patients, RCTs are now being carried out to analyse whether modifying the conventional RT strategy with hypofractionated, or low dose whole brain RT will provide extra survival benefit for these patients (Ryu et al., 2014; Adamson et al., 2009). Ionizing radiation induces single and double strand breaks in the DNA of dividing cells and hence it is obvious that it will have a significant deleterious effect on normal brain tissue as well. In addition, radiation causes various side effects like headaches, vomiting, cognitive disturbances, memory loss, worsening neurological deficits and damage to BBB leading to oedema (Adamson et al., 2009; Harter et al., 2014).
1.4.3 Chemotherapy

Chemotherapy for GBM has been the most varied and disappointing strategy in the history of cancer therapeutics, with numerous laboratory research and RCTs dating back to 1970s (Walker et al., 1978). The benefits offered by chemotherapy for GBM patients have been questioned for many decades but various RCTs involving different chemotherapeutic regimen shows that patients receiving chemotherapy along with radiation have a significant survival benefit when compared with patients receiving radiation alone (Anton, 2012; Cairncross et al., 1998). Certain patients are reported to have remarkable and prolonged responses to chemotherapy and new agents are being developed that targets molecular markers which are promising and may extend the efficacy of chemotherapy (Olson et al., 2014). Chemotherapy can be applied at different stages during treatment. Neoadjuvant CT- after diagnosis before surgery or any other treatment; Concurrent CT– in parallel with radiation; Adjuvant CT– after surgery/radiation as a follow up treatment; Recurrent CT – At the time of tumour relapse (Figure 1.4) (Parney and Chang, 2003). Chemotherapeutic agents are of two types - Cytotoxic agents cause cell death in tumour mass by damaging DNA or other cellular contents thereby inducing apoptosis or Cytostatic agents that alter biological mechanisms of tumour like proliferation, invasion, angiogenesis, etc., that inhibits tumour growth. They can be used on their own or in combination with other agents. For example, the use of cytostatic agents used along with cytotoxic agents can block biological mechanisms like resistance or growth factor pathways that help the cytotoxic agents to work better (Parney and Chang, 2003).
1.4.3.1 Temozolomide

A breakthrough study in 2005 by Stupp and colleagues demonstrated that a regimen of parallel doses Temozolomide (TMZ) with surgery and radiation followed by adjuvant TMZ improved the overall survival, progression free survival and two-year survival rates of GBM patients significantly (Stupp et al., 2005). Since then Stupp protocol became the standard therapy for GBM patients with or without modifications. Temozolomide (TMZ), a second-generation imidazotetrazine derivative is a drug of interest since 1990s and is at present the first line chemotherapeutic agent for treating GBM following surgery and radiation (Villano et al., 2009). Being lipid soluble, TMZ is rapidly absorbed with a nearly 100% bioavailability including the CNS and it readily crosses the blood-brain barrier achieving high concentrations in the cerebrospinal fluid. TMZ is an oral alkylating agent that degrades at physiological pH to 3-methyl-
(triazen-1-yl) imidazole-4-carboxamide (MTIC) that methylates the DNA at O\textsuperscript{6} position of guanine residue resulting in DNA lesions. During subsequent replication, the methylated guanine adducts pair with thymidine instead of cytidine that activates ineffective mismatch repair and eventually leads to apoptosis (Villano et al., 2009; Malcolm et al., 2002; Roos et al., 2006).

However, DNA damage caused by TMZ could be corrected by DNA excision repair enzyme O\textsuperscript{6} methyl guanine DNA methyl transferase encoded by the gene MGMT that leads to resistance to TMZ and other alkylating drugs (Sarkaria et al., 2008). It is also shown that patients have different levels of MGMT for DNA repair mechanisms. Treatment with TMZ saturates the existing enzyme activity and results in further DNA damage. However, MGMT positive GBM patients have a high expression of the enzyme that works beyond the saturation point (Nagane et al., 2007; Hegi et al., 2008). The current regimen for TMZ when given in combination with RT is 75 mg/m\textsuperscript{2}/day for 6 weeks. Following completion of RT, patients receive adjuvant TMZ at a dose of 150 mg/ m\textsuperscript{2}/day for 5 days followed by 23 days rest. This 4 weeks cycle is repeated for at least 6 cycles. However, because of MGMT mediated resistance and recurrence of GBM, the median PFS time is only 7 months and the 2-year survival rate is only 27%. In those cases, increasing the dose of TMZ (Dose Dense- DD –TMZ regimen) to a level that would saturate the enzymatic activity of MGMT would benefit the MGMT positive patients as well as patients with recurrent GBMs.
Fig 1.4 (A) Structure of Temozolomide (B) Mechanism of action and repair of Temozolomide induced DNA damage (Adapted from Liu et al., 2006).
In addition to MGMT, recent understanding of the diverse mechanisms responsible for resistance and the role played by various subtypes of GBM has resulted in the evaluation of new therapies in combination with TMZ (Sarkaria et al., 2008; Tentori and Graziani, 2002). Various clinical trials are being conducted at different phases to verify the effectiveness of TMZ in combination with other drugs like protein tyrosine/serine-threonine kinase inhibitors, interferons, conventional drugs, EGFR and VEGF inhibitors, etc.,. So far, none of them showed realistic benefits compared to temozolomide treatment alone (Ren et al., 2009). Thus, TMZ has shown a remarkable improvement in patient survival; however TMZ simply does not have the answer for GBM.

1.4.3.2  Gliadel wafers

Gliadel wafers are biodegradable polymers loaded with carmustine, designed to deliver local chemotherapy in the peritumoural surgical bed. The wafer consists of a biodegradable polymer disc that measures 1mm in thickness, 200mg in weight and 1cm in diameter and carries 3.85% BCNU by weight (Brem et al., 1995). The wafers are implanted in the surgical cavity and as the polymer degrades, the drug is slowly released resulting in local concentrations 100 times higher than systemic administration (Panigrahi et al., 2011). Gliadel is currently approved for treating recurrent glioblastoma and as an adjunct for surgery and radiation in newly diagnosed GBM patients (Panigrahi et al., 2011; Aoki et al., 2007). Patients treated with carmustine wafers after surgery and radiation showed a significant increase in median survival rate and follow up studies for 2-3 years demonstrated a survival advantage in these patients compared to a
placebo group (Westphal et al., 2003). From the encouraging preliminary results, many ongoing studies adopted a multimodality approach to use BCNU wafers in combination with other drugs (McGirt et al., 2009). A multicentered, phase III double, blind study that combined the use of Gliadel wafer with temozolomide increased the median survival of GBM patients to 21 months (McGirt et al., 2009). Although more than 20,000 procedures with carmustine wafers have been carried out since 1997, the use of gliadel wafers for intracranial treatment is limited by fatal cerebral oedema, seizures, infections and the cost of wafers (Gallego et al., 2007; Weber & Goebel, 2005).

1.5 Chemoresistance in GBM

1.5.1 Drug efflux mechanisms

1.5.1.1 Blood brain barrier

Although significant development has been made in the development of drugs against specific targets, no progress has been made in the treatment outcomes of chemotherapy for GBM mainly because most of the drugs discovered are unable to cross the BBB (Sugiyama et al., 1999). BBB is a complex network made up of endothelial cells, pericytes and astroglia that lines the vessels of the central nervous system (CNS). BBB isolates the CNS from systemic circulation by regulating the passage of materials between the brain intravascular compartment and the cellular compartment. Since brain is a delicate organ BBB protects the brain from bacteria, toxic substances and large molecules that are present outside the CNS and provide selective access to molecules that are needed for normal functioning of CNS (Schlosshauer, 1993; Bentivoglio and
Kristensson, 2014). This property of the BBB also restricts the entry of most pharmaceutical agents into the brain and this is therefore a major obstacle in chemotherapy of GBM. In general, any drugs or small molecules, which are water soluble or lipophilic in nature and less than 400Da, can readily cross the BBB by passive transport. However, 98% of molecules greater than 200Da are prevented from crossing the BBB (Ricci et al., 2006). The pharmaceutical agents also face additional barriers present in the BBB which are the efflux pumps like P-glycoprotein (P-gp) and multi-drug resistance associated protein family (MRP) that transport these molecules actively back to the vascular compartment (Kusuhara and Sugiyama, 2001).

1.5.1.2 P-glycoprotein

P-gp plays important physiological roles in a wide range of tissues like BBB, capillaries, brain, kidney, liver, intestine and adrenal glands. Among all gliomas, GBM cells are reported to have high levels of P-gp that contributes to the intrinsic resistance of GBM (Ling, 1995). P-gp is a 170-kDa membrane protein encoded by the ABCB1 gene and belongs to the ATP binding cassette super family (ABC) transporters (Gros, et al., 1986). P-gp aids in transport of neutral and cationic hydrophobic compounds, natural product anticancer agents, including vinca alkaloids, anthracyclines, and taxanes such as vincristine, paclitaxel, doxorubicin, epirubicin, cepharanthine, cytarabine, bisantrene, etoposide, vinblastine, actinomycin, etc., (Gottesman et al., 2002). The transport of drug is coupled with two step hydrolysis of ATP. The first hydrolysis
of ATP to ADP is needed to bring about a conformational change to form a transition state intermediate of P-gp complex followed by the extrusion of drug. The second hydrolysis ensures that ADP bound to P-gp is dissociated and the conformation of P-gp is restored to its original state (Leonessa 2003, Ambudkar, 2003; Sarkadi, 2006). Despite 30 years of research in P-gp, no clear therapeutic strategy has been identified to tackle the drug efflux pump in tumours.

1.5.1.3 Multidrug resistance associated protein (MRP)

The MRP family of transporter proteins are organic ion transporters found in many mammalian tissues and consists of 9 members, MRP 1-9. Some of the family members especially MRP1 is shown to be expressed in brain capillaries. They carry anionic substances such as methotrexate and neutral drugs in association with acidic ligands like sulphate, glucoronide or glutathione. MRP1 is a 190-kDa protein that has 17 transmembrane domains (TMDs) and has a core similar to the P-gp. The range of resistance conferred by MRP 1 extends to anthracyclines, antifolates, vinca alkaloids but MRP cannot resist taxanes, mitoxantrone or cisplatin (Tanaka et al., 1999). MRP 1 expression is thought to be significantly affected by chemotherapy where a higher expression rate is observed in samples after chemotherapy. Other members of the family MRP 2-6 have been correlated with resistance to variety of drugs in different mechanisms but their relevance with GBM is not well established (Rudas et al., 2003; Leonard, 2003; Konig et al, 2005).
1.5.2 DNA damage repair mechanisms

1.5.2.1 MGMT

The DNA repair enzyme MGMT also known as O\textsuperscript{6}-alkylguanine-DNA alkyltransferase (AGT), plays an important role in resistance to TMZ and other alkylating agents. The chemoresistant activity of MGMT has been shown in several cell culture and xenograft studies. The DNA damage induced by addition of methylated adducts by TMZ and other alkylating agents can undergo single-step error free reversal in the presence of MGMT (Esteller et al., 2000). MGMT forms a covalent bond between the alkylated bases and an internal cysteine residue within its active site. It then directly transfers the alkyl group from the DNA to the cysteine thereby restoring the structural integrity of methylated bases in the DNA (Payne et al., 2005). Thus, MGMT can confer resistance to the cytotoxicity induced by alkylating agents and increased MGMT expression is associated with \textit{in vitro} and \textit{in vivo} GBM resistance to first line drug TMZ, other nitrosoureas and alkylating drugs (Dolan et al., 1990). However, in this process, MGMT undergoes self degradation through the ubiquitin-proteasome pathway after binding alkyl groups from DNA and hence the cells have to re-synthesize MGMT molecule (Chen et al., 1998; Esteller and Herman, 2004). Research has shown that the expression levels of MGMT are not the same in all patients and differ with individuals as well as with the grade and aggressiveness of the tumour. Epigenetic inactivation of MGMT gene by promoter hypermethylation results in low or no MGMT expression and lowers cellular ability to carry out DNA repair (Esteller and Herman, 2004). Hence, GBM patients lacking tumour MGMT expression benefit from treatment with
alkylating drugs and show prolonged survival compared to MGMT-positive GBM patients. For example in the case of first line drug TMZ, a median survival of 21.7 months was seen among patients with a methylated promoter who received TMZ + radiotherapy compared to patients with a unmethylated promoter who had a median survival of just 12.7 months with the same treatment. Therefore, MGMT promoter methylation status has been shown to predict clinical response to alkylating agents (Hegi et al., 2005). The above facts suggested that inactivation of MGMT using inhibitors could improve treatment outcomes and made MGMT a suitable target for intervention. An inhibitor like O\(^6\)-benzylguanine (O\(^6\)-BG) that irreversibly inactivates MGMT is shown to enhance BCNU and TMZ cytotoxicity in MGMT-positive glioma cells both in vitro and in vivo but the therapeutic potential of adding O\(^6\)-BG to TMZ-treatment has so far also been discouraging (Jaeckle et al., 1998).

1.5.2.2 DNA Mismatch repair

The second important mechanism of resistance against chemotherapy is the DNA mismatch repair mechanism (MMR). MMR is a normal DNA repair pathway that maintains genomic integrity by correcting replication errors, which escape the DNA polymerase proof reading process (Hickman and Samson, 1999). MMR acts as direct sensors to DNA damage signalling through ATR checkpoint and this property is therefore considered as an important tumour suppressor mechanism. Absence of a functional MMR system due to mutations in the protein complexes results in unnoted drug-induced DNA damage that contribute to further mutagenesis and reduced anti-tumour activity of
methylating anticancer drugs (Fink et al., 1998). For example in the case of TMZ treatment DNA polymerase mispairs O\( ^6 \)-meG with thymine (instead of C opposite) and a normal MMR system should recognise base pair by MutS\( \alpha \) a heterodimeric complex made of two MMR proteins HMSH2 and HMSH6. This in turn will interact with MutL\( \alpha \) to excise the damaged strand of DNA. However, O\( ^6 \)- meG remains and binds with another thymine thereby activating multiple cycles of MMR. Futile cycling of MMR, continuous DNA re-synthesis and failure to get past O\( ^6 \)- meG halts DNA replication and leads to checkpoint activation finally causing breaks in DNA strand (Taverna et al., 2000). Thus, cytotoxicity induced by TMZ largely depends on functional MMR and MMR mutated or deficient cells are shown to be more than 100-fold more resistant to alkylating agents like TMZ.

1.5.2.3 Base excision repair and PARP-1

Endogeneous DNA damage resulting from methylation, deamination, hydroxylation and ROS are repaired by the DNA base excision repair system (BER). The BER pathway also repairs DNA base lesions induced by drugs like TMZ and other alkylating agents (Frosina, 2000). Apart from O\( ^6 \)-meG, several other DNA adducts as N\( ^7 \)-methylguanine, O\( ^3 \)-methyladenine and N\( ^3 \)-alkyladenine are produced by drugs. These alkylated adenine or guanine bases are repaired by the BER pathway in two steps. Firstly a lesion specific DNA repair enzyme alkyladenine DNA glycosylase (AAG) removes those bases from the DNA strand leavng single nucleotide gaps known as AP sites since they are either apurinic (devoid of A or G) or apyrimidinic (devoid of C or T). In
the second step the AP site is recognised by an AP-endonuclease (APE1) that makes an incision in the strand and fills the single nucleotide gap by DNA polymerase-β (pol-β)-mediated DNA synthesis and DNA ligase that seals the nick in the strand (Trivedi, 2005; Frosina, 2000). Poly-ADP-ribose polymerase-1 (PARP1) is an important enzyme in the BER pathway with zinc finger DNA binding nuclease activity and acts as a sensor of DNA damage. It facilitates repair of single stranded or double stranded breaks in the DNA by coordinating with other pathways like BER and MMR (de Murcia et al., 1997). Inhibition of PARP is shown to suppress the function of BER pathway and significantly enhance the cytotoxicity of alkylating drugs in GBM cells (Tentori et al., 2002). In addition, it is also shown that most GBM specimens have very high basal levels of PARP expression especially after treatment with TMZ (Wharton et al., 2000; Cheng, 2005). Both BER and PARP expression are correlated to suppression of MMR activity that further worsens chemoresistance.

1.5.3 Apoptosis resistance

Dysregulation of apoptosis is an additional chemoresistance mechanism in GBM. An apoptotic response to DNA damage induced by chemotherapeutic drugs can be disrupted by various apoptosis-interrupting mechanisms like loss of p53 function, overexpression of Bcl-2 or Bcl-XL, or upregulation of EGFR. p53 is well known for its central role in mediating apoptosis by detecting DNA damage and acting as a transcription factor that regulates the genes involved in pro-apoptotic signaling pathways (Fels et al., 2000; Karmakar et al., 2006). Additionally cytoplasmic p53 is known to regulate mitochondrial membrane
permeability by acting as a pro-apoptotic BH3 domain protein that releases cytochrome c and eventually inducing caspases for cell death (Willis and Adams, 2005). Target genes of p53 include BCL2, BAX, EGFR, MDM2 and BIM. Although p53 plays a central role, the resistance mechanism is largely mediated by increased expression of Bcl-2 family members that suppress the drug-induced apoptosis. The BCL-2 family consist of both pro apoptotic proteins like Bax, Bak and Bcl-Xs and anti apoptotic proteins such as Bcl-2 and Bcl-XL. Increased expression of Bcl2 and Bcl-XL due to p53 dysregulation will inhibit Bax and Bak thereby inhibiting the intrinsic apoptosis pathway. Hence, the balance between the level of pro and anti apoptotic BCL2 family members plays a vital role in apoptosis. Based on the analysis of patient tumour samples it was reported earlier that, inactivation of p53 occurs in 87% of GBMs, associated with the upregulation of Bcl-2 and suggested to be an important mechanism in the pathogenesis and resistance of GBM (Newcomb et al., 1997).

1.6 GBM Cancer stem cells

Among the several challenges for successful treatment of GBM, difficulty in combating the resistant cancer stem cell population has been the prime focus of many researchers in recent years. It is well established that most solid tumours have heterogeneous group of cancer cells in addition to vasculatures, inflammatory cells and other stromal components (Hanahan and Weinberg, 2011). As discussed earlier GBM shows remarkable hierarchy and heterogeneity in both morphological and differentiation statuses of the cells. Traditionally heterogeneity is believed to be a result of regional variations in the
subpopulations of the tumour microenvironment or due to progressive genetic and epigenetic alterations. This was known as stochastic model of GBM where it was considered that GBM has multiple clones of cells (Nowell, 1976). The strongest clone continually grows and surpasses other clones while the heterogeneous nature was attributed to the residual weaker clones (Nowell, 1976).

For many years parallelism existed between tumour biology and stem cell biology because of several reports highlighting the importance of stem cell genes and their roles in cancer. Rudolf Virchow, also known as the father of pathology, suggested in 1858 that cancer is a disease of embryonic like tissue. Evidence accumulated in the last decade from studies on haematopoietic and solid cancers like breast, brain, colorectal, prostate and lung has suggested the existence of a small subpopulation of cancer cells known as cancer stem cells (CSCs) (Lapidot et al., 1994; Bonnet and Dick, 1997; Al-Hajj et al., 2003; Hemmati et al., 2003; Singh et al., 2004; Galli et al., 2004; Dalerba et al., 2007; Schatton et al., 2008). They were named so because of their ability to differentiate into other types of cells in the tumour as well as the ability to self renew giving rise to more CSCs. They share similarities with normal stem cells in critical characteristics such as cell surface markers and embryonic signalling mechanisms (Reya et al., 2001; Vescovi et al., 2006; Bao et al., 2006; Rosen and Jordan, 2009; Park and rich, 2009; Heddleston et al., 2010; Frank et al., 2010). Aggressive tumours like GBM are well recognized for the presence of poorly differentiated cells and hence their names contain the term ‘blastoma’. Similar to other malignancies the existence of such small population of cells in
GBM called GBM cancer stem cells (GBM CSCs) were identified and characterized by several groups beginning from 2003. GBM CSCs are functionally able to self-renew, differentiate in vitro into cells of CNS and display high tumourigenic potential in vivo when compared with non-stem tumour population (Hemmati et al., 2003; Galli et al., 2004; Singh et al., 2004; Bao et al., 2006; Lee et al., 2006). So far, it has been identified that these GBM CSCs exhibit a wide range of NSC markers such as CD133, CD44, CD90, the intermediate filament NESTIN, transcription factors SOX2, OCT4, NANOG and BMI1, increased aldehyde dehydrogenase activity (ALDH) and the RNA binding protein MUSASHI. There are many controversies concerning the characterization of GBM CSCs, their origin, markers and their exact role in GBM pathogenesis and hence they are referred to as ‘Stem-like cells’ (SLCs) by some researchers. Much of the ongoing research in GBM is focussed on establishing the crucial role of GBM CSCs in initiation, progression, and angiogenesis, resistance to therapy and systematic relapse of GBM and developing GBM CSCs as potential targets for treatment.

1.6.1 GBM CSCs and therapeutic resistance

Despite maximal resection, GBMs are extremely resistant to conventional radiotherapy and chemotherapy with rapid recurrence (Furnari et al., 2007; Wen and Kesari, 2008). Although there is a controversy between the hierarchical relationship of CSC population and the entire mass of tumour cells, it is widely accepted that CSCs contribute to the repopulation of tumour after conventional therapies (Park et al., 2010). By far radiotherapy is considered the most efficient
non-surgical therapeutic measure for GBMs, but it is quite disappointing that these CSC populations are widely resistant to radiation. It has been demonstrated that CD133⁺ CSCs are more resistant to radiation when compared to the parental population (Bao et al., 2006). GBM CSCs are shown to preferentially activate DNA damage checkpoint response like ataxia-telengectasia mutated kinases (ATM), Rad17, Chk1 and Chk2 kinases in response to DNA damage induced by radiation (Bao et al., 2006). Checkpoint activation enables CSCs to rapidly repair damaged DNA and recover from genotoxic stress. More recently another essential CSC factor BMI1 was shown to interact with these checkpoint proteins and non-homologous end joining (NHEJ) repair proteins to achieve efficient recruitment of DNA damage response machinery during radiation. Various other signalling pathways like Notch, Wnt/β-catenin signalling, ROS/MAPK has been linked to decreased radiosensitivity of CSCs (Diehn et al., 2009; Woodward et al., 2007). Treatment for GBMs also includes adjuvant chemotherapy with primary chemotherapeutic molecule TMZ an oral methylating agent (Stupp et al., 2005; Wen and Kesari, 2008). The mechanism of TMZ induced cytotoxicity, MGMT mediated resistance to TMZ, and involvement of promoter methylation has been described earlier. Resistance to TMZ and invariable tumour recurrence regardless of MGMT status indicates the presence of a TMZ-resistant population in GBM. Various findings suggest that GBM CSCs can induce resistance to TMZ in a MGMT dependent and MGMT independent mechanism. For example in a genetically engineered GBM mouse model exposure to TMZ significantly enriched a population with CSC properties (Bleau et al., 2009). In addition, in vitro exposure of GBM NS cultures to TMZ showed that the CD133⁺
population is significantly resistant to TMZ and other drugs. On the contrary, other researchers have also shown that TMZ preferentially depletes CSCs in sphere cultures and inhibits their tumorigecity. However, it was also found that TMZ can eliminate only the MGMT− CSC population. MGMT expressing CSCs were not inhibited by TMZ. (Liu et al., 2006; Bleau et al., 2009; Beier et al., 2007; Clement et al., 2007). CSCs are known to have several other potential mechanisms like expression of multidrug resistance genes such as MDR1, BCRP1 and ABCG2. CD133+ CSCs express high levels of drug transporters, antiapoptotic genes including FLIP, BCL-2 and BCL-XL compared to CD133− cells (Hirschmann-Jax et al., 2004; Schatton et al., 2008). Consequently, CSCs are resistant to TMZ, carboplatin, VP16 and Taxol drugs. Future design of chemotherapy and other treatment procedures largely depends on a better understanding of GBM CSCs resistance mechanisms.

Fig 1.5 Normal neural progenitor cells and cancer stem cells. Neural stem/progenitor cells that harbour mutations can give rise to resistant GBM CSCs, which act as the source for cancer recurrence. (Figure adapted from Zhang et al., 2009).
1.6.2 Origin of GBM CSCs

In an effort to understand the GBM CSCs and their roles in GBM, it is necessary to understand the source of these GBM CSCs. Many theories have been proposed and evidenced in recent years and great progress has been made in the understanding of GBM and the origin of CSCs. Due to their striking similarities with normal neural stem cells (NSCs) it was believed that GBM CSCs might have resulted from genetic and epigenetic changes in neural stem/progenitor cells (Sanai et al., 2005; Safa et al., 2015). Although a definitive proof is still lacking, GBM CSCs were also named as tumour initiating cells (TICs) or glioma initiating cells (GICs) due to the belief that GBM tumour may be initiated and maintained by these uncommitted self-renewing population of cells (Safa et al., 2015).

1.6.2.1 Neural stem cells as source of GBM CSCs

The search for GBM cells with stem like properties is based upon the knowledge gained from the normal neural stem cells (NSCs) in the brain. NSCs are multipotent self renewing cells found in the sub ventricular zone, dentate gyrus or subcortical zone of brain that generate differentiated cells of the brain (Doetsch et al., 1999). They can be grown in vitro as suspended cell aggregates called neurospheres (NS) using a serum free media supplemented with stem cell growth factors. When they are exposed to serum, they generate all types of differentiated cells of the brain (Nunes et al., 2003). Since GBM CSCs isolated from GBM samples and grown in a similar manner share very definitive characteristics with NSCs and faithfully reproduced the tumour in xenograft models it was considered that an altered NSC could be the cell of origin for
GBM. This was also because NSCs survive indefinitely and reproduce over a longer period making them suitable candidates that can accumulate mutations and lead to the first GIC (Rao et al., 1998; Sanai et al., 2005). In addition, the GBM CSC model also follows a hierarchical organization similar to the normal tissue generation and turnover where the number of stem cells would be very low. This was true with GBM as the amount of CSCs in GBM were only less than 1% of the total population (Lee et al., 2006). In an effort to establish the fact whether GBM or a first GBM CSC or TIC may arise from altered NSCs, researchers developed murine models of gliomagenesis. The first and most genetically faithful models were the xenograft models (Singh et al., 2004). Early studies used CD133 positive GBM CSC population as side-population method and considered them as pure CSCs. It was found that as few as 100 CD133\(^+\) cells could faithfully recapitulate the parent tumour in immunodeficient mice whereas more than a million CD133\(^-\) cells cannot induce tumours (Beier et al., 2007). These studies initially proved that GBM CSCs could be the likely source of gliomagenesis and NSCs could be the source of tumour initiating GBM CSC. However, subsequent studies proved that gliomagenesis could also happen with CD133\(^-\) cell population, which clearly highlighted the need for better markers and further studies (Wang et al., 2008).

Many researchers focussed on the subventricular zone (SVZ) and other stem cell niches in murine models. Genetic studies involving NSCs in the SVZ like p53 mutations, KRAS and Akt mutants, PDGFR alterations, PTEN/p53 recombination, EGFR amplification, infecting NSCs with oncogenic viruses, HCMV infection, etc have clearly given rise to GBM (Sanai et al., 2005). A number of radiographic studies in patients revealed that GBM formation occurs
near the periventricular areas in majority of the cases. Other GBMS that are present supratentorially can be attributed to the migration of TIC before establishing the tumour (Jackson et al., 2006). Therefore, it seems reasonable that NSCs or transiently dividing neural progenitors located in the SVZ regions are likely candidates for cellular origin of GBM and hence selective targeting of these altered NSCs/GBM CSCs will effectively reverse chemoresistance and provide therapeutic benefit in GBM treatment.

1.6.2.2  
Epithelial to mesenchymal transition induced GBM CSCs

Epithelial-to-mesenchymal transition (EMT) is an evolutionarily conserved cellular process that has multifunctional roles in embryogenesis, tissue repair and wound healing. The epithelial cells are normally immobile due to their polarized organization and adherence to the basement membrane. During EMT process the polarity and cell to cell junctions of the epithelial cells are lost which results in cytoskeletal rearrangement and change of shape leading to the acquisition of mesenchymal phenotype. These cells are characterized by increased proliferation, increased cell migration, invasion and synthesis of components that can degrade the basement membrane. Increasing evidence suggest that progression of most cancers is associated with acquisition of the mesenchymal phenotype which enable the cells to infiltrate surrounding tissue and migrate to distant sites leading to metastasis and also lead to the acquisition of stem cell characteristics (Kalluri et al., 2009; Acloque., 2009). Milestone studies in GBM, breast cancer, pancreatic cancer and colon cancer suggest that mesenchymal cells produced by EMT inside the tumour
microenvironment account for the stem cell phenotypes in solid tumours and thus can explain the heterogeneity, hierarchical nature of CSCs and clonal evolution in tumours. These studies also highlighted that EMT is a major molecular event that is required for progression of early stage benign tumour into a malignant phenotype that has invasive and metastatic capabilities (Brabletz et al., 2001). EMT can be readily observed by the loss of epithelial markers like E-cadherin, cytokeratins and tight junction proteins such as claudins and occludins. On the other hand EMT phenotypes show increase in markers like N-cadherin, vimentin, matrix metalloproteinases, fibronectin, etc., (Lee, 2006). In addition to these markers EMT phenotypes also show markers like ALDH, CD133 and nestin that are originally thought to be specific for NSCs or GBM CSCs. Various stem cell developmental pathways and transcription factors like Wnt, Notch, Hedgehog, SOX2, OCT4, NANOG and β-catenin are also observed in the EMT phenotypes in addition to suppression of p53 mediated apoptosis and expression of resistance proteins such as ABC, P-gp and MRP.

Recent reports in GBM research also seem to link this phenomenon to GBM progression, invasion and intrinsic resistance. Members of Twist, Snail, ZEB and other EMT activators are shown to augment GBM motility and invasion both in vitro from patient samples and in vivo using animal models (Mikheeva et al., 2010; Qi et al., 2012; Kahlert et al., 2013). The recent addition of a mesenchymal subgroup to GBM classification also supports this phenomenon and highlights the clinical importance of EMT (Louis et al., 2007). Patients with mesenchymal subtype of GBM have significantly low overall and event free survival rates (Verhaak et al., 2010). Tumours in these patients are
characterized by enormous motility, aggressive invasion, increased distribution and multifocal presentation in addition to intrinsic resistance to chemoradiation treatments. Mesenchymal cells themselves are pluripotent cells with stemness and also can grow as spheres like NSCs that have the ability to differentiate into different types of cells of the CNS (Li et al., 2015). Hence some researchers believe that the mesenchymal population is the CSC population in tumours as they are dedifferentiated from cancer cells. But on the other hand, evidences for NSC origin of GBM CSCSs are quite strong. The existence of CSCs in the proneural subtype of GBM and their origins from progenitors of NSCs are well established (Lei et al., 2011). Hence some researchers refer to the mesenchymal group as ‘stem-like cells’ but not true CSCs.

1.6.2.3 **EMT induced by tumour microenvironment**

EMT and the resulting mesenchymal phenotype displaying all the CSC features are thought to be induced, supported and maintained by the tumour microenvironment. The tumour cells in the microenvironment are provided a supportive framework by several factors like ECM, hypoxia, acidity, cancer associated fibroblasts (CAFs), cancer associated macrophages (CAMs), endothelial progenitor cells (EPCs), stromal cells, soluble growth factors, numerous cytokines, and nutritional gradients and modified metabolism (Vaupel, 2004; Ariztia et al., 2006). The multiple signals received from the neighbouring cancer cells and stromal cells trigger expression of transcription factors like TGF-β, HIF1α, HIF2α, NF-κB, EGF, WNT, NOTCH and β-catenin that initiates EMT pathways leading to invasion, quiescence, progression, metastasis and chemoresistance (Polyak and Weinberg, 2009).
1.7 Hypoxia

The concentration of oxygen is tightly regulated in cells and tissues due to its essential role in different biological processes. Low levels of oxygen in cells or tissues, referred to as hypoxia, results in extensive transcriptional effects involving multiple downstream signalling leading to pro-apoptosis or pro-angiogenesis pathways. Hypoxia is the most common feature in solid tumours and one of the most pervasive microenvironmental stresses associated with tumour progression, invasion, angiogenesis, metabolic alterations and resistance to therapy (Favaro et al., 2011). Usually when the tumour size reaches above 2mm³ the ever expanding cancer cells experience poor oxygen diffusion, chaotic vasculature and irregular blood resulting in a flux in the oxygen tension (Carmeliet and Jain, 2011). It is estimated that hypoxia (1% or less O²) or anoxia (<0.01% or no detectable O²) is a predominant feature in more than 60% of locally advanced solid tumours which clinically contributes to high risk of metastasis, tumour recurrence, reduced overall survival rate and high mortality (Carmeliet and Jain, 2011; Evans, 2004).

Compared to normal cells, neoplastic cells employ various abnormal oxygen sensitive mechanisms to control transcriptional and post-transcriptional changes in gene expression that allows them to adapt to a hostile, hypoxic environment for survival. These can be done by either engaging specific metabolic reprogramming to survive under hypoxia, by producing an environment favouring de novo angiogenesis or by activating a motility program such as EMT to escape the hostile environment and colonize in a different environment.
(metastasis). This clearly explains the contribution of hypoxia to the malignant and aggressive behaviour of extensively hypoxygenated tumours like GBM. The normal physiological $O_2$ concentration in a healthy brain ranges between 12.5% and 2.5%. However majority of GBM tumours displayed either mild hypoxia ranging from 2.5% to 0.5% or severe hypoxia with $O_2$ concentrations reduced upto 0.1% (Evans, 2004; Pistollato et al., 2010).

1.7.1 Molecular biology of hypoxia inducible factors

Cellular responses to hypoxic conditions are controlled by a family of oxygen sensitive proteins known as hypoxia inducible factors (HIFs) which are one of the most critical transcriptional regulators that help tumour cells to adapt to a hypoxic microenvironment. So far three different HIFs are identified in humans namely HIF1$\alpha$, HIF2$\alpha$ and HIF3$\alpha$, however HIF1$\alpha/\beta$ is the most commonly reported transcription factor involved in hypoxic response (Majmundar et al., 2010). HIFs are known as master regulators under hypoxia because of their extreme ability to undergo conformational changes to varying oxygen concentrations. HIFs are heterodimeric transcription factors that consist of an oxygen sensitive HIF-$\alpha$ subunit and a constitutively expressed HIF-$\beta$ subunit. The functional HIF is produced when the $\alpha$ and $\beta$ subunits are assembled together by post transcriptional modifications which largely depend on stabilization of oxygen dependant $\alpha$ subunit that is well studied in the case of HIF1$\alpha$ (Philip et al., 2013). In the presence of oxygen the HIF1$\alpha$ subunit is hydroxylated on proline positions of the polypeptide by a group of enzymes called prolyl-hydroxylases (PHDs) namely PHD1, PHD2 and PHD3. The
hydroxylation of HIF1α subunit by PHDs is an oxygen requiring reaction, which also needs a-ketoglutarate as substrate. Hydroxylated HIF-1α is ubiquitinated by another enzyme called von Hippel-Lindau (VHL) tumour suppressor ubiquitin ligase. Ubiquitination of HIFα subunit will mark this protein ubiquitin dependant protein degradation and therefore prevents the α subunit to form a functional dimer with β subunit. In addition to the proline hydroxylation by PHDs another oxygen dependant hydroxylation mechanism mediated by the factor-inhibiting HIF1α (FIH) can hydroxylate the aspartine^803 that prevents the interaction between HIF1α and its transcriptional co-activator (Kallio et al., 1999). The HIF-1β subunit is an aryl hydrocarbon receptor nuclear translocator (ARNT) which is constitutively in the nucleus of many cell types (Kallio et al., 1999). Under hypoxic conditions, the hydroxylation reaction cannot happen effectively and leads to the stabilization of HIF-1α subunit. It is then translocated into the nucleus where it forms an active transcription factor heterodimer with HIF-1β. The dimer of HIF1-α/β with the help of p300/CREB binding protein (CBP) coactivators will bind to transcriptional enhancer elements normally known as hypoxia response element (HRE) which are identical sequences (5’-RCGTG-3’; R=A or G) present in the promoter/enhancer regions of target genes. This in turn enhances transcription of target genes involved in cellular adaptations to hypoxia (Philip et al., 2013; Majmundar, et al., 2010).

HIF-2α, which is also known as endothelial PAS domain protein 1, EPAS1 due to its restricted expression in endothelial cells is also an oxygen regulated transcription factor that has been shown to share several transcriptional target genes with HIF-1α. This includes genes like VEGF, TE-1, Tie2, Ang-2 and Flt-1
with promoter/enhancer regions that has homologous DNA-binding sequence binding sites for both HIF-1α and HIF-2α mediated HREs (Holmquist-Mengelbier et al., 2006). Despite their similarities, the restriction of HIF-2α expression in vasculature highlights the dominant role played by HIF-2α in the induction of tumour organs. In addition HIF-2α is known to have several unique transcriptional targets such as Oct4 and transforming growth factor-α (TGF-α) which are different from the canonical pro-angiogenic hypoxic response suggests an important and specific role for HIF-2α in the regulation of stem cell pathways. Multiple recent experimental studies have revealed that high expression of HIF2α important is required for maintaining the CSC phenotype and tumourigenesis in GBM, neuroblastoma, renal cancer, and non-small cell lung cancer (Li et al., 2009; Mazumdar et al., 2010; Helczynska et al., 2008). However the role of HIF2α is still not fully elucidated.

The third HIF isoform is HIF-3α and very little is known about its function and regulation. A small number of studies have shown that HIF-3α has a limited expression pattern in eyes, kidneys, lung and cerebellum and some studies have described several splice variants of HIF-3α. These variants are thought to be dominant-negative regulators of HIF-1α and 2α isoforms and potentially direct transcriptional targets of HIF-1α under hypoxia (Gu et al., 1997). Over expression of HIF-3α isoforms in vascular cells is shown to decrease VEGF expression under hypoxia, which confirms the regulatory function of HIF-3α in HIF signalling pathway (Heidbreder et al., 2007; Augstein et al., 2010).
1.7.2 Hypoxia induced EMT and CSCs

Hypoxia has been well known as a factor that regulates and maintains the stem cell sub-population in normal tissues during development (Dunwoodie, 2009). Recent experimental evidences indicate that hypoxia and HIF signalling also plays a pivotal role in phenotype and function of CSCs by enhancing the self-renewal capacity and maintenance of undifferentiated state of CSCs. CSCs maintained under hypoxic conditions retain the undifferentiated phenotype whereas those exposed to normoxia readily undergo differentiation (Ezashi, et al., 2005; Zeng et al., 2011). It has been shown that hypoxia is able to achieve this by activating signalling pathways that are associated with normal stem cells, including Sox2, Oct4 and Notch-1 signalling. This activation of the signature stem cell genes in CSCs may be the important reason for the association of hypoxia with increased tumour aggressiveness resulting in poor clinical outcomes. This is also consistent with recent findings in glioma CSCs where hypoxia promotes CSC phenotypes and contributes to tumour aggressiveness. It is also now understood that hypoxic areas or necrosis within the solid tumours like GBM may act as a niche where CSCs can reside (McCord et al., 2009). Therefore, it is likely that tumours may originate and develop from mutation acquired by normal stem cells or from non-stem cell population due to hypoxic microenvironment. In fact, there is substantial evidence showing that CSCs may arise from epithelial cells through hypoxia induced EMT especially in breast cancer (Jiang, Tang and Liang, 2011). But the precise mechanism involved by which hypoxia mediates the EMT-CSC phenotype is not fully understood. It has been documented that hypoxia-
mediated CSC phenotypes are regulated by HIF proteins, especially HIF-1α- and HIF-2α- signalling pathways that activates Sox2, Oct4, Nanog, cMyc, Notch-1, CD133, CD44 and other CSC markers. But in the very initial stages of hypoxia survival becomes the priority for cells experiencing hypoxic stress and cannot carry out aerobic respiration (Heddleston et al., 2010). It is HIF-1α which helps these stressed cells to switch primarily to glycolysis rather than oxidative metabolism and adapt to that environment. HIF-1α mediated expression of pyruvate dehydrogenase kinase completely blocks decarboxylation of pyruvate to acetyl coA. HIF1 also mediates expression of GLUT transporters to enhance the uptake of glutamine which can be converted to isocitric acid and to α-ketoglutarate by IDH1/IDH2 pathways. α-ketoglutarate is a strong inhibitor of PHDs that keeps the HIF-1α signalling active (Lisy and Peet, 2008). HIF1 signalling effectively communicates with stromal cells and CAFs to help the starving tumour cells by producing and uploading lactate in the microenvironment through Warburg metabolism which is used by cancer cells to protein and energy production. Although HIF-1α induces angiogenesis and finally achieves supply of oxygen and nutrients the cells still seem to adhere to Warburg metabolism and is thought to contribute to mimic persistent hypoxia activation and hypoxia induced metaplastic switch (Semenza, 2003). On the other hand HIF-2α is shown to be highly expressed in CSCs unlike HIF-1α which is expressed both in stem and non stem cell populations (Skuli et al., 2009). Recent investigations show that conditional loss of HIF-2α is shown to affect the self-renewal capacity of CSCs and suppress EMT. Meanwhile forced expression of HIF-2α in non stem cell population is shown to activate various stem cell features like Oct4, Nanog, CD44, CD133, Sox2 and c-Myc with
Figure 1.6 A) Fate of HIF1α under normoxia and hypoxia (Carroll et al., 2005). B) Hypoxia induced EMT as an alternate way of inducing CSCs in GBM.
increased tumourigenicity (Hedleston et al., 2010). These facts collectively underscore the role of HIF-1α and HIF-2α in adaptation of cancers under hypoxia. However if the metabolic switch is not sufficient, additional adaptations are activated by HIF-1α to promote the escape of cancer cells from the primary tumour through EMT program. Multiple studies demonstrate that hypoxia induces EMT characteristics in a variety of cells and EMT is tightly regulated by HIF-signalling pathways. A complex molecular crosstalk between hypoxia-induced pathway and EMT has not yet fully understood. During the acquisition of EMT phenotype, several transcription factors such as ZEB1, ZEB2, Snail1, Slug, Twist1 and TCF3 are shown to be critical mediators of EMT program and the activation of HIF-1α and HIF-2α can induce these transcription factors. In addition hypoxia and HIF signalling could increase the expression of EMT-associated inflammatory cytokines such as TNF-α and interleukins and activate other EMT-associated signalling pathways such as TGF-β, Notch, NF-κB, Wnt/β-catenin, and Hedgehog (Jiang et al., 2011; Graham et al., 2008). HIFs are also shown to facilitate motility and invasion through direct or indirect regulation of cell-matrix interactions and genes such as LOX, Hey1, Hes1 and MMPs (Chen et al., 2010). Unlike HIF-1α, HIF-2α can operate under moderate hypoxic levels or even under physiological oxygen concentrations and seems to participate in acidity induced CSC phenotypes. It is postulated that HIF-1α is required during acute or transient hypoxia whereas HIF-2α may play a significant role during prolonged or chronic hypoxia. It is evident from certain cancers that during chronic hypoxia they switch from HIF-1α metabolism to HIF-2α by degrading HIF-1α using an E3 ubiquitin ligase known as SART1 (Koh et al., 2011; Koh et al., 2008). This switch enables the cells to acquire more
mesenchymal features and motility in addition to expression of EMT related genes. Thus the differential expression of HIF-1α and HIF-2α on stem cell and non-stem cell population coupled with duration of hypoxia in the microenvironment could be the deciding factor in CSC population and tumour aggression (Koh et al., 2011).

1.8 Nuclear Factor kappa B (NF-κB)

NF-κB is a family of structurally related transcription factors that regulates a large number of normal cellular processes such as immune and inflammatory responses, growth and apoptosis. Over three decades of research has demonstrated that NF-κB is ubiquitously expressed in almost all cell types and tissues. NF-κB is thought to play a pivotal role in multiple physiological and pathological processes by regulating variety of genes that has NF-κB binding sites in their promoters or enhancer regions (Oecninghaus et al., 2009). Due to its ability to induce gene expression in numerous genes the activity of NF-κB is tightly regulated at multiple levels.

The NF-κB family consists of five structurally related and evolutionarily conserved proteins namely RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Chen and Ghosh, 1999). All components share a highly conserved amino terminal Rel homology domain (RHD) which is a sequence made up of 300 amino acids that is responsible for DNA binding, dimerization, and interaction with IκB. NF-κB proteins are divided into two classes based on the synthesis and transactivation properties of sequences from C-terminal to the
The class I NF-κB proteins include NF-κB1 and NF-κB2 which are synthesized as large precursors p105 and p100 respectively. Both the proteins contain an N-terminal RHD and a long C-terminal transactivating domain with a series of ankyrin repeats. Both p105 and p100 is converted to mature DNA binding proteins p50 and p52 respectively by removing transactivating part of the C-terminal domain with the help of ubiquitin/proteasome pathway. The class II NF-κB proteins include RelA (p65), RelB and c-Rel, which are synthesized in their mature forms and no processing is required. Their C-terminal contains transactivating domain but it does not have the inhibitory ankyrin repeats (Karin et al., 2002). All these 5 different NF-κB proteins can form homodimers within themselves or heterodimers with other members (Barkett and Gilmore, 1999). These homodimers or heterodimers start transcription when they bind specifically to DNA sites which are called κB sites which are made up of 9-10 base pairs with the base sequence 5'-GGGRNNYYCC-3'; where R= purine; Y= pyrimidine; N= any nucleotide (Nishikori, 2005). This distinct ability of REL/NF-κB proteins to form dimers enables them to bind to different κB sites or to the inhibitor proteins like IκBα. The heterodimer made up of p50/RELA is known as the prototypical NF-κB complex. NF-κB dimers are held in the cytoplasm in an inactive form by members of another family of proteins called IκB (inhibitors of κB) that prevents the nuclear translocation of the dimers (Karin et al., 2002). The IκB inhibitory protein family contains seven members namely IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and the carboxyl-terminal regions of NF-κB1 (p105) and NF-κB2 (p100). The IκB proteins have multiple ankyrin repeats at their C-terminal domains through which they associate with the RHD of NF-κB proteins and retain them in the cytoplasm (Nishikori, 2005).
1.8.1 The NF-κB activation pathways

Under normal conditions of cells, the activation of NF-κB is a tightly regulated process that depends on degradation of IκB through specific phosphorylation mediated by IκB kinase (IKK) (Ghosh et al., 2002). The IKK complex plays an important role upstream of NF-κB signal transduction cascade. The IKKs complex is comprised of two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2) and a regulatory element IKKγ also known as NF-κB essential modulator (NEMO) (Ghosh et al., 2002). The IKK-dependent activation of NF-κB can take place in two ways: classical (canonical) pathway and the alternative (non-canonical) pathway. The classical NF-κB pathway is triggered when the cells are stimulated by inducers such as TNFα, T-cell receptors (TCR), B-cell receptors (BCR), Toll-like receptor (TLR) or interleukin-1 receptors (IL-1R) (Karin et al., 2000). IκBα is phosphorylated at specific sites Ser32 and Ser36 by IKKβ of the IκB kinase complex. The role of IKKα in the classical pathway is not confirmed, but recent studies suggest prior activation of IKK complex depends largely on IKKα specifically the phosphorylation and activation of IKKβ. (Karin et al., 2000). Phosphorylation of IκBα triggers rapid polyubiquitination at Lys21 and Lys22 sites of IκBα and stimulates subsequent degradation of this cytoplasmic inhibitor by 26S proteasome complex, that liberates NF-κB (RelA/p50) and unveils the nuclear localization signal present on RelA promoting the nuclear translocation of NF-κB dimers to the nucleus (Karin et al., 2000). The alternative pathway involves the inducible proteolytic processing of p100. The p100 protein binds to N’-terminal of RHD of RelB and holds the dimer inactive in the cytoplasm. Signal from NF-κB inducers like LTβ and lymphotoxin
B and B cell activating factor (BAFF) and CD40L via NF-κB inducing kinase (NIK) results in the phosphorylation of p100 by IKKα. This results in ubiquitylation and partial proteolytic degradation of p100 by 26S proteasome resulting in p52. This generates RelB/p52 heterodimers that is translocated into the nucleus as transcription factors (Bonizzi et al., 2004).

1.8.2 NF-κB and cancer chemoresistance

NF-κB is an important transcription factor associated with many hallmarks of cancer development like proliferation independent of any growth factors, inhibition of apoptosis, never-ending replication, invasion and metastasis (Baldwin et al., 2001; Karin, 2002). NF-κB is also known to play important role in tumour initiating cells. NF-κB is constitutively activated in a variety of hematologic and solid tumours, by multiple mechanisms including mutations in upstream regulatory components of this pathway. It has been shown that cytokines such as TNFα, IL-1, IL-6, and IL-8 mediates constitutive activation of NF-κB that maintains the chronic inflammation in these tumours (Ghosh et al., 2002; Chen et al., 2004). NF-κB plays an instrumental role in regulating the expression of anti-apoptotic proteins as well as suppression of multiple apoptotic or proapoptotic proteins (Patel et al., 2000; Arlt et al., 2001). The anti-apoptotic proteins regulated by NF-κB are inhibitor of apoptosis proteins IAPs, FLICE, cFLIP, Bcl-XL, Bfl-1, TRAF1 and TRAF2. IAPs (c-IAP1, c-IAP2, and XIAP) inhibit effector caspases and suppress both extrinsic and intrinsic pathways of apoptosis (Deveraux and Reed, 1999). c-FLIP associates with
Figure 1.7 Role of NF-κB in cancer. NF-κB activation pathways and important functions of NF-κB target genes in promoting cancer cell survival and chemoresistance (Adapted from Viennois et al., 2013).

TNFR to block caspase 8 activation. Anti-apoptotic members of the bcl-2 family (Bcl-XL, A1) stop the release of cytochrome-c and inhibit caspase-9 activity (Cheng et al., 2000). TRAF proteins are known to obstruct the caspase cascade at the TNFR1 level through amplification of NF-κB activation. Another
mechanism by which NF-κB blocks apoptosis is by inhibiting JNK activation and accumulation of ROS (De Smaele et al., 2001). In some tumours, cells exposed to radiation or treated with chemotherapeutic drugs enhance the NF-κB activation which inhibits the apoptosis induced by treatment and subsequent acquisition of chemo and radioresistance. This drug or radiation induced activation of NF-κB combined with inherent increased NF-κB activity prior to treatment in tumour cells could be a significant obstacle to effective cancer therapy (Brach et al., 1991). In addition, recent studies have demonstrated that drug resistance genes like MDR1, P-gp, MGMT and BCRP have NF-κB binding sites in their promoter regions confirming the role of NF-κB in chemoresistance (Wang et al., 1999).

1.8.3 Role of NF-κB in hypoxia induced EMT

Although HIF signalling is important for cellular response under hypoxia, more than 20 different transcription factors are functional in the interplay between hypoxia, EMT and CSC phenotypes to induce resistance and metastasis. Among them, NF-κB is thought to play a crucial role in inducing CSC population under hypoxic conditions by functioning as an important transcription factor that activates various EMT and metastasis promoting genes (Chua et al., 2007; Connelly et al., 2007). For cancer epithelial cells to undergo EMT several developmental transcription factors together have to repress epithelial gene expression. For example E-cadherin gene transcription is thought to be suppressed by Snail and Slug. Two other transcription factors ZEB1 and ZEB2 together with Twist and SMAD are also known to regulate E-cadherin and EMT
programme. Multiple lines of evidence suggest all these transcription factors are controlled upstream by NF-κB signalling (Nieto, 2002; Culleres et al., 2006; Kajita et al., 2004; Chua et al., 2007; Yang et al., 2004; Sosic et al., 2003). It has been shown that the region between -194 and -78 of SNAI1 promoter is activated by GSK3 inhibition stimulating the transcription of Snail in a NF-κB dependant fashion (Min et al., 2008). Slug downregulates the expression of epithelial E-cadherin, claudins and occludins and it is thought be controlled by an aryl hydrocarbon receptor (AhR) (Zhang et al., 2006). AhRs are known to transactivate a variety of environment responsive genes including Slug, which in turn is shown to be regulated by c-REL of NF-κB family. ZEB family of proteins are known for their role in EMT by disintegrating cell-cell junctions in epithelial cells and induction of mesenchymal markers. Studies from various cancers suggest that p65 subunit of NF-κB directly regulates ZEB family which are very important mediators of NF-κB signalling during EMT. Twist is another EMT gene that helps the cancer cells to undergo intravasation to systemic circulation and hence promoting invasion and metastasis. Twist is a direct transcriptional target of NF-κB and is shown to be evolutionarily conserved in this way. It has also been shown that Twist expression and activation induced by chemotherapeutic agents in certain cancers largely depended on p65 and c-REL containing NF-κB complexes (Yang et al., 2004). In addition to above factors NF-κB dependant expression is also observed in mesenchymal cytoskeletal protein Vimentin, invasive collagenases MMP2 and MMP9 which are important elements of EMT phenotypes (Kang and Massague, 2004).
HIFs regulate more than 150 genes including NF-κB and many studies have indicated that hypoxia resulted in enhanced NF-κB signalling pathway in cancer cells (Melvin et al., 2011; Devries et al., 2010). NF-κB is primarily required as an anti apoptotic machinery to enable a hypoxic cell to survive. On the other hand, multiple experimental evidences suggest that NF-κB regulates HIFs owing to the presence of NF-κB binding site in their promoter regions (Cockman et al., 2006). Together HIF and NF-κB signalling pathways orchestrate EMT and maintain the stem cell phenotype in tumour microenvironment. Further experiments and validations are required to precisely understand the interplay between hypoxia, HIFs, NF-κB, EMT, CSC phenotypes and cancer chemoresistance. However, it is reasonable to hypothesize that hypoxia induced NF-κB could be the key in maintenance of CSCs during the development and progression of tumours. The pivotal role of the NF-κB pathway and the fact that NF-κB is constitutively activated in a large number of malignancies, strongly imply that inhibiting NF-κB would be a promising strategy in cancer therapy.

1.9 Disulfiram

1.9.1 Pharmacology of Disulfiram

Disulfiram (DS) also known as tetraethylthiuram disulfide was developed from thiocarbamide by a German chemist, M. Grodzki in the year 1881 (Grodzki et al., 1881). DS was first used as a catalyst in the rubber industries to accelerate the vulcanization of rubber and other synthetic rubber products (Eneanya et al., 1981). DS was introduced in the clinics in early 1940s by two British physicians
as a drug to tackle scabies (Gordon et al., 1942). Two Danish physicians Hald and Jacobsen reported in 1945 that ingestion of alcohol after treatment with DS would lead to highly unpleasant reactions, which led to the use of DS as an antialcoholism drug (Eneanyn et al., 1981). Clinically DS is shown to be a very safe drug with very less toxic effects (even after high doses of 300 to 500 milligrams a day) an excellent bioavailability where 20% of the administered drug remains in the body for up to two weeks (Sauna et al., 2005). Structurally DS is made up of two molecules of diethyldithiocarbamate molecules attached together by a disulfide bridge. It has a low molecular weight of 296Da (Sauna et al., 2005). When DS is metabolized, the breakdown of disulfide bridge by a rapid reduction reaction converts DS into its respective thiol groups namely diethyldithiocarbamate (DDC). If administered orally DS is rapidly absorbed from the gastrointestinal tract. More than 80% of oral dose is generally absorbed but a little amount of this is reduced in the stomach and DDC is liberated which is less stable in stomach acid and decomposes to diethylamine and carbon disulphide. However DDC is a strong chelating agent of metals and rapidly forms complex with metal ions like cupric ions to yield DDC-copper (Cu(DDC)_2). Cu(DDC)_2 is more stable in stomach acid that allows its absorption in the upper intestinal tract. Hence a combination of copper with DS is thought to improve the bioavailability of the parent drug. After crossing the intestinal mucosa into the blood stream, DS rapidly disappears below the limit of detection. This process is very quick and can happen within 4minutes *in vitro* (Cobby et al., 1977). *In vivo* the glutathione reductase system in RBCs can readily reduce DS, which causes the rapid disappearance of DS from blood stream.
The DDC formed could be metabolised further by four different reactions namely glucuronidation, nonenzymatic degradation, methylation, and oxidation. Glucuronidation is the main detoxification mechanism of DS where DDC is conjugated with glucuronic acid in mammals and about half of the given dose of DS is excreted in the urine as glucuronide metabolite. Nonenzymatic degradation DDC is a pH dependent mechanism where DDC is rapidly decomposed to diethylamine and carbon disulphide in an acidic environment (Stromme et al., 1966). The resulting diethylamine may be further degraded to simpler compounds such as ammonia and acetaldehyde. About 0.05% of the given dose of DS and its DDC form can be methylated to form a methyl ester of DDC (MeDDC). Another type of methylation of DDC observed was the S-methylation, which accounted for about 27% of metabolism of the given dose (Cobby et al., 1977., Gessner et al., 1972). Enzymes such as esterases targets MeDDC derivative to generate a methyl mercaptan which is further oxidized to sulfate and formaldehyde. Since production of sulfate from other thioester compounds is a rapid process the S-methyl compound oxidation to produce inorganic sulfate by cleavage of methyl mercaptan (Canellakis et al., 1953., Maw et al., 1954). Oxidation reaction involving DDC under atmospheric oxygen condition, results in the reoxidation of DDC back to DS. However it is rare process that accounts for only about 4% of DDC and can be frequently affected by oxidases in the body (Strume, 1965).
1.9.2 Disulfiram and treatment

1.9.2.1 Anti-alcoholism

DS has been used for treating alcoholism for more than 60 years (Kampman, 2009). In healthy adults, ethanol is eliminated in the liver by a group of enzymes known as alcohol dehydrogenases that results in the formation of acetaldehyde. Acetaldehyde is an extremely toxic compound which is instantly converted to less toxic acetate by the enzyme ALDH. DS and its metabolites such as Me-DDC irreversibly inhibit the enzyme ALDH1 by oxidation of sulfhydryl groups to form irreversible internal S-S bonds (Vallari et al., 1982). Alcohol consumption after taking DS results in a dramatic increase of acetaldehyde in the plasma, which mediates histamine release and leads to distressing physical symptoms collectively known as disulfiram ethanol reaction (DER). Typical DER will result in tachycardia, hypotension, nausea, vomiting, flushing of the skin owing to cutaneous vasodilatation particularly on the trunk, tachypnoea, palpitations, anxiety and headache. DS was sold under the trade name Antabuse that was prescribed to well-motivated alcoholics who wished to quit heavy drinking. It was a well-tolerated drug but produced extreme adverse effects beyond safety levels if alcohol was ingested during antabuse treatment.

1.9.2.2 Anti-cancer treatment

Repurposing old drugs for new uses is an established shortcut between the lab and clinics. The anticancer activity of DS is long known. The use of DS for anticancer treatment gained attention after a case report by Lewison in 1977 about an alcoholic breast cancer (BC) patient with multiple bone metastases showed spontaneous regression after the use of Antabuse-DS for her alcohol
addiction. Earlier in vivo studies showed that oral administration of Benzo[α]pyrene induced tumours in 90% of animals, but animals with 1% DS was added to their diet did not develop tumours even after 30 weeks. Another experiment in Wistar rats showed that administration of DS in the diet in parallel with N-n-butyl-N-(4-hydroxybutyl) nitrosamine (BHBN) significantly reduced the induction of urinary bladder cancer by BHBN. A phase II clinical trial with 5 year follow up study that used DDC as an adjuvant in 64 late stage BC patients reported 13 relapses and 12 deaths in control group, whereas only 6 relapses and 5 deaths were reported in DDC group. The OS at 6 years was 81% in DDC group with 76% disease free survival rate while the OS in the control group was only 55%. A single case study of a patient with ocular melanoma and hepatic metastasis reported that a combination of oral DS and zinc gluconate induced reduction in hepatic metastases and produced clinical remission. She also continued the dose of DS therapy for 53 months with negligible side effects. Moreover various high-throughput cell-based screening studies of known drugs and drug like small molecules have identified DS as a potential therapeutic agent for prostate cancer, triple negative breast cancer and GBM.

1.9.3 Mechanism of Action

1.9.3.1 Role of Reactive Oxygen Species and Copper in anti cancer effect of DS

Reactive oxygen species (ROS) are biologically essential highly reactive species of partially reduced unpaired electrons of oxygen such as superoxide (O2-), hydrogen peroxide (H2O2), hydroxyl radical, and peroxynitrite. ROS are generated mainly from superoxides that are formed in mitochondria by reaction
of O2 with electrons leaked out from the respiratory chain (Schafer et al., 2001). ROS can also be generated by reactions involving NADPH oxidase complexes or from biochemical reactions in peroxisomes and cytochromes (Perry et al., 2000). A small increase in intracellular ROS levels will promote cell proliferation and differentiation. However massive increase in the amounts of ROS will result in irreversible damage to lipids, proteins and DNA, leading to apoptosis (Boonstra et al., 2004; Schafer et al., 2001; Perry et al., 2000). ROS could induce DNA lesions through oxidized bases, strand breaks or by adding adducts (Randerath et al., 1996; Lloyd et al., 1997). Excessive ROS is known to damage the mitochondrial membrane and release cytochrome c eventually activating the apoptotic cascade of proteins such as caspases to induce cell death (Hensley et al., 2000). Therefore, the levels of ROS are kept under balance by ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2, and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase (Boonstra et al., 2004). Cancer cells are known to have high intracellular ROS levels because of their high metabolic activity and increased oxidative stress. Under such increased ROS stress, cancer cells are known to adapt by increasing the expression of cell survival proteins, modification of protein structures, enhancing transcription factors and activate genes that control cell growth and differentiation (Davies et al., 1999). For example, up-regulation of the expression of antioxidant enzymes such as SOD, catalase and peroxidases and activation of the glutathione system (GSSG/2GSH) and thioredoxin system has been reported as adaptive mechanisms in cancer cells to maintain cellular redox balance (Schafer et al., 2001; Sasada et al., 1996). Another mechanism is to activate the transcription
factors like NF-κB, HIF1α, HIF2α and other cell signalling molecules such as MAPK and JNK to modulate cell growth and survival (Martindale et al., 2002). Therefore further increase in ROS levels after exposure to anti-cancer drugs will lead cancer cells to apoptosis.

Cu is an essential trace element in humans and plays important roles in respiratory and metabolic reactions. The redox potential of Cu makes it an useful element as biological cofactor, but also can react with oxidizing agents to undergo Fenton’s reaction and generate ROS mediated toxicity that can irreversibly damage cellular components including lipids, proteins, DNA resulting in cell death. The movement of Cu in and out of the cells is tightly regulated by a Cu transporting integral termed Ctr1. DS is a strong chelator of divalent metal ions such as Cu, iron (Fe), nickel (Ni), zinc (Zn), gold (Au), cadmium (Cd), etc (Morrison et al., 2010). Described as a Cu ionophore, DS binds primarily to Cu and can increase the cellular deposition of Cu leading to cytotoxic effects, a mode of action known as Cu overload. Cu can exist in cells only as protein bound forms and hence Cu overload disrupts the functions of proteins. In addition, Cu bound with DS is also a well established cytotoxic complex on its own, reported to induce apoptosis in many cancer cells by several mechanisms.

According to the Cu overload theory the excess Cu mediates cytotoxicity through various mechanisms. As mentioned earlier, cancer cells have high ROS levels due to higher metabolic activity. They also accumulate Cu to meet their metabolic demands, which result in greater intracellular Cu concentration than non-malignant cells. Cu interferes with the ROS withstanding glutathione
system of tumour cells and depletes the antioxidant defences in them. In the presence ROS in cancer cells Cu ions can cycle between two ionic forms, Cu(II) and Cu(III), to induce further ROS by the Fenton reaction. High Cu levels within tumour cells also activate NF-κB, triggering anti-apoptotic factors which decrease this effect but the DS/Cu complex both increases ROS and inhibits NF-κB, so the overall effect seems that Cu can push the oxidative stress in cancer cells beyond the threshold limit leading to cell damage or sensitise them to other anticancer drugs. This loophole can also be used as a selective target mechanism because chelation of excess Cu in cancer cells by DS itself will be toxic to cancer cells than normal cells. (Guo et al., 2009; Liu et al., 2012; Yip et al., 2011).

1.9.3.2 Overriding resistance to chemotherapy

DS was shown to inhibit P-glycoprotein (P-gp) mediated drug efflux of vinblastine and colchicine in vitro by modifying thiol groups in cysteine residues of P-gp. Another finding suggests that the multidrug transporter-mediated resistance in Candida albicans is reversed by DS using the same mechanism.
Furthermore, metabolites of DS, such as *DDC, MeDDC-sufoxide and MeDDC-sulfone*, inhibited the ATPase activity of P-gp stimulated verapamil treatment. Progression of a primary tumour largely depends on angiogenesis. DS demonstrates extensive antiangiogenic activity both *in vitro* and *in vivo* resulting in suppression of gliomas and Lewis lung carcinoma tumours that further led to clinical trials of DS in lung cancer (ClinicalTrials.gov Identifier NCT00312819). Another work using chicken embryos revealed that DS/Zn complex interacts with MMP2 and MMP9 to inhibit angiogenesis. DS is shown to downregulate the release of MMP2, MMP9, VEGF and TNFα in osteosarcoma cells. Tumour angiogenesis like normal angiogenesis requires VEGF and FGF to use Cu as a co factor. Addition of any Cu chelators like penicillamine has been shown to inhibit angiogenesis. It was hypothesised that DS also acts in a similar fashion to remove Cu from blood vessels during tumour vascularization. A recent finding in GBM reported the effect of DS/Cu complex on the DNA repair enzyme MGMT that confers resistance to alkylating agents like TMZ. It was found that DS modified Cys145, a critical cysteine site for DNA repair leading to the loss of MGMT from tumour cells through ubiquitin proteasome pathway in a dose dependant manner.

1.10 **Targeting CSCs with Disulfiram**

1.10.1 **Proteasome / NF-κB inhibition**

The ubiquitin proteasome pathway is the principle mechanism for controlled degradation of proteins in the cell. The proteasome enables rapid clearance of proteins, which is a critical process in tightly regulated mechanisms like cell
cycle, inflammation, apoptosis, transcriptional activation, and ageing. This breakdown of proteins is also critical for recycling the raw materials for new protein synthesis. Thus, the proteasome system helps to maintain the circadian rhythm and homeostasis of the cell. As mentioned in detail in section 1.8 the proteasome pathway plays an important role in the activation of NF-κB, which is a predominant transcription factor activated in many malignant tumours. The inactive form of NF-κB is held in the cytoplasm by its inhibitor IκB until any external stimuli triggers the phosphorylation of IκB. Phosphorylated IκB is poly-ubiquitinated by ubiquitin ligases, which is recognised by the proteasome and finally degraded. This releases NF-κB, which is then translocated to the nucleus and binds to its target genes in order to drive their transcription. NF-κB is known to play multiple roles in cancer chemoresistance and considered to be one of the prime targets for anticancer drug development. DS/Cu complex is a potent inhibitor of NF-κB pathway primarily by its proteasome inhibitory action. To be more specific DS/Cu inhibits the E3 ubiquitin ligase of the proteasome system and impeding the breakdown of IκBα, eventually stopping NF-κB release and translocation to the nucleus. DS/Cu is also known to interact with sulfhydryl groups of REL homology domain to inhibit NF-κB independent of the proteasome system.

1.10.2 ALDH inhibition

In recent years, CSCs have become an attractive target to improve treatment outcomes in resistant tumours. ALDH has been identified as a functional marker of CSCs and known for its role in chemoresistance in wide range of cancers. ALDH is a superfamily containing 19 functional isoenzymes in 11 families and 4
subfamilies. They are NADH dependant enzymes involved in detoxification of exogenous and endogenous aldehydes as well as xenobiotics like cyclophosphamide. Overexpression of ALDH isoenzymes in cancer cell lines can induce chemoresistance to a wide range of conventional anticancer drugs in vitro. Also knocking down ALDH genes has been shown to inhibit the growth of cancer cell lines and sensitizes CSCs to anticancer drugs. None of the available detection methods is specific for a particular ALDH isoenzyme because of the overlap of the same substrates for different ALDH isoenzymes. ALDHs also act as ROS scavengers thereby reducing the drug or radiation induced oxidative stress which could explain the high amounts of ALDH in CSCs and their resistant nature. Different ALDH isoenzymes are reported to be expressed for different cancer types. It is hypothesized that multiple ALDH isoenzymes are coexpressed in the CSCs derived from the same cancers. Therefore targeting an individual ALDH subtype may not be efficient in eliminating the CSC population within a tumour. DS is a pan-ALDH inhibiting molecule that targets ALDH enzyme activity rather than ALDH isoenzyme and thus could be an ideal agent to target CSCs. Therefore, inhibition of ALDHs by DS/Cu will result in ROS accumulation and ROS related cytotoxicity in CSCs. Although ALDH is one of the potential targets of DS, several other mechanisms are still not understood.
1.11 AIMS and OBJECTIVES

Resistance to chemotherapy is a major obstacle in improving the therapeutic outcome of GBM patients. The majority of drugs do not cross the BBB which results in poor bioavailability of anticancer drugs in the brain. In addition to various intrinsic resistance mechanisms, GBM contains a very small population of cancer cells expressing CSC markers that promote therapeutic resistance. It is still unclear whether GBM CSCs are derived from neural progenitor cells or differentiated cells. Many recent studies have elucidated that intra-tumoural hypoxia induces CSC phenotypes in tumour via epithelial-to-mesenchymal transition (EMT). Hypoxia inducible factors (HIFs) are master transcriptional regulators under hypoxic conditions. However NF-κB, another key transcription factor, is also highly up-regulated in hypoxia induced CSCs and is proved to play an important role in cancer chemoresistance. There are no clear insights on how HIFs and NF-κB together orchestrate the anti-apoptotic signalling, chemo-radiation resistance and maintenance of stemness in GBM CSC phenotypes. GBM is resistant to all available anticancer drugs and GBM CSCs play a major role in chemoresistance of GBM. Therefore, development of drugs that cross BBB, targets GBM CSCs in addition to eliminating non-CSC tumour population is of significant clinical importance. The ultimate aim of my study is to determine the anticancer efficacy of Disulfiram and copper in vitro and in vivo.
This project was designed to meet the following objectives:

- To understand the relationship between CSCs and chemoresistance in GBM CSC cultures and to investigate the nature and origin of CSCs isolated from cell lines.

- To determine whether hypoxia and its underlying mechanisms can induce CSCs in GBM and lead to resistance.

- To understand the role of HIFs and NF-κB, in bridging hypoxia and CSC-related chemoresistance in GBM cells.

- To examine the cytotoxic effect of DS and Cu on GBM cell lines, especially on GBM CSCs and to investigate the molecular anticancer mechanisms of DS/Cu in vitro.

- To examine the tumour inhibiting effect of newly developed formulation of DS using orthotopic xenograft GBM mouse models.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Labware, equipment, reagents, enzymes and kits

10, 25, 50 and 100 mL polystyrene tubes, 6-well, 24 well and 96-well flat-bottom tissue culture plates with lids, Tissue culture Cell+ flasks with PE ventilation caps (T25, T75, T175), Filtropur V50.0.2 Vacuum Filter (500 mL), Cell scraper, 1.5mL and 0.5mL eppendorf tubes, sterile disposable pipettes, (5mL, 10mL, 25mL), (Sarstedt Ltd., Leicester, UK).

AccuGel 40% (19:1 acrylamide:bis), 30% acrylamide:bis-acrylamide (37.5:1), 10× Electrophoresis buffer - Tris/Glycine/SDS, 10× Transfer buffer - Tris/Glycine, 10×TAE buffer, 10XTBE buffer, EZ-ECL chemiluminescence detection kit for horse radish peroxidise solution A and B (GeneFlow, UK-National Diagnostics, Yorkshire, UK).

NuPAGE ® LDS Sample Buffer (4x), SeeBlue plus 2 pre-stained protein markers, Geneticin (G418), N2® supplement (100x), B-27® supplement (50x), Trizol, Optimem, Lipofectamine transfection reagent (Life technologies-Invitrogen Ltd., Paisley, UK)

Boyden chamber assay kit (Cell biolabs inc. San Diego, USA)

Basic fibroblast growth factor- human (hFGF) (R&D systems, Albingdon, UK)

Matrigel (BD biosciences, Oxford, UK)

Boyden chambers, BD Falcon Chamber slides, BD Falcon tissue culture dishes, Corning 24 well plates for transwell inserts (Corning, MA, USA – distributed by VWR, Sussex, UK)

Confocal microscope (Carl Zeiss Laser Scanning Systems LSM 510), Inverted light microscope (Nikon, Japan)

Clean air laminar flow hood (Telstar, Netherlands)

CO₂ incubator (Sanyo, Japan)
Micropipettes – Biopette™ Autoclavable pipettes (Labnet international inc., USA) and Pipetman™ autoclavable pipettes (Gilson inc., Luton, UK)

Micropipette tips refill cartridges (1mL, 200μL) and Nucleasefree filtered tips (1mL, 10μL, 40μL, 200μL) (Alpha laboratories, Eastleigh, UK)

Amersham Hybond™ - P (PVDF membrane), Amersham Hybond TM - N+ (GE Healthcare, Buckinghamshire, UK)

ADLH-ALDEFLUOR assay kit, Hypoxia incubator chamber (Stem cell technologies, Cambridge, UK)

BamH I and Hind III enzymes and associated buffers (New England Biolabs).

BD FACSCalibur™ Flow Cytometer, CellQuest™ Pro, Cell wash, FACS Clean, FACS Rinse, BD Falcon FACS tubes (BD Biosciences, Oxford, UK).

Mini-protean western blot tank with lid and gel plate adapters, 1.0mm and 1.5mm glass plates, 15 well and 10 well combs, gel cutter, buffer dam (balance plate for running single gel), Bio-Rad protein assay kit II, Power pack for electrophoresis (Bio-Rad Laboratories, Hertfordshire, UK).

Complete easy packs-Protease inhibitor cocktail tablets, phospho-stop phosphatase inhibitor tablets, Annexin-V apoptosis kit, Rapid Ligation kit (Roche Diagnostics Ltd., East Sussex, UK)

Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, Foetal Calf Serum (FCS), L-Glutamate (200 mM), Penicillin-Streptomycin (10,000U/mL penicillin and 10,000U/mL Streptomycin), 10xTrypsin, Sterile Phosphate Buffer Saline (PBS) (0.0067M PO4), DNase and RNase free Ultrapure water (BioWhittaker® Lonza, Walkersville, USA - Lonza, Slough, UK).

Enhanced Chemiluminescence Western Blot Signal Detection Kit (Amersham Biosciences, UK).

EZ-ECL chemiluminescence detection kit for horse radish peroxidases solution A and B (Thermo fisher scientific, UK).
Fuji Medical X-Ray Film Super RX (13x18) (Fujifilm UK Ltd., Bedfordshire, UK)

Special Gas (1%O₂, 5%CO₂ in Nitrogen) – (BOC gases, UK)

Heraeus Multifuge 3SR laboratory centrifuge with microtitre carrier plate adapter max 4 plates (Thermo Fischer scientific, Longborough, UK)

HypoxyprobeTM-1 Plus Kit supplied by Hypoxyprobe Inc (Burlington, MA, USA)

Marvel dried milk (Marvel, Dublin, Ireland)


Nalgene Cryoware™ Cryogenic vials (Labware, Roskilde, Denmark)

Polylysine-coated slides (VWR, Lutterworth, UK)

Qiaquick gel extraction kit, plasmid maxiprep kit, plasmid miniprep kit (Qiagen, West Sussex, UK)

Restriction enzymes and associated buffers, pfu polymerase, Luciferase assay kit, Gel shifting assay kit, 1Kb DNA ladder, 100Kb DNA ladder, Dual Luciferase Assay reagents, Access RT-PCR system (Promega UK Ltd., Southampton, UK)

RNase A (100 mg/mL) (Qiagen Ltd., West Sussex, UK)

Semi-dry transfer unit (Hoefer, Inc., Holliston, USA)

Semi-dry transfer unit (Amersham Biosciences, UK)

Sigma 1-15k refrigerated microtube centrifuge (Sigma Laborzentrifugen GmbH, Germany)

Stuart® 3 block heater (Bibby Scientific Ltd., Straffordshire, UK).

Insulin (10mg/mL), 45% D-Glucose, human EGF, Heparin, poly-HEMA, Disulfiram, Cu Chloride (CuCl₂), Vincristine (VCR), Paclitaxel (PAC), Doxorubicin (DOX), Temozolomide (TMZ), minisart plus (0.20 μm) filter,
99.9% Dimethylsulfoxide (DMSO), Tris HCl, Tris Base, Tween 20, EDTA, Hygromycin B, Ammonium persulfate (APS), Sodium Dodecyl Sulfate (SDS, 98.5% GC grade), Methanol, Isopropanol, Glycine, HEPES, Sodium Chloride (NaCl), Thymidine, KH₂PO₄, Sodium hydroxide (NaOH), Nonident p40, Ethidium Bromide, LB broth, polydIdC (PdIdC), Triton X-100, Agarose, 3MM filter paper, Propidium iodide (powder), Tetramethylethlenediamine (TEMED), fixer/replenisher, developer/replenisher, Kodak medical X-ray film, DL-dithiothreitol (DTT) solution (1 M in H₂O) (Sigma Aldrich Company Ltd., Dorset, UK)

Multiskan Ascent and Multidrop 384 (Thermo Fisher Scientific Inc., Leicestershire, UK)

2.1.2 Antibodies

MACS- Flow cytometry CD133-PE conjugated antibody, (Miltenyi Biotec Ltd, Surrey, UK)

BD FACS CD44-FITC conjugated antibody (BD biosciences, UK)

p-p65 (S276) antibody (Abcam, Cambridge, UK)

Horse radish peroxidase conjugated secondary anti-mouse and anti-goat antibodies (Amersham Biosciences, UK)

Sox2, Oct4, Nanog, Vimentin, N-Cadherin (Cell Signalling, Herts, UK)

Enhanced ChemiLuminescence (ECL)™ anti-mouse antibody (Ab), ECL™ anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK)

HIF2α antibody (Novus Biologicals, CO, USA)

p65, BCL-2, BAX, E-Cadherin, Nucleolin, ALDH1A1, ALDH3A1, HIF1α, IκBα, MDR1 antibodies (Santa Cruz Biotechnology Inc., Heidelberg, Germany)
ALDH2, ALDH1A3, Anti-vinculin monoclonal antibody, Anti-tubulin monoclonal antibody, FITC-conjugated anti-mouse IgG antibody (Sigma Aldrich Company Ltd., Dorset, UK)

### 2.1.3 Cell lines

Human Glioblastoma multiforme cell lines: U87MG, U251MG and U373MG (Prof John Darling, BTUK neurooncology lab, University of Wolverhampton, UK)

Human normal breast epithelial cell line: MCF10A (American Tissue Culture Collection, Rockville, USA)

Normal Human Astrocytes (Dr. Tracy Warr, BTUK neurooncology lab, University of Wolverhampton, UK)

Normal human vascular endothelial cells, HeCV (Prof Wenguo Jiang, University of Cardiff, UK)

Human endothelial cell line: EAhy926 (Dr Angel Armesilla, University of Wolverhampton, UK)

WI38 –Lung fibroblasts (Dr Weiguang Wang, University of Wolverhampton, UK)

### 2.1.4 Buffers

**Complete protease inhibitor**

A (25x) complete protease inhibitor (Roche Diagnostics Limited, West Sussex, UK) was prepared by dissolving 1 tablet of complete protease inhibitor in 25 mL of distilled water, and stored at -20°C until use.
**Sorensen's Glycine Buffer**

Glycine buffer solution (500 mL) was prepared by dissolving 3.75 g glycine and 2.92 g NaCl in distilled water. The pH of the glycine buffer was adjusted to pH 10.5 using 5 M NaOH. The glycine buffer solution was stored in room temperature until use.

**RIPA Buffer for Whole Protein Extraction**

10x RIPA Buffer was prepared by the list below (pH 7.4), and RIPA Buffer was stored in -20°C until use.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Conc.</th>
<th>W/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Tris HCl</td>
<td>25 mM</td>
<td>395 mg</td>
</tr>
<tr>
<td>• SDS</td>
<td>0.1%</td>
<td>0.1 g</td>
</tr>
<tr>
<td>• Triton X-100</td>
<td>1%</td>
<td>1 mL</td>
</tr>
<tr>
<td>• Sodium deoxycholate</td>
<td>0.5%</td>
<td>0.5 g</td>
</tr>
<tr>
<td>• NaCl</td>
<td>0.15 M</td>
<td>0.88 g</td>
</tr>
<tr>
<td>• EDTA</td>
<td>1 mM</td>
<td>37.2 mg</td>
</tr>
<tr>
<td>• Sodium orthovanadate</td>
<td>1 mM</td>
<td>18.4 mg</td>
</tr>
<tr>
<td>• Leupeptin</td>
<td>1 µM (1 - 10 µg/mL)</td>
<td>1 mg</td>
</tr>
<tr>
<td>• Aprotinin</td>
<td>1 µM (1 - 10 µg/mL)</td>
<td>1 mg</td>
</tr>
<tr>
<td>• PMSF</td>
<td>1 mM</td>
<td>17.4 mg</td>
</tr>
</tbody>
</table>

**Separating buffer**

Separating buffer was prepared using the following recipe and the pH was adjusted to 8.8 and stored in room temperature until use.

- Tris base 45.4 g
- SDS 1 g
- Add 250 mL distilled water
Stacking buffer

Stacking buffer was prepared using the following recipe and the pH was adjusted to 6.8 and stored in room temperature until use.

- Tris base 15 g
- SDS 1 g
- Add 250 mL distilled water

Running buffer

Running buffer was prepared by mixing 100 mL of 10X stock solution with 900 mL distilled water.

Buffer A for Nuclear Protein Extraction

Buffer A was prepared using the following recipe and stored at -20°C until use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
<th>Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM hepes</td>
<td>500 mM</td>
<td>200 μL</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td>2M</td>
<td>50 μL</td>
</tr>
<tr>
<td>0.1 mM EGTA (pH 8.0)</td>
<td>500 mM</td>
<td>2 μL</td>
</tr>
<tr>
<td>0.1 mM EDTA (pH 8.0)</td>
<td>500 mM</td>
<td>2 μL</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.5 M</td>
<td>20 μL</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>25 ×</td>
<td>500 μL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>9.2 mL</td>
<td></td>
</tr>
</tbody>
</table>

Buffer C for Nuclear Protein Extraction

Buffer C was prepared using the following recipe and stored at -20°C until use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
<th>Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM hepes</td>
<td>500 mM</td>
<td>60 μL</td>
</tr>
<tr>
<td>0.4 M NaCl</td>
<td>5 M</td>
<td>120 μL</td>
</tr>
<tr>
<td>1 mM EGTA</td>
<td>500 mM</td>
<td>3 μL</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>500 mM</td>
<td>3 μL</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.5 M</td>
<td>3 μL</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>25 ×</td>
<td>75 μL</td>
</tr>
<tr>
<td>5% glycerol (v/v)</td>
<td>100%</td>
<td>75 μL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1161 μL</td>
<td></td>
</tr>
</tbody>
</table>
**Tris-buffered saline Tween-20 (TBS-T) – Wash Buffer**

A stock (10x) TBS-T solution was prepared as given below and the pH was adjusted to 7.4

- 12.11 g Tris base
- 81.8 g NaCl
- 1 litre of distilled water

The (1x) TBS-T buffer was then prepared by mixing 100 mL (10x) TBS-T, 900 mL distilled water and 500μL of Tween-20.

**Transfer buffer**

Transfer buffer was prepared by mixing 100 mL of 10x stock solution, 200mL of methanol and 700mL distilled water. This solution was stored at room temperature until use.

**Blocking buffer for western blot**

For western blot the blocking buffer used was 5% (w/v) milk prepared by dissolving 5g Marvel milk powder in 100 mL of (1x) TBS-T. 2%BSA (w/v) in TBST was used as blocking buffer for phosphorylated antibodies.

**Blocking buffer for Flow cytometry and ICC**

For flow cytometry procedure involving staining of live cells 4%FBS in PBS was used as blocking buffer. For procedures involving fixed cells and to increase cell permeability PBS containing 1%BSA (w/v) and 0.01% (v/v) Triton-X 100 was used as blocking buffer.
2.2 Methods

2.2.1 Routine Cell culture (ATT or NOR culture)

**Serum-containing medium**

Serum-containing medium consists of DMEM medium with 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin and 10% Foetal Calf Serum (BioWhittaker® Lonza, Walkersville, USA).

**Serum-free medium**

Serum-free medium consists of DMEM medium with 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin (BioWhittaker® Lonza, Walkersville, USA).

**Trypsin**

Working solution of trypsin (1x) was prepared by diluting stock solution of trypsin (10x) in sterile PBS (BioWhittaker® Lonza, Walkersville, USA).

**Neurosphere medium**

The selective medium for isolating neurospheres or neural stem cells consist of DMEM F-12 medium (500mL) with 1%v/v L-glutamine, % (v/v) penicillin-streptomycin, 5mL N-2 Supplement, 10mL B-27 Supplement, 10ng/mL hEGF, 10ng/mL hFGF, 20ug/mL Insulin, 20ug/mL heparin, 3.1mL 45% D-Glucose. The media was filter sterilised and stored at 4°C until use. However the media was not stored for extended periods due to its less stable ingredients.

**Freezing Medium for cryogenic storage of cell samples**

Freezing medium was prepared by mixing 90% foetal calf serum with 10% DMSO and stored at 4°C until use.
Recovering Cell Lines from Liquid Nitrogen Storage

Cells were recovered from liquid nitrogen storage, rapidly defrosted in 37°C water bath, and then transferring the 1 mL content of the cryovial into a 75 cm² tissue culture flask containing 19 mL of serum-containing medium. The cells were incubated at 37°C and allowed to attach and recover overnight. The following day media was changed to get rid of any unattached or dead cells and to ensure that there is no DMSO in the culture.

Trypsinization of Adherent Cell Lines

Any spent cell culture medium was removed from the tissue culture flask, rinsed with 5 mL of sterile PBS, and 2 mL of trypsin was added and spread evenly to cover the adherent cells. Cells with trypsin were incubated at 37°C in incubator, gently tapped in between and checked regularly under the microscope for detachment. When the cells were completely detached, 2 mL of serum-containing medium was added to neutralize the trypsin and the cells were re-suspended thoroughly by pipetting the 4 mL volume up and down. The contents were transferred in to a tube and cells were suspended for culture by centrifugation at 880g for 5 minutes.

General Cell Line Maintenance

Cell cultures were regularly checked under the microscope (2-3 times a week) for cell density, and changes in the colour of the medium. When there is a change in the colour of the medium but the cell density is low, (defined as adhered cells are spread out and very few cells are touching each other),
the medium was removed and replaced with fresh serum-containing medium. When there was a change in the colour of the medium and the cells were confluent, (defined as cells are fully grown and only very few gaps are left between the cells), cells were sub-cultured. Sub-culturing was done by trypsinizing the cells. The cell pellet collected after trypsinization and centrifugation was re-suspended thoroughly in 5 mL of fresh serum-containing medium. 1 mL of this cell suspension was then added to two 75 cm² tissue culture flasks with 19 mL of serum-containing medium incubated at 37°C with 5% CO₂, with the tissue culture flask lying down. When they are fully grown again, the same process of subculture was applied. The cells can be enlarged in a number of flasks based on requirements or used for experiments or collected and stored at -20°C as pellets.

Preparing Cell Lines for Liquid Nitrogen Storage

Cells were trypsinised and the cell pellet was collected by spinning down for 3 minutes at 800 g. The cell pellet was then re-suspended in freezing medium and aliquot into 1 mL/labelled-cryovial. Each cryovial was then wrapped in tissue paper, put into a disposable labelled-glove and placed in -80°C overnight. The following day, the cryovials were removed from the gloves and tissue papers, and transferred into liquid nitrogen (-180°C) for long-term storage. The locations of the samples stored in liquid nitrogen are recorded in the liquid nitrogen storage log.
2.2.2 Sphere cell culture

Poly-HEMA coating for sphere cell culture

The poly HEMA crystals were dissolved overnight (poly HEMA (poly-2-hydroxyethyl methacrylate Sigma ref P3932) under agitation at 10 mg/mL in 95% ethanol (100% EtOH doesn’t work). The solution was filter sterilized and stored at 4°C for several months. The following volumes were used for coating the plates and flasks

- 4 mL for T75
- 2 mL for T25
- 0.4 mL 6 wells
- 0.3 mL 24 wells
- 30 µl 96 wells

The coated plates/flasks were then dried in a 50-60°C incubator and can be kept at RT for several months. Before using those coated flasks/plates for culturing the cells they were thoroughly rinsed twice with sterile water or PBS (critical step as trace of free poly HEMA can be toxic).

Growing cells as Neurospheres (NS culture)

The U87MG, U251MG and U373 MG cells were trypsinised from an adherent culture flask and counted using haemocytometer. For neurosphere cultures, 10,000 cells/mL were grown in selective NS medium with all growth factors. The cells were cultured in low adherence flasks or multi-well plates provided by Corning, USA. Alternatively the cells can also be grown in poly-HEMA coated low adherence flasks or plates that are prepared in our lab using the above protocol. The cells were incubated for 5-7 days and allowed to form
spheres with intermittent replacement of fresh media. After incubation the cells were photographed and observed for sphere formation and used for further stem cell experiments.

**Growing cells as spheres with serum containing medium (SUS culture)**

Alternatively to the NS culture with serum free selective medium we tried to grow the U87MG, U251MG and U373 MG cells as suspension cultures in serum containing medium. The cells were trypsinised from an adherent culture flask and counted using haemocytometer. About 10,000 cells/mL were grown in normal DMEM serum containing medium without any growth factors in low adherence flasks or multi-well plates. The cells were incubated for 5-7 days and allowed to form spheres with intermittent replacement of fresh media. The cells were later photographed and observed for any evidence of sphere formation under non-selective medium.

**2.2.3 Hypoxic Cell culture (HYP culture)**

All three GBM cell lines U87, U251 and U373 grown in adhered condition (ATT) were subcultured and the flasks were placed under hypoxic conditions to grow the cells as HYP cultures. This method was a simple adaptation of normal cell culture using a tightly sealed chamber that contains low O₂ levels and can be placed inside the 37°C incubator. The cells were cultured in dishes, flasks or plates and placed inside this chamber. The gasket and seals were tightly secured and hypoxia special gas mixture (1%O₂, 5%CO₂ and
rest N\textsubscript{2}) was passed into the chamber through inlet tube. The outlet tube was left open was at least 2-3 minutes in order to flush out the normal O\textsubscript{2} containing air inside the chamber. The reduction in O\textsubscript{2} was measured using an O\textsubscript{2} meter placed inside the chamber. The outlet is closed tightly using the clamps provided. After ensuring that the special gas has filled the chamber and the inlet was also quickly secured using the clamps. The chamber was now placed in a non-CO\textsubscript{2} incubator at 37\degree C for 5-7 days. Intermittent feeding of the cells was done every 2-3 days and the flasks were flushed with gas to remove any O\textsubscript{2} introduced from the replaced medium before placing them back to the chamber. The cells grown in these conditions were processed quickly during experiments to ensure maximum reliability. In addition all these ATT-HYP cells were compared with cells grown under normoxia for 5-7 days in parallel with same initial cell number (ATT-NOR) for experiments. The chambers were also monitored for any leaks and gassed everyday to ensure that there is proper induction of hypoxic conditions.

2.2.4 Cytotoxicity assay

Stock MTT solution
5 mg/mL MTT solution (500 mL) was prepared by adding 2.5 g of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 500 mL of sterile PBS, mixed to dissolve using a magnetic stirrer, with the bottle wrapped in foil to protect the MTT from direct light. This MTT solution was then filtered sterilized, wrapped in foil and stored at 4\degree C until use.
MTT assay

MTT assay is one of the widely used methods to evaluate the effect of anti-cancer drugs on cell viability. Cells were cultured in 96-well plates (1 × 10^4/well) and left overnight at 37°C. Cells were exposed to different concentrations of anti-cancer drugs like VCR, TMZ, PAC, DOX, DS/Cu prepared by a two-fold serial dilution using culture medium i.e. 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM. One column of cells was used as negative control and other column was used as carrier control for carriers like DMSO. After removing the spent media from the wells, the medium (200 μL) with different concentrations of drug was distributed in each well. The plates were then incubated at 37°C for desired time. Normally there are 3 wells with the same drug concentration and cell type in each plate and the experiment was done with triplicate plates at the same time.

The experiment was repeated three times with triplicate plates to calculate the standard deviation. After incubation time, 50 μL of MTT solution was added to the wells and the plates were wrapped in the aluminium foil and incubated for 3-4hrs at 37°C. The solution was removed after incubation and 80μL of 99.9% DMSO and 20μL glycine buffer was added. The OD values of each well in the plates were read by a multi-well plate reader spectrophotometer at a wavelength of 540 nm. The readings were tabulated and %viability can be calculated in order to determine the IC50 values for each drug and each cell line.
MTT assay for sphere cells (NS and SUS)

Since the NS and SUS cells were suspended they get washed away while dosing with drug containing medium or removing spent medium following addition of MTT. Hence we followed a modified MTT method for these sphere cells. The sphere cells NS and SUS from all three GBM cell lines used were trypsinised and dissociated into individual cells. They were then reseeded in poly HEMA coated 96 well flat bottom plates in their corresponding culture medium at a cell density of 5000 cells/well and cultured overnight. They are grown overnight at a volume of 100 μL/ well. They were then dosed with drug containing medium (2X concentration to compensate already present 100 μL) on top with another 100 μL with appropriate drugs under study. The cells were treated for 120 hours and MTT reagent was added to the wells. After incubation with MTT reagent, the plates were spun down at 800 rpm for 5 mins in a plate centrifuge and media is discarded. The spheres which appear like purple crystals are dissolved by adding DMSO and the OD is measured using a plate reader. The % viability and corresponding IC50 values for each drug was calculated.

MTT assay for Hypoxic cells

MTT assay for hypoxic cultures were carried out very similar to that of ATT NOR cells. Prior to seeding in 96 well plates the cells were grown under HYP for 5 days. These HYP cells were quickly trypsinised and seeded at a cell density of 1000 cells per well and incubated for another 48hrs before dosing with drugs. ATT- NOR cells grown in parallel was used as control. After
treatment with drugs for desired time the cells were subjected to MTT assay as mentioned above and the cell viability was calculated in order to determine any difference in IC\textsubscript{50} values of HYP cultures.

2.2.5 SDS-PAGE / Western Blot

10% Ammonium Persulphate (APS)

10% Ammonium Persulfate (APS) (Sigma Aldrich Company Ltd., Dorset, UK) was prepared by dissolving 0.1 g APS in 1 mL of distilled water. This was prepared fresh before use.

Preparation of Separating Gel

Separating gel was prepared by following the recipe below

**Separating Gel: (normally use 10% gel.)**

1) 30% 19:1 Acrylamide:bis

<table>
<thead>
<tr>
<th>Component</th>
<th>10%</th>
<th>8%</th>
<th>7.5%</th>
<th>6.5%</th>
<th>6%</th>
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</thead>
<tbody>
<tr>
<td>Separating buffer</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>30% 19:1 acrylamide:bis</td>
<td>5.9</td>
<td>4.3</td>
<td>4.0</td>
<td>3.4</td>
<td>3.2</td>
</tr>
<tr>
<td>H2O</td>
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<td>7.2</td>
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<tr>
<td>10% (w/v) ammonium persulphate (µL)</td>
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<td>120</td>
<td>120</td>
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<td>120</td>
</tr>
<tr>
<td>TEMED (added before pouring gel) (µL)</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

2) 40% 19:1 Acrylamide:bis

<table>
<thead>
<tr>
<th>Component</th>
<th>10%</th>
<th>8%</th>
<th>7.5%</th>
<th>6.5%</th>
<th>6%</th>
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<tbody>
<tr>
<td>Separating buffer</td>
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<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>40% 19:1 acrylamide:bis</td>
<td>4.4</td>
<td>4.3</td>
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<td>3.2</td>
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<tr>
<td>H2O</td>
<td>6.5</td>
<td>6.2</td>
<td>6.4</td>
<td>7.0</td>
<td>7.2</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulphate (µL)</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>TEMED (added before pouring gel) (µL)</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
About 4.5mL was used for one gel with 1.0mm spacer plates and 7mL was used for 1.5mm spacer plates. After adding the gel a small amount of isopropanol (200μL) was added to cover the top of the separating gel solution, was added on top of the separating gel to get rid of air bubbles and form a flat layer. The gel was left to set for 20 mins.

**Preparation of Stacking Gel**

Stacking gel was prepared by following the recipe below

**4% Stacking gel:**

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking Buffer</td>
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<tr>
<td>30% (19:1) acrylamide</td>
<td>1.6 mL</td>
</tr>
<tr>
<td>H2O</td>
<td>6.2 mL</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>250 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

When the gel was set the isopropanol from the separating gel was removed and washed with distilled water. The stacking gel mix was added above the separating gel and a comb was immediately inserted on the top of the stacking gel, to form wells for protein loading. Care must be taken to avoid air bubbles and the gel was left to set before removing the comb.

**Collection of whole cell lysates and proteins**

Whole cell proteins were collected from the cells by washing the cell pellets with PBS and adding 100-200μL (less or more depending on the size of pellet) of RIPA buffer with protease and phosphatise inhibitors to the pellet. The cells were thoroughly suspended and completely lysed using the RIPA
buffer and then centrifuged for 20mins at 4°C at 14000rpm. The supernatant collected is the whole protein extract and they were stored in clearly labelled tubes at -80°C until use.

**Cytoplasmic and Nuclear Protein Extraction**

Nuclear protein extracts were prepared by washing the cells in PBS and resuspending them in 100μL of buffer A. The mixture was left on ice for 15 minutes, to which, 25 μL 10% NP-40 (Sigma Aldrich Company Ltd., Dorset, UK) was added and then centrifuged for 30secs at 10,000rpm. The supernatant was collected (cytoplasmic protein) and the pellet was re-suspended in 500 μL buffer A to completely wash any cytoplasmic proteins, centrifuged again for 30secs at 10,000rpm, and the supernatant was removed. The pellet was then re-suspended in 50-100 μL of buffer C, left to spin on a rotator at 4°C for 1 hour, then centrifuged for 5 minutes at 14,000rpm and the supernatant was collected as nuclear protein and stored in clearly labelled tubes at -80°C until use.

**Protein Concentration Measurement**

A serial standard using BSA protein was prepared by serially diluting the 3.0mg/mL BSA solution in RIPA buffer to the following concentrations: 3.0mg/mL, 1.5 mg/mL, 1.0 mg/mL, 0.75 mg/mL, 0.50 mg/mL, 0.25 mg/mL and 0mg/mL (30μL RIPA buffer). The BioRad protein estimation reagent mix A/S was prepared by adding 20μL of reagent S to every 1mL of reagent A and mixed using vortex mixer. 5μL of the BSA standards (duplicates) and the 5μL of protein samples were pipetted into a 96-well flat-bottomed plate, in
triplicates, to which 25μL of A/S reagent mix was added. 200μL of reagent B was then added and incubated at room temperature for 10 minutes. The absorbance was read at 650nm using a multiwall plate reader spectrophotometer and protein concentration of the samples was calculated from plotting the absorbance versus protein concentration of the BSA serial standard.

**SDS-PAGE Electrophoresis**

The previously prepared SDS-PAGE gels were assembled into the electrophoresis tank using the cassette adapter and soaked in the tank loaded with 1 x running buffer. The protein samples were prepared by mixing 50-100μg of quantified protein extracts with loading buffer (4x), 1μL of 1M DTT and distilled water to make up a final volume of 30 μL in a 0.5 mL eppendorf. The samples were heated using a PCR block heater at 96°C for 10 minutes then centrifuged quickly at 10,000g for 10 seconds and stored on ice until use. The wells were washed to remove any debris and the samples were loaded into the wells. 10μL of pre-stained protein was loaded as a control in one well. The gel was then run at 200V for 1 hour until the protein marker clearly separates.

**Blotting**

3MM blotting paper was cut into 9 x 10cm size and soaked in transfer buffer. PVDF membrane was cut into 8 x 4.5 cm size and briefly dipped in methanol and soaked in transfer buffer. A sandwich of SDS-PAGE gel on top of the PVDF membrane between 3 layers of the wet blotting papers was setup in
the semi-dry transferring unit. Protein was transferred from the SDS-PAGE gel to the PVDF membrane by applying 20V and 200mA current for 1 hour and 30 minutes.

**Blocking of the membrane**

5% fat-free milk in 1x TBS-T solution was used to block the protein-containing PVDF membrane for 1 hour on a rocker to prevent non-specific binding of primary and secondary antibodies to the protein-free areas of the PVDF membrane.

**Antibody Staining**

5 mL of primary antibody according to the dilutions given in Table 2.1 was prepared in the blocking buffer. After blocking the PVDF membrane with blocking buffer for at least 1 hour, the membrane was placed in a clean plastic bag and sealed around the corners using a heat sealer. The primary antibody was poured into the bag, sealed, and left overnight at 4°C on a shaker. Care was taken to ensure that the antibody solution completely covers the surface of the membrane and there were no air bubbles inside the bag. The following day, the primary antibody was removed for reuse from the bag and the PVDF membrane was washed twice with TBST (1x) for 10 minutes each. The PVDF membrane was then placed face up in a tray containing 10mL of secondary antibody (diluted as in Table 2.1) and incubated at room temperature for at least 1 hour on a shaker. The PVDF membrane was then washed twice with TBS-T (1x) for 10 minutes. The
membrane was immediately transferred to a prepared x-ray film exposure cassette and processed for signal detection.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p65 (S276)</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Sox2</td>
<td>1:1000</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>Oct4</td>
<td>1:1000</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>Nanog</td>
<td>1:500</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>HIF2α</td>
<td>1:500</td>
<td>Novus biologicals</td>
</tr>
<tr>
<td>HIF1α</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>p65</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>BCL-2</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>BAX</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>1:500</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>ALDH2</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>ALDH1A3</td>
<td>1:2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>ALDH3A1</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>IκBα</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>1:500</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1:2000</td>
<td>Cell signalling technology</td>
</tr>
<tr>
<td>Tubulin</td>
<td>1:8000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>1:1000</td>
<td>Santa cruz biotechnology</td>
</tr>
<tr>
<td>Secondary Anti-Rabbit</td>
<td>1:5000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Secondary Anti-Mouse</td>
<td>1:5000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Secondary Anti-Goat</td>
<td>1:2000</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

Table 2.1 Dilutions ratios for all the primary and secondary antibodies used in the work given in μL. The antibodies were prepared in 5% (w/v) fat free milk. 5% (w/v) BSA was used for antibodies against phosphorylated proteins.
Signal detection

The membrane after washing was placed in a cassette layered with cling film paper. In the dark room, about 2-3mL of EZ-ECL solution was prepared by mixing solution A with solution B at a 1:1 ratio and added to PVDF membrane. PVDF membrane in the A/B mixture solution was left for 3-5 minutes and then any excess solution was removed using a tissue paper. The PVDF membrane was then wrapped in cling film and then exposed to a Fuji medical x-ray film in complete dark conditions. The length of exposure depends on the strength of the signal. The film after exposure was developed in a tray containing developer solution and briefly rinsed in water and then fixed in a tray containing fixer solution. The bands were seen as dark spots due to chemiluminescent light affecting the x-ray film. Intensities of bands indirectly represent the protein content in the wells. This will implicate the expression level of protein under study.

2.2.6 Flow Cytometry

Flow cytometry for fixed cells

70% ethanol was prepared by diluting absolute ethanol in PBS or water. Any ATT, HYP, NS and SUS cells under study were trypsinised and pelleted by centrifugation at 800 g for 5 minutes. The supernatant was removed and the cells were washed with 5mL of sterile PBS for at least two times. The final cell pellet was re-suspended in 200μL of sterile PBS. While placing the tubes on a vortex mixer 2 mL of 70% ethanol was slowly added to the cell suspension, and fixed for 10 minutes. After 10 minutes the fixed cells were pelleted by centrifugation at 800 g for 5 minutes, the ethanol was removed
and the cell pellet was washed with sterile PBS. Following this process the fixed cells were permeabilized, blocked, stained and analysed according to ICC protocol. For FACS analysis involving intracellular proteins like Sox2, Oct4 and Nanog the collected cells were fixed with ethanol and permeabilized using FACS blocking buffer containing tritonX100 and BSA. The primary antibody was diluted at 1:500 in blocking buffer and incubated overnight at 4°C. The following day cells were washed with PBS to remove any unbound primary antibody and then incubated with fluorescent tagged secondary antibody prepared in blocking buffer at the dilution 1:1000 for 1 hour in room temperature. After incubation the cells were washed and resuspended in flow buffer and analysed using FACS.

**Hypoxyprobe assay**

HP assay was performed using fixed cells. Full instructions are available in the kit. The cells grown as ATT, HYP, NS or SUS were treated with hypoxyprobe reagent for 2-4hrs. The cells were then trypsinised, fixed with ethanol, blocked and stained with anti-HP FITC conjugated monoclonal antibody prepared in blocking buffer. The cells were then washed with PBS, resuspended in flow buffer and analysed using FACS.

**Flow cytometry for live cells**

Alternative to the use of fixed cells the cells can also be used as live cells. After collecting the cells by trypsinising, the cells were stained with antibodies prepared in flow buffer. Flow buffer contains serum and hence helps to maintain live cells. We have used live cell staining method for CD133 and
CD44. Cells were collected, resuspended in flow buffer containing the CD133 or CD44 antibody and incubated in 4°C for 15 minutes. After incubation the cells were washed with fresh flow buffer and analysed by FACS. The CD133 is a PE conjugated antibody and hence analysed using FL2 red range filter in the FACS machine. The CD44 is a FITC conjugated antibody and hence FL1 was used as filter for green range. The stained cells should be analysed quickly to avoid any non specific binding.

**ALDEFLUOR assay**

The ALDH- ALDEFLUOR reagent was prepared according to the instruction in the kit. The cells collected after trypsinising were stained with the reagent suspended in assay buffer and incubated at 37°C for 30 mins. The cells were washed and analysed using FL1-green filter in FACS.

**2.2.7 Confocal microscopy**

**Fluorescence labelling of sphere cells**

Sphere cells were washed twice with PBS gently and trypsinised to individual cells. The cells were subjected to cytospin at 800rpm for 3mins using a funnel attachment in the poly-lysine coated slides. The cells attached to the slides were fixed with 70% ethanol or methanol acetone (50:50) mix. Fixed cells were washed twice with PBS, and then the cells were blocked with 5% BSA containing triton X 100 for 30 minutes at room temperature to block non-specific binding of immunoglobulin. The cells were incubated with the primary antibodies for 1 hour at room temperature, washed three times with PBS and then labelled with the secondary antibodies for 1 hour in the dark, then the
cells were washed three times in PBS. For HP the direct conjugated antibody was used to bind to HP. Cover slips were mounted onto slides using anti-fade mounting medium. Images were captured using laser-scanning microscope.

**Fluorescence labelling of ATT cells**

Cells from flasks were harvested by trypsinization and 0.5x10^4 cells were seeded on a sterile 8-well chamber slide over night at 37°C for immunofluorescence experiment. For HP experiment the cells were incubated with HP reagent for 2 hours before fixing the slides. The staining protocol is very similar to that of sphere cells but the cells don’t have to go through cytopsin step as they are grown in chamber slides. The chamber walls are carefully removed with forceps and the cells were then fixed, permeabilized and stained with appropriate antibodies. Images were taken after mounting the slides with a cover slips and mounting medium.

**2.2.8 Luciferase reporter gene assay**

**Cell culture**

Cells were seeded at a density of 5 x 10^4/100 μL of medium/well on to 96 well flat bottom white opaque plates and allowed to grow overnight at 37°C.

**Lipofectamine Transfections**

The following day, two sets of transfection solutions were prepared as described below which contains the control pGL3-Basic vector in one tube and the test luciferase reporter gene vectors in another. Both solutions
contain an internal control which is pRL-SV40- renilla. The cells were transfected using these vectors prepared in Lipofectamine 2000. **Solution I:** According to requirements in total number of wells, 0.2 μg/well of Luciferase reporter plasmid DNA and 0.002 μg/well pRL-SV40 (Renilla) DNA was diluted in 25 μL/well of opti-MEM medium without serum. **Solution II:** 10μL of lipofectamine 2000 was diluted in 250μL of opti-MEM for each plasmid vector and incubated at room temperature for 5 minutes. Solution I and solution II were mixed in equal amounts and incubated at room temperature for 20 minutes. After incubation the mix was aliquoted equally to all the wells with cells and the plates were incubated at 37°C, 5% CO2 for 48 hours. Luciferase test was carried out after incubation time.

**Luciferase assay test & analysis**

The medium was removed after 48 hours, and the wells were washed with 1x PBS. 100 μL of 1x lysate buffer was added to each well and incubated at room temperature for 10 minutes on a rocker followed by the addition of 30μL Luciferase assay reagent II and read in a luminometer. The reading taken corresponds to the firefly luciferase activity. This was followed by the addition of 30 μL Stop & Glo reagent and read again which corresponds to renilla activity. The readings of the luciferase activity in each well were normalized using the pRL-SV40 Renilla value using the formula: \( \text{Ln} = \frac{\text{L}}{\text{R}} \) where Ln: normalised luciferase activity; L: value of luciferase activity and R: value of Renilla activity. Finally Ln was further standardized by transcriptional activity of the control (pGL3-Basic), using the formula: \( \text{RLU} = \frac{\text{Ln}}{\text{pGL3-basic}} \) (RLU: relative luciferase unit).
2.2.9 Molecular biology protocols

2.2.9.1 Preparation of LB plates

25 g LB broth powder and 20 g agar was added to 1 L of deionised water to prepare LB agar medium. The medium was autoclaved and allowed to cool to 50°C. 50 μg/mL ampicillin was added to the medium and mixed well. Approximately 15 mL/plate were poured into 10 cm2 Petri dishes and were allowed to set at room temperature. The plates can be stored at 4°C.

2.2.9.2 Transformation of competent bacterial cells

DH5α component E.Coli bacteria was purchased from invitrogen and stored as 50μL aliquots at -80°C. An aliquot of DH5α cells was thawed on ice and 500ng of DNA was added and incubated on ice for another 15 minutes. The cells were subjected to heat shock for 90 seconds at 42°C and replaced on ice for 2 minutes. After this 1 mL of SOC medium was added to the cells and incubated at 37°C for 1 hour in a shaking incubator. 100μl of this bacterial culture was spread using an L-rod onto prepared LB plate and incubated overnight at 37°C. The next day plates a single colony of positively selected cells was transferred to LB broth for plasmid preparation.

2.2.9.3 Preparation of plasmid DNA

Mini-preps

QIAGen miniprep kit was used to do mini-prep purification following the manufacturer’s instructions. Cells were collected by centrifugation at 12,000g for 5 minutes and re-suspended in 100μL of re-suspension buffer I (50 mM
Tris-HCl, 10 mM EDTA, pH 8.0 containing 100 μg/mL RNase A). 100μL of lysis buffer II (200 mM NaOH, 1% (w/v) SDS) was added to the cells, mixed and incubated for 5 minutes with gently up and down. 100μL of neutralisation buffer III (3 mM potassium acetate, pH 5.5) was then added to stop lysis. Following centrifugation for 10 minutes at 12,000 g, the supernatant was transferred to mini-prep columns. The columns were spun for 1 minute at 12,000 g and washed two times with 750 μL wash buffer (1.0 mM NaCl, 50 mM Tris-HCl, pH 7.0, 15% (v/v) isopropanol). The bound DNA was then eluted into a clean 1.5 mL eppendorf tube with 100 μL DNase, RNase-free H2O.

Maxi-preps
Plasmid DNA was extracted on a larger scale using the Qiagen QIAfilter kit following manufacturer’s instructions. 350 mL of transformed bacterial culture was centrifuged for 15 minutes at 6,000 g at 4°C. The cell pellet was re-suspended in 10 mL of pre-cold buffer P1 (50 mM Tris-HCL pH 8.0, 10mM EDTA, 100 μg/μL Rnase A). 10 mL buffer P2 (200 mM NaOH, 1% (w/v) SDS) was added to the cells, mixed well by inverting the tube few times and incubated for 10 minutes at room temperature. Buffer P3 (3.0 M potassium acetate pH 5.5) was added to neutralise the reaction. The solution was quickly applied to a QIAfilter cartridge and left for 10 minutes at room temperature. Meanwhile, a Qiagen tip 500 was equilibrated by the addition of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol). The lysed cells were added onto the equilibrated tip and allowed to pass through the filter. 60 mL of buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0,
15% (v/v) isopropanol) was used to wash the column. 15 mL of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol) was added to elute the DNA which was finally precipitated by adding 10.5 mL isopropanol followed by centrifugation at 12,000 g for 30 minutes at 4°C. 5 mL of room temperature 70% (volume ratio) ethanol was added to the pelleted DNA and centrifuged for 15 minutes at 12,000 g at 4°C. The supernatant was carefully discarded and the pellet was air-dried before re-suspension in 0.5-1 mL of DNase, RNase-free H2O.

### 2.2.9.4 Quantification of DNA

DNA was quantified by checking the absorbance of a 1:40 dilution of the sample at 260 nm. 1 unit of absorbance at this wavelength was taken to correspond to 50 μg/mL of double stranded DNA. The final concentration of the sample could be determined using the formula:

\[
\text{Sample concentration} = \text{OD 260} \times \text{dilution factor (40)} \times 50 \mu\text{g/mL.}
\]

### 2.2.9.5 Digestion of DNA with restriction endonucleases

Depending on the manufacturer, 1-2 units of restriction endonuclease enzyme was added to 1 μg of DNA along with the specified buffer for the optimum activity of the enzyme in use, making the final volume of reaction to be 20 μL. The mixture was incubated at 37°C for a minimum of 2 hours.

### 2.2.9.6 Agarose gel electrophoresis of DNA

The digested DNA samples were diluted in 5 × DNA loading buffer. They were run on 1.0% (w/v) agarose gel prepared by dissolving 100g of agarose in 100mL 1 × TAE buffer 5ul of 2.5 mg/mL ethidium bromide and allowed to
set in a horizontal gel tank. Samples were loaded along with 10ul of 1kb DNA ladder and the gel was run at 100V for 1 hour. The DNA fragments were visualized under ultraviolet light. The size of each fragment was assessed by comparing with the molecular weight of different fragments of the DNA ladder.

2.2.9.7 DNA Purification from agarose gels
The desired DNA fragments were cut from the gel with a sterile disposable scalpel blade inside the UV transilluminator hood. Excised gel fragments were then transferred to a sterile tube and purified using the QIAQuick gel extraction kit according to the manufacturer’s instructions. The fragments were dissolved in buffer QG followed by the addition of isopropanol at 37°C. The solution was transferred onto a purification column and centrifuged. 20-30 μL of sterile water was used to elute the bound DNA from the purification column.

2.2.9.8 Ligation of DNA
The digested PCR or DNA fragments were ligated onto the digested vector DNA using a Rapid DNA ligation kit. A molar ratio of 1:3 to 1:8 (vector to PCR product/DNA fragment) was used in a reaction volume of 10 μL containing 1 unit of ligase and the supplied buffer. The mixture was incubated at room temperature for 1 hour or alternatively at 4°C overnight.

2.2.9.9 DNA sequencing
DNA sequencing analysis was done using the Sequencing Service at the School of Life Sciences, University of Dundee, Scotland.
2.2.10 Stable transfection of cell lines

About $0.5 \times 10^6$ cells/well were cultured in 6 well-plates with 2 mL medium and incubated overnight. In a tube containing $250 \mu$L of OPTI-MEM, 10ul of lipofectamine 2000 was added and incubated for 5mins at RT. To another tube containing $250 \mu$L of OPTI-MEM, 10 μg of plasmid DNA was added. After incubation both the solutions were mixed together and incubated for another 20mins at RT. The mix was added to cells in corresponding wells and incubated at 37°C for 24 hours and then sub-cultured in cell culture dishes with fresh DMEM medium containing 250 μg/mL G418 or/and 50 μg/mL hygromycin as selection agent. The cells were allowed to grow for 10-15 days for selection of positive clones. They were routinely checked and fed with fresh selection medium every two days. When the positive cells form colonies of more than 50 cells, the colonies were picked up by trypsinizing using colony cloning cylinders and the single colony was transferred to 24 well plates. Once the cells establish themselves in 24-well plates they were transferred to 25cm² flasks containing a selective medium to enlarge the population and screened by western blot for expression of target gene product. In total about 50-72 clones were picked up for every transfection and the two clones confirmed with best expression was chosen for further experiments.
2.2.11 Migration assay

Scratch or wound healing assay is a popular cheap assay that can be used to study migration of cells. A confluent plate generated from initial seeding of 0.5x10^6 cells in 6 well plates of any type of attached cells NOR or HYP is “wounded” by scraping off a thin straight line area of cells using a 200 μL plastic pipette tip. Pictures of the scratch area were taken at 0hr, 24hrs and 48 hrs. Cell migration can be monitored microscopically, as cells travel from the undamaged regions into the scratched region. %migration can be calculated by measuring the decrease of the uncovered region at different time points until the “wound” is closed using the image J software. Cell migration can be observed as single cells or loosely connected population (mesenchymal cells) or as sheets of cells (epithelial cells).

Calculation using image J software:

The images were converted to same size and resolution on a single sheet using MS powerpoint and loaded as Jpeg file. The pixels of the uncovered regions were calculated in the 0hr picture drawing an outline manually around the scratch seen in the picture and a numerical value is obtained (A). The pixels for 24hr picture is also calculated by manually drawing free form lines around the uncovered regions and a sum of all the numerical values for uncovered region is taken. The value of uncovered region at 24hr (B) is subtracted from the 0hr value (A) to give the value of region that has been covered by migration of cells (C). The percentage migration can be then calculated using the formula below
A=Total cell free region at 0hr  
B=Total cell free region at 24hr  
Total migrated region C = A - B  
Percentage Migration = C / A x 100%

2.2.12 Invasion assay

300 ul of warm, serum-free media was added to the cell culture inserts and incubated at room temperature for 1 hour in order to rehydrate the basement membrane layer of the insert. The media was carefully removed after the incubation period. 300 µL of cell suspension with 0.5-1x10^6 cells/mL in serum-free media was added to the inside of each insert. 500 µL of media with 10% fetal bovine serum was added to the lower well of the plate. After incubation for 48 hours, media was carefully aspirated from the insert. Using wet cotton-tipped swabs, cells were removed from the interior of the insert. The insert was then dipped in a well containing 400 µL of cell stain solution and incubated for 10 minutes at room temperature. After several times of gentle washing in a beaker of water, the inserts were allowed to air dry. The inserts were then transferred to a well with 200 µL of Extraction Solution and incubated for 10 minutes in an orbital shaker. 100 µL from each sample was transferred to a 96-well plate and OD was measured at 560nm. The OD values are presented as invasion index. The OD for control is taken as 100% and the relative increase or decrease in invasion of the sample is given by calculating the percentage difference in invasion.
2.2.13 Sphere-formation assay: 6-well plate format

Cells were collected from spheres or attached cells by trypsinization and were counted using a haemocytometer. For each group, 1000 cells were mixed in 2mL medium in a 15mL tube. Drug was added into each tube and cells were treated for the designed time period. Cells were centrifuged and medium discarded. Cells were then washed with 1xPBS once and resuspended into 6mL of fresh medium. Cells were seeded into HEMA-coated and 2-times water rinsed 6-well plate (2mL/well). Cells were cultured for 7 days and the total number of spheres in all wells was counted.

2.3 Statistical Tests

Microsoft Excel 2010 data analysis tools were used to perform all statistical data analysis. The cut-off chosen for statistical significance between samples was p=0.05. Values less than 0.05 indicate that the difference between the samples is statistically significant. P-value <0.001 denotes that the difference between the samples is extremely significant, and p≥0.05 defines that the difference between the two samples is not statistically significant. The statistical test’s- t-test: paired two samples for means’ was used to determine the statistical significance between GBM cell lines that were tested under same conditions. The statistical test “Anova: single factor” was used to determine the statistical significance of the same GBM cell line that was tested under varying conditions.
Chapter 3

Characterisation of Spheroids from
GBM cell lines
3.1 Introduction

3.1.1 The GBM CSCs and Sphere cell culture

Tumours like GBM are complex tissues that comprise a heterogeneous collection of cells associated with the neoplastic compartment, vasculature, inflammation, stroma and ECM (Furnari et al., 2007). This heterogeneity can be explained by the CSC hypothesis which proposes that tumours contain a small subset of CSCs that are able to undergo asymmetric division that generates a cellular hierarchy representing the tumour bulk while maintaining the CSC population itself (Ward and Dirks 2007). CSCs have been isolated from a variety of cancers including many gliomas such as GBM (Li et al., 2007; Al-Hajj et al., 2003; Singh et al., 2004). Accumulated evidence suggests that these GBM CSCs play important roles in GBM initiation, angiogenesis, maintenance, invasion, aggression and resistance to chemo and radiation therapy. Targeting these GBM CSCs in addition to treatments that eliminate the tumour bulk will be a better strategy to improve the survival rates in GBM patients. GBM CSCs have been mentioned in numerous studies with different terminologies like glioma stem cells, glioma initiating cells, glioma propagating cells, stem-like cells, resistant GBM subpopulation, multipotent glioma cells, transdifferentiated cells, etc. The terminology used raises confusion regarding the cell of origin and their specific function (Eyler and Rich, 2008; Bao et al., 2006; Liu et al., 2006).
In order to study and understand the biology and role of CSCs in GBM, researchers needed a system, where they can be propagated. The earliest known system was developed by Reynolds and Weiss in 1992 to isolate neural stem cells from mouse that were able to grow as neurospheres in a serum-free culture medium supplemented with growth factors like EGF and bFGF. When the neurospheres were dissociated and re-plated as single cells they developed new neurospheres. It was observed that most differentiating and differentiated cells died under these serum-free conditions, whereas the neural stem cells were enriched by the growth factors (Vescovi et al., 2006). When the CSC theories stepped in to GBM research, methods to culture and enlarge the stem cell-like population was adopted from the methods that were originally used to culture normal neural stem cells. This is because the discovery of neural stem cells has influenced the thought among scientists that brain tumours originate from transformed neural stem or progenitor cells (Reynolds and Weiss, 1992; Singh et al., 2004). Applying the same conditions as that of NSC culture, a population of cells that formed tumour spheres were isolated from human GBM cells. These cells are capable of differentiation into neural and glial lineages in addition to self-renewal capacities (Galli et al., 2004; Ignatova et al., 2002; Lee et al., 2006; Singh et al., 2004). Furthermore, the cells from the tumour spheres faithfully reproduced the primary tumour when injected into the brains of immunodeficient mice which imply the stem-like and tumour initiating abilities of these cells. Similar kinds of sphere culture models were established in a variety of cancers such as leukemia, breast cancer, colon cancer, prostate cancer, ovarian cancer and pancreatic cancer (Bonnet and Dick, 1997; Al-Hajj et al., 2003; Tang et al., 2009).
Therefore, when culturing putative tumour stem cells from GBM, the clonogenic spheroid models known as “Neurosphere assay” are often used for preserving tumour stem-like cells in serum-free medium (Lee et al., 2006). The GBM cells are grown in suspension in a culturing medium that has to be serum-free. It is known from normal NSC culture that serum causes irreversible differentiation of neural stem cells (Gage et al., 1995). This serum-free medium should contain neurobasal medium with added EGF and bFGF which are growth factors that induces proliferation and selection of multipotent, self-renewing, expandable stem-like cells from tumours (Galli et al., 2004; Ignatova et al., 2002; Lee et al., 2006). In the study by Lee et al., (Lee et al., 2006) it was observed that dissociated GBM cells from short term cultures grown as suspension in NS medium, successfully selected CSCs and formed neurospheres expressing stem cell markers. But when they cultured those selected cells in serum containing medium, they noted irreversible differentiation of the CSCs into neural and glial cell lineages which is very similar to the irreversible differentiation observed in NSCs under the serum conditions (Gage et al., 1995). Thus the sphere culture system employed to grow NSCs and CSCs attempts to avoid differentiation by reducing the secretion of differentiation factors and evading adherence mediated stimulation of differentiation. NS cultures are excellent models to study CSCs since they enrich the population with pathological signature and retain the genetic profile of actual tumour (Miki and Rhim, 2007).
3.1.2 Identification of GBM CSCs from culture

Although self-renewal, differentiation, and tumorigenicity of a population is used as a signature identity for CSCs they are usually identified precisely by their cell surface markers. With the discovery of fluorescence-activated cell sorting and magnetic bead isolation of cells based on surface markers a lot of research has focussed on identification and validation of CSC markers in many types of tumours. Nowadays a wide range of stem cell markers are used to identify CSCs and they differ based on tumour types. CD133, CD44, ABC transporter, CD90, etc are commonly used for many tumours (Zhu et al., 2009; Hwang-Verslues et al., 2009; Ho et al., 2007). For example CD44hi/CD24low pattern and ESA are used in breast cancer, while MDR1, CD166 and CD44 are used for prostate cancer CSCs (Fillmore and Kuperwasser, 2008). Currently leukaemias are the only malignancies known to have the universally accepted panel of CSC markers (Gilbert and Ross, 2009).

Several markers are hypothesised as unique for GBM CSCs /Glioma stem cells which are mostly based on NSCs. Nestin expressed in NSCs was thought to be a valuable marker but is a cytoplasmic protein, which makes it difficult to use for sorting the CSC population (Lendahl et al., 1990; Ehrmann et al., 2005). Although Nestin, CD133, CD90, ALDH, Notch, Sox2, Bmi1, Musashi1, A2B5, Oct4 and Nanog are used for identification of GBM CSCs, currently there is no universally accepted panel of CSC markers for isolation and purification of a GBM CSC population (Strojnik et al., 2007; Uchida et al., 2000; Ying et al., 2011; Singh et al., 2004). In this study we used ALDH, CD133, Sox2, Oct4 and
Nanog as markers of pluripotency and stem cell characteristics shown by cells cultured as spheres in comparison to adhered GBM cell lines.

3.1.2.1 **CD133 (Prominin-1)**

CD133 is a glycosylated transmembrane protein, with five transmembrane (TM) domains and 2 extended extracellular loops expressed in a variety of non-pathogenic tissue including brain with unknown function. CD133 was initially used as a marker for haematopoietic stem cells and later identified as a surface marker for NSCs (Miraglia et al., 1997; Uchida et al., 2000). Subsequently CD133 was applied to GBM CSCs to isolate populations with enhanced stem cell characteristics from different CNS tumours (Singh et al., 2003; Singh et al., 2004). CD133+ cells from GBMs have high capacity to undergo multilineage differentiation, form neurospheres in culture, and express high levels of NSC genes similar to signalling pathways in normal NSCs (Liu et al., 2006). CD133+ cells show elevated expression of embryonic genes nestin, Msi-1, Notch, Oct4, Sox2 and have the ability to reproduce a tumour that resembles the patient’s tumour in animal models (Ma et al., 2008; Miki et al., 2007). These tumours initiated by CD133+ GBM can be serially transplanted into NOD-SCID mice and faithfully reproduce the tumour. It was noted that, injecting as less as 100 CD133+ cells resulted in tumour formation while over 100,000 CD133− injected cells did not produce any tumours (Singh et al., 2004). Recent reports reveal that CD133− cells still exhibit CSC characteristics like self-renewal, multilineage differentiation, and tumour formation in xenograft models (Beier et al., 2007; Jaksch et al., 2008). Moreover it is now known that CD133 was not expressed
in about 40% of freshly isolated GBM tumours (Beier et al., 2007) which underlines the limitation of using CD133 for selecting GBM CSCs (Son et al., 2009). Although CD133 is still used as a means of isolating cell population with enhanced stem cell properties, it is not considered to be a universal GBM CSC marker.

### 3.1.2.2 ALDH Activity as a Universal CSC Marker

In addition to using cell surface markers for CSC identification, certain functional characteristics of stem cells were used for CSC detection and isolation. In 2007, groundbreaking studies by Cheung et al., (2007) and Ginestier et al. (2007) showed that increased aldehyde dehydrogenase (ALDH) activity measured by the ALDEFLUOR assay, originally used for the isolation of hematopoietic stem cells, can be used for the isolation of CSCs from leukaemia and breast cancer. They demonstrated the potential applicability of quantifying ALDH activity using ALDEFLUOR in solid tumours. Cells isolated based on increased ALDH activity had tumourigenic and self-renewal properties of CSCs (Ginestier et al., 2007).

In the following years, reports from various researchers established the successful use of ALDH activity as a CSC marker in many cancers like lung, liver, brain, colon, cervical, pancreatic, skin, prostate, head and neck, bone, bladder and thyroid cancers and it was suggested that ALDH activity can be used as a universal CSC marker (Boonyaratankornkit et al., 2010; Basak et al., 2009; Carpentino et al., 2009; Chu et al., 2009; Clay et al., 2010; Deng et
al., 2010; Li et al., 2009; Ma et al., 2008; Wang et al., 2010; Su et al., 2010; Todaro et al., 2010; van den Hoogen et al., 2010). In addition ALDH is considered to play an important role in chemoresistance of CSCs by detoxifying drugs and maintaining quiescence and low ROS levels that minimizes therapy induced ROS-DNA damage. However the ALDH activity measured by ALDEFLUOR in various tissues may differ and identification of specific ALDH isoforms from a family of 19 enzymes is difficult to correlate with the increased activity found in various solid tumours (Black and Vasiliou, 2009).

3.1.2.3 SOX2

Sox [SRY (sex determining region Y)-box] 2 belongs to the Sox family of high mobility group domain-containing transcription factors that are expressed in many tissues during development. Sox-encoding genes are highly expressed in the developing nervous system especially Sox2 which is expressed in undifferentiated neural precursor cells functions as a specific marker for neural development (Pevny and Nicolis, 2010). Sox2 is highly expressed in embryonic and adult neural stem cells present in the subventricular zone or in the subgranular layer and its expression persists until the cells differentiate into mature neural or glial cells. These stem cells with high expression of Sox2 are characterized by self-renewal and multipotency along with the expression of the neural stem cell marker nestin and glial marker GFAP (Tanaka et al., 2004). In addition to stem cells in the nervous system, Sox2 is also a bona fide marker of stem and progenitor cells of the stomach, breast, cervix, liver, bone marrow, testes, and several glands (Arnold et al., 2011). Sox2 is also well-known for its
role as an important transcription factor unique to embryonic stem cells and foetal progenitor cells found in the developing embryo. Several animal model studies with forced expression of Sox2 reveal that Sox2 is very important for preventing neuronal development and maintenance of stem cell state (Favaro et al., 2009; Ferri, 2004).

3.1.2.4 OCT-4

Oct4 (Oct3/4 or POU5F1) belongs to the POU family of transcription factors and plays a crucial role in the maintenance of self-renewal and pluripotency in ESCs. Oct4 is commonly found in unfertilized oocytes, blastocysts, germ cells, embryonic carcinoma cells and embryonic germ cells (Nichols et al., 1998). Studies have demonstrated that high expression of Oct4 sustains a pluripotent undifferentiated stem cell state and a further two-fold increase in Oct4 will push the cells to very primitive endoderm and mesoderm-like cells. On the other hand even a 50% loss of Oct4 induces stem cells to undergo differentiation, producing a heterogenous population (Nichols et al., 1998; Niwa et al., 2000). This suggests that Oct4 is regulated at a precise level to maintain lineage-specific ESC differentiation. Moreover Oct4 is an important transcription factor involved in the recent research area of reprogramming differentiated somatic fibroblasts into embryonic pluripotent cells known as inducible pluripotent stem cells (iPSC). Oct4 is the only transcription factor shown to be required exogenously among the several combinations of transcription factors needed to make iPSCs. This indicates that Oct4 may act as a ‘gatekeeper’ of pluripotency in differentiated cells (Stefanovic and Pucéat, 2007). Expression of Oct4 is seen
in number of cancers and its expression has been correlated with aggressive
tumour grade and decreased survival rates (He, 2012; Chen et al., 2012). It has
been demonstrated that ectopic expression of Oct4 in normal breast cells leads
to the generation of cells showing features of CSCs with tumour-initiating and
colonization abilities in mouse models of breast cancer (Beltran et al., 2011).
Presence of Oct4 in CD133- lung cancer cells attributes them to CSC properties
and increases the sub population of CD133+ cells (Chen et al., 2008). In
addition to this Oct4 is also shown to be expressed in acquired EMT
phenotypes which enhanced tumourigenicity and drug resistance of prostate
cancer (Kong et al., 2010).

3.1.2.5 Nanog

Nanog is a homeodomain-containing transcription factor that plays a major role
in the maintenance of pluripotency and self-renewal in embryonic stem (ES)
cells (Chambers et al., 2003) (Pan and Thomson, 2007). Along with Oct4 and
Sox2, Nanog is also one of the key transcription factors that have the ability to
reprogram human somatic fibroblast cells into pluripotent embryonic stem cell-
like cells known as iPSCs (Park et al., 2007). The expression of Nanog is
regulated at transcriptional level by Oct4/Sox2 heterodimers (Kuroda et al.,
2005). Although the Oct4/Sox2 heterodimers are positioned at a higher level in
the regulatory network of ES cell hierarchy, Nanog possesses unique properties
that regulate key stemness-associated transcriptional factors and hence is
considered to be indispensable to maintain ES cell identity and biology
(Chambers et al., 2003). Higher Nanog expression is observed in cancer stem
cells than in non-stem cancer population of several germ cell tumours and other solid tumours, including breast, lung, brain, cervix, prostate, kidney, colon, oral and ovarian cancers (Lee et al., 2011; Ibrahim et al., 2012; Bussolati et al., 2008; Niu, 2011). Several correlation studies indicate that Nanog-positive cancer stem cells also express high levels of the CSC surface marker and represent only <2% of the total cancer cell population (Ibrahim et al., 2012). For example, a high level of Nanog expression is observed in CD133+ or CD44+ cancer cells than CD133− or CD44− ones (Xu et al., 2012). It was also shown in prostate cancer cells that increased Nanog results in upregulation of CD133 and ALDH1. In addition, many functional studies through ectopic overexpression of Nanog have demonstrated that Nanog is not only a CSC marker, but also functions to drive a subpopulation of cells to adopt a stem like phenotype with clonal growth and tumour regenerative capacity, thereby promoting CSC-like characteristics in several cancers (Jeter et al., 2011; Ibrahim et al., 2012).

### 3.1.3 CSCs from cell lines

In addition to the isolation of CSCs from tumour tissues and primary cultures, it is also now evident that CSCs can be isolated from established tumour cell lines. Generally, the cells growing as cell lines containing vigorously dividing tumour cells are considered to be terminally differentiated cells. Additionally these cell lines were cultured in serum containing medium as monolayers for a very long time (haematopoietic malignancies are exceptions). These cell lines are used in various studies for decades and they have undergone various
genetic, epigenetic and morphological changes in order to adapt to the in vitro culture conditions. Li et al., (2008) reported that although established gliomas cell lines conserved some features of primary tumour, they had significantly higher number of recurrent aberrations which are not found in primary tumours. Therefore cultured cell lines are very unlikely to contain CSC population. Recent validated studies report the isolation of CSCs from established tumour cell lines of breast cancer (Fillmore and Kuperwasser, 2008), prostate cancer (Luk et al., 2011), ovarian cancer (Shi et al., 2010), melanoma (Chandrasekaran and DeLouise, 2011), colon cancer (Fan et al., 2011), brain tumour (Qiang et al., 2009) and the list seems to be expanding further. All the above studies used sphere cell culture and specialised stem cell selection media with growth factors to enrich the CSC phenotypes growing as spheres. However, the differentiation potential of the CSCs derived from cell lines is not well established. Although it is unclear how CSCs can be extracted from cell lines, the presence of CSCs or stem like cells in these sphere cultures is unquestionable. This is based on the fact that cells from these sphere cultures also express very high levels of CSC markers equivalent to the spheres isolated from primary tumours or short term cultures. In addition they are also shown to express various proteins and transcription factors involved in embryonic signalling and stem cell survival pathways. These cell lines based on CSCs are very much comparable to CSCs from primary sources since they also show high tumourigenic capacity in animal models (Fehlauer et al., 2006).
3.1.4 Rationale and aims of this study

The above findings stating the isolation of CSCs from cell lines have led to interesting questions and uncertain solutions. Can CSCs be re-induced from tumour cells cultured for a long-term under *in vitro* conditions? Does the sphere culture system serve as a selection media or induce CSCs from non-stem cells? Why CSCs are found in some cancer cell lines and not in others?

It is not known whether some residual CSCs exist in cell lines or the culture condition in selective medium enhanced the enrichment of CSC phenotypes. It is also not known whether growing these cells as spheres in suspension results in cellular aggregation and subsequent activation of dedifferentiation pathways to convert differentiated cells back to stem-like phenotype. Recent evidence suggest that overcrowding in solid tumours caused by bulk tumour cells and resultant hypoxic and microenvironmental conditions leads to EMT pathways and induces stem cell phenotypes (Polyak and Weinberg, 2009). Some studies also mentioned that sphere culture conditions mimic the tumour microenvironment by inducing mesenchymal cells under low oxygen conditions (due to hypoxic core in sphere cell aggregates) and mediate CSCs and resistance phenotypes in tumour models (Bjerkvig *et al*., 1990). There are various correlations between hypoxia induced stem cells and chemoresistance nature of the tumours that leads to failure of therapy and relapse. If EMT is the answer for CSC phenotypes in cell lines, then we have to know the true role of the selection medium in CSC sphere culture.
The main goal of this study is to understand whether the sphere culture can mimic the actual CSCs found in GBM which are known to be the utmost contributors of therapy resistance in GBM. To address this, we cultured the established glioblastoma cell lines as spheres in two different culture conditions. On one hand the cells were grown as neurospheres with neural CSC selection medium without serum, containing all the growth factors. On the other hand cells were grown as suspension in normal complete medium as used for monolayer cultures with serum. We examined the expression of generally accepted CSC markers in these sphere cells and compared their chemoresistance ability to that of adherent monolayer cultures by cytotoxicity assays using conventional anticancer drugs used for GBM therapy.

### 3.2 Experimental design

Detailed information on materials, products, manufacturers and methodologies used for the entire study has been described in chapter 2. The following are specific experimental designs and methods used for this part of the study.

#### 3.2.1 In vitro neurosphere and suspension sphere culture

Three established GBM monolayer cell lines U87 MG, U251MG and U373MG were used in this study. Routine subculture methods were followed for the maintenance of the attached monolayer cultures. Two individual groups of three GBM cell lines U87 MG, U251MG and U373MG were cultured in ultra low adherence poly-HEMA coated T75 flasks at a cell density of 10,000 cells/mL. One group was grown in conventional stem cell enrichment medium without and
with growth factors. The other group was cultured in normal DMEM with 10% serum. The cells were incubated at 37°C for 7 days with necessary media replacement every 3 days. The cells were allowed to form spheres and subjected to further experiments after trypsinisation and dissociation of spheres. The cells grown as monolayer are labelled as attached (ATT), spheres grown suspended in selective medium are labelled neurosphere cultures (NS) and spheres grown suspended in serum containing medium are labelled as suspension cultures (SUS). The sphere cells were photographed and can be trypsinised and reseeded for further passage or collected at the end of 7 days. Since they are derived from cell lines the sphere cells can be generated from adherent cells at any time.

### 3.2.2 Flow cytometric analysis of CSC markers

Cells from all three types of culture conditions ATT, NS and SUS from all three cell lines were trypsinised and dissociated into single cells. The cells were counted and 2.5x10^5 cells were analysed with corresponding CSC markers. ALDH activity was measured by staining with ALDH substrate and assay buffer for 30 mins at 37°C and analysed according to the instructions provided with the ALDEFLUOR kit. For detection of CD133 cell surface marker the cells were incubated with anti-CD133-PE conjugated antibody for 15-20 mins and then analysed. For intracellular embryonic stem cell marker proteins like Sox2, Oct4 and Nanog about 1x10^6 cells were fixed with 100% ethanol and blocked using FACS blocking buffer that includes TritonX100 to make the cells permeable to the antibody. The cells were then stained with the corresponding primary
antibody diluted (1 in 1000) in blocking buffer and left overnight. The primary antibody was washed and the cells were incubated with appropriate FITC tagged secondary antibody (1 in 5000) diluted in blocking buffer. All cells were washed with PBS after incubation and re-suspended in corresponding buffers and examined using BD FACS Calibur and Cell Quest Pro™ Software (BD bioscience). An increase in the expression of CSC markers is quantified by measuring increase in fluorescence.

3.2.3 MTT cytotoxicity assay for GBM monolayer cells

Three GBM cells lines U87 MG, U251MG and U373MG were cultured overnight in flat bottomed 96 well plates at a cell density of 5000 cells/well in triplicates. These ATT cells were then exposed to first line GBM drug TMZ and other conventional anticancer drugs VCR, PAC and DOX in the following concentrations (conc). TMZ (highest conc- 500μM serially diluted down to 3.9μM); VCR (highest conc- 50nM serially diluted down to 0.39nM); PAC (highest conc- 50nM serially diluted down to 0.39nM); DOX (highest conc- 250nM serially diluted down to 1.9nM). The cells were treated for 120 hours and subjected to MTT analysis. The % cell viability at different concentrations was calculated and the corresponding IC_{50} values were calculated for each drug.

3.2.4 MTT cytotoxicity assay for Sphere cells derived from GBM cell lines

The sphere cells NS and SUS from all three GBM cell lines used were trypsinised and dissociated into individual cells. They were then reseeded in
poly HEMA-coated 96-well flat bottom plates in their corresponding culture medium at a cell density of 5000 cells/well and cultured overnight. Since the cells were suspended they were grown overnight at a volume of 100 μL/ well. They were then dosed with drug containing medium on top with another 100 μL with appropriate drugs under study as follows: TMZ (highest conc- 500μM serially diluted down to 3.9μM); VCR (highest conc- 50nM serially diluted down to 0.39nM); PAC (highest conc- 50nM serially diluted down to 0.39nM); DOX (highest conc-250nM serially diluted down to 1.9nM). The cells were treated for 120 hours and MTT reagent was added to the wells. After incubation with MTT reagent, the plates were spun down at 800 rpm for 5 mins in a plate centrifuge and media is discarded. The spheres which appear like purple crystals were dissolved by adding DMSO and the OD was measured using a plate reader. The % viability and corresponding IC₅₀ values for each drug were calculated.

### 3.2.5 Western blot analysis of stem cell proteins

ATT, NS and SUS cells from all three cell lines were trypsinised and collected as pellets. The whole cell protein in the cells were extracted with RIPA buffer, quantified and separated by SDS PAGE. The expression levels of embryonic CSC markers like Oct4, Sox2 and Nanog were analysed using appropriate primary and secondary antibodies as mentioned in the western blot section of chapter 2.
3.3 Results

3.3.1 High levels of CSC markers are observed in both NS and SUS cells

When compared to the ATT monolayer culture, both the NS cells and SUS cells isolated from all three GBM cell lines used in this study showed remarkable increase in all the CSC markers tested. Figure 3.1 shows the general morphology of ATT, NS and SUS cells from U87 MG, U251MG and U373MG. It can be observed that SUS grown with serum formed bigger and tighter spheres than the cells grown in NS medium. This may be because, in the presence of serum the cells dividing outside formed a healthy tighter shell outlining the cells inside similar to the tumour microenvironment. The results of FACS analysis are shown in Figure 3.2. The measurement of ALDH activity by ALDEFLUOR assay showed statistically significant increase in ALDH activity in the NS and SUS population of all three cell lines (Figure 3.2). Similarly expression of the cell surface marker CD133 increased significantly in NS and SUS population as shown in Figure 3.3. Although the function and reliability of CD133 marker is still uncertain, it is arguable that most neural progenitor cells have characteristic expression of CD133 on their surface. Both ALDH and CD133 in sphere cells indicates that the CSC phenotypes present in the NS and SUS population is similar to neural progenitor cells. Embryonic stem cell proteins such as Sox2, Oct4 and Nanog are important transcriptional factors that play a major role in regulation of stemness, pluripotency and stem cell renewal pathways. The expression of these proteins is usually restricted to embryonic stem cells and other progenitor cells as they are known to be switched off after differentiation.
Our results clearly indicated increased expressions of Sox2, Oct4 and Nanog in NS and SUS cells in comparison to ATT cells as shown in Figure 3.4, 3.5 and 3.6 respectively. Furthermore Western blot analysis confirmed the presence of high levels of these proteins in NS and SUS cells derived from all three GBM cell lines (Figure 3.7). The \textit{in vitro} culture of sphere cells using expensive selective medium is basically adopted from normal neural stem cell culture. The cells grown in this system known as Neurospheres (NS) were thought to be CSCs selected by the special medium. But our results showed that the cells grown with serum in normal culture medium also can select or support the growth of GBM CSC like cells. We did not observe any significant difference in the CSC characteristics between the NS and SUS cultures, thus questioning the use of expensive media for growing CSCs.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{morphology}
\caption{Morphology of GBM spheres.} All three GBM cell lines grown as ATT, NS and SUS cultures. Cells grown as suspensions in low attachment conditions using both selective NS medium and normal DMEM with serum formed spheroids. Images taken after 7 days of culture (x40 magnification).\end{figure}
Fig 3.2 High ALDH activity detected in NS and SUS sphere cells. ALDH activity measured by ALDEFLUOR assay using FACS analysis. The bar chart displays the percentage of ALDH cell populations. n= 9, **p<0.01
Fig 3.3 High CD133 expression detected in NS and SUS sphere cells. CD133 immunostaining using PE conjugated anti-CD133 measured by FACS analysis. The bar chart displays the percentage of CD133 cell populations. n= 9, **p<0.01
Fig 3.4 NS and SUS sphere cells express high levels of embryonic CSC marker SOX2. Expression of SOX2 determined by immunostaining using SOX2 antibody and FITC conjugated secondary antibody and measured by FACS analysis. The bar chart displays the percentage of SOX2 expressing cell populations. n= 9, *p<0.05; **p<0.01
Fig 3.5 NS and SUS sphere cells express high levels of embryonic CSC marker OCT4. Expression of OCT4 determined by immunostaining using OCT4 antibody and FITC conjugated secondary antibody and measured by FACS analysis. The bar chart displays the percentage of OCT4 expressing cell populations. n= 9, **p<0.01
Fig 3.6 NS and SUS sphere cells express high levels of embryonic CSC marker NANOG. Expression of NANOG determined by immunostaining using NANOG antibody and FITC conjugated secondary antibody and measured by FACS analysis. The bar chart displays the percentage of NANOG expressing cell populations. n= 9, **p<0.01
3.3.2 The NS and SUS cultured cells are highly resistant to anticancer drugs

Although the NS and SUS cells displayed several CSC characteristics, we were not certain whether these cells play a role in chemoresistance of GBM like the CSCs present in vivo which are well known for their intrinsic chemoresistance. GBM CSCs evade the damage caused by chemotherapeutic agents through various mechanisms like drug efflux, quiescence, anti-apoptosis and enhanced DNA repair and leads to recurrence and poor survival rates in patients. In this study we tested the first line drug TMZ and three other conventional anticancer drugs VCR, PAC and DOX on both monolayer ATT and sphere cells NS and SUS population. We hypothesized that if the cells that display GBM CSC characteristics are resistant to multiple drugs, we can use the CSCs isolated

Fig 3.7 Expression of embryonic stem cell markers in NS and SUS cells. Western blot analysis of embryonic stem cell proteins Sox2, Oct4 and Nanog in ATT, NS and SUS cells from all three GBM cells. Tubulin was used as a loading control.
from established cell lines to study chemoresistance mechanisms and develop
targets. Our MTT cytotoxicity results show that both NS and SUS cultures are
extremely resistant to first line drug TMZ even at very high concentrations used
in this study (500 μM) while the non stem ATT cells are comfortably killed by
TMZ. Similar results were observed on treatment with other drugs like VCR,
PAC and DOX where the ATT cells were killed at low concentrations and NS
and SUS survived at high concentrations. Figure 3.8 A,B and C shows the
morphology of cells ATT, NS and SUS from all three cell lines respectively after
drug exposure. Figure 3.9 A, B and C which displays the results of U87, U251
and U373 cell lines respectively shows the cell viability curves of ATT, NS and
SUS for different drugs in comparison to each other. There is a clear difference
between the curves of ATT and NS or SUS which explains the low IC50 values
for ATT cells and increased IC50 values of NS and SUS cells mentioned in
Table 3.1

<table>
<thead>
<tr>
<th>3.8 A</th>
<th>-VE</th>
<th>Vincristine 50nM</th>
<th>Paclitaxel 50nM</th>
<th>Doxorubicin 50nM</th>
<th>Temozolomide 500μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 ATT</td>
<td></td>
<td></td>
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<tr>
<td>U251 ATT</td>
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<tr>
<td>U373 ATT</td>
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</tbody>
</table>
**Fig 3.8** Morphology of ATT, NS and SUS cells after treatment with conventional anticancer drugs. (A) Attached monolayer cultures are effectively killed by all drugs whereas (B) NS and (C) SUS cultures survived even under very high concentrations of drugs. Cells were exposed to drugs for 120 hours and images were taken at 40X magnification.
Fig 3.9  In Vitro Cytotoxicity of conventional anticancer drugs on U87 ATT, NS and SUS cells. The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay. The cell viability curves clearly show a significant difference in the dose response between ATT and Sphere cells.
Fig 3.10 *In Vitro* cytotoxicity of conventional anticancer drugs on U251 ATT, NS and SUS cells. The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay. The cell viability curves clearly show a significant difference in the dose response between ATT and Sphere cells.
Fig 3.11 In Vitro cytotoxicity of conventional anticancer drugs on U373 ATT, NS and SUS cells. The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay. The cell viability curves clearly show a significant difference in the dose response between ATT and Sphere cells.
Table 3.1 IC50 values of conventional anticancer drugs on GBM cell lines.

<table>
<thead>
<tr>
<th></th>
<th>VCR (nM)</th>
<th>PAC (nM)</th>
<th>DOX (nM)</th>
<th>TMZ (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>U87 att</strong></td>
<td>2.50 (0.76)</td>
<td>3.02 (1.41)</td>
<td>36.83 (7.7)</td>
<td>15.69 (1.81)</td>
</tr>
<tr>
<td><strong>U87 NS</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>&gt;2000**</td>
<td>&gt;500**</td>
</tr>
<tr>
<td><strong>U87 Sus</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>892.3 (14.8)**</td>
<td>&gt;500**</td>
</tr>
<tr>
<td><strong>U251 att</strong></td>
<td>2.52 (0.74)</td>
<td>2.77 (1.27)</td>
<td>41.92 (7.99)</td>
<td>13.3 (0.64)</td>
</tr>
<tr>
<td><strong>U251 NS</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>&gt;2000**</td>
<td>&gt;500**</td>
</tr>
<tr>
<td><strong>U251 Sus</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>958.72 (32.4)**</td>
<td>&gt;500**</td>
</tr>
<tr>
<td><strong>U373 Att</strong></td>
<td>2.71 (0.43)</td>
<td>2.04 (0.2)</td>
<td>46.51 (6.26)</td>
<td>40.45 (11.21)</td>
</tr>
<tr>
<td><strong>U373 NS</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>&gt;2000**</td>
<td>&gt;500**</td>
</tr>
<tr>
<td><strong>U373 Sus</strong></td>
<td>&gt;50**</td>
<td>&gt;50**</td>
<td>&gt;2000**</td>
<td>&gt;500**</td>
</tr>
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The values in the table indicate the IC50s for different drugs on GBM cells. SD is given in parenthesis. n=9 **p<0.01.

3.4 Discussion

Stem cells possess two unique features: pluripotency and self-renewal, and it has been revealed that all tissues contain tissue-specific stem cells which continuously produce differentiated counterparts of the tissue to maintain its structure and function. The discovery of cancer stem cells that behaved in a similar manner to normal stem cells turned out to be a breakthrough in the area of cancer resistance and relapse (Dick et al., 2008). GBM was the first solid tumour from where self-renewing, multipotent, tumourigenic CSCs were isolated (Singh et al., 2003). It is clearly understood from various studies that GBM
CSCs are responsible for the invasive growth and GBM recurrence due to their chemoresistant nature. In an effort to study and target the therapeutic resistance of GBM CSCs the CSC culture system was used to isolate CSCs from patient tumour samples or short term cultures.

The GBM CSC culture system is adapted from the normal neural stem cell culture system. The growth factor-supplemented serum-free spheroid culture is widely accepted as a gold standard method to keep the CSCs in an undifferentiated state under *in vitro* conditions. It is also referred that serum free conditions will purge the differentiated progenies, and selectively enrich the CSC population (Lee *et al*., 2006). The major drawback of the serum-free enrichment system is that it is very expensive (approx. £150 for a 500mL bottle). Additionally this system does not exactly mimic the physiological conditions and there is no clear information on the origin/source of the CSCs growing in sphere culture. Another paradox for the NS system is the belief that CSCs once exposed to serum will differentiate irreversibly in to their progenies. So people supporting this selection hypothesis do not use established cell lines as a source for CSCs. They consider the cell lines to be fully differentiated cells grown for many years under serum containing culture conditions. On the contrary there are several reports on isolating CSCs from established cell lines. They show all CSC characteristics including initiation of tumour in animal models.

Our results also indicate that when the cells from established GBM cell lines such as U87, U251 and U373 are grown as NS in selective media they form
spheres and exhibit several CSC characteristics like CD133, ALDH, Sox2, Oct4, and Nanog. If this is the case it appears like most of the established cell lines have a residual population of CSCs that can be enriched and activated by this growth factor enriched medium and proper culture conditions. But on the other hand growing the cell lines as spheres in normal DMEM+10% serum conditions also induces better spheres and enhanced all the above mentioned CSC characteristics. Therefore from the results of our study and information from literature we can be definite that stemness in GBM CSCs is not determined by the culture medium. Also we can be sure that the serum free culture system is not mandatory for GBM CSC culture.

Therefore there must be another common factor in this culture method that induces and maintains the CSC phenotypes from established cell lines. The most important feature of this culture system is growing the cells in low adherence conditions that allow the cells to grow as spheres. It is well known that cells grown in suspension are more likely to aggregate with each other and form clumps. No matter how low the cell number is, unless they are grown as single cell suspension, the aggregation of the suspended cells is inevitable (Jensen et al., 2011). In our experiments we followed the standard NSC culture methods and therefore we used a cell density of 10,000 cells/mL. The cells aggregate together and form clumps which eventually become sphere-shaped in about 5-7 days after which the CSC populations become apparent. If the culture media does not induce GBM CSCs the other possible mechanism could be a stress-induced phenotype change that occurs in the spheres. If we have a closer look at the sphere cells they clearly have a hypoxic core and a necrotic
region surrounded by healthy cells. The cells in the centre of the sphere may have been subjected to severe hypoxia and nutrient deprivation that causes necrosis in the middle. This matches very closely with in vivo GBM tumour conditions where there is extensive hypoxia and necrosis due to lack of nutrients and oxygen in the centre of rapidly growing tumour mass (Jensen et al., 2011).

There are several recent evidence indicate that hypoxia can induce a pathway known as EMT which allows the fully differentiated cells to dedifferentiate to mesenchymal cells that shows CSC characteristics. If this is true, then it is very likely that all sphere cultures will eventually result in hypoxic core due to aggregation which will induce CSCs/mesenchymal cells through EMT program. This could be the possible reason why both serum and non serum GBM sphere cultures can show CSC phenotypes in them. Even if cells are grown as single cell suspension, the same scenario will apply to it after the cells divide and join together to form spheres. Under physiological conditions tumour cells under hypoxia reprogram to mesenchymal cells to facilitate migration, metastasis or to produce growth factors to initiate angiogenesis. Unlike the NSCs, the monolayer-cultured non-CSCs and sphere-cultured CSCs are interconvertible by oxygen concentrations. Hypoxia induces EMT and the cells can migrate out of the tumour and follow another program called MET where the mesenchymal cells become differentiated epithelial cells. This could be the reason why when grown as spheres, established cell lines get converted to mesenchymal phenotype and revert back to epithelial when they are adhered back to monolayer culture. We also had a question how some researchers isolated NS
CSCs from fresh samples or by growing them as single cell suspension. This is probably because they are enriching the mesenchymal or CSC phenotypes induced by physiological hypoxia in the primary tumour. A detailed analysis of how hypoxia can induce CSCs in GBM cell line derived spheres is given in chapter 4.

Despite optimal therapies GBM remains to be a fatal malignancy mainly due to their resistance towards conventional therapy and recurrence (Furnari et al., 2007). Studies from our group and many others have confirmed that GBM CSCs promote therapeutic resistance and relapse (Liu et al., 2012; Bao et al., 2006; Johannessen et al., 2009; Liu et al., 2006). In this study we also established that cell line-derived NS and SUS GBM CSCs show high level of chemoresistance compared to the ATT non-stem cell population. The exact mechanisms of GBM CSC chemoresistance are unknown, but probably due to a combination of slow cell cycle kinetics, resistance to oxidative damage, DNA repair mechanisms, avoiding apoptosis, hypoxia activated mechanisms and multidrug resistance proteins (Diehn et al., 2009; Bao et al., 2006; Liu et al., 2006). GBM CSCs probably remain in a quiescent state and may be more resistant to anticancer drugs that target rapidly dividing cells. It is found that cell cycle profiles of human breast CSCs had an extended G2 phase and hence more resistance to induction of apoptosis (Harper et al., 2010). The current standard chemotherapeutic agent known to improve survival in newly diagnosed GBM is TMZ, an oral methylating agent that induces DNA damage by methylated guanine adducts (Stupp et al., 2005). But invariable tumour recurrence after TMZ therapy point towards the presence of TMZ-resistant
subpopulation in GBM that express the repair enzyme MGMT which removes the DNA adduct. It is reported that MGMT is expressed in GBM sphere cultures isolated from patient samples so GBM CSCs also contribute to the chemoresistance to TMZ (Johannessen et al., 2009; Liu et al., 2006; Paranjpe et al., 2013). So we hypothesised that the expression of MGMT is elevated after growing the monolayer into spheres that results in resistance of NS and SUS to TMZ. But even after several experiments with positive controls we were not able to identify any MGMT expression in both NS and SUS GBM CSCs. This indicates that probably there are MGMT independent mechanisms present in GBM CSCs that renders the drug ineffective both in vitro and in vivo. This could be the possible reason why many MGMT negative GBM patients also develop resistance to TMZ and end up in tumour relapse.

GBM CSCs also express multidrug resistance proteins like P-gp, BCRP and ABCG2, whose drug efflux functions aid survival of GBM CSCs. When compared with autologous CD133− cells, GBM CSCs that are CD133+ are shown to express an array of anti-apoptotic proteins including FLIP, BCL-2 and BCL-XL that helps in survival after treatment (Liu et al., 2009). In addition to MGMT and other resistance proteins, GBM CSCs may have various other mechanisms like enhanced DNA repair, ALDH activity, oxidative stress balance, etc that can drive multidrug resistance to cytotoxics such as TMZ, BCNU, CCNU, carboplatin, Vincristine, Palitaxel, Docetaxel, Doxorubicin, VP16 and other drugs. Another mechanism of resistance in NS and SUS is thought to be hypoxia induced by cell aggregation and sphere formation. Hypoxia is widely acknowledged as a contributing factor in chemoresistance of solid tumours.
Some studies highlight that hypoxia induced EMT can drive chemoresistance (Harris, 2002). Other studies point out that the chemoresistant effects of CSCs are largely mediated by hypoxia inducible factors (HIFs) and other transcription factors highly expressed in GBM CSCs (Tanase et al., 2013). Our findings also indicate a means through which oxygen tension in NS and SUS culture systems could affect GBMs responsiveness to drugs. We have explained these facts in detail in the chapter 4.

### 3.5 Conclusions

To conclude, the CSC-like cells in GBM cell lines are probably induced by spheroid cultures. The culture medium or the source of initial GBM does not affect the formation of GBM CSCs in sphere cultures. The isolated GBM CSCs were resistant to chemotherapeutic drugs. It may be hypoxia that drives these CSC phenotypes by epithelial cell dedifferentiation. The NS and SUS cultures can mimic the physiological conditions in the tumour and possibly provide us with various insights on how hypoxia could drive CSCs. It appears like the GBM CSCs acquire resistance through a number of potential mechanisms that may vary between individuals and cancer types. A better understanding of these mechanisms will improve our existing knowledge about GBM CSCs and guide us towards future therapeutic targets.
Chapter 4

Hypoxia drives chemo-resistant CSCs in GBM spheres through EMT
4.1 Introduction

In the previous chapter (chapter 3) the existence of a subpopulation of cancer stem-like cells in spheroid-cultured (NS and SUS) GBM cell lines has been demonstrated. The most widely used method for identifying CSCs like surface markers and embryonic markers tested on these NS and SUS cells provide definitive evidences that they closely resemble normal neural stem cells and are capable of self-renewal. It is also apparent that they are resistant to various drugs and could be the possible sources of tumour relapse. However, the reliability of these markers and their relationship to the phenotype of CSCs from our study and many others is still questioned mainly because several reports show that these cells comprise as many as 25% of the total population disputing the idea that CSCs are very rare populations (Kelly et al., 2007; Quintana et al., 2008). On the other hand there are several questions to be answered about using serum containing medium and isolating CSCs from established cell lines (Lee et al., 2006). We compared the growth of spheres in media with serum and without serum and suggested that the sphere cells form as a result of cell aggregation rather than CSC selection by the medium. We hypothesised that due to aggregation there is a nutrient deficient hypoxic condition in the middle of the spheres that could possibly drive these epithelial cells to reprogram into CSC/stem like phenotype that bring about these CSC characteristics. We also proposed that an established process called “hypoxia induced EMT” could coordinate the entire reprogramming events occurring in the sphere cells. In this chapter I will discuss the possible mechanisms of how hypoxia can drive EMT and analyse the possibilities of these mechanisms in our NS and SUS CSCs from GBM cell lines.
4.1.1 Hypoxia

Cancer cells undergo numerous genetic and epigenetic changes that allow them to acquire various features that help them adapt to environmental changes. It is becoming increasingly clear that the interdependent interactions between the tumour and its microenvironment play a key role in the initiation and progression of tumours. One such aspect of the tumour milieu that modulates behaviour of tumour cells is oxygen. The progression of a tumour is normally maintained by enough supply of O$_2$ and nutrients through tumour associated vasculature. As most tumours proliferate rapidly and grow bigger in size, they undergo episodes of aberrant vasculature which then fail to meet the metabolic requirements of the growing tumour. Especially hypoxia, a condition of O$_2$ deprivation that compromises normal cellular function, is a common phenomenon experienced by many malignancies, particularly the fast growing tumours (Hulleman & Helin, 2005; Louis et al., 2007; Preusser et al., 2006).

Hypoxic tumours activate angiogenic pathways that results in improper neo-vascularisation which forms immature, leaky and dilated blood vessels with blind ends, intravascular shunts and excessive branches (Bar, 2010). Hence the growing tumour is subjected to repetitive cycles of hypoxic stress which push them to undergo epigenetic, metabolic, morphological and functional modifications and eventually change their phenotype. As a survival mechanism these altered cells escape and move away from the stressful environment through invasion, migration and metastasis. Thus hypoxia is a well known contributing factor for tumour invasion and metastasis, resistance to radiation and chemotherapy and often associated with poor survival rate in patients (Bar, 2010; Li et al., 2009; Mashiko et al., 2011).
4.1.2 Hypoxia Induced EMT

The poor patient outcome associated with hypoxia is probably due to the phenotypic change of cells induced by hypoxia in order to escape the hostile environment. Recent evidence suggest that hypoxia induced EMT could possibly be the mechanism through which the distressed differentiated cells in the tumour microenvironment can escape the tumour. They achieve this by switching themselves through the EMT program to an early stage mesenchymal phenotype which has more motility and multipotent properties. Following this change these cells can move out through leaky vasculature of tumours and spread locally or carried as circulating tumour cells to distant metastatic sites.

EMT is a complex cellular programme by which epithelial cells get rid of their differentiated features like cell–cell adhesion, apical–basal polarity and immotile nature, and alternatively acquire mesenchymal features like motility, invasiveness and increased resistance to apoptosis. EMT is characterized by various signature features like down-regulation of E-cadherin, relocation of zonula occludens-1 (ZO-1), down-regulation and nuclear translocation of β-catenin and up-regulation of mesenchymal markers such as vimentin, fibronectin and N-cadherin (Gregory et al., 2008; Thiery, 2002; Lee, 2006; Hugo et al., 2007). Although the idea of EMT was initially met with scepticism, increasing evidence has demonstrated its vital role in cancer progression and metastasis (Brabletz et al., 2001; Christiansen, 2006). Tumour cells with mesenchymal phenotype that escapes the primary tumour are believed to undergo reverse transition at the site of metastasis to regain the pathology of epithelial cells through another controlled process known as Mesenchymal-to-
Epithelial Transition (MET). MET is a crucial step by which metastatic tumour cells grow and establish at the secondary site. This return to an epithelial state involved the formation of cell–cell interactions between normal cells and the migrated cancer cells, allegedly mediated by E-cadherin bridges formed between them (Christiansen, 2006). It is still unknown whether any specific growth factors or cytokines present in the normal tissues are ever involved in actively inducing MET. Another possible way MET could happen is due to the absence of signals, cytokines and growth factors similar to that of primary tumours like those induced by hypoxia, tumour stromal cells and inflammation that helped them to maintain the EMT phenotype. Metastatic cancer cells may simply fall back to an epithelial state as a consequence of reversion in epigenetic transcriptional mechanism (Moustakas and Heldin, 2007; Fischer et al., 2006; Strutz et al., 2002). Under normal processes like embryogenesis, EMT is strictly controlled by time and space to ensure accurate homing and prevents abnormal development by reversion of cells to non-invasive phenotypes after migration to an intended location (Hay, 1995; Oft et al., 1998; Brabletz et al., 2001). But cancer cells undergoing EMT does not have intended target location and induce independent autocrine loops of growth signals to avoid apoptosis which leads to pathological conditions (Gotzmann, 2004). EMT is regulated by many transcriptional repressors like Snail, Slug, TWIST and the ZEB family which repress expression of E-cadherin and the key signalling pathways which induce EMT include the TGF-β, Wnt, and Notch signalling pathways that control the above transcription factors (Batlle et al., 2000; Yee et al., 2010). However the EMT and MET pathways in cancer biology is still not completely elucidated.
4.1.3 Hypoxia induced EMT in GBM

GBM is one such malignancy in which hypoxic regions, hypoxia-induced necrosis and neovascularization are very common and are thought to play an important role in pathological diagnosis. Indeed hypoxic necrotic foci associated with pseudopalsading cells is a WHO recommended classification feature for GBM. The physiological O$_2$ concentration in the brain ranges from 0.5% to 7% (Bar et al., 2010). Using the method of EF5 binding and Eppendorf needle electrodes, Evans and colleagues measured O$_2$ levels in gliomas. They noted that the hypoxia levels varied drastically between patients and they grouped them as mild hypoxia (0.5% to 2.5% O$_2$), moderate hypoxia (0.1% to 0.5% O$_2$) and severe hypoxia/anoxia (0.1% O$_2$ or less). They noted that the patients with moderate hypoxia had reduced survival while the patients with severe hypoxic gliomas had the worst outcome among all (Evans et al., 2008). Many other studies also showed that increased levels of hypoxic regions are associated with invasive, resistant phenotype of GBM that has very poor patient survival (Sathornsumetee et al., 2008; Vaupel and Mayer, 2007).

Although hypoxia or microenvironment induced EMT is considered as a major modulator of metastasis formation in many epithelial solid tumours, there are only very few reports on EMT process occurring in neuroepithelial environment or in CNS tumours. In spite of being extensively hypoxygenated tumours, there is no robust evidence for the existence of EMT process or its role in invasion or progression in the case of GBMs. Recent analysis of The Cancer Genome Atlas (TCGA) data by Cheng and colleagues, demonstrated that GBM has a strong
association between an EMT expression signature and the expression of the CSC markers and early recurrence following initial treatment (Cheng et al., 2012). The activity of EMT transcription factors like SNAI1, TWIST1, ZEB2 are reported to be significantly elevated in GBM tissue compared to normal brain and positively correlate with tumour grading. These TFs are thought to promote invasion and proliferation in GBM by modulating genes such as MMPs, HGF, FGF, Wnt and β-catenin (Yang et al., 2010; Mikheeva et al., 2010). These TFs are known to suppress the epithelial marker E-cadherin and in parallel increase the mesenchymal markers like N-cadherin, vimentin and cadherin-11. But E-cadherin expression is very rare in both normal and glioma tissues of the brain (Motta et al., 2008). On the other hand, N-cadherin and cadherin-11 are frequently expressed at a low level in normal brain tissue and their expression is elevated in malignant gliomas (Asano et al., 2004). The loss of E-cadherin and its correlation with glioma’s grade was reported in only two studies and therefore there is no definitive evidence for classical cadherin switch in malignant gliomas, except in some specific cases (Motta et al., 2008; Lewis-Tuffin et al., 2010). However, Lu and colleagues recently reported an alternative cadherin switch in GBMS, different from the classic E-cadherin-to-N-cadherin switch (Lu et al., 2012). They observed that another atypical member of the cadherin family called T-cadherin was downregulated when Snail and N-cadherin increased in GBM cells. T-cadherin expression is absent in most high grade gliomas including invasive GBMs and is also associated with a poorer prognosis (Andreeva and Kutuzov, 2010). Zeb-1 has been shown to suppress T-cadherin to aid the acquisition of EMT phenotype and it has been
demonstrated that ectopic overexpression of T-cadherin in GBM cells inhibits proliferation, migration and invasion (Huang et al., 2003).

It is becoming increasingly evident that EMT plays an important role in regulating GBM CSCs, GBM invasion and its intrinsic resistance but a large part of it remains unexplored due to complex feedback loop systems and missing links between them. It is likely that the elevation of mesenchymal signature genes in GBM could be due to an ongoing EMT process due to hypoxia or could be the reminiscent of an earlier mesenchymal shift during gliomagenesis. Due to the remarkable comprehensive analysis of TCGA data by Philips and colleagues, the WHO classification system has added the mesenchymal like GBM as another subtype of GBM owing to the solid evidences of EMT in GBM (Phillips et al., 2006; Louis et al., 2007). Given the established role in progression of other hypoxygenated solid tumours, signalling pathways like NF-kB, HIFs, notch, hedgehog-GLI, WNT/β-catenin, EGFR, RTKs and PI3K-AKT may play important roles in hypoxia induced EMT program of GBM. All these facts together indicate that GBM share several features with EMT but more research and perfect evidence are required to propose this as a therapeutic target in GBM.

4.1.4 Hypoxia induced EMT and CSCs

It is interesting to know that hypoxia induced EMT mesenchymal cells carry molecular characteristics similar to progenitor or stem cells and are thought to initiate or propagate tumour acting as seeds for relapse of tumour after therapy.
Multiple evidence from researchers working on various solid tumours argue that the CSC phenotypes inside a tumour are probably these EMT induced mesenchymal cells. EMT phenotypes are often confused with cancer initiating mutated progenitor cells and hence are referred to as tumour initiating cells or cancer stem like cells. Joo et al., (2008) showed that a mesenchymal subtype genetic signature was seen specifically in CD133-negative GBM CSCs when compared with CD133-positive GBM CSCs and they initiated tumours with an invasive growth. Similarly, Lottaz et al., (2010) demonstrated that GBM CSCs which are positive for the EMT marker CD44 carry a mesenchymal gene signature and produced enhanced invasive growth in vitro and in vivo compared with CD44 negative cells regardless of CD133 expression.

Many researchers still believe that a mutated progenitor cell could become a CSC and establish a tumour, while other believe that the EMT phenotypes are resistant stem like population or metastasis initiating cells induced by microenvironment and may not be the actual CSCs that initiated the tumours. Regardless of whether CSCs arise from progenitors or are induced by an EMT program, it is well established that hypoxia supports the survival and maintenance of these CSCs. A hypoxic niche is well known for its role in maintaining normal NSCs in the CNS. This raises the possibility that if CSCs are present in GBM, the intra-tumoural hypoxia provides the hypoxic niche for these cells along with cytokines and growth factors from the tumour stroma to maintain their undifferentiated state (Panchision, 2009).
4.1.5 Markers used in this study to identify EMT phenotypes

4.1.5.1 CD44:

CD44 is a principal transmembrane cell surface glycoprotein that acts as a receptor for hyaluronan (HA). CD44 plays a vital role in alteration and degradation of HA which leads to migration and invasion of cancer cells and consequently metastasis (Rodgers, 2006; Nagano and Saya, 2004). Under hypoxic conditions HA is degraded into smaller subunits by enzymes called hyaluronidases. HA fragments promote angiogenesis and also can bind with CD44. The binding of HA-CD44 activates the cytoplasmic tail of CD44 and interact with actin cytoskeleton through a group of linked proteins called ezrin-radixin-moesin (ERM) proteins. Interactions between CD44 and cytoskeletal components modulate the adhesion of cancer cells to each other as well as to endothelial cells and increase the migratory potential of the cells (Mori et al., 2008). CD44-HA binding is also known to activate EMT program through TGF-β pathway and by activation of Rho-GTPases and protein kinase C and subsequently various CSC genes like Nanog, OCT4, Wnt and β-catenin. CD44 also interacts with MMPs and increases the activity of MMP-dependant degradation of ECM thereby promoting invasion and metastasis (Chetty et al., 2012). The expression of CD44 has been associated with more metastatic and resistant phenotypes of hepatocellular carcinoma, breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer and ovarian cancer. The significance of CD44 in GBM CSCs has not been studied clearly but it is thought that EMT phenotypes expressing high levels of CD44 could be the
potential reason behind aggressive nature of GBM. Thus CD44 has been suggested as an important differentiator of epithelial versus mesenchymal characteristics (Mani et al., 2008).

4.1.5.2 Cadherin Switch

Cadherins are a superfamily of adhesion molecules that play vital roles in embryogenesis, tissue recognition and maintaining epithelial structure and tissue architecture. E-cadherin is a calcium-dependent cell adhesion molecule that mediates cell-cell adhesion, one of the hallmarks of epithelial cells. Another well-characterized cadherin is N-Cadherin which is expressed in many cells and predominantly in mesenchymal cells. The interaction between cadherins results in stable and strong adhesive forces between cells and is thought to be regulated by environmental signals, growth factors and cytokines. Accumulating evidence from many epithelial cancers suggests that multiple changes in cadherin expression occur during tumour growth that results in tumour progression. Particularly a phenomenon known as “cadherin switching” play a critical role during the process of EMT where E-cadherin is switched off and N-cadherin is switched on resulting in the conversion of nascent tumour cells from an epithelial to a mesenchymal phenotype. The loss of E-cadherin has been proved to be an important reason for the disruption of tight cell-cell contact during EMT leading to an invasive/metastatic state (Maeda, 2005; Baum, Settleman and Quinlan, 2008; Nieman, 1999). In addition, the mesenchymal N-cadherin becomes upregulated that promotes a motile state in cancer cells allowing the dissociation of individual cells from primary tumour and their
interaction with endothelial cells. This enhances migration and extravasation of
tumour cells to a distant metastatic site through circulation. Therefore the switch
between the classic E and N cadherins is used as a hallmark of EMT and the
acquisition of mesenchymal phenotype (Hazan et al., 2004).

4.1.5.3 Vimentin

Vimentin is a major constituent of intermediate filaments that comprise the
cytoskeletal proteins along with tubulin and actin microtubules. Vimentin is
predominantly expressed in mesenchymal cells and are often used as a marker
of cells undergoing EMT or cells derived from the mesenchymal origin (Satelli
and Li, 2011). It is a major cytoskeletal component of mesenchymal cells
because of its dynamic nature and its ability to offer flexibility in shape of cells
thereby increasing their mobility (Satelli and Li, 2011). Moderate to strong
vimentin expression was found in normal human brain especially in the
ependymal cells, meninges and choroid plexus (Yamada et al., 1992). Expression of vimentin in GBM cells was shown to be dependent on the cellular
density and chemo/radio treatment and is shown to mediate integrin trafficking
which is vital for propelling GBM malignancy (Fortin et al., 2010). In other CNS
tumours like meningiomas, schwannomas and neurofibromas vimentin
expression was associated with the migration potential and infiltrative/invasive
nature of these tumours (Bouamrani et al., 2010; Kawahara et al., 1988).
4.1.6 Rationale and aims of this study

The conventional sphere culture system is based on the above mentioned fact that GBM CSCs are preserved in the tumour microenvironment by hypoxia and hence can be isolated, enriched and studied using suitable serum-free selective medium. But we believe that when the cells grown in sphere culture system the aggregation of cells will first induce hypoxia and activate hypoxia mediated EMT in vitro that results in mesenchymal-CSC phenotypes. These cells are further maintained and protected by self renewal and embryonic signalling pathways activated under hypoxic environment. The main goal of this study is to show that CSC phenotypes induced in NS and SUS cultures of GBM cell lines are due to hypoxia induced EMT. Although the spheres are grown under normal O$_2$ conditions, spheroids of larger size become hypoxic due to diffusion gradient (Lee et al., 2006). We aim to prove that any established cell lines grown under sphere culture method will have the same effect and result in CSCs which are mesenchymal cells. By this way we would like to implicate that the GBM CSCs that are chemoresistant in actual primary tumour could possibly be mesenchymal cells and also establish the fact that hypoxia is the key target.

If hypoxia is the problem, we would like to know whether hypoxia can induce EMT and CSCs while the cells are grown as attached monolayer. We aim to do this by culturing monolayer of GBM cell lines under normoxic and hypoxic conditions and determine whether there are any signs of EMT or CSC characteristics under hypoxic conditions. If there are mesenchymal changes observed we also would like to examine whether these cells are resistant to
anticancer drugs. By this way we can be sure whether or not the hypoxia induced mesenchymal cells are the true culprits in GBM chemoresistance. In this chapter we will discuss the possible mechanisms of how hypoxia can drive EMT and analyse the possibilities of these mechanisms in our NS and SUS CSCs from GBM cell lines.

4.2 Experimental design

Detailed information on materials, products, manufacturers and methodologies used for the entire study has been described in chapter 2. The following are specific experimental designs and methods used for this part of the study.

4.2.1 Culturing cells under hypoxic conditions

The ATT monolayer cells of all three GBM cell lines U87, U251 and U373 were grown under hypoxic conditions using STEMCELL Technologies Hypoxia incubator chamber gassed with mixture of 1%O\textsubscript{2}, 5%CO\textsubscript{2} and remaining N\textsubscript{2} from a custom made BOC gas cylinder. The cells for culture were placed in the chamber and sealed after purging the O\textsubscript{2} in the chamber with the above mentioned low O\textsubscript{2} gas for 4 minutes. Cells were grown for 5-7 days depending on growth rate before subjecting to experiments. The cells were fed with fresh media every 2-3 days and to make sure the residual O\textsubscript{2} in the media doesn’t affect the experiment, the media in the flask was gassed before closing and then placed in the chamber to continue hypoxic conditions. An O\textsubscript{2} meter was always inside the chamber to monitor any leaks or increase in O\textsubscript{2} levels during
the process. Any processing or handling of the hypoxic cells during culture or for experiments was done quickly every time to achieve maximum reliable results. The cells grown under hypoxia will be referred in this study as **ATT-HYP** while the normoxic cells will be referred as **ATT-NOR**. For example –U373 ATT-HYP or U373 ATT-NOR.

### 4.2.2 Determining the presence of hypoxia in sphere cells and hypoxic cell cultures

The presence of hypoxic status in the cells was determined using the HypoxyprobeTM-1 plus Kit supplied by Hypoxyprobe Inc (Burlington, MA, USA) following the supplier’s instruction. The kit was used for both immunocytochemistry (ICC) assay as well as quantification using flow cytometry (FACS). The NS and SUS cells were cultured for 7 days and treated with Hypoxyprobe (HP) reagent for 24 hours. Since the sphere cells are suspension cultures they were spun at 800 g for 3 minutes to spread the spheres onto Polylysine-coated slides. The cells were fixed with methanol and stained with FITC conjugated monoclonal antibody against HP for 1 hour, washed, mounted and then images were taken using a confocal microscope. The measurement of hypoxia status in ATT-HYP culture was done similarly to that of NS and SUS cells using the Hypoxyprobe kit. But the cells were cultured in 8-well chamber slides at ATT-NOR/HYP conditions and labelled with HP for 24 hours. For flow cytometric analysis, the NOR, HYP, NS and SUS cells were grown in 25cm² flasks and after treatment with HP reagent were trypsinized, counted, fixed with methanol and stained with the FITC anti-HP antibody. The hypoxic population
was detected using a FACS Calibur flow cytometer with 488 nm blue laser and standard FITC 530/30 nm bandpass filter. Any experiments involving HYP cell culture, NS or SUS was compared with ATT normoxia cells grown in parallel with same initial cell number and incubation periods. In both the cases of ICC or FACS, cells without HP but stained with FITC antibody were used as suitable negative and staining controls.

Additional confirmation for hypoxia status can be done by western blot analysis of HIFs. HIFs are highly oxygen sensitive proteins and their degradation by PHDs is immediately stopped as a response to low O₂ levels, hence the functional HIF proteins will translocate to nucleus of the cells. If hypoxia is present in cells grown as NS and SUS, there will be an increased expression and nuclear translocation of HIF1α and HIF2α in these cells. The same phenomenon applies to cells grown under hypoxia. ATT-NOR, ATT-HYP, NS and SUS cultures of all three GBM cell lines were harvested and the nuclear protein in the cells was extracted by following the protocol for nuclear protein extraction, quantified and separated by SDS PAGE. The expression of HIF1α and HIF2α was analysed using appropriate primary and secondary antibodies as mentioned in the western blot section of chapter 2.

4.2.3 Analysis of EMT markers in sphere and hypoxic cells

To establish the fact that hypoxia induces EMT three important EMT markers such as CD44, vimentin and E-Cadherin to N-cadherin switching were tested for ATT-HYP, NS and SUS cells with ATT-NOR cells as control. CD44 cell surface
marker expression was analysed using FACS flow cytometry protocol. The other markers vimentin, E-cadherin and N-cadherin were analyzed by western blot to detect protein level expression. If EMT was happening under hypoxic conditions and sphere cell cultures, we expect an increase in the expression of CD44, N-cadherin and vimentin and a decrease in the level of E-cadherin expression.

### 4.2.4 Determining mesenchymal properties cells

Cells that have undergone EMT and acquired a mesenchymal phenotype display increased migratory and invasive characteristics. To confirm if the cells grown under hypoxia are truly mesenchymal in nature we planned to carry out *in vitro* wound healing assay (scratch assay) for migration and transwell invasion assay (Boyden chamber assay) to determine invasion. But unfortunately the cells grown under hypoxia divided very slowly and hence a confluent tightly packed monolayer was not achieved in order to carry out a scratch assay. Hence we performed the migration assay using Boyden chamber without matrigel. The cells grown under NOR and HYP in 25cm² flasks for 6 days were quickly subjected to Boyden chamber migration assay according to the manufacturer’s recommended protocol and instructions in chapter 2. The cells under NOR and HYP were subjected *in vitro* cell invasion assay as described in chapter 2 using 8.0µM pore size transwell filter inserts coated with matrigel in 24 well plates. Briefly, the invasion rate of HYP and NOR cells that invaded through matrigel basement matrix was assessed according to the manufacturer’s recommended protocol.
4.2.5 Analysis of CSC markers in hypoxic cells

We already established the fact that NS and SUS cells have increased CSC characteristics in chapter 3. If hypoxia-induced EMT resulted in CSCs in sphere cells and if EMT happened in the cells grown under hypoxic conditions, we expect that there should be an increase in the CSC characteristics of the ATT-HYP cells. So we examined all CSC markers tested for NS and SUS cells like ALDH, CD133, Sox2, Oct4 and Nanog using immunofluorescence protocol for FACS as mentioned in chapter 2. An increase in expression of CSC markers is quantified by measuring increase in fluorescence. Additional confirmation for the expression of Sox2, Oct4 and Nanog embryonic CSC markers was done by western blot analysis. ATT-NOR and ATT-HYP cells of all three GBM cell lines were compared for their expression of the above markers. If hypoxia induced any phenotypic changes in cells grown as monolayers and generated any cells with CSC characteristics, then we can implicate that hypoxia in the NS and SUS cells is the true reason behind CSC characteristics in sphere cultures.

4.2.6 MTT cytotoxicity assay for hypoxic cell cultures

Although we assumed that hypoxic conditions can induce CSCs through EMT, the true aspect we are interested in is the chemoresistance induced by CSCs. If cells grown under hypoxia showed CSC characteristics, we aim to assess whether they mimic the chemoresistant nature of CSCs. To determine the effect of hypoxia on drug sensitivity, the cells were cultured in 1% oxygen condition at a cell density of $1 \times 10^3$ cells/well in 96-well plate for 4 days and exposed to
anticancer drugs for another 120 hours before MTT assay. TMZ and other conventional anticancer drugs VCR, PAC and DOX were used in the same concentrations as used for NS and SUS MTT assay. A parallel MTT assay was performed for cells under normoxic condition.

4.3 Results

4.3.1 GBM sphere cells contain high proportion of hypoxic cells

Hypoxyprobe analysis reveals that a high proportion of hypoxic cells are present in both NS and SUS cells in comparison to the ATT monolayer culture. This was true for NS and SUS from all three GBM cell lines (Figure 4.1). The quantification by FACS analysis shows a statistically significant increase in the hypoxic population in sphere cells and there was no significant difference between NS and SUS cells (4.1 bar chart). A closer look at the FACS results also show that NS and SUS cells have two populations probably the outer normoxic cells and inner hypoxic cells. ICC and confocal imaging of cells treated with hypoxyprobe stained with anti HP-FITC antibody and propidium iodide counter stain clearly show bright green cytoplasmic staining (Figure 4.2) indicating hypoxia in majority of the cells. Furthermore western blot results showed a significant increase in the nuclear translocation of HIF1α and HIF2α in NS and SUS cells whereas no increase was observed in ATT cells (Figure 4.3). These results clearly indicate that hypoxia is an important characteristic of sphere cell culture due to aggregation of cells and also there are no differences between the cells grown as spheres using expensive special medium or with normal medium.
**Fig 4.1 Hypoxic population in GBM sphere cells:** Determination of hypoxic status in cells by Hypoxyprobe staining and FACS analysis. The bar chart displays the presence of statistically significant number of hypoxic cells in NS and SUS than ATT cells (n=6; **p<0.01)
Fig 4.2 *In situ* detection of hypoxic cells in GBM spheres using Hypoxyprobe staining.
ICC staining of ATT, NS and SUS cells from all three GBM cell lines using Hypoxyprobe kit shows that the sphere cells have increased proportion of hypoxic cells (Green+FITC) in comparison to the ATT cells. PI-(Red) was used as nuclear counter stain. (x40 magnification).

Fig 4.3 Western blot analysis of HIFs in GBM sphere cells: Western blot analysis shows that the sphere cells have increased expression and nuclear translocation of both hypoxia inducible factors (HIF1α and HIF2α) in comparison to the ATT cells. Nucleolin was used as loading control.
4.3.2 GBM sphere cells express EMT markers

FACS analysis for expression of CD44 cell surface marker show that NS and SUS cells from all three GBM cell lines had increased level of CD44 expression when compared to ATT cells (Figure 4.4). CD44 is an important cell surface marker in EMT activated cells and mesenchymal cells. We further used western blot analysis to determine the expression of other EMT markers like vimentin and cadherin switching. Our results (Figure 4.5) show that NS and SUS have increased expression of mesenchymal markers vimentin and N-cadherin in comparison to the ATT cells which clearly indicates the presence of mesenchymal population in the sphere cells. On the other hand there was a significant loss of the epithelial marker E-cadherin in both NS and SUS cells which further confirms the phenotype change to mesenchymal origin. The ATT cells retained E-cadherin and no increase in vimentin or N-cadherin was observed in them.
Fig 4.4 High expression of CD44 in GBM cell line-derived NS and SUS cells. The expression of CD44 in ATT and GBM spheres was detected using CD44-FITC conjugated antibody in FACS analysis. The bar chart above displays statistically significant increase in the CD44 expression in GBM spheres than ATT cells. n=9; **p<0.01

Fig 4.5 Western blot analysis of EMT markers in GBM sphere cells. Western blot analysis using whole cell lysates of ATT and sphere cells clearly displays that the sphere cells have decreased expression of epithelial marker E-cadherin and increased expression of mesenchymal markers (N-cadherin and vimentin) in comparison to the ATT cells indicating EMT activation in these cells. Tubulin was used as loading control.
4.3.3 The effect of hypoxia on GBM cell lines cultured in monolayer

The attached monolayer cultures of all three GBM cell lines grown under hypoxic conditions was compared with attached monolayer grown under normoxia using the hypoxyprobe analysis. The results obtained through FACS analysis quantification showed significant increase in proportion of hypoxic cells in ATT-HYP cultures than ATT-NOR cells (Figure 4.7). The HP results from ICC shows clear increase of hypoxic population in ATT-HYP cells with bright green HP-FITC staining and PI counter staining (Figure 4.6). The normoxic cells have a clear cytoplasm with prominent nucleus that stained red with PI.

Although HP can detect hypoxic conditions the true background of the study was to induce hypoxia mediated activation of cellular pathways. This is indicated by the expression of various hypoxia dependant factors in the nucleus. Further analysis for the confirmation of hypoxia through nuclear translocation of HIFs was done by western blot and the results indicate a clear increase in the translocation of HIFs in ATT-HYP cells compared to that of ATT-NOR cells which is shown in Figure 4.8. These results confirms the successful generation of hypoxia in ATT-HYP cells by growing them in hypoxic chamber with 1%O₂ and any results obtained from hypoxic cell cultures are comparable to that of hypoxic population in the NS and SUS cultures.
Fig 4.6 Hypoxic population was detected in hypoxia-cultured GBM cell lines. The hypoxic cells were detected by Hypoxyprobe staining and FACS analysis. The bar chart displays the presence of statistically significant number of hypoxic cells in GBM cells cultured under hypoxia than normoxia (n=6; **p<0.01)
Fig 4.7 Hypoxic cells in hypoxia-cultured GBM cell lines detected by in situ ICC Hypoxyprobe staining. ICC staining using Hypoxyprobe kit shows that all three GBM cell lines grown under Hypoxic conditions (less than 1 % O$_2$) have increased proportion of hypoxic cells (Green+FITC) in comparison to the cells grown under Normoxic conditions cells. PI-(Red) was used as nuclear counter stain.

Fig 4.8 Western blot analysis of HIFs in hypoxia-cultured GBM cell lines. Western blot analysis of nuclear extracts from ATT-NOR and ATT-HYP cells of all three GBM cell lines shows that the hypoxic cultures have increased expression and nuclear translocation of HIF1$_\alpha$ and HIF2$_\alpha$ in comparison to the normoxic cells. Nucleolin was used as loading control.
4.3.4 Hypoxia induces EMT in GBM cell lines

We hypothesized that hypoxia induces EMT to give rise to CSC characteristics in tumour cells. Cells grown as ATT-HYP are tested for the expression of mesenchymal cell surface marker CD44 and other EMT markers. FACS analysis shows a statistically significant increase in the expression of CD44 in ATT-HYP (Figure 4.9). In addition western blot results in Figure 4.10 shows the increased expression of other mesenchymal proteins like N-cadherin and vimentin in ATT-HYP cells. Very similar to that of sphere cells the ATT-HYP cells lost the epithelial marker E-cadherin. Whereas in cells grown under normoxia there was no loss of E-cadherin and hence they remain epithelial in nature. These results indicate that if hypoxia can transform the cells into a more mesenchymal phenotype and can be reversed to epithelial nature if the cells were returned back to normoxia. This also indicates that accumulated hypoxia due to aggregation of cells is probably the reason behind the acquisition of EMT phenotypes leading to CSC characteristics in NS and SUS cells.
**Fig 4.9 Expression of CD44 in hypoxia-cultured GBM cell lines.** FACS analysis using CD44-FITC conjugated antibody shows increased expression of CD44 cell surface marker in all three hypoxic GBM cell lines in comparison with their normoxic counterparts. *n=6, **p<0.01*

**Fig 4.10 Western blot analysis of EMT markers in hypoxia-cultured GBM cell lines.** The results clearly displays that in comparison with their NOR counterparts the HYP cells have decreased expression of E-cadherin and increased expression of N-cadherin and vimentin indicating EMT activation in the HYP cells. Tubulin was used as loading control.
4.3.5 Hypoxic cells show increased migration and invasion

Further confirmation of hypoxia-induced EMT and transformation of cells into mesenchymal phenotype was done by analysing the migratory potential of cells under hypoxia. The images taken using the inverted microscope for Boyden chamber migration assay and the OD measured from the experiment enable us to calculate the %migration between ATT-NOR and ATT-HYP cells. Results from the above analysis (Figure 4.11 A and B) showed significant increase in the migratory potential of cells grown under hypoxic conditions. Mesenchymal phenotypes are also known to have increased invasion potential that can be measured by transwell matrigel invasion assay. Cells with mesenchymal characteristics have the potential to produce MMPs that can digest the ECM or substance like matrigel that enables them to invade the gel and move out of the matrix towards chemoattractants like serum or growth factors. The cells that invaded, crossed the matrix and moved out of the pores in transwell were fixed and stained while attached to the filter. Images taken from the above experiment shown in Figure 4.12A indicate a remarkable increase in the invasion potential of cells grown under hypoxia. We also lysed the stained cells according to the protocol and measured the OD at 540nm using a multiwell plate reader. The results given as % invasion (Figure 4.12B) confirm the increased invasion potential of hypoxic cell cultures which is statistically significant.
**Fig 4.11** Hypoxia enhances the migratory potential of hypoxia-cultured GBM cell lines. Migration ability was determined using Boyden chamber migration assay. A) Images show migration of cells through the pores in transwell membrane (x40 magnification) indicating EMT. B) Migration index shows statistically significant increase in the migratory potential of HYP cultures in comparison to their respective NOR counterparts. $n=4$; **$p<0.01$**
Fig 4.12 Hypoxia enhances the invasive activity of hypoxia-cultured GBM cell lines.

A) Images from matrigel invasion assay showing penetration of cells through matrigel coated membrane indicating EMT (x 40 magnification). B) Invasive index shows statistically significant increase in the invasive potential of cells grown under hypoxic conditions in comparison to that of Normoxic cells. n=6; **p<0.01
4.3.6 Hypoxic Cells show increased CSC characteristics.

To show that hypoxia induced EMT and the mesenchymal phenotypes are the driving force behind CSC characteristics in GBM sphere cells, we tested the ATT-HYP cultures for the presence of CSC markers. For all the results below we have compared ATT-HYP cells with ATT-NOR cells grown in parallel. Our results from FACS analysis confirmed that all three GBM cell lines U87, U251 and U373 grown as ATT-HYP show statistically significant increase in the universal stem cell marker ALDH and neural progenitor marker CD133 (Figure 4.13 and 4.14). The expression of embryonic stem cell proteins like Sox2, Oct4 and Nanog that regulate stemness, self-renewal and pluripotency are also significantly increased which is evident from FACS data of HYP cultures from all three GBM cell lines (Figure 4.15 A, B and C). The expression of these proteins is restricted to progenitor cells and they are switched off in fully differentiated cells. In addition, results from western blot analysis confirm the protein level increase in the expression of these embryonic markers (Figure 4.16). In addition to stem cell markers, results from growth curve experiments for cells grown under hypoxia (Figure 4.17) showed significant decrease in growth rate and doubling time indicating quiescence which is an important stem cell feature. These results confirm the fact that an increased hypoxic stress can induce EMT program to convert a subpopulation of cells into mesenchymal phenotypes that display all stem cell like characteristics which are interpreted as CSCs when they arise from tumour cells. This implicates that although NS and SUS are grown under normal O$_2$ conditions developed hypoxia and resulted in CSC like cells which are more likely to be mesenchymal cells.
**Fig 4.13 High ALDH activity detected in hypoxia-cultured GBM cell lines.** ALDH activity was measured by ALDEFLUOR assay using FACS analysis. The bar chart displays the statistically significant increase in the ALDH activity in HYP cells in comparison to their respective NOR counterparts. n=9; **p<0.01
Fig 4.14 High level of CD133 expression detected in hypoxia-cultured GBM cell lines.

Expression of CD133 was measured by CD133 immunostaining using FACS analysis. The bar chart displays the statistically significant increase in the expression of CD133 in HYP cells in comparison to their respective NOR counterparts. n=9; **p<0.01
Fig 4.15 (A) Expression of Embryonic CSC markers in normoxic and hypoxic cultures of U87MG. Embryonic CSC markers measured by ICC using FACS analysis. The bar chart below displays all three embryonic CSC markers e.g. SOX2, OCT4 and NANOG are increased in HYP cultures in comparison to the NOR counterparts. n=6, **p<0.01
Fig 4.15 (B) Expression of Embryonic CSC markers in normoxic and hypoxic cultures of U251MG. Embryonic CSC markers measured by ICC using FACS analysis. The bar chart below displays all three embryonic CSC markers e.g. SOX2, OCT4 and NANOG are increased in HYP cultures in comparison to the NOR counterparts. n=6,**p<0.01
Fig 4.15 (C) Expression of Embryonic CSC markers in normoxic and hypoxic cultures of U373MG. Embryonic CSC markers measured by ICC using FACS analysis. The bar chart below displays all three embryonic CSC markers e.g. SOX2, OCT4 and NANOG are increased in HYP cultures in comparison to the NOR counterparts. \( n=6,^{**}p<0.01 \)
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**Fig 4.16 Western blot analysis of embryonic stem cell markers in hypoxia-cultured GBM cell lines.** Western blot analysis of embryonic stem cell proteins Sox2, Oct4 and Nanog in NOR and HYP cells from all three GBM cells. Tubulin was used as a loading control.

**Fig 4.17 Growth curve for Normoxic and Hypoxic cultures of GBM cell lines.** Growth curve experiment using U251 and U373 GBM cell lines show very slow growth rate under hypoxic conditions in comparison to their respective NOR cultures.
4.3.7 Hypoxic cells are highly resistant to anticancer drugs

GBM CSCs are very well known to be the reason behind chemoresistance of GBM. Although hypoxia induces EMT and increases CSC marker expression in GBM cells, we need to know whether those cells are chemoresistant like the CSCs isolated from NS and SUS. In this study we tested the first line drug TMZ and other three conventional anticancer drugs VCR, PAC and DOX on both ATT-NOR and ATT-HYP cells from all three GBM cell lines. We hypothesized that if the cells that display GBM CSC characteristics are resistant to multiple drugs, we can confirm that hypoxia induced EMT is the true reason in driving GBM CSCs and therefore can be potential treatment targets. Our MTT cytotoxicity results show that ATT-HYP cells are extremely resistant to first line drug TMZ even at very high concentration used in this study (500 μM) while the ATT-NOR cells are comfortably killed by TMZ. Similar results were observed on treatment with other drugs like VCR, PAC and DOX where the ATT-NOR cells were killed at low concentrations and ATT-HYP survived up to high concentrations. Figure 4.18 A, B and C displays the cell viability curves results of U87, U251 and U373 cell lines respectively under ATT-NOR in comparison to ATT-HYP for different drugs in comparison to each other. There is not a clear difference between the curves of two conditions in all three cell lines which explains the low IC\textsubscript{50} values for ATT-NOR cells and increased IC\textsubscript{50} values of ATT-HYP cells as mentioned in Table 4.1 and bar charts below the viability curves.
Fig 4.18 (A) Hypoxia induces chemoresistance in U87MG cells. The ATT-Normoxia and ATT-Hypoxia cells were analysed by MTT assay after exposure to TMZ, VCR, PAC and DOX. The cell viability curves clearly show significant difference in the dose response between the Normoxia and Hypoxia cultures. The bar chart below shows the elevated IC\textsubscript{50} values for HYP cells indicating increased drug resistance of the cells grown under hypoxic conditions. (\(n=9\) **p<0.001)
Fig 4.18 (B) Hypoxia induces chemoresistance in U251MG cells. The ATT-Normoxia and ATT-Hypoxia cells were analysed by MTT assay after exposure to TMZ, VCR, PAC and DOX. The cell viability curves clearly show significant difference in the dose response between the Normoxia and Hypoxia cultures. The bar chart below shows the elevated IC50 values for HYP cells indicating increased drug resistance of the cells grown under hypoxic conditions. (*n=9 **p<0.001)
Fig 4.18 (C) Hypoxia induces chemoresistance in U373MG cells. The ATT-Normoxia and ATT-Hypoxia cells were analysed by MTT assay after exposure to TMZ, VCR, PAC and DOX. The cell viability curves clearly show significant difference in the dose response between the Normoxia and Hypoxia cultures. The bar chart below shows the elevated IC_{50} values for HYP cells indicating increased drug resistance of the cells grown under hypoxic conditions. (n=9 **p<0.001)
4.4 Discussion

CSCs have been found in a variety of different cancers such as leukaemia (Bonnet & Dick, 1997), colon cancer (Zhang et al., 2004), breast cancer (Al-Hajj et al., 2003) and brain cancer (Singh et al., 2004). With the discovery of the neural stem cells (Reynolds & Weiss, 1992) it also became plausible that brain tumours could be derived from the transformation of neural stem cells or progenitor cells (Singh et al., 2004). When culturing putative tumour stem cells, spheroid models are often used especially in a selective serum free neurosphere medium and the cells grew as neurospheres. Under these serum-free conditions most differentiating and differentiated cells died, whereas the GBM CSCs are protected. But studies from our group and several others showed that CSCs can be isolated from fully differentiated GBM cell lines. Our results from chapter 3 also indicated that GBM cell lines grown as spheres in normal serum containing culture medium can also produce CSCs with no significant differences from that of NS cells. Several studies associate tumour hypoxia with poor patient outcome and resistance to therapies (Bar, 2011; Li et al., 2009; Mashiko et al., 2011). In line with this, one of the hallmarks of GBMs is the presence of necrosis, occurring as a consequence of poor oxygenation and nutrition because of rapid tumour growth and formation of vessel thrombosis (Hulleman & Helin, 2005; Louis et al., 2007; Preusser et al., 2006). The role of hypoxia in normal stem cell biology is well established. Hematopoietic stem cells, for example, colonize hypoxic niches within the bone marrow, where they are maintained quiescent by hypoxia-induced proteins such as osteopontin (Stier et al., 2005; Nilsson et al., 2005). Severe hypoxia inhibits the differentiation of normal NSCs (Santlli et al., 2010). Also, hypoxic conditions
reduce differentiation in human embryonic stem cell cultures while not affecting their proliferation (Ezashi et al., 2005). Hypoxia is also known to improve the generation of induced pluripotent stem (IPS) cells (Yoshida et al., 2009).

With above facts in mind we hypothesized that the only common factor between the NS and SUS is hypoxia due to cell aggregation and proliferation. Hypoxia can drive CSCs as shown in other cancers through a mechanism known as EMT. We analysed our NS and SUS cells from GBM cell lines for presence of hypoxia using HP. HP contains pimonidazole hydrochloride which is reductively activated in hypoxic cells. The activated intermediate forms stable covalent adducts with thiol (sulphydryl) groups in proteins, peptides and amino acids. The antibody reagent MAb1 binds to these adducts allowing their detection by immunochemical means. The results obtained showed a remarkable increase in hypoxic cell populations in both NS and SUS. Furthermore cellular responses to O₂ concentrations are tightly regulated by PHDs that degrade HIFα subunits of HIF transcription factors under normal O₂ concentrations. PHDs become inactive under hypoxia thereby releasing HIFα subunits to join with HIFβ subunits and translocate into the nucleus to form functional HIF TFs that activates hypoxia responsive genes. Analysing our NS and SUS with their nuclear protein by western blot has shown increased nuclear translocation of HIF1α and HIF2α TFs. This is a very strong evidence for the presence of hypoxic status in sphere cells.

EMT is a collection of trans-differentiation program that occur during embryogenesis and cancer cells exploit the same principle to drive invasive and aggressive tumour that results in poor patient outcome. Hypoxia is one of the
physiological mechanisms that can induce EMTs in tumours through multiple distinct mechanisms. Hypoxia-induced EMT has been reported in a wide range of solid tumours recently but evidences in GBM are still not solid. These facts pushed us to find out if hypoxia induced EMT can occur in GBM cells giving rise to CSC traits. With the confirmation of hypoxic status of GBM spheres we wanted to determine if there is any hypoxia driven EMT mediated phenotypic change occurring to those cells. Our results clearly established that cells under NS and SUS conditions display an increased vimentin and N-cadherin expression indicating switching to a mesenchymal phenotype while losing E-cadherin which is a hallmark of epithelial phenotype. With these results we confirmed that hypoxia induced EMT is probably the reason behind CSC characteristics of cell line-derived GBM spheres. But this was not settled completely because if hypoxia is the key player, we wanted to know if inducing hypoxia to any cells grown as attached monolayer will have the same EMT and CSC effect that eventually drives resistance to anticancer drugs.

To study this we grew cells under hypoxia as attached monolayer cultures and compared it with normoxia in parallel. We confirmed the successful induction of hypoxia in our hypoxic cultures by performing HP analysis and subsequent confirmation of HIFs nuclear translocation. With successfully grown hypoxic cultures we searched for evidence of EMT by analysing all the EMT markers. We found significant increase in EMT when cells are grown under hypoxia indicating a true change to a mesenchymal phenotype. We further confirmed the mesenchymal characteristics through increased invasion and migration properties of hypoxic cell cultures. Although we established that hypoxia can certainly drive EMT to induce mesenchymal phenotype, we wanted to know
whether these are the cells responsible for CSC characteristics in sphere cells. Our results for analysis of CSC markers clearly established that hypoxic cell cultures undergoing EMT show increased stemness. In addition to cell surface CSC markers, embryonic signalling pathways and quiescent behaviour of these cells were also evident from the expression of embryonic stem cell proteins and a much slower proliferation rate under hypoxia.

However the main aim of our work was to understand the molecular mechanisms behind CSC induced chemoresistance to anticancer drugs. GBM normally recurs intracranially and tumour recurrence happens due to several reasons including chemoresistance. Hypoxia induced EMT driving the invasive and migratory phenotype may help the tumour cells to penetrate the nearby tissue and escape the hostile environment leading to tumour recurrence. So we wanted to know if hypoxia induced EMT mediated CSCs are the true culprits driving recurrence and resistance to drugs in order to develop effective methods to target them for therapy. The MTT analysis results showed significant increase in the resistance of hypoxic cells to all four drugs tested. Hence it can be confirmed that in NS and SUS cells the CSCs are induced by hypoxia which play an important role in chemoresistant nature of GBM CSCs. If this is the case in actual GBM in patients the same phenomenon can happen where GBM CSCs can develop in the hypoxic niche of the tumours. Several studies relate hypoxic nature of GBM as prognostic factor for response to therapy. In addition several correlation studies also highlighted that extensively hypoxygenated nature of GBM could be the reason behind increased invasive and aggressive nature of GBM tumours. This could be possibly due to the mesenchymal characteristics acquired by GBM during progression and evidences are
emerging to support the EMT nature of GBM. A good example is the inclusion of an additional mesenchymal subtype in the classification of GBM by WHO. Under in vitro conditions culturing of cells is usually performed at 21 % O$_2$ as attached monolayer. But when cells are grown as spheroids of large sizes they become hypoxic even if cultured in normoxia because of a diffusion gradient (Fehlauer et al., 2005). Low oxygen levels in different tumour types are believed to increase the population of CSCs and to promote a stem-like state (Bar et al., 2010; Heddleston et al., 2009; Saigusa et al., 2011; Soeda et al., 2009; Wang et al., 2011; Xing et al., 2011; Yeung et al., 2011). This is similar to results obtained for embryonic stem cells showing that low oxygen levels promote maintenance of pluripotent potential, and maintenance of the cells in an undifferentiated stem cell state (Ezashi et al., 2005; Heddleston et al., 2009). The existence of tumour stem cells has been suggested to be restricted to perivascular niches and hypoxic areas within the tumour (Heddleston et al., 2009) explaining the poor outcome and therapeutic resistance seen in these hypoxic tumours. In addition to obtaining a more in vivo like metabolic milieu when culturing cells in hypoxic conditions, hypoxia also seems to promote the existence and propagation of tumour stem cells (Heddleston et al., 2009; McCord et al., 2009; Soeda et al., 2009). Several studies thus reported an increase in spheroid diameter, cell proliferation and number of spheroids (Heddleston et al., 2009; McCord et al., 2009; Soeda et al., 2009) when culturing spheroids in hypoxic compared to normoxic conditions. In a study from Kolenda et al., spheroids obtained from a glioblastoma short term culture and the commercial glioblastoma cell line U87MG were cultured in both normoxia and hypoxia (Kolenda et al., 2010). Interestingly, a significant increase in the
expression of the proposed stem cell markers CD133, Podoplanin and Bmi-1 was found in both types of spheroids when cultured in hypoxia. Furthermore, a study by Heddelston et al. (Heddelston et al., 2009) proposed that a phenotypic shift from non-stem to stem-like cells was obtained when culturing tumour cells in hypoxia. On the more mechanistic level, the spheroid formation in hypoxia has been shown to be affected by the hypoxia inducible factors as shown in studies by Li et al. (Li et al., 2009) and Méndez et al. (Mendez et al., 2010). Knockdown of HIF altered spheroid formation in glioma spheroids, resulting in smaller and fewer spheroids.

4.5 Conclusions

To conclude, GBM CSCs in NS and SUS are induced by hypoxia resulting from their growth as suspended spheres. The culture medium or the source of initial GBM does not affect the formation of GBM CSCs in sphere cultures. Development of CSCs in NS and SUS is due to the dedifferentiation of epithelial to mesenchymal phenotypes by hypoxia-induced EMT process. This is evident from hypoxic cultures acquiring mesenchymal phenotype and eventually CSC properties without growing them as spheres. Transition into CSC like phenotypes also leads to resistance which indicates that accumulated hypoxia could be the key reason behind chemoresistance in GBM. Overall these findings suggest that culturing of cells as spheroids provides important in vivo like hypoxia conditions that are optimal when studying the stem cell biology and chemoresistance of brain tumours. Further understanding of the mechanisms of hypoxia-induced EMT will guide us towards therapeutic targets.
Chapter 5

The role of hypoxia inducible factors in hypoxia induced EMT and GBM CSCs
5.1 Introduction

Hypoxia is one of the basic biological phenomena that has an intricate association with the development and progression of a variety of solid tumours. Hypoxia can activate the EMT program in tumours through several mechanisms mediated by HIFs, HGF, SNAI1, TWIST, Notch or NF-κB pathways, and induction of other epigenetic DNA modifications (Vooijs et al., 2008). Although several factors are reported to be important for hypoxia induced EMT, Hypoxia-inducible factors (HIFs) are widely recognized to function as the primary and master transcription factors in regulating hypoxia responsive genes. HIFs are known to play critical roles in tumour invasion, cell proliferation, metastasis, angiogenesis, chemo-radio resistance, stem cell maintenance and survival (Bruick, 2001). Hypoxia activates self-reinforcing positive-feedback loops involving HIFs and various other signalling pathways that stabilize the expression of EMT-inducing transcription factors in the nucleus in order to maintain the mesenchymal state (Radisky et al., 2005).

Although both EMT and hypoxia on their own are considered as crucial processes that favours invasion and metastasis of cancer cells, the two events are long known to have some molecular mechanisms in common. Increasing amount of evidence reveals that, alterations of oxygen level in the tumour microenvironment and subsequent activation of hypoxic signalling through HIFs acts as important triggers and modulators of the EMT, which is now believed to be the convergence point between hypoxia and cancer.
5.1.1 The regulatory role of hypoxia and HIF pathway in EMT

The acquisition of EMT during tumour progression is known to modulate cell adhesion capacity, which leads to increased cell migration and invasion, resulting in tumour aggressiveness (Hugo et al., 2007). In the process of EMT, several TFs such as ZEB1, ZEB2, Snail1, Snail2 (also known as Slug), Twist1 and E47 are shown to be critical mediators of various EMT inducers in different cell lines including cancer cells (Jiang et al., 2011). The hypoxia-induced EMT is tightly mediated by HIFs which are TFs that activates of E-cadherin transcription repressors and hence reprograms epithelial cells to a more mesenchymal phenotype, leading to the promotion of invasive potential in tumours (Chang et al., 2011). This complex molecular crosstalk between hypoxia-induced pathways and EMT has not been fully understood. Several aspects of potential molecular mechanisms are still being studied, as reviewed recently by Jiang et al., in 2011. One proposed mechanism is that the activation of HIF-1 and 2 can up-regulate EMT associated TFs or repressors such as Twist, Snail, Slug, and SIP1/ZEB2 as observed in many carcinomas (Sun et al., 2009; Welford et al., 2011; Katoh et al., 2009). Another possible mechanism is that hypoxia and HIF pathway activate further downstream signalling pathways related to EMT such as TGF-β, Notch, NF-κB, Wnt/β-catenin, and Hedgehog (Zavadil et al., 2004; Yang et al., 2006; Vincent et al., 2009). Other proposed mechanisms like the regulation of EMT-associated inflammatory cytokines by hypoxia and HIF pathway such as increased expression of TNF-α, IL-6 and IL-1β are shown to play positive roles in inducing EMT phenotype or characteristics (Sullivan et al., 2009). Finally, hypoxia and HIF pathway is also shown to interact directly or
indirectly with proteins and enzymes that mediate communications between cells and ECM to facilitate motility and invasion and induce EMT. This also can be mediated through the regulation of LOX/LOX2, Hey1, Hes1, and uPA expression (Erler et al., 2006). Recent data also suggest that other signalling pathways such as VEGF and epigenetic regulators also play central roles in hypoxia-induced EMT of cancer cells. For example, hypoxia or HIF can induce HDCA3 which is reported to be an important requirement for EMT program (Yang et al., 2006; Wu et al., 2011). In addition to various citations of HIF1 pathway in regulating EMT, one in vitro and in vivo study in non-small cell lung showed that HIF-2α can also promote the expression of EMT genes such as ZEB1, Snail, SIP1, and vimentin and enhance tumour growth in xenograft mouse models (Kim et al., 2009). There is increasing evidence for an important role of HIF-2α in pathological conditions. Therefore, targeting both HIF1 and HIF2 pathway along with inhibitors of EMT could provide a therapeutic benefit in the future.

5.1.2 The regulatory role of hypoxia and HIFs in CSCs

When hypoxia induced EMT results in CSC phenotypes in the tumour population, it is also the responsibility of hypoxia to maintain the CSC phenotypes. Several studies have provided robust evidence that hypoxia and HIF signalling pathway play crucial roles in the regulating the function of CSCs, contributing to tumour aggressiveness and chemoresistance (Hermann et al., 2010; Creighton et al., 2010). It has been widely recognized that hypoxia is an important factor that regulates and maintains the normal stem cell population in
the normal tissues or during embryonic and adult development (Dunwoodie, 2009). Emerging experimental evidence indicates that the same function of hypoxia is also applicable for GBM CSC phenotypes, where hypoxia enhances the self-renewal capacity and helps in the maintenance of undifferentiated state of CSCs. This is further confirmed by experiments involving CSCs under normoxic and hypoxic conditions where it is shown that hypoxic CSCs robustly retained the undifferentiated phenotype, whereas normoxic CSCs entered the differentiated state. Hence CSC phenotypes induced by hypoxia are reversible when they re-grow under normoxic conditions, a process consistent with the EMT-MET changes that occur after tumour seeding at a distant metastatic site (Li et al., 2010).

This activation of signature CSC genes by hypoxia or HIF signalling may be one of the major reasons behind the CSC supporting function of hypoxia. Emerging evidence suggests that hypoxic areas within a tumour or the areas of necrosis behave as a niche where CSCs reside (Li et al., 2010).

It is recognized that HIF proteins, specifically HIF-1α and HIF-2α participates in the transcriptional activation and regulation of HIF targets, such as Oct4 and other CSC markers thereby mediating signalling pathways leading to hypoxia induced CSC phenotypes (Heddleston et al., 2009). Experiments in neuroblastomas has shown that hypoxia is able to maintain the stem-like phenotypes by activating signalling pathways that are associated with undifferentiated phenotypes of normal stem cells, including Sox2, Oct4, Nanog and Notch-1 signalling (Bao et al., 2006). Recent studies have demonstrated
that HIF-1α have several target genes such as Oct4, Nanog, c-Myc, Notch-1, and CD133, in various cancer cells including GBM CSCs (Covello et al., 2006; Mathieu et al., 2011) whereas knock-down of HIF-1α attenuates the hypoxia-induced CD133+ CSCs (Wang et al., 2011). These results collectively suggest the central role of HIF-1α as a primary hypoxia responsive factor that is required for the maintenance of the CSC phenotypes.

Several other recent investigations are also focused on the role of HIF-2α in the regulation of CSCs. It has been shown that HIF-2α is highly expressed in CSCs of neuroblastoma, glioblastoma, renal cell carcinoma, and breast carcinoma (Pietras et al., 2008; Pietras et al., 2010). The expression of HIF-2α also increased expression of CD133 (Bar, 2010) and the tumorigenic capacity of GBM CSCs (Li et al., 2009). Conditional loss of HIF-2α resulted in vessel abnormalities, decreased proliferation and reduced self-renewal of CSCs resulting in suppression of tumour growth and metastasis (Skuli et al., 2009). Ectopic over-expression of HIF-2α induces CSC markers such as Oct4, Nanog, Sox2, and c-Myc, and augments the tumorigenic potential of the non-CSC population (Heddleston et al., 2009). It is interesting to note that HIF-2α are highly active in low pH environments and function in an oxygen-independent manner and regulate CSCs (Hjelmeland et al., 2010). These findings strongly suggest that HIF-2α may be the pathological HIF required for the phenotype and function of CSCs while HIF1 could be the primary responsive factor for hypoxia.
5.2 Rationale and aims of this study

From the findings of chapter 3 and chapter 4 we established the fact that isolation of GBM CSCs does not require special culture medium because they are not selected CSCs, but instead are induced by hypoxia through EMT when grown as sphere cultures. We also confirmed that CSCs induced by hypoxia are the real culprits in chemoresistant nature of GBM CSCs. If this is the case according to literature HIFs should play a predominant role in inducing stemness in our NS and SUS cultures. But in recent years there has been a lot of debate over the primary HIF involved in CSCs. This is due to the following findings of differential expression and role of HIF-1α and HIF-2α between non-stem cells and CSCs. HIF-2α has attracted the attention of researchers in recent years and has also been shown to be significantly present in the population of CSCs, and remarkably induced by hypoxia (Li et al., 2009). In comparison, HIF-1α is found in both stem and non-stem tumour cells and is only stabilized and activated under severe hypoxic conditions. It is hypothesised that HIF-1α is involved in the adaptation to acute/transient hypoxia and HIF-2α to chronic/prolonged hypoxia (Li et al., 2009). Recent experimental data has shows that cancer cells highly expressed E3 ubiquitin ligase factor known as hypoxia associated factor (HAF) which decreases during acute hypoxia, but increased following prolonged hypoxia. HAF is shown to inhibit HIF-1α activity by directly binding to HIF-1α protein and marking it for degradation by ubiquitination in an O₂ and pVHL-independent mechanism. Whereas binding of HAF to HIF-2α, increases HIF-2α transactivation without causing its degradation. Thus, HAF switches the hypoxic response of the cancer cells from
HIF-1α dependent to HIF2α -dependent transcription and activates CSC and EMT related genes. This switch to HIF-2α-dependent gene expression mediated by HAF also promotes highly aggressive and rapidly expanding tumour stem cell population, resulting in increased tumourigenic potential in vivo (Koh et al, 2008; Koh et al, 2011). However, the exact molecular mechanisms of interplay between HIF-1α and HIF-2α in regulation of CSC require further in-depth investigation.

Thus in this study we were interested to establish the role of HIF1α and HIF2α in hypoxia induced EMT and related stemness and to find out whether HIF1α or HIF2α functions as the driving force behind chemoresistant GBM CSCs. We also would like to ascertain whether HIF1α or HIF2α could be the ultimate target to reverse chemoresistance in GBM.

5.3 Experimental Design

Detailed information on materials, products, manufacturers and methodologies used for the entire study has been described in chapter 2. The following are specific experimental designs and methods used for this part of the study. Although we studied the CSC and hypoxic characteristics using all three GBM cell lines U87, U251 and U373, due to the limitations of time, funding and resources we used only the U373MG GBM cell line in this study.

5.3.1 Stable transfection U373GBM cells with HIF1α and HIF2α

HIFs are tightly regulated by O₂ concentrations and are active only under hypoxia. We aimed to carry out ectopic overexpression of both HIF1α and
HIF2α in GBM cell lines in order to compete with the action of PHDs and thereby achieving high nuclear translocation of HIFs under normoxic conditions and eventually driving the HIF target genes. cDNA for HIF1α and HIF2α were obtained from Origene, (Cambridge, UK) and recombinant vectors were constructed by inserting the cDNA into pCMV6/Neo mammalian expression vector. U373 GBM cells were cultured (1x10^6 cells/well) in 6 well plates without antibiotics overnight. 4ug of empty vector pCMV6 and recombinant vector with HIF1α or HIF2α were introduced into the cells separately using Lipofectamine™ 2000 reagent. The transfected cells were incubated at 37°C for 24 hours selected for 7-10 days in a selective medium containing G418 250ug/mL. Colonies of cells were picked up, enlarged and screened for over-expression of target gene in comparison with mock transfected clones using western blot. For the overexpression of HIF1α and HIF2α confirmation was done by analysing the nuclear translocation of HIFs using western blot from nuclear extract of transfected cells. At least two positive clones with high nuclear translocation of HIFs were selected for each gene and subjected to further analysis. The mock transfected cells containing empty pCMV6 vector was grown in parallel for all experiments and used as controls for HIFs expression. All cells were grown under the selecting medium containing G418 throughout culturing time.

5.3.2 Analysis of CSC markers in HIF transfected cells

We already established the fact that NS, SUS and HYP cells have increased CSC characteristics in chapter 3. If these CSC characteristics are driven by either HIF1α or HIF2α then we expect that there should be an increase in the CSC characteristics of the cells with increased expression of HIFs. So we
examined all CSC markers tested in previous experiments like ALDH, CD133, Sox2, Oct4 and Nanog using immuno-fluorescence protocol for FACS as mentioned in chapter 2. An increase in expression of CSC markers is quantified by measuring increase in fluorescence. Additional confirmation for the expression of Sox2, Oct4 and Nanog embryonic CSC markers was done by western blot analysis. The HIF1\(\alpha\) or HIF2\(\alpha\) expressing clones were compared for their expression of the above markers with mock cells. If either of HIFs increased CSC characteristics, then we can implicate that they are the key TF in driving the stemness in the NS, SUS and HYP cells.

### 5.3.3 Determining mesenchymal properties of HIF-transfected clones

If HIF1\(\alpha\) or HIF2\(\alpha\) truly drives EMT signalling pathways and results in an EMT program, the positive clones should express high EMT markers. In other words the positive clones should be growing as mesenchymal phenotypes. In order to determine the mesenchymal properties and to determine which HIF induces EMT three important EMT markers like CD44, Vimentin and E-Cadherin to N-cadherin switching were tested for both HIF1\(\alpha\) and HIF2\(\alpha\) positive clones with Mock cells as control. CD44 cells surface marker expression was analysed using FACS flow cytometry protocol. The rest of the markers vimentin, E-cadherin and N-cadherin were analyzed by western blot to detect protein level expression. In addition cells that have undergone EMT and acquired a mesenchymal phenotype should display increased migratory and invasive characteristics. To confirm if the HIF positive clones are truly mesenchymal in nature we carried out \textit{in vitro} wound healing assay (scratch assay) for migration...
and transwell invasion assay (Boyden chamber assay) to determine invasion. For scratch assay cells were grown in a 6 well plate and incubated for 24h. The cells were serum starved for 24h and a scratch wound was made using a 200 µl pipette tip to create a cell-free linear uniform gap. The progress of cell migration into the scratch was photographed at regular intervals following the protocol mentioned in chapter 2. The HIF transfected cells were subjected in vitro cell invasion assay as described in chapter 2 using 8.0uM pore size transwell filter inserts coated with matrigel in 24 well plates. Briefly, the invasion rate of Mock and HIF transfected cells that invaded through matrigel basement matrix was assessed according to the manufacturer’s recommended protocol.

5.3.4 MTT cytotoxicity assay for hypoxic cell cultures

The true aspect we are interested in for our study is the chemoresistance induced by CSCs. If cells transfected with HIFs activated EMT and resulted in CSC phenotypes they should also show the chemoresistant characteristics of CSCs. We aim to assess whether the cells with increased HIF expression could mimic the chemoresistant nature of CSCs. To determine the drug sensitivity, the cells were cultured in at a cell density of 2.5 x 10^3 cells/well in 96-well plate for overnight and exposed to anticancer drugs for another 120 hours before MTT assay. TMZ and other conventional anticancer drugs VCR, PAC and DOX were used in the same concentrations as used for sphere cell MTT assay and hypoxic conditions MTT assay. A parallel MTT assay was performed for mock transfected cells.
5.4 Results

5.4.1 Stable transfection of U373 GBM cell line with HIF1α

U373 GBM cell lines were transfected with pCMV6/ HIF1α recombinant vector and empty pCMV6 vectors and clones were selected and enlarged in media containing 250μg/mL G418. The selected clones were screened for overexpression of HIF1α protein by western blot analysis in the cytoplasmic level using the mock transfected cells as control. Positive clones were selected enlarged and screened once again to select cells with high level of nuclear translocation of HIF1α functional transcription factor using the nuclear extracts of selected cells. Western blot results in Figure 5.1 shows the cytoplasmic and nuclear level HIF1α protein expression. As seen in the figure, when compared to mock two clones namely C10 and C17 had the highest HIF1α expression levels and were selected for further experiments.

![Western blot analysis](image)

Fig 5.1 Expression status of HIF1α protein in HIF1α transfected U373MG GBM cell lines. Western blot analysis shows increased expression and nuclear translocation of HIF1α under normoxia conditions in selected clones C-10 and C-17 in comparison to that of Mock transfected cells. Tubulin and Nucleolin was used as loading control.
5.4.2 HIF1α induces CSC characteristics in GBM cells

In order to analyse if HIF1α can play role in inducing CSC traits we tested the presence of CSC characteristics in U373/pCMV6/HIF1α positive clones C10 and C17. For all the results below we used U373/pCMV6/Mock as a control which was grown along in parallel along with the HIF1α clones C10 and C17. As seen from the FACS analysis data in figure 5.2 HIF1α expressing clones showed statistically significant increase in the expression level of universal CSC marker ALDH which was detected by ALDEFLUOR activity. In addition to ALDH the expression of another cell surface CSC marker CD133 which is specific to neural progenitor cells was analysed by FACS using PE-conjugated CD133 antibody. The results as shown in figure 5.2 displays an increased expression of CD133 in HIF1α clones C-10 and C-17 in comparison to the mock transfected cells. In addition the expression of embryonic CSC markers namely Sox2, Oct4 and Nanog also increased remarkably in the HIF1α expressing clones than the mock cells (Figure 5.3). These proteins which are restricted to progenitor cells are important for stem cell maintenance, renewal and pluripotency. Our results indicate that all CSC characteristics are increased just by a simple overexpression of HIF1α protein under normoxia, implying that HIF1α may play a pivotal role in driving these CSC related proteins and thereby induce CSC characteristics in cells when they are subjected to hypoxic conditions.
Fig 5.2 High ALDH activity and CD133 expression were detected in HIF1α transfected U373MG cell lines. ALDH activity and expression of CD133 were measured by ALDEFLUOR assay and CD133 immunostaining using FACS analysis, respectively. The bar chart displays the percentage of ALDH and CD133 cell populations. n= 9, **p<0.01
Fig 5.3 Expression of Embryonic CSC markers in HIF1α transfected U373MG cell lines.

CSC markers were measured using FACS analysis. The bar chart below displays the statistically significant increase in all three embryonic CSC markers like SOX2, OCT4 and NANOG in HIF1α positive clones. n=6 **p<0.01
5.4.3 HIF1α transfected cells displayed mesenchymal properties

To establish the role of HIF1α and EMT we analysed all the EMT markers and invasive and migratory properties of U373/pCMV6/HIF1α positive clones C10 and C17 in comparison to the mock transfected cells. FACS analysis for the EMT cell surface marker CD44 showed an increased expression of CD44 in C10 and C17 clones with high HIF1α (Figure 5.4). In addition, expression of other mesenchymal proteins like N-cadherin and vimentin also increased in HIF1α clones which is evident from the western blot results shown in Figure 5.5. Very similar to that of sphere cells and ATT-HYP cells with increased HIF1α lost the epithelial marker E-cadherin. Whereas in control mock transfected cells there was no loss of E-cadherin and hence they remain epithelial in nature. These results indicate that high levels of HIF1α can transform the cells into a more mesenchymal phenotype which could be the reason behind EMT phenotypes leading to CSC characteristics.

Further confirmation for the role of HIF1α signalling in inducing EMT phenotypes was done by analysing the mesenchymal based migration and invasion potential of these HIF1α positive clones (Figure 5.6A). The images taken using the inverted microscope for wound healing scratch assay were analysed using Image J software. This program measures the migratory distance and calculates the area and pixels for regions with no migrations as numerical value which enables us to calculate the %migration between HIF1α clones and Mock cells. Results from the above analysis (Figure 5.6B) showed significant increase in the migratory potential of cells when there is high HIF1α.
expression. Invasion potential of these cells measured by matrigel invasion assay and the images taken from that experiment is shown in Figure 5.7A. There is a clear increase in the invasion potential of HIF1α clones C10 and C17 in comparison to the mock cells. We also lysed the stained cells according to the protocol and measured the OD at 540nm using a multiwall plate reader. The results were given as % invasion (Figure 5.7B) confirms the increased invasion potential of cells with high HIF1α expression.

Fig 5.4 Expression of CD44 in HIF1α transfected U373 cell lines: FACS analysis using CD44-FITC conjugated antibody show increased expression of CD44 cell surface marker in HIF1α positive clones C10 and C17 in comparison to Mock transfected cells. n=9 **p<0.01
**Fig 5.5 Expression of EMT markers in HIF1α transfected U373 cell lines.** EMT markers were detected from whole cell lysates of HIF1α transfected clones and mock cells using western blotting assay. Tubulin was used as loading control.

5.6A)
5.6 B)

![Graph showing migration rate of U373-pCMV6-HIF1α- cells](image)

**Fig 5.6** HIF1α enhances the migration potential of U373MG cells *in vitro*. A) Migration ability of HIF1α transfected U373 GBM cell line determined by the images from (x100 magnification) wound healing assay. The images show increased migration of HIF1α clones into the scratch when compared to the mock cells. B) Rate of migration (%) determined by analysis of wound healing assay images using image J software shows statistically significant increase in the migratory potential of HIF1α clones when compared to the mock cells. *n=6, **p<0.01*

5.7 A)

![Images of cell cultures](image)
**Fig 5.7 HIF1α enhances the invasive activity of U373MG cells in vitro.** The invasive activity of GBM cells was detected using matrigel Invasion assay. A) Morphology of the membrane penetrated GBM cells (×40 magnification). B) Invasive index shows statistically significant increase in the invasive potential of HIF1α transfected cells in comparison to mock cells. n=6; **p<0.01

### 5.4.4 HIF1α expression did not induce multidrug resistance in GBM cells.

From above results we know that HIF1α expression can definitely induce EMT and results in CSCS characteristics. So we speculated that high HIF1α expression plays an important role in promoting chemoresistant GBM CSCs. To determine this we measured the drug sensitivity of HIF1α positive clones compared to that of Mock cells using MTT analysis. Although HIF1α cells displayed increased CSC properties they did not show any significant increase in resistance to any of the four drugs tested. As seen from the results shown in Figure 5.8 there was no real difference between the drug sensitivity curves and the IC50 values of HIF1α clones and Mock cells are shown in the corresponding bar charts Figure 5.8B. The HIF1α transfected clones treated with the first line drug TMZ showed a slight increase in the IC 50 for C17 and also a very minor
resistance trend. But at higher doses the cell viability curve dropped even below the mock. The slight variation seen in IC50 values for TMZ was not statistically significant due to an increased standard deviation. However the IC50 of HIF1α transfected cells to other drugs namely DOX, VCR and PAC was even lower than the mock cells. The curves also indicated that cells with HIF1α were more sensitive to these drugs contradictory to that of various literature resources as well as resistance shown by cells grown under hypoxic conditions. These results indicate that HIF1α may play a role in inducing CSC features through HIF1α mediated signalling pathways but do not play a role in chemoresistance in hypoxia induced EMT.

5.8A)
Fig 5.8 In vitro cytotoxicity of TMZ, VCR, PAC and DOX in HIF2α transfected U373MG cell lines. (A). Cell viability curves after the cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay. B) The bar charts display the IC50s of different drugs in the transfected cells. n=9 **p<0.01
5.4.5 Stable transfection of U373 GBM cell line with HIF2α

U373 GBM cell lines were transfected with pCMV6/ HIF2α recombinant vector and empty pCMV6 vectors and clones were selected and enlarged in media containing 250ug/mL G418. The selected clones were screened for overexpression of HIF2α protein by western blot analysis in the cytoplasmic level using the mock transfected cells as control. Positive clones were selected enlarged and screened once again to select cells with high level of nuclear translocation of HIF2α functional transcription factor using the nuclear extracts of selected cells. Western blot results in Figure 5.9 shows the cytoplasmic and nuclear level HIF2α protein expression. As seen in the figure, when compared to mock two clones namely C14 and C26 had the highest HIF2α expression levels and were selected for further experiments.

5.4.6 HIF2α induces CSC characteristics in GBM cells

We already established the fact that HIF1α can drive an EMT program and result in mesenchymal phenotypes with increased CSC characteristics. But HIF1α was not able to mimic the chemoresistant characteristic of the CSCs. Under hypoxic conditions HIF2α is also known to induce EMT. Hence we hypothesised that HIF2α could be another possible transcription factor that can result in CSC phenotypes with chemoresistance characteristics. In order to analyse if HIF2α can play role in inducing CSC traits we tested the presence of CSC characteristics in U373/pCMV6/HIF2α positive clones C14 and C26. For all the results below we used U373/pCMV6/Mock as a control which was grown
along in parallel along with the HIF2α clones C14 and C26. As seen from the FACS analysis data in Figure 5.10 HIF2α clones C14 and C26 showed statistically significant increase in the expression level of universal CSC marker ALDH and neural progenitor marker CD133 in comparison to the mock transfected cells. In addition the HIF2α transfection also induced the expression of embryonic CSC markers namely Sox2, Oct4 and Nanog increased remarkably in the HIF2α expressing clones than the mock cells (Figure 5.11). These proteins which are restricted to progenitor cells are important for stem cell maintenance, renewal and pluripotency. Results from western blot analysis for in Figure 5.12 also confirm the protein level expression of these embryonic markers.

U373-pCMV6/HIF2α

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Fig 5.9 Expression status of HIF2α protein in HIF2α transfected U373MG GBM cell lines. Western blot analysis shows increased expression and nuclear translocation of HIF2α under normoxia conditions in selected clones C-14 and C-26 in comparison to that of Mock transfected cells. Tubulin and Nucleolin was used as loading control.
**Fig 5.10** High ALDH activity and CD133 expression were detected in HIF2α transfected U373MG cell lines. ALDH activity and expression of CD133 were measured by ALDEFLUOR assay and CD133 immunostaining using FACS analysis, respectively. The bar chart displays the percentage of ALDH and CD133 cell populations. n= 9, **p<0.01
Fig 5.11 Expression of Embryonic CSC markers in HIF2α transfected U373MG cell lines.

The CSC markers were measured using FACS analysis. The bar chart below displays the statistically significant increase in all three embryonic CSC markers like SOX2, OCT4 and NANOG in HIF2α positive clones. $n=6$ **$p<0.01$**
Fig 5.12 Western blot analysis of embryonic stem cell markers in HIF2α transfected U373MG cells. Western blot analysis shows increased expression of embryonic stem cell proteins Sox2, Oct4 and Nanog in HIF2α transfected clones C14 and C26 in comparison to Mock. Tubulin was used as a loading control.

5.4.7 HIF2α transfected cells displayed mesenchymal properties

To establish the role of HIF2α and EMT we analysed all the EMT markers and invasive and migratory properties of U373/pCMV6/HIF2α positive clones C14 and C26 in comparison to the mock transfected cells. FACS analysis for the EMT cell surface marker CD44 showed an increased expression of CD44 in C14 and C26 clones with high HIF2α expression (Figure 5.13). In addition expression of other mesenchymal proteins like N-cadherin and Vimentin also increased in HIF2α clones which is evident from the western blot results shown in Figure 5.14. Very similar to that of sphere cells and ATT-HYP cells with increased HIF2α expression lost the epithelial marker E-cadherin. Whereas in control mock transfected cells there was no loss of E-cadherin and hence they
remain epithelial in nature. These results indicate that if HIF2α expression can transform the cells into a more mesenchymal phenotype which could be the reason behind EMT phenotypes leading to CSC characteristics.

Further confirmation for the role of HIF2α signalling in inducing EMT phenotypes was done by analysing the mesenchymal based migration and invasion potential of these HIF2α positive clones. The images taken using the inverted microscope for wound healing scratch assay (Figure 5.15A) were analysed using Image J software. This program measures the migratory distance and calculates the area and pixels for regions with no migrations as numerical value which enables us to calculate the %migration between HIF2α clones and Mock cells. Results from the above analysis (Figure 5.15B) showed significant increase in the migratory potential of cells when there is high HIF2α expression. Invasion potential of these cells measured by matrigel invasion assay and the images taken from that experiment is shown in Figure 5.16A. There is a clear increase in the invasion potential of HIF2α clones C14 and C26 in comparison to the mock cells. We also lysed the stained cells according to the protocol and measured the OD at 540nm using a multiwall plate reader. The results were given as invasion index (Figure 5.16B) which confirms the increased invasion potential of cells with high HIF2α expression.
Fig 5.13 Expression of CD44 in HIF2α transfected U373 GBM cell lines. FACS analysis using CD44-FITC conjugated antibody shows increased expression of CD44 cell surface marker in HIF2α transfected clones C14 and C26 in comparison with mock cells. n=6, **p<0.01

Fig 5.14 Expression of EMT markers in HIF2α transfected U373 GBM cell lines. The EMT markers were detected from whole cell lysates of HIF2α transfected clones and mock cells using Western blotting assay. Tubulin was used as loading control.
Fig 5.15 HIF2α enhances the migration potential of U373MG cells in vitro. A) Migration ability of HIF2α transfected U373 GBM cell line determined from the images (x100 magnification) of wound healing assay. The images show increased migration of HIF2α clones into the scratch when compared to the mock cells. B) Rate of migration (%) determined by analysis of wound healing assay images using image J software shows statistically significant clear increase in the migratory potential of HIF2α clones when compared to the mock cells. 

*n=8, **p<0.01*
Fig 5.16 HIF2α enhances the invasive activity of U373MG cells in vitro. The invasive activity of the GBM cells was detected using matrigel Invasion assay. A) Morphology of the membrane penetrated GBM cells (×40 magnification). B) Invasive index shows statistically significant increase in the invasive potential of HIF2α transfected clones in comparison to that of mock cells. n=6; **p<0.01

5.4.8 HIF2α expression did not induce multidrug resistance in GBM cells.

From above results we know that HIF2α expression can definitely induce EMT and results in CSCS characteristics. So we speculated that high HIF2α expression plays an important role in promoting chemoresistant GBM CSCs. To
determine this we measured the drug sensitivity of HIF2α positive clones compared to that of Mock cells using MTT analysis. The expression of HIF2α in these cells induced a significant resistance to the first line drug TMZ. Many studies in TMZ mediated resistance refers to the activation of resistance gene MGMT that confers resistance to alkylating agents, specifically to TMZ. MGMT is one of the major reasons for chemotherapy failure in GBM at present because TMZ is the only drug known to improve the survival rate in GBM by a few months. We were interested to study whether HIF2α can drive the activation of MGMT gene. But after several trials we did not detect any MGMT increase in these HIF2α positive clones. This could be an important finding because in some cases of GBM nowadays MGMT independent resistant nature has been reported in patients. Possibly HIF2α could be one of the reasons behind this phenomena according to our results. But HIF2α did not increase the resistance for other three drugs tested namely PAC, VCR and DOX. Although HIF2α cells displayed increased CSC properties they did not show any significant increase in resistance to any of the four drugs tested. As seen from the results shown in Figure 5.17 there was no real difference between the drug sensitivity curves and the IC50 values of HIF2α clones and Mock cells shown in the corresponding bar charts. These results indicate that HIF2α may play a role in inducing CSC features through HIF2α mediated signalling pathways but do not play a role in chemoresistance in hypoxia induced EMT.
**U373MG/pCMV6/HIF2α TMZ**

**U373-pcMV6-HIF2α VCR**

**U373-pcMV6-HIF2α PAC**

**U373-pcMV6-HIF2α DOX**

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**U373MG/pCMV6/HIF2α**

**TMZ IC50**

**U373-pcMV6-HIF2α VCR IC50**
5.5 Discussion

Hypoxia is one of the physiological mechanisms that can induce EMTs in tumours through multiple distinct mechanisms. In the previous chapter we had shown that both HIF1α and HIF2α nuclear translocation increased in both hypoxic and sphere cell cultures. With above facts in mind we hypothesized that either HIF1α or HIF2α could be the possible driving factor behind hypoxia EMT leading to chemoresistance. We studied the EMT and CSC inducing abilities of HIF1α and HIF2α individually through ectopic overexpression of these TFs by stable transfection of U373MG GBM cell line. Cellular responses to O₂ concentrations are tightly regulated by PHDs that degrades HIFα subunits of HIF transcription factors under normal O₂ concentrations. PHDs are extremely O₂ sensitive and allow the binding of HIFα subunits with HIFβ subunits only
under severe O$_2$ deprivation. But stable transfected clones of GBM cells overexpressed significant amount of HIF1$\alpha$ and HIF2$\alpha$ under normoxic conditions so that they can outcompete the amount of PHDs and translocate into the nucleus to form functional HIF TFs that activates hypoxia responsive genes. We had picked about 48 clones for each successful transfection and screened them to select two clones with the highest expression and nuclear translocation of HIFs. Two positive clones and one mock transfected clone was analysed for both HIF1$\alpha$ and HIF2$\alpha$ separately. Confirmation for the nuclear translocation of HIFs in the selected clones was determined with their nuclear protein by western blot. Several studies have previously shown that HIFs modulates the process of EMT by regulating the expression of MET associated TFs such as TWIST, SNAIL, SLUG and ZEB. Constitutive expression of HIFs was shown to induce EMT in various cell lines by increasing their invasive and migratory potential. However, there is no solid evidence for EMT in GBM which encouraged us to find out if hypoxia induced EMT can occur in GBM cells giving rise to CSC traits via HIF regulated EMT. With confirmation of higher HIF1$\alpha$ nuclear translocation in clones compared to mock, we wanted to determine if there is any EMT mediated phenotypic change occurring to those HIF1$\alpha$ clones. Our results clearly established that cells with elevated HIF1$\alpha$ display an increased CD44, vimentin and N-cadherin expression indicating switching to a mesenchymal phenotype while losing E-cadherin which is a hallmark of epithelial phenotype. Along with these results we also confirmed through the migration and invasion assays that these cells have increased migratory and invasion potential which is another trademark characteristic of mesenchymal phenotypes. We finally confirmed the CSC characteristics of these cells by
increased expression of CSC markers like ALDH, CD133, SOX2, Nanog and Oct4. Bar et al., (2010) reported that they could recapitulate the hypoxia-dependent increase in CD133-positive population under normoxic conditions simply by over expressing an oxygen stable form of HIF-1α thus suggesting that HIF-1α is sufficient to mediate EMT. Yang et al. (2008) reported that HIF1α directly activates TWIST which in turn represses E-cadherin. They mentioned that reduced HIF1α could revert these cells to a pre-hypoxic status where all the mesenchymal markers are replaced with epithelial markers. Chen et al., (2010) showed that HIF1α could activate SNAIL, SIP1 and SLUG in order to repress E-cadherin and activates MMPs that leads to cell migration and breakdown of ECM.

Sahlgren et al., (2008) reported the effects of constitutively activated HIF1α pathway in cervical, colon, GBM, ovarian and breast cancer cell lines. They reported the activation of Notch signalling by HIF1α which is a known activator of SNAIL, LOX and ZEB EMT TFs leading to increased motility and invasiveness of the tumour cells. In addition to Notch, HIF1α target genes include other CSC factors like oct4, c-Myc, Nanog and CD133. Several consistent studies with various cell lines and CSCs has shown the CSC enhancing effects of HIF1α and on the other hand several evidences are also presented for attenuation of CSC phenotypes with conditional knock down of HIF1α (Chen et al., 2007; Soeda et al., 2009; Li et al., 2010., Mathieu et al., 2011; Covello et al., 2006; Gordan et al., 2008). All these studies along with our obtained results suggest that HIF1α is required for the activation of EMT and subsequent maintenance of the CSC phenotypes. But this was not settled
completely, because the main aim of our work was to understand the molecular mechanisms behind CSC induced chemoresistance to anticancer drugs. Many studies in recent years, demonstrated the contribution of HIF-1 to drug resistance in a range of neoplastic cells (Sullivan et al., 2008; Brown et al., 2006; Hao et al., 2008; Liu et al., 2008; Sasabe et al., 2007). One of the earliest observations of HIF1α and drug resistance was done by Wenger and colleagues where the efficacy of carboplatin and etoposide was enhanced by inactivation of HIF-1α in mouse embryonic fibroblasts (Unruh et al., 2003). Most of these studies focused mainly on the reversal of hypoxia-induced drug resistance by targeting HIF-1α through RNA interference or inhibitors. However some studies in neuroblastoma and lung adenocarcinoma cells could not reproduce the fact that inactivation of HIF-1α had positive effect on drug response (Chang et al., 2006; Hussein et al., 2006). Meanwhile some studies in fibrosarcoma, gastric cancer or breast carcinoma cells reported that inhibiting HIF-1α even under normoxic conditions had positively enhanced the sensitivity of chemotherapeutic agents showing the resistance-mediating effect of HIF-1α under normoxia (Brown et al., 2006; Li et al., 2006; Liu et al., 2008; Rohwer et al., 2010).

Our results indicated that none of the HIF1α positive clones showed resistance to anticancer drugs which was completely contradictory to our results obtained from HYP and sphere cells. Despite the fact that HIF1α induced significant changes to activate CSC phenotypes it failed to trigger any drug resistance which is a clinically significant hallmark characteristic of CSCs. From the above facts reported by several groups the overall conclusion of these observations is
that HIF-1α has the ability to promote drug resistance in cancer cells through various reported mechanisms like drug efflux, defective apoptosis, DNA damage inhibition and reduced ROS activation. However, there are some reports that indicated that HIFs are not necessary for inducing drug resistance. We even observed that some of the HIF1α clones were even more sensitive to the drugs that were tested. This could be probably due to the variations in the tumour cell types and limitations of cell line models for transfection experiments. There are even some contradictory reports indicating that the expression of HIF1α could enhance the susceptibility of tumour cells to chemotherapy. A recent study by Evens et al. (2010) showed that increased HIF-1α expression in patients with diffuse large B-cell lymphoma treated with rituximab and anthracyclines had improved progression-free survival and overall survival rates (Evens et al., 2010). This indicates that HIF1α may not be the only factor responsible for chemoresistance mediated by CSCs and hence targeting HIF1α necessarily reverse chemoresistance in GBM CSCs.

The above findings pushed us to understand the status of other HIF increased under hypoxia which is HIF2α. Although both HIF1α and HIF2α TFs activate the HRE-dependant gene expression, there is considerable difference in their trans-activation domains that indicates the requirement of distinct transcriptional co-activators and unique targets for each of them. But unlike the well studied HIF1α, the role of HIF2α in hypoxia mediated signalling pathways is not well established. We wanted to know if our HIF2α transfected clones will have the same EMT and CSC effect that eventually drives resistance to anticancer drugs. In our results we found that HIF2α overexpression led to a decrease in
the expression of epithelial marker E-cadherin and an increase in the expression of EMT markers like CD44, Vimentin and N-cadherin indicating the switching of HIF2α positive clones to a mesenchymal phenotype. Many studies reported that HIF2α in addition to HIF1α or independently can also activate the target genes involved in EMT like TWIST, ZEB, SNAIL and SIP1 (Kim et al., 2009). In addition we also observed that the HIF2α positive clones had increased CSC characteristics evident by the presence of markers like CD133, ALDH, Sox2, Oct4 and Nanog. Heddleston et al used a similar approach with oxygen stable form of HIF-2α and found an increased percentage of CD133-positive cells with HIF2α whereas CD133-negative cells did not express HIF2α. This HIF-2α expression also resulted in increased expression of CSC factors like cMyc, Nanog and Oct4 (Heddleston et al., 2010). Multiple recent studies in CSC isolated from GBM, neuroblastoma, renal cancer and non-small lung cancer show that HIF2α is required for the maintenance of the CSC phenotype and its functions (Kim et al., 2009; Li et al., 2009; McCord et al. 2009; Pietras et al., 2008). Li et al., (2009) examined the expression of HIF2α mRNA in cells extracted from primary GBM patients and xenografts of GBM cell lines and showed that HIF-2α mRNA is expressed highly in CD133-positive cells than in CD133-negative cells, whereas HIF-1α mRNA was uniformly present in both CD133-positive and CD133-negative cells. They also showed that HIF-2α mRNA levels increased significantly in response to hypoxia in CD133-positive cells, and its levels were stable even after return to normoxia. Similar findings were made by McCord et al., where HIF-2α protein was present even under physiological O₂ levels (7%). whereas HIF-1α was barely detected. This indicates that CD133-positive GBM cells can maintain their CSC signature even
at physiologic oxygen levels due to enhanced activation of HIF-2α. Seidel et al., (2010) showed that the presence of HIF-2α but not HIF-1α can significantly induce the expression CSC panel of genes generating a side population.

In contrast to numerous reports on the well-established role of HIF-1α in anticancer therapy, the significance of HIF-2α in chemoresistance is remarkably understudied and poorly understood. One of the earlier studies in head-and-neck cancer samples by Harris et al., in 2002 threw light on the role of HIF2α in response to anti-proliferative therapy. The expression of HIF-2α highly correlated with an incomplete response to chemoradiotherapy of these cancers (Koukourakis et al., 2002). But various recent studies analyzed the role of HIF2α on a functional level. Bertout et al., used human clear cell renal cell carcinoma (ccRCC) cells that expressed HIF-2α under normoxia but lacks VHL- and HIF-1α to show that functional inhibition of HIF-2α significantly enhanced the sensitivity of these cells (Bertout et al., 2009). It was also shown that inhibition of HIF-2 promoted p53-mediated apoptosis by ROS accumulation in these cells (Rohwer et al., 2010). In our experiments with HIF2α transfected GBM clones, we observed HIF2α can significantly increase the resistance to the first line drug TMZ. However we did not observe multidrug resistance in these HIF2α clones like the CSCs. These cells were in fact sensitive to the drugs like DOX, VCR and PAC. These results were similar to that of HIF1α clones, indicating that HIF2α might be an important factor in activating EMT and CSC characteristics, but does not play a significant role in hypoxia induced chemoresistance. The possible explanation for our observations could be the
fact that there may be another important resistance factor equally important as that of HIFs which is activated under hypoxia. ALDH which is a universal CSC marker could be one of the possible factors in chemoresistance that can be thought of, due to several reports made in literature regarding the role of ALDH in chemoresistance. In our experiments both HIF1\(\alpha\) and HIF2\(\alpha\) transfected cells carried significantly enhanced ALDH activity, but still no resistance was observed. So there must be other TFs like NF-kB, TGF-beta, TNF-\(\alpha\), etc, that could possibly link up to the resistance induced under hypoxia. Further studies are required to confirm these facts.

5.6 Conclusions

Therefore, the overall conclusion from this study could be that when there is reduced oxygen environment for a given cell type, both HIF-1\(\alpha\) and HIF-2\(\alpha\) will be activated which directly or indirectly activates the expression of hundred of genes. Some of these targets overlap between these two factors due to the similar DNA binding sequence due to structural homology. Therefore the biological consequences of hypoxia in cells largely depend on which HIF is active at any given time and what targets are being regulated by them. While HIF-2\(\alpha\) protein is more stable and widely expressed range of \(O_2\) levels, HIF-1\(\alpha\) is tightly regulated by \(O_2\) levels and is expressed in cells experiencing moderate hypoxia. Despite these differences and not playing important role in chemoresistance in GBM cells tested, we cannot deny the fact that both HIF-1\(\alpha\) and HIF-2\(\alpha\) are crucial for EMT, CSCs, tumour motility and invasion suggesting that pharmacological inhibition of these factors in addition to suppression of chemoresistance factors holds great promise in targeting GBM CSCs.
Chapter 6

The role of NF-κB in hypoxia induced EMT and GBM CSCs
6.1 Introduction

Our findings from chapter 5 about the role of primary hypoxia response genes HIF1α and HIF2α in hypoxia induced EMT and CSCs has revealed that HIF1α and HIF2α play a central role in altering the epithelial phenotype of cells to more mesenchymal phenotype. We also showed that HIF1α and HIF2α mediated phenotype alteration leads to CSC characteristics in cells indicating the possible role of these signalling pathways in hypoxia induced CSCs of GBM. Although they expressed CSC markers and had invasive characters, these cells failed to induce significant amount of chemoresistance or multidrug resistance, which is an important feature of CSCs. Although several literature findings supported the role of HIF1α and HIF2α in inducing chemoresistance and projected them as important targets to improve GBM therapy, we did not find convincing evidence from our in vitro model. Additionally, these findings do not match with the findings from NS, SUS and HYP cells that displayed both resistance and CSC traits, indicating that there are some other factors involved in regulating the responsiveness to drugs. Since chemoresistance of GBM CSCs is the aspect we are interested in for our study we had to search for another possible factor that should be regulated by hypoxia but must be independent of HIF1α or HIF2α signalling. This could possibly be another transcription factor that is expressed in parallel to HIFs at a higher level than HIFs. There are more than 20 different transcription factors involved directly or indirectly in hypoxic sensitivity and one suitable TF candidate could be NF-κB which is a common TF reported to be upregulated in several types of cancers.
The activation of NF-κB by hypoxia has been known for more than a decade in a wide variety of cells (Koong et al., 1994). Several studies have suggested that hypoxia could activate NF-κB signalling pathway in a wide range of cancer cells. The NF-κB is well-known for its function as a critical transcription factor in a wide range of cancers that regulates the expression of several genes involved in cancer cell survival, proliferation, invasion, migration, angiogenesis and tumour metastasis, leading to resistance to chemo-radiotherapy, tumour relapse and poor prognosis (Baldwin, 2001). Additionally, emerging data hint that NF-κB may also play an important role in EMT and CSCs, contributing to aggressiveness of tumours. Hence it is reasonable to speculate that hypoxia induced NF-κB activation may contribute to the maintenance of CSCs and EMT during the development and progression of tumours especially because NF-κB signalling pathways are well-studied chemoresistant factors and are known to enhance the induction of EMT phenotype and maintenance of stem cell phenotype. NF-κB is a structurally conserved family of heterodimeric transcription regulators which play important roles in large number of normal cellular processes namely immune response, inflammation, cellular growth and apoptosis. They are typically activated following stimulation of cells with pro-inflammatory cytokines such as TNF-α and IL-1β, antigens, bacterial liposaccharides, growth factors, UV light, and ROS (Ghosh et al., 2002; Chen and Greene, 2004). Since NF-κB is a primary regulator of inflammatory and anti-apoptotic gene expression it is speculated that NF-κB activation is very essential for a hypoxic cell to inhibit apoptosis that enable them to survive through the period of hypoxic insult (Koong et al., 1994). Moreover, hypoxic activation of NF-κB signalling pathway is mediated by the regulation of gene
expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, adhesion molecules, enzymes, and pro-inflammatory enzymes which finally result in a hypoxic inflammatory response (Taylor et al., 2008).

6.1.1.1 NF-κB and EMT

Multiple evidence from literature point out that NF-κB is a master transcriptional regulator of several other developmentally important EMT inducing transcription factors. These factors like Snail, Slug, ZEB and Twist are part of the growing list of genes that are recognised to be direct transcriptional targets of NF-κB which upon activation lead to suppression of various epithelial genes like cadherins, claudins, occludins and muc1 (Nieto, 2002; Culleres et al., 2006; Kajita et al., 2004; Chua et al., 2007; Yang et al., 2004; Wang et al., 1997; Kanegae et al., 1998; Takeda et al., 1999; Sosic et al., 2003). Additionally NF-κB is also known to directly regulate end stage mesenchymal genes such as Vimentin and MMPs (Lilienbaum et al., 1990; Chua et al., 2007; Himelstein et al., 1997; Connelly et al., 2007).

6.1.1.2 NF-κB and HIF signalling

It has been shown previously by many researchers that HIF-1α is required for the activation of NF-κB, which suggest HIF-mediated regulation of canonical NF-κB signalling by directly binding to the promoter region of NF-κB (Cockman et al., 2006). Some of the earlier findings also support that hypoxia regulates the NF-κB pathway through activation of IKK, specifically via IKK-β (Melvin et al., 2011; Cockman et al., 2006; Devries et al., 2010). The activation of NF-κB
mediated by HIF-1α has been shown to coincide with cancer cell survival, invasion, and tumour progression and therefore shown to be very important in the modulation of hypoxic tumour microenvironment (Scortegagna et al., 2008; Nam et al., 2011; Yeramian et al., 2011). Besides HIF-mediated activation of NF-κB, evidences from large number of experimental studies indicate that NF-κB could directly regulate HIF pathway in tumourigenesis. NF-κB could control the expression of HIFs and its downstream signalling pathways within the tumour microenvironment because the promoter region of HIF1α gene contains active NF-κB binding site at position −178/−188 (Belaiba et al., 2007, Bonello et al., 2007; Van et al., 2008). It is speculated that NF-κB drives HIF-1 signalling pathway to maintain the basal levels of HIF-1α under normoxia condition, and further increase the levels of HIF-1α under hypoxia (Cockman et al., 2006). Unlike the well studied interaction between HIF-1α and NF-κB, the link between NF-κB in HIF-2α still remains largely unexplored. In one study by Bracken et al., (2005) it was found that NF-κB interact with IKK-γ which leads to the transcriptional activation of HIF-2α. It was also shown that NF-κB could modulate HIF-2α protein levels indirectly by inducing HIF-1β mRNA levels. Saito et al., (2010) studied skeletal growth and osteoarthritis, where they found HIF-2α as a central transactivator in the ossification of chondrocytes and skeletal muscle development. They performed a screening for various TFs that could regulate the upstream of HIF-2α and found that NF-κB is the most potent activator of HIF-2α in the majority of the cells. They also identified an NF-κB motif in the promoter region of EPAS1 (HIF-2α gene) which is particularly found to be regulated by RelA. He et al., (2014) reported the link between NF-κB and
HIF-2α signalling in kidney cells where they found that NF-κB induced HIF2α is responsible for the protective effects of renal cells against oxidative injuries. Although considerable evidences exist from various sources for the presence of cross talk between NF-κB and HIF signalling the molecular mechanism behind this process has only been partly understood. Both NF-κB and HIF signalling pathways demonstrate different degree of sensitivity under hypoxia and it is evident from various studies they are interdependent on each other.

6.2 Rationale and aims of the study

With the findings from chapter 5 that HIF1α and HIF2α did not play a significant role in GBM resistance to anticancer drugs, we looked out for a suitable factor that was elevated under hypoxic conditions and also plays a role in EMT, CSCs and chemoresistance. Extensive evidence suggests that aberrant NF-κB activation could play a key role in various stages of tumour development by driving CSCs through EMT. Although a wealth of data is available to demonstrate the association between NF-κB and GBM, very few studies have focussed on the role of NF-κB mediated EMT in GBM. Especially in the case of GBM, studies always focus either on the relation between hypoxia and CSCs or hypoxia and NF-κB the link between hypoxia-NF-κB-EMT and CSCs is not studied well so far. In this study we aim to evaluate the role NF-κB in hypoxia induced EMT signalling and generation of CSC phenotypes in cells grown as NS, SUS or HYP. We would also like to establish its role in resistance to anticancer drugs to find out whether NF-κB functions as the driving force behind
chemoresistant GBM CSCs. There are possibilities that HIFs and NF-κB can be either connected to each other or unrelated in their modes of actions. We aim to determine whether HIFs are the driving force behind enhanced NF-κB activity or whether NF-κB is expressed as a separate hypoxia responsive transcription factor. We would like to understand the mechanism of action of the interplay between the regulation of NF-κB and HIF. By achieving the above mentioned aims we would like to ascertain whether NF-κB could be the ultimate target to reverse chemoresistance in GBM.

6.3 Experimental Design

Detailed information on materials, products, manufacturers and methodologies used for the entire study has been described in chapter 2. The following are specific experimental designs and methods used for this part of the study. Although we studied the CSC and hypoxic characteristics using all three GBM cell lines U87, U251 and U373, due to the limitations of time, funding and resources we used only the U373MG GBM cell line for all the functional work related NF-κB p65 ectopic overexpression.

6.3.1 Determining NF-κB activity in GBM spheres and hypoxic cells

The high NF-κB activity in NS, SUS and HYP cells from all GBM cell lines can be determined by analysing the nuclear translocation of NF-κB-p65 subunit which is an indicator of the NF-κB transcriptional activity. This can be done by western blot using nuclear protein extracts from all cells under study. ATT-GBM under NOR was used as a control for all samples of NS, SUS and HYP cells.
6.3.2 Stable transfection U373GBM cells with NF-κB-p65

NF-κB transcriptional activity is tightly controlled by various factors. In this study we aim to carry out ectopic overexpression of NF-κB-p65 in GBM cell lines in order to compete with the action of NF-κB repressor IκB-α and thereby achieving high nuclear translocation of and eventually driving the target genes of NF-κB signalling pathway. cDNA for NF-κB-p65 was obtained from OriGene™, UK and recombinant vectors were constructed by inserting the cDNA into pcDNA3.1(+)hygromycin mammalian expression vector. U373 GBM cells were cultured (1x10^6 cells/well) in 6 well plates without antibiotics overnight. 4ug of empty vector pcDNA3.1 and recombinant vector with NF-κB-p65 gene were introduced into the cells separately using Lipofectamine™ 2000 reagent. The transfected cells were incubated at 37°C for 24 hours selected for 7-10 days in a selective medium containing hygromycin 50ug/mL. Colonies of cells were picked up, enlarged and screened for over-expression of target gene in comparison with mock transfected clones using western blot. For the overexpression of NF-κB-p65 confirmation was done by analysing the nuclear translocation of p65 using western blot from nuclear extract of transfected cells. At least two positive clones with high nuclear translocation of p65 were selected for each gene and subjected to further analysis. The mock transfected cells containing empty pcDNA3.1 vector was grown in parallel for all experiments and used as controls for HIFs expression. All cells were grown under the selecting medium containing hygromycin throughout culturing time.
6.3.3 Luciferase reporter gene assay for NF-κB activity

Any increase in NF-κB transcriptional activity in GBM cells used in this study can be determined by luciferase reporter gene assay. Transfections of cells (1 × 10^4/well) were cultured in 96-well were performed using Lipofectamine 2000 reagent. The NF-κB and pGL3-Basic luciferase reporter vectors were co-transfected with pSV40-Renilla DNA, an internal control for normalization of the transcriptional activity of the reporter vectors. 24 hours after transfection, the cells were lysed and luciferase activity was determined using Dual Luciferase Assay kit protocol as mentioned in chapter 2. However, this method cannot be used for NS and SUS sphere cells due to their suspended culture conditions.

6.3.4 Analysis of CSC markers in NF-κB-p65 transfected cells

We already established the fact that NS, SUS and HYP cells have increased CSC characteristics (Chapter 3 and 4) possibly driven by HIFs (Chapter 5). In this study to establish whether these CSC characteristics are driven by NF-κB signalling we analysed the p65 clones for their expression of CSC markers like ALDH, CD133, Sox2, Oct4 and Nanog using immuno-fluorescence protocol for FACS as mentioned in chapter 2. Additional confirmation for the expression of Sox2, Oct4 and Nanog embryonic CSC markers was done by western blot analysis. If p65 transfected cells showed increased CSC characteristics than the control mock cells, then we can implicate that NF-κB could be a key TF in driving the stemness in the NS, SUS and HYP cells in addition to HIFs.
6.3.5 Determining mesenchymal properties of NF-κB-p65 clones

If NF-κB truly drives EMT signalling pathways and results in an EMT program, the positive clones should express high EMT markers. In other words the positive clones should be growing as mesenchymal phenotypes. In order to determine the mesenchymal properties, three important EMT markers like CD44, Vimentin and E-Cadherin to N-cadherin switching were tested for both p65 positive clones with Mock cells as control. CD44 cells surface marker expression was analysed using FACS flow cytometry protocol. The rest of the markers vimentin, E-cadherin and N-cadherin were analyzed by western blot to detect protein level expression. In addition cells that have undergone EMT and acquired a mesenchymal phenotype should display increased migratory and invasive characteristics. To confirm this we carried out in vitro wound healing assay (scratch assay) for migration and transwell matrigel invasion assay (Boyden chamber assay) to determine invasion following the protocol mentioned in chapter 2. Briefly, the invasion rate of Mock and NF-κB transfected cells that invaded through matrigel basement matrix was assessed according to the manufacturer’s recommended protocol.

6.3.6 MTT cytotoxicity assay for NF-κB-p65 transfected cells

The true aspect we are interested in for our study is the chemoresistance induced by CSCs. If cells transfected with NF-κB activated EMT and resulted in CSC phenotypes they should also show the chemoresistant characteristics of CSCs. We aim to assess whether the cells with increased NF-κB activity could
mimic the chemoresistant nature of CSCs. To determine the drug sensitivity, the cells were cultured in at a cell density of $2.5 \times 10^3$ cells/well in 96-well plate for overnight and exposed to anticancer drugs for another 120 hours before MTT assay. TMZ and other conventional anticancer drugs VCR, PAC and DOX were used in the same concentrations as used for sphere cell MTT assay and hypoxic conditions MTT assay. A parallel MTT assay was performed for mock transfected cells.

6.4 Results

6.4.1 High levels of NF-κB was observed in both NS and SUS cells

When compared to the ATT monolayer culture, both the NS cells and SUS cells isolated from all three GBM cell lines used in this study showed remarkable increase in the expression of NF-κB p65 protein. Figure 6.1 shows the western blot analysis from whole protein of ATT, NS and SUS cells which clearly shows an increased expression of NF-κB in NS and SUS in comparison to ATT cells. It can also be observed from Figure 6.1 that an increased nuclear translocation of NF-κB p65 is evident by detection of NF-κB proteins in the nuclear extracts of NS and SUS cells. In addition to this the NF-κB inhibitory molecule IκB-α is down regulated at the cytoplasmic level in NS and SUS samples which confirms the degradation of IκB-α proteins by IKK leading to increased nuclear translocation of NF-κB in these cells. Tubulin and Nucleolin were used as loading control.
Figure 6.1 Western blot analysis of NF-κB in GBM sphere cells: Western blot analysis shows that the sphere cells have increased expression and nuclear translocation of NF-κB-p65 and decreased expression of IκB-α in comparison to the ATT cells indicating high NF-κB activity. Nucleolin was used as loading control.

6.4.2 High levels of NF-κB was observed in hypoxic cells

Cells grown as ATT-HYP are tested for the increased expression and nuclear translocation of NF-κB by western blot analysis. Figure 6.2 show that all three GBM cell lines grown under ATT-HYP conditions show a significant increase in the expression of NF-κB p65 protein in the cytoplasm. In addition the western blot using nuclear extracts from these cultures showed significant nuclear translocation of NF-κB p65 in comparison to their corresponding ATT-Normoxia cultures. Tubulin and Nucleolin were used as loading control.
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Figure 6.2 Western blot analysis of NF-κB in hypoxia cultured GBM cell lines: Western blot analysis shows that the hypoxic cultures have increased expression and nuclear translocation of NF-κB in comparison to the normoxic cells. Nucleolin and tubulin were used as loading control.

6.4.3 Stable transfection of U373 GBM cell line with NF-κB-p65

U373 GBM cell lines were transfected with pcDNA3.1/ NF-κB-p65 recombinant vector and empty pcDNA3.1 vectors and clones were selected and enlarged in media containing 50ug/mL hygromycin. The selected clones were screened for overexpression of NF-κB-p65 protein by western blot analysis in the cytoplasmic level using the mock transfected cells as control. Positive clones were selected enlarged and screened once again to select cells with high level of nuclear translocation of NF-κB-p65 functional transcription factor using the nuclear extracts of selected cells. Western blot results in Figure 6.3 shows the cytoplasmic and nuclear level NF-κB-p65 protein expression in selected clones. The NF-κB transcriptional activities of positive clones were verified using the luciferase reporter gene assay which is shown in figure 6.4. As seen in the
figure, when compared to mock two clones namely C1 and C4 had the highest p65 expression, nuclear translocation and transcriptional activity and were selected for further experiments.

Fig 6.3 The expression status of NF-κB-p65 protein in p65 transfected U373MG GBM cell lines. Western blot analysis shows increased expression and nuclear translocation of NF-κB-p65 protein in selected clones C1 and C4 in comparison to that of Mock transfected cells. Tubulin and Nucleolin was used as loading control.

Fig 6.4 High NF-κB transcriptional activity in NF-κBp65 transfected U373MG GBM cell lines. NFκB transcriptional activity was determined by luciferase reporter gene assay. C1 and C4: p65 transfected clones.
6.4.4 NF-κB p65 transfected clones displayed mesenchymal properties

To establish the role of NF-κB signalling in EMT we analysed all the EMT markers and invasive and migratory properties of p65 transfected positive clones C1 and C4 in comparison to the mock transfected cells. FACS analysis for the EMT cell surface marker CD44 showed a statistically significant increase in the expression of CD44 in C1 and C4 clones with high NF-κB activity (Figure 6.5). In addition expression of other mesenchymal proteins like N-cadherin and Vimentin also increased in p65 clones which is evident from the western blot results shown in Figure 6.6. Clones C1 and C4 with increased NF-κB activity lost the epithelial marker E-cadherin very similar to that of sphere cells and ATT-HYP. Whereas in control mock transfected cells there was no loss of E-cadherin and hence they remain epithelial in nature. These results indicate that high NF-κB activity can transform the cells into a more mesenchymal phenotype which could be the reason behind EMT phenotypes leading to CSC characteristics.

Further confirmation for the role of NF-κB signalling in inducing EMT phenotypes was done by analysing the mesenchymal based migration and invasion potential of these p65 positive clones. The images taken using the inverted microscope for wound healing scratch assay were analysed using Image J software. This program measures the migratory distance and calculates the area and pixels for regions with no migrations as numerical value which enables us to calculate the percentage migration between p65 positive clones and Mock cells. Results from the above analysis (Figure 6.7A and B) showed significant increase in the migratory potential of cells when there is high
NF-κB activity. Invasion potential of these cells measured by matrigel invasion assay and the images taken from that experiment is shown in Figure 6.8. There is a clear increase in the invasion potential of p65 clones C1 and C4 in comparison to the mock cells. We also lysed the stained cells according to the protocol and measured the OD at 540nm using a multiwall plate reader. The results were given as invasion index (Figure 6.8 - bar chart) confirms the increased invasion potential of cells with increased NF-κB activity.

Fig 6.5 Expression of CD44 in NFκB transfected U373 GBM cell lines. FACS analysis using CD44-FITC conjugated antibody shows increased expression of CD44 cell surface marker in p65 transfected clones C1 and C4 in comparison with mock cells. n=6, **p<0.01
Fig 6.6 Expression of EMT markers in NFκB transfected U373 GBM cell lines. The EMT markers were detected from whole cell lysates of NFκB transfected clones and mock cells using western blotting assay. Tubulin was used as loading control.
6.7 B)

**Fig 6.7 NFκB enhances the migration potential of U373MG cells in vitro.** A) Migration ability of NFκB transfected U373 GBM cell line determined from the images (x100 magnification) of wound healing assay. The images show increased migration of NFκB clones into the scratch when compared to the mock cells. B) Rate of migration (%) determined by analysis of wound healing assay images using image J software shows statistically significant increase in the migratory potential of NFκB clones when compared to the mock cells. *n=8, **p < 0.01*

6.8 A)
Fig 6.8  NFκB enhances the invasive activity of U373MG cells in vitro. The invasive activity of the GBM cells was detected using matrigel Invasion assay. A) Morphology of the membrane penetrated GBM cells (×40 magnification). B) Invasive index shows statistically significant increase in the invasive potential of NFκB transfected clones in comparison to that of mock cells. n=6; **p<0.01

6.4.5 High NF-κB activity induces CSC characteristics in GBM cells

In order to analyse if NF-κB can play role in inducing CSC traits we tested the presence of CSC characteristics in NF-κB p65 positive clones C1 and C4. For all the results below we used Mock as a control which was grown in parallel along with the p65 clones. As seen from the FACS analysis data in Figure 6.9 p65 over expressing clones showed statistically significant increase in the expression level of universal CSC marker ALDH and neural progenitor marker CD133 when compared with mock transfected cells. In addition the expression of embryonic CSC markers namely Sox2, Oct4 and Nanog also increased remarkably in the p65 over expressing clones than the mock cells (Figure 6.10).
These proteins which are restricted to progenitor cells are important for stem cell maintenance, renewal and pluripotency. Results from western blot analysis for in Figure 6.11 also confirm the protein level expression of these embryonic markers.

**Fig 6.9** High ALDH activity and CD133 expression were detected in NFkB transfected U373MG cells. The ALDH activity and expression of CD133 were measured by ALDEFLUOR assay and CD133 immunostaining using FACS analysis, respectively. The bar chart displays percentage of ALDH and CD133 cell populations. n= 9, **p<0.01
Fig 6.10 Expression of Embryonic CSC markers in NFκB transfected U373MG cells. The CSC markers were measured using FACS analysis. The bar chart below displays the statistically significant increase in all three embryonic CSC markers like SOX2, OCT4 and NANOG in NF-κB positive clones. $n=6$ **$p<0.01$
Fig 6.11 Western blot analysis of embryonic stem cell markers in NFκB transfected U373MG cells. Western blot analysis shows increased expression of embryonic stem cell proteins Sox2, Oct4 and Nanog in NFκB transfected clones C1 and C4 in comparison to Mock. Tubulin was used as a loading control.

6.4.6 NF-κB induced multidrug resistance in GBM cells.

From above results we know that NF-κB activity can definitely induce EMT and results in CSCS characteristics. In addition previous result indicated that HIFs mediated signalling induced EMT and CSC phenotypes but not resistance. So we speculated that high NF-κB activity may play an important role in promoting chemoresistant GBM CSCs. To determine this we measured the drug sensitivity of p65 positive clones compared to that of Mock cells using MTT analysis. The cells were exposed to drugs for a period of 120hrs and then subjected to MTT assay. In addition to increased CSC properties, the p65 positive clones C1 and C4 also displayed statistically significant resistance to the first line drug TMZ and other conventional drugs like VCR and PAC. However, high NF-κB activity did not induce any significant resistance when treated with the drug DOX. As seen from the results shown in Figure 6.12 there is a clear difference between the drug sensitivity curves and the IC50 values of NF-κB clones and Mock cells.
treated with TMZ, VCR and PAC. But the IC50 values and sensitivity curve for DOX does not follow this resistance trend and has no change with NF-κB activity.

This indicates that additional mechanisms along with p65 may be involved in resistance to DOX. These results indicate that elevated NF-κB activity may play an important role in inducing CSC features and chemoresistance through NF-κB signalling pathways in hypoxia induced EMT.
**Fig 6.12** In vitro cytotoxicity of TMZ, VCR, PAC and DOX in NF-κB transfected U373MG cell lines. (A). Cell viability curves after the cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay. B) The bar charts display IC50s of different drugs in the transfected cells. n=9 **p<0.01

### 6.5 Interplay between HIFs and NF-κB signalling

Since both HIFs and NF-κB activities increased stemness in GBM cells, we were not able to confirm which is the master transcriptional factor in regulating hypoxia induced EMT and generating CSCs under hypoxic condition of NS and SUS cells. There are possibilities that HIFs and NF-κB could be connected to each other or unrelated in their mode of actions. In addition, it is evident from literature that NF-κB can regulate HIFs. The resistance pattern displayed by
HIFs did not match with the NS and SUS characteristics. Whereas NF-κB was able to induce resistance to three out of four drugs that we tested and thus gaining a upper hand as an important TF. In order to determine whether HIFs are the driving force behind enhanced NF-κB activity or whether NF-κB is expressed as a separate hypoxia responsive transcription factor we had to use the HIF1α, HIF2α and NF-κB p65 transfected U373 GBM cell lines and compared each other’s expression between them. If HIF1α or HIF2α regulates NF-κB activity, the transfected clones containing high HIF1α or HIF2α activity should have increased levels of NF-κB nuclear translocation and transcriptional activity. We can determine this using western blot analysis of nuclear extracts from HIF transfected cells. In addition, any increase in NF-κB transcriptional activity due to increased levels of HIF signalling in the HIF transfected clones can be determined using the luciferase reporter gene assay. Similarly if NF-κB is the driving factor behind HIF1α or HIF2α, the p65 transfected clones with high NF-κB activity should have increased nuclear translocation of HIF TFs in their nucleus. This can again be confirmed using western blot analysis of nuclear extracts from p65 transfected clones. This would help us to figure out among these three transcription factors which one could be the master regulator of hypoxia induced GBM CSCs that drives chemoresistance in GBM. Our results for the above experiment indicated that HIF1α positive clones C10 and C17 with high HIF signalling did not show any increase in the expression or nuclear translocation NF-κB. Furthermore no change in NF-κB transcriptional activity was observed in these clones as confirmed by reporter gene assay. Very similar results were obtained for HIF2α positive clones C14 and C26 with no increase in nuclear translocation or NF-κB transcriptional activity (Figure 6.13). On the
other hand NF-κB positive clones C1 and C4 showed increased nuclear translocation of both HIF1α and HIF2α indicating that NF-κB could be drive HIFs protein expression (Figure 6.14). Direct evidence for the transcriptional level regulation can be achieved by performing a TF binding site analysis using online analytical tools. These results confirm that NF-κB could possibly be the primary hypoxia response gene that drives HIF1α and HIF2α to induce stemness characteristics in GBM cells grown as spheres or under hypoxic conditions.

**Fig 6.13** NF-κB activity in HIF1α and HIF2α transfected GBM cell lines. A) Western blot analysis and B) Luciferase reporter gene assay results show that both HIF1α and HIF2α transfected U373 cells do not increase NF-κB nuclear translocation and transcriptional activity.
Fig 6.14 High expression of HIF1α and HIF2α in NF-κB transfected U373MG cells. Western blot analysis of nuclear extracts from NF-κB transfected cells show increased nuclear translocation of HIF1α and HIF2α in C1 and C4. Nucleolin was used a loading control.

### 6.6 Interplay between NF-κB and ALDH

From the results of our previous chapters we identified that GBM sphere cells and Hypoxic cultures have increased ALDH activity. ALDHs are a family of detoxifying enzymes that contains 19 different isotypes and are known to play protective roles against toxic molecules and ROS. High ALDH has been recognized as a hallmark characteristic of CSCs in variety of cancers including GBM. Evidences from different cancers indicate that specific iso-enzymes of ALDH namely ALDH1A1, ALDH1A3 and ALDH3A1 are frequently associated with detoxification of drugs, like vincristine, paclitaxel, epirubicin and cyclophosphamide. It is hypothesized that multiple ALDH isoenzymes are coexpressed in the CSCs derived from the same cancers. In this study we aimed to identify whether the commonly mentioned ALDH isoenzymes play a role in ALDH activity and chemoresistance of GBM. Another important fact that we wanted to analyse was that both HIF1α and HIF2α transfected cell lines had increased ALDH activity. But neither of them showed multi drug resistance. So we wanted to find out whether ALDH truly play a role in chemoresistance of GBM CSCs. It has been speculated that NF-κB can drive different members of
the ALDH family to aid in the maintenance of CSCs thereby increasing ALDH activity and chemoresistance of GBM CSCs. So we also wanted to analyse whether there is any relation between NF-κB and ALDH expression in CSCs. Firstly Western blot analysis of three ALDH isoenzymes namely ALDH1A1, ALDH1A3 and ALDH3A1 was performed to analyse the expression of the above ALDH isoenzymes in GBM sphere cells and NF-κB-p65 transfected clones. Our results from western blot analysis indicated that both NS and SUS cells isolated from all three GBM cell lines showed increased expression of ALDH1A3 and ALDH3A1 isoenzymes in comparison to their respective ATT-monolayer cultures. But much surprisingly the expression of ALDH1A1 was found to be low in both NS and SUS than the ATT cells (Figure 6.15).

![Western blot analysis of different ALDH isoenzymes in GBM sphere cells](image)

**Fig 6.15 Expression of different ALDH isoenzymes in GBM sphere cells.** Western blot analysis of whole cell lysates from ATT and sphere cells of the three GBM cell lines show increased expression of ALDH1A3 and ALDH3A1 and decreased expression of ALDH1A1 in NS and SUS cells. Tubulin was used as a loading control.

Our results were contradictory to numerous research papers that mentioned ALDH1A1 as the key chemoresistance factor among ALDH enzymes (Kahlert et al., 2012; Wang et al., 2013; Duong et al., 2012; Song et al., 2014; Schaefer et al., 2012). When we tested the NF-κB transfected cell lines for the above three ALDH enzymes we found that the expression pattern exactly matched with that
of NS and SUS cells. Both NF-κB clones C1 and C4 had increased ALDH1A3 and ALDH3A1 but there was a downregulation of ALDH1A1 in these clones when compared to mock transfected cells (Figure 6.16).

![Western blot analysis of whole cell lysates from NF-κB transfected GBM cells show an increased expression of ALDH1A3 and ALDH3A1 and decreased expression of ALDH1A1 in NF-κB transfected cells. Tubulin was used as loading control.](image)

**Fig 6.16 Expression of different ALDH isoenzymes in NFκB transfected GBM cells.** Western blot analysis of whole cell lysates from NF-κB transfected GBM cells show an increased expression of ALDH1A3 and ALDH3A1 and decreased expression of ALDH1A1 in NF-κB transfected cells. Tubulin was used as loading control.

So in order to determine the role of ALDH in chemoresistance we overexpressed all the above isoenzymes in U373 GBM cell line by stable transfection. Three vectors pCDNA3.1(+)-ALDH1A1, pCDNA3.1(+)-ALDH1a3 and pCMV6-ALDH3A1 were and their corresponding empty vectors were transfected in to U373 cell line by following the stable transfection protocol. 48 clones were picked for each gene after selection with neomycin. The cells transfected with empty vectors were used as control and the positive clones were screened by western blot for the cells with highest protein level expression of the target genes. One mock transfected clone and two positive clones with high expression of the respective genes were picked out for each isoenzyme and any increase in ALDH activity was detected by routine ALDEFLUOR assay. The cytotoxicity of all the drugs used in our study TMZ, VCR, PAC and DOX
was tested using MTT cytotoxicity assay as mentioned above. Western blot results shown in Figure 6.17A shows the successful overexpression of all the above three ALDH isoenzymes in U373 GBM cell line. We also confirmed the increase in ALDH activity of these transfected clones Figure 6.17B and C. Our results show that any ALDH isoenzyme could increase the ALDH activity that can be detected by ALDEFLUOR, not just ALDH1A1, as mentioned by many research papers (Li et al., 2010; Sladek et al., 2002). As seen from the results shown in Figure 6.17 it was confirmed that both ALDH1A3 and ALDH3A1 which are highly expressed in GBM spheres and p65 clones can significantly increase the ALDH activity. We tested these ALDH transfected cells for their sensitivity to four drugs TMZ, VCR, PAC and DOX. The results of the MTT cytotoxicity assay are shown in Figure 16.18 (ALDH3A1) and Figure 16.19 (ALDH1A3) and Figure 16.20 (ALDH1A1). The corresponding IC50 values are shown in Table below each cytotoxicity curve. It can be clearly seen from the results that the clones expressing high ALDH1A3 (C15 and C30) and ALDH3A1 (C30 and C39) showed significant resistance to the first line drug TMZ and other drugs VCR and PAC. But the resistance towards DOX was not statistically significant but a resistance trend could be observed with the IC50 values. This pattern of resistance is matching very well with NF-κB transfected clones where resistance to DOX was not observed. Surprisingly the isoenzyme ALDH1A1 which was downregulated in both GBM spheres as well as p65 clones also showed significant increase in chemoresistance to all the four drugs tested. Especially ALDH1A1 transfected clones showed significantly higher resistance to DOX.
Fig 6.17 Stable transfection of ALDH isoenzymes in U373 GBM cell line. (A) Western blot analysis of ALDH1A3, ALDH3A1 and ALDH1A1 transfected U373 positive clones with increased expression of respective isoenzyme. Tubulin was used as a loading control. (B) High ALDH activity was detected by ALDEFLUOR assay in cells transfected with ALDH1A1, ALDH3A1 and ALDH1A3. (C) Bar chart represents percentage of cells with ALDH activity. n=6, **p<0.01
Fig 6.18 *In vitro* cytotoxicity of TMZ, VCR, PAC and DOX in ALDH3A1 transfected U373MG cell lines. The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay.

Table 6.1. IC50s of TMZ, VCR, PAC and DOX in ALDH3A1 transfected U373MG cell lines

<table>
<thead>
<tr>
<th></th>
<th>ALDH3A1</th>
<th>Mock</th>
<th>Clone 30</th>
<th>Clone 39</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temozolomide</td>
<td>26.37 (2.47)</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>2.00 (0.57)</td>
<td>19.99 (0.59)**</td>
<td>9.23 (1.75)**</td>
<td></td>
</tr>
<tr>
<td>(nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.29 (0.69)</td>
<td>7.57 (2.11)**</td>
<td>15.27 (3.29)**</td>
<td></td>
</tr>
<tr>
<td>(nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>16.77 (2.97)</td>
<td>23.75 (2.65)**</td>
<td>30.17 (4.12)**</td>
<td></td>
</tr>
<tr>
<td>(nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The figures in the table represent the IC50 values. SD shown in parenthesis. N=9; **p<0.01
**In vitro** cytotoxicity of TMZ, VCR, PAC and DOX in ALDH1A3 transfected U373MG cell lines. The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay.

Table 6.2. IC50s of TMZ, VCR, PAC and DOX in ALDH1A3 transfected U373MG cell lines

<table>
<thead>
<tr>
<th>ALDH1A3</th>
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<th>Clone 15</th>
<th>Clone 30</th>
</tr>
</thead>
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<tr>
<td>Temozolomide</td>
<td>26.37</td>
<td>867.88</td>
<td>642.37</td>
</tr>
<tr>
<td>(μM)</td>
<td>(2.47)</td>
<td>(135.9)**</td>
<td>(53.01)**</td>
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<tr>
<td>Vincristine</td>
<td>2.00</td>
<td>6.47</td>
<td>5.98</td>
</tr>
<tr>
<td>(nM)</td>
<td>(0.57)</td>
<td>(3.67)**</td>
<td>(2.73)**</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.29</td>
<td>6.29</td>
<td>5.17</td>
</tr>
<tr>
<td>(nM)</td>
<td>(0.69)</td>
<td>(0.63)**</td>
<td>(0.66)**</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>16.77</td>
<td>22.05</td>
<td>24.90</td>
</tr>
<tr>
<td>(nM)</td>
<td>(2.97)</td>
<td>(11.53)</td>
<td>(18.1)</td>
</tr>
</tbody>
</table>

The figures in the table represent the IC50 values. SD shown in parenthesis. N=9; **p<0.01
**Fig 6.20 In vitro cytotoxicity of TMZ, VCR, PAC and DOX in ALDH1A1 transfected U373MG cell lines.** The cells were exposed to different drugs for 120 hours and subjected to MTT cytotoxicity assay.

**Table 6.3 IC50s of TMZ, VCR, PAC and DOX in ALDH1A1 transfected U373MG cell lines.**

<table>
<thead>
<tr>
<th>ALDH1A1</th>
<th>Mock (µM)</th>
<th>Clone 30 (nM)</th>
<th>Clone 39 (nM)</th>
</tr>
</thead>
<tbody>
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<td>Temozolomide</td>
<td>26.37</td>
<td>145.09</td>
<td>107.30</td>
</tr>
<tr>
<td></td>
<td>(2.47)</td>
<td>(15.62)**</td>
<td>(2.35)**</td>
</tr>
<tr>
<td>Vincristine</td>
<td>2.00</td>
<td>14.86</td>
<td>28.31</td>
</tr>
<tr>
<td></td>
<td>(0.57)</td>
<td>(2.06)**</td>
<td>(7.86)**</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.29</td>
<td>17.09</td>
<td>13.91</td>
</tr>
<tr>
<td></td>
<td>(0.69)</td>
<td>(4.38)**</td>
<td>(1.79)**</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>16.77</td>
<td>254.59</td>
<td>225.03</td>
</tr>
<tr>
<td></td>
<td>(2.97)</td>
<td>(41.72)**</td>
<td>(35.49)**</td>
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</table>

The figures in the table represent the IC50 values. SD shown in parenthesis. N=9; **p<0.01
6.7 Discussion

Extensive evidence in the past three decades have indicated that aberrant sustained activation of NF-κB induces cell signalling implicated in various stages of tumourigenesis as well as in resistance to chemo-radiation therapy (Greten et al., 2004; Baldwin et al., 2001). NF-κB is a key transcription factor involved in inflammatory responses of both innate and acquired immunity. The link between inflammation and cancer is a long known fact to biologists and has been established as the seventh hallmark of cancer. Accumulated evidence over many years suggests that many types of cancers have hypoxia induced chronic inflammation which is one of the very early stages in solid cancers. Thus NF-κB activation could possibly be a response towards inflammation and is a well known anti-apoptotic factor. Hence it is speculated that NF-κB is one of the primary hypoxia response genes recruited by tumour cells in order to avoid apoptosis and survive the hostile environment leading to tumour development (Coussens and Werb, 2002). NF-κB is also shown to help tumour adaptation to hypoxia and survival by up-regulating VEGF to initiate neoangiogenesis and vascularization followed by tumour progression (Xie et al., 2010; Yoshida et al., 1999).

NF-κB is an important transcription factor with more than 200 target genes. The nuclear translocation and transcriptional activity of NF-κB p65 subunit is tightly regulated by a family of IκB proteins that forms dimer with p65 and mask the nuclear localization sequence. The stable transfected clones that we used in this study expressed high cytoplasmic levels of NF-κB-p65 that could compete
with IκB-α activity and therefore has increased nuclear translocation of p65 subunits. This in turn has enhanced the NF-κB transcriptional activity as evident from our reporter gene results. With confirmation of high NF-κB activity in p65 transfected clones we wanted to determine whether ectopic overexpression of p65 could activate EMT pathways. Our results clearly indicated a drop in epithelial marker E-Cadherin and an increase in mesenchymal markers. Moreover the p65 transfected cells displayed increased migratory and invasion potential along with CSC characteristics indicating that these cells clearly are mesenchymal phenotypes. The recruitment of NF-κB plays a central role in cancer progression by regulating various aspects of EMT and metastasis (Huber et al., 2004). Several developmentally important transcription factors that are shown to induce EMT by repressing epithelial gene expression are regulated either directly or indirectly by NF-κB.

One of the most well-known transcription factors regulated by this way are the Snail and Slug (Nieto, 2002) which are direct inactivators of epithelial genes like E-cadherin, claudins, occludins, and muc1 (Ohkubo and Ozawa, 2004; Martinez-Estrada et al., 2006). They also enhance the expression of genes like fibronectin and metalloproteinase (MMPs) that are associated with a mesenchymal and invasive phenotype (Jorda et al., 2005). Snai1 transcription is directly activated by the NF-κB which is evident by the presence of a NF-κB binding region between -194 and -78 bp in the human SNAI1 promoter (Barbera et al., 2004). In addition, SNAI1 mRNA levels during EMT can be reversed by inhibition of NF-κB signalling. Similar to Snail, Slug is also required for the EMT and also downregulates the expression of the epithelial claudins and occludins.
genes (Kajita et al., 2004). Recent studies have identified that the Slug gene is a target for the aryl hydrocarbon receptor (AhR) which transcriptionally regulates slug only with co-expression of c-Rel. Thus, NF-κB may indirectly regulate the expression of Slug via the AhR (Shin et al., 2006; Belguise et al., 2007). Twist is also part of the growing list of genes with a role in repression of E-cadherin and induction of EMT (Kang and Massague, 2004; Yang et al., 2004). Twist is an evolutionarily conserved NF-κB target gene (Wang et al., 1997; Kanegae et al., 1998; Takeda et al., 1999; Sosic et al., 2003). In Drosophila, twist is recognised as a direct transcriptional target of the NF-κB protein (Pan et al., 1991). The expression of Vimentin in the p65 clones is a clear-cut evidence of mesenchymal phenotype because expression of vimentin is often described as the end stage progression of EMT pathway, which represents a completely dedifferentiated state in tumour cells that are highly proliferative and invasive (Satelli and Li, 2011). The vimentin promoter is comprised of a NF-κB binding site between nucleotides -239 and -197 bp (Lilienbaum et al., 1990; Lilienbaum and Paulin, 1993). NF-κB is responsible for the activation of promoter transcription of MMPs like MMP-9 and MMP-2 (Himelstein et al., 1997; Yoshizaki et al., 2002; Vu and Werb, 2000) that enables rapid degradation of collagen in the basement membrane leading to tumour invasion and metastasis.

Although many chemotherapeutic agents have been developed in the recent past for treating GBM patients, chemoresistance becomes the major obstacle to the cytotoxic effects of anti-cancer drugs. One of the important reasons behind this is the activation of NF-κB in response to treatment with cytotoxic drugs.
NF-κB is shown to induce resistance against a variety of chemotherapeutic agents like the topoisomerase I inhibitor SN38 and the topoisomerase II inhibitor, vinblastine, Doxorubicin, irinotecan, cyclophosphamide, docetaxel and many other drugs (Cusack et al., 1999). Our MTT results for the p65 transfected clones show an increase in chemoresistance to the first line GBM drug TMZ and other drugs like PAC and VCR. However we did not see any significant increase in resistance to DOX. This may be because of a different mechanism of resistance for DOX in GBM sphere cells which are significantly resistant to DOX. Like GBM sphere cells and HYP cultures the p65 clones are resistant to TMZ. But we did not find any evidence of MGMT protein expression even after several trials. This could be very likely due to TMZ resistance induced by NF-κB being MGMT-independent. In addition, this can explain why some GBM patients who are MGMT-negative also develop resistance to TMZ (Gaspar et al., 2010).

It has been reported that constitutive NF-κB activation is a reason for tumour cells escaping apoptosis, which is also related to the development of drug resistance in cancer cells (Patel et al., 2000; Arlt et al., 2001; Mabuchi et al., 2004).

It has been believed that the most important function of p53 tumour suppressor gene is its ability to induce apoptosis, and disruption of this process can promote tumour progression and chemoresistance (Tergaonkar et al., 2002). Activated NF-κB inhibits chemotherapy-induced stabilization and activation of p53, which results in resistance to chemotherapy (Tergaonkar et al., 2002; Webster and Perkins, 1999). High NF-κB directly blocks caspase activation or enhance the expression of genes encoding anti-apoptotic proteins to promote
cell survival. The expression of BCL-XL and BFL1 play important roles in the survival effect of NF-κB in response to chemotherapeutic drugs in human lung carcinoma cell line (Cheng et al., 2000). In addition, recent studies have demonstrated that NF-κB has a consensus-binding site for the human MDR1 (Bentires-Aljet et al., 2003). This finding further supports the vital role for NF-κB in chemoresistance. All these data indicate that NF-κB is a crucial target for cancer therapy and future cancer prevention.

Our results from chapter 5 and chapter 6 show that both HIF1α and HIF2α as well as NF-κB activities increased stemness in GBM cells. All three TFs induced EMT and resulted in a mesenchymal phenotype showing CSC characteristics, indicating the possibility that any of these transcription factors could play a role in regulating hypoxia induced EMT and generating CSCs under hypoxic condition of NS and SUS cells. There are other possibilities like a loop system existing between HIFs and NF-κB or they could be totally unrelated in their mode of actions. There are many findings that indicate NF-κB can regulate HIFs, but at the same time several findings also indicate that HIFs could regulate NF-κB signalling (Melvin et al., 2011; Cockman et al., 2006; Devries et al., 2010). But the interesting results we obtained with chemoresistance which is an important characteristic GBM CSCs showed that the resistance pattern displayed by HIFs did not match with the NS and SUS characteristics. On the other hand NF-κB was able to induce resistance to three out of four drugs and could be considered as an important TF. But that will not settle the question of master transcriptional regulator. In order to determine whether HIFs are the driving force behind enhanced NF-κB activity or whether NF-κB is expressed as
a separate hypoxia responsive transcription factor we analysed the expression of each of the above transcription factors under the influence of another using the HIF1α, HIF2α and NF-κB p65 transfected U373 GBM cell lines.

The interesting results obtained showed us clearly that neither HIF1α nor HIF2α regulated the NF-κB activity, but the NF-κB p65 transfected clones showed enhanced HIF1α and HIF2α activity. In addition, results from the luciferase reporter gene assay clearly indicated that there is no increase in NF-κB transcriptional activity due to increased levels of HIF signalling in the HIF transfected clones. This helped us to come to a conclusion that among these three transcription factors, NF-κB could be the master regulator of hypoxia induced EMT and GBM CSCs that drives chemoresistance in GBM. It has been shown previously by many researchers that HIF-1α is required for the activation of NF-κB. Scortegagna et al., showed that functional loss or deficiency of HIF-1α decreases p65 expression and NF-κB activation, whereas higher levels of HIF-1α resulted in the activation of NF-κB through hyper phosphorylation of IκBα and phosphorylation of p65 (Scortegagna et al., 2008).

The molecular mechanism behind this process has been partly understood through several studies, which suggest HIF mediated regulation of IKK signalling which further then regulates canonical NF-κB signalling. In particular, some studies has mentioned that IKK-β is activated by hypoxia and this activation is thought to be mediated by a PHD domain present in IKK-β, which are known targets for hydroxylation by PHD1 (Cummins et al., 2006; Oliver et al., 2009). But some other literature data support that fact that hypoxia regulates
the NF-κB pathway through activation of IKK, specifically via IKK-β, without the involvement of any HIFs. In addition to IKK, FIH is also considered to regulate NF-κB under hypoxic condition through hydroxylation of NF-κB proteins such as p105 and IκB-α (Cockman et al., 2006). From the evidence listed above and various other studies it appears that hypoxic activation of NF-κB pathway is partly dependent on HIF-1α. It is considered that functional HIF-1 proteins may directly interact with NF-κB proteins, enhancing their binding at the promoter region of NF-κB target genes (Cockman et al., 2006; Devries et al., 2010).

Besides HIF-mediated activation of NF-κB, evidences from large number of experimental studies indicate that NF-κB could directly regulate HIF pathway in tumourigenesis. NF-κB could control the expression of HIFs and its downstream signalling pathways within the tumour microenvironment because the promoter region of HIF1α gene contains an active NF-κB binding site at position−178/−188. Any mutations introduced at this site of HIF-1α promoter region leads to the loss of hypoxia-induced HIF-1α activation (Bonello et al., 200). Moreover, siRNAs mediated blocking of NF-κB activity results in decreased HIF-1α activity. TNF-α induced activation of NF-κB results in increased levels of HIF1α mRNA and protein (Belaiba et al., 2007; Van Uden et al., 2008). It has also been shown that NF-κB up-regulates HIF-1 signalling pathway via IKK-β which is consistent with other recent findings that knockdown of IKK-β decreases HIF-1α activity (Jiang et al., 2010) suggesting the importance of IKK signalling HIF-1α activation. Even under the absence of hypoxia, cytokines especially TNF-α and IL-4 can also activate HIF-1α activity through NF-κB dependent mechanism (Cockman et al., 2006; Jiang et al.,
Therefore, it is speculated that NF-κB drives HIF-1 signalling pathway to maintain the basal levels of HIF-1α under normoxia condition, and further increase the levels of HIF-1α under hypoxia. Another interesting study has shown that NF-κB also can activate HIF-2α signalling through the interaction with IKK-γ which leads to recruitment of CBP/p300 for transcriptional activation of HIF-2α (Bracken et al., 2006). Furthermore, NF-κB is also known to regulate HIF-3α, a negative regulator of HIF signalling pathway but the role of NF-κB mediated HIF-3 signalling in the tumour microenvironment is not fully understood (Augstein et al., 2010). A recent clinical study in gastric cancer patients revealed that the expression of HIF-1α is positively connected with the expression of p65. The experiment showed that over-expression of IκB-αM which is a super-suppressive mutant form of IκB-α suppresses HIF-1α expression, angiogenesis, and tumour growth. This is consistent with earlier findings indicating that the HIF and NF-κB pathways induce EMT in a p65 and HDAC dependant manner to contribute towards breast cancer metastasis (Nam, 2006; Bendinelli et al., 2009). Taken together, it is understood that the interplay between HIF and NF-κB signalling pathways is crucial to promote tumour aggressiveness, but the precise molecular crosstalk between these complex pathways still requires profound investigation.

ALDH is a well known CSC marker used for isolating CSCs from various solid tumours and haematological malignancies including GBM. The role of ALDH in drug resistance was mentioned in many studies involving sphere cell cultures and drug resistant cell lines (Magni et al., 1996, Moreb et al., 1998). Initially it was reported that the ALDH activity measured by ALDEFLUOR assay in CSCs
is due to a specific isoenzyme in the ALDH family known as ALDH1A1 (Storms et al., 1999). But later several other isoenzymes were also mentioned as factors for increasing ALDH activity and resistance in CSCs (Marcato et al., 2011; Brocker et al. 2011). Although some studies mentioned ALDH1A1 as important resistance mediator in GBM to the first line drug TMZ (Schafer et al., 2012), our results showed that ALDH1A1 is down regulated in all NS and SUS cultures. Hence we investigated the presence of three commonly mentioned ALDH isotypes namely ALDH1A3 and ALDH3A1 in GBM sphere cells. We noted that whereas the ALDH1A3 and ALDH3A1 were highly increased in NS and SUS cultures as well as increased the ALDH activity in GBM CSCs. We were interested to know if NF-κB is playing a master role in regulating various genes under hypoxia induced EMT, could it possibly be a regulator in inducing ALDH isoenzymes in GBM spheroids cultures. Our results showed that NF-κB transfected clones C1 and C4 had decreased ALDH1A1 and increased ALDH1A3 and ALDH3A1 which matched precisely with the ALDH expression profile of GBM sphere cells. This indicates that in addition to the role of inducing EMT and mediating CSCs, NF-κB plays an important role in regulating HIFs and ALDH isoenzymes under hypoxic conditions.

NF-κB also possibly regulates the ALDH activity and therefore could be the master regulator of hypoxia induced EMT and CSCs. But further studies are required to determine any direct involvement of NF-κB in regulating ALDH genes. In order to investigate the role of these three ALDH isoenzymes in resistant nature of GBM spheroids we used transfected U373 GBM cells with expressing high amounts of ALDH1A1, ALDH1A3 and ALDH3A1. From the
above results we showed that any ALDH can increase the ALDH activity in GBM cells. These results indicate that regardless of the protein level of different isoenzymes the resistance to drugs largely depends on the enhanced ALDH activity rather than the subtype. It can be speculated that if one ALDH isoenzyme level drops the expression of any other isoenzyme could compensate the increase in ALDH activity and lead to resistance. For example although ALDH1A1 level is low in GBM sphere cells and NF-κB transfected clones, forced overexpression of this isoenzyme leads to resistance. This proves that the presence of this isoenzyme will definitely increase resistance to the cancer cells as shown by many researchers. We have shown in chapter 3 and 4 that GBM sphere cells and HYP cultures are resistant to DOX. However, the NF-κB transfected clones, ALDH1A3 and ALDH3A1 expressing clones were not significantly resistant to DOX. But the ALDH1A1 expressing clones are significantly resistant to DOX. This gives us a hint that out of the 19 different members of the ALHD family, the presence of any other ALDH isoenzyme or a combination of many which are not tested in this study could possibly be the responsible factor for GBM sphere cells and HYP cell resistance to DOX.

Another factor is that these models used for our study are expressing ALDH isoenzyme at an extremely high level and the resistance achieved may be because of the presence of high level of enzymes that could easily detoxify the drugs. This also indicates the fact that ALDH activity not simply serves as a passive CSC marker but also could play a major diagnostic and prognostic role in GBM. Being a detoxification and antioxidant agent ALDH could play an important role in protecting the drug-tolerant CSC subpopulation from the
elevated levels of reactive oxygen species (ROS) in these cells, or potential toxic effects of drug induced ROS. Hence pharmacological inhibition of ALDH activity could lead to accumulation of ROS to very high toxic levels, resulting in DNA damage and apoptosis specifically within this CSC population.

Since HIF transfected cell lines did not induce enough NF-κB we can also hypothesise that lack of enough NF-κB activity in these cells may be related to the lack of resistance which indirectly may indicate the lack of ALDH mediated resistance in these cells. But further experiments and confirmations are required to verify this reason behind the sensitivity of HIF transfected cells. But we can positively correlate these results with the resistance of NS and SUS cells because of the extremely high ALDH activity induced by hypoxia through activation of NF-κB. Or it is very likely that the presence of other resistance factors like the ABC transporters or P-glycoprotein could also be present in the sphere cells that provide them with multi drug resistance. Even if that is the case, there is a wealth of literature indicating that NF-κB regulates these MDR proteins in cancer cells. Hence targeting the overall enzyme activity of ALDH in addition to suppression of the master regulator NF-κB could be a promising strategy to reverse the intrinsic chemoresistant nature of GBM cells as well to target CSCs.

### 6.8 Conclusions

To conclude we can say that NF-κB is highly expressed under reduced oxygen environment in both GBM spheres and hypoxic cultures. The above findings
point out that the biological consequences of hypoxia largely depend on NF-κB which includes regulation of HIFs which are the primary hypoxia response genes. NF-κB could be the master regulator of EMT, stemness and chemoresistance and therefore responsible for the dedifferentiation of epithelial to mesenchymal phenotypes in GBM sphere cells and GBM hypoxic cultures. Various survival mechanisms and other anti-apoptotic pathways activated by NF-κB in addition to enhanced ALDH activity possibly driven by NF-κB could be the key factors behind chemoresistant nature of GBM. Hence Targeting NF-κB and ALDH activity could be a promising strategy to target CSCs and reverse chemoresistance in GBM.
Chapter 7

Disulfiram targets hypoxia induced GBM CSCs by modulating NF-κB pathway
7.1 Introduction

Our results from all previous chapters have pointed out that the mesenchymal cells that are induced by hypoxia associated EMT process could be the chemoresistant tumour cell population that mimics CSCs in GBM sphere cultures. GBMs are extensively hypoxygenated tumours and it is very likely that in GBM patients this hypoxia induced mesenchymal phenotypes which display all characteristics of CSCs play a key role in the survival, invasion and resistance of GBM cells. Our findings of chapter 5 indicated that the biological consequences of hypoxia largely depend on NF-κB which includes regulation of HIFs which are the primary hypoxia response genes. Hence, NF-κB could be the master regulator of EMT, stemness and chemoresistance and therefore responsible for the dedifferentiation of epithelial to mesenchymal phenotypes in GBM sphere cells and GBM hypoxic cultures. Various survival mechanisms and other anti-apoptotic pathways activated by NF-κB in addition to enhanced ALDH activity possibly driven by NF-κB could be the key factors behind chemoresistant nature of GBM. Thus, targeting NF-κB and ALDH activity could be a promising strategy to target CSCs and reverse chemoresistance in GBM.

7.1.1 NF-κB pathway as a therapeutic target in GBM

Accumulated evidence over the past decade indicates that inhibiting NF-κB activation results in reversal of chemoresistance in a variety of cancer cells (Wang et al., 1999; Patel et al., 2000; Jones et al., 2000; Arlt et al., 2001; Cusack et al., 2001; Flynn et al., 2003; Guo et al., 2004; Mabuchi et al., 2004; Banerjee et al., 2005; Kwon et al., 2006; Kim et al., 2006; Tapia et al., 2007;
Sors et al., 2008; Murtaza et al., 2009). Although studies involved with the upstream or downstream inhibition of NF-κB pathway employs several novel compounds designed with various systems and computational models, two mains strategies to block NF-κB are well known: 1) IκK inhibitors and 2) Proteasome inhibitors. Owing to the central role of IκK-β in regulating NF-κB activation through phosphorylation and degradation of Iκ-B, a number of compounds such as IMD-0354, TPCA-1, IKI-1, PF-184, PS1145, etc were designed to inhibit IκK-β. Other widely used NF-κB inhibitors like BAY-11-7082 and sulfasalazine suppresses both IκK-α and IκK-β (Weber et al., 2000; Weitsman et al., 2006). A recent study involving a novel IκK-β inhibitor EC-70124 showed that blocking NF-κB induces senescence in GBM stem cells (Nogueira et al., 2011). Another recent work by Zanotto-Filho et al., (2011) also showed that inhibition of NF-κB pathway using a wide range of NF-κB inhibitors like BAY-11-7082, MG132, parthenolide, Arsenic, Curcumin or by siRNA mediated knock down selectively enhanced cell death in GBM cells, worked synergistically with anticancer drugs and reversed chemoresistance in these GBM cells. Proteasome inhibitors are other effective NF-κB inhibitors that act by interrupting the 26S proteasome degradation system. In the case of NF-κB, the degradation of Iκ-B by proteasomes in response to phosphorylation by IκK leads to enhanced NF-κB activation in tumour cells. Inhibition of the 26S proteasome will lead to stabilization of Iκ-B thereby suppressing NF-κB activity (Nakanishi and Toi., 2005). Proteasome inhibitors such as bortezomib and MG132 are considered to be promising therapies because inhibition of the proteasome degradation results not only in the inhibition of NF-κB pathway but
also a wide range of other targets leading to dysregulated cellular pathways and induction of apoptosis (Orlowski et al., 2002). Due to the successful and promising results from clinical trials which started 10 years ago, more than 5 novel proteasome inhibitors have been introduced for clinical trials in 2012. But as with many other anticancer drugs, the proteasome inhibitors will also probably face the obstacle of resistance. Bortezomib an established proteasome inhibitor has already entered the decline phase due to resistance by MDR and other ABC drug efflux mediated resistance mechanisms (Orlowski and Kuhn, 2008; Lu and Wang., 2013). Additional mechanisms like mutations in the bortezomib binding sites of β-5 proteasome subunit and overexpression of PSMB5 proteins are also thought to mediate resistance to proteasome inhibitors (Kale et al., 2012).

Although inhibition of NF-κB through proteasome inhibitors might be beneficial, the high amounts of ABC efflux transporters, MGMT, p-glycoprotein and high ALDH activity present in GBM CSCs has to be tackled in addition to inhibition of NF-κB. None of the anticancer drugs, kinase inhibitors, proteasome inhibitors or angiogenesis inhibitors are promising for GBM. There is an urgent need for the development of new drugs that can target these multiple resistance mechanisms of GBM. With increasing incidence rates of GBM every year, we cannot afford to develop drugs that will cost around US$1 billion and take decades of research and testing. More than 25,000 FDA-approved drugs with well established safety and pharmacokinetic profiles are currently available on the market. Together with their derivatives they make a vast library of potential therapeutic compounds. Repurposing such old drugs for treatment of GBM
would reduce the time and cost for drug development to a greater extent. (Chong and Sullivan, 2007)

7.1.2 Repurposing Disulfiram for GBM treatment

Among several compounds which are repurposed into treatment of cancer, disulfiram has the strongest evidence for potential anticancer activity (Cvek, 2008). Disulfiram (DS) or tetraethylthiuram disulfide which is well known as Antabuse®, has been used for treatment of alcohol addiction for more than 60 years. DS is considered to be a well tolerated drug with very little adverse side effects. At present two clinical trials for DS in GBM are in progress (www.clinicaltrials.gov, identifiers NCT01907165 and NCT01777919). Much of the seminal work on the anticancer property of DS comes from our group led by Prof. Weiguang Wang here at the University of Wolverhampton. The multiple anticancer activities of DS were demonstrated in several preclinical studies involving leukemia, melanoma, breast, and colon, prostate, cervical and lung cancers (Kast et al., 2013).

Studies from our group and others report that DS is cytotoxic to GBM cell lines and inhibits self-renewal of GBM CSCs. Multiple studies using in vitro models of GBM have demonstrated that DS targets GBM through proteasome and ALDH inhibition (Liu et al., 2012; Lovborg et al., 2006; Cvek., 2008). It is widely regarded that the resistant ALDH expressing CSC population in tumours are susceptible to inhibition by DS thereby eliminating tumours or sensitize them to conventional therapy including several anticancer agents. Recently it has also
been shown that DS inhibits the growth of TMZ-resistant cells isolated from patients regardless of MGMT expression levels (Hothi et al., 2012; Triscott et al., 2012). Another study from Paranjpe et al. (2013) using T98G GBM cells that are resistant to TMZ has shown that DS can specifically inhibit MGMT protein in cell lines and xenografts implanted subcutaneously (Paranjpe et al., 2013). Choi et al. recently demonstrated that DS can effectively cross the BBB in mice and reduce atypical teratoid rhabdoid tumours (AT/RT) by targeting AT/RT CSCs (Choi et al., 2014). AT/RT is a very rare paediatric brain tumour and they reported that AT/RT CSCs were more sensitive to DS than clinically used drugs. DS was shown to remarkably reduce ALDH in vitro and in vivo and inhibit tumour cell proliferation in these tumours and prolonged the survival of mice with no adverse effects. Potential mechanisms of anti-cancer activity by DS such as inhibition of MDR efflux proteins, ALDH, elevating ROS, proteasome inhibition and inhibition of NF-κB pathway have been discussed in detail in chapter 1.

7.2 Rationale and aims of this study

Although potential mechanisms of the anti-cancer activity of DS was described by our and various other groups, it is still not understood which are the primary, secondary or underlying targets of DS mediated anticancer effect. It is still not known which pathways are important to DS toxicity. Nishikawa et al., (2013) believe that ALDH inhibition is the primary mechanism, while other studies mention that proteasome inhibition followed by NF-κB inhibition is the key factor (Cvek., 2008). But a lot of uncertainties are still surrounding the role of ALDH in
chemoresistance. Some studies mention elevated ROS or interaction with mitochondria or inhibition of certain kinases as the reasons for DS induced cytotoxicity. Since DS and its metabolites diethyldithiocarbamates react with several proteins inside the cells and binds to many metal compounds present in enzymes, it is very difficult to determine the primary mechanism of action. In this study we would like show that DS-mediated cytotoxicity is largely dependent on Cu. Previous results from our group demonstrated the importance of using Cu in combination with DS for enhanced cytotoxicity of DS in different cancer cells including GBM. Preliminary in vitro results from our laboratory indicated that DS alone cannot successfully eliminate CSCs or resistant populations of GBM and other cancers. This is mainly because the CSC population possesses high ALDH activity and high antioxidant capacity which acts as ROS scavengers and reduce oxidative stress in addition to the antiapoptotic activity of NF-κB (Moreb et al., 2012). Therefore, inhibition of ALDHs and NF-κB by DS alone will not lead to enough ROS accumulation leading to cell death in CSCs. Although proteasome inhibition to target NF-κB and ALDH is effective in suppressing the non CSC cancer cells, we believe that an additional ROS trigger is required to induce apoptosis in CSCs. Cu plays a crucial role in the generation of reactive oxygen species (ROS) that induce apoptosis in cancer cells. The entry of Cu into cells is strictly regulated by the transmembrane Cu transporter protein Ctr1. DS is a strong divalent metal ion chelator, and hence form a complex with Cu that enhances the transport of Cu into cancer cells. DS/Cu is a very stronger ROS inducer than Cu or DS alone (Nobel et al., 1995). Free DS that enters the cells can also chelate with intracellular Cu (II), forming a DS/Cu complex and triggers ROS mediated apoptosis. Cancer tissues possess higher levels of Cu
in comparison to normal cells (Mulay et al., 1971; Yip et al., 2011) and thus high Cu concentration in tumour tissue under physiological conditions can be used to selectively target cancer cells with DS. In the in vitro part of this study we aim to reverse chemoresistance in GBM sphere cells and hypoxic cultures by inhibiting ALDH and NF-κB activity in addition to triggering ROS through addition of Cu. We want to demonstrate that DS/Cu complex induces cell death in both GBM CSC (NS and SUS) and non-CSC population and HYP cultures by intrinsic pathway of apoptosis. We also would like to test the cytotoxicity of DS/Cu on the NF-κB and ALDH transfected clones that were resistant to conventional anticancer drugs. Although the in vitro anticancer activity of DS/Cu has provided promising results for many cancers, there are very few successful cases reported in clinical trials. This inconsistency is mainly attributed to the very short half-life of DS in the bloodstream of approx 2-4 mins (Eneanya et al., 1981 and our unpublished data). This would be a major drawback for GBM treatment as this time is not enough for DS to cross the BBB and hence reduce the bioavailability of DS in GBM tumours. Nanotechnology provides a cutting edged drug delivery system which may pave the path for translation of DS into cancer indication. Our previous study shows that liposome encapsulated DS demonstrated stronger anticancer efficacy in breast cancer model (Liu et al., 2014). In this study, I examined a novel long circulating new formulation of poly (lactic- co-glycolic acid)-PLGA encapsulated disulfiram nano particles developed in our lab. In order to determine the efficiency of this PLGA-DS nano particles to cross the BBB, we used orthotopic xenograft mouse models of GBM generated using GFP tagged U87 cell lines. To meet the requirement of Cu we used copper gluconate salt which is approved by FDA as a safe substance and
widely available as a dietary supplement. By achieving the above aims we would like to show that DS/Cu targets GBM CSCs and reverse chemoresistance by modulating hypoxia-NF-κB axis and is the first drug that can simultaneously suppress multiple mechanisms of GBM CSCs like MDR, NF-κB, proteasomes, ALDH and induce ROS to induce apoptosis.

7.3 Experimental Design

Detailed information on materials, products, manufacturers and methodologies used for the entire study has been described in chapter 2. The following are specific experimental designs and methods used for this part of the study.

7.3.1 MTT cytotoxicity assay

Three GBM cells lines U87 MG, U251MG and U373MG were cultured overnight in flat bottomed 96 well plates at a cell density of 5000 cells/well in triplicates. These ATT-Normoxia cells were then exposed to DS with doses ranging from 1000nM serially diluted down to 7.8nM. A uniform dose of 10μM Cu was maintained in all the wells. This is achieved by serially diluting DS in a 10μM Cu containing medium. The cells were incubated at 37°C for 72hours. After incubation the cells were subjected to MTT analysis. The cell viability (%) at different concentrations was calculated and the corresponding IC_{50} values for DS/Cu were calculated. The same method was performed for ATT-HYP cultures in parallel.

Similarly the cytotoxicity of DS/Cu on NF-κB-p65, ALDH1A3, ALDH1A1 and ALDH3A1 transfected clones was determined by MTT assay. All these clones
were seeded at cell number of 5000 cells/well in 96 well plates in parallel to their corresponding mock cells. The cells were treated with serially diluted DS and 10μM Cu and MTT analysis was performed as mentioned above and the IC50 values were calculated from cell viability (%) at different concentrations. The sphere cells NS and SUS from all three GBM cell lines used were trypsinised and reseeded in poly HEMA-coated 96-well flat bottom plates in their corresponding culture medium at a cell density of 5000 cells/well and cultured overnight. Since the cells were suspended they were grown overnight at a volume of 100 μL/ well. They were then dosed with medium containing double strength DS dilutions (to make it 1x strength including already present 100μl medium) on top with another 100 μL. Again the concentration of Cu was kept at 10μM. The cells were treated for 72 hours and MTT reagent was added to the wells. After incubation with MTT reagent, the plates were spun down at 800 rpm for 5 mins in a plate centrifuge and media is discarded. The spheres which appear like purple crystals were dissolved by adding DMSO and the OD was measured using a plate reader. The % viability and corresponding IC50 values for DS/Cu was calculated.

7.3.2 Western blot analysis

1x10⁶ Cells of ATT, HYP, NS and SUS cells from all three cell lines were cultured in 25cm² flasks under appropriate conditions overnight and treated with 1μM DS and 10μM Cu for 24 hours. After treatment the cells were collected, washed with PBS and stored as pellets. The whole cell protein in the treated cell pellets were extracted with RIPA buffer, quantified and separated by SDS PAGE. The expression levels of anti apoptotic protein Bcl2 and pro-apoptotic
protein Bax were analysed using appropriate primary and secondary antibodies as mentioned in the western blot section of chapter 2. In addition to the above apoptotic proteins, the expression level of NF-κB in these treated cells was also determined using appropriate NF-κB –p65 antibody.

7.3.3 Sphere formation assay

GBM NS and SUS sphere cells from all three GBM cell lines grown in poly-HEMA coated 25cm² flasks for 7 days were collected by trypsinization and were counted using a haemocytometer. For each group, 10000 cells were mixed in 2mL medium in a 10mL tube. The cells were treated in the tube with 1μM DS and 10μM Cu for 4hours. DMSO was added to control tubes. After incubation, the cells were centrifuged and the drug containing medium was discarded. Cells were then washed twice with 1xPBS and resuspended into 2mL of fresh medium. Cells were then seeded into poly-HEMA-coated and 2-times water rinsed 6-well plate (2mL/well). Cells were cultured for 7 days and the spheres in all wells were photographed at x40 and x100 magnifications. Another experiment for long term treatment with DS/Cu was done with a similar protocol. The ATT cells of all three GBM cell lines were trypsinised and seeded in poly-HEMA coated 6 well plates. They were left to form NS and SUS cells in the 6 well plates for 7 days with intermittent feeding with fresh medium. After they formed spheres the medium was replaced with DS/Cu containing medium (DS 1μM and Cu 10μM). One group of cells from the same plate was used as a control. The cells were continually treated with DS/Cu 72 hours. After 72 hours the effect of DS/Cu on the NS and SUS cells were photographed.
7.3.4 Cytotoxicity of DS/Cu on a panel of normal cell lines

MTT cytotoxicity assay was performed to determine the cytotoxicity of DS/Cu on normal cell lines. Different immortalized normal cell lines like NHA (Normal human astrocytes), MCF10A (normal breast), HECV (Human umbilical vein endothelial cell line), WI38 (Human lung fibroblasts), EA.hy926 (Human endothelial cell line) were experimented in parallel with three GBM cell lines U87, U251 and U373. About 5000 cells per well were treated with serially diluted DS (1μM down to 7.8nM) and 10μM Cu for 1 hour. After incubation for 1 hr the drug containing medium was removed, and the cells were washed twice with 1xPBS and replaced with drug free medium. The cells were then incubated for 72 hrs and subjected to MTT analysis. The cell viability of normal cell lines at a concentration of DS1μM /Cu 10μM was compared with that of cancer cells.

7.3.5 Preparation of PLGA-DSF nanoparticles

We used a polyester named Poly(D,L-lactide-co-glycolide) (PLGA) to prepare a new formulation of DS loaded nanoparticles. Due to lack of time this part of the work was done with the help of Dr. Zhipeng Wang, post-doctoral research fellow in our group. The nanoparticles, loaded with DS, were prepared by an emulsion–solvent evaporation method. 200 mg of PLGA and 20, 40, 50, 100 or 150mg DSF were dissolved in 10mL of dichloromethane, and then mixed with 20mL of 2.5% PVA aqueous solution. This mixture was homogenized for 1 min by vortex and then sonicated using a microtip probe sonicator set at 70% power output (XL 2002 Sonicator® ultrasonic liquid processor) for 3, 4 or 5 min to produce the oil-in-water emulsion. The organic phase was evaporated for 5h at room temperature. The nanoparticles were recovered by ultracentrifugation.
(10,000 rpm, 20 min, Hitachi). The nanoparticles were washed thrice with water. The purified nanoparticles were freeze-dried in 5% sucrose. In order to evaluate the surface morphology of the NPs, scanning electron microscopy was performed using a high resolution scanning electron microscope (JEOL JSM T330A). A drop of the nanoparticle samples were mounted on metal stubs and coated with a gold/palladium thin film by sputtering for 60 seconds, with a 15 mA current, using a SPI Module sputter coater system. Images were obtained at an acceleration voltage of 15 kV.

7.3.6 In vitro half-life of Disulfiram

Liposome encapsulated DS was obtained from our collaborators in Shenyang Pharmaceutical University, China. 100 μL of free DS, Lipo-DS, or PLGA-DS, all at the concentration of 3mg/mL, was added to 300 μL of horse serum and shaken at 37°C. At different time intervals the Eppendorf tubes were collected and the proteins were precipitated by adding 300 μL of absolute methanol. The supernatant was subjected to HPLC analysis.

7.3.7 Effect of PLGA-DS nano particles on orthotopic GBM xenograft mouse models

All experiments were done with the kind help of our collaborators in Prof. Xiuwu Bian’s lab at Third Military Medical University, Chongqing, China. Five-week-old female BALB/c Nu/Nu athymic nude mice (Biotechnology & Cell Biology Shanghai, China) were housed under pathogen-free conditions according to Fourth Military Medical University (FMMU), China animal care guidelines and the animal experiments were reviewed and approved by the
Ethical Committee of FMMU. Nude mice were anesthetized with an intraperitoneal injection of 0.6 mL of a stock solution containing chloral hydrate (7 µl/g). The surgical site was shaved and prepared with 70% ethyl alcohol and iodine-containing solution. Intracranial injection was performed at the position of parietal midline with a double outer canthus connection intersection 0.5 cm at the right and rear bias. 2×10⁵ U87-Luciferase-GFP cells in 5 µl PBS were delivered using a 25µL micro-syringe drilled to a depth of 0.5cm. 10 days later, mice were randomly subdivided into three groups (8 mice/group) and treated 3 times/week for 3 weeks, e.g. control, Cu-Glu 6mg/kg p.o. + empty NPs i.v., Cu-Glu 6mg/kg p.o. plus PLGA-DSF 10mg/kg i.v. At the end of the experiment, mice were photographed by an in vivo imaging system, sacrificed and brains were removed, and subjected to further analysis.

7.4 Results

7.4.1 DS/Cu targets GBM CSCs in vitro

Firstly we tested the toxicity of DS/Cu to ATT-normoxia cells to determine whether DS/Cu can eliminate the non-CSC population. From the results shown in Fig 7.1 it was very clear that DS/Cu comfortably eradicated the GBM cells at a very low concentration of nanomolar levels. The treatment was done for 72 hours. We have also tried short treatment of DS/Cu for all the three GBM cell lines. We found that just 1 hour treatment of DS/Cu and then release will induce apoptosis in these cells but at a slightly higher concentration of DS. We treated the ATT-HYP cultures with DS/Cu which showed resistance towards all anticancer drugs tested in the previous experiments. The results shown in figure
7.1B clearly reveal that there is no resistance shown towards DS/Cu by ATT-HYP cultures which is also evident from the very similar IC50 values for both NOR and HYP as shown in bar charts. In the other part of this study we tested the cytotoxicity of DS/Cu on GBM sphere cells isolated from U87, U251 and U373 cell lines. These spheres were proved to be highly resistant to all the four drugs that we tested earlier. But our MTT results for DS/Cu on these sphere cells did not show any evidence of resistance. DS/Cu was cytotoxic to these spheres at very low nanomolar levels after 72 hours treatment (Figure 7.2). We did not observe any significant difference in the cytotoxicity ranges between ATT and NS and SUS cells for DS/Cu treatment. However from the cytotoxicity curves shown in Fig 7.2 it is very clear that in all three cell lines tested there is a trend where the NS cultures were the most sensitive cells and the SUS cells needed a higher dose of DS. However the IC50 value calculated shows us that the dose of DS for SUS cells is around 1μM DS which is still a very low dose given the fact that DS is generally a well tolerated drug with very little side effects.

![Image of cell cultures](image_url)
Figure 7.1 DS/Cu induced cytotoxicity in GBM cell lines. A) Morphology of cells treated with 1μM DS and 10μM Cu for 1hr and released for 72hr (40x magnification). B) *In vitro* cytotoxicity of DS/Cu on ATT-NOR and ATT-HYP GBM cell lines. Bar charts display IC50 values of DS/Cu on normoxia and hypoxia cultures.
**Figure 7.2 In vitro cytotoxicity of DS/Cu on GBM sphere cells.** The cells were exposed to DS/Cu for 72 hrs and subjected to MTT analysis. Bar charts display the differences in IC50 values of DS/Cu on ATT, NS and SUS cells.
7.4.2 DS/Cu abolished the sphere forming ability of GBM cells

The NS and SUS cells from GBM cell lines under culture for 7 days which expresses all CSC, EMT and chemoresistant characteristics were subjected to the sphere formation assay. Unlike the MTT cytotoxicity assay which treats these sphere cells in DS/Cu containing medium for 72 hrs the sphere formation assay exposes the cells to DS/Cu only for a short period of 4hrs. The cells were dissociated and exposed to a dose of 1\(\mu\)M DS and 10\(\mu\)M Cu for 4 hours. The cells were then removed from the drug containing medium and washed twice with PBS to remove any drugs present. The cells were reseeded into appropriate drug free medium and allowed to form spheres in polyHEMA coated 6well plates. The results of this experiment is shown in figure 7.3. Our results shows that the untreated cells can form spheres again after dissociation of spheres and reseeding them back to suspended cultures. But the cells treated with DS/Cu for just four hours did not form any spheres even after 7 days incubation time indicating the effectiveness of DS/Cu against the cells with CSC characteristics. We observed that some of these cells were still alive with intact plasma membrane but they lost the ability to aggregate together and form spheres again. In addition the majority of cells underwent apoptosis. On the other hand we observed that DS alone or Cu alone did not affect the sphere formation indicating the importance of DS/Cu complex in targeting these cells. When we repeated the experiment in a modified manner by exposing the sphere cells without dissociation for 72 hours and without releasing the drug we observed that DS/Cu induced apoptosis and eradicated the spheres completely as shown in the last column of fig 7.3.
Figure 7.3 DS/Cu abolished sphere forming ability of GBM cells. NS and SUS cells were exposed to DS 1µM/Cu 10µM for 4 hrs and cultures in drug free medium for 7 days or continuously treated for 72 hrs w/o release to observe sphere formation. Images were taken at x40 magnification.
7.4.3 DS/Cu inhibits NF-κB and is cytotoxic to NF-κB and ALDH transfected GBM cells

DS/Cu is a well known inhibitor of NF-κB pathway through the inhibition of proteasomes (Cvek, 2008). We performed western blot analysis of proteins collected from cells under all 4 culture conditions ATT, HYP, NS and SUS after treatment with DS1μM/Cu10μM for 24 hours. Our results indicated that treatment with DS/Cu significantly inhibits the NF-κB protein levels in these cells (Fig 7.4). We also tested the cytotoxicity of DS/Cu towards the NF-κB transfected clones which were previously shown to be resistant to different anticancer drugs. The cells were treated for one hour with DS/Cu. After exposure to DS/Cu the cells were washed with PBS twice and then replenished with fresh drug free medium. Our MTT results shown in figure 7.5 indicated that DS/Cu induced cell death in NF-κB transfected clones and they did not show any signs of resistance to DS/Cu. After this short treatment DS probably inhibited both ALDH and NF-κB in these clones that led to Cu mediated ROS accumulation leading to apoptosis.

We also tested the cytotoxicity of DS/Cu on U373 cells transfected with three ALDH iso-enzymes namely ALDH1A1, ALDH1A3 and ALDH3A1. All these ALDH transfected cells were proved to show resistance to different drugs tested. We observed very similar results for this experiment where a short 1 hr exposure of these cells to DS/Cu induced cytotoxicity at low nanomolar levels as shown in fig 7.6. This could be again probably due to the inhibition of ALDH activity in these cells and paving way for ROS mediated apoptosis.
Fig 7.4 Expression status of NF-κB-p65 protein in DS/Cu treated GBM cell lines. Western blot analysis shows reduced expression of NF-κB-p65 protein after treatment with 1μM DS and 10μM Cu for 24hrs. Tubulin was used as a loading control.

Fig 7.5 In vitro cytotoxicity of DS/Cu on NF-κB-p65-transfected GBM cell lines. The cells were exposed to DS/Cu for 1hr and cultured with drug free medium for 72hrs and subjected to MTT analysis. Bar chart displays differences in IC50 values of DS/Cu between Mock and NF-κB-p65 transfected clones.
**Fig 7.6** *In vitro* cytotoxicity of DS/Cu on ALDH1A1, ALDH1A3 and ALDH3A1 transfected GBM cell lines. The cells were exposed to DS/Cu for 1hr and cultured with drug free medium for 72hrs and subjected to MTT analysis. Bar chart displays differences in IC50 values of DS/Cu between Mock and transfected clones.
7.4.4 DS/Cu induces Apoptosis in GBM cells

From the above results we found that in addition to targeting non CSC-ATT cells, treatment with DS/Cu induces cell death and eliminates the resistant population in GBM spheres as well HYP cultures. We determined whether this cell death is induced through apoptosis by western blot analysis of Bax and Bcl2 proteins in ATT, HYP, NS and SUS treated with DS/Cu. The majority of cancer cells and resistant population express high level of anti-apoptotic protein Bcl2 and low level of pro-apoptotic protein Bax. The disturbance of this homeostatic balance is one of the reasons behind resistant nature of cells. The expression of Bcl2 is directly regulated by NF-κB. On the other hand Bax can be activated by various factors including ROS. Our results (Figure 7.7) indicate that when the cells are treated with DS/Cu Bcl2 is suppressed and Bax is increased indicating that DS/Cu kill the cells by inducing intrinsic pathway of apoptosis.

7.4.5 DS/Cu is less toxic to normal cells

We hypothesised that by using copper in combination with DS we can selectively target GBM cells and CSCs because of the already high intracellular Cu levels in GBM cells. We performed MTT cytotoxicity assay with a panel of normal cell lines and three GBM cell lines in parallel. The results in Figure 7.8 shows the cell viability (%) of normal cell lines in comparison to the three GBM cell lines at a dose of 1µM DS and 10µM Cu. It is clearly evident that the normal cells had increased survival rates after treatment with DS/Cu than the GBM cells indicating the selective killing of GBM cells by DS/Cu.
Fig 7.7 Western blot analysis of alteration in BAX and BCL-2 in DS/Cu treated GBM cell lines. Western blot analysis shows the simultaneous inhibition of BCL2 and induction of BAX after treatment with 1μM DS and 10μMCu. Tubulin was used as a loading control.
Fig 7.8 DS/Cu is less toxic to normal cells. In vitro cytotoxicity of DS/Cu on a panel of normal and GBM cell lines. The cells were exposed to DS/Cu for 1hr and cultured with drug free medium for 72hrs and subjected to MTT analysis. The bar chart displays the difference in cell viability (%) between normal cells and GBM cells at a concentration of 1µM DS+10µM Cu.

7.4.6 PLGA-DS nanoparticles have increased serum half-life than free DS

We performed an in vitro assay for determining the half life of PLGA encapsulated DS by studying the release and stability of these nanoparticles in serum. The results shown in figure 7.9 clearly show that free DS disappears from the serum in less than 2 minutes time. We used a liposome encapsulated DS as a comparative tool for release studies. We can clearly see that LipoDS is stable in serum for about 2hrs. But this formulation usually produces bigger size particle and are not suitable for crossing BBB and also from the figure it is clear that the release of DS is not efficient with LipoDS. In addition two hours time may not be enough to increase the bioavailability in brain. The new PLGA nanoparticles formulation of DS significantly enhanced the half life of DS for
about 24 hours. The releasing efficiency of DS from the nanoparticles is good and high compared to that of LipoDS. Hence PLGA-DS NPs should be small enough and provide sufficient time to cross BBB and possibly enhance the bioavailability of DS in brain.

**Figure 7.9 Scanning electron micrograph of PLGA-DS Nanoparticles.** SEM images of PLGA and PLGA-DS nanoparticles show an uniform size and shape of the nanoparticles indicating the high quality of synthesis.

**Figure 7.10** PLGA-Nanoparticle encapsulation increased the serum half life of DS. *In vitro* drug release studies analysed using HPLC indicate increased serum half life for PLGA-DS nanoparticles than free DS or lipoDS.
7.4.7 PLGA-DS NPs inhibits GBM in orthotopic xenograft mouse models

The ability of DS/Cu to target GBM in vivo was examined using PLGA-DS-NPs and orthotopic xenograft mouse models of GBM. We administered a dose of PLGA-DS 10mg/kg intravenously along with 6mg/kg Copper gluconate orally. The GFP Luciferase tagged U87MG cells used for inducing tumours successfully generated tumours in all test animals 7.10A. The figure clearly indicates that tumours generally developed in both the hemispheres of the brain represented by a bright green fluorescence in the image. The treatment was done three times a week for 3 weeks. The results obtained are shown in figure 7.10C which clearly shows that tumour almost disappeared in the group of mice treated with PLGA-DS NPs. The GFP imaging shows a small region of tumour in one of the treated animals but there is a significant reduction in tumour size. To show that the killing is not by Cu we had a separate group treated only with copper gluconate. The results clearly showed that Cu did not have any effect on the tumour in any of the animals. Figure 7.10B. But since this was a very preliminary trial experiment involving less animals per group we did not measure all facts like survival rates and progression free survival. Based on this preliminary data we are conducting another big experiment at present involving more animals to gather detailed in vivo data on the effectiveness of our PLGA formulation against GBM.
Figure 7.11 PLGA-DS-NPs inhibited GBM in orthotopic xenograft models. Tumour bearing mice were randomly subdivided into 3 groups (4 mice/group) A) control; B) copper gluconate (CuGlu) 6 mg/kg p.o + Empty NPs i.v.; C) PLGA-DS NPs 10mg/kg i.v. + CuGlu 6 mg/kg p.o; The drugs were administered 3 times/week for successive 3 weeks. The tumours were photographed using in vivo imaging system that detects green fluorescence produced by tumour cells expressing luciferase GFP.


7.5 Discussion

Currently available chemotherapeutic drugs improve the survival rate in GBM only by a small margin in combination with surgery and radiotherapy. This is due to various reasons like blood–brain barrier (BBB) limiting the access of drugs to brain and intrinsic resistance mechanisms of GBM cells to anticancer drugs. Therefore, there is an urgent need for the development of new drugs that effectively penetrate the BBB and target GBM cells. CSCs are one of the important reasons behind the failure of chemotherapy in GBM. We hypothesised and provided evidence that the CSCs isolated from GBM cell lines are highly resistant to anticancer drugs and these CSCs are probably induced by hypoxia through EMT. We showed that hypoxia also activates an important TF NF-κB, which has been reported to be overexpressed in a diverse range of malignancies like leukemia, lymphoma, breast, colon, lung and ovarian cancers (Karin et al., 2002).

The association between GBM and NF-κB has been well known for many years through various studies indicating the upregulation of NF-κB in response to various stresses in tumours including hypoxia. We showed that the transcription factor NF-κB plays an important role in inducing CSCs through hypoxia induced EMT and therefore could be an important target in reversing chemoresistance in GBM. Although there are several NF-κB specific inhibitors available in the market, many of them do not show promising results in clinical trials (Orlowski and Kuhn, 2008). This is because of other resistance mechanisms like p-gp, MDR, MRP that prevents the action of drugs. In addition NF-κB also regulates
anti apoptotic mechanisms that enhance survival in GBM cells and GBM CSCs (Nogueira et al., 2011). Elevated ALDH activity displayed by GBM CSCs could be another factor for chemoresistance and we showed that NF-κB also plays a role in regulating ALDH expression. Hence simultaneously suppressing multiple resistance mechanisms of these CSCs should be a promising approach and may improve the prognosis of GBM patients. Recently it has been reported that the anti-alcoholism drug DS and its dithiocarbamate derivatives are cytotoxic in vitro to a variety of cancer cells but not to normal cells and hence may have a potential in cancer therapy (Cvek et al., 2008).

ALDH is well known for its involvement in detoxification of a wide range of aldehydes which otherwise generates high ROS inside the cells leading to DNA damage and cell death. ALDH is also known for its ROS scavenging activity reducing oxidative stress (Moreb et al., 2012). Cancer cells have high intracellular ROS levels due to increased metabolic rates. So any conventional drug that elevates the ROS levels can easily target the cancer cells. But the chemoresistant CSC population maintains very low ROS levels by increasing the ALDH activity which is a trademark characteristic of stem cells. ALDH activity has been recognized as a universal functional marker of CSCs and recent evidence suggests that ALDH may not only function as a surrogate marker but also be a target for therapeutic drugs (Skrott and Cvek, 2012). This is because stem cells use the high ALDH levels as a natural mechanism to avoid DNA damage by UV or other stress responses including anticancer drugs. Hence the conventional drugs that elevate the ROS levels simply do not target the CSCs. As an anti alcoholism drug DS specifically inhibits ALDH enzymes
levels in patients’ blood. Our results from chapter 5 shows that transfection of GBM cell lines with ALDH cDNA can induce chemoresistance to conventional anticancer drugs like TMZ, VCR, PAC and DOX. It has been reported by various researchers that knocking down ALDH genes could sensitize CSCs to chemotherapeutic drugs in prostate, ovarian, breast, GBM and lung cancer cell lines (Moreb et al., 2012). Our results also indicate that more than one ALDH isoenzymes are expressed together in the CSCs and the loss of ALDH activity by knocking down one ALDH could probably be compensated by another isoenzyme. Hence targeting a single ALDH isoenzyme may not be efficient in eliminating the CSC population.

DS is a pan- ALDH inhibitor that suppresses ALDH activity rather than individual isoenzyme, and thus is an ideal agent to target ALDH in CSCs. Although targeting ALDH is one of the promising strategies to reverse chemoresistance in CSCs, that doesn't seem to be enough as CSCs have several other mechanisms of resistance which explains why many commercial ALDH inhibitors do not induce apoptosis in CSCs. This also explains why DS alone can inhibit ALDH but cannot produce any cytotoxic effects in CSCs. Results from our group have shown that the cytotoxicity of DS is highly copper (Cu)-dependent. DS in combination with Cu enhances the cytotoxic effects of conventional anticancer drugs (Yip et al., 2011; Liu et al., 2012). My results from the sphere formation assay shown in figure 7.3 also indicate that DS alone or Cu alone does not affect the GBM spheres and hence leaving CSCs intact. But the combination of DS with Cu completely eradicates the sphere cell population. Similar results have been shown previously by our group in breast cancer; colon
cancer and GBM cell lines highlighting the importance of addition of Cu in DS cytotoxicity (Yip et al., 2011; Liu et al., 2012; Guo et al., 2010). Cu is an important trace element that plays a vital role in mitochondrial redox reactions and generates ROS that bring about apoptosis in cells. The transport of Cu into the cells is strictly regulated by the Cu transporting transmembrane protein Ctr1. DS is a strong chelator of metal ions, and can combine with Cu to form a DS/Cu complex. DS is known to enhance the transport of Cu into cancer cells by forming complex outside the cells or DS chelates intracellular Cu after penetrating into the cells forming a DS/Cu complex. DS/Cu is a much stronger inducer of ROS than Cu alone. (Nobel et al., 1995). So inhibition of ALDH by DS and generation of ROS by DS/Cu complex will lead to accumulation of ROS in CSCs thereby inducing apoptosis in these cells.

But ROS generated by any mechanism is like a double-edged sword. It can also induce various anti-apoptotic mechanisms among which activation of NF-κB is an important mechanism by which CSCs evade ROS mediated apoptosis (Liu et al., 2012). In addition to the role in hypoxia mediated EMT leading to CSCs, NF-κB also plays an important role in survival of these CSC population (Liu et al., 2014). This is the reason why inhibition of NF-κB is crucial for successful anticancer treatment. Consequently a compound that can simultaneously inhibit ALDH, inhibit NF-κB and elevate ROS would significantly improve cytotoxic effects mediated by ROS. DS is a specific inhibitor of NF-κB pathway by inhibiting the proteasome degradation pathway (Cvek, 2008). In addition to NF-κB pathway, proteasome degradation will also have an effect on various other cellular proteins causing a total imbalance and hence leading to cell death. Our
results after treatment with DS/Cu clearly show that NF-κB protein levels are suppressed. Simultaneously BCL2 an anti-apoptotic factor and a direct target of NF-κB were suppressed leading to enhanced pro-apoptotic protein BAX. BAX can also be induced by elevated ROS generated by DS/Cu complex leading to binding of voltage dependant anion channel in mitochondrial membrane leading to apoptosis (Skrott and Cvek, 2012). Moreover none of the NF-κB transfected clones survived after treatment with DS/Cu. Very similarly the cells that are transfected with different ALDH cDNAs were also killed by DS/Cu treatment.

In addition to these our results showed that DS/Cu selectively targets GBM cells and spares normal cells. This could be again because of the variations in Cu levels between normal cells, cancer cells and CSCs. Compared with normal tissues, high Cu levels present in cancer cells (Mulay et al., 1971; Yip et al., 2011). DS transports Cu into the cells and elevate the Cu levels in the cells. At the same time inhibition of ALDH and NF-κB will help the DS/Cu complex to accumulate ROS levels beyond the threshold limit thereby inducing cell death. But the Cu level in normal cells does not increase high enough to push the ROS beyond the threshold. The same strategy could possible apply between CSCs and normal stem cells leading to selective targeting of CSCs and cancer cells by DS/Cu. It has been previously shown that DS does not affect cells in the kidney, gut or bone marrow while potentiating the cytotoxic effects of conventional anticancer drugs (Hacker et al., 1982; Bodenner et al., 1986). The prospective clinical application of DS for treatment of GBM is hindered by the currently available oral formulation of DS which is unstable in the acidic gastric environment and has a very short half-life of less than 2mins. Even after a
500mg dose of DS, its metabolites cannot be detected in blood because of very low systemic concentration (Johansson, 1992). This short half-life may present a serious challenge for the use of DS in GBM treatment as the time is too short for the drug to cross BBB and accumulate in the brain environment. Therefore, we developed a new formulation of DS with longer half-life using PLGA-nano encapsulation technique that increased the half life of DS up to 24 hours. This also will ensure that DS can cross BBB and sufficiently dose can reach the brain. We successfully tested this new formulation of PLGA-DS nanoparticles in orthotopic xenograft mouse models and achieved significant reduction in tumour levels of treated mice.

Fig 7.12 Proposed mechanisms of selective killing of GBM cells by DS/Cu
It is very likely that DS/Cu eliminated the CSC population as well as non CSC population in the brain effectively thus reducing tumour size. Although we propose the mechanism to be simultaneous inhibition of resistance proteins, NF-κB pathway and ALDH activity in addition to ROS elevation, there may be still many unknown mechanisms involved behind the success of DS/Cu. Further studies are required to confirm these findings. Once the DS/Cu and its formulations are fully characterized and tested, proper clinical trials are required. The prognosis for GBM patients still remains poor and development of drugs for BM is very slow. Hence repurposing of FDA approved drugs like DS with very little known side effects will cut down the cost and time required for development of new drugs besides providing therapeutic benefit for GBM patients.

7.6 Conclusion
To conclude we can say that DS/Cu effectively targets the CSC and non CSC population in GBM cells in vitro. The new formulation of PLGA encapsulated DS nanaoparticles with increased serum half life can effectively cross BBB and inhibits GBM in vivo. The possible mechanisms of action could be by simultaneous inhibition of NF-κB and ALDH and increased generation of ROS by DS/Cu complex. DS/Cu is probably one of the best combinations of drugs that can efficiently target different CSC resistant mechanisms simultaneously. As an FDA approved drug with all clinical and preclinical data available, further studies with various formulations could quickly translate DS in to GBM therapeutics.
Chapter 8

Discussion
8. General Discussion

GBM is the most common malignant primary brain tumour in adults, and has the worst prognosis among all human cancers. Even after aggressive treatment measures including surgery, radiotherapy and chemotherapy, the median survival among patients with GBM is only 12-15 months (Reardon and Arvold, 2014). Only about 3-5% of patients have an overall survival rate of 2 years (Parsons et al., 2008). The reason behind such poor outcome is because most of the GBM patients develop a refractory disease with several characteristics that hinder therapeutic options and results in high rates of relapse. These include heterogeneity, highly infiltrative and invasive nature and presence of resistant population including CSCs. Currently available chemotherapeutic agents including the first line drug TMZ, increases survival only by a small margin however most patients develop resistance to these agents. This is partly due to the blood–brain barrier (BBB) which limits drug access and also because of the intrinsic resistance nature of GBM cells to many anticancer drugs (Sugiyama et al., 1999). Therefore, developments of new drugs that can cross the BBB and target GBM cells are of significant clinical importance for the treatment of GBM. While demand for better cancer therapies is high the rate of drug development is slow and expensive (average 15 years and US$1.5bn per drug) and only 5-25% of anticancer drugs in clinical development actually reach the market mainly due to toxicity of these agents (Walker et al., 2009). Hence the idea of finding new uses for existing drugs also known as ‘repurposing’ has emerged in recent years. Because of the already existing safety profiles and pharmacokinetics information, any newly identified use of old drugs can be
quickly translated to phase-II clinical trials within two years and at a low cost of $17m (Marshall, 2011; Chong and Sullivan, 2007). The drug we used in this study Disulfiram, the old anti-alcoholism drug is one best example for a repurposed drug which has been reported to have significant potential in cancer therapy.

Previously published research from our group in different cancers has already shown various mechanisms of DS induced cytotoxicity. These include inhibition of ALDH, inhibition of proteasome, inhibition of NF-κB pathway, targeting MDR p-gp, and inducing ROS-MAPK pathway of apoptosis. In the body DS is metabolised into DTC which is shown to chelate with metal ions like Cu or Zn to form complexes that can elevate the ROS in cells. Our published data also revealed the importance of Cu in DS mediated cytotoxicity (Yip et al., 2011; Liu et al., 2012). DS/Cu was also shown to sensitise the cancer cells and potentiate the cytotoxic effects of other conventional anticancer drugs (Liu et al., 2012; Triscott et al., 2012; Yip et al., 2011; Liu et al., 2013; Liu et al., 2014; Chen et al., 2006). In this study I intended to repurpose DS for GBM treatment by understanding the in vitro and in vivo efficiency of DS in targeting GBM cells, specifically GBM CSCs.

In order to do this, it is indispensable to have a complete understanding about the role and mechanisms of GBM CSCs mediated resistance of GBM. GBM CSCs are a very small population of cancer cells expressing CSC markers that promotes therapeutic resistance. Although there is wealth of evidence supporting the role of GBM CSCS in initiation, progression and relapse of GBM
there is a still an ongoing debate concerning the origin and nature of GBM CSCs. It is still unclear whether GBM CSCs are derived from neural progenitor cells or from differentiated cells. I was interested to fully elucidate the the nature of GBM CSCs and their resistance mechanisms. Many research groups mentioned that GBM CSCs are a special group of cells that can be isolated only from fresh tumour samples of GBM from patients. Additionally it was claimed that isolation of GBM CSCs also required specific low attachment spheroid growth conditions and special growth factor-supplemented serum-free selective growth medium which was is adapted from the normal neural stem cell culture system. This method is widely accepted as a gold standard method to keep the CSCs in an undifferentiated state under in vitro conditions. It is also referred that serum free conditions will purge the differentiated progenies, and selectively enrich the CSC population (Lee et al., 2006). The major pitfalls of the serum-free enrichment system are that it is very expensive, it differs from the physiological conditions and there is no clear evidence for source of the CSCs growing in sphere culture. Another factor to consider is that CSCs once exposed to serum will differentiate irreversibly in to their progenies. Researchers supporting this selection hypothesis do not use established cell lines as a source for CSCs. But on the other hand several studies also reported the isolation of CSCs from established cell lines of GBM. They showed all CSC characteristics including initiation of tumour in animal models (Charafe-Jauffret et al., 2009; Kondo et al., 2004; Collins, 2005; Setoguchi, 2004).

Our results in this study also demonstrated that when the cells from established GBM cell lines such as U87, U251 and U373 are grown as NS in selective
media they form spheres and exhibit several CSC characteristics such as CD133, ALDH, Sox2, Oct4, and Nanog. If this is the case it appears like most of the established cell lines have a residual population of CSCs that can be enriched and activated by this growth factor enriched medium under specific growth conditions. But on the other hand we also demonstrated that growing these cell lines as spheres in normal DMEM+10% serum conditions also induced better spheres and enhanced all the above mentioned CSC characteristics. Therefore we concluded that stemness in GBM CSCs is not determined by the culture medium and hence serum free culture system is not mandatory for GBM CSC culture.

We found that hypoxia is the common factor that existed in all GBM sphere cultures. Many recent studies have elucidated that intra-tumoural hypoxia induces CSCs phenotypes in tumour via epithelial-to-mesenchymal transition (EMT) (Batlle et al., 2000; Yee et al., 2010; Polyak and Weinberg, 2009; Salnikov et al., 2012). We provided substantial evidence for EMT that occurred in both NS and SUS cultures indicating that culture medium or the source of the initial GBM does not affect the formation of GBM CSCs in sphere cultures. Instead it is the mesenchymal phenotypes which are induced by hypoxia are the true culprits in driving resistance. We provided evidence for the above statement by showing that cells grown as attached monolayers under hypoxia in normal medium acquired mesenchymal phenotype and eventually CSC properties without growing them as spheres. This transition into CSC like phenotypes also led to remarkable increase in the chemoeresistance towards various drugs which indicated that accumulated hypoxia could be the key
reason behind chemoresistance in GBM. This transition to mesenchymal phenotype also played an important role in enhancing the migration invasion and metastatic potential of these cells. This can also apply to physiological conditions of GBM in patients where tumour cells experience extensive hypoxia in the central region of the tumour bulk. Under such hypoxic conditions added to several other microenvironmental stresses the fully differentiated GBM cells could reprogram themselves to mesenchymal cells to facilitate migration, metastasis or to produce growth factors to initiate angiogenesis in order to survive and escape the hostile environment. Unlike the NSCs, the monolayer-cultured non-CSCs and sphere-cultured CSCs are interconvertible by oxygen concentrations (Biddle et al., 2011; Chen et al., 2012). Hypoxic stress induces EMT and the cells can migrate out of the tumour and follow another program called MET where the mesenchymal cells become differentiated epithelial cells. This could be the reason why when grown as spheres, established cell lines get converted to mesenchymal phenotype and revert back to the epithelial phenotype when they are adhered back to monolayer culture. Physiologically this could be a very important mechanism that happens in all solid tumours where cells constantly under hypoxic stress are undergoing EMT to move away from the hostile environment and undergo MET to establish in the new region. This could be the possible reason why GBMs has multiple spots of local metastasis or highly infiltrative in nature that leaves the tumour with no margins (Szeto et al., 2009). The survival signalling pathways activated for this interconversion adds up to the already existing intrinsic resistance rendering chemotherapy useless. But we also had questions to ourselves about how some people can isolate NS CSCs from fresh samples. This is probably
because they are enriching the already existing mesenchymal or CSC phenotypes induced by physiological hypoxia in the primary tumour. Thus we concluded that GBM sphere cell culture using selective medium or normal medium could provide a suitable model for studying this hypoxia induced resistant cells. Although under physiological conditions there are a lot of other microenvironmental factors that play their role in chemoresistance to drugs, these sphere cell cultures from GBM cell lines could provide us with physiologically relevant model for studying drug uptake, resistance mechanisms and EMT phenotypes.

After establishing the fact that hypoxia is the key driver of CSCs and resistance in GBM sphere cells I analysed the possibilities of various transcription factors which are likely to be involved in activating various signalling pathways leading to this hypoxia induced EMT programme eventually to chemoresistant GBM CSCs. We had to do this because we had to understand who the master driver under hypoxic conditions is and whether DS/Cu can target this upstream activator of CSCs in order to inhibit CSC population. If not upstream we wanted to analyse what are the downstream regulators that are activated and I also wanted to understand if DS/Cu can blocking these important factors. Hypoxia inducible factors (HIFs) are master transcriptional regulators under hypoxic conditions (Rohwer and Cramer, 2011). In this study we analysed the role of two HIFs namely HIF1α and HIF2α. The DNA binding and the transcriptional activity depends of these ubiquitously expressed proteins depend largely on nuclear translocation of HIFα subunits. These elements are tightly regulated and constantly degraded by PHDs which prevents nuclear localization under
high concentrations by of $O_2$. So in order to study them we used U373MG cell line transfected with HIF1$\alpha$ and HIF2$\alpha$ cDNA that drastically increased the cytoplasmic proteins levels of HIFs and compete with the PHDs ultimately leading to increased HIF1 or HIF2 signalling pathways. Both HIF1$\alpha$ and HIF2$\alpha$ will be activated under reduced $O_2$ environment which directly or indirectly activates the expression of hundred of genes. Some of these targets overlap between these two factors due to the similarities in their DNA binding sequence. Therefore the biological consequences of hypoxia in cells largely depend on which HIF is active at any given time and what targets are being regulated by them (Hedleston et al., 2010). Our results demonstrated that increased expression of both HIF1$\alpha$ and HIF2$\alpha$ could play vital roles in driving the EMT programme. We found that expression of HIF2$\alpha$ induced resistance to TMZ. However we did not find significant evidence to confirm the involvement of both HIFs in multidrug resistance which is a clinically important characteristic of GBM CSCs. But we cannot deny the fact that both HIF1$\alpha$ and HIF2$\alpha$ are crucial for EMT, CSCs, tumour motility and invasion suggesting that pharmacological inhibition of these factors in addition to suppression of chemoresistance factors holds great promise in targeting GBM CSCs.

However NF-κB, another key transcription factor, is also highly up-regulated in hypoxia induced CSCs and is proved to be playing an important role in cancer chemoresistance (Cummins et al., 2005). NF-κB is a key TF involved in various normal and pathological conditions of cells and is widely associated with several diseases. Although the link between inflammation, NF-κB and cancer has been studied for decades, the detailed molecular mechanisms and pathways linking
hypoxia, NF-κB and CSCs are not fully elucidated. There is also a large amount of literature evidence that suggest that HIFs can activate NF-κB through interaction with components of NF-κB pathway (Yeramian et al., 2011; Cummins et al., 2006). While there are some evidences which suggest that NF-κB could be the factor activating HIFs (Bonello et al., 2007). In my study the activation of NF-κB was observed in all sphere cell models and in cells under hypoxic conditions. This suggested that NFκB which is known to be a TF for more than 200 genes could play an important role in determination of CSC traits. But there are no clear insights on how HIFs and NF-κB together orchestrates the anti-apoptotic signalling, chemo-radiation resistance and maintenance of stemness in GBM CSC phenotypes. In order to study this in detail, I transfected the U373MG cell line with NFκB p65 cDNA in order to enhance the cytoplasmic levels of NF-κB that can compete with IkB and gain nuclear translocation. NF-κB pathway activation was also confirmed in this p65 transfected cells. Our results revealed that the NF-κB p65 transfected clones induced EMT pathway to convert these cells to a mesenchymal phenotype, expressed high levels of stem cell markers and displayed evidence of multidrug resistance to drugs like TMZ, PAC and VCR. In order to establish the link between HIFs and NF-κB, we analysed the nuclear translocation and transcriptional activity of NF-κB in HIF1α and HIF2α transfected cells. None of the HIF transfected clones had increased NF-κB activity indicating that neither HIF1α nor HIF2α could be direct drivers of NF-κB p65. On the other hand we found that NF-κB transfected clones significantly enhanced the expression and nuclear translocation of both HIF1α and HIF2α indicating that NF-κB could be a
direct driver of these genes. Therefore we concluded that NFκB could possible play the master role in regulating hypoxia induced EMT, stemness and chemoresistance by activating various genes directly or through activation of HIF signalling. In addition to NF-κB mediated resistance factors all CSCs possess high levels of ALDH activity which is a universal stem cell marker and has been implicated with chemoresistance for many types of cancers (Sladek et al., 2002; Liu et al., 2014). ALDHs are a group of detoxification enzymes that has 19 different isoforms. High ALDH expressing cells have been associated with enhanced xenograft tumour formation in mice and chemotherapeutic resistance by cell detoxification leading to resistance towards drugs like cyclophosphamide, cisplatin, docetaxel, temozolomide and doxorubicin (Sladek et al., 2002). Initially, ALDH1A1 was thought to be the isoform associated with the CSC phenotype, but later several other phenotypes are also shown to play a role in resistance (Marcato et al., 2011). Since DS is a specific inhibitor of ALDH we thought that ALDH inhibition could be the reason behind the ability of DS to reverse chemoresistance and induce cytotoxicity. So this means that ALDH as reported by many others could be the important target in reversing chemoresistance of GBM CSCs. We screened our GBM sphere cells for the expression of three commonly mentioned ALDH isoenzymes namely ALDH1A1, ALDH3A1 and ALDH1A3. We found that GBM sphere cells had increased expression of ALDH1A3 and ALDH3A1 but had clearly downregulated expression of ALDH1A1 in comparison to that of non-CSC GBM cells. Since NF-κB clones were resistant to drugs and displayed enhanced ALDH activity, we screened them for the above three isotypes. Excitingly, the same pattern of ALDH expression in GBM spheres was reproduced in NF-κB transfected
clones. Although we couldn’t provide detailed or direct evidence for NF-κB in transcription of ALDH genes we hypothesised that NF-κB may play a key role in regulatory link in this pattern of ALDH expression. In order to find out whether ALDH isoforms play any role in chemoresistance of GBM CSCs we transfected the U373MG cell line with cDNAs of all the three ALDH isoforms and selected the clones with very high expression of these isoenzymes. We tested these ALDH clones for chemoresistance and found that all the ALDH isoenzymes induced resistance towards drugs like TMZ, PAC, VCR and DOX. We concluded that regardless of the isoenzyme the high ALDH enzyme activity induced by the presence of high levels of any isoform could be the reason behind chemoresistance driven by ALDH. We also pointed out that if any one isoform is targeted, the presence of another isoform could still maintain the ALDH activity to retain chemoresistance. Due to redundancy in activity by some isoforms it very difficult to pinpoint or target independent isotype. Hence targeting the whole ALDH activity rather than subtype is a better strategy to target CSCs.

DS is able to suppress GBM tumour development through multiple mechanisms. As a single compound DS is shown to be a inhibitor of drug resistance proteins like P-gp and MRP-1. It is a well established proteasome inhibitor that leads to inhibition of NF-κB pathway. DS is also known to be a pan ALDH inhibitor that suppresses ALDH activity (Liu et al., 2014). Several pieces of evidences from our research group and various other groups working on repurposing DS for cancer treatment have provided solid evidences to prove that DS could be the drug that has the ability to simultaneously target multiple
chemoresistance mechanisms. Data from our research group provides convincing evidences that the cytotoxicity of DS is dependent forming complexes with Cu or other metal ions like Zn (Liu et al., 2014). We chose Cu because it plays a critical role in redox reactions and in generates free radicals in combination with DS (Turnlund et al., 1998). Cu has a very long history in cancer treatment with records early as 400 B.C. (Gelarie, 1913; Hieger, 1926). Cu exists in two forms in the body, oscillating between the cuprous (Cu1+) and cupric (Cu2+) forms, with Cu2+ found widely. The transport of Cu into the cells is strictly regulated by Ctr1, a Cu transporter protein (Kim et al., 2008). Hence treatment with Cu alone does not result in cytotoxicity which is clearly evident from our previously published results as well as results shown in treatment of GBM CSCs in this study. Copper initiates oxidative damage through generation of the most powerful oxidising hydroxyl radicals (OH) from hydrogen peroxide (H2O2). Hydroxyl radicals are capable of reacting with every biological molecule resulting in DNA damage and apoptosis through accumulation of ROS. Not only DS and but also it metabolic derivative, DTC can form a complex Cu. In fact it was shown the enhancement of Cu levels in the cells was achieved by transport of Cu into the cells by DS/Cu complex in a Ctr1 independent mechanism (Cen et al., 2002; Cen et al., 2004).

But it is well understood that GBM tissues possess significantly higher (approx 44-fold) levels of Cu owing to their high metabolic rate and a high need for Cu in redox reactions than the surrounding normal brain tissues (Margalioth et al., 1983; Yoshida et al, 1993). Therefore additional Cu intake by DS/Cu could push the generation of ROS in GBM cells beyond the threshold limit thereby inducing
oxidative damage and apoptosis. This enables us to develop a strategy to selectively target GBM cells sparing normal brain tissue. Since brain is a highly delicate organ, selective killing by any anticancer drug is a vital requirement and DS can effectively achieve this with the strategy involving Cu. My results using a panel of normal cell lines compared to that of cancer cell lines demonstrated that DS/Cu is highly cytotoxic to GBM cells but less toxic to normal cells. In addition DS/Cu can kill GBM cells at very low nanomolar levels and hence we can target tumour cells without any system toxicity or worsening side effects which is extremely rare or impossible to be achieved by any present day anticancer drugs.

Although all the above mentioned mechanisms of DS/Cu apply for GBM cells the major aim of my work was to see the anti-GBM CSCs effect of DS/Cu. My in vitro data comparing the NS and SUS sphere cells of GBM showed that there is no big difference in the cytotoxicity ranges of DS/Cu on these cells and the results are very much comparable. In addition we found that DS/Cu treatment abolishes NF-κB in ATT, HYP and in sphere cells indicating that NF-κB could be one of the primary targets. As mentioned earlier the possible mechanism of DS/Cu cytotoxicity on these cells could be by elevating ROS levels. But we need to consider that cells possessing CSC characteristics are known for their very low ROS levels due to high ALDH activity. Being a well known pan ALDH inhibitor DS/Cu comfortably induced cell death in GBM CSCs because elevation of ROS is possible by bringing the ALDH down. The ALDH transfected GBM cells that showed resistance to all drugs tested earlier did not show any form of resistance to DS/Cu treatment. It is known that elevated ROS levels also
activate NF-κB pathway and anti-apoptotic machinery related to it. But DS/Cu being an effective inhibitor of NF-κB pathway this mechanism of evasion by CSCs simply does not work. Earlier work by our group in breast cancer, colon cancer and GBM has shown that in addition to ALDH and NF-κB inhibition and accumulation of ROS, treatment with DS/Cu resulted in the persistent activation of JNK and p38 MAPK pathways that promote ROS-induced apoptosis in all these cancer cells (Liu et al., 2012, Liu et al., 2014; Junttila et al., 2008). ROS is known to activate ERK pathway downstream that plays essential role in survival and proliferation. But the ROS generated by DS/Cu did not activate the ERK pathway. ROS-activated JNK and p38 pathways induce apoptosis via mitochondrial apoptotic pathways in which the balance between pro-apoptotic BAX and anti-apoptotic BCL2 plays a major decisive role (Junttila et al., 2008).

From my results from the untreated GBM cells including ATT, HYP, NS and SUS it well understood that the balance between BAX/BCL2 is inclined towards resistance of apoptosis where BCL2 expression is higher. In fact very high BCL2 expression was noted in the sphere cells and HYP cells compared to the non CSC counterparts indicating their resistant nature. The treatment with DS/Cu reversed this balance to express more BAX and suppress BCL2. It is also known that BCL2 is a direct target of NF-κB pathway (Gloire et al., 2006). So it is evident that inhibition of NF-κB pathway by DS/Cu resulted in suppression of BCL2 leading to BAX increase by ROS.

Although DS shows remarkable anticancer activity in vitro its clinical application in patients is highly limited by its bio-instability. The half-life of DS in the bloodstream is only less than 4 minutes due to its rapid degradation and extensive
metabolic conversion (Cobby et al., 1977). Therefore an effective delivery system was required to protect DS in bloodstream that can extend its half life to provide enough time to cross the BBB and reach the GBM tissue. Our collaborators in Shenyang Pharmaceutical University, China developed a liposome encapsulated DS that improved the half life of DS to about 2 hours. But for GBM we had practical problems of delivering Lipo DS because of its size and probably insufficient time to reach the brain. So we had to think about further increasing the half life and decreasing the size of the delivering substance. Dr. Zhipeng Wang, post-doctoral research fellow in our group recently developed PLGA-coated DS nanoparticles which extended the half life up to 24 hours and are relatively small particles measuring in the nanometer ranges. We tested on orthotopic GBM xenograft mouse models. The results demonstrated that DS has remarkable potential of eliminating GBM tumours in the animal models. The results were achieved with very low dose of 10mg/kg dose of PLGA-DS and 6mg/kg of oral copper gluconate supplement. The normal dose of free DS used for alcoholism treatment ranges between 300-500mg. We are continuing further experiments to have an enlarged animal study to demonstrate the effectiveness of PLGA-DS-nanoparticles.

The tumour microenvironment plays a vital role in promoting the progression of GBM. My data suggest that hypoxic microenvironment could result in heterogeneous population due to interconversions and transitions between different cell phenotypes. Further studies are required to understand the molecular mechanism of hypoxia induced CSCs and its relevance to physiological tumour conditions. The role of NF-κB and HIFs has to be studied
in further details using techniques that can functionally knock out or knock down these genes and other important factors involved in the origin of GBM CSCs. We also aim to do chemical inhibitor studies to block the above pathways individually to further understand the relationship between hypoxia, NF-κB and CSCs. If NF-κB acts as a master regulator of hypoxia induced stemness and resistance, it will be interesting to see whether inhibition of NF-κB in hypoxic cultures will stop the cells from being converted to mesenchymal phenotypes. Further studies are ongoing to analyse all 19 isoforms of ALDH in GBM CSCs to understand the regulators of these isoenzymes.

In order to successfully treat tumours like GBM novel combination therapies targeting different phenotypes in different regions of the tumour microenvironment are necessary. Formulating the existing drugs in nanoparticles (NPs) will have unique advantages in delivering multiple anticancer drugs to tumour sites without systemic side effects. In addition it is possible to change existing pharmacokinetics and bio-distribution of drugs using NPs. There are several attempts made by researchers to formulate DS into gold NPS, lipid nanoemulsions, dendrimer NPS, polymeric micelles, etc. Since PLGA-DS NPs are working in vivo will do further analysis using these NPs. However our lab is also generated polymeric micelle NPs of DS. It will be very interesting to compare the effectiveness of both these NPS to select the best delivery system with high efficiency and low toxicity for the treatment of GBM.

In summary, GBM contains CSC population that are highly resistant to anticancer drugs. These CSCs are probably hypoxia induced EMT phenotypes
with acquired mesenchymal characteristics that results in aggressive, chemoresistant and infiltrating nature of GBM. NF-κB along with HIF signalling orchestrates the various signalling pathways leading to EMT programme under hypoxic conditions and hence are important targets in eradicating GBM CSCs. ALDH activity resulting from the acquisition of CSC characteristics also play a major role in chemoresistance. DS is a specific inhibitor of NF-κB pathway and a pan ALDH inhibitor. DS forms a complex in combination with Cu, which was highly cytotoxic to GBM cells, hypoxic cells and also chemoresistant GBM CSCs. DS has a very short half life in blood stream which limits its clinical applications as an anticancer drug. The new formulation of PLGA-DS-nanoparticles developed in our lab with extended half life significantly reduced GBM in xenograft models of GBM at very low doses in combination with oral copper gluconate. This also indicated that the new formulation effectively crossed the BBB. DS is a safe, well-understood, FDA approved drug that has been used in the anti-alcoholism clinic for over 60 years. DS does not show any systemic toxicity or severe side effects because of the low toxicity to normal cells. We have demonstrated that DS has excellent anticancer activity in a wide range of cancers, including the most aggressive form of colon, breast, and liver cancer and triple negative breast cancer. Hence DS should be translated to clinics at the earliest at least for treatment of tumours like GBM where there are no successful therapeutic options at present.
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