ROLE AND REGULATION OF MYC IN GLIOBLASTOMA MULTIFORME CELL DIFFERENTIATION:
IMPLICATIONS IN TUMOR FORMATION

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1.0 BACKGROUND

1.1 Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most aggressive and malignant brain cancer in adults (1). It is considered to be one of the deadliest of human cancers. Clinical outcome of GBM is very poor; surgical resection of the tumor followed by chemo- and radiation therapy fails to prolong the median survival of the patient beyond 15 months (5-7). There are two distinct routes of GBM development: de novo and progressive. De novo (or primary) GBM occurs in older patients who present with the disease without any prior history of brain tumors (8). Whereas, patients who have progressive (or secondary) GBM present with a history of low grade astrocytoma that later develop into GBM over a period of 5 to 10 years (9). A recent study using clinically defined de novo and progressive GBM tumors, has revealed that the progressive GBM tumors are heterogeneous in genetic characteristics although they have similar morphological phenotypes (10). However, both de novo and progressive forms of the disease demonstrate identical clinical and histopathological characteristics such as
necrosis, pseudopalisading nuclei and microvascular proliferation, which are hallmarks of GBM (Figure 1).
Figure 1. Two subtypes of GBM tumors (1). Primary GBM tumors occur suddenly without any prior history of tumors. Secondary GBM tumors develop from low grade astrocytomas over a period of 5 to 10 years. Primary and secondary GBM tumors are clinically indistinguishable.
Some of the common genetic alterations that are found in GBM tumors are listed in Table 1 (1).

Table 1: Common genetic alterations in GBM tumors (1)

<table>
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<th>Genetic alterations</th>
<th>Frequency</th>
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<tr>
<td>EGFR amplification</td>
<td>40-50%</td>
</tr>
<tr>
<td>Deletion or mutation in both copies of chromosome 9 in a region that encodes CDK inhibitors CDN2A/p16 &amp; CDKN2B/p15</td>
<td>30-40%</td>
</tr>
<tr>
<td>Homozygous inactivation of Rb gene on chromosome 13</td>
<td>&gt;14%</td>
</tr>
<tr>
<td>Amplification of cdk4 gene segment of chromosome 12</td>
<td>15%</td>
</tr>
<tr>
<td>Overexpression of Cyclin D1/Cyclin D3</td>
<td>some</td>
</tr>
<tr>
<td>Mutations/deletions of p53 gene</td>
<td>25%</td>
</tr>
<tr>
<td>Amplification of MDM2 locus on chromosome 12</td>
<td>5-12%</td>
</tr>
<tr>
<td>Deleted/mutated ARF locus on chromosome 9</td>
<td>30-40%</td>
</tr>
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According to World Health Organization (WHO) classification scheme, GBM is categorized as grade IV astrocytoma (11). Gliomas of different grades differ in their genotypic and phenotypic characteristics from each other (1). The prognostic outcome and patient survival of each of the glioma grades also differ dramatically from somewhere around 10 years in grade I astrocytoma to 9-12 months in GBM (12). Although by definition, gliomas are infiltrative brain tumors, the capacity to infiltrate is the highest in GBM tumors (13). GBM tumor cells also possess the highest proliferation
ability (1). Among the gliomas, only GBM tumors display angiogenesis and tissue necrosis (14). A plethora of knowledge regarding the characteristic genetic changes in GBM has resulted in many clinical trials however; the median patient survival has not changed significantly.

### 1.2 Brain Tumor Initiating Cells

Inefficacy of current therapeutic modalities and understandings from other human cancers has resulted in a shift in approach to GBM research to focus on the tumor cells that are able to initiate and maintain the tumor instead of studying the bulk of the tumor tissue. These cells are thus called the brain tumor initiating cells (BTICs) and the underlying concept is termed Cancer stem cell (CSC) hypothesis due to the functional similarities between the initiator cells and normal stem cells. This hypothesis voices that only a small rare fraction of tumor cells dispersed in the bulk of the tumor tissue possess the ability to maintain and regenerate the cancer. These cells are called the CSCs (or the tumor-initiating cells) (15-19). The idea of CSCs slowly and steadily developed from observations in hematopoietic cancers such as lymphomas and leukemias (20-23), and spread across non-hematopoietic (solid) cancers like breast (24-30), prostate (19, 31-33) and colon cancers (34-38). It is a relatively new idea in the field of cancer research. The notion of brain CSCs emerged from the observations made by Steindler et al. in 1962.
Their group generated clonogenic neurosphere-forming precursor cells from GBM tumor tissue resected from patients (39). Neurosphere formation was a functional characteristic of neural stem cells (NSCs) that was exploited by researchers in developmental neurobiology to demonstrate the presence of the NSCs (40).

Normal NSCs possess the ability to self-renew and generate progenitors, which can give rise to cells of neuronal and glial lineage (41, 42). The BTICs also display similar characteristics, namely the ability of limitless self-renewal, proliferation, and differentiation into multiple lineages (Figure 2) (10, 43).
Figure 2. A minor fraction of GBM tumor cells resemble normal NSCs. Both populations form neurosphere in *in vitro* culture. Under defined conditions, these cells are able to differentiate into multiple lineages including neuronal, oligodendroglial and astrocytic lineage. The differentiated cells can be identified using lineage specific markers such as MAP2ab in neurons, MAP4 in oligodendrocytes and GFAP in astrocytes.
However, unlike normal neural stem cells, this minor population of BTICs can form tumors upon transplantation in appropriate hosts. The BTICs are speculated to be the resident neural stem/progenitor cells that normally remain quiescent but possess the ability to become active under conditions of stress and insult to the brain (44). The quiescent normal neural stem/progenitor cells have characteristically long lifespan which facilitate their probability of acquiring and accumulating multiple mutations over years that ultimately transform these cells and initiate tumors (45). Another line of evidence emerges from studies on glioma variants, namely, oligoastrocytomas and gliosarcomas. These tumors have characteristic biphasic tissue patterns. In oligoastrocytomas, there are areas with astrocytic and oligodendrocytic differentiation. In gliosarcomas, there is distinct gliomatous and mesenchymal differentiation. The presence of different cell types in the same tumor suggests that either independent transforming events occurred in each of the terminally differentiated cell types or an undifferentiated multipotent progenitor cell underwent transforming changes resulting in cancerous cells of multiple lineages (1). A layer of complexity is added to this issue by the knowledge that stem/progenitor cells may be later recruited at the site of tumorigenic lesion in the brain (46). Reports have demonstrated that normal NSCs possess the ability to localize and graft into sites of lesion and can be used to deliver may therapeutic agents (41). This information highlights the possibility that quiescent normal NSCs present in the subventricular zone and dentate gyrus of the hippocampus, which are the stem cell niches in the brain (47, 48), may be mobilized by any lesion and migrate to new locations and later get transformed to initiate
tumors. Thus, the question regarding the origin of the cell(s) responsible for brain tumor initiation is elusive and is a topic of extensive investigation.

An alternative hypothesis holds that transforming mutations in differentiated cells, astrocytes in case of GBM, trigger their de-differentiation into a multipotent state. The transformed and dedifferentiated cells then initiate brain tumors. Studies demonstrate that mature astrocytes in brain dedifferentiate into an undifferentiated radial glia-like cell. To this end it is noteworthy that radial glia appear during early embryonic development and it was believed to terminally differentiate into astrocytes until such observations of dedifferentiation of astrocytes into glia-like cells were made. Thus, mature glial cells such as astrocytes are also considered to be potential candidates for the cell of origin of GBM tumors.

GBM tumor-derived CSCs are found to continuously self-renew in vitro, and give rise to new but similar tumors in vivo as monitored by xenograft assays (49-51). Reports have also claimed these cells to be resistant to chemotherapy and radiation therapy (52-54). Although, the cell of origin of the tumor is still a debatable topic, there is a section of researchers who believe that brain tumor stem cells initiate and maintain the tumor (55-57). This leads to the prediction that elimination of the bulk of the tumor (shown in blue in Figure 3) may spare the diffusely distributed minor population of CSCs (shown in yellow in Figure 3), and thus, causes the re-growth of the tumor. The significance of the CSC hypothesis in GBM tumor biology and patient outcome is immense. Most of the chemotherapeutic efforts fail because the initiator cells have active ABC transporters (58-61) that expel the drugs and thereby survive. Thus, brain tumor stem cell-targeted
therapy is required to eliminate GBM tumors and prevent their recurrence (Figure 3). So, this is currently the topic of active research in brain cancers (62-64).
Figure 3. CSC-targeted therapy. The CSC population (shown in yellow) is a rare fraction of the total cancerous cells. They remain dispersed in the tumor and are resistant to conventional therapies like chemotherapy and radio-therapy. Surgical removal of the bulk of the tumor (shown in blue) tends to spare the CSCs which then regenerate the tumor with more aggressive phenotype, and are thus responsible for the recurrence of the disease. Therapeutic approaches to eliminate the CSC population can potentially prevent the recurrence of the cancer while the conventional therapies including chemotherapy and radiation facilitate removal of the differentiated cancer cells.
1.3 Markers of CSCs

Since BTICs functionally resemble normal NSCs, it is can be speculated that normal stem cell markers may be present in the so-called cancer stem cell population in GBM tumors. Efforts are still under way to identify expression of stem cell markers in the cancer cell population and classify them as putative CSCs. Expression of specific cell surface markers would then enable prospective isolation of the CSC population for intensive analysis of their cellular and molecular biology, which is critical for development of CSC-targeted therapy. Recently the normal stem cell marker Cluster of Differentiation 133 (CD133) glycoprotein has been reported to be expressed by CSC population, while other potential candidates including SSEA-1 expression remain to be investigated.

**SSEA-1:** SSEA-1 (stage specific embryonic antigen-1) or FAL (fucose N-acetyl lactosamine) also called Cluster of Differentiation 15 (CD15) is a carbohydrate moiety that is expressed by pluripotent mouse blastocysts, embryonic stem cells, primordial germ cells and adult bone marrow-derived pluripotent stem cells (65, 66). SSEA-1 is secreted by progenitor cells present in the embryonic nervous system, into their extracellular matrix where it binds Wnt-1, a critical modulator of stem cell (54).

1.3.2 **CD133: a transmembrane glycoprotein:** CD133 glycoprotein expression distinguishes normal hematopoietic stem cells from the non-stem cell population. Recently, brain tumor stem cell fraction has been putatively isolated from gliomas
including GBM tumors, based on their cell surface expression of CD133 glycoprotein (50). However, other reports claim that the CD133 glycoprotein expressing cells are radio-resistant and they form highly vascular tumors, in transplantation assays; nevertheless, cells that do not express CD133 glycoprotein also form tumors in xenograft assays, but the tumors are not comparably vascular (67).

A definitive and reliable marker is yet to be identified for isolation of CSCs. However, methods, based on functional characteristics of CSCs, have been developed for studying and characterizing these cells (68, 69). Such methods include focus-forming assay or tumorsphere or neurosphere assay in which the putative stem cells grow as clonal cluster of cells generated from division of the parent cells (70). Adenosine-5’-triphosphate (ATP)-binding cassette (ABC) drug efflux transporter activity has also been utilized to isolate CSCs which are then referred to as the ‘side population’ (35, 60, 61, 71-73).

### 1.4 Neurosphere Assay

Neurosphere assay is a procedure wherein neural tissue is dissociated physically into a single cell suspension, and cultured in serum-free selective conditions in the presence of defined growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) to yield clusters of cells that are enriched in adult neural stem
cells and their immediate progeny, called transiently dividing cells (40). The
differentiated cells and the cells that are committed to differentiation die in this condition
(39). Although, this method enables identification of presence of cancer stem cell
population in culture, it is not beneficial in isolation of the initiator cells since the cells
continuously divide asymmetrically thereby generating both CSCs and committed
progenitor cells at any given time making it a heterogeneous population (74).

1.5 Isolation of ‘Side Population’: A Function of ABC Transporters

ABC drug efflux transporters are expressed by endothelial cells and parenchymal
cells in the brain (75). GBM tumors cells also express ABC transporters like ABC-1 that
is up-regulated in response to radiation and temozolomide administration, which may
contribute to chemotherapy and radiation resistance of GBM tumors (76). In other solid
tumors, these ABC drug efflux transporters are responsible for multi-drug resistance
(MDR) (77). ABC transporters have also been used to isolate the rare stem cell fraction
called the ‘side population’ in glioma, lung cancer, and breast cancer cell lines (17, 78,
79). However, a recent report claims that most of the C6 glioma cells are CSCs although
they were not CD133 positive nor the side population (61). The side population is
isolated based on efflux of the dye, Hoechst 33342. Reports now show that Hoechst
33342 is detrimental to the clonogenicity and proliferation potential of the C6 glioma
cells that were otherwise clonogenic and tumorigenic (61). These evidences highlight the inefficiency of these isolation methods.

The currently available methods of isolation are not definitive, thus making it practically impossible to study a pure population of CSCs. A different approach is to initiate the differentiation process in the CSCs so that the differentiated cancer cells then can be eliminated by the conventional therapeutics. With this understanding, it is necessary to investigate and clearly understand the mechanisms underlying the differentiation and de-differentiation of GBM cells. Current literature suggests that combination of four genes namely octamer-3/4 (oct3/4), SRY (sex determining region Y)-related HMG box 2 (sox2), Krüppel-like family of transcription factors 4 (klf4) and myelocytomatosis oncogene (c-myc) is capable of generating pluripotent stem cells from differentiated fibroblast cells (80, 81). A recent report has identified c-myc as the molecule that confers an undifferentiated state to non-tumorigenic differentiated astrocytes, thereby rendering them tumorigenic (82). These findings imply a potential requirement of c-Myc (hereafter referred to as Myc according to latest nomenclature) tumorigenesis by maintaining an undifferentiated phenotype of GBM cells.

1.6 Myc: A Prototypic Oncoprotein

1.6.1 Myc- the oncoprotein: Myc, formerly known as c-Myc, is a transcription factor that heterodimerizes with its binding partner Max or Mad to activate or inhibit
transcription of target genes. Myc was first described in 1982 as the cellular homologue to the transforming sequence of the avian myelocytomatosis (myc) retrovirus (83). Myc is a crucial regulator of cell proliferation and cell fate decision (84). Some reports also suggest that Myc triggers the apoptotic cascades (85-87). Myc deregulation is often evident in a variety of human malignancies (88-92). Although, it was first identified as the main transforming agent in human Burkitt’s lymphoma, subsequent studies have identified Myc deregulation in many human solid cancers including cervical, colon and breast cancers, small cell lung carcinomas, osteosarcomas and glioblastomas and liquid cancers including myeloid leukemias (88-91, 93-95). Overexpression of Myc oncoprotein has been strongly implicated in many human cancers (95, 96). The cause of overexpression in some cases is gene amplification or translocation to regions of high transcriptional activity (92, 97). In lymphoblastic leukemias, abnormalities in the protein degradation pathway of Myc, mainly controlled by Ser\textsubscript{62} and Thr\textsubscript{58} phosphorylation is responsible for its overexpression (88). Myc stabilization has been reported in four GBM cell lines contributing to their uncontrolled proliferation (98-100). 

1.6.2 Myc involvement in cellular differentiation: Myc is down-regulated in terminally differentiated cells that exit the cell cycle, since it is a strong inducer of cellular proliferation (101). It has been found that untransformed acinar cells that regenerate the pancreas following acute pancreatitis, also express Myc, demonstrating the up-regulation of the oncoprotein in mitotically active cells. These cells also have a five-fold increase in villin expression, a marker of embryonic pancreas (93). Collectively, these findings indicate that the acinar cells dedifferentiate to a relatively undifferentiated state that
express Myc. Myc is also over expressed in many embryonic tissues (102). myc mRNA was found to correlate with the differentiation status of the malignant human promyelocytic leukemia cell line, HL-60 that express Myc in the undifferentiated state only. Myc transcript expression is lost when the cell line is treated with dimethyl sulfoxide (DMSO) to induce differentiation of the cells into mature granulocytes (103). Campisi et al. have shown myc and c-ras expression in relation to cell cycle of untransformed cells (104). More recent evidence demonstrates that expression of myc oncogene in differentiated murine astrocytes expressing glial fibrillary acidic protein (GFAP) loses the marker expression and forms tumors when injected into mouse (82). These studies suggest a critical role for Myc in cellular differentiation in a wide variety of cells including pancreatic cells and astrocytes; however, the precise mechanism of myc action remains unclear.

1.6.3 Myc alters chromatin structure: In addition to the conventional functions described above, recently Myc has been found to alter global chromatin structure (105, 106). Myc function is required for the maintenance of active chromatin, since loss of Myc leads to chromatin condensation (106). It remains to be determined if this newly identified function of Myc contributes further to its transforming potential.

1.6.4 Myc- a DNA replication factor: Recent findings have identified Myc as a DNA replication factor that promotes DNA replication by a nontranscriptional mechanism as shown in Figure 4 (2). Thus, Myc deregulation causes DNA damage predominantly during S phase of the cell cycle. This indicates a novel role of Myc in DNA replication and also suggests an additional function of Myc in cell growth and tumor formation.
Figure 4. Myc is involved in replication initiation. The origins of replication are licensed in the early growth phase 1(G1) by the origin recognition complex (ORC)-dependent assembly of the pre-replication complexes on the DNA. Protein kinases promote the initiation of replication by stimulating cell division cycle 45 (Cdc45)- and mini chromosome maintenance complex proteins (MCM2-7)-dependent unwinding of the origin. Myc potentially affects the process at a stage between the pre-replication complex formation and the beginning of replication initiation. synthesis phase of cell cycle (S), Cell division cycle 6 (Cdc6), pre-replicative complex (pre-RC), Chromatin licensing and DNA replication factor 1 (Cdt1), Cyclin-dependent kinases involved in the S-phase of cell cycle (S-CDKs), (2)
Figure 5. Cellular functions of Myc. Some of the known cellular functions of Myc include induction of proliferation, alteration of chromatin structure, participation in DNA replication as well as maintenance of an undifferentiated phenotype and suppression of differentiation.
Since Myc deregulation can be deleterious for the cell, Myc levels are tightly controlled to maintain normal cellular phenotype (3). Myc is regulated at all possible stages of its life cycle from synthesis to degradation. However, better perception of Myc regulation warrants an understanding of both myc gene and its protein products.

1.6.5 Structure of Myc: Three exons comprise the myc gene. Exon I is a long untranslated sequence, while the second and third exons contain the protein coding sequence. In human myc, transcription is initiated mainly from two promoters P1 and P2 located in the first exon at a distance of 174 nucleotides from each other. Transcripts of 2.4 and 2.2 kb are generated from the promoters P1 and P2 respectively. A consensus TATA box element is located in both promoters; however, only the P2 promoter contains a strong consensus initiator element (Inr) (107, 108). The majority of myc transcripts arise from the P2 promoter in almost all the tissues and cell lines. Both P1 and P2-initiated mRNA code for myc proteins of 67 kDa and 64 kDa, called Myc 1 and Myc 2, respectively. Myc 1 contains an extra 14 amino acids at the amino terminus of the protein. Recently, a short isoform of Myc has been identified called Myc-S, that arises from a translation initiation site at about 100 amino acids downstream of the amino terminus. Myc-S lacks the Myc transcription activation function (109). The products of the myc gene are nuclear phosphoproteins that are highly conserved among different species (93). Myc 2, the 64 kDa protein is predominantly found in almost all the tissues and cell lines, and it plays crucial roles in cellular proliferation, whereas Myc 1 protein mediates cellular quiescence in COS cells (110).
1.6.6 Regulation of Myc oncoprotein: Myc oncoprotein is a strong modulator of cellular proliferation, cell fate decisions, and apoptosis (111, 112). So Myc oncoprotein levels are tightly regulated in normal cells, (3). Myc protein is controlled at various stages namely, transcription (initiation and elongation), post-transcription (mRNA stability and translation) and post-translation (protein stability) (113). Myc oncoprotein is regulated differentially depending on the proliferative status of the cell (114). Recent findings have implicated the ubiquitin proteasome pathway in the degradation of Myc in vivo (115). Myc is unstable with a short half-life in quiescent cells; however, during cell cycle entry, and in vitro serum stimulation transiently stabilizes the oncoprotein, so that it accumulates in the cell (116). Stabilization of Myc protein is mediated by activated Ras (3). Activated Raf, that is a downstream effector of activated Ras, stabilizes Myc through the phosphorylation events mediated by the Raf-mitogen-activated ERK kinase (MEK)-external signal-regulated kinase (ERK) signaling pathway shown in Figure 6 (3, 117).
Figure 6. Life cycle of Myc. Myc oncoprotein is transiently stabilized by growth stimulatory signals in normal cell. Ras is activated by growth inducing signals in the cells which also stimulate myc gene transcription. Activated Ras operates via two parallel effector systems. Raf/MEK/ERK pathway stabilizes Myc by phosphorylating Myc on serine 62 (S62-P). Phosphoinositide 3-kinase PI3K/AKT pathway inhibits glycogen synthase kinase 3 beta (GSK3β) which otherwise phosphorylate Myc on threonine 58 (T58-P) thereby destabilizing Myc protein. T58-P-Myc is unstable as it is subjected to ubiquitination followed by proteasomal degradation (3).
Growth stimulatory signal

\[ \text{Myc} \]

\[ \text{Raf/MEK} \]

\[ \text{Ras} \]

\[ \text{Erk} \]

\[ \text{Myc}^{S62-P} \]

(Stable)

\[ \text{GSK3}\beta \]

\[ \text{Myc}^{S62-P, T58-P} \]

(Unstable)

\[ \text{Ubiquitin} \]

Proteasomal degradation
Another molecule that is critically involved in cancer is Stat3 (118). Currently it is being considered a potential therapeutic target in many cancers (119-121). Its role in GBM is yet to be completely unraveled; however, it has been reported to be involved in normal astrocyte development (122).

1.7 Signal Transducer and Activator of Transcription 3 (Stat3)

1.7.1 STAT family: Stat3 belongs to a family of transcription factors that are collectively called Signal Transducers and Activators of Transcription (STATs). Extracellular signaling polypeptides that interact with specific cell surface receptors initiate the activation of Stats as described in Figure 7 (4). In response, these transcription factors become active by getting phosphorylated on signature tyrosine residues (123). The activation of Stats leads to their dimerization by reciprocal SH2 phosphotyrosine interaction (124). The Stat dimers then enter the nucleus and function as a transcription factor by interacting with their cognate DNA elements called GAS, IFN-γ-activated sequence, and thereby modifying their target gene expression (125). The Stat-family of proteins was first identified as transcription factors that were activated in interferon-treated cells (4, 126, 127); however, later they were found to be induced by many other signaling molecules (128). Seven mammalian STAT genes have been reported to be located in three chromosomal clusters. In humans, Stats 3, 5A and 5B, which are involved in cancer, map to chromosome 12, bands q13 to q14-1. Stats 1, 3, 4, 5A and 5B
range from 750 to 795 amino acids in length, however, differential splicing result in a number of additional proteins (125).

1.7.2 Stat activation in cancer: In addition to Stats 3 and 5, Stat 1 is also activated by many receptor tyrosine kinases in a variety of cancer cells (118, 125). Growth factor receptors like EGFR and PDGFR that have intrinsic tyrosine-kinase activity also activate Stats through a mechanism that is similar to that of cytokine receptor signaling (128-130). In normal cells, Stat activation is strictly regulated; however, tumor cells have over-activated Stats (131). In tumor cells, Stat activation can result due to constitutive activation of tyrosine kinase-activity of growth factor receptors (118). Constitutive activation of the receptor tyrosine kinase activity can be caused by mutations affecting the receptor structure, receptor overexpression or constitutive ligand-receptor interaction due to over-supply of ligand from autocrine or paracrine sources (131). Reports suggest that Stat3 and Stat5 are over-activated in many human solid and liquid cancers (118).
**Figure 7.** Stat activation and function. Stats are activated in response to cytokine (C) or growth factor (GF) signaling via receptor tyrosine kinases (RTKs) or receptor-associated to tyrosine kinases like Janus kinase (JAK). JAKs phosphorylate the receptors at their cytoplasmic domain and docking sites are formed. Stats are latent in the cytoplasm and they dock on the phosphorylated cytoplasmic domain of the receptors. Dissociation of the Stats follows their phosphorylation on tyrosine residues which then facilitate Stat-Stat homo- or hetero-dimerization which renders them active (P-Stat). Active Stat dimers translocate to the nucleus and recognize and interact with cognate elements called interferon (IFN)-γ-activated sequence (GAS) on their target genes to activate transcription (4).
Transcriptional activation of target gene
1.7.3 **Role of Activated Stat3 and Myc in astrocyte differentiation:** Activated Stat3 has been found to suppress apoptosis in GBM cell lines by induction of anti-apoptotic factors Bcl-2, Bcl-XL, MCL-1 (132, 133). Although activated Stat3 has been reported to induce Myc oncoprotein transcriptionally in hematopoietic cells like anaplastic large cell lymphoma (ALCL) (134), the two proteins have opposing effects in astrocytic development. Activated Stat3 induces astrocytic differentiation (Figure 8) (135), whereas Myc renders an undifferentiated status to differentiated astrocytes (Figure 9 a) (82). Myc being a mitotic stimulus also induces apoptosis (136, 137) whereas activated Stat3 serves as a survival factor in GBM cells (138). These findings highlight the contrary functions of these two transcription factors in the context of both normal astrocytes and GBM cells (Figure 9 b) where Myc serves an oncogenic function by inhibiting differentiation and up-regulating proliferation. On the other hand, Stat3 functions both as an oncogene by providing survival signals, and it acts as a tumor-suppressor by promoting astrocytic differentiation. A recent report has also demonstrated that Stat3 is capable of having dual functions in brain tumor cells depending on the type of mutation present in the cells (139). Stat3 function in astrocytic differentiation is mediated via leukemia inhibitory factor receptor β (LIFRβ) that is responsive to PTEN-Akt-FOXO pathway, and in brain tumor cells which harbor a PTEN mutation, Stat3 function is impaired. Thus, Stat3 serves as a brain tumor-suppressor by promoting astrocytic differentiation. On the other hand, Stat3 forms a complex with the oncogenic epidermal growth factor receptor III variant (EGFRIIIv) to promote transformation of glial cells (139).
**Figure 8.** Activated Stat3 is involved in GFAP expression during normal development of astrocytes. During normal development of astrocytes from undifferentiated neuroepithelial cells, Leukemia inhibitory factor (LIF) and/or Interleukin-6 abbreviated IL-6 (of the IL-6 family of cytokines) mediated- and Bone morphogenetic protein (BMP)-mediated signaling synergistically function to up-regulate the differentiated astrocyte-marker, glial fibrillary acidic protein (GFAP) expression. Stat3 activated by IL-6 and Small mothers against decapentaplegic (SMAD) complexes activated BMPs assemble at the GFAP promoter bridged by the transcription cofactor, p300.
**Figure 9.** Myc renders differentiated astrocytes tumorigenic. (a) Myc overexpression in normal murine astrocytes causes loss of GFAP expression in them and renders them tumorigenic. (i) Normal murine neural stem cells generate tumors in mice model when Ras and Akt are over-expressed in them. (ii) Expression of Ras and Akt in differentiated astrocytes is not sufficient to develop tumors from them. (iii) Addition of Myc along with Ras and Akt enables tumor formation from the differentiated astrocytes in mice. The differentiated astrocytes loose their expression of GFAP upon expression of Myc. (b) Myc and Stat3 have contrasting functions. Myc induces apoptosis while Stat3 promotes survival by suppressing apoptosis. Myc is also well known to maintain an undifferentiated state in cells and block differentiation whereas Stat3 is found to facilitate differentiation of neural progenitors towards an astrocytic lineage.
1.8 **Significance of the Current Investigation**

The current study was based on the hypothesis that Myc is associated with GBM tumorigenesis, and its interplay with activated Stat3, modulates GBM cell differentiation thereby affecting tumor formation. Three specific aims were pursued to test this hypothesis. First, the level of Myc protein was monitored in GBM cells to determine any correlation between Myc and tumorigenicity of the cells. Secondly, the relationship between Myc and activated Stat3 was explored to determine if a regulatory mechanism exists between these two transcription factors. Finally, the mechanism of Myc-mediated suppression of GFAP, a differentiation marker, was investigated. These studies will enhance our mechanistic understanding of GBM tumorigenesis by highlighting the functions of molecules, such as Stat3 and Myc which are involved in signaling networks implicated in cancer and normal development. The information we will obtain will be critical for development of effective therapeutic approaches in GBM. The experiments described in this dissertation have been designed to delineate the mode of regulation of Myc, a molecule that is involved in GBM tumors; and, the mechanism of action of Myc in GBM cell de-differentiation by assessing the regulation of differentiated astrocyte marker, GFAP. This approach of studying GBM tumors is of utmost importance due to the dynamic state of the GBM-initiating cells with regards to their differentiation status and marker expression. To achieve the aim of understanding the differentiation of the tumorigenic GBM cells, it is compelling to investigate the precise mechanism of differentiation (Figure 10).
The overall goal of this dissertation is to explore the mechanism underlying GBM cell differentiation and de-differentiation focusing on molecules like Myc and Stat3 that are intricately involved in normal differentiation and de-differentiation processes. Since brain CSCs, unlike the rest of the brain cancer cells, are resistant to both chemotherapy and radiation therapy, they evade the conventional therapeutic regimen against GBM tumors. Precise mechanistic understanding of GBM cell differentiation will facilitate the development of effective therapy.

**Figure 10.** Overall aim of study. The overall goal of this dissertation is to explore the mechanism underlying GBM cell differentiation and de-differentiation focusing on molecules like Myc and Stat3 that are intricately involved in normal differentiation and de-differentiation processes. Since brain CSCs, unlike the rest of the brain cancer cells, are resistant to both chemotherapy and radiation therapy, they evade the conventional therapeutic regimen against GBM tumors. Precise mechanistic understanding of GBM cell differentiation will facilitate the development of effective therapy.
2.0 METHODS AND MATERIALS

Cell lines, primary cultures and reagents: Breast cancer cell line MCF-7 and GBM cell lines U87, U251 were cultured in DMEM from GIBCO-BRL (NY) with 10% heat-inactivated fetal bovine serum (FBS) from Life Technologies Inc, (MD), 2 mM L-glutamine and 50 mg/L of penicillin and streptomycin. Colon cancer cell line, Caco-2 was grown in MEM supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, and 20% FBS. Primary glioma cell cultures, CCF-334, CCF-310 were obtained from Cleveland Clinic (CC) Tumor Bank following NIH and CC policies. The primary cultures were maintained in a special media comprised of DMEM supplemented with serum replacement-2 50X, G5 supplement 100X from, 50 mg/ml of penicillin and streptomycin and EGF Hu Rec (0.02 µg/ml) and bFGF Hu Rec (0.02 µg/ml) from Sigma. Primary glioma cell culture CCF-252 was maintained in the special media supplemented with 0.5% FBS and CCF-247 was cultured in the special media supplemented with 10% FBS. Sta-21 was purchased from BIOMOL International, L.P. (Plymoth Meeting, PA).

Plasmids: DN-Stat3 and CA-Stat3 constructs were prepared by sub cloning wild-type Stat3 into pcDNA3.1-V5/HisA expression vector, followed by site-directed mutagenesis using the Quick Change kit from Stratagene (La Jolla, CA). One of the putative Stat3
DNA-binding elements was mutated in the GFAP-Luc construct by site-directed mutagenesis using the Quick Change kit from Stratagene (La Jolla, CA) to generate dGAS-Luc. Primer sequences are given in Table 2. Myc promoter-luciferase reporter constructs, HBM-Luc, SNM-Luc and XNM-Luc were generated and gifted by Dr. Linda Penn, University of Toronto (140). The dominant negative mutant of Smad4, DeltaCSmad4 was a gift from Dr. Philip Howe, Cleveland Clinic.

**Flow cytometry:** Cells were washed with 1x PBS and trypsinized to make a single cell suspension. The cells were washed once with FACS wash buffer (1x PBS, 1% BSA and 0.02% sodium azide, filtered through 0.2 micron). 20 µl/ml of the CD133/1-PE antibody (Miltenyi Biotech, Mouse IgG1 isotype) or IgG1-PE (BD Biosciences) isotype control antibody was mixed with the cells and incubated in dark for 30 mins at room temperature. The cells were then washed twice with 500 µl of FACS wash buffer. Then the cells were resuspended in 400 µl of FACS buffer containing 1x PBS, 1% BSA and 0.02% sodium azide, filtered through 0.2 micron by vortexing briefly and maintained on ice in dark until the analysis. FACScan was used to measure the fluorescence and the results were analyzed with FLOJO software.

**Neurosphere assay:** Adherent cells were mildly trypsinized and non-adherent cells were treated with Accutase (Sigma) to make a single cell suspension. The cells were washed with their respective media followed by 1x PBS. The cells were then resuspended in serum–free DMEM. 1 million cells (about 30% confluence) were cultured in 10-cm
plates at 37 C, with 5% CO$_2$. After 5 days the plates were checked for presence of neurospheres. The neurospheres were then documented using an inverted microscope.

**RNA isolation:** 70% confluent cell plates were used for RNA isolation. RNA was isolated using TRIZOL reagent (Gibco-BRL). The attached cells were lysed directly in the 10 cm plate by adding 1 ml of TRIZOL reagent and passing the cells through a pipette for several times. Suspension cell cultures were pelleted first and then lysed with TRIZOL reagent and passing through the pipette. The samples were transferred to sterile RNA grade capped-tubes. Incubated the homogenized samples at room temperature for 5 mins for complete dissociation of the nucleoprotein complexes and then 0.2 ml of chloroform was added for every 1 ml of TRIZOL reagent. The tubes were shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 mins. The tubes were then centrifuged at 12,000 X g for 15 mins at 4°C. The aqueous phase containing the RNA was transferred carefully to fresh RNA grade tube and isopropyl alcohol (0.5 ml per 1 ml of TRIZOL reagent) was added to it. The samples were incubated at room temperature for 10 mins and then centrifuged at 12,000 X g for 10 mins at 4°C. The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75% ethanol per 1 ml of TRIZOL reagent. The samples were mixed and then centrifuged at 7,500 X g for 5 mins at 4°C. The supernatant was discarded and the RNA was air-dried at room temperature for 20 mins. The RNA pellet was dissolved in RNase-free water by incubating at 60°C for 10 mins.
**Reverse Transcription-Polymerase Chain Reaction:** One microgram of total RNA was reverse-transcribed using the gene-specific primers. The total reaction volume was 50 µl. The primers used for RT-PCR analysis are given in Table 2.

**Table 2: List of primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>P1: 5’-sequence</th>
<th>P2: 5’-sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCD133</td>
<td>5’-AACGCTCTTTGGTCTCCTTCTCTTGAT-3’</td>
<td>5’-TGTTGCTGTTTTATTATTCTGA-3’</td>
</tr>
<tr>
<td>hGFAP</td>
<td>5’-GGGTGGCTTCATCTGCTTTGCTTG-3’</td>
<td>5’-GCTCGGCGCTCTACGTCTCCTCA-3’</td>
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<td>hMYC</td>
<td>5’-AGGCGCTGCGTTGCTTGATG-3’</td>
<td>5’-CTGCGACCCGGAGACGAGACC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CCATGGAGAAGGCTGGG-3’</td>
<td>5’-CAAAGTTTGTCATGGATGACC-3’</td>
</tr>
<tr>
<td>dGAS-Luc</td>
<td>5’-CCAGCCCCCCTCAATGCCCCCAAGAGAAGGCCCCATTGAGCAG-3’</td>
<td>5’-CCTGCTCAATGGGCTTCTCTTGAGCATTTGGGGGTGG-3’</td>
</tr>
<tr>
<td>DN-STAT3</td>
<td>5’-CCAGGTAGCGGCTGCCCTAGCTGAAGACCAAGTTATCTGTG-3’</td>
<td>5’-CACAGATAAACCTGGGTCTTCAGGGAATGGGGAGCGGTACCTG-3’</td>
</tr>
<tr>
<td>CA-STAT3</td>
<td>5’-CATCATGGGTATAAGATCATGGATTTGTACC</td>
<td>5’-CACAGATAAACCTGGGTCTTCAGGGAATGGGGAGCGGTACCTG-3’</td>
</tr>
</tbody>
</table>

*Note: Mutated sequences are underlined in the table above.*
**CDNA synthesis and real-time PCR:** iScript select cDNA synthesis kit (BIORAD) was used for cDNA synthesis from 2 µg of total RNA. Random hexamer primers, supplied in the kit, were used to synthesize a cDNA pool. The cDNA pool was then used in real time-PCR (Applied BioSystems) using Myc-specific primers (Table 2).

**Immunocytochemistry:** The cells were cultured on plastic cover slips for 4 days before staining. The cells were washed with 1x PBS. 4% paraformaldehyde was used to fix the cells. Then the cells were permeabilized with 0.5% Triton X100. Polyclonal rabbit anti-GFAP (DAKO Cytomation), polyclonal rabbit anti-Myc (Epitomics) primary antibodies and anti-rabbit Alexa 488-tagged secondary antibody were used for immunostaining. Fluorescence was detected using fluorescent microscope.

**Western blot analysis:** Western blot analysis was performed as described previously (138). Monoclonal anti-Myc antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA) recognized one isoform of Myc protein and polyclonal anti-Myc antibody bought from Epitomics recognized both the isoforms. Antibodies against cyclin A, cyclin E2 and p21 were from Santa Cruz Biotechnology, and were gifts from Dr. Alexandru Almasan. Anti-β-actin antibody was purchased from Santa Cruz Biotechnology. ECL developing reagent system was from GE Technologies.

**Electrophoretic mobility shift assay (EMSA):** Cells were collected and whole cell extracts were prepared and 10 micrograms of it was then used to perform EMSA as
described (138). 0.2 ng of $^{32}$P-labeled c-fos gene promoter-derived high affinity SIE (top strand: 5’-TCGACATTTCCCGTAAATC-3’) was used as radioactive probe for EMSA (141).

**Cell proliferation assay:** 0.05 X $10^6$ cells were seeded in 24 well-plate in quadruplicate in DMEM supplemented with 10% FBS. After 4 days the cells were detached with trypsinization and washed and stained with 0.4% trypan blue. After 5 mins of incubation, the unstained viable cells were counted with a hemocytometer under a light microscope.

**Transfections of cells and generation of stable clones:** U87 cells were transfected using Lipofectamine 2000 and U251 cells were transfected using Lipofectamine PLUS reagents from Life Technologies Corporation. Stable clones were selected in neomycin (G418) treatment (150 µg/ml) for 15 days.

**Luciferase reporter assay:** The cells were cultured in 6 well-plates for 24 hours in antibiotic-free conditions. Transfection was done using Lipofectamine 2000 for U87 cells and Lipofectamine PLUS for U251 cells. The cells were co-transfected with the effector plasmids and the luciferase reporter plasmids. The cells were grown at 37°C for 48 hours and then washed with 1x PBS. Then the cells were lysed in 200 ul of 5X lysis buffer (Promega Corporation) for overnight at -80°C. The cell debris were pelleted by centrifuging at 14,000 X g for 20 mins at 4°C. To 20 µl of supernatant solution, 50 µl of luciferase assay substrate was added and the luminescence was measured using a
luminometer. Next, 50 µl of Stop and Glo reagent (Promega Corporation) was added to the same wells in the plate reader to measure the renilla luciferase activity for normalization.

**Chromatin immunoprecipitation assay:** U251 cells were cross-linked by treating with 1% final concentration of formaldehyde solution in 1x PBS for 10 mins at 37°C. The reaction was stopped using glycine solution in 1x PBS for 5 mins. The cells were then washed thoroughly with 1x PBS and nuclear extracts were prepared. The nuclei were then sonicated to generate chromatin fragments of approximately 500 to 800 bp. 30 µl of the sonicated chromatin was treated with RNase A and Proteinase K followed by de-cross-linking. The DNA was isolated and quantified. The chromatin was precleared with mixture of proteinA-sepharose and proteinG-sepharose that were blocked with 1 mg/ml of bovine serum albumin and 1 mg/ml of Salmon sperm DNA. 10% of the precleared chromatin was used as input control. Equal amount of the precleared chromatin fragments were subjected to immunoprecipitation with either anti-Stat3 polyclonal antibody (Santa Cruz Biotechnology) or with anti-actin polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitation and subsequent RNase A and Proteinase K treatments, de-cross-linking and DNA isolation were performed as described previously (142, 143). The isolated DNA was then subjected to PCR analysis using GFAP promoter-specific primers. The PCR products were resolved on a 1% agarose gel and stained with ethidium bromide to visualize under ultraviolet light.
3.0 IDENTIFICATION OF MOLECULES ASSOCIATED WITH TUMORIGENICITY OF GBM CELLS

3.1 Abstract

According to the cancer stem cell hypothesis, only a rare subset of cancer cells called CSCs is able to initiate and maintain the tumor. To identify molecules associated with the tumorigenic potential of GBM cells, I have used GBM cell lines and primary cultures of GBM tumor cells to study the cell surface expression profile of CD133. CD133, a transmembrane glycoprotein has been reported recently to be the marker of brain CSCs. I have also determined the level of expression of Myc, a transcription factor that has been shown to have oncogenic effect in many cancers. Flow cytometric analyses of GBM cells showed that cell surface expression of CD133 glycoprotein is not correlated with the tumorigenic ability of the GBM cell line, U87 that formed big tumors in a rodent xenograft assay. I also report that U87 cells have higher steady state level of Myc protein as compared to U251 another GBM cell line that formed smaller tumor than U87. Interestingly, an inverse correlation between Stat3 activation level and Myc expression was also observed in these two GBM cell lines. Collectively, these data show
that CD133 glycoprotein expression on the cell surface is not required for the tumorigenic ability of GBM cells. Moreover, Myc oncoprotein expression is higher in tumorigenic GBM cells that have low levels of constitutive activation of Stat3.

### 3.2 Introduction

GBM tumors comprise of a heterogeneous population of cells with regards to tumorigenic potential. A minor subgroup of cancer cells, termed the brain tumor initiator cells with sustained proliferation and self-renewal capabilities initiate and maintain the tumors (49). These cells are diffusely distributed in and around the tumor making their effective removal by surgery inefficient. This knowledge demands the characterization of the initiator cells in terms of the cellular and molecular differences that bestow them with tumorigenic potential. Molecular markers are required that can distinguish the tumorigenic cells from the non-tumorigenic counterparts. A cell surface marker, CD133 has been reported to mark the initiator cells in brain tumors including GBM (50). CD133 is a five-pass transmembrane glycoprotein that is expressed by normal hematopoietic stem cells, neural stem cells, as well as acute myeloid leukemia (AML) progenitor cells (144). Any functional relevance of the cell surface expression of CD133 to tumorigenicity is yet to be defined; however, CD133 expression is not related to overall survival of AML patients (144).
Myc is a potent transcription factor that either activates or inhibits a wide range of target genes (145). Myc regulates crucial cellular processes such as proliferation, apoptosis and inhibition of terminal differentiation of the cells via these effectors (93). In normal condition, Myc is tightly regulated and its deregulation leads to cellular abnormalities that can culminate into cancers (108). Altered Myc expression has been reported in many human cancers such as breast cancers, colon cancers, cervical cancers, small cell lung carcinomas, osteosarcomas, myeloid leukemias and GBM (93). Aberrant stabilization of Myc oncoprotein in GBM cells has been reported earlier (99) and recent findings demonstrate that Myc bestows an undifferentiated status to normal differentiated murine astrocytes that lose the differentiated astrocytic marker, GFAP expression and form tumors when implanted in rodents (82). It remains to be investigated if Myc expression has a correlation to tumorigenicity of human GBM cells.

Activation of Stat3, a transcription factor, has been reported to be involved in normal astrocytic development where it induces differentiation of neuroepithelial cells to mature astrocytes (122). Stat3 is constitutively activated in all GBM cell lines and primary cultures of GBM tumor cells examined (133). It has been reported that activated Stat3 provides survival support to a GBM cell line, U87 by inducing expression of anti-apoptotic factors such as mcl-1 (138). Activated Stat3 has been shown to induce myc gene expression in breast cancers (134, 146), but its relation to GBM tumors remain to be elucidated.

To determine the molecules associated with GBM tumorigenicity, I have used GBM cell lines, with different tumor forming abilities, and human GBM tumor-derived
primary cultures to profile their cell surface expression of CD133 glycoprotein, Myc oncprotein expression levels and the level of Stat3 activation and its relation with Myc expression.

It is demonstrated here that steady state level of Myc protein is greater in highly tumorigenic U87 cells as opposed to that in less tumorigenic U251 cells. The cell surface expression of CD133 glycoprotein did not correlate with tumorigenic potential of GBM cell lines although CD133 glycoprotein was expressed by some primary GBM tumor cell cultures. Most importantly, there appears to be an inverse relationship between Myc expression and Stat3 activation in GBM cells.
3.3 Results

3.3.1 GBM cell lines exhibit differential tumorigenicity

Previous observations in our laboratory have suggested that GBM cell lines, U87 and U251 have different tumor-forming ability. To eliminate any clonogenic variation, the tumorigenic potential of our clones of U87 and U251 cell lines were determined by heterotopic rodent xenograft assay. Equal number of U87 cells and U251 cells were injected in the right flank of athymic nude mice and their tumor sizes were followed over a period of 3 months. All 6 mice that were injected with U87 cells developed large tumors in their flanks and the mice had to be sacrificed due to morbidity (solid black bar in Figure 11a). In contrast, 2 out of the 3 mice that were injected with U251 cells developed relatively smaller tumors in their right flanks compared to the U87 cell-derived tumors (white bar in Figure 11a). The U251 cell-derived tumors did not grow further even after 3 months.
Figure 11. (a) GBM cell lines have different tumorigenic abilities. Equal numbers of U251 and U87 cells mixed with matrigel were injected in the right flank of athymic mice. Tumor volume was monitored weekly. The data represents the average tumor volume in each group 4 weeks post-injection. Tumor volume was calculated using the formula; length X width^2 X 0.4. (b) Growth of GBM cells in vitro. U251 and U87 cells were grown in the presence of 10% serum for 4 days and the number of cells were measured at day 0 and on day 4. The viable cells were counted after staining with trypan blue. The data represent average cell number from 4 independent experiments. The error bars represent standard deviations.
To determine the growth kinetics of these two cell lines *in vitro*, the cells were cultured in 10% serum-containing media for 4 days, and then counted the viable cells by trypan blue dye exclusion assay. 0.05 X 10^6 cells were grown in 12-well plates. U251 cells grew almost three-times more than U87 cells measured after 4 days (Figure 11b). The doubling time of U251 cells was 17 hours whereas that of U87 cells was 43 hours. Collectively, these data suggest that the growth of U251 cells *in vitro* and *in vivo* differ markedly. U87 cells were slow growing in culture but *in vivo* these cells proliferated rapidly to form large tumors within 4 to 5 weeks. In contrast, U251 cells, that grew very rapidly *in vitro*, had relatively low proliferation capacity *in vivo*, indicating differences in the cellular and molecular mechanisms functional during *in vivo* and *in vitro* growth. One possible reason for the difference may be the growth in presence of serum, which is known to contain various defined and undefined growth promoting agents. Another factor that possibly contributed to the different growth kinetics of the cells is their p53 status. U87 cells express wild-type p53, whereas U251 cells have a mutated form of p53(147). Overexpression of wild-type p53 in both cell lines caused suppression of cell growth in culture (148) thereby suggesting growth inhibitory effect of wild-type p53. Thus, wild-type p53 in U87 cells may contribute to their slower growth in culture compared to U251 cells, which have the mutated p53.
3.3.2 GBM cells form neurospheres in culture

Neurosphere forming ability is one of the characteristics of normal neural stem cells and neural precursor cells (149-152). Reports have also claimed that brain CSCs form neurospheres in culture (51, 153). To determine the neurosphere forming ability of GBM cell lines, U87 and U251 cells were cultured in serum-free conditions for 5 days. U87 cells were able to form neurospheres in culture within 5 days of serum-starvation, whereas U251 cells did not form neurospheres (Figure 12 and supplementary Figure 1).
Figure 12. GBM cells form neurospheres in *in vitro* culture. GBM cell line U87, primary GBM cultures CCF-334, CCF-310 and CCF-252 were grown in serum free conditions for 4 days and then the cells were checked for presence of neurospheres. A representative picture is shown from three similar experiments.
Primary cultures derived from GBM patient tumors (gifts from Drs. Gene Barnett and Michael Vogelbaum, Cleveland Clinic) were examined for neurosphere forming ability. CCF-334 and CCF-310 were maintained in serum-free media, and they grew as neurospheres only. The cells were dissociated into a single cell suspension and plated in fresh media. After 4 days the cells were tested visually for the presence of neurospheres. Although the cells attached to the tissue culture plate initially, after one day the neurospheres started forming and the cells finally grew only as neurospheres (Figure 12). This was in contrast to U87 cells, which had both adherent cells and neurosphere-forming cell populations. CCF-252 cells were cultured in 0.5% serum. These cells resembled U87 cells in the neurosphere assay with the presence of two populations, attached cells and neurospheres (Figure 12).

3.3.3 U87 cells do not express CD133 glycoprotein on their surface

U87 cells have the ability to form neurospheres in culture and tumors when transplanted in athymic mice, thus it is reasonable to believe that they contain a cancer stem cell population.

Recent reports suggested that CD133, a cell surface glycoprotein expression distinguishes brain CSCs from the non-stem cell fraction (50). Hence, the cell surface expression of CD133 glycoprotein was monitored in GBM cells by flow cytometry. The
results were normalized for auto-fluorescence of the cells and antibody isotype non-specific binding. The normalized data demonstrated that U87 and U251 cell lines lacked CD133 positive population (Figure 13b and c). The auto fluorescence and the isotype control antibody reactivity of these cells were higher than that of the CD133 antibody. The colon carcinoma cell line Caco-2 was used as a positive control since it had very high expression of the cell surface glycoprotein CD133 (Figure 13a).

Next, the primary cultures derived from GBM patient tumors were tested for CD133 glycoprotein expression as these cells also formed neurospheres in culture. GBM tumor-derived primary culture CCF-334 was found to contain about 13 +/- 5 % cells expressing CD133 glycoprotein on their cell surface (Figure 13d). CCF-310, CCF-252, and CCF-247 lower fluorescence due to CD133 antigen-antibody reaction than the combined percentage of auto fluorescence and the isotype control antibody reactivity. These results demonstrate lack of expression of CD133 glycoprotein on the surface of these cells (Figure 13e).
**Figure 13.** CD133 glycoprotein expression in GBM cells. (a) Caco-2 cells express CD133 so they used as positive control. GBM cell lines U251 (b) and U87 (c) lack CD133 expression. Primary culture of GBM tumor, CCF-334 (d) contains CD133 expressing cells. The GBM cells were washed with 1x PBS and 1% BSA and stained with or without either CD133-PE or IgG-PE isotype control. The cells were then washed twice with 1x PBS and 1% BSA to remove unbound antibody and then run through the flow cytometer to measure the PE tagged cells. (e) Tabulation of the auto fluorescence, isotype control and CD133 expressing GBM cells. The difference represents the percentage (%) of cells expressing CD133 glycoprotein after deducting the auto fluorescence and isotype control from it. Results represent one of three independent experiments.
Ungated
U87+IgG1
Event Count: 20000

Ungated
U87+IgG1
Event Count: 20000

Ungated
U87+CD133 PE
Event Count: 20000

Ungated
U87+CD133 PE
Event Count: 20000
<table>
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<tr>
<th>Cell lines &amp; primary cultures</th>
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<th>U251</th>
<th>CCF-334</th>
<th>CCF-310</th>
<th>CCF-247</th>
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<tr>
<td>Cells</td>
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<td>15.70</td>
<td>2.13</td>
<td>1.03</td>
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<td>0.13</td>
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<tr>
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<td>0.88</td>
<td>0.49</td>
<td>0.89</td>
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<td>-14.09</td>
<td>+13.34</td>
<td>-1.22</td>
<td>-0.84</td>
<td>-0.26</td>
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</table>
To investigate if CD133 mRNA was expressed in these GBM cells that lacked cell surface expression of the glycoprotein, CD133 transcript expression was measured in the GBM cell lines and primary cultures. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as endogenous control for polymerase chain reaction (PCR) (Figure 14). U251 cells (lane 1 in Figure 14) expressed higher levels of CD133 transcript compared with U87 cells (lane 2 in Figure 14), whereas, the primary cultures, which did not have cell surface expression of CD133 glycoprotein, expressed CD133 transcript (Figure 14). CCF-334, which contained about 13% cells expressing CD133 on their cell surface also contained higher levels of CD133 mRNA compared with CCF-310, CCF-252, and CCF-247 which contained decreasing levels of CD133 mRNA (compare lanes 5, 6, 4 and 3 in Figure 14). Although the RT-PCR method that was used for this analysis is only semi-quantitative, it provides preliminary information regarding CD133 mRNA expression in GBM cells. These data indicate that there are differences in CD133 processing at the post-transcriptional level. Most importantly, these results suggest that cell surface expression of CD133 glycoprotein expression is not correlated to the tumorigenic potential of GBM cell lines since the tumorigenic GBM cell line U87 that formed large tumors in athymic mice compared with U251 cells (Fig. I-1a) has lower CD133 mRNA levels than that of U251 cells, even though they lack CD133 glycoprotein expression on their cell surface.
Figure 14. CD133 mRNA expressions in GBM cells. Total RNA was isolated from the cells as described in the methods section; 1-2 µg of total RNA was used in PCR analysis with primers specific for CD133 and GAPDH transcripts. The products from 35 cycles of RT-PCR were resolved on a 1% agarose gel.
3.3.4 **GBM cell lines have different levels of GFAP expression**

GFAP is considered as a marker for differentiated astrocytes and their expression is also reported in GBM tumors (154-156). Expression of GFAP in C6 glioma cell line suppresses growth of the glioma cell line (157). GFAP expression in U87 and U251 cell lines was determined by immunocytochemistry. U251 cells (panel 1 in Figure 15a) showed extensive immunoreactivity for GFAP whereas very little staining for GFAP was detected in U87 cells (panel 2 in Figure 15a). U251 cells showed clearly cytoplasmic distribution of GFAP. This is consistent with previous reports about GFAP being an intermediate filament that has cytoplasmic distribution (158).

To further examine the expression of GFAP in GBM cells, I determined GFAP transcript levels by RT-PCR. The data demonstrated that although the gfap gene was active in U87 cells (lane 2 in Figure 15b), the transcript level was very low, which correlated with the protein expression. Although the method is semi-quantitative, these data suggest that GFAP mRNA expression is lower in U87 cells than that in U251 cells (compare lanes 1 and 2 in Figure 15b). This is consistent with the GFAP protein expression profile of these two GBM cell lines. β-actin was used as the endogenous control (Figure 15b). Primary cultures of GBM patient tumors also expressed varying levels of gfap transcript comparable to that in U87 cells (compare lanes 3-6 to lane 2 in Figure 15b). Collectively, these results suggest that GBM cell lines and primary cultures vary in the differentiation status suggested by the difference in expression levels of the astrocytic differentiation marker, GFAP.
**Figure 15.** GFAP expression in GBM cell lines. (a) Cytoplasmic Immunostaining of GFAP protein with anti-GFAP antibody. Rabbit polyclonal anti-GFAP antibody was used. (b) GFAP mRNA expression in GBM cells. Total RNA was isolated from the cells as described in the methods section. 1-2 µg of total RNA was used in PCR analysis with primers specific for GFAP transcript. The products from 35 cycles of RT-PCR were resolved on a 1% agarose gel. β-actin was used as control.
3.3.5 Myc expression in GBM cells

Myc oncoprotein deregulation has been critical in both solid cancers (92, 96) and hematopoietic malignancies (88, 89, 159). Genetically engineered mouse with constitutive Myc expression have been reported to be highly prone to tumorigenesis. This study also suggest that Myc protein levels affected the tumor latency and growth kinetics (91). Myc levels also influence the type of malignancies that develop in the transgenic mice. Previous studies have shown aberrant stabilization of Myc protein in human glioma cell lines due to prolonged half life of the protein (90). Recently, Lassman et. al. used a mouse model to show that expression of Myc along with activated Ras and Akt in differentiated astrocytes suppressed their GFAP expression (82). Importantly, these cells were able to form tumors in the rodent xenograft assay, unlike the differentiated astrocytes that did not express Myc but expressed activated Ras and Akt (82). These findings highlight critical involvement of Myc in cancers including GBM. However no reports mentioned if Myc expression was correlated with tumorigenicity of human GBM cells. This prompted us to investigate the expression of Myc in both the highly tumorigenic and low or non-tumorigenic GBM cell lines U87 and U251 respectively.

Western blot analysis demonstrated that U87 cells (lane 3 in Figure 16a) expressed detectable levels of Myc, whereas U251 cells (lane 2 in Figure 16a) expressed very little Myc oncoprotein. The cells were grown in serum-starved conditions for 24 hours before they were harvested to minimize the effect of serum since serum was reported to be an inducer of Myc expression (160). β-actin was used as ensure that equal
amounts of protein was loaded in each well (Figure 16a).
Figure 16. Myc expressions in GBM cells. (a) GBM cell lines have different levels of Myc oncoprotein expression. Monoclonal anti-mouse antibody was used for immunoblot analysis. (b) myc mRNA was expressed by both U87 and U251 cells at a comparable level. (c) U87 cells were heterogeneous with respect to the level of Myc protein expression. Rabbit polyclonal anti-Myc antibody was used to detect anti-Myc immunoreactivity in U87 cells which was visualized under a fluorescence microscope.
Immunocytochemical staining with anti-Myc antibody showed that U87 cells were heterogeneous with respect to the level of Myc immunoreactivity (Figure 16c).

In order to determine if the myc mRNA levels correlated to the protein expression in GBM cells, the myc transcript expression was monitored by RT-PCR analysis using the total RNA isolated from the GBM cell lines. The results show that myc transcript was expressed at a comparable level in both U87 and U251 cell lines (comparing lanes 1 and 2 in Figure 16b), although U87 cells express higher Myc protein levels than U251 cells. This indicated that Myc may be undergoing different post-transcriptional modifications in the two GBM cell lines.

To further investigate this phenomenon, I tested Myc message and protein expression in the primary cultures derived from GBM patient tumors. Interestingly, the results demonstrate that the levels of myc mRNA was comparable in the different primary cultures (lanes 4, 5, 6 and 7 in Figure 17b) but their Myc protein expression was very different ranging from very low levels in CCF-247 to high expression in CCF-252 (compare lanes 1 and 2 in Figure 17a). These data suggest that although myc mRNA is synthesized in most GBM cells, however, the steady state level of the protein is dependent on post-transcriptional modifications in cell type-specific manner.
**Figure 17.** Myc expression in primary cultures derived from GBM tumors. (a) Primary cultures of GBM patient tumors expressed varying levels of Myc oncoprotein. Monoclonal anti-Myc antibody was used for immunoblot analysis. (b) GBM cell lines and primary cultures expressed comparable levels of myc mRNA. Total RNA was isolated from cells that were pre-conditioned by 24 hours of serum starvation. 1-2 µg total RNA samples were used to perform 35 cycles of RT-PCR analysis using primers specific for myc mRNA. The RT-PCR product was then resolved on 1% agarose gel.
a

IB: Myc

IB: β-Actin

b

myc

β-Actin
3.3.6 Myc protein expression is inversely correlated to the Stat3 activation level in GBM cells

Constitutive Stat3 activation has been reported in almost all GBM cells (138). Reports also indicated that activated Stat3 supports survival of U87 cells \textit{in vitro} by inducing anti-apoptotic genes, such as Mcl-1 (132). On the other hand, Myc has been recognized as an inducer of apoptosis in many different cell types and the Myc-induced apoptotic pathway can be dissected from the pathways that regulate Myc-induced cell cycle progression although there are effectors common to both pathways (86, 87, 137). These reports suggest contrary roles of activated Stat3 and Myc oncoprotein in GBM, therefore, I wanted to investigate whether Stat3 activity and Myc expression had any correlation in GBM cells.

Electrophoretic mobility shift assays revealed that Stat3 activation in U251 cells (lane 2 in Figure 18a) was higher than that in U87 cells (lane 1 in Figure 18a), while western blot analysis showed that Myc protein expression was lower in U251 cells (lane 1 in Figure 18b) than that in U87 cells (lane 2 in Figure 18b), thereby suggesting an inverse correlation between Stat3 activation status and myc protein expression levels in these two GBM cell lines (Figure 18a and b).

To further explore this correlation, Stat3 activation status and steady state levels of Myc protein were determined in GBM tumor-derived primary cultures. The results demonstrated that the primary cultures with high Myc protein expression maintained a
low level of Stat3 activation. For example, CCF-252 had very low level of Stat3 DNA-binding activity (see lane 1 of Figure 18c), but had a relatively high steady state level of Myc protein (see lane 2 of Figure 18d), whereas, CCF-247, that had high Stat3 DNA-binding activity (shown in lane 2 of Figure 18c), displayed low steady state level of Myc protein (see lane 1 of Figure 18d). These data collectively suggest an inverse correlation between Stat3 activation status and Myc protein expression level in GBM cells.
Figure 18 Myc protein expression and Stat3 DNA-binding activity are inversely correlated in GBM cells. (a) U251 cells had high levels of activated Stat3 monitored by the DNA-binding activity of Stat3 by electrophoretic mobility shift assay (EMSA). Total protein was extracted from the cells and then 10 µg of the total protein was incubated with $^{32}$P-radiolabelled hSIE probe for 20 mins and then the DNA-protein complex was resolved on a 5% native polyacrylamide gel. (b) U251 cells had low expression of Myc oncoprotein compared to that in U87 cells. Total protein was extracted from the cells by lysis using a modified RIPA buffer. 50 µg of the total protein was resolved on a 10% SDS-polyacrylamide gel. Monoclonal anti-Myc (C-33) antibody was used to detect endogenous Myc protein levels. β-actin was used as loading control. (c) GBM patient tumor-derived primary cultures had varying levels of activated Stat3 levels. Protein was prepared as described above in (a) and 10 µg of protein was subjected to EMSA. (d) Myc protein was expressed at different levels in the primary cultures derived from GBM tumors. The protein was extracted as in (b) and 50 µg of the protein was used for the western blot analysis for Myc expression and β-actin as loading control.
3.4 Discussion:

GBM cell lines are useful as a model to study different cellular and molecular mechanisms since they are easily available and may be cultured readily in vitro. However, primary GBM cultures that are derived from patient tumors are difficult to obtain and maintain in culture. The primary cultures are therefore, useful in validating observations while the detailed analyses are done using the established GBM cell lines that possess the fundamental characteristic of tumorigenicity. Thus, I have used GBM cell lines to perform the majority of the experiments in this dissertation. The primary GBM cultures were used for validation purposes.

Recent studies have shown that GBM tumor cells are distinguishable on the basis of their cell surface expression of a glycoprotein, CD133 (161). CD133 glycoprotein-expressing cells are functionally different from their CD133 glycoprotein-non-expressing counterparts in their tumor-forming ability in rodent xenograft assays (50). Only 100 CD133-expressing cells are able to form tumors in nude mice, while even 1 million non-expressing cells failed to do so in a parallel experiment (50). This indicates that cell surface expression of CD133 glycoprotein is potentially correlated to tumorigenicity of GBM cells. However, another study demonstrated that GBM tumor cells that do not express CD133 glycoprotein on their surface also form tumors that are relatively less angiogenic than those formed by CD133 expressing GBM tumor cells (67). The current results support the latter finding by demonstrating that the cell surface expression of
CD133 glycoprotein is not a reliable marker of all brain tumor-initiating cells since tumorigenic GBM cell line U87 does not contain CD133 glycoprotein expressing cell population.

Maintenance, and thus characterization of brain tumor-initiating cells has been elusive because the cancer cells are in a dynamic process with regards to their differentiation status. The differentiation pathway, that was previously believed to be irreversible, is now recognized as a reversible process and responsive to the microenvironment. Stemness is considered to be a function not an entity (162, 163). CSCs are believed to behave similarly, responding to their microenvironment and modifying their characteristics to serve the required function. Intensive research has identified three essential functions that cancer stem cell have to maintain for their self-sufficiency; namely, limitless proliferation, survival and maintenance of an undifferentiated phenotype. Therefore, it is imperative to identify functionally relevant molecules that are associated with stemness of the tumor cells.

Myc expression, in combination with activated Ras and activated Akt, is able to confer tumorigenicity to differentiated astrocytes, which thereby loose the expression of GFAP, the marker for differentiated astrocytes (82, 164). Recent studies also demonstrate that the expression of four transcription factors, namely, Oct3/4, Sox2, KIf4 and Myc, in human adult dermal fibroblasts reprogram the former into pluripotent stem cells (80). Overexpression of myc in the induced pluripotent stem (iPS) cells caused tumorigenesis when the iPS cells were injected into nude mice (80, 81, 165). The overexpression of the rest of the three genes excluding myc in MEFs lead to formation of iPS cells since MEFs
have low level of endogenous Myc expression which is 20% of that expressed in mouse ES cells (166). Collectively, these studies indicate that Myc is one of the transcription factors required for the reprogramming of an adult cell into a pluripotent stem cell. Overexpression of Myc increases tumorigenic potential of the iPS cells. Therefore, it was important to determine if Myc expression was correlated to tumorigenicity of the GBM cells.

My results suggest that Myc oncoprotein is expressed in some GBM cells, although comparable levels of myc transcript are expressed in most GBM cell lines and primary cultures. Most importantly, tumorigenic GBM cell line U87 expresses higher levels of Myc oncoprotein compared with U251 cell line that is found to form smaller tumors than U87 cells. These findings suggest that Myc oncoprotein expression is positively correlated to tumorigenic potential of GBM cells. My results are further corroborated by recent observations by Nakagawa et al (166). They have demonstrated that expression of three transcription factors namely Oct3/4, Sox2 and Klf4 in MEFs that already have endogenous Myc expression induces generation of pluripotent stem (iPS) cells.

Interestingly, the mice generated from these iPS cells showed significantly lower tumor incidence as compared to mice generated from iPS cells that were transduced with myc-expressing retrovirus (166). These evidences suggest that Myc overexpression increases tumorigenicity of the cells. Previous observations suggest that Myc is a potent inducer of apoptosis. Myc-mediated inhibition of NFκB activation or c-FLIP gene transcription may contribute to Myc-mediated apoptosis (85).
Our laboratory has previously reported that activated Stat3 supports the survival of GBM cell line U87. So I wanted to determine if there was a relationship between the expression levels of Myc oncoprotein and Stat3 activation levels in GBM cells including U87. My results demonstrate that the GBM cell lines and primary GBM cultures that have high level of Stat3 activation have low levels of Myc expression. These data suggest an inverse correlation between Stat3 activation levels and Myc oncoprotein expression in GBM cells. Apparently, such an inverse relationship is appropriate with regards to the functions of these two transcription factors, Myc and Stat3 in cells of astrocytic lineage.

In normal development, activated Stat3 is known to induce differentiation of neuroepithelial cells into astrocytes (122). Myc protein is suppressed in terminally differentiated cells (167-169). While the undifferentiated cells like embryonic stem cells in mouse and humans express Myc protein (80, 81, 166). Most importantly, the finding that expression of Myc oncoprotein in differentiated astrocytes result in loss of the differentiated astrocyte marker, GFAP corroborates the function of Myc as a molecule that induces de-differentiation of astrocytes (82). Thus, Stat3 and Myc serve opposite functions with regard to differentiation of cells towards the astrocytic lineage.

Myc has also been implicated as a potent mitogen that induces apoptosis (86, 87). On the other hand, activated Stat3 has been accepted as a survival signal in GBM cells (132, 133, 138). These findings indicate opposite functions of Myc and activated Stat3 in GBM cells and thereby lend support to the observations described in the current chapter of this dissertation. They also raise important question whether activated Stat3 has a regulatory effect on Myc in GBM cells? This question is addressed in the next chapter.
4.0 REGULATION OF MYC EXPRESSION IN GBM CELLS

4.1 Abstract:

Previous findings suggest that activated Stat3 and Myc serve contrary functions in cells of astrocytic lineage. My observations in the first chapter indicated that steady state levels of Myc protein and activated Stat3 were inversely correlated in GBM cells. The experiments in this chapter were designed to investigate if activated Stat3 had a regulatory effect on Myc protein expression. The data from this study demonstrated that Myc oncoprotein expression was negatively regulated by activated Stat3. myc transcript level remained unaffected by activated Stat3, suggesting a post-transcriptional regulation. Furthermore, the effect of activated Stat3 on Myc was reflected in the levels of Myc-target proteins including Cyclin A, Cyclin E2, and p21 as well as Myc-regulated functions, such as proliferation of GBM cells.
4.2 Introduction:

Under normal conditions, Myc protein is tightly controlled because its deregulated expression is detrimental for the cell (170). Myc is a potent oncoprotein that is elevated in many cancers including breast cancer (171). In most cancers, myc gene regulation is abrogated leading to elevated Myc protein levels (89). However, Myc overexpression has been reported in some primary GBM patient tumors (172). Anomalous stabilization of Myc protein resulting in an elevation of the protein level has been observed in four GBM cell lines (99). The results in the first chapter demonstrated that highly tumorigenic GBM cell line, U87 had higher Myc expression compared with U251 that had low tumorigenic potential. However, the level of activated Stat3 was higher in U251 cells in comparison with that in U87 cells, suggesting an inverse relation of activated Stat3 level with Myc protein expression in these cells. Since activated Stat3 induces myc gene expression in anaplastic large cell lymphoma (ALCL) cells (134), the observations mentioned above, instigated the question whether activated Stat3 regulated Myc expression differently in GBM cells. To address this question, a dominant negative mutant and a constitutively active mutant of Stat3 were used. To further corroborate the findings, a small molecule inhibitor, Sta-21 was employed to suppress the DNA-binding activity of activated Stat3 in order to study Myc-target proteins. Expression of Myc-target proteins were also monitored in Sta-21 treated cells and compared to GBM cells that were transfected with myc gene causing Myc overexpression in them. Collectively, the
data suggest that Myc protein expression was suppressed by activated Stat3 in GBM cells. myc transcript level was unaltered by changes in activated Stat3 levels indicating a post-transcriptional regulatory mechanism. Moreover, the levels of Cyclin A, E2, and p21, downstream targets of Myc were altered in response to changes in the level of activated Stat3, which resembled the effect of over-expressing Myc in the same cell line. Modulation of activated Stat3 level also affected cellular proliferation, a Myc-regulated function.

4.3 Results:

4.3.1 Myc expression is down-regulated by activated Stat3

The results described in chapter 1 of this dissertation indicated that the level of Myc protein expression was inversely correlated to the level of Stat3 activation in GBM cells, so I hypothesized that activated Stat3 down-regulates Myc protein in GBM cells. To test this hypothesis, I used pharmacologic and molecular genetic approaches for suppressing Stat3 activation in GBM cells and then determined the effects on Myc protein expression. First, a G418-selected stable clone of U87 cell line expressing a
dominant negative form of Stat3 (DN-Stat3) that was previously generated in our laboratory was used to determine steady state level of Myc protein.

DN-Stat3 was generated by site-directed mutation of a tyrosine residue at 705 to phenylalanine thereby impairing the phosphorylation of Stat3 at Tyr\textsubscript{705} (134, 138). (Figure 19a) When expressed in the cell, this mutated form of Stat3 functions as dominant-negative form for the endogenous Stat3 because it can bind to phosphorylated tyrosine residues on the cytokine or growth factor receptors using the SH2 domain, but are unable to get phosphorylated on the Phe\textsubscript{705} residue thus they remain docked to the cytoplasmic domain of the receptor tyrosine kinases preventing activation of the endogenous Stat3 molecules.

The DNA-binding activity of endogenous Stat3 was suppressed in U87 cells that stably expressed the DN-Stat3 mutant compared to the empty vector-transfected cells (compare lanes 1 to 2 in Figure 19b). The same DN-Stat3 expressing clone of U87 cell line had higher Myc protein expression compared to the untransfected U87 cells determined by immunoblot analysis using anti-Myc antibody (compare lane 3 to 2 in Figure 19c). β-actin was used as a loading control (Figure 19c). U251 cells have been used as a positive control for Stat3 DNA-binding activity in Figure 19b. The breast cancer cell line, MCF-7 was used as a positive control for Myc protein expression in Figure 19c.
Figure 19 Dominant negative (DN) Stat3 upregulates Myc oncprotein. (a) Schematic representation of Stat3, DN-Stat3 and CA-Stat3 mutant forms. DN-Stat3 has a tyrosine to phenylalanine mutation at 705 position. CA-Stat3 was developed by replacing alanine at 662 and asparagine at 664 to cysteine. (b) DN-Stat3 expression in U87 cells suppressed the Stat3 DNA-binding activity compared to the empty vector transfected U87 cells. Total protein was salted out from U87 cells stably transfected with DN-Stat3 or the empty vector and U251 cells. 10 µg of the protein was incubated with radiolabelled hSIE probe for Stat3 DNA-binding reaction. The DNA-protein complex was then resolved on a native polyacrylamide gel. (c) Myc expression is increased in the cells expressing DN-Stat3 compared to the untransfected cells. Total protein was isolated by lysing cells in modified RIPA buffer. 50 µg of the total protein was then resolved on a 10% SDS-polyacrylamide gel. The protein was transferred onto a PVDF membrane and probed with anti-Myc and anti-β-actin antibodies. β-actin was used to ensure equal amount of protein in each lane.
To test the converse of the above observation, I generated G418-selected cell lines from U87 cells that stably expressed a constitutively active mutant form of Stat3 (CA-Stat3). CA-Stat3 construct was generated in our laboratory, by site-directed mutagenesis of Stat3 where Ala\textsubscript{662} and Asn\textsubscript{664} residues within the C-terminal region of the SH2 domain were mutated to cysteine so that they form disulfide bond thereby forming Stat3 dimers independent of upstream phosphorylation events (173). All the clones derived from U87 cell line were assayed for V5 expression since the CA-Stat3 mutant was tagged with V5 epitope. Three of the clones which expressed moderate level of CA-Stat3-V5 protein were selected for further screening (Figure 20a). The CA-Stat3-V5 protein-expressing clones were tested for their Stat3 DNA-binding activity by EMSA to establish the function of CA-Stat3 mutant (see Figure 20b). The clones that showed increased DNA-binding activity were assayed for Myc protein expression. Myc oncoprotein expression was decreased in CA-Stat3 expressing clones of U87 cells compared to that in empty vector expressing clones and the untransfected cells (compare lanes 2, 3 and 4 to lane 1 in Figure 20b). β-actin was used to ensure equal protein content in each lane (Figure 20a and b).
Figure 20 CA-Stat3 suppresses Myc protein expression in GBM cells. (a) Western blot analysis of V5 tagged CA-Stat3 (CA-Stat3-V5) protein expressed in U87 cell-derived clones. (b) U87 cell-derived clones that expressed CA-Stat3-V5 protein also showed increased Stat3 DNA-binding activity and decreased Myc protein expression. β-actin was used to ensure equal protein content in each lane. Western blot analysis and EMSA were done as described in Figure 11 and detailed in the methods section.
Pharmacological agent was used to further corroborate the observations using molecular genetic approach. Sta-21, a small molecule inhibitor of Stat3 was used to study the regulation of Myc protein expression by activated Stat3 in GBM cells. Sta-21 was reported to inhibit the DNA-binding activity of Stat3 in breast cancer cell lines (174-176). To determine the efficiency of Sta-21 in inhibiting Stat3 DNA-binding activity, I tested Sta-21 on the whole cell extracts of two GBM cell lines U251 and U87. 30 µM of Sta-21 treatment for 20 mins at room temperature was sufficient to reduce the DNA-binding function of Stat3 in whole cell extract of U87 cells by 50% (Figure 21b) compared to that in DMSO treated whole cell extract of the same cell line (see lanes 1 and 2 in Figure 21a). The same dose of Sta-21 was used for treating the whole cell extract of U251 cells for 20 mins; and Stat3 DNA-binding activity was decreased by 24% (Figure 21b) compared to DMSO treated whole cell extract of the same cell line as vehicle control (see lanes 3 and 4 in Figure 21a). It is also noteworthy that Stat3 activation level was high in U251 as compared to U87 cells so 30 µM of Sta-21 may not be sufficient to suppress the DNA-binding activity any further. Next, the efficiency of Sta-21 in inhibiting the DNA-binding activity of Stat3 in vivo was determined. U87 cells were treated with 30 µM of Sta-21 or with equal volume of DMSO in serum-free condition for 48 hours. The cells were harvested post-treatment by scraping, and subjected to protein extraction. The resulting whole cell extract was incubated with radio-labeled DNA probe and the DNA-protein complex was then resolved on a native polyacrylamide gel for EMSA. The results demonstrated 51.34% reduction (Figure 21d) in the Stat3 DNA-binding activity in the
Sta-21 treated cells compared to the cells that were treated with or without equal volume of DMSO (compare lanes 3 to 2 and 1 in Figure 21c).
**Figure 21.** Sta-21 suppresses the DNA-binding activity of Stat3 in GBM cells. (a) Stat3 DNA-binding activity is reduced upon Sta-21 treatment of the protein extracted from U87 and U251 cells. Untreated U87 cells and U251 cells were harvested by scraping and then total protein was extracted by salting out. 10 µg of the total protein was treated with 30 µM of Sta-21 for 20 min and then further incubated with radio-labeled hSIE probe for another 20 min at room temperature. The DNA-protein complex was resolved on a 5% native polyacrylamide gel. (b) Quantification of Stat3 DNA-binding activity *in vitro*. N=2 (c) Stat3 DNA-binding activity is reduced in U87 cells treated with Sta-21 compound. U87 cells were treated with 30 µM of Sta-21 compound in serum-free media for 48 hours. The cells were scraped and total protein was extracted by salting out. 10 µg of protein was incubated with radiolabelled hSIE for 20 min at room temperature and the DNA-protein complex was resolved on a 5% native polyacrylamide gel. (d) Quantification of Stat3 DNA-binding activity *in vivo*. Results described as mean intensity and error bars indicate standard deviations. N=3
Whole cell extracts; 10µg per lane

U87 U251
- + - +

Sta-21; 30µM; 30 mins; RT

1 2 3 4

Whole cell extracts of U87 cells
(Serum-free media)

DMSO (Vehicle)
Sta21 (30µM)

1 2 3

Untreated DMSO Sta-21 (30 µM)
Arbitrary units of intensity
After establishing that Sta-21 suppressed Stat3 DNA-binding activity of U87 cells, the expression level of Myc in response to Sta-21 treatment was determined. Western blot analysis of Sta-21 treated U87 cells showed elevated Myc expression by 38.46% (Figure 22b) as compared to that in DMSO treated samples (compare lane 1 to lane 2 and 3 in Figure 22a). Both molecular genetic and pharmacological approaches demonstrated similar results that suggest an inhibitory effect of activated Stat3 on Myc expression levels in U87 cells.
**Figure 22** Sta 21 up-regulates Myc protein expression in U87 cells. (a) U87 cells were treated with 30 µm of Sta-21 compound in serum-free media for 48 hours. The cells were scraped and total protein was extracted by lysing the cells in modified RIPA buffer. 60 µg of protein was resolved on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membrane was then probed with polyclonal anti-Myc antibody. β-actin was used as loading control. (b) Quantification of Myc expression in U87 cells is shown in (a). Results are described as mean intensity and error bars indicate standard deviations. The results shown represent one of four independent experiments (N=4).
a

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DMSO (7.5µL)
Sta-21 (30µM)

Myc
β-Actin

b

Arbitrary units of intensity

n=4

26.236
10.543
0
5
10
15
20
25
30
35
40

DMSO
STA-21

38.46%

10.50
26.23

Arbitrary units of intensity

DMSO
STA-21
4.3.2 **Activated Stat3 negatively regulates Myc expression in primary cultures of GBM patient tumors**

In the first chapter of this dissertation I have demonstrated that primary cultures of GBM patient tumors have activated Stat3 and the level of Myc expression is inversely correlated to the Stat3 activation level. So, I hypothesized that activated Stat3 suppresses Myc protein expression in the primary cultures of GBM cells too. To test this hypothesis, the effect of down-regulating the DNA-binding activity of Stat3 in a primary culture of GBM patient tumor, CCF-252 was determined. (Figure 23) Sta-21 treatment of CCF-252 in serum-free conditions for 48 hours resulted in elevated Myc protein expression monitored by immunoblot analysis of the total cell lysate (compare lane 1 to lanes 2 and 3 in Figure 23). The polyclonal anti-Myc antibody was used for detecting both the isoforms of Myc protein since the monoclonal anti-Myc antibody does not recognize the isoforms efficiently. β-actin was used as loading control to ensure equal protein in each lane. MCF-7 cell lysate (lane 4 in Figure 23) was used as a positive control for Myc protein expression.
Figure 23 Sta-21 increases Myc expression in primary culture of GBM patient tumor, CCF-252. CCF-252 culture was treated with 30 μM of Sta-21 in serum-free special media described in methods and material section. The cells were scraped and then lysed with modified RIPA buffer to extract the total protein. 60 μg of the total protein was resolved on a 10% SDS-polyacrylamide gel and probed with polyclonal anti-Myc antibody. β-actin was used to ensure equal protein content in each lane.
4.3.3 Activated Stat3 affects Myc-regulated target proteins and Myc-regulated function

To further corroborate the observation that activated Stat3 inhibits Myc protein expression, the levels of the downstream targets of Myc including cyclin A (101), cyclin E2 (177) and p21 (178), were investigated after suppressing Stat3 activity in U87 cells. Myc has been found to induce cyclin A (179), so cyclin A protein level was determined in U87 cells that expressed DN-Stat3 stably. WB analysis of total cell lysate of DN-Stat3 expressing cells showed elevated Myc protein level; and cyclin A protein expression was 55.44% higher (Figure 24b) than that in empty vector-transfected cells (compare lanes 1 and 2 in Figure 24a). The expression levels of cyclin E2 that is known to be induced by Myc (180, 181) and p21 that has been reported to be inhibited by Myc (182) were also determined. Cyclin E2 protein steady state level was found to be 25.92% higher (Figure 24d) in Sta-21 treated U87 cells, compared with its DMSO treated counterparts (compare lanes 1 and 2 in Figure 24c). Steady state level of p21 protein was 64.16% lower (Figure 24e) in Sta-21 treated cells compared with that in DMSO treated U87 cells (compare lanes 1 and 2 in Figure 24c). β-actin was used as loading control (Figure 24a and c).
Figure 24. Downstream targets of Myc are affected by changes in Stat3 activity. (a) Cyclin A expression was increased in DN-Stat3-expressing U87 cells measured by immunoblot analysis. (b) Quantification of the intensity of the Cyclin A band shown in (a). (c) Sta-21 treated U87 cells had higher expression of Cyclin E2; and lower expression of p21 protein compared to that in DMSO treated cells. (d) Quantification of intensity Cyclin E2 band shown in (c). (e) Quantification of intensity of p21 band shown in (c). β-actin was used to ensure equal protein content in each lane. Western blot assays were performed as described in Figure II-1. Bands detected on the western blots were quantified using NIH Image J software. (f) gfap mRNA expression was decreased in Sta-21 treated U87 cells compared to DMSO treated cells. U251 cell in lane 1 of (f) was used as a positive control for gfap mRNA expression. Total RNA was isolated from the Sta-21 treated or DMSO treated and untreated cells. The gfap-specific primer set was used in reverse transcriptase-PCR analysis to detect gfap mRNA expression.
U87; G418 selection; Serum starved; 48 hrs

Cyclin A

β-actin

V5 vector DNSTAT3/V5

U87; G418 selection; Serum starved; 48 hrs

0.844
3.255

DMSO Sta-21
Arbitrary units of intensity

5.759
3.695

DMSO STA-21
Arbitrary units of intensity

9.842
17.752

V5 vector DNSTAT3/V5

Arbitrary units of intensity
It was previously reported that Myc expression suppresses differentiation and helps maintain the cell in an undifferentiated state (183-185). Overexpression of Myc in differentiated astrocytes resulted in loss of GFAP protein expression (82). GFAP is an intermediate filament protein that is expressed in differentiated astrocytes (184, 185), so the level of GFAP transcript expression was determined in U87 cells that were treated with Sta-21. The product generated from 35 cycles of reverse transcription PCR analysis using primers specific for GFAP mRNA showed that U87 cells that were treated Sta-21 lost their GFAP transcript expression (compare lane 2 to 3 in Figure 24f). GAPDH was used as an endogenous control (Figure 24f).

Myc is known to be a potent regulator of cell proliferation; so, I hypothesized that activated Stat3-mediated negative regulation of Myc will be reflected in proliferation of GBM cells. To test this hypothesis, I monitored the proliferation of U87 cells that expressed CA-Stat3 mutant described above by trypan blue dye exclusion assay. Equal number of cells was cultured in 10% serum-containing media for a period of 4 days. On the fourth day the cells were collected and stained with 0.4% trypan blue dye. The unstained viable cells were counted on a hemocytometer. U87 cell line-derived stable clones expressing CA-Stat3 mutant form of Stat3 showed decreased proliferation as compared to the cells that were transfected with the empty vector and the untransfected cells (Figure 25).
**Figure 25** CA-Stat3 expressing clones of U87 cell line have decreased cell proliferation. Equal number of untransfected U87 cells (U87-parental), empty vector transfected and CA-Stat3 transfected stable clones of U87 cell line were plated in quadruplet in 24-well plates and allowed to grow. After 4 days, the cells were collected by trypsinization and stained with 0.4% trypan blue solution for 5 mins. The unstained viable cells were counted with a hemocytometer. Results represent the average cell number and error bars indicate standard deviation. N=3
4.3.4 Effect of Stat3 inhibitor resembles that of Myc overexpression in GBM cells

To further confirm the observation that activated Stat3 regulates Myc expression in GBM cells, myc was over-expressed in U87 cells, and the level of Myc targets was measured. Since the data described above suggest that activated Stat3 suppresses Myc protein expression in U87 cells, I wanted to determine if the effect of down-regulating Stat3 activity would be similar to up-regulating Myc protein expression in U87 cells. To address this question, cyclin A and p21 protein levels were monitored in U87 cells. Immunoblot analysis of U87 cells over-expressing Myc and U87 cells treated with Sta-21 demonstrated that cyclin A protein expression was increased in these cells compared to the cells that were transfected with the pcDNA3.1 empty vector and treated with DMSO respectively (Figure 26a). p21 protein expression was decreased in U87 cells that over-expressed Myc and U87 cells that were treated with Sta-21 as compared to the cells that were transfected with pcDNA3.1 and the cells treated with DMSO, respectively (Figure 26b). These results demonstrated that treatment of Sta-21 resembled the effect of Myc overexpression on cyclin A and p21 proteins, which are known to be regulated by Myc. Collectively, these findings demonstrated that activated Stat3 negatively regulated steady state level of Myc protein in GBM cells; and this regulation was conveyed downstream of Myc to its target proteins which, in turn, altered the Myc-regulated functions like cell proliferation.
Figure 26. Myc overexpression in U87 cells had similar effects as suppression of activated Stat3 by Sta-21 treatment. (a) p21 level altered similarly in both U87 cells treated with Sta-21 and U87 cells over-expressing Myc. (b) Cyclin A level responded similarly in U87 cells that were treated with Sta-21 and those that over-express Myc. β-actin was used to ensure equal protein loading in each lane. Western blot analyses were performed as described in Figure 19.
a

<table>
<thead>
<tr>
<th>vector</th>
<th>myc</th>
<th>DMSO</th>
<th>Sta-21</th>
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IB: Cyclin A

IB: β-Actin

1 2 3 4

b

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<tr>
<th>Sta-21</th>
<th>DMSO</th>
<th>myc</th>
<th>vector</th>
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IB: Myc

IB: p21

IB: β-Actin

1 2 3 4
4.3.5 Activated Stat3 regulates Myc post-transcriptionally

Under normal conditions, Myc oncprotein has a very short half life (108) and it can be regulated at many different stages like transcription, post-transcription, translation, post-translation and degradation (3, 115, 186). Myc deregulation is often found in cancers (89, 93, 159, 171). In breast cancer cells, Stat3 has been shown to up-regulate Myc expression at the transcriptional level (94, 187, 188). So, I investigated if activated Stat3 down-regulated Myc protein expression by inhibiting myc gene transcription in GBM cells. To address this issue, myc transcript levels were monitored by conventional RT-PCR and by real-time RT-PCR in U87 cells following 48 hours of Sta-21 treatment under serum-free conditions. The RT-PCR analysis showed no difference between the myc mRNA levels in U87 cells treated with Sta-21 or DMSO or no treatment at all (compare lanes 1 to 2 and 3 in Figure 27a). This result was confirmed by quantification of the mRNA levels by real-time RT-PCR analysis (Figure 27b).

To further explore if activated Stat3 had any effect on myc transcription, myc promoter activity was monitored in U87 cells in response to altered levels of Stat3 activation. To investigate the effects of activated Stat3 on myc promoter, a human myc promoter-driven luciferase reporter construct (140) (gift from Dr. Linda Penn in University of Toronto) was used. The construct named HBM-Luc is comprised of -2052 to +34 sequences of the human myc gene relative to P2, one of the major transcription
start site, subcloned upstream of the firefly luciferase reporter sequence in pGL2basic vector (Figure 28a). The other major transcription start site, P1 is also present in the construct. Co-transfection of the HBM-Luc reporter construct and either empty vector, DN-Stat3 or CA-Stat3 constructs did not alter the myc promoter-driven luciferase reporter activity in U87 cells (Figure 28b). The effect of DN-Stat3 on SNM-Luc and XNM-Luc were also determined (data not shown). However, the reporter activity remained unchanged in both SNM-Luc and XNM-Luc co-transfected cells. HBM-Luc construct most closely resembled the endogenous myc promoter in the cells. These results demonstrated that myc promoter was not regulated by activated Stat3 in GBM cells. Collectively, these observations suggested a post-transcriptional mechanism of negative regulation of Myc protein expression by activated Stat3 in GBM cells. These findings were consistent with the observation that GBM cells differed in the Myc protein expression levels even though they had comparable myc mRNA levels demonstrated in the first chapter, thereby indicating a difference at a post-transcriptional level.
Figure 27 Activated Stat3 suppresses Myc at post-transcriptional level. (a) Total RNA was isolated from U87 cells that were either treated or untreated with Sta-21 or DMSO. 1-2 µg of the RNA was then used in 40 cycles of reverse-transcriptase PCR employing myc-specific primers to determine myc mRNA expression. (b) Total RNA was isolated as described in (a). cDNA pool was synthesized from the total RNA using random primers. The cDNA was then used to quantify myc mRNA with myc-specific primers using the SYBR green I method of real time PCR analysis. GAPDH was used as endogenous control in both of the assays.
**Figure 28** myc promoter activity is unaltered by changes in activated Stat3 levels in GBM cells. (a) Structure of myc promoter-driven Luciferase construct. Human myc gene is shown schematically with the three exons I, II, and III. P1 and P2 are the two major transcription start sites. The sequence from -2052 to +34 relative to the P2 was subcloned into the pGL2basic vector upstream of the Luciferase coding sequence. H stands for *Hind*III, S stands for *Sca*I, X stands for *Xho*I and N stands for *Nae*I. (b) myc promoter-driven luciferase activity was unchanged by modifications in Stat3 activation levels in GBM U87 cells. The HBM-Luc construct was used for measuring myc promoter activity. Luciferase assay was done as described in the methods section. N=3
4.4 Discussion:

The findings reported here suggest that activated Stat3 negatively regulated Myc oncoprotein at the post-transcriptional level, since it did not alter myc mRNA levels in GBM cells. Since activated Stat3 is crucial in astrocytic differentiation of neuroepithelial cells into mature astrocytes (189), whereas Myc maintains an undifferentiated phenotype (102, 103), my observations are consistent with their apparent contrasting functions in cells of astrocytic lineage. Myc has been reported to be up-regulated in other cancers by activated Stat3 (188). There have been reports of Myc having cell type-specific mode of regulation by other molecules such as PDGF in fibroblasts (113); Regulation of Myc in GBM cells is likely to represent another example of tissue-specific modification to best serve the functional requirements of the tissue. My studies described in this chapter have provided the basis for a potential model of regulation of Myc in GBM cells (Figure 29). According to this model, a yet unknown factor may be responsible for mediating the negative regulatory effect of activated Stat3 on steady state level of Myc protein. Given the opposite functions served by Myc and Stat3 with regards to differentiation of cells of astrocytic lineage, the effector is likely to be a factor that is induced by activated Stat3 when the relatively undifferentiated cells perceive differentiation signals. In turn, this factor then suppresses Myc protein expression so as to enable differentiation of the cell.
**Figure 29** Model of Myc-regulation by activated Stat3 in GBM cells. Activated Stat3 potentially suppresses Myc protein expression via a third factor likely to be associated with GBM cell differentiation, thereby supporting differentiation. Myc in turn inhibits GBM cell differentiation measured by GFAP expression. Myc also facilitates cell proliferation *via* target proteins including Cyclin A, E2, and p21. The inhibition of Myc protein by activated Stat3 is consequently reflected in the Myc target proteins and Myc-regulated functions.
5.0 REGULATION OF GFAP EXPRESSION IN GBM CELLS: ROLE OF MYC IN DIFFERENTIATION

5.1 Abstract:

Myc oncoprotein has been implicated in rendering an undifferentiated phenotype to differentiated astrocytes which consequently lose their expression of the differentiation marker, GFAP. However, the precise mechanism of suppression of GFAP expression was not known. In this chapter, I show that overexpression of p300, a transcription cofactor, induced the GFAP promoter-driven luciferase activity in U251 cells synergistically with IL-6 treatment. IL-6 induces Stat3 activation in these cells. Site-directed mutation study revealed that loss of one of the potential Stat3 binding sites on the GFAP promoter led to inhibition of the p300-induced GFAP promoter induction. Interestingly, overexpression of Myc suppressed the basal GFAP promoter activity. Also, when co-expressed with p300, Myc suppressed the p300-induced GFAP promoter activation in GBM cells. These results suggest that the GFAP promoter is positively regulated by p300 and IL-6 and their effects are dependent on DNA-binding activity of Stat3. In addition, Myc inhibits the GFAP promoter activity thereby suppressing GFAP expression in GBM cells.
5.2 Introduction:

Glial fibrillary acidic protein (GFAP) is the most prominent intermediate filament protein in differentiated astrocytes (185, 190-192). Although the GFAP-null mice are viable, missense mutations in the coding region of the GFAP gene appeared to be correlated to Alexander disease, a neurodegenerative disorder (185, 191). GFAP expression is considered a phenotypic signature of gliomas (193, 194). Reports have also claimed that the extent of GFAP expression decreases with the increase in degree of malignancy of the astrocytomas (195). Myc, on the other hand, is closely associated with undifferentiated phenotype of cells ranging from embryonic stem cells to tissue-specific progenitor cells (166). In medulloblastoma, the most common primary brain tumor in children, Myc expression has been found to be correlated with death of the patients due to tumor progression (92). In situ hybridization studies of gliomas and glioma cell lines have shown that topographic distribution of Myc and GFAP mRNA expression are mutually exclusive (196). Recently, it is reported that Myc overexpression in differentiated astrocytes led to the loss of GFAP expression and development of a relatively undifferentiated phenotype (82). Although these studies suggest an inverse relation between GFAP and Myc expression, there is no clear evidence regarding any regulatory mechanism between Myc expression and GFAP expression. I demonstrate that in U251 cells, the 2.2 kB promoter of GFAP gene was regulated by p300 in synergy with Stat3 which was activated by IL-6. The p300-induced GFAP promoter activity was
dependent on the DNA-binding activity of Stat3. Interestingly, both the basal level and p300-induced GFAP promoter function were dramatically inhibited by Myc overexpression.

5.3 Results:

5.3.1 p300 and IL-6 synergistically increases GFAP promoter activity in GBM cells

GFAP expression is one of the most commonly used markers of differentiated astrocytes and tumors of astrocytic origin (194, 197, 198). However, the mechanism of GFAP regulation in GBM cells is not clearly understood. A paradigm of molecular events observed during normal astrocyte development shows that GFAP promoter is regulated by a transcription complex comprising of activated Stat3 (activated by IL-6), Smad1/Smad4 (activated by BMPs) and p300 (135). I have hypothesized that a similar transcription complex functions on the GFAP promoter in GBM cells. To test this hypothesis, a GFAP promoter-driven luciferase reporter construct was transfected into the GBM cell line U251 to monitor GFAP promoter function.

In an effort to test the involvement of each of the components of the regulatory
machinery, experiments were performed to determine the effects of each of the components mentioned above on the GFAP promoter-driven luciferase reporter activity. A dose of 100 ng/ml of BMP4 was used since the same was reported to induce GFAP expression in primary human GBM cells (199). Treatment of U251 cells transfected with GFAP-luc construct for 4 hours and 24 hours did not show much difference from untreated control, although 48 hours of treatment increased the GFAP promoter activity by 1.4 fold (Fig 30a). So, for the rest of the studies BMP4 treatment was conducted for 48 hours.

Next, the effect of IL-6 on GFAP promoter activity was determined in U251 cells. 20 ng/ml of IL-6 treatment was effectively used in previous experiments; so, this concentration was used to determine the time required to induce any change in GFAP promoter. There was an increase in GFAP promoter activity within 24 hours of treatment although at earlier time points there was a fluctuation in the promoter response with consecutive increase and decreases, which almost followed a cyclical pattern (Figure 30b). The 24 hour time-point was used for the rest of the experiments.

During normal astrocyte development, p300, a transcription co-factor has been found to be an important element in the transcription complex formation event. In order to test whether p300 contributes to the regulation of GFAP promoter in GBM cells, either p300 or the empty vector pCI was co-transfected with GFAP-luc in U251 cells. Co-transfection of 2 µg of p300 plasmid with the GFAP-Luc construct induced consistent increase in the reporter activity by about 2-fold over the basal expression level (Figure 30c). Given that GBM cells have been reported to produce and secrete IL-6 and BMPs, it
is not surprising that exogenous administration of these two cytokines did not have conspicuous effects. However, p300 appeared to be limiting in GBM cells, and expression of p300, therefore, increased GFAP promoter activity significantly.

The next set of experiments aimed at investigating the effects of p300 combined with either IL-6 or BMP4 or both. p300 and GFAP-luc constructs were co-transfected in U251 cells and either 20 ng/ml of IL-6 or 100ng/ml of BMP4 was added to the cells and harvested 24 or 48 hours post-treatment respectively. Luciferase assay showed that although p300 alone induced GFAP promoter activity by two-fold, in combination with IL-6, it increased the function of GFAP promoter even further to almost 2.5 fold (solid gray bars in Figure 30d); while in combination with BMP4 (dotted black bar in Figure 30d) or with both IL-6 and BMP4 there was no such increase (diagonal striped bar in Figure 30d). The GFAP promoter induction by p300 and IL-6 was suppressed by BMP4 treatment (diagonal striped bar in Fig. 30d). It still remains elusive why the p300-induced GFAP promoter activity was inhibited by BMP4 stimulation. A recent finding that BMP receptor dysfunction due to epigenetic silencing of the BMPR1B in a subset of GBM cells results in impairment of the BMP-mediated JAK/Stat-dependent astrocytic differentiation pathway (200, 201) may support an alternative argument that BMP4-mediated pathway may not be functional in these GBM cells. This seems unlikely, since BMP4 treatment did affect the GFAP promoter activity in U251 cells suggesting operational BMP receptors.
**Figure 30.** p300 induces GFAP promoter activity in GBM cells. (a) Time-course of BMP4 treatment at a concentration of 100 ng/ml. (b) Time-course of IL-6 treatment at a concentration of 20 ng/ml. (c) p300 co-transfection with the reporter construct. (d) IL-6 along with p300 increases the promoter activity. (e) BMP4 treatment of the cells for 48 hours did not increase the GFAP promoter activity, instead it suppressed the IL-6 and p300-induced increase in GFAP promoter activity. For all the experiments described above, the cells were harvested 48 hours post-transfection and subjected to Luciferase assay. Renilla luciferase (pRLTK) reporter construct was co-transfected in each condition as an internal control. The transfections were repeated three times and the average normalized data are reported here. The firefly luciferase activity was normalized against the renilla luciferase activity and the normalized fold induction is shown with the error bars representing standard deviation. N=3 *p<0.005, **p<0.003
Normalized Folic Acid Induction

- **No treatment**
- **20 ng/ml hIL-6 treatment**

**c**

**d**

- **No treatment**
- **20 ng/ml hIL-6 treatment**

**Normalized Percentage**

- **pCI**
- **p300**

* * *
5.3.2  Smad4 is required for GFAP promoter activity in GBM cells:

In normal astrocyte development, Smad1 and Smad4 complex is involved in induction of GFAP promoter activity downstream of BMP signal (202). Smad4 functions as a common binding partner to Smads 1, 5, 8 downstream of BMPs (199) and also to Smads 2 and 3 which are activated by TGFβ. I investigated if Smad4 was needed for GFAP promoter activity in GBM cells. A dominant negative mutant of Smad4 called deltaCSmad4 was co-expressed in U251 cells either with the GFAP-luc reporter alone or with p300. Analysis of luciferase reporter activity showed that expression of deltaSmad4 suppressed both the basal level of GFAP promoter activity and the p300-induced GFAP promoter function dramatically as seen in the black bars (Figure. 31). These results suggest that Smad4 is essential for GFAP promoter function and also for the effect of p300 on GFAP promoter in GBM cells. However, Smad4 functions as a dimer with one of the other Smads and the potential candidate here is Smad1.
**Figure 31.** Smad4 function is essential for GFAP promoter activation. GFAP promoter-driven Luciferase (GFAP-luc) and renilla Luciferase reporter (pRLTK) constructs were co-transfected with either pCI vector or p300 in duplicate sets in U251 cells. One set was transfected with deltaCSmad4, the dominant negative mutant of Smad4, while the other set was not. The cells were harvested 48 hours post-transfection and subjected to Luciferase assay. The Luciferase activity was normalized against the renilla luciferase activity and the normalized fold induction is reported. Error bars represent standard deviation. N=3 *p<0.004
5.3.3 A putative Stat3 DNA-binding site is required for p300-induced GFAP promoter function in GBM cells:

Nine putative Stat3 DNA-binding sites were identified on the GFAP promoter and one of them was reported to be involved in transcription of GFAP gene during normal astrocyte development (203). In an attempt to explore if the same site was important in GFAP promoter function in GBM cells, the reported potential Stat3 DNA-binding site was mutated by site-directed mutagenesis to abrogate Stat3-recognition in the GFAP promoter-driven luciferase reporter construct (Figure 32a). When the mutant reporter construct was transfected either alone or with p300, GFAP promoter activity, as measured by luciferase reporter assay, was reduced by 66.6% from the basal level and the p300-driven native GFAP promoter activity (Figure 32b). This suggested that Stat3 binding was involved in the activation of the GFAP promoter in GBM cells. The remaining promoter activity in the presence of the mutant construct indicated that other Stat3-binding sites might be functional, thus contributing to the remaining GFAP promoter activity in GBM cells.
Figure 32. Putative Stat3 DNA binding site on the GFAP promoter contributes to GFAP promoter activity. (a) The schematic representation of the molecular species that have been found to affect GFAP promoter activity in GBM cells in the vicinity of the GFAP promoter. The red dashes indicate the site-directed mutation that destroyed the Stat3 DNA binding site on the GFAP promoter Luciferase construct. (b) Schematic representation of GFAP-Luc and dGAS-Luc mutant constructs with the GAS and mutated dGAS sequences. (c) U251 cells were co-transfected with either pCI vector or p300 plasmid and either the GFAP promoter-driven Luciferase reporter gene or the mutated construct that is demonstrated in (a). 48 hours after transfection the cells were harvested and subjected to Luciferase reporter assay. Renilla luciferase (pRLTK) reporter construct was co-transfected in each condition as an internal control. The transfections were repeated three times and the average normalized data are reported here. The firefly luciferase activity was normalized against the renilla luciferase activity and the normalized fold induction is shown with the error bars representing standard deviation. N=3 *p<0.001
5.3.4 **Stat3 physically binds the GFAP promoter in GBM:**

The involvement of Stat3 DNA-binding site in GFAP promoter function raised the question: Does Stat3 physically interact with the GFAP promoter in GBM cells? The experiment performed to address this question was chromatin immunoprecipitation (ChIP) assay with or without IL-6 treatment of U251 cells followed by PCR analysis of the pulled-down chromatin fragments bearing the GFAP promoter. Anti-Stat3 antibody was used to pull down the formaldehyde cross-linked chromatin fragments derived from U251 cells, and the DNA was isolated and examined for the presence of GFAP promoter fragments. The interaction between activated Stat3 and GFAP promoter was studied under two conditions, either with or without treatment with 20 ng/ml of IL-6 for 20 mins since IL-6 was previously reported to increase Stat3 activation in U251 cells (204). Sterile water was used as the template to ensure uncontaminated PCR reaction. The de-crosslinked chromatin was used as template for PCR in the input lanes and both of them showed the product demonstrating that the chromatin samples contained GFAP promoter. For each experimental condition, no treatment or 20 mins of IL-6 treatment, no antibody control and an antibody isotype control were used to eliminate the possibility of non-specific interaction of the antibody. The no-antibody control and rabbit IgG isotype-control antibody did not generate any PCR product. Under no treatment condition, Stat3 failed to bind to and pull down any GFAP promoter, however, following IL-6 treatment, Stat3 interacted detectably with GFAP promoter in GBM cell line, U251 and a PCR
product was observed that lined up with the product in the input lanes. To further ensure that only the antibody was not capable of generating a PCR product, I did mock immunoprecipitation with the rabbit IgG and anti-Stat3 antibodies without the chromatin samples. The mock immunoprecipitate did not generate any PCR product. This showed that Stat3 interacts with GFAP promoter strongly enough to be detected only in the presence of IL-6 (Figure 33).
Figure 33. Stat3 physically interacts with the GFAP promoter in vivo following IL-6 treatment. 70% confluent U251 cells were crosslinked with formaldehyde treatment. The reaction was stopped by glycine treatment and the cells were washed thoroughly with PBS. Then the cells were harvested and nuclear lysate was prepared followed by sonication to fragment the chromatin. Equal amount of chromatin (quantified as described in methods and materials section) was used to immunoprecipitate with anti-Stat3 antibody. Rabbit IgG antibody was used as a negative control for immunoprecipitation. The controls and the Stat3 immunoprecipitate were de-crosslinked and the DNA was isolated from the protein and then subjected to PCR reaction using primers designed to identify the amplified fragments of GFAP promoter. The PCR products were then resolved on a 1% agarose gel and visualized by ethidium bromide staining.
5.3.5 Myc suppresses GFAP promoter activity:

To elucidate the mechanism of suppression of GFAP expression by Myc, the effect of Myc on GFAP promoter activity was investigated. Since Myc is known to function as a transcription factor (205), I hypothesized that Myc inhibits GFAP promoter activity in GBM cells. To test this hypothesis, myc was co-transfected with GFAP promoter-driven luciferase construct and p300 construct or the empty vector, pCI in which p300 was cloned. Luciferase reporter assay demonstrated that Myc expression inhibited the both the basal level of GFAP promoter activity and the p300-induced GFAP promoter function (Figure 34a). Next, it was determined whether IL-6 treatment of the GBM cells rescued the Myc-mediated inhibition of GFAP promoter activity. Co-transfection of the respective plasmids and 24 hours of IL-6 treatment followed by luciferase reporter assay demonstrated that IL-6 failed to rescue Myc-mediated suppression of the basal level and p300-induced GFAP promoter function (Figure 34b).
Figure 34. Myc suppresses p300 and IL-6-induced GFAP promoter activity in GBM cells. (a) U251 cells were co-transfected with either 2.0 ug of pCI vector or p300 or 0.5 ug of myc and p300 both and GFAP promoter driven luciferase construct (GFAP-luc) and renilla luciferase gene (pRLTK) as an internal control. (b) U251 cells were transfected as described in (a) and one set was treated with 20 ng/ml of IL-6 after 24 hours of transfection while the other set was not. The cells were harvested at 48 hours post-transfection and luciferase assay was performed with equal protein quantity in each well in a 96 well-plate. The GFAP promoter-driven firefly luciferase activity measured was normalized with the renilla luciferase activity measured. The data are presented as fold induction over control. Transfections were performed in two separate experiments and the average fold-inductions from three separate data points per condition are presented. The error bars represent standard deviations. N=3 *p<0.003
a

Vector p300

0.5 ug empty vector
0.5 ug Myc

Normalized Fold Induction

b

pCI p300

NT, hIL6; 24 hrs
hIL6+Myc, 0.5 ug

Normalized Fold Induction
5.4 **Discussion:**

Myc maintains an undifferentiated cellular state (206, 207). In normal astrocytic cells, Myc is found to induce de-differentiation as monitored by loss of GFAP, the marker of differentiated astrocytes (82). The mode of regulation of GFAP expression in GBM was not known. So I tried to elucidate the mechanism of GFAP expression by investigating the regulation of GFAP promoter. This study has identified some important modulators of GFAP expression in GBM cells like p300 and IL-6. It has also demonstrated a novel regulatory mechanism by which Myc oncoprotein inhibited GFAP promoter function in GBM cells. My findings provide a mechanistic basis to the previous observations in which Myc was found to cause loss GFAP expression in differentiated astrocytes (82). These observations also suggest that p300 may be limiting in GBM cell differentiation because overexpression of p300 in GBM cell caused an increase in GFAP promoter activity. A paradigm of GFAP promoter regulation in normal murine astrocyte development has suggested that p300 functions as a bridge between two classes of transcription factors, Stat3 and Smads downstream of IL-6 and BMP respectively (135). This may be true for GFAP promoter function in GBM cells too, since similarities in signaling events between development and cancer are not rare (208, 209). The downstream effector of IL-6, Stat3 DNA-binding activity was shown to be necessary for GFAP expression, since abrogation of one of the potential Stat3 recognition sites on GFAP promoter reduced the GFAP promoter activity. Moreover, the chromatin
immunoprecipitation studies showed that Stat3 physically associates with the GFAP promoter in GBM cells following IL-6 stimulation. GFAP promoter function was also dependent on the intact Smad4 activity since a dominant negative mutant of Smad4 inhibited GFAP promoter activity significantly. Smad4 serves as the common link between BMP-signal specific-Smads, Smad1, Smad5, and Smad8, and TGFβ-pathway specific-Smads namely Smad2 and Smad3 (210). Studies in normal murine brain development have shown that unlike BMPs, TGFβ1 that activates Smad2 and Smad3, does not induce astrocytic differentiation(202). Given this, it would be intriguing to determine which of the Smads forms complex with Smad4 and is thus critical for GFAP expression in GBM. This work has also led to the identification of the function of Myc in suppression of GFAP expression in GBM cells that can potentially occur during astrocyte differentiation too. However, it would be very interesting to explore the role of Myc in the various events associated with differentiation. The close association of GFAP expression with astrocyte differentiation makes it interesting to investigate if GFAP expression is a consequence of the global cellular modifications induced by differentiation or if GFAP expression has any causal effect on astrocyte differentiation.
Figure 35. Proposed model for Myc-mediated suppression of GFAP expression in GBM cells. Myc inhibits the function of the transcription complex formed at the GFAP promoter in GBM cells. The constituents of the transcription complex are activated Stat3, p300 and Smad 4. Activation of Stat3 is mediated by IL-6 signaling while Smad complex formation is mediated by BMP signaling. Stat3 DNA-binding activity is required for GFAP promoter activity.
DISCUSSION AND FUTURE PERSPECTIVES

6.1 DISCUSSION

The prognosis of GBM remains extremely poor even after decades of research on the biology of these tumors. Recent findings have triggered a shift in focus onto a rare subset of GBM tumor cells which resemble normal stem cells in their self-renewal and multipotent abilities and are thus termed brain CSCs. These cells are also known as the brain tumor initiator cells since they are implicated in tumor initiation and maintenance. However, the dynamic nature of the differentiation status of these initiator cells have made it very difficult to culture the prospectively isolated pure population in order to identify their differential biology with respect to their non-initiator counterparts. A parallel approach to fight this disease is to induce differentiation in the relatively undifferentiated cells. This requires the identification of important modulators of differentiation mechanism in GBM cells. In this regard, Myc, oncoprotein was found to confer tumorigenicity to differentiated murine astrocytes which also lost the expression of GFAP, a marker of differentiated astrocytes. However, the function and regulation Myc in human GBM cells was not well understood. We hypothesized that Myc is associated with GBM tumorigenicity and its interplay with Stat3 modulates GBM cell
differentiation and thereby affects tumor formation. The results described here demonstrate differential Myc protein expression in GBM cell lines and primary cultures although myc transcript levels were comparable in all the GBM cells. Moreover, Myc protein expression was inversely correlated to the level of activated Stat3 in the GBM cells. The results further show a negative regulatory effect of activated Stat3 on steady state levels of Myc protein in GBM cells. Importantly, this study also showed that Myc inhibits the GFAP promoter activity in GBM cells thereby providing a mechanistic basis to previous observations (82). Myc is reported to be a critical transcription factor that maintains an undifferentiated state of cells (80, 81, 84, 165, 166). This study has uncovered a novel mode of regulation of Myc, and it has identified a mechanism by which Myc suppresses the expression of GFAP, a protein that is upregulated during astrocyte differentiation. Collectively, these results support our hypothesis and enhance our knowledge about GBM cells and the events that occur during GBM cell differentiation.

Recent studies had suggested that the brain tumor initiator cells exclusively expressed a cell surface glycoprotein named CD133 although its functional significance was not known. This initiated efforts to isolate and target these CD133-expressing cells as a therapeutic intervention. However, the current work has demonstrated that both tumorigenic and non-tumorigenic GBM cell lines lacked the cell surface expression of CD133 glycoprotein, although some GBM tumor-derived primary cultures contained CD133 glycoprotein-expressing population. This is consistent with some previous reports stating that cell surface expression of CD133 glycoprotein may be present on some CSCs
(211-213), however all CSCs do not express this marker (67). Thus, CD133 glycoprotein expression was found to be a non-reliable marker for distinguishing brain tumor initiating cells from the non-initiator brain tumor cells.

GFAP expression is a marker of normal differentiated astrocytes (214). GFAP is also expressed in the majority of GBM tumor cells (215). However, the level of GFAP expression decreases with the degree of malignancy of the disease (195) which supports the observations described here. GFAP expression was found to be higher in the non-tumorigenic GBM cells compared to that in the tumorigenic ones.

Understanding the mechanism of GBM cell differentiation is important for so as to design effective therapeutic strategies (see Figure 10). Efforts are underway to develop treatment regimens on the basis of GBM cell differentiation (216, 217). A recent study has shown that GBM tumor-derived initiator cells differentiate into cells of multiple lineages including astrocytes and neurons upon treatment with BMP-4 and fail to form xenograft tumors in mice. Although the GBM cell line, U87, did not respond to BMP-4, the effect of this cytokine on GBM tumor-derived initiator cells indicate that agents that differentiate GBM cells may function as tumor suppressors (199, 218). Another report however, points out that there may be mutations in the differentiation pathways operational in GBM cells that may alter the response of these cells to differentiation-inducing agents such as BMP family of cytokines. They observed that one of the BMP receptors, BMPR1B, was not functional in some of the GBM tumor-derived initiator cells which therefore did not differentiate in response to BMP-2/4 treatment, instead, those cells showed increased rate of proliferation (200, 201). This highlights the need for
extensive understanding of the mechanism of differentiation of GBM cells for implementing effective therapeutic regimens.
Figure 36. Role and regulation of Myc in GBM cell differentiation. Differentiated GBM cells express GFAP. DNA-binding activity of activated Stat3 is important for GFAP expression. Myc suppresses GFAP expression by inhibiting the GFAP promoter activity. Activated Stat3 down-regulates steady state level of Myc protein in GBM cells. The undifferentiated GBM cells that lack GFAP expression promote tumorigenesis, whereas differentiated cells have less tumorigenic potential.
6.2  FUTURE PERSPECTIVES

The work described here also begs important questions such as; how does activated Stat3 exert an inhibitory effect on steady state level of Myc protein? A putative differentiation inducing factor may be involved in mediating the inhibitory effect of activated Stat3 on Myc protein expression (see Figure 29). So it may be interesting to screen the list of genes that are responsive to activated Stat3 and then cross-check them for candidates that have negative regulatory effect on the steady state level of Myc protein.

It would be interesting to investigate the mode of action of Myc-mediated suppression of the GFAP promoter. Myc is known to function in synergy with critical binding partners such as Max that is associated with Myc-mediated gene activation in many cancers (219-228). Myc and Mad are also reported to modify acetylated-deacetylated states of chromatin which then affects the gene expression (105, 106, 229). Possible involvement of Myc-binding partners could be monitored to elucidate their role in GBM cell differentiation. In this regard, recent reports have indicated critical role of microRNA (miRNA) clusters in GBM tumor biology mainly because many miRNAs are either upregulated or downregulated in GBM tumor tissue compared to their normal brain tissue and untransformed astrocytes that have lower expression.

MiRNAs are 22 nucleotide long regulatory RNAs that have been found to modulate the development and differentiation of animal cells (230).
These RNA molecules are believed to base-pair to their recognition elements in the 3’ untranslated region (3’ UTR) and prevent protein translation by either physically binding to the mRNA or by initiating the degradation of the mRNA (231). Recent observations pointed out that miRNA expression profiles were capable of predicting the class and outcome of cancers in patients (232). miRNAs have also been reported to function as oncogenes and tumor-suppressor genes thus highlighting their importance in cancer research (233).

MiRNAs have also been found to be involved in gliomas including GBM (234). Most importantly, a miRNA known as miR-21 has been suggested to serve as an antiapoptotic element in GBM tumor by suppressing the expression of critical pro-apoptotic genes. A recent study showed that inhibition of miR-21 expression causes activation of caspases and leads to apoptosis of many GBM cell lines. The interplay of Myc and miR-21 may also be an area of research since Myc is known to be an inducer of apoptosis and so may be antagonistic to the function of miR-21. The functional significance of most of the miRNAs that are altered in GBM (Table 3 in Appendix) is not known yet, however, they may have important roles in GBM tumorigenesis thus it will be interesting to study the functions of miRNAs in this disease.

BMP-mediated signaling pathway is involved in GFAP promoter activation as is evident from the requirement of Smad4 for the induction of GFAP promoter activity in GBM cells. However, Smad4 functions only in conjunction with other Smad family members and this raises the question, which Smad is involved in GFAP promoter activation in GBM cells.
Supplementary data

Figure 37. U251 cells do not form neurospheres in culture.
Table 3: Role of MicroRNAs in GBM

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Function in GBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17</td>
<td>Acts together with Myc to accelerate tumor development</td>
</tr>
<tr>
<td>miR-21</td>
<td>Antiapoptotic; Highly expressed in GBM tumors (5- to 100-fold)</td>
</tr>
<tr>
<td>miR-10a</td>
<td>Upregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-10b</td>
<td>Upregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-96</td>
<td>Upregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-221</td>
<td>Strongly Upregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-128</td>
<td>Downregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-181a</td>
<td>Downregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-181b</td>
<td>Downregulated in GBM tumors</td>
</tr>
<tr>
<td>miR-181c</td>
<td>Downregulated in GBM tumors</td>
</tr>
</tbody>
</table>
APPENDIX III:

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ARF</td>
<td>alternate reading frame product</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CD133</td>
<td>Cluster of Differentiation 133</td>
</tr>
<tr>
<td>CD15</td>
<td>Cluster of differentiation 15</td>
</tr>
<tr>
<td>Cdc6</td>
<td>Cell division cycle 6</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Chromatin licensing and DNA replication factor 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>ERK</td>
<td>External signal-regulated kinase</td>
</tr>
<tr>
<td>FAL</td>
<td>Fucose N-acetyl lactosamine</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor2</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>G1</td>
<td>Growth phase 1</td>
</tr>
<tr>
<td>GAS</td>
<td>IFN-γ-activated sequence</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>Klf4</td>
<td>Krüppel-like family of transcription factors</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>myc</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis oncogene product (protein)</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MCM2-7</td>
<td>mini chromosome maintenance complex 2-7 proteins</td>
</tr>
<tr>
<td>MAP4</td>
<td>Microtubule associated protein 4</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated ERK kinase</td>
</tr>
<tr>
<td>NSC</td>
<td>Normal neural stem cells</td>
</tr>
<tr>
<td>Oct3/4</td>
<td>Octamer-3/4</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replicative complex</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>Stage specific embryonic antigen-1</td>
</tr>
<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y)-related HMG box 2</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>S-CDKs</td>
<td>Cyclin-dependent kinases involved in the S-phase of cell cycle</td>
</tr>
<tr>
<td>SMAD</td>
<td>Small mothers against decapentaplegic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>3’ UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
</tbody>
</table>


181. Li XN, Shu Q, Su JM, Perlaky L, Blaney SM, Lau CC. Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone


